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EVALUATION OF SNAIL MUCIN DISPERSED IN *Brachystegia* GUM GEL AS A WOUND HEALING AGENT

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ABSTRACT

Snail mucin was obtained from the mucilage of Archachatina marginata (Family Arionidae). The wound healing effect of the snail mucin was evaluated with special attention to the effect when combined with honey in Brachystegia eurychoma gel preparation. Brachystegia eurychoma gum, snail mucin and honey were combined in different concentrations in the treatment of wound made by excision model in rats. It was observed that mucin when combined with honey and in the Brachystegia eurychoma gel heals faster than when used alone. Brachystegia eurychoma gum was also observed to effect fast healing of the wounds when used alone. Complete healing was observed in 15 days post treatment. Honey in combination with mucin as well as the Brachystegia eurychoma gel should be harnessed in pharmaceutical formulations for the treatment of wounds. Brachystegia eurychoma gum in right combinations with mucin and honey for wound healing, prevents bacteria infection, scar formation and promotes regeneration of hair follicles.

Keywords: Snail mucin, Honey, *Brachystegia eurychoma* gum, Gel, Wound healing

INTRODUCTION

Wound healing is an important process involving tissue repair and regeneration. A wound is described as a break in the continuity of tissue from violence or trauma and is regarded as healed if there is a restoration of the wound site or inflamed tissue to normal condition (Adikwu and Ikejiuba, 2005). Tremendous advancements have been made in understating the process of wound healing. The cell types and the order in which they appear in the wound have been established. Many growth factors and their functions have been elucidated. Despite the advances in understanding the science of wound healing, many more steps are yet to be discovered.

An incision wound created by scalpel, trauma resulting from a bullet, or tissue death caused by a myocardial infection all undergo a similar and predictable reparative process. Understanding how the body repairs damaged tissue and what factors influence the wound healing process helps the surgeon ensure an acceptance outcome from surgery (Alvarez and Gilbreath, 1982).

The 3 categories of wound healing are primary, secondary and tertiary Primary wound healing involves healings or closure of a wound within hours of its creation. Secondary wound healing involves no formal wound closure or healing; the wound closes spontaneously by contraction and reepithalization. Tertiary wound healing or closure, are also known as delayed primary closure, involves initial debridement of the wound for an extended period and then formal closure with suturing or by another mechanism (McCarty, 1998).

The use of honey in the treatment of wound has been rediscovered, is becoming increasing interest as more effect of its effectiveness has been proved (Zumla and Lutat, 1989). The clinical

observations recorded are that infection is rapidly cleared, inflammation, swelling and pain are quickly reduced, odour is reduced, sloughing of necrotic tissue is induced, granulation and epithelisation are hastened, and healing occurs rapidly with minimal scarring. The antimicrobial properties of honey prevent microbial growth in the moist healing environment created. Unlike other topical antiseptics, honey causes no tissue damage. In animal studies it has been demonstrated histologically that it actually promotes the healing process. It has a direct nutrient effect as well as drawing lymph out to he cells by osmosis. The stimulation of healing may also be due to the acidity of honey. The osmosis creates a solution of honey in contact with the wound surface which prevents the dressing sticking, so there is no pain or tissue damage when dressings are changed. There is a controlled clinical trial that have proven honey more effective than silver sulfadiazine and a polyurethane film dressing for the treatment of wound made be burns. Honey can also be used for wound dressing (Zumla and Lutat, 1989).

Many procedures have been used, but in most of the reports it was use to clean the wound first. Many described honey as having a cleansing and deriding action on wounds. The necrotic tissue is being removed, before dressing wounds with honey. Some used rigorous cleansing procedures, scrubbing with a soft toothbrush followed by hydrogen peroxide, saline rinse (Wadi *et al.*, 1987), betadine and another saline rinse, dilute Dakin solution on wound bed and alcohol on the surrounding skin (Subrahmanyam, 1993), or the wound was cleaned with Eusol or aqueous 1 % chlorhexidine (Obaseki-Ebor *et al.*, 1983). Some reported cleaning the wounds before dressing. One cleaned with gauze.

