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Evaluation of Wound Healing Properties of Nigerian *Archacatina marginata* mucin and Its Combination with Honey in Wistar Rats

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Abstract

The widespread existence of unhealed wounds has had a great impact on public health and economy especially in developing countries with high indices of mortality and morbidity rates associated with wound due to poor health care delivery system. This study evaluated the wound healing properties of snail mucin and snail mucin-honey (M-H) formulations in different ratios (50:50, 70:30 and 30:70) on excision wound model in wistar rats. Thirty rats of both sexes, grouped into six, were used for the study. Physico-chemical analysis of the honey was performed and the mineral contents of honey, snail mucin and M-H were also analysed. The anti-microbial properties of the samples were also investigated using four organisms, (*S. aureus, E.coli, P.aureginosa* and *P. mirabilis*) isolated from wounds. The antioxidant properties of mucin and M-H were also analysed. The results of the analysis showed that the pH and viscosity of honey were 4.46 ± 0.10 and 4.89 ± 0.10 pas/sec respectively while its peroxidase and glucose oxidase were 1.20% ± 0.10 and 4301.00 ± 0.10mg/dl respectively. There was significant increase of K level and low level of Se in M-H formulation compared to mucin only. Vitamin A, E and C contents were found to be appreciably higher in honey than in mucin. The activities of SOD inhibition, catalase and glutathione peroxidase were significantly higher in M-H formulation compared to mucin only. There was an increased *E. coli* susceptibility to M-H formulations (70:30 and 50:50) compared to the control drug (0.5% Amoxicillin) and also an increased *P. mirabilis* susceptibility to M-H formulation (30:70) compared to the control drug (untreated group). The significant increases in *S. aureus* and *P.aureginosa* susceptibilities to the control drug compared to other samples were observed. As from the ninth day of the study, there was significant reductions in the wound areas of the treatment groups especially in M-H formulations (50:50 and 70:30) compared to negative control group. Although mucin only showed greater capacity for wound healing compared to negative control, results showed that M-H formulations especially 70:30 (group E) had better wound healing capacity compared to mucin only, and even the positive control. By nineteenth day of treatment, complete healing was observed in M-H formulations (50:50 and 70:30). Honey in combination with mucin should be harnessed in pharmaceutical formulations for the treatment of wounds in right combination to aid wound healing, prevent bacterial infection, scar formation and promote regeneration of hair follicles.

Keywords

Archacatina Maginata, Snail Mucin, Honey, Excision Wound, Antimicrobial

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1. Introduction

Wound healing is the process by which skin or other body tissue repairs itself after trauma. In undamaged skin, the epidermis (surface layer) and dermis (deeper layer) form a protective barrier against the external environment. When the barrier is broken, an orchestrated cascade of biochemical events is set into motion to repair the damage [1]. Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix proteins, remodeling of new parenchymal and connective tissue and collagen deposition [2]. Wound healing begins at the moment of injury and involves both resident and migratory cell populations, extracellular matrix and the action of soluble mediators. Wound healing is crucial in order to bring back skin integrity and it is also a complex and dynamic process with a predictable pattern.

Snails produce mucin in a very large quantity, which is often referred to as slime. Mucins are a family of large glycosylated proteins (50% w/w carbohydrate) and are of group of nitrogenous substances secreted by mucous glands.

Figure 1. Nigerian Archacatina marginata.

They are the major macromolecular components of the mucous secretions that coat delicate epithelial surfaces in animals where they provide protection from microbial and physical damage, and are responsible for the viscoelastic properties of mucous secretions. Snail mucin has been documented to contain glycosaminoglycans reported to be of great value in wound healing and repair [3]. It has also been reported to contain antimicrobial proteins [4]. A bactericidal glycoprotein known as achacin, obtained from the body surface mucus of *Archacatina marginata* (African giant snail) has been reported to kill both Gram-positive and Gram-negative bacteria by attacking the cytoplasmic membrane of the cell [5]. The use of snail mucin obtained from snail mucus secretions for wound healing has also been well documented [6, 7].

Honey is a sweetener for foods and a powerful medicinal tool for centuries. It has simple sugars that are absorbed directly into bloodstream without digestion [8]. Honey's greatest medicinal potential is its application as topical agent to wounds and skin infections [9]. Honey has anti-inflammatory, antioxidant and immune boosting property. Much of the therapeutic properties of honey are due to the high sugar concentration and the resulting osmotic effect, low pH and acidity [10] and due to hydrogen peroxide generated from the oxidative conversion of glucose to gluconic acid by glucose oxidase upon dilution. Honey applied to wounds, burns and ulcers promotes faster healing by clearing infections and rendering sterility, through promotion of tissue growth and regeneration, and preventing dehydration of the infected site [11].

Since wound management has become a major problem and the use of antibiotics in the management of wounds has resulted in drug resistance, this study became imperative for the need of alternative treatment using snail mucin and its combination with honey.

2. Materials and Methods

2.1. Sample Collection

The snails, (*Archacatina marginata*) were purchased from Eke Awka Market Awka, in Anambra state, Nigeria and were classified by Dr. Okeke, J. J. (a Zoologist) in the department of Zoology, Nnamdi Azikiwe University, Awka, Anambra state. Morphological characters of collected land snails were recorded and specimens photographed. The snail identification key used in this work was as stated and provided [12] and in line with [13] and includes such features as; shape of snail, Diameter, Number of size and spatial arrangement, whorls, umbilicus whether it is imperforate, perforate implicate or rimate, Teeth (their size and spatial arrangement) and Spiral striae.

Honey was procured locally from Nsukka central market, Enugu State, Nigeria.

Albino rats were obtained from the Animal house of the Department of Pharmacology, University of Nigeria, Nsukka.

2.2. Physico-Chemical Analysis of Honey

2.2.1. Determination of pH of Honey

Ph was measured by Electrometric method using laboratory pH meter (Hanna model H1991300) as described by [14].

2.2.2. Determination of Viscosity of Honey

Viscosity of honey was measured by Electrometric method using Laboratory Viscometer (Hanna model H1991300), as described by [14]. 5mls of honey was measured into a test tube and its viscosity measured with the viscometer.

2.2.3. Determination of H_2O_2 in Honey

The ability of the sample (honey) to scavenge H_2O_2 was assessed by the method of [15].

Procedure

5.075g of aliquot (honey) was weighed out in a 500ml conical flask and distilled water was added to make up to 250mls and stirred well with a stirrer to mix well. 25mls of the aliquot was added to 250mls distilled water. 10mls conc. H_2SO_4 was added to the mixture and titrated with 0.3N $KMnO_4$. The titre value was taken.

% H_2O_2 in the honey calculated thus; Volume of $KMnO_4$ X N X 0.01701 X 1000

Weight of Sample (honey)

Where N = Normality.

0.01701 = Constant.

2.2.4. Determination of Glucose Oxidase Activity in Honey

Principle

It is an enzymatic indicator test based on the Trinder reaction quantified by the formation of a pink quinoneimine dye.

Glucose oxidase

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconic acid} + H_2O_2$$

Peroxidase

$$2\ H_2O_2 + \text{Phenol} + \text{4-aminoantipyine} \rightarrow \text{Red Chinonimin} + 4H_2O$$

Procedure

The sample was stabilized at 6 ^{O}C for 24 hours and diluted in the ratio 1:2 with 0.9 5 NaCl and the result got was multiplied by a factor of 2.

