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NOVEL ZINC OXIDE NANO-HYDROGEL AND MUCIN CO-FORMULATION FOR DIABETIC WOUND HEALING: A SYNERGISTIC APPROACH

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ABSTRACT

Present investigation aims to evaluate the diabetic wound healing property of Zinc Oxide nanoparticles and snail mucin from Achatina marginata, and to compare the synergistic effects of the Zinc Oxide Nano-Hydrogel and mucin co-formulated product in a diabetic rat model. Excision wound model was inflicted on five groups of three diabetic rats each. Group V received dermal application of blanc hydrogel (negative control), Group IV received 1% w/w gentamycin ointment as positive control, Group III received a combination of 0.5 % w/w Zinc Oxide nanoparticles and 1 % w/w mucin in Hydrogel. Group II received 1 % w/w Zinc Oxide nanoparticles in hydrogel, while group I receive 1% w/w mucin in hydrogel alone. The parameters observed were epithelialisation period and percentage wound contraction. The combination of Zinc Oxide Nano-Hydrogel and mucin formulation was observed to significantly heal the diabetic wound within twelve days showing a synergistic wound healing property. The standard gentamycin ointment had a lower wound healing activity compared to all other test groups. Mucin alone showed the highest percentage of wound contraction while the combination of mucin and Zinc Oxide Nano-Hydrogel showed a relatively high percentage of wound contraction with a short epithelialisation period. The results of present research indicate the significant wound healing of Zinc Oxide Nano-Hydrogel and Mucin co-formulated product in experimental animal groups in diabetic condition.

KEYWORDS: Zinc Oxide nanoparticles; Mucin; Synergistic; Diabetic; Epithelialization; Wounds.

INTRODUCTION

Study of the skin and wound healing has been going on for many centuries. The skin, which is the largest organ of the human, serves as a protective material against all forms of environmental aggressive factors such as temperature changes, invasion of biopathogens, dehydration and inner cell abrasion. The structure of the skin is highly complicated, but dynamic in nature. Anatomically, it is made up of three main layers, namely; epidermis, dermis and subcutaneous lipid layer (hypodermis). A wound is a distortion or break in the continuation of the skin epithelium which could be superficial or deep and caused by some underlying factors such as cut, chemical factors and trauma. This results in alteration either in the function or in the structural integrity of the skin respectively. The regeneration of an intact functional skin is a complex process with significant challenges. Wound healing can take quite some time and this is significant when fresh wound turns to chronic wound. Observed pathophysiology changes during wound formation are normally attributed to conditions such as pathogenic infections, age, sex hormones, stress, alcohol, smoking, nutrition and pathological conditions like diabetes. The wound healing process involves highly articulated four phases of biological events which includes but not restricted to the following; homeostasis involving vascular constriction, platelet aggregation, degranulation and fibrin formation. Inflammation marked by neutrophil infiltration, monocytes infiltration as well as macrophage and lymphocyte infiltration. The third phase is proliferation involving re-epithelization including collagen synthesis, angiogenesis and extracellular matrix formation. Traditional medicine has been used for time immemorial by the local population in developing countries. Traditional therapies involve the use of herbal and animal derived compounds. Traditional herbs that have been implicated in wound healing include *Aloe vera*, *Moringa oleifera*, *Colendula officinalis, Curcuma longa*, and *Vernonia amygdalina*. Living organism that has been indicated in wound healing is maggot and leech therapy. Adikwu and Alozie, (2007) have reported an observed complete healing of wounds using snail mucin. Zinc oxide products have been used in wound healing and these agents include Bacitracin and Zinc Oxide (Cicatrin [R]) which is a topical combination of the antibiotic Bacitracin. Diabetes affects hundreds of millions of people worldwide. Diabetic individuals

exhibit a documented impairment in the healing of acute wounds. This population is amenable to develop chronic non-healing diabetic foot ulcers (DFUs) which are estimated to occur in 15 % of all persons with diabetes. Amongst the metal oxide nanoparticles, Zinc oxide nanoparticle has received increasing attention in recent years and has been used in many biomedical applications. They are reported to poses antimicrobial activity against pathogenic organisms. Wound patients presents at the clinics on a daily basis, but of all the cases seen by physicians, diabetic wound is the most costly and life depreciating complications of diabetes mellitus that has kept the interest of health care at oblivion gaze and there is no cost effective therapy for this condition yet. This research ensured cost effective, safe and accessible therapy in diabetic wound management.