Snail mucin is used for wound healing (Adikwu and Ikejiuba, 2005).

It is rich in glycosamoglycans which has been shown to possess wound healing properties (Glade, 1990). It is also used in the removing of keloid scars. Snail mucin is extracted from land snails. The compound acts as biological activator of the elimination of dead and damaged skin cells and the renewal of healthy cells. It also controls bacteria (Kim *et al.*, 1996).

Snail mucin prevents, diminishes and eliminates stretch marks (*Striae atrophica*, *Striae distensea*) and scars. Snail mucin utilizes biological activators of mammalian skin growth factors. It dissolves damaged collagen skin cells, triggers the renewal of collagen, elastin and the production of glycosaminoglycans (GAGS) and proteoglycans from within the deep layers of the skin (Kim *et al.*, 1996).

GAGS are complex polysaccharides (sugar chains) that participate in the regulation of physiological processes through their interactions with proteoglycans and with a wide variety of proteins. The loss of glycosaminoglycans from the skin weakens the supportive inter-cellular skin (Kim *et al.*, 1996).

GAGS and poteoglycans have large water holding capacity, occupy a large space in the extracellular matrix and fill most of the intercellular space between the collagen and elastin fibres. They play a critical role as shock absorbents and provide binding, hydrating and swelling pressure to tissues enabling them to withstand compressional force and prevent tearing and scaring of the deep layers of the skin during pregnancy outgrowth, growth spurts during adolescence, overstretching by body building (in association with steroids) or over stretching by more than average weight gain. They also play a vital role in cell proliferation, migration and adhesion. Proteoglycans and GAGS are found to be prominent molecules during wound healing through their influential role in cell – cell and cell – matrix interactions (Kim *et al.*, 1996).

The majority of gels are complex and the detailed chemical composition of some of them has not yet been elucidated. In general, they are high molecular weight carbohydrate polymers formed around a central unit of *D*-galactose of *D*-galacturonic acid linked together by sugar units (Hutchins and Singiser, 1955). Substances frequently called gels are hydrocarbons of high molecular mass, petroleum products, rubber latex, synthetic polymeric gums, balms and resins, which have sticky or gelly nature. Dispersion of polysaccharides, acids and gels containing them present characteristics which render them suitable for application as laxatives agents for treatment of hypoacidity, vehicles for masking the taste of alkaloid preparations, bases for medicated jellies (Hutchins *et al.*, 1955). Besides their inherent emulsifying and stabilizing properties, some gels like, gel Arabic, their demulcent and emollient characteristics led to a number of uses, from the stabilization of emulsions, suspensions, to the formation of tablets and pills. However, gels such as tragacanth and agar are known to reduce the bacterial effect of incorporated preservatives (Taub *et al.*, 1958).

Gels are also used in dairy product manufacture; for example, in ice creams as stabilizers. In ceramic industry, gels may be used for binding, thickening and as a fixing agent for enamels and porcelains. In the textiles industry, gels find use as pigment dispersing aid and above all as a thickening gent for colour printing pastes. The *Brachystegia* gum used here in the formulation of gels has been evaluated in the formulation of various pharmaceutical products.

MATERIALS AND METHODS

Chemicals: Acetone (Merck), diazepam injection (10 mg/2 ml, Tiajin Medicines and Health Products), methylated spirit (Hardis and Dromedras) and distilled water was obtained from an all glass still. The mucin was obtained from a batch prepared in our laboratory following earlier established procedures (Adikwu, 2005). Purified honey was obtained from the local market and diluted with sterile, distilled water to obtain a viscosity grade that was equivalent to that stated in the Pharmaceutical Codex (1979). Original honey was also purchased from commercial sources and prepared to meet pharmaceutical standards.

Animals: Albino rats of either sex weighing between 184 – 230 g were used during the study. Animals were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. Before and after the surgery, the animals were housed individually in a partition metal cage and they were fed *ad libitum* on standard commercial pellet diet and water.

***Brachystegia eurycoma* gum:** *Brachystegia eurycoma* seeds were purchased from Ogie market, Nsukka, Enugu State, Nigeria. The seeds were identified by Mr. Ozioko of Department of Crop Science, Faculty of Agriculture, University of Nigeria, Nsukka.

