Trop J Nat Prod Res, January 2022; 6(1):109-116 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Evaluation of Honey-Based Pharmaceutical Preparations for the Management of Diabetic Wounds

*Bisola O. Ojo, Nkechi V. Enwuru, Udoma E. Mendie

Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Lagos, Nigeria

ARTICLE INFO

Article history:
Received 20 October 2021
Revised 20 January 2022
Accepted 28 January 2022
Published online 03 February 2022

ABSTRACT

Management of Diabetic wounds is a financial burden globally. Investigations into cheaper and alternative dressings to manage these wounds especially in developing countries like Nigeria is crucial. Honey was used to treat wounds since ancient times till nowadays. This present study aimed at preparing honey with other actives and assay its antimicrobial properties and wound healing activity; in-vitro and in-vivo, also to compare its effectiveness against sugar syrup. Raw honey obtained was processed and characterized. Eight formulations F1 to F4 containing 66.7% syrup and either chlorhexidine or aloe-vera or both and F5 to F8 containing 75% Honey with or without the other actives were prepared, characterized, and investigated on their antimicrobial and wound healing properties in a diabetic rat model. The honey sample was found to be of good quality from the physicochemical and microscopical analysis carried out. Formulation F8 containing Honey 75%, Aloe vera 1%, and chlorhexidine 0.02% showed the highest antimicrobial activity with none of the formulations showing any significant activity against the fungal isolate. The wound healing effects of the formulations were comparative but honey-containing formulations showed better diabetic wound healing activity with formulation F8 giving total and complete healing of the diabetic wound. Honey in conjunction with Aloe vera and chlorhexidine has better antibacterial and wound healing activity than syrup, according to this study, and could be utilized as a safe and effective natural diabetic wound healing treatment.

Keywords: Aloe vera, Antimicrobial activity, Chlorhexidine, Diabetic wounds, Honey, Syrup.

Introduction

Chronic wounds are a predominant health care challenge all around the planet.[1] Disruptions in any part of the process of wound healing result in chronic wounds. Diabetic wounds are lesions of the skin in diabetic patients which develop usually on the lower limbs (particularly the foot). A large number of Diabetic chronic wounds degenerate eventually to gangrenes due to various factors, leading to amputation of the affected limbs.[2] It is reported from multicenter research of the six major tertiary hospitals, that Nigeria which is the most populous country in Africa has the greatest burden of diabetes and diabetic wounds.[3] Diabetic wounds occurring with other complications of diabetes were reported to be the highest cause of death in diabetic patients.[4] Several approaches have been developed and adapted in the treatment of diabetic wounds, their goal is to create a moist environment that promotes granulation, autolytic processes, angiogenesis, and more rapid migration of epidermal cells across the wound base.[5]

A myriad of topical agents is available incorporating several antiseptic products; Silver, Iodine, Chlorine, Biguanide compounds, and Hydrogen peroxide, there are also dressings incorporating several natural products; Honey, Essential oils, and natural extracts.[6,7] A combination of these agents, which are cheaper, naturally sourced, and efficacious could promote faster diabetic wound healing and eventually reduce the economic, clinical, and humanistic outcomes of wound care.

*Corresponding author. E mail: boladapo@unilag.edu.ng
Tel: +2349091178874

Citation: Ojo BO, Enwuru NV, Mendie UE. Evaluation of Honey-Based Pharmaceutical Preparations for the Management of Diabetic Wounds. Trop J Nat Prod Res. 2022; 6(1):109-116. doi.org/10.26538/tjnpr/v6i1.18

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Honey is a well-known antimicrobial agent whose antioxidants fight against infections and reduce Reactive Oxygen Species (ROS) and inflammations in wounds, it has broad-spectrum antimicrobial activity against wound pathogens which is attributed to its high acidity, osmotic effects, hydrogen peroxide, and nitric oxide. Honey also facilitates autolytic wound debridement mediated by protease enzymes and osmotic activity.[8]

Honey-based hydrogel dressing was found to have the highest antimicrobial and burn wound healing [9], Honey as a wound dressing was also found to improve wound healing and significantly reduce the rate of amputation in diabetic wounds.[10]

Aloe vera has been used for its moisture-retaining, wound healing, and antiseptic properties to treat burn wounds, post-operative wounds, cracked nipples, psoriasis, and chronic wounds.[11]

A study found that the presence of Aloe vera gel (AVG) in Hydrogel dressings significantly improved the water absorption and the in-vitro degradation rate of films for chronic wounds, this is favorable since a moist environment is required for wound healing[12] and some authors compared AVG with Nigella sativa oil (NSO) in induced Diabetic ulcer wounds and found that AVG reduced inflammation and improved re-epithelization better than NSO.[13]