10uml of sample was pipetted into test tube. The working reagent consisted of 1ml blank and 1ml sample. They were thoroughly mixed and diluted further to 0.04ΔA/minute in reagent grade water for the assay, incubated for 10 minutes at 37^{o}C to achieve temperature equilibrium. 0.1 ml of the diluted enzyme was added and increase in A_{546} was read against reagent blank at 546nm. The absorbance was determined as follows:

$$\text{Units/mg} = \Delta A_{546}/\text{min}$$

11.3X mg enzyme/ml reaction mixture.

2.3. Mucin Isolation and Preparation

Isolation and purification of mucin was done according to the method of [16].

The snails were housed in wood timber boxes (35 cm width x 30 cm height), weighing about 1.89kg. The shells of the *Archacatina marginata* were washed, broken at the tip end using a rod glass and the clear liquid which exuded from the snail was allowed to flow directly into a falcon tube. The snails were 'mechanically stressed' to encourage secretion. Altogether, 225mls of mucus were harvested from 9 snails. The mucus collected was mixed with 4 volume of water (900mls) and shaked overnight at room temperature (RT) using a shaker. After stirring, the mixture was centrifuged at 11000 rpm for 30 minutes at 4°C. After centrifugation, the supernatant was decanted and collected into a clean container and the residue discarded. Water soluble fraction (WSF) was collected. The supernatant was then precipitated with 3 volumes of cooled ethanol (3495mls) at 20°C for 2 hours. Due to large volume of the supernatant, separation was done using separating funnel. Collection of precipitate was made by centrifugation at 2900 rpm for 30 minutes. Before precipitation, ethanol and WSF were cooled down at 20°C for 20 minutes. Precipitate with ethanol was kept at 20°C for experimental study on albino wistar rats.

2.4. Formulation of the Combination Therapy

Honey was mixed with prepared snail mucin in the following ratios;

1 (50:50).....10g mucin + 10g honey + 10ml distilled water

2 (70:30).....14g mucin + 6g honey + 10ml distilled water

3 (30:70).....6g mucin + 14g honey + 10 ml distilled water

2.5. Mineral Analysis of Honey, Snail Mucin and Mucin-Honey (M-H) (Cu, K, Se and Zn)

Heavy metal analysis was conducted using Varian AA240 Atomic Absorption Spectrophotometer according to the method of [14].

Principle

Atomic Absorption Spectrophotometer's principle is based on the sample being aspirated into the flame and atomized when the AAS's light beam is directed through the flame into the monochromator and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wavelength, a source lamp composed of that element is used

making the method relatively free from spectral or radiational interferences.

The amount of energy of the characteristics wavelength absorbed in the flame is proportional to the concentration of the element in the sample.

Procedure

Wet digestion of sample.

2g of the sample was weighed into a digestion flask and 20ml of the acetic acid prepared by mixing (650ml of conc HNO_3; 80ml of perchloric acid; 20ml of conc H_2SO_4). The flask is heated until a clear digest was obtained. The digest was diluted with distilled water to the 100ml mark. The sample was thoroughly mixed by shaking and 100ml of it transferred into a glass beaker of 250 ml volume, to which 5ml of conc. Nitric acid was added and heated to boil till the volume is reduced to about 20 ml, by adding conc nitric acid in increments of 5ml till all the residue was completely dissolved. The mixture was cooled, transferred and made up to 100ml using metal free distilled water. The sample was aspirated into the oxidizing air- acetylene flame. When the aqueous sample was aspirated, the sensitivity for 1% absorption was observed.

Preparation of Reference solution.

A series of standard reference solutions in the optimum concentration range was prepared, the reference solutions were prepared daily by diluting the single stock element solutions with water containing 1.5ml concentrated nitric acid/litre. A calibration blank was prepared using H_2O_2 + Nitric acid except for the metal stock solutions. Calibration curve for each metal was prepared by plotting the absorbance of standards versus their concentrations

2.6. Determination of Vitamin Contents of Honey, Snail Mucin and Mucin-Honey (M-H)

2.6.1. Estimation of Vitamin C

Ascorbic acid was analyzed by spectrophotometric method as described by [17].

Principle

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2, 4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540nm.

Extraction of ascorbic acid

10 mg/l of Ascorbate was extracted from 1ml of the samples (honey, snail mucin and M-H) using 4% TCA and the volume was made up to 10ml with the same. The supernatant obtained after centrifugation at 2000rpm for 10 minutes was shaken vigorously using a cyclomixer and kept for 5minutes.

Procedure

Standard ascorbates (0.5ml) of the supernatant were taken. The volume was made up to 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37^0C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5mls of 8.55 sulphuric acid in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance read at 540nm in a spectrophotometer (Genesys 10-S, USA). Calibration curve was used to estimate the concentration (appendix 1).

2.6.2. Estimation of Vitamin E

Tocopherol was estimated in the samples by the Emmerie-Engel reaction as reported by [18].

Principle

The Emmerie-Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols, which, with 2, 2'-dipyridyl, forms a red colour. Tocopherols and carotenes are first extracted with xylene and read at 460nm to measure carotenes. A correction is made for this after adding ferric chloride and read at 520nm.

Extraction of vitamin E

Procedure

Into 3 stoppered centrifuge tubes, 0.5ml of sample, 1.5ml of the 10mg/l standard and 1.5ml of water were pipetted out separately. To all the tubes, 1.5ml of ethanol and 1.5ml of xylene were added, mixed well and centrifuged. Xylene (1.0ml) layer was transferred into another stoppered tubes. To each tube, 1.0ml of dipyridyl reagent was added and mixed well. Ferric chloride solution (0.33ml) was added to all the tubes and mixed well. The red colour developed was read exactly after 15 minutes at 520nm in a spectrophotometer, (Genesys 10-S, USA). The concentration of tocopherol in the sample was calculated using the calibration curve.

2.6.3. Estimation of Vitamin A

Vitamin A was estimated by the method of [19].

Principle

The assay is based on the spectrophotometric estimation of the colour produced by vitamin A acetate or palmitate with TCA.

Procedure

0.1ml of sample was mixed with 1.0ml of saponification mixture (12% alcoholic mixture) and refluxed for 20 minutes at 60°C in the dark. The tubes were cooled and 20ml of water added and mixed well. Vitamin A was extracted twice with 10ml of petroleum ether at room temperature. The two extracts were pooled and washed thoroughly with water. Anhydrous sodium sulphate was added to remove excess moisture. An aliquot of the extract (0.1ml) was taken and evaporated to dryness at 60°C. The residue was dissolved in 1.0ml of chloroform. Standards (vitamin A palmitate) of concentrations ranging from 0-15mg were pipetted into a series of test tubes.

The volume in all the tubes was made up to 1.0ml with chloroform. TCA reagent (2.0ml0 was added rapidly, mixed and absorbance was read immediately at 620nm in a spectrophotometer, (Genesys 10UV, USA). The same procedure was repeated for the sample tubes also. Vitamin A content was expressed as mg/l using the calculation in the calibration curve.

2.7. Anti-Oxidant Assays

2.7.1. Superoxide Dismutase (SOD) Assay

SOD was assayed according to the method of [20].

Preparation of Enzyme Extract

The different samples (mucin, mucin + honey) 0.1ml were mixed with 3.0ml of Potassium Phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were used for the assays.