MATERIALS AND METHOD

Chemicals and reagent

De-ionized Milli Q water, analytical-grade zinc nitrate, sodium hydroxide, and acetone were obtained from Merck, Germany and Oxoid, Hampshire, United Kingdom. Chemicals were used without any further purification.

Plant material

Fresh leaves of *Solanum torvum* L. were collected at the Pharmacognosy garden of the University of Port Harcourt, Nigeria in July 2019. Plant samples were identified by Dr. Ekeke Chimezie of the Department of Plant Science and Biotechnology Unit of University of Port Harcourt and voucher specimen number (UP/PHCOG00056) were kept in the departmental herbarium.

Solanum torvum L. leaf extract preparation

The leaves were washed three times with tap water followed by de-ionized water. They were further dried away from direct sun light for 1 week. The dried leaves were boiled in the analytical-grade water for 45 min at 100 °C. The dark brown extract was filtered to remove insoluble fractions and macromolecules. Near ultra-filtration was then ensured using 0.45-µm sintered glass funnel and the resultant extract was stored in refrigerator at 4 °C until use. The extract obtained afforded the polyphenols and amino phyto-compounds (protein) which acted as the reducing and capping agent.

Green synthesis of ZnONPs

The green synthesis was done following the method of *Ezealisiji et al* (2019). A 200 ml of aqueous zinc nitrate solution (0.5M) was mixed with 20 ml of the aqueous leaf extract of *Solanum torvum* L. and subsequently treated with 1.0 M sodium hydroxide (10 ml). The ions which initiated the reaction were afforded by the zinc nitrate in de-ionized water. The reaction mixture was incubated with constant stirring in the dark at 60 °C to avoid photo-catalysis. An observed off-white colour marked the formation of ZnONPs at the end of 24 h. The resultant product was further purified by centrifugation and washed in double-distilled water and ethanol, respectively, dried and kept in an amber-coloured sample bottle until use. The resulting Zinc oxide nanoparticles were characterized using Scanning Electron Microscope, Transmission Electron Microscope, Zeta sizer and X-ray Difractometer.

Extraction of mucin

Snail mucin was extracted from the African giant snail *Archachatina fulica* following the method of Adikwu.M.U (2007). The snail shells were cracked and their fleshy bodies removed from the shells with the aid of a metal rod. Excretory materials accompanying the bodies were removed. A total weight of 20 g of the snail bodies was subjected to washing by squeezing off the slime from the fleshy bodies repeatedly into a pool of 100 ml of water and decanted. This procedure was repeated 2 more times to give a total decanted pool of 1 L. Mucin was precipitated out of the pooled washings using 2 L of chilled acetone. The precipitate was filtered and lyophilized to give brownish flakes. The dried flakes were blended in an electric blender to give the mucin powder.

Zinc oxide nano–hydrogel and mucin composite preparation

Hydrogel preparation was done following *Ezealisiji et al* (2019) method. A 2.0 kg of *Ipomoea batatas* (sweet potatoes) was purchased from the local market in Port Harcourt, Nigeria. Peeling, washing, wet milling and further washing with water were ensured to remove the starch. The resulting cellulosic fibre was dried in a hot air oven, pulverized and passed through a 180-µm stainless steel sieve. The product was treated with sodium hypochlorite (3.5% w/v), washed in ethanol (98% w/v) and basified to get the hydrogel. A 0.5g quantity of ZnONPs and 1.0g of mucin powder was then infused into 95 g of the hydrogel. Both were triturated to homogeneity in a porcelain mortar and the amount made up to 100 g of the preparation with the hydrogel. Further trituration to ensure homogeneity was done.

Animal handling

Fifteen Wistar rats weighing 200–250 g obtained from the Animal House of the Department of Pharmacology, University of Port Harcourt, were used for the present study. The animals were quarantined for a period of one week to ensure stabilization before use. They were fed with standard rodent feed (Nigeria) and table water *ad libitum*. The animals were housed in an animal cage maintained at a room temperature of 25 ± 1 °C and 50 ± 8% relative humidity with an alternating light–dark cycle, (*Adikwu MU 2006)* . All animal procedures were reviewed and approved by the local institutional use of animal Ethics Committee of the University of Port Harcourt, Nigeria.