Chlorhexidine which has been in use since the 18th century is a bis-biguanide, available as the acetate (diacetate), hydrochloride, and gluconate salts, it has broad antibacterial activity, low mammalian toxicity, and a strong affinity for binding to skin and mucous membranes, it is currently commercially available as an antimicrobial tulle-gras dressing (TGD) for wound dressing and as an Irrigation solution Irrisept®.[14–16]

Chlorhexidine, serving as an antimicrobial to treat and prevent infection at different concentrations and as an anti-protease agent, reducing the activity of matrix metalloproteinase and bacterial proteases in chronic wounds.[17] AVG extract, a natural product with several constituents that promote wound healing, and Honey with multiple roles as antimicrobial, mild debrider, deodorizer, and antioxidant activity. Although at concentrations ≥0.04% chlorhexidine was found to be cytotoxic to mucous membranes inducing an

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

inflammatory reaction and retards the granulation of tissue formation, inhibits cell growth, proliferation, and collagen synthesis[18], honey and *Aloe vera* have counter effects and using these three ingredients together will provide a synergistic environment to promote maximum wound healing in the complex environment of chronic wounds.

Combining these three ingredients will provide a synergistic environment to promote maximum wound healing in the complex environment of chronic wounds.

This study intends to develop a novel chlorhexidine dressing gel that is formulated with Aloe vera extract and honey. This dressing should provide faster healing of diabetic chronic wounds, reduce hospital stay, and improve quality of life due to the double antibacterial activity of chlorhexidine and honey, as well as the wound healing properties of Aloe vera.

Materials and Methods

Sources of Raw materials

The honey was obtained from Ibadan, Oyo State, Nigeria in January 2020, and identified in the Pharmacognosy department of the Faculty of Pharmacy, University of Lagos. Aloe vera powder (LTLH121104, Rioto Botanical, China). Chlorhexidine gluconate (237854443, Prachi Pharma Pvt Limited, India) Soybean-Casein Digest Medium/Tryptone soy broth and Agar, Saboraud dextrose agar, Mannitol Salt Agar, Cetrimide agar, and Mueller Hinton agar were purchased from Sigma Aldrich. All the chemicals used in this study were of analytical grade.

Purification of Raw Honey

Honey was preheated to 40°C, strained using nylon cloth to remove solid particles, filtered /clarified using sintered glass funnel, and indirectly heated at 65°C for 30 minutes in the oven followed by rapid cooling to protect its natural color, flavor, enzyme content, and other biological substances. The purification and physicochemical characterization were carried out using the method from Azonwade *et al*.[19]

Microbiological characterization of honey

Microbial limits test was carried out based on the methods in the United States Pharmacopoiea (USP).[21]

Total Aerobic Microbial Count (TAMC)

A pour plate method was used to count the colonies of microbial growth, 10g each of honey sample was suspended and made up to 100mL using phosphate buffer (pH 7.2) as a diluent to make a 1:10 dilution. 1:100 dilution was also prepared by taking 1mL from the previous dilution and making it up to 10mL. 1mL of each dilution was pipetted onto each of two sterile Petri dishes, 19mLs of Tryptone Soybean Agar (TSA) medium that was prepared, autoclaved, and cooled was added. The Petri dishes were covered and the sample was mixed with the agar by rotating the dishes and allowed to solidify at room temperature. They were incubated at 37^{o}C for 72hrs. The plates were examined every 24hrs and the number of colonies of microorganisms per gram was obtained.

Total Combined Yeast and Mold Count (TCYMC)

After dilution as above 1mL of each dilution was pipetted onto each of two sterile Petri dishes, 19mL of Saboraud Dextrose Agar (SDA) medium that was prepared, autoclaved, and cooled was added. The Petri dishes were covered and the sample was mixed with the agar by rotating the dishes and allowed to solidify at room temperature. They were inverted and incubated at 20-25^{o}C for 5 days. The plates were examined and the number of colonies of microorganisms per gram was obtained.

Specific Test for Staphylococcus aureus

The dilution (1mL each) was pipetted onto each of two sterile Petri-dishes, 19mL of Mannitol Salt Agar (MSA) medium that was prepared, autoclaved, and cooled was added. The Petri dishes were covered and the sample was mixed with the agar by rotating the dishes and allowed to solidify at room temperature. They were incubated at 37^{o}C for 24hrs. The plates were examined and the number of colonies of microorganisms per gram was obtained.