Assay

The assay mixture contained 1.2ml sodium pyrophosphate buffer, 0.1ml PMS, 0.3mls NBT, 0.2mls of the Enzyme preparation and water in a total volume of 2.8mls. The reaction was initiated by the addition of 0.2mls NADH. The mixture was incubated at 30°C for 90 seconds and stopped by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0mls of n-butanol, allowed to stand for 10minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a Spectrophotometer, (Genesys 10-S, USA).

$$\text{SOD \%} = \frac{\text{sample OD} - \text{control OD}}{\text{Sample OD}} \times 100 = \text{X\% Inhibition}$$

50% Inhibition = 1 unit enzyme

Where OD= Optical Density

2.7.2. Catalase Assay

Catalase activity was assayed following the method of [21].

Principle

The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

Procedure

Preparation of Enzyme Extracts

A 20% homogenate of the sample was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay.

Assay

H_2O_2- Phosphate buffer (3.0mls) was taken in a cuvette, followed by the rapid addition of 0.1ml of enzyme extracts and mixed thoroughly. The time required for a decreased in absorbance by 0.05 units was recorded at 360nm in a spectrophotometer, (Genesys 10-S, USA). The enzyme solution containing H_2O_2-free phosphate buffer served as control.

One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 360nm by 0.05 units.

$$\text{Catalase umol/ml} = \frac{\text{Abs Change X Total Reaction Volume}}{\text{Sample Volume X Extinction Coefficient}}$$

Where abs= Absorbance

Extinction Coefficient = 0.394umol^{-1}ml^{-1}

2.7.3. Glutathione Peroxidase Assay

The method proposed by [22] was adopted for assaying the activity of peroxidase.

Principle

In the presence of hydrogen donor, pyrogallo or dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallo or dianisidine to a coloured product called purpurogalli can be followed spectrorphotometrically at 430nm.

Procedure

Preparation of Enzyme Extract

A 20% homogenate was prepared in 0.1M phosphate buffer (pH 6.5) from the sample, clarified by centrifugation and the supernatant was used for the assay.

Assay

To 3.0ml of pyrogallo solution, 0.1ml of GSH, and 0.1ml of

the enzyme extract were added and the spectrophotometer was adjusted to read zero at 430nm. To the test cuvette, 0.5ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer, (Genesys 10-S, USA). One unit of peroxidase is defined as the change in absorbance/ minute at 430nm.

$$\text{Glutathione Peroxidase (umol/ml)} = \frac{\text{abs change X total reaction volume}}{\text{Sample volume X extinction coefficient}}$$

Extinction coefficient = $0.266 umol^{-1}ml^{-1}$

2.8. Antimicrobial Analysis

Test Organisms

A total of four clinically implicated wound pathogenic bacteria namely: *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were obtained from the Reference Laboratory section of Applied Microbiology and Brewing Department of Nnamdi Azikiwe University Awka, Anambra State, Nigeria. These organisms were maintained on Nutrient Broth for 24 hours under aseptic condition

2.8.1. Total Bacterial Plate Count (TBC)

Ten-fold serial dilutions of the test micro bacteria were done and 0.1 ml of the appropriate dilutions were plated out on Nutrient Agar and incubated for 24 hours. Thereafter, the developing colonies were counted and the Total Bacterial Plate Count calculated thus:

$$\text{TBC (CFU/ml)} = \frac{\text{(N) x 10 x D}}{\text{V}}$$

Where: TBC = Total Bacterial Plate Count.

V = Volume of inoculums plated.

N = Number of colonies, developing on plate, that were counted.

D = Dilution factor.

CFU/ml= Colony Forming Unit per millilitre.

2.8.2. Antibacterial Susceptibility Test

This was determined by the modified method described by [23]. Plates that had confluent and/or semi-confluent growth were selected for the antimicrobial susceptibility tests. The disk diffusion method was used to assay the effect of the extracts on the various microorganisms. Mueller-Hinton Agar was used for the bacteria. Twenty four hours broth cultures of the test organisms were serially diluted, then the stock broth culture, 10^{-1} and 10^{-2} dilutions were used to seed the bacterial plates. Then, 0.1ml of the appropriate dilution of the broth culture of each microorganism was uniformly spread using a sterile glass spreader on the surface of the media, and sterile filter papers were soaked in the extracts (Mucin, 50 % Honey + 50 % Mucin, 70 % Honey + 30 % Mucin, 30 % Honey + 70 % Mucin) and placed on two points on each petri dish. Incubation was done at room temperature for 24 hours. Clear zones of inhibition around the wells indicated antimicrobial activities of the extracts against the test organisms. The diameter of the zone of inhibitions were measured and recorded in millimeter. All experiments were done in triplicates. Negative controls were set up with sterile water and positive controls were set up using 0.5% Amoxicillin

2.9. Experimental Animal Model

2.9.1. Housing and Management of Animals

Thirty Albino rats of both sexes, which had not been used for any studies and weighing between 168g to 210g, were used for this study. They were housed in the animal house of Chris-Vic farms in Mgbakwu, Awka North, Anambra state, Nigeria. The animals were exposed to 12 hour light and dark cycle with free access to water and feed. They were kept individually in a conducive, healthy environment in clean steel-gauzed cages (under laboratory conditions) for the period of the experiment and fed on standardized animal feed and water for a period of 2 weeks for acclimatization. The experimental procedures involving the animals and their care were in line with the approved guidelines by Nnamdi Azikiwe University, Awka research and ethical committee.

2.9.2. Animal Groupings

The animals were randomly assigned into six groups of five rats each comprising of both sexes per group, each rat in a group was individually caged in a cubicle and separated from other groups.

The animals were grouped thus:

Group A: negative control, untreated wounded rats.

Group B: positive control, wounded rats treated with Neomycin cream.

Group C: wounded rats treated with 100% mucin only (20ml) in 10 ml H_2O.

Group D: wounded rats treated with combination formulation 1 (CF1) (50% mucin + 50% honey).

Group E: wounded rats treated with combination formulation 2 (CF2) (70% mucin + 30% honey).

Group F: wounded rats treated with combination formulation 3 (CF3) (30% mucin + 70% honey).

2.9.3. Excision Wound Model (Surgical Infliction of Wounds on the Rats)

The experimental rats were inflicted with excision wounds according to the method of [24]. The animals were anaesthetized with 1ml of intravenous ketamine hydrochloride (120mg/kg body weight). After the rats were anaesthetized, about 1.8 diameter circle was shaved with surgical blade on the pelvic anterior region. The excision was conducted after the target area was applied with antiseptic solution, (methylated spirit). A full thickness of the excision wound of circular area of about 2.54 cm^2 and 0.2 cm depth was created along the markings with a surgical blade in the shaved region by lifting the rat's skin following the marked area. The rats were put into their individual cages and allowed to recover from the anaesthesia. The dressing of the wound commenced 24 hours post wounding.