Diabetes induction in rats

After acclimatization, the weights of the rats were determined and baseline glucose level was determined

after overnight fasting prior to the induction of diabetes. Alloxan monohydrate was administered intraperitoneal with 150mg/kg body weight dissolved in ice cold normal saline. After three days the rats with blood glucose level greater than 16mmol per litre were considered diabetic and used for the experiment.

Preparation of wound site in experimental animals

The Wister rats were anaesthetized with diazepam (0.2 mg/kg body weight) and the dorsal hairs on the skin of the animals were shaved with a sterilized razor blade. A circle of diameter 20 mm was marked on each right side of the thigh of the animal's skin surface, and the skin dissected out. 70% ethanol was used for sterilization of the wound area and cotton wool soaked in normal saline was used to damp the wound surface to maintain haemostasis.

Determination of the rate of wound healing

Treatment was initiated immediately after the excision was made by applying the test sample in gel, on the wound and then once every day for 16 days. All the gels were liberally applied topically using sterile cotton wool. The wound area of each animal was measured with pair of divider and transparent metre rule while the animals were under diazepam anaesthesia on the days following post-surgery.

Treatment protocol

Group I were treated with 1% w/w Mucin in hydrogel, group II were treated with 0.5% w/w ZnONP in hydrogel, group III were treated with hydrogel of composition 1% w/w Mucin, and 0.5% w/w ZnONP, group IV served as positive control and were treated with 1% w/w gentamicin ointment, group V rats served as a negative control and were treated with blank hydrogel formulation. Each evaluation was carried out in triplicate.

All the procedures followed World Health Organization (WHO) Procedures for Biomedical Research Involving Animal Subjects, 1982.

Assessment of wound healing

The diameter of the wounds was measured with a transparent meter rule. The diameter was obtained by measuring horizontally and vertically, and then the mean was taken as the diameter. The area was calculated and recorded as initial wound area which is the wound area for day. The area was obtained using the formula: $A=\pi r^2$. Where A = Area, π = 22/7 and r^2= diameter. The rate of wound contraction for each rat was determined by using the formula: W_0- W_1. Where W_0= initial wound area and W_1= wound area of each measuring day. The percentage wound contraction was calculated thus: % wound contraction = $[(C_0 - C_T) / C_0] \times 100$; Where C_0 and C_T are the wound sizes at the initial time (0) and time 'T', respectively. Epithelialization time was noted as a number of days required for the first scar of the wound to fall off. Complete wound healing was determined by the total number of days required for the second scare to fall off.

Statistical analysis

All experiments were performed in replicate of n=3 for validity of statistical analysis. Results were expressed as mean±SEM. ANOVA, Pearsean colleration and Student *t*-test were performed on data set. 95% confidence interval was considered that is $p \leq 0.05$ to ascertain the significant differences of wound healing among the three products.

RESAULTS

Characterization of Zinc Oxide nanoparticles

X-ray diffraction (XRD) pattern of the green synthesized sample of Zinc Oxide nanoparticles was recorded at the Department of Chemistry, Rhodes University Nanotechnology Center. Bruker d8 Advanced X-ray diffractometer was employed using cu K$\propto$ radiation (λ = 1.5408 Å) 40 kV, 2θ/θ scanning mode. Data were recorded within the 2θ range of 10 ^{o} – 100 ^{o} in a step proceeding of 0.0204 degree. The diffractogram (fig x) presented with six peaks at 2 θ value of 18.204^o, 20.937^o, 23.672^o, 30.108^o, 34.608^o and 40.206^o, corresponding to (020), (022), (022), (111), (120), and (200) integer on the 'hkl' planes, respectively. The above information reveals spherical but discreet crystalline nature of the particulate matter. These results were in agreement with matched data from International Centre for Diffraction Data (ICDD). Optimum Bragg reflection was obtained at 2θ of 18.402^o to predict full width half maximum (FWHM) value as the average size of the Zinc Oxide nanoparticles was found to be 28.24 nm (fig. 1) using the following Debye – Scherer equation:

$$D = \frac{K\lambda}{\beta Cos\theta}$$

Where D is the thickness of the nanocrystal, K is a constant; λ is the Bragg's angle 2θ. This result was in agreement with the one obtained from Photon correlation microscopy (Zeta Sizer) which gave 28.1 ± 6.369 nm average particle sizes. Presence of crystallized Phyto-chemicals (Capping and Stabilizing agents) on the surface of the Zinc oxide nanoparticles were justified by the observed unassigned crystalline peaks (42.8^o, 48.2^o, and 51^o) as recorded on the XRD parttern. A Transmission Electron Microscopy image revealed Zinc Oxide nanoparticle average size of 28.0 ± 02.1 nm, (fig. 3). The UV – Visible Spectroscopic analysis of synthsized Zinc oxide nanoparticles , showed a single sharp peak corresponding to Zinc oxide nanoparticles at 359 nm (fig. 2). The DLS particle sizes of Zinc oxide nanoparticles were observed to be 24 nm (fig. 4).