Specific Test for Pseudomonas aeruginosa

The dilution (1mL each) was pipetted onto each of two sterile Petri-dishes, 19mL of Cetrimide Agar medium that was prepared and autoclaved at 45^{o}C was added. The Petri dishes were covered and the sample was mixed with the agar by rotating the dishes and allowed to solidify at room temperature. They were incubated at 37^{o}C for 24hrs. The plates were examined and the number of colonies of microorganisms per gram was obtained.

Preparation of syrup and honey-based formulations with Aloe vera and chlorhexidine.

Preparation of Sucrose syrup.

British Pharmaceutical codex (BPC) 1973

Sucrose	667 g
Purified water	to 1000 g

Heat together until dissolved and add sufficient boiling purified water to produce 1000 g. A suitable preservative or mixtures of preservatives may be added.

Preparation of syrup- and honey-based products

The formulations were prepared using the formula in Table 1. Honey was used at 75% based on previous work carried out where maximum activity was found at that concentration[9]. Honey, chlorhexidine, syrup, and Aloe vera powder with different concentrations were added to the previous mixture with uninterrupted stirring till they dispersed in the hydrogel. The necessary amount of polyethylene glycol and preservatives methyl and propylparaben are added. The final weight was completed to 100mL with the aqueous solution. The final formulations were packed in wide-mouth glass containers covered with a screw plastic lid and placed in the refrigerator to complete the formation of hydrogel.[9]

Table 1: Composition of Honey and syrup hydrogel formulae (%w/w)

Code	Honey (w/w%)	Syrup (%w/w)	[a]CHXg (%v/v)	[b]AVP (%w/v)	[c]PG	[d]MP	[e]PP	Water to (mL)
F1	-	66.7	-	-	5	0.03	0.02	100
F2	-	66.7	0.02	-	5	0.03	0.02	100
F3	-	66.7	-	1.0	5	0.03	0.02	100
F4	-	66.7	0.02	1.0	5	0.03	0.02	100
F5	75	-	-	-	-	0.03	0.02	100
F6	75	-	0.02	-	-	0.03	0.02	100
F7	75	-	-	1.0	-	0.03	0.02	100
F8	75	-	0.02	1.0	-	0.03	0.02	100

a-chlorhexidine gluconate, b-Aloe vera powder, c-polyethylene glycol, d-methylparaben, e-propylparaben.

Physicochemical Evaluation of Formulations
Visual examination; The prepared honey formulations were examined for their color, consistency, homogeneity, and existence of lumps by visual check after they were set in the container.

pH determination; One g of each formulation was weighed and mixed with 25 mL of purified water. The pH of the mixture was measured using a pH meter (Orion Research, Inc., USA), which was calibrated before each use with buffer solutions 4.0 and 7.0. Experiments were carried out in triplicates and average values were calculated.[9]

Viscosity measurement; A Brookfield viscometer DV-E (Brookfield Engineering Laboratories, Middleboro, MA) was used with a concentric cylinder spindle #29 to determine the viscosity of the different topical formulations. The tests were carried out at 30.8°C. The spindle was rotated at 20 rpm values. All measurements were made in triplicate.[22]

In vitro antimicrobial examination of honey and syrup formulations
Test organism- Clinical microbial isolates of Gram-negative (*Pseudomonas aeruginosa, Escherichia coli*), Gram-positive (*Staphylococcus aureus, Proteus* spp.), and Yeast (*Candida albicans*) organisms usually obtained from chronic wounds. A series of morphological, physiological, and conventional biochemical tests were performed to identify the selected microorganisms. The fungi were identified following growth on appropriate media and morphological and microscopic characteristics.

Antimicrobial Evaluation
Antimicrobial assay of the formulations was performed by agar well diffusion method in Mueller Hinton Agar (MHA) plates. The test organisms were inoculated in Nutrient broth and incubated overnight at 37°C to adjust the turbidity to 0.5 McFarland. MHA plate was lawn cultured with standardized microbial culture broth. Four wells of 12 mm were bored in the inoculated media with the help of a sterile cork-borer (12 mm). Each well was filled with 50 µL of different formulations. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37°C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm.[23]

In vivo wound healing study
Animals and Experimental Design: Sixty rats of either sex aged 16 weeks old weighing 150 - 200 g purchased from Joss Rattery® breeds Ibadan Oyo State were used in this study. The animals were kept in polycarbonate cages housed in well-aerated rooms, maintained under controlled temperature (30 ± 2 °C), relative humidity (45 ± 10%), and a 12 h light and dark cycle. They had access to a standard rodent chow diet and clean drinking water ad libitum. This study followed the National Institutes of Health guide for the care and use of laboratory animals (National Institute of Health, 2011). All the experiments accorded with the Institution Guidelines and were approved by the College of Medicine University of Lagos Health Research Ethical Committee CMUL/HREC with HREC Number CMUL/ACUREC/06/21/861.