2.9.4. Animal Treatment

The formulations were applied topically on the inflicted wounds using a cotton bud. Then, wound areas were measured at 2 days interval up to 21 days. Each of the preparations according to the groupings was applied to cover the surface of the wound on alternate days. The initial diameter of the circular wound was measured and monitored to evaluate the rate of healing. The diameter of the wound was the average of the vertical and horizontal diameters of the wound area. Recordings of the wound areas were measured on graph paper and calculated. The animals were fed regularly and their drinking water changed on daily basis. All the procedures followed WHO Procedures for Biomedical Research involving animal subjects (1982). The day of eschar falling, after wounding, without any residual raw wound was considered as the period of epithelization [25]. The rate of healing between the treated and the control groups was calculated by comparing healed wound area on respective days with healed wound area of control groups. The period of epithelization, were noted. Significance in wound healing of the test groups was derived by comparing healed wound area on respective days with healed wound area of the positive control group. The period of epithelization, were also noted. The wound areas on subsequent days were compared with the wound areas on the first day and the percentage contraction calculated. The rate of wound contraction was calculated as given in the formular below;

$$\% \text{ Wound Contraction} = (\text{Healed Area}^* \div \text{Total Wound Area}) \times 100$$

Where: Healed Area*= Original Wound Area- Present Wound Area

2.9.5. Statistical Analysis

The means of wound area measurements between groups at different time intervals were compared using one-way ANOVA followed by Tukey's post-hoc tests. One-way ANOVA was used to examine the mean differences in wound healing between the groups in excision wound model. Data were analyzed using SPSS (Version 12.0, Chicago, USA). Means values were significant at $P < 0.05$ for all analyses.

3. Results

3.1. Honey Analysis

The pH and viscosity of honey were 4.46 ± 0.10 and 4.89 ± 0.10 pas/sec respectively while the level of hydrogen peroxide was 1.20% ± 0.10.

Table1. Physico-chemical properties of honey.

Parameter	Sample
pH	4.46±0.10
Viscosity (pas/sec)	4.89±0.10
H_2O_2 (%)	1.20±0.10

Values are means ± SD of triplicate determinations.
The glucose oxidase activity in honey was 4301±0.1mg/dl.

Table 2. Glucose oxidase activity in honey.

Parameter	Sample (honey)
Glucose Oxidase (mg/dl)	4301±0.1

Values are means ± SD of triplicate determinations.

3.2. Mineral Contents of Honey, MUCIN and Mucin- Honey (50:50)

It was observed that Potassium was highest in honey, followed by mucin-honey and mucin only contained the least amount. Selenium was highest in mucin only followed by honey while mucin-honey had the least amount. Zinc was highest in mucin only. Copper was highest in mucin-honey (50:50), followed by in mucin only while honey had the least, (Figure 2).

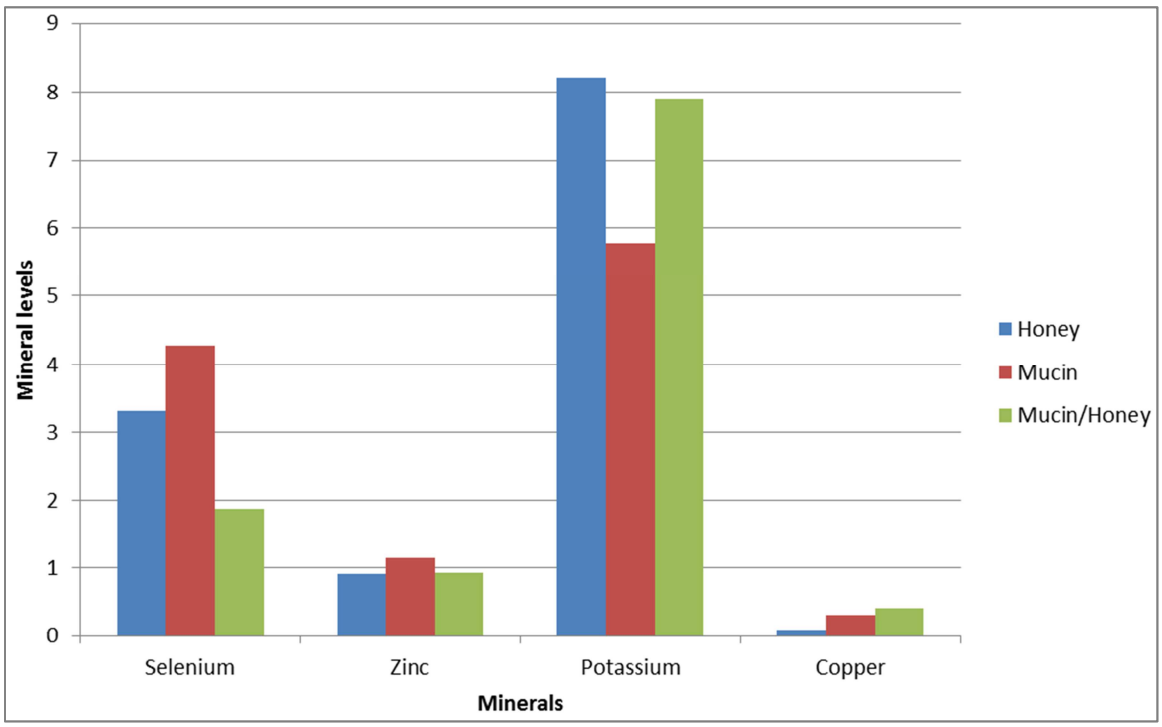

Figure 2. Mineral contents of honey, mucin and mucin-honey (50:50).

3.3. Vitamin Analysis

3.3.1. Vitamin Contents in Mucin

Results showed presence of Vitamin A, E and C in mucin. It was observed that of all the vitamins quantified in mucin, vitamin C was found to be the highest followed by vitamin A and vitamin E was the least.

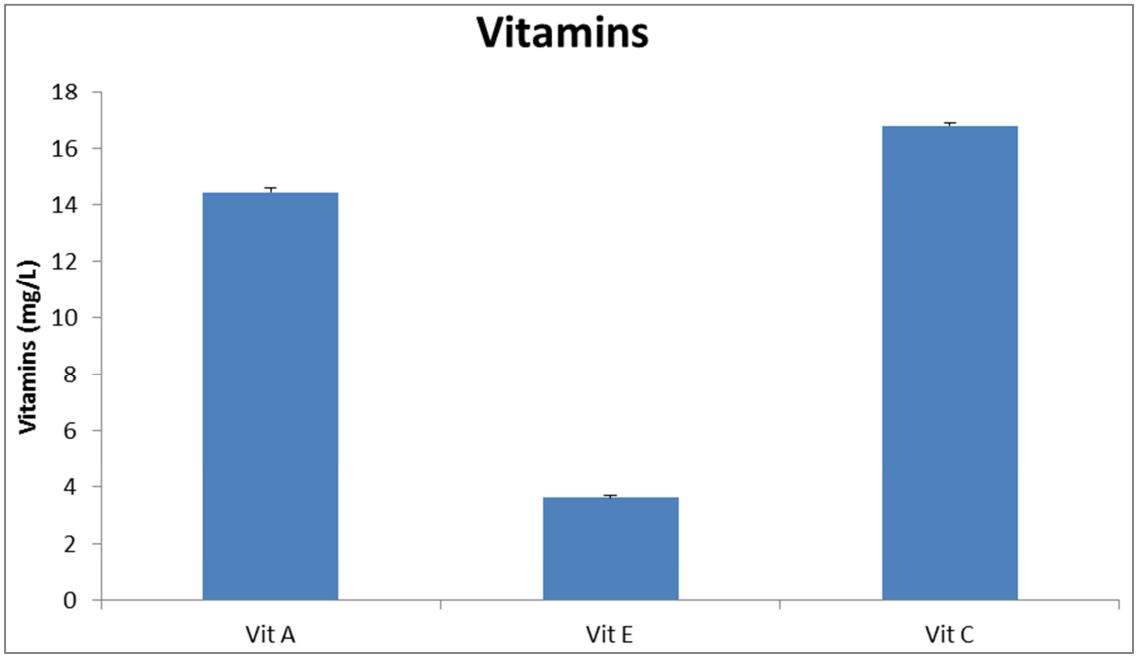

Figure 3. Vitamin contents in mucin.