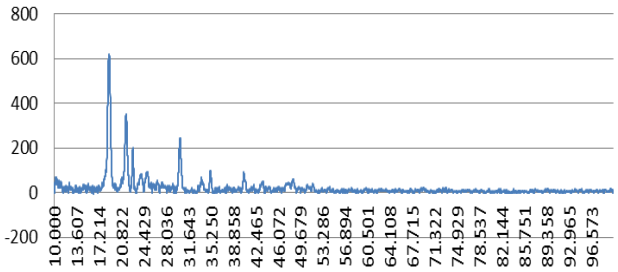


Fig. 1: XRD analysis of synthesized ZnO NPs which captured six major Bragg reflections corresponding to the spherical crystalline nature of the nanoparticles.

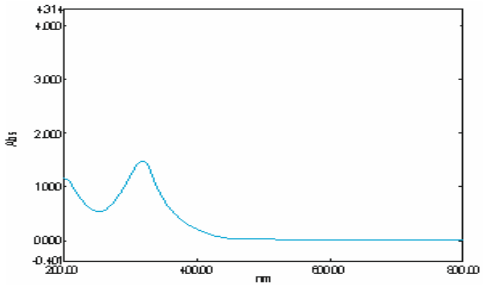


Fig 2: UV-vis spectroscopic analysis of synthesized Zinc Oxide NPs, a single peak corresponding to Zinc Oxide nano particles (359 nm).

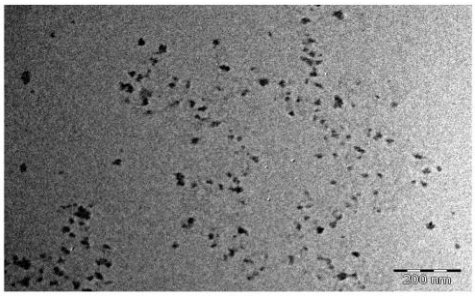


Fig. 3: *a* TEM image of zinc oxide nano particles.

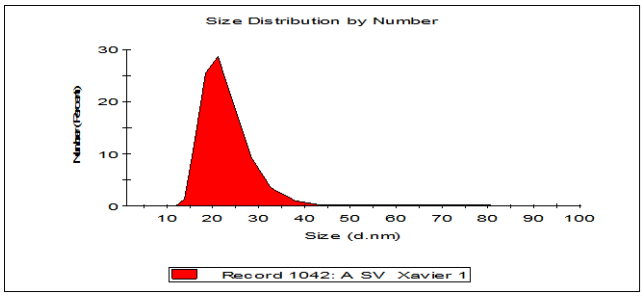


Fig.4: DLS Particle size distribution of synthesized ZnONPs.

Table 1: Effect of Mucin, zinc oxide nanoparticle, Mucin+Zinc oxide nanoparticle, Versus the positive control (1% Gentamicin ointment) and the Negative control (Blank hydrogel) on excision wound model.

Days	Group I (Mucin)	Group II (ZnO NP)	Group III (Mucin+ZnO NP)	Group IV (Gentamicin)	Group V (Blank hydrogel)
0	20.00±0.00 (0%)	20.00±0.00 (0%)	20.00±0.00 (0%)	20.00±0.00 (0%)	20.00±0.00 (0%)
2	16.33±0.33 18%)***	15.66±0.66 (22%)**	15.66±0.33 (22%)***	18.66±0.33 (7%)***	16.00±0.57 (20%)
4	10.00±0.57 (50%)**	13.00±0.57 (35%)**	10.66±1.20 (47%)*	17.00±0.57 (15%)**	12.66±1.20 (37%)
6	8.33±0.33 (58%)**	9.33±1.33 (53%)	8.00±0.57 (60%)**	15.66±0.33 (22%)	9.00±1.53 (55%)
8	5.66±0.33 (73%)*	5.66±0.66(72%)*	3.68±0.33 (82%)**	7.33±1.20 (63%)	6.33±0.88 (68%)
10	2.33±0.33 (88%)	3.00±0.00 (85%)	0.33±0.33 (98%)	3.00±0.57 (85%)	3.66±0.33 (82%)
12	1.66±0.02 (92%)	0.66±0.33 (97%)	0.00 (100%)	0.72±0.33 (95%)	1.87±0.33(90%)