Induction of Diabetes
Animals were acclimated for 1 week before diabetes induction. Diabetes was induced by injecting alloxan monohydrate intraperitoneally (150 mg/kg body weight) at 48h intervals in rats fasted overnight. To monitor the blood glucose levels, the blood sample was obtained by tail clipping method, and glucose levels were checked using pre-calibrated Accu-Chek Active® glucometer with blood glucose levels of 250 mg/dL or higher were considered diabetic.[13]
The average random blood glucose level of normal rats was found to be 156 mg/dL whereas 48 h after alloxan administration the random blood glucose levels were 418.3 mg/dL. Such animals were considered severely diabetic and selected for wound healing studies.

Diabetic rats were taken and distributed in ten different groups with each group containing four diabetic rats (n = 4). The distribution of the diabetic rats was done via simple randomization. All the diabetic rats in all experimental groups were exposed to the same environmental conditions. The rats had a mean body weight of 155.51 ± 2.1 g at the start of the experiment.
Wounds incised on the rats' dorsal surface with a diameter of 10mm were covered with the varying formulations F1, F2, F3, F4, F5, F6, F7, F8, the animals placed in group 9 were diabetes-induced but not treated with formulations, while those in group 10 were not diabetes-induced and not treated, each wound was then covered with sterile gauze. Using a sterile cotton swab, all treatments were applied topically on each mouse once daily for fourteen days. Excision wound margin was measured with a Vernier caliper. Wound contraction was measured in each 2 days interval, until complete wound healing and expressed in percentage of healed wound area. The evaluated surface area was then employed to calculate the percentage of wound contraction; the initial size of the wound is 10 mm^2.

$$\%\ Wound\ Contraction = \frac{Initial\ wound\ size - Specific\ day\ wound\ size}{Initial\ wound\ size} \times \frac{100}{1}$$

Histopathology
The animals were euthanized on day 14, and the injured area was sampled by trimming to include the dermis and hypodermis. The trimmed skin layers were fixed in 10% Neutral Buffered Formalin (NBF). Representative sections were then stained with hematoxylin and eosin (H&E) for histology.[13]

Statistical analysis
The data were presented as mean ± standard deviation of three experimental values for every variable and analyzed by one-way ANOVA, p-value ≤ 0.05 was considered statistically significant.

Results and Discussion

Chronic wounds management is approached with the basic tenets, acronym TIME,[24] honey and syrup provide a moist environment for wound healing, their effectiveness in managing infection and improving wound contraction was investigated with regards to diabetic wounds. For honey to be used in wound healing its quality has to be ascertained and it needs to be processed and purified.

Organoleptic characteristics of raw honey
The honey sample used in this study was a golden-brown color which is considered more nutritious as reported by.[25] More so, it has a sweet taste and sweet aroma which is a characteristic of honey obtained from honeydew bees.[26]

Microscopy of honey
The Honey sample contains several pollen grains from different plant species as shown in Figure 1, these pollens are similar to those found by three different studies on honey gotten from that region (Oyo State, southwestern, Nigeria).[27–29] The pollen grains observed were also checked against the Polen 23 database to obtain the possible pollen grains present in the sample. The microscopy carried out shows to a certain degree that the honey was indeed obtained from the southwestern region and is of good quality relating to the number of pollen grains observed.

Microbial limits
The honey sample met the USP's specified standards[21] as shown in Table 3, indicating that the honey sample is suitable for use in the formulation of cutaneous wound healing preparation.

Physicochemical characteristics of honey
The moisture content of honey is an important factor contributing to its stability. Higher moisture content could lead to undesirable honey fermentation during storage caused by the action of osmo-tolerant

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

yeasts and resulting in the formation of ethyl alcohol and carbon dioxide.[30] Moisture content is also an indicator of the maturity of the honey.[31] According to the results in Table 2, the moisture content of the honey sample was 19.50 which falls below the standard limits specified by the official books USP and BP. Honey with pH < 3.5 is susceptible to spoilage and according to the Codex Alimentarius honey within the standard limits pH (3.5 – 5.5) are considered acceptable, at lower values, < 3.5 indicates that the honey sugar has been fermented into organic acids. The pH of the honey used in this study was 4.5 as shown in Table 2 which agrees with the acceptable standards,[31] and showed that the acidic content of honey controls honey spoilage and maintains its flavor

Ash represents the mineral residue of the honey after incineration, it offers the possibility of knowing the overall mineral content of the honey and the value obtained is 1.18g/100g which falls within the permissible limits according to the Codex Alimentarius.[20,26]