Twelve kg of *Brachystegia* seeds, was weighed out, roasted, and soaked in water. The effect of soaking was to increase the moisture content of the seed thereby increasing the swelling index. It was allowed to stand for 12 hours. The outer coat was then peeled off and the cotyledons were washed with tap water and dried for 12 hours in the oven (Manesty F) set to a temperature of 40 °C.

The dried seeds were collected and milled using a hammer mill (Retsh GMBH 5657 Ham) fitted with sieve no. 0.5. This was to reduce the particle size. The powder material obtained was further passed through sieve no. 60 aperture size and the fines (1.4 kg) were collected and these were used in the precipitation process after dispersion in 2 litres of water. The powder dispersion gave a good viscous mixture which hydrated properly within 24 hours and gave enough gum on precipitation. The dispersion was aided with a glass rod stirrer and then homogenized using a Silverson mixer.

The mixture was then stored for 24 hours to aid hydration of the powder. The dispersion was centrifuged and the supernatant was collected and reserved while the sediment was discarded. The introduction of acetone into the collected supernatant in the ratio of 1:1 resulted in the complete precipitation of the gum. The mixture obtained was centrifuged, the supernatant decanted while the sediment which is the brachystegia gum was collected.

The precipitated gum was further washed in acetone for 10 min, and then dried to a constant weight at a temperature of 40 °C in an oven. The dried flakes of the gum were reduced in size using an end runner mill (Pascal Engineering H) and were passed through sieve of aperture size 0.355 mm. The pulverized gum was weighed. A yield of 30 % was recorded.

Formulation of Gel: The gels were formulated according to the general formula (Table 1). Snail mucin was poured into a beaker containing 50 ml of distilled water which was stirred with a rod until the mixture was a viscous gel. Then brachystegia gum powder and honey were also added to the mixture and the stirring continued until a uniformly mixed gel was obtained. The gel was then transferred into sterilized plastic containers with cover. This same procedure was used in all the groups according to their various components (Table 1).

Table 1: Composition of the gel

Groups	Quantity of <i>Brachystegia</i> gum (g)	Quantity of snail mucin (g)	Quantity of honey (ml)	Volume of distilled water (ml)
A	200	100	7.4	50
B	200	200	14.8	50
C	200	300	22.2	50
D	200	-	-	50

Wound Healing Studies: A total of twenty rats were used for these studies. The animals were divided into five groups of four animals each, and were caged in a partition cage. The animals were anaesthetized with diazepam injection intramuscularly at the dose of 5 mg/kg body weight respectively, and the wound healing was carried out following the excision wound model. The formulations were applied on the inflected wound using a cotton bud. Then, wound areas were measured at 2 days interval up to 15 days. The animals were fed regularly and their drinking water was changed on daily bases. The wound areas on subsequent days were compared with the wound areas on the first day and the percentage contraction were calculated thus: Percentage wound contraction % = $(W_{A0} - W_A) \times 100 / W_{A0}$ Where: W_{A0} = Wound area on day 0 and W_A = Wound area on subsequent days.

Data Analysis: The data obtained were analyzed using the Student's *t*-test at the 5 % level of

significance. Standard deviations of the results were also calculated.

RESULTS AND DISCUSSION

Wound healing process generally has 3 phases. They are the inflammatory phase, the proliferative phase and the maturational phase. The inflammatory phase is characterized by homeostasis and inflammation. Collagen exposed during wound formation, activates the clotting cascade (both the intrinsic and extrinsic pathways) initiating the inflammatory phase (Mazzotta, 1994).

After injury to tissue occur, the cell membrane damaged from the wound formation, releases thromboxane A2 and prostaglandin 2 – alpha, a potent vasoconstrictor. This initial response helps to limit haemorrhage. After a short period, capillary vasodilatation occurs secondary to local histamine release, and the cells of inflammation are able to migrate to the wound bed. The timeline for cell migration in a normal wound healing process is predictable. When the gel containing snail mucin is applied, it helps in arousing the immune system leading to various immune reactions and processes leading to protein proliferation at the point of injury hastening the wound closure. This is because the immune properties of the body at the point of the wound saw snail mucin as foreign.