3.3.2. Vitamin Contents in Honey

Results showed presence of Vitamin A, E and C in honey. It was observed that of all the vitamins quantified in mucin, vitamin C was found to be the highest followed by vitamin A and vitamin E was the least.

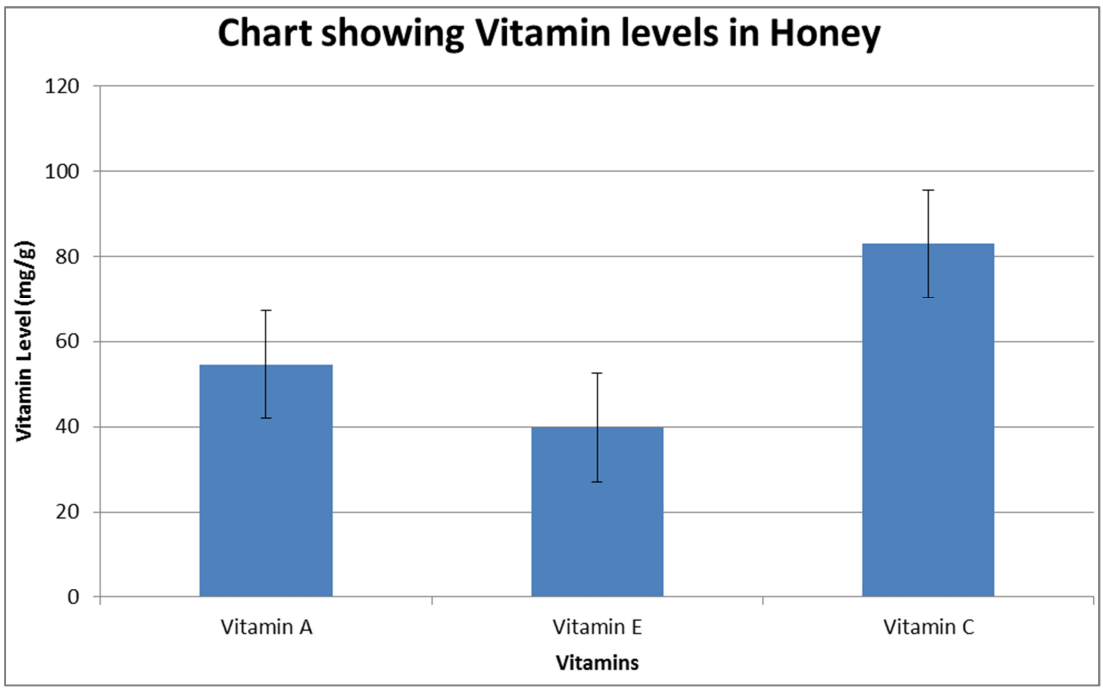

Figure 4. Vitamin contents in honey.

3.4. Antioxidant Assays

3.4.1. Superoxide Dismutase Assay

The % SOD inhibition of mucin and mucin- honey (50:50).

It was observed that the % SOD inhibition of mucin-honey was much higher than the % SOD inhibition of mucin only.

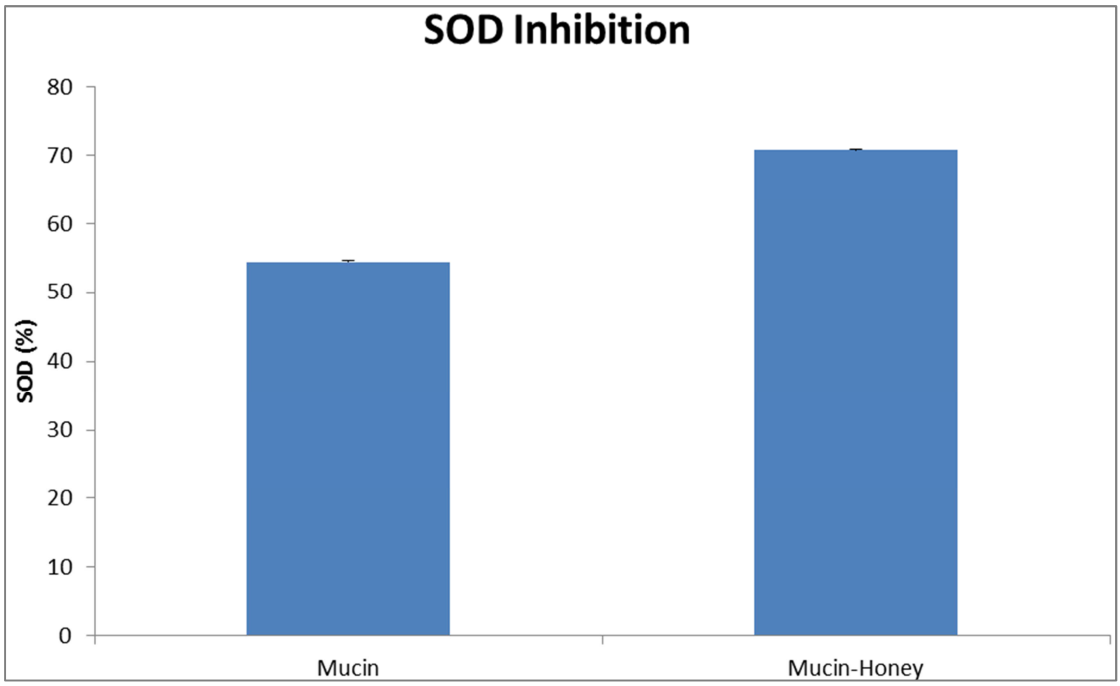

Figure 5. % SOD inhibition of mucin and mucin- honey (50:50).

3.4.2. Catalase Assay

The catalase activity of mucin and mucin- honey (50:50).

It was observed that the catalase activity of mucin-honey was much higher than the catalase activity of mucin only.