Fructose and glucose constitute the primary sugars in all honey samples, and in the honey of good quality, the fructose content should exceed that of glucose as observed from the results obtained in Table 2, 41.58g/100g as compared to 27.73g/100g. The sum of the fructose and glucose content as reducing sugar obtained was 67.47g/100g which falls above the acceptable limits of >60g/100g.[32]

In addition to the sum of fructose and glucose, other important factors that relate to honey quality include the fructose/glucose (F/G) ratio, in this study, the F/G ratio is 1.49, the F/G ratio indicates the ability of honey to crystallize. Even though honey has less glucose than fructose, it is the glucose that crystallizes when honey granulates because it is less soluble in water than fructose. When the fructose/glucose ratio is high, honey remains liquid. Honey crystallization is slower when the fructose/glucose ratio is more than 1.3 and it is faster when the ratio is below 1.0.[32]

Hydroxymethylfurfural (HMF) is a quality indicator that indicates the freshness and purity of honey. A higher HMF value indicates that the honey has been overheated, aged, or stored under poor conditions for too long and the observed HMF value in Table 2 was 10.33mg/kg which falls way below the acceptable limit of 80mg/kg.

All the values obtained for the honey sample indicate that the honey is of good quality, is fresh, has not undergone fermentation and crystallization, and was stored under good conditions after harvesting.

Physicochemical properties of prepared formulations

Formulations F1 and F2 prepared with syrup appear colorless while F3 and F4 appear light brown due to the Aloe vera extract present in the samples while F5 to F8 all have the characteristic color of Honey. All samples are sticky because of the high sugar content and they all appear homogenous as indicated in Table 4.

Healthy, intact skin has a slightly acidic pH ranging from 4.0 to 6.0. This is an important aspect of the skin's barrier function since it regulates bacterial flora and prevents infection. When a wound occurs, the skin's acidic milieu and pH are disrupted, exposing the more neutral pH (7.4) of the underlying tissue. The pH of chronic wounds is in the alkaline range from 7.15 to 8.63 and also most bacterial organisms grow best at pH values of 6.5 to 7.0. The pH values of formulations F1 to F4 shown in Table 4 are in the ranges of 6.23 to 6.42 close to neutral while F5 to F8 from 5.61 to 5.81 slightly acidic and within the pH range of the skin, which indicates that the honey formulations will help to limit bacterial infection to promote wound healing. An acidic pH environment is considered to be beneficial, by increasing fibroblast proliferation and migration and also regulating bacterial colonization.[33]

In-vitro antimicrobial activity of prepared formulations

The antibacterial activity of the eight prepared formulations (F1, F2, F3, F4, F5, F6, F7, and F8) was tested against the most common chronic wound microorganisms; *Pseudomonas aeruginosa*, *Staphylococcus aureus* (American Type Culture Collection) ATCC25923, *Escherichia coli* ATCC25922, *Proteus* spp., and *Candida albicans* using the agar wells diffusion method in triplicates. Controls used in this experiment were all the active ingredients in their concentrations Honey (75%), Chlorhexidine (0.02%), Aloe vera (1%), and Syrup (66.7%) (H, C, A, S).