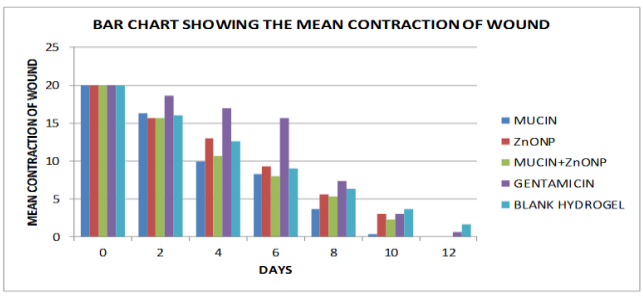


Fig 5: Bar chart showing the mean contraction of wound.

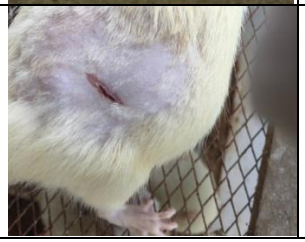
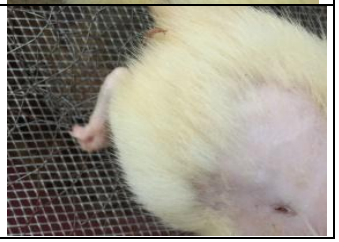
DAY	MUCIN	NANO	MUCIN+NANO	GENTAMICIN	HYDROGEL
0					
6					
10					
12					

Fig 6: Photograph of the excision wound model on the Albino rats and the healing effect of different products.

DISCUSSION AND CONCLUSION

Discussion

The process of wound healing is a self-initiated activity involving the regeneration of skin tissues through an organised cascade of events. Achieving homeostasis, reducing blood loss and preventing the entrance of potential pathogens into the body is the primary event in wound healing under normal circumstance *(Doughty and Sparks, 2007)*. The stage is followed by inflammatory, proliferative and the remodelling phases. This process can be impaired in the presence of a chronic illness such as diabetes. Treatment of chronic wounds cost huge amount of money. Beyond the financial cost this also results to psychological issues to the patients and the families. The evaluation of the wound healing effect of Mucin and Zinc Oxide nanoparticles produced an interesting analytical data, as different topical applications obviously enhanced wound contraction and epithelialization though at different contraction and epithelialization period. A comparism of each product with the blank hydrogel showed different levels of positive significant values, indicating that they had an appreciable wound healing effect when compared with the blank hydrogel.

At day 2 post-wounding, a highly significant reduction ($P < 0.01$) in wound contraction was observed among all treated groups with a maximum reduction($P < 0.001$) in group I and III. Mucin and Zinc Oxide nanoparticles show a more significant difference ($P < 0.005$) In wound contraction when compared to the combination product ($P < 0.05$). It can be said that Mucin, Zinc Oxide nanoparticles and the combination product is good in treating the inflammatory phase of wound as they showed higher percentage of wound contraction in the first four days post-wounding. Different drugs preferentially affect various phases of wound healing this is supported by the work done by *Velmurugan et al, (*2012) where *Gossypium herbacium* was used in treating excision and incision wound in rats. A highly significant reduction ($P < 0.005$) was prominent in group III on day eight. A two way analysis of variance done in order to compare the zinc oxide nanoparticle, Mucin, and the combination of the product (group III) showed significant values at $P < 0.01$. From the table the effect of the interaction between group III and group I, group III and group II is given by the probability value of ($P < 0.001$) thus, the mean effect of Mucin and Zinc Oxide nanoparticle is statistically significant. From the correlation table, an observed relationship exist between group I (Mucin), group II (Zinc Oxide nanoparticle) and group III (combination of group I and II). The correlation value (r) shows that, the relationship between group I and group III, group II and group III, was seen to be $r= 0.992$ which is a perfect relationship between the two drug effect. From the table, (Table 1) the test was checked at 1% error at 99% confidence interval. The significant value of 0.000 which was seen to be lower than our error term $\alpha=0.01$, thus leading to a conclusion that there is a correlation (statistical relationship) between Mucin, Zinc Oxide nanoparticle and a combination between Mucin and Zinc Oxide nanoparticle.