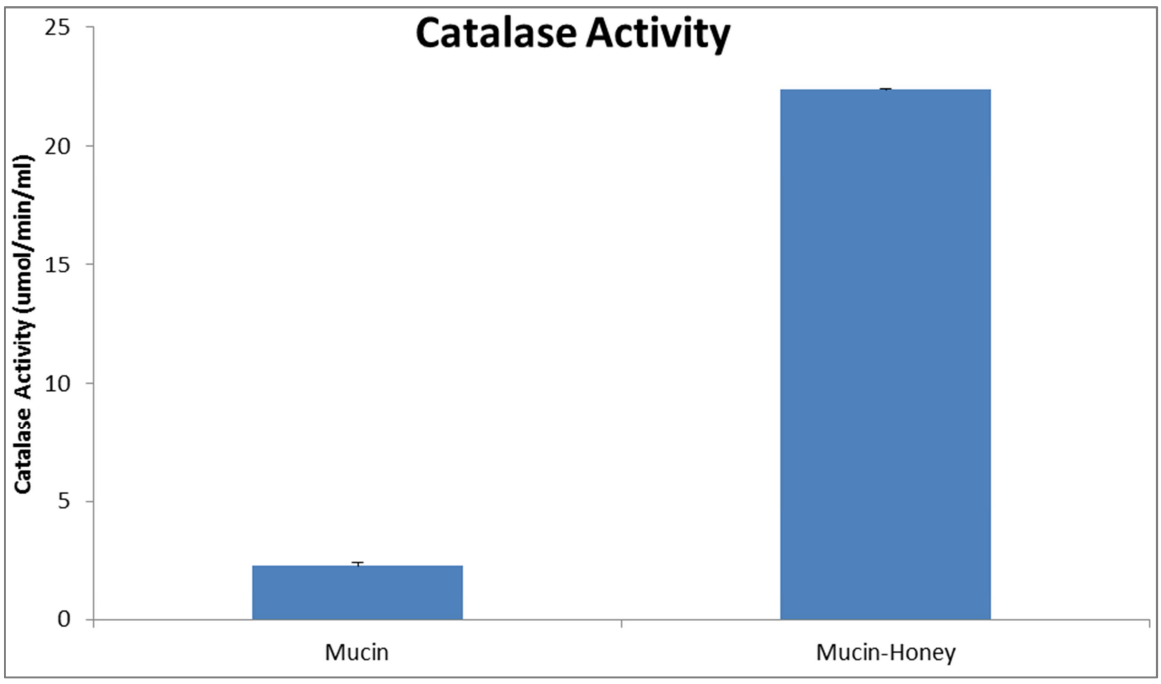

Figure 6. Catalase activity of mucin and mucin- honey (50:50).

3.4.3. Glutathione Peroxidase Assay

Glutathione Peroxidase Activity of mucin and mucin- honey (50:50).

The Glutathione Peroxidase activity of mucin-honey was slightly higher when compared to that of mucin only.

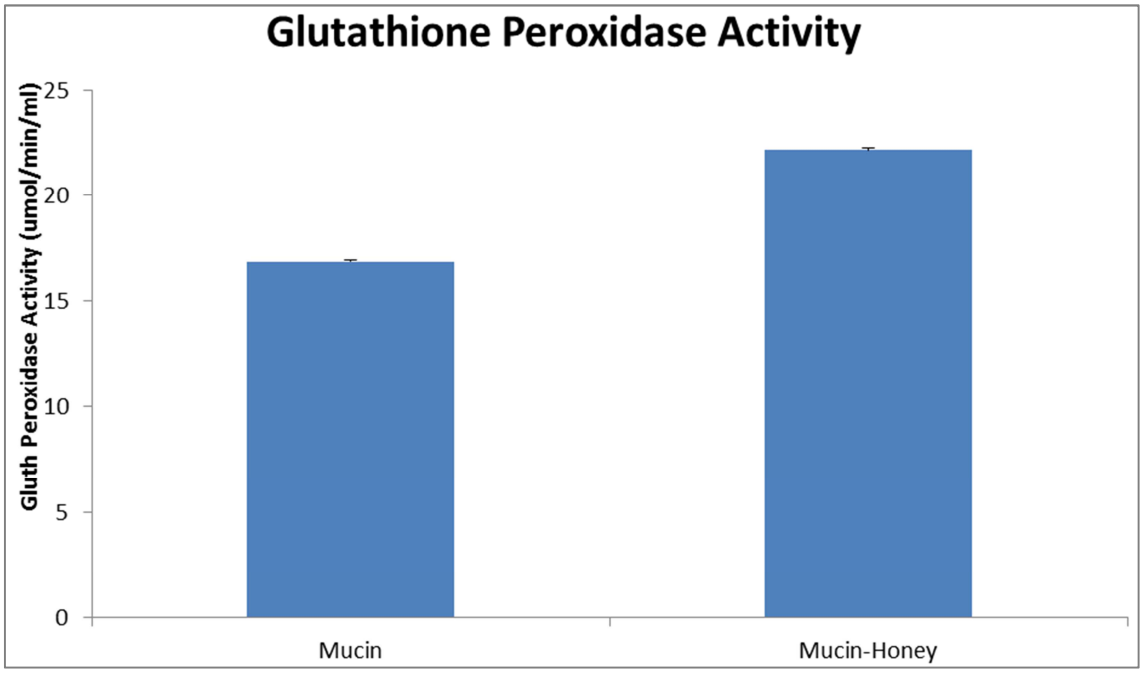

Figure 7. Glutathione Peroxidase Activity of mucin and mucin honey (50:50).

3.5. Microbial Study

3.5.1. Total Bacterial Plate Count

The total bacterial plate count was done using 10^{-1} and 10^{-2} dilutions. It was observed that 10^{-2} dilutions showed better results than 10^{-1}. Total of four pathogenic bacteria namely: *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were used for this study (Table 3).

Table 3. Total Bacterial Plate Count.

Total Microbial Plate Count (CFU/ml)					
S/N	Isolates	Stock	10^{-1} (dilution)	10^{-2} (dilution)	Mean TBC
		Mean	Mean	Mean	
1	*S. aureus*	TNTC	TNTC	253	$2.53x10^6$
2	*E.coli*	TNTC	TNTC	281.3	$2.81x10^6$
3	*P. aeruginosa*	TNTC	TNTC	250.7	$2.51x10^6$
4	*P. mirabilis*	TNTC	287.5	229.3	$2.29x10^6$

Where, TNTC = Too Numerous To Count.
TBC = Total Bacterial Count.

3.5.2. Anti-microbial Susceptibility Screening

Anti-microbial susceptibility screening of mucin and mucin – honey in different concentrations showed that except for the control, mucin and honey in the ratio of 50:50 had the greatest antimicrobial activities against *S. aureus* and *E.coli* while mucin-honey in the ratio of 70:30 showed the greatest antimicrobial activity against *E.coli* even when compared against the control. Mucin-honey (30:70) showed the greatest antimicrobial activity against *P. mirabilis* even when compared with the control. Mucin only had the susceptibility against all the wound organisms tested.

Table 4. Anti-microbial Susceptibility Screening.

Zones of inhibition (mm)					
Isolates	Mucin	M-H (50:50)	M-H (70:30)	M-H (30:70)	+ve Control
E coli	Nil	22.82±0.09	31.88±0.09	Nil	20.02±0.01
S. aureus	8.00±0.02 28.09±0.07	Nil	21.26±0.04	51.07±0.09	
P. aeruginosa	5.97±0.15	17.35±0.04	11.73±0.15	13.04±0.03	25.63±0.07
P. mirabilis	6.67±0.04	12.88±0.09	Nil	33.09±0.08	20.19±0.10

Data are means of triplicate determination ± SD. Positive control is Amoxicillin (0.5%). The negative control used was sterile water which showed no inhibition.

3.6. Animal Study/Wound Healing Analysis

3.6.1. Percentage Wound Healing

Percentage wound healing of the different groups during the entire duration of the study are as shown in table 5.

Group B (Neomycin), Group D (M-H 50:50) and Group E (M-H70:30) exhibited above 50% wound healing by first week of wound induction while the control group, (group A) showed 22.43% level of wound healing.

By the second week of the study, Groups B, D, E and F showed above 90% level of wound healing while group A exhibited only 56.27% level of wound healing. Mucin only, (group C), exhibited 75.85% level of wound healing.

By the third week of the study, all the treatment groups except mucin only (group C), exhibited 100% level of wound healing while group A showed 97.34% level of wound healing.

Table 5. Percentage (%) Wound healing per week.