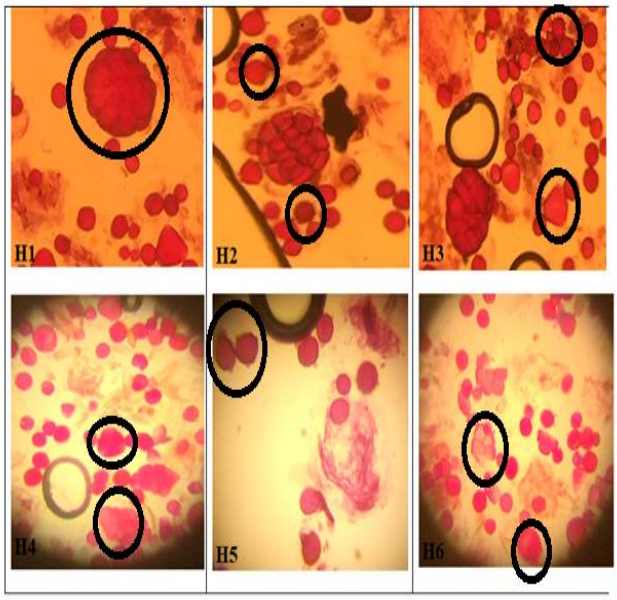

Figure 1: Microscopy of the honey sample showing the different pollen grains

Table 2: Physicochemical characteristics of Honey sample

Test	Standards (Bp, Usp, Codex Alimentarius)	Mean Results
Moisture content	≤ 20% (BP, USP)	17.63 ± 0.23
Refractive index	≥1.487 (BP, USP)	1.49 ± 0.01
Ph	3.5-6.1 (Codex Alimentarius)	4.50 ± 0.18
Viscosity	At 30.8°C and 20rpm (mPas) No fixed limit	8596 ± 5.51
Ash	Not more than 1.2g/100g (Codex Alimentarius)	1.18 ± 0.04
Total sugars	No less than 60g/100g (sum of fructose and glucose) [26]	73.35 ± 1.04
Reducing sugars	No less than 60g/ 100g (sum of fructose and glucose) [26]	67.47 ± 3.42
Fructose	No fixed limit	41.58 ± 0.88
Glucose	No fixed limit	27.73 ± 0.17
Fructose/Glucose ratio	No fixed limit	1.49 ± 0.03
Hydroxymethyl furfural	≤80mg/kg (USP, BP)	10.33 ± 0.30

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

This is to compare the antimicrobial activity of the formulations against their actives and to also know which active is responsible for the antimicrobial activity. Honey and chlorhexidine, of all the controls, exhibited any antimicrobial activity with honey being only effective against *Staphylococcus aureus* and *Escherichia coli*, while chlorhexidine showed no activity against the fungal isolate. Although, honey has been shown to exhibit antibacterial activity against *Pseudomonas aeruginosa* and Gram-negative organisms[34,35] the honey used in this investigation had no effect against the strain used hence the activity of the formulations on the microorganisms are mostly due to the chlorhexidine used. Aloe vera and Syrup showed no antimicrobial activity, this could be due to the major activity of Aloe vera being primarily wound healing. According to the results (Table 5), formulation F1 with syrup as an active ingredient exhibited no antimicrobial activity against the bacterial isolates and fungal isolates.

Table 3: Microbial limits for honey sample

Sample	**Mean plate count ([a]cfu/mL)**		**Absence of organism in 1mL**	
	[b]TAMC	[c]TCYMC	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
Honey	5.5×10^1	1×10^1	0	0
USP Standard	10^2	10^1	0	0

a=colony forming units, b=Total Aerobic Microbial Count, c=Total Combined Yeast, and Mold Count

Table 4: Physicochemical properties of prepared formulations

Formulations	**Parameters**				
	General appearances	**Homogeneity**	**Texture**	**Ph**	**Viscosity at 30.8°C (mPas)**
F1	Colorless			6.23 ± 0.12	104.51 ± 0.49
F2	Colorless			6.43 ± 0.08	100.20 ± 0.46
F3	Light brown	Homogenous	Sticky	6.23 ± 0.04	125.11 ± 0.16
F4	Light brown			6.25 ± 0.41	128.25 ± 0.42
F5	Golden brown			5.61 ± 0.03	1015.75 ± 0.68
F6	Golden brown			5.81 ± 0.51	1062.46 ± 2.69
F7	Golden brown			5.67 ± 0.47	1036.60 ± 0.54
F8	Golden brown			5.78 ± 0.04	1096.24 ± 3.50

Table 5: Antibacterial activity of the eight formulations

Formula	**Inhibition zone (mm) ± SD**				
	Staphylococcus aureus	***Candida albicans***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Proteus spp.***
F1	-	-	-	-	-
F2	20 ± 0.1	-	-	16 ± 1.2	-
F3	12 ± 0.1	-	-	-	15 ± 0.1
F4	26 ± 0.6	-	-	21 ± 0.6	-
F5	15 ± 1.2	-	-	41 ± 1.2	33 ± 1.2
F6	21 ± 0.5	-	23 ± 0.5	41 ± 1.7	34 ± 2.5
F7	15 ± 0.1	-	-	39 ± 1.2	32 ± 1.2
F8	23 ± 0.6	16 ± 1.1	20 ± 0.5	47 ± 0.6	39 ± 1.7
H	16 ± 0.1	-	-	40 ± 1.2	-
C	20 ± 1.7	-	21 ± 0.6	29 ± 2.8	14 ± 0.7
A	-	-	-	-	-
S	-	-	-	-	-

Syrup-based formula; F1, F2, F3, F4, honey-based formula; F5, F6, F7, and F8, and controls H- Honey, C- Chlorhexidine, A- Aloe vera, S- Syrup) against commonly associated wound microbial strains, determined by Agar wells diffusion test. (The values are means of 3 replicates ± Standard deviation)

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Formulation F4 containing all three actives; syrup, chlorhexidine, and Aloe vera had the widest zone of inhibition against *Staphylococcus aureus* and *Escherichia coli* and all syrup formulations were ineffective against *Candida albicans* and *Pseudomonas aeruginosa*.