Platelets, the first response cell, release multiple chemokines, including epidermal growth factor (EGF), fibronectin, fibronogen, histamine, platelet – derived growth factor (PDGF), serotonin, and von Willebrand factor. All these are closely related to the immune processes that can be encouraged by the presence of snail mucin. These factors help stabilize the wound through clot formation. These mediators control bleeding and limit the extent of injury. Platelet deggranulation also activates the complement cascade, specifically C5a, which is a potent chemoattractant for neutrophils.

The inflammatory phase continues, and more immune response cell migrate to the wound. Neutrophils are the next substances to migrate to the wound and it is for debris scavenging. Neutrophils, along with the mucin and honey, kill bacteria and decontaminate the wound from foreign debris.

The next cells present in wound are leukocyte and the macrophages (monocytes). The macrophage referred to as orchestrator, which is essential for wound healing, numerous enzymes and cytokines are secreted by the macrophage. These are collagenases, which deride the wound, interleukins and tumors necrosis factor (TNF) which stimulate fibroblasts (produce collagen) and promote angiogenesis and transforming growth (TGF) which stimulates keratinocytes.

The second stage of wound healing is the proliferative phase. Epithelization, angiogenesis, granulation tissue formation and collagen deposition are the main steps in this anabolic portion of wound healing, Epithelization occurs early in wound repair, if the basement membrane remains intact.

Table 2: Mean wound healing size (cm)*

Groups	Day 3 (cm)	Day 5 (cm)	Day 7 (cm)	Day 9 (cm)	Day 11 (cm)	Day 13 (cm)	Day 15 (cm)
A	1.8 ±0.03	1.5 ±0.03	1.1 ±0.09	0.5 ±0.09	0.3 ±0.05	0.08 ±0.05	0.0 ±0.0
A	1.8 ±0.05	1.6 ±0.05	1.3 ±0.05	0.6 ±0.06	0.4 ±0.07	0.23 ±0.09	0.025 ±0.03
C	1.9 ±0.03	1.8 ±0.03	1.5 ±0.05	0.9 ±0.06	0.7 ±0.05	0.43 ±0.08	0.15 ±0.06
D	1.6 ±0.03	1.3 ±0.05	0.7 ±0.05	0.2 ±0.05	0.05 ±0.03	0.0 ±0.00	0.0 ±0.00
E	2.0 ±0.03	1.9 ±0.06	1.9 ±0.06	1.6 ±0.06	1.5 ±0.11	1.1 ±0.1	1.2 ±0.10

*All the animals in each group have the same diameter of wound which is 2 cm.

Table 3: Mean percentage wound healing contraction on days post-surgery

Groups	Day 3 (%)	Day 5 (%)	Day 7 (%)	Day 9 (%)	Day 11 (%)	Day 13 (%)	Day 15 (%)
A	12.50 ±1.44	25.00 ±2.04	43.75 ±4.27	76.25 ±4.27	86.25 ±2.39	96.25 ±2.39	100.00 ±0.00
A	11.25 ±2.39	21.25 ±2.39	36.25 ±2.39	71.25 ±3.15	80.00 ±3.54	88.75 ±4.27	98.75 ±1.25
C	5.00 ±2.04	12.50 ±1.44	26.25 ±2.39	53.75 ±3.15	66.25 ±2.39	63.00 ±18.81	92.50 ±3.23
D	18.75 ±1.25	33.75 ±2.39	63.75 ±2.39	88.75 ±2.39	97.50 ±1.44	100.0 ±0.00	100.00 ±0.00
E	1.25 ±1.25	5.00 ±2.89	7.50 ±1.44	20.00 ±2.89	26.25 ±5.54	46.25 ±5.15	25.50 ±4.79

The epithelial cell migrates upwards in the normal pattern. The epithelial progenitor cells blow the wound and the normal layers of epidermis are restored in 2 – 3 days. If the basement membrane has been destroyed, then the wound is reepithelized from the normal cells in the periphery and from the skin appendages, if intact (e.g., hair follicles and sweat glands). The adhesive gel, containing the mucin and honey, helps in providing an additional layer of coverage that could prevent wound infection. This leads to the higher level of healing noted for the three combinations as shown in Tables 2 and 3. The results obtained for Brachystegia gum alone was also significant ($p < 0.05$).