Period	Group A	Group B	Group C	Group D	GroupE	Group F
(Week)	(No Treatment)	(Neomycin)	(Mucin)	(CF1)	(CF2)	(CF3)
1st	22.43	54.41	41.51	60.69	72.08	53.13
2nd	56.27	90.44	75.85	93.90	96.47	92.58
3rd	97.34	100.00	98.87	100.00	100.00	100.00

3.6.2. Wound Areas

It was observed that the wound areas for both the control and treatment groups got smaller as the study progressed from the first day to the twenty-first day of the study (figure 6). By the end of the study, all the treatment groups showed no wound areas compared to the control that showed very little wound area.

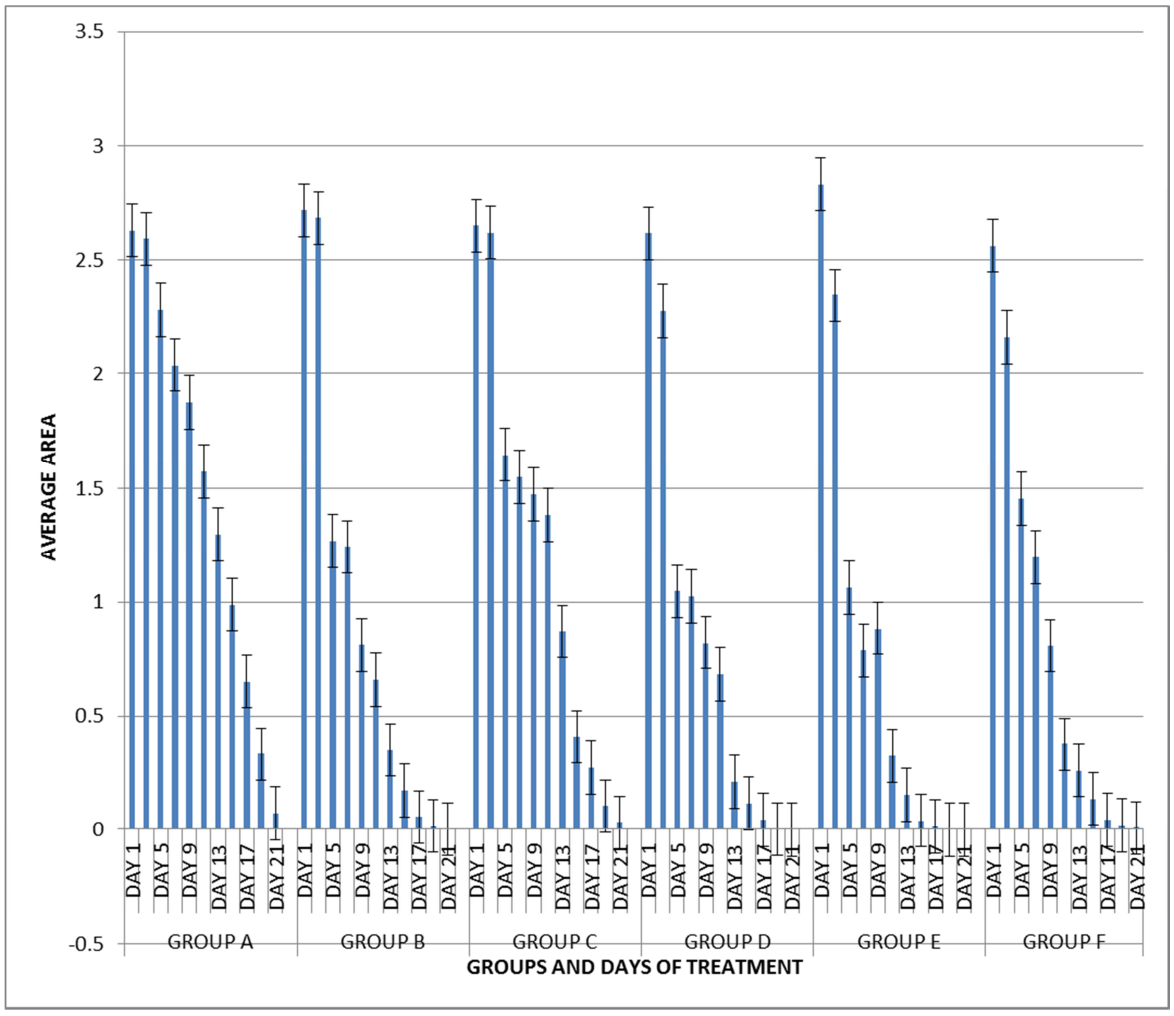

Figure 8. Average areas (cm^2) of wounds of the control and treatment groups.

4. Discussion

Results of physico-chemical properties of sample (honey) showed that the mean values of pH, Viscosity and hydrogen peroxide were 4.46±0.1, 4.89±0.1pas/sec and 1.2*0±0.1 % respectively, Table* 1 which is in consonance with the values obtained for honey by e [26]. Honey is mildly acidic with a pH of 3.2 and 4.5. [26, 27]. The low pH alone is inhibitory to many pathogenic bacteria, [26, 27]. This relatively acidic pH level inhibits the growth of many bacteria, [10, 28].

The Glucose oxidase level of honey was found *to be 4301±0.1mg/dl in Table* 2 which is high. Ghaderi and Afshar, [27] reported in their work that honey contains Glucose oxidase which converts glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide, an antibacterial agent in honey, is generated by glucose oxidase, an enzyme that bees add to the collected nectar stored in honeycombs. The enzyme is inactive under the low level of free water present in honey, but becomes active if the honey becomes diluted, as with wound exudate, [29]. Collagen film dressings that contained glucose oxidase were found to promote wound healing in a rat diabetic model apparently by increasing levels of reactive oxygen species (ROS) in the wounds. Medicinal grade honey, which has been claimed to promote healing of chronic wounds [30] has also been shown to contain H_2O_2, possibly again by the action of glucose oxidase [31] On the other hand, excessive ROS have been thought to be involved in the pathogenesis of chronic wounds [32]. ROS can cause damage by reacting with nucleic acids, protein and lipids, inducing a loss of function and tissue damage. In most of the world's honeys, the antibacterial activity beyond that which is due to the osmolarity a3ivated by dilution with body fluids. When honey is used, hydrogen peroxide of concentration as low as 1 mmol/l will slowly be released and acts as an antiseptic. Various groups have shown that H_2O_2 plays an important role in wound healing. Non-phagocytes have been shown to produce H_2O_2 after wounding which can attract neutrophils [33] as well as promote reinnervation of the peripheral sensory axons in a zebrafish model of wound healing.

As ROS such as H_2O_2 are inherently damaging, perhaps, low concentrations of H_2O_2 will promote healing by acting as a

signaling molecule while high concentrations would delay healing by causing oxidative damage.

The results of mineral analysis showed the presence of copper, potassium, selenium and zinc in the samples, (honey, snail mucin and mucin-honey in figure 1). This to some extent agrees with the study by [34] who stated that minerals may be present in honey with Potassium being the highest while [35] reported the presence of Calcium, Phosphorous, Potassium, Magnesium, Sodium, Iron, Zinc and Manganese in snail mucin.

There was statistically significant difference ($P < 0.05$) in the mean Copper contents of the samples. Copper content of mucin was found to be the highest while that of honey was the least. Copper is a required co-factor for cytochrome oxidase, for cytosolic anti-oxidant superoxide dismutase, and for the optimal cross-linking of collagen, [36, 37, 38]. Cross linking of collagen is an important part of wound repair. Copper is vital for maintaining the strength of the skin, blood vessels, and epithelial and connective tissue throughout the body. A balance between copper and zinc is important since both minerals are absorbed in the small intestine.