Honey formulations F5 to F8, all exhibited good antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Proteus* spp. with the widest zones of inhibition seen with F8 containing three actives honey, Aloe vera, and chlorhexidine. Formulation F6 containing honey and chlorhexidine and F8 were the only formulations that exhibited antibacterial activity against *Pseudomonas aeruginosa* with F6 having the widest zone of inhibition, this could be attributed to the presence of chlorhexidine which was the only active effective against *Pseudomonas aeruginosa*. Formulation F8 exhibited better antibacterial activity against all bacterial isolates with little to no activity against the fungal isolate.

Honey is noted for its broad antimicrobial activity against Gram-positive and Gram-negative bacteria and several authors have established its activity against *Staphylococcus aureus*.[35,36] Honey's antimicrobial activity is influenced by its botanical and entomological origin and also on the resistivity of the microorganism and a study on honey from different sources in Nigeria found that honey sourced from Oyo State (which was where the honey used in this study was obtained from) was less effective against some Gram-negative bacteria especially *Pseudomonas* spp.[37] similar to results obtained from this study, hence the importance of combining with other actives like chlorhexidine to confer maximum antimicrobial coverage.

Findings from this study are also similar to findings from a study with Sri Lankan bee honey with no effect on *Pseudomonas aeruginosa* and any *Candida* spp.[38]

This shows that combining honey with chlorhexidine exhibited better activity against *Pseudomonas aeruginosa*, an organism usually implicated in chronic diabetic wounds.

In-vivo wound healing evaluation

This study was conducted on albino Wistar rats with an equal number of males and females and treatments were applied daily for fourteen days. Measurement of wound diameter was the main criterion for indication of progressive healing where wound edge contraction was expressed as a decrease of the original wound diameter. All measurements were recorded every two days for fourteen days. The control group was not induced with diabetes and was treated with antiseptic gauze. The excision wound model in Figure 2 showed that at the end of the treatment period, all wounds treated with prepared formulations had good wound healing as evidenced by wound contraction compared to control, the maximum wound diameter contraction was reached by F8. This shows that honey 75%, Aloe vera 1%, and chlorhexidine 0.02% formula showed the best healing properties compared to the other formulations. Of the syrup containing formulations F1 to F4, F3 and F4 showed equal wound contraction on the 8th day compared to F2 and F1, but on the 14th-day wounds treated with F1 showed better wound-healing activity. Wounds of the diabetes-induced but not treated (cleaned with normal saline) rats got infected by the third day but later showed some degree of healing, on the last day of observation the wound was still infected although there was a small percentage of wound contraction.

Wounds treated with formulations F5 to F8 containing honey all showed progressive wound healing with varying rates of wound contraction.