Angiogenesis, stimulated by TNG-alpha is marked by endothelial cell migration and capillary formation. The new capillaries deliver nutrients to the wound and help maintain the granulation tissue bed. The migration of capillaries into the wound bed is critical for proper wound healing. The granulation phase and tissue deposition require nutrients supplied by the capillaries, and failure for this to occur result in a chronically unhealed wound.

The final phase of wound healing is the maturational phase. Fibroblasts differentiate and produce ground substance and then collagen. The ground substance is deposited into the wound bed; collagen is then deposited as the wound undergoes the final phase of repair. Many cytokines are involved in proliferative phase of wound repair, which include insulin like growth factor (IGF). The wound undergoes contraction, intimately resulting in a smaller amount of apparent scar tissue.

The entire process is a dynamic continuum with an overlap of each phase and continued remodelling. Collagen deposition continues for a prolonged period, but the net increase in collagen deposition plateaus after 10 days. This depends on the size and depth of wound. The gel, containing the mucin helps in keeping the wound moist, enabling all the biochemical processes to take place. The gum gel particularly helps to maintain contact of the mucin and honey with the wound surface due to its adhesive property.

Proper wound healing involves complex interactions of cell cytokines working in concert. In recent years, more chemical mediators integral to this process have been identified. The sequential steps and specific processes have not been fully differentiated. When examining the process of wound healing, one should identify the major steps and know the important mediators. The mucin in the preparation is reported to contain glycosaminoglycans which have been reported to be of value in wound healing and repair (Glade, 1990; Kim *et al.*, 1999). Apart from the mucin, honey too has been reported to possess wound healing properties.

Honey was common form of wound dressing in ancient times (Forest, 1982). Excessive heating of honey should be avoided because the glucose oxidase enzyme in honey which produces hydrogen peroxide, a major component of the antibacterial activity of honey, is very readily inactivated by heat. Honey can be made very fluid by warming at 37 °C if vigorous stirring is not sufficient (Armon, 1980).

It has been reported from various studies on the usage of honey as a dressing for infected wounds that the wounds become sterile in 3 – 6 days. Others have reported that honey is effective in cleaning up infected wounds. It has also been reported that honey dressing halt advancing necrosis (Bloomfield, 1973).

Honey has also been found to act as a barrier preventing wounds from becoming infected (Seymour and West, 1951). It prevents cross-infection, and allows burn wound tissue to heal rapidly uninhibited by secondary infection (Yang, 1944).

It has been observed that under honey dressings sloughs, necrotic and gangrenous tissue separated so that they could be lifted off painlessly, and others have noted quick and easy separation of sloughs and removal of crust from a wound (Bose, 1982). Rapid cleansing and chemical or enzymic debridement resulting from the application of honey to wounds have also been reported, with no scar forming on burns. Several other authors have noted the cleansing effect of honey on wounds. It has also been noted that dirt is removed with the bandage when honey is used as a dressing, leaving as clean wound (Mcinerney, 1990). Honey has also been reported to give deodorization of offensively smelling wounds. These properties of honey when combined with those of mucin (Wei and Bobeck, 2005) can have very positive consequences on wound healing. The rapid wound healing noted in this study may suggest a synergistic effect of the honey and the mucin.

Conclusion: It could be concluded from the above results that *Brachystegia* gum when combined with snail mucin and honey in low concentrations gave appreciable results in terms of wound heading. Finally, from the results, it can strongly be advised that *Brachystegia* gum mixed with mucin and honey should be used in the preparation or formulations of topical drugs in the treatment of wounds. The advantage of this combination is that the components are all natural products with little or no known side effects but has high cicatrizant activity.