There was significant difference ($P < 0.05$) in the Potassium content of the samples. Potassium content of honey was found to be the highest while that of mucin was the least.

There was significant difference ($P < 0.05$) in the Selenium content of the samples. Selenium has antioxidant property which is necessary for wound healing. Selenium content of mucin was found to be the highest while that of mucin-honey (50:50) was the least.

There was no significant difference ($P > 0.05$) in the Zinc content of the samples. Zinc is a co-factor for both RNA and DNA polymerase, and a zinc deficiency causes a significant impairment in wound healing [39]. The vitamin analysis of mucin and honey showed presence of Vitamin A, C and E. The vitamins A, E and C are markedly higher in honey than in mucin (figure 2). This agrees with the study conducted by [40, 41] that honey contains vitamins (and phenolic compounds) in trace quantities. Some of the phenolic compounds in honey responsible for antioxidant effect are flavonols, flavones, flavonones, benzoic and cinnamic acids.

Vitamin A (retinol) shows potent anti-oxidant and anti-inflammatory effects. Vitamin A deficiency leads to impaired wound healing. The biological properties of vitamin A include anti-oxidant activity, increased fibroblast proliferation, modulation of cellular differentiation and proliferation, increased collagen and hyaluronate synthesis, and decreased MMP-mediated extracellular matrix degradation [41].

Vitamin C has many roles in wound healing, and a deficiency in this vitamin has multiple effects on tissue repair. Vitamin C deficiencies result in impaired healing, and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, and increased capillary fragility. Also, vitamin C deficiency leads to an impaired immune response and increased susceptibility to wound infection [38].

Vitamin E, an anti-oxidant, maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. Vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds. Animal experiments have indicated that vitamin E supplementation is beneficial to wound healing [41] and topical vitamin E has been widely promoted as an anti-scarring agent. However, clinical studi es have not yet proved a role for topical vitamin E treatment in improving healing outcomes [42].

The antioxidant assays showed the presence of catalase, SOD and Glutathione peroxidase in (figures 5, 6, 7). There was significant increase ($P < 0.05$) in % SOD inhibition by mucin-honey formulation compared to mucin only (Figure 5). This also might be due to honey's antioxidant activity. SOD inhibition is a measure of the antioxidant activity of a sample. These results suggest that mucin-honey formulation has more antioxidant activity than mucin only. It has been demonstrated that application of antioxidants to wounds decreases inflammation and the main mechanism of action of honey in improving the healing of burns has been found to be through its antioxidant activity [43].

There was a significant increase ($P < 0.05$) in the catalase activity of mucin- honey formulation compared to mucin only (Figure 6). This might be as a result of antioxidant capacity of honey as contributed to the formulation as indicated in the works of [44]. Honey has been found to contain significant antioxidant activity attributed to glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids, proteins, [45, 44]. The antioxidant activity of honey probably also contributes to its anti-inflammatory properties because ROS act as messengers to give feedback amplification of the inflammatory response [46] and this process can be blocked by phenolic antioxidants [47]

There was statistically significant increase ($P < 0.05$) in the Glutathione Peroxidase activity of mucin-honey formulation compared to mucin only (Figure 7). This also suggests greater antioxidant activity of mucin-honey formulation compared to mucin only as a result of greater antioxidant activity of honey Another benefit of the anti-inflammatory

action of honey is that it decreases oedema, thus decreasing the pressure on the microvasculature of wound tissue that otherwise restricts the availability of oxygen and nutrients required for growth of tissue for wound repair [48].

The samples showed various degrees of antimicrobial effects against tested wound organisms, namely; E. coli, P. aeruginosa, P. mirabilis and S. aureus (Table 4).

There was statistically significant ($P < 0.05$) increase in P. mirabilis susceptibility in mucin-honey formulation (30:70), compared to the control drug used (Table 4). While, there was statistically significant increase in S. aureus susceptibity in control drug when compared to mucin only and mucin-honey combination. There was statistically significant increase ($P < 0.05$) in P. aeruginosa susceptibility to the control drug compared to mucin only and mucin-honey formulation. There were statistically significant increases ($P < 0.05$) in E. coli susceptibility to mucin-honey formulation (70:30) and mucin-honey formulation (50:50) compared to the control drug. This agrees with the study conducted by [49] that using a medical-grade honey, wound pathogens, including those with high levels of innate or acquired antibiotic resistance, were killed by 4.0–14.8% honey, which is a concentration that can be maintained in the wound environment. Resistance to honey could not be induced under conditions that rapidly induced resistance to antibiotics [49]. Honey inhibits a broad spectrum of bacterial species. The alcohol extracts of honey exhibit an inhibitory effect to array of bacterial species including aerobes and anaerobes, Gram positives, and Gram negatives [50]. Honey has powerful antimicrobial effects against pathogenic and non- pathogenic micro-organisms (yeasts and fungi), even against those that developed resistance to many antibiotics. The antimicrobial effects could be bacteriostatic or bactericidal depending on the concentration that is used [51] as reported by [52].

Sugar draws water out of bacterial cells and, as long as the sugar does not become too diluted by the wound fluid, the growth of bacteria is inhibited [52]. The additional bioactivity within the honey itself allows for continued inhibition of bacterial growth even when the osmolarity has been diluted below the point where it should cease to be inhibitory. Snail mucin has been reported to have antimicrobial activity. [6] have suggested that due to its surfactant activity it prevents bacteria attaching to host cells.

From the ninth day to the nineteenth day of the study, there were statistical significant reductions in the wound areas of the all the treatment groups including the positive control compared to negative control (group A). From the ninth day of the study, there were significant reductions in the wound areas of M-H 50:50, M-H 70:30 and M-H 30:70 formulations compared to mucin only and negative control (group A). Mucin only showed non-significant reduction in wound area compared to negative control (group A) on the ninth day. Also, group B showed significant reduction in wound area compared to negative control (group A).

Although mucin only showed greater capacity for wound healing compared to negative control, results showed that M-H formulations especially M-H 70:30 (group E) had better wound healing capacity compared to mucin only, and even the positive control. In a study by Adikwu, it was reported that snail mucin from the giant African snail, Archacatina marginata, (Family Arionidae) has wound healing effect. The extract (mucin) remarkably increased the wound healing capacity of CicatrinR powder, [6]. The antioxidant activity of honey probably also contributes to its anti-inflammatory properties because ROS act as messengers to give feedback amplification of the inflammatory response [46] and this process can be blocked by phenolic antioxidants [49]. It has been demonstrated that application of antioxidants to wounds decreases inflammation and the main mechanism of action of honey in improving the healing of burns has been found to be through its antioxidant activity [47].

It is possible that the presence of the minerals and vitamins analyzed in this study together with the antioxidant enzymes which are present in honey and mucin might have contributed to the greater wound healing capacity of the formulation.

5. Conclusion

The results of this study had shown that mucin, and mucin-honey formulations have wound healing properties, but mucin-honey formulation showed better wound healing property than mucin only. This might be as a result of their content of antioxidant enzymes, (SOD, Catalase and Glutathione peroxidase), minerals (Cu, K, Se and Zn), and vitamins (A, C and E). The antimicrobial properties of the samples also must have contributed to the wound healing properties of the samples.

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