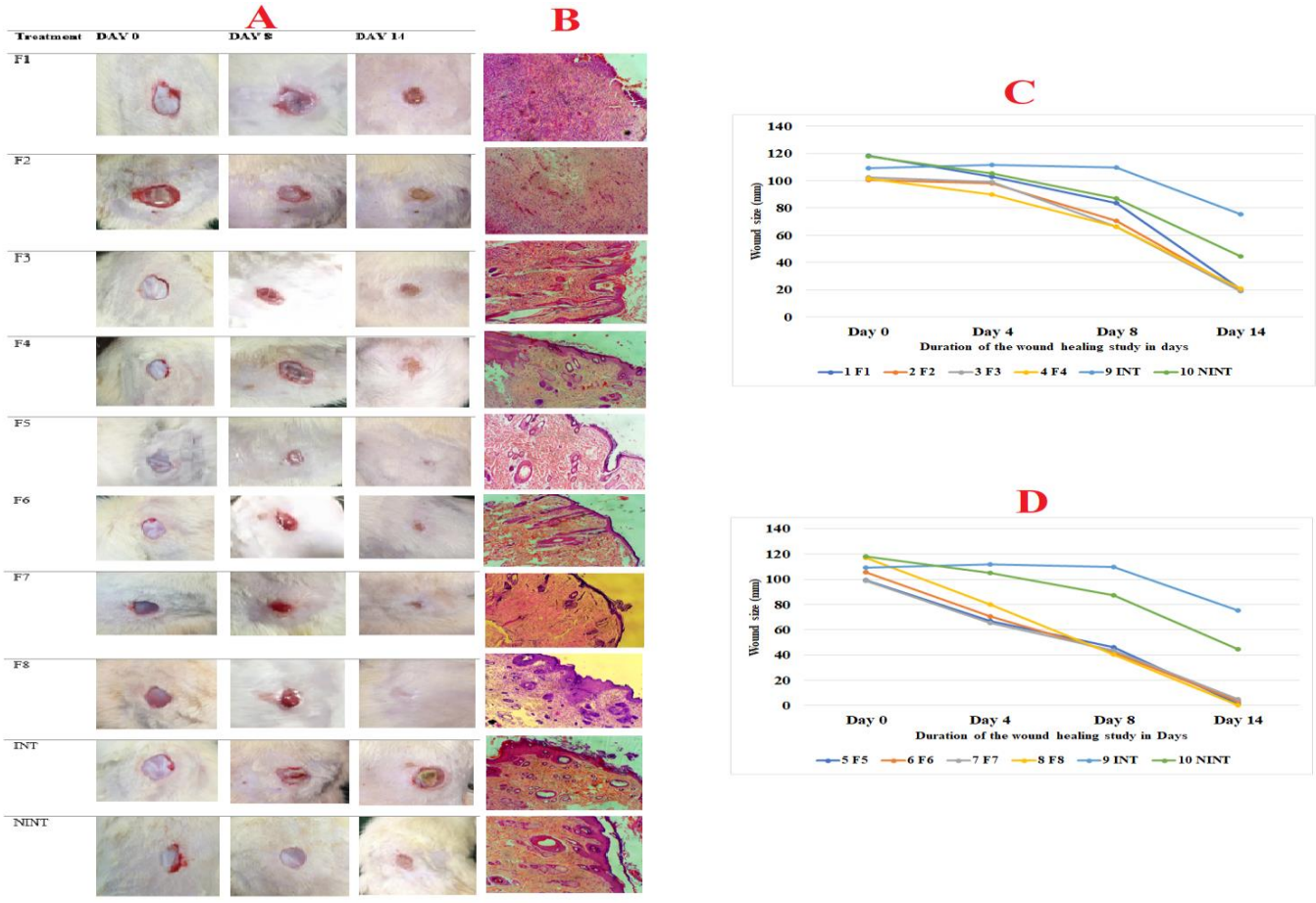

Figure 2: (A) Representative images of wounds as days elapsed showing the marked difference between varying syrup and honey formulations F1, F2, F3, F4, F5, F6, F7, F8, no treatment group covered with gauze (INT & NINT) **(B)** Representative sections stained with hematoxylin and eosin 14 days post-treatment (H&E) (C) Wound contraction ratio of wounds treated with syrup (F1 – F4) and honey (F5 – F8) based formulation and controls N = 4, $p < 0.001$.

This is similar to studies carried out on burns wounds using honey hydrogel formulations where They exhibited better wound healing activity than with formulations F1 to F4 indicating that Honey is an excellent agent for wound healing and in combination with Aloe vera and chlorhexidine its wound healing capacity is increased and antimicrobial coverage widens.

There were no significant differences between the syrup groups and honey groups as seen in Figure 2, however, the wound areas in the honey groups were significantly smaller than in the syrup groups, while both groups showed significantly better healing than the control groups. This is similar to results obtained from studies comparing sugar (syrup) and honey on open and infected wounds where honey was more effective than sugar dressings.[39]

In samples, F1 to F4 treated with syrup and combinations of chlorhexidine and Aloe vera powder showed infiltration by dense aggregates of inflammatory cells in the underlying dermis with the epidermis showing keratinized lesions.

There was less intense inflammation in samples F5 to F8 with fibroblast infiltration being more prominent. Re-epithelialization of the dermis was more complete in the honey treated group than in the syrup treated and control groups, with the control group being highly inflamed and fibrotic.

Formulations F5 and F8 showed the best wound healing with almost complete or complete healing with the epidermis and dermis showing regeneration and containing sebaceous glands and hair follicles. These findings are similar to previous studies which made evident that honey promotes the re-epithelization of wounds.[40–42]

There was significant healing of wounds by time in all groups ($p < 0.001$), no significant difference between groups treated with honey ($p > 0.005$) and between groups treated with syrup ($p > 0.005$) while using two-way ANOVA, as well as a significant time x treatment interaction ($p < 0.001$) at day 14 of treatment. There was a significant healing difference between formulations F8 and control groups ($p<0.0007$).

Conclusion

The findings from this study showed that honey-based formulations had better antimicrobial and wound healing activity. Formulation F8 containing 75% of honey, 1% aloe vera and chlorhexidine 0.02% exhibited good antimicrobial activity against the tested bacteria. It also exhibited the best wound healing activity in the diabetic rat model. Therefore, this formulation may be used in the management of diabetic wounds.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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