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REFERENCES

ADIKWU, M. U. and IKEJIUBA, C. C. (2005). Some physicochemical and wound healing properties of snail mucin. *Bolletino Chimico Farmaceutico*, 144: 1 – 8.

- ADIKWU, M. U. (2005). Evaluation of snail mucin motifs as rectal absorption enhancer for insulin in non-diabetic rat models. *Biological and Pharmaceutical Bulletin*, 28(9): 1801 – 1805.
- ALVAREZ, G. M. and GILBREATH, R. L. (1982). Effect of dietary thiamine on intermolecular collagen cross-linking during wound repair: a mechanical and biochemical assessment. *Journal of Trauma*, 22: 20 – 24.
- ARMON, P. J. (1980). The use of honey in the treatment of infected wounds. *Tropical Doctor*, 10: 9.
- BLOMFIELD, R. (1973). Honey for decubitus ulcers. *Journal of American Medical Association*, 224: 906.
- BOSE, B. (1882). Honey or sugar in treatment of infected wound? *Lancet*, 329 (April 21): 963.
- FORREST, R. D. (1982). Early history of wound treatment. *Journal of the Royal Society of Medicine*, 75: 198 – 205.
- GLADE, M. J. (1990). Polysulphated glycosaminoglycans accelerates net synthesis collagen and glycosaminoglycans by arthritic equine cartilage tissues and chondrocytes. *American Journal of Veterinary Research*, 51: 779 – 785.
- HUTCHINS, H. H. and SINGISER, R. E. (1955). New gums for pharmaceutical jellies. *Journal American Pharmaceutical Association, Practical Pharmaceutical Education*, 16: 226 – 230.
- KIM, Y. S. Y. Y., CHANG, I. M., TOIDA, T., PARK, Y. and LINHARDT, R. J. (1996). A new glycosaminoglycan from the giant African snail *Achatina fulica*. *Journal of Biological Chemistry*, 271: 11750 – 11755.
- MAZZOTTA, M. Y. (1994). Nutrition and wound healing. *Journal of American Podiatric Medical Association* 84: 456 – 462.
- MCCARTY, M. F. (1998) Glucosamine for wound healing. *Medical Hypotheses*, 47: 273 – 275.
- Mcinerney, R. J. F. (1990). Honey a remedy rediscovered. *Journal of the Royal Society of Medicine*, 83: 127.
- OBASEKI-EBOR, E. E., AFONYA, T. C. and ONYEKWELI, A. O. (1983). Preliminary report on the antimicrobial activity of honey distillate. *Journal of Pharmacy and Pharmacology*, 35: 748 – 749.
- PHARMACEUTICAL CODEX (1979). The Pharmaceutical Press, London, 415 pp.
- SEYMOUR, F. L. and WEST, K. S. (1951). Honey – its' role in medicine. *Medical Times*, 79: 104 – 107.
- SUBRAHMANYAM, M. (1993). Honey impregnated gauze versus amniotic membrane in the treatment of burns a prospective randomized study. *British Journal of Plastic Surgery*, 46(4): 322 – 323.
- TAUB, A., MEER, W. A. and CLAUSEN, L. W. (1958). Conditions for the preservation of gum tragacanth jellies. *Journal American*

- Pharmaceutical Association, Practical Pharmaceutical Education*, 47: 235 – 238.
- WADI, M., AL-AMIN, H., FARAOUQ, A., SASHEF, H. and KHALED, S. A. (1987). Sudanese bee honey in the treatment of suppurating wound. *Arab Medico*, 3: 16 – 18.
- WEI, G. X. and BOBEK, L. A. (2005). Human salivary mucin MUC7 12-mer-L saliva, and 12-mer-D peptides: enhancement of activity with protease inhibitor cocktail or EDTA, and cytotoxicity to human cells. *Antiimicrobial Agents and Chemotherapy*, 49: 2336 - 2342.
- YANG, K. L. (1944). The use of honey in the treatment of nonspecific ulcers, and small wounds. *Chinese Medical Journal*, 62: 55 – 60.
- ZUMLA, A and LUTAT, A. (1989). Honey a remedy rediscovered. *Journal of the Royal Society of Medicine*. 82: 384 – 385.