It can be deduced that Mean±SEM values of the wound area recorded for each product decreased as the number of days of exposure increased (fig.6). While percentage wound contraction increased with increased in the number of days of exposure to the products this was elucidated in the bar charts (fig.5). The information from the percentage wound contraction showed that group I and group III gave the highest contraction rate at 50% and 47% respectively on day four hence could be said to be very effective at the inflammatory phase of wound healing. The contraction of wound with Zinc oxide nanoparticle alone became significant from day eight with 72% wound contraction as against the negative control with 68% contraction. The positive control Gentamicin 1% was observed to show significant contraction from day ten with percentage contraction of 85% as against 82% of the blank hydrogel; the positive control showed a slow onset of action as compared to other test substances this is clearly seen from day two, down to day six on the chart. From table 1, Contraction rate was seen to be maximum in mucin from day eight and a 92 % wound healing on day twelve. Zinc oxide nano particles showed 97 % while the combination product also showed complete wound healing on day twelve. From fig 2, average wound contraction for each product observed graphically, showed that though all the test products showed relatively good wound healing property and group I (Mucin) having the best contraction property, the combination of mucin and zinc oxide nanoparticle showed a significant synergistic contraction effect with time.

Garg, (2000) stated that wound epithelialization is another parameter used in evaluation of wound and it occurs when epithelial cells covers the wound bed providing protective coverage for the new tissue. The epithelialization period which was monitored by noting the number of days required for the eschar to fall off from the wound surface without leaving a raw wound behind. The data from the research showed that group III animals have lesser day of wound epithelialization than animals treated with other products. The potato hydrogel was used as vehicle due to its emollient effect on wound surface and also it is a good carrier for nanoparticles. The abnormal observed wound healing effect seen with the blank hydrogel as when compared with the standard gentamicin could be attributed the excellent emollient effect of the hydrogel. Combination of Mucin and Zinc Oxide nanoparticles having shown an average of 100% wound contraction on the 12^{th} day, as other products will require longer period of exposure to give a 100% epithelialization. In the inflammatory phase, micro-organism are phagocytised and removed and pro inflammatory substances are released that cause the migration and division of cells involved in the proliferative phase. Angiogenesis, collagen deposition,

granulation tissue formation, epithelialization and wound contraction occur in the proliferative phase *(Midwood et al. 2004)*, in the remodelling phase, collagen is remodelled and realigned along tension lines and cells that are no longer needed are removed by apoptosis.

Previous works support that the snail mucus principally contains allantoin, collagen, elastin and glycolic acid. Allantoin, or 5-Ureidohydantoin, derives from the uric acid transformation by the enzyme uricase. Glycolic acid, or alfa-Hydroxyacetic acid, has an excellent capability to penetrate skin and is capable to increase collagen synthesis. Snail mucin from *Archachatina marginata* (Family Ariondiae) has been reported to have antimicrobial activity Adikwu *et al* have suggested that due to its surfactant activity it prevents bacteria attaching to host cells. Zinc oxide nanoparticle has been found in addition to having the ability to induce reactive oxygen species (ROS) generation, which can lead to cell death when the antioxidative capacity of the cell is exceeded, also has anti-diabetic property *(Xia T, et al. 2006; Ryter S W et al. 2007; Long TC, et al. 2006; Lewinsky N, et al 2008)*. Zinc promotes hepatic glycogenesis through its actions on the insulin pathways and thus improves glucose utilization *(Jansen et al.2009; Pawlak K et al 2012; Hojyo S et al.2016)* . Zinc is also known to keep the structure of insulin and has a role in insulin biosynthesis, storage and secretion *(Chausmer 1998; Tomlinson ML et al. 2008; Prasad AS 2014; Brayner R et al. 2006; Gammoh NZ and Rink L 2017)*. The combination of Mucin and Zinc oxide nanoparticle could have shown a synergistic effect through combined anti-oxidation, increased collagen synthesis anti-diabetic effect; reducing blood glucose level at the sight of infection hence discourage the thriving and accumulation of micro-organism.

CONCLUSION

In conclusion, this research shows that mucin from *Archatina marginata* and Zinc Oxide nanoparticles has good wound healing effect and effective in preventing bacteria action. The combinatination of the two products has synergistic effect seen more in wound re-epithelialization when compared to wound contraction, and effective for treatment of all phases of wound healing in diabetic condition.

Conflict of Interest: The authors report no conflicts of interest

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