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Evaluation of the Antibacterial Properties of Nigerian Propolis Hydrogel

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Abstract | **Original Research Article**

Due to the rise in resistance of synthetic antibiotics, researchers have centered on identifying antimicrobial properties of agricultural wastes. Propolis was harvested from the scraping of bee hives in the Agricultural garden of the University of Benin, using standard techniques and macerated in absolute ethanol for 2 weeks in a dark room, at room temperature. The preparation was concentrated and stabilised in 5% Tween 80. The hydrogel were prepared at different concentrations of Propolis and Carbopol. Physical properties of the formulation were evaluated by an independent panel of three researchers. Physicochemical properties of the formulation (pH, spreadability, depth and viscosity) were evaluated, and analyzed. The antibacterial spectrum of propolis were evaluated against different gram- negative and gram-positive organisms by agar well diffusion method. The percentage yield of Propolis was 50.5%. Quantitative study indicated the presence of Alkaloid (351.6mg/g), Flavonoid (300mg/g), Phenolics (50mg/g), Tannin (10.9mg/g) and Saponin (0.199mg/g). The pH of the formulation was 6.73 – 6.6 (p-value = 0.04); spreadability was 4.72 – 3.17 (p-value = 0.64); depth was 18.67 – 14.83 (p-value < 0.001); viscosity was highest in formulations of 2% Carbopol (72,802 – 72,122 mPas.s). The stability studies revealed that preparations stored in the refrigerator had a better profile (pH of 6.86 – 4.51; spreadability of 3.25 – 5.57; viscosity of 7997 – 72,802 mPas.s) over 90-days period. The inhibition zone diameter against *Staphylococcus aureus* was between 17-21mm, 16.5-21mm for *Streptococcus pyrogen*, 0-7.5mm for *Staphylococcus epidermis*, 14.5-18mm for *Escherichia coli*, and between 16.5-20mm for *Pseudomonas aeruginosa*. All batches of the hydrogel did not elicit inhibitory effect against *Staphylococcus epidermidis.* The minimum inhibitory concentration was determined, for *Staphylococcus aureus* it was at 62.5mg/ml concentration; for *Streptococcus pyrogen,* it was at 125mg/ml concentration; for *Staphylococcus epidermis* it was at 500mg/ml; for *Escherichia coli,* it was at 500mg/ml, and for *Pseudomonas aeruginosa* it was at 62.5mg/ml.

Keywords: Propolis, Antibacterial, Natural Products, Hydrogel Formulation, Phytochemical Analysis.

1. INTRODUCTION

The rise in synthetic antibiotic resistance prompted researchers to investigate the antibacterial properties of plants in order to counteract the challenges presented by synthetic antibiotic resistance (Sarita *et al.*, 2019; Smith *et al*., 2021; Zhang & Li, 2022) and hence this research. It can also be noted that most synthetic antibiotics have toxic adverse effects despite their strong pharmacological actions which most medicinal plants and agricultural wastes lack due to their source from nature (Subramani *et al.*, 2014; Ahmed *et al*., 2023; Kumar *et al*., 2020). Herbal medicine, also known as phytomedicine, is a field of traditional medicine that deals with the diagnosis, prevention, and treatment of diseases using plants or plant parts. Medicinal plants contain active ingredients known as phytochemicals or secondary metabolites, which are responsible for the pharmacological activities of most plants, they serve as a source for the development of novel medications in traditional medicine. In addition to morphine, early drugs like cocaine, codeine, digitoxin, and quinine were also isolated from medicinal plants, some of which are still used today (Newman *et al.,* 2000; Butler, 2004; Samuelsson, 2004; Patel & Singh, 2021; Torres *et al*., 2022). Secondary metabolites can be defined as non-nutritive plant compounds that have disease-preventive and protective qualities (Adegboyega and Oyewole, 2015). These phytochemicals include alkaloids,

saponins, anthraquinone, terpenoids, tannins etc (Subramani *et al.*, 2014).

Herbal medicine is still utilized today; it is estimated that over 75% of the world's population, particularly in underdeveloped countries, uses herbal medicines as primary health care and first-line disease treatment (Qazi and Molvi, 2016). Extensive research has revealed that propolis from *Apis mellifera* has a wide range of pharmacological actions ranging from antibacterial, immunomodulatory, anticancer, anti-inflammatory, antioxidant, antiviral which is a justification for this study (Orsatti *et al*., 2010a; Sforcin *et al.,* 2002a; Búfalo *et al*., 2009c; Gomes *et al*., 2021; Li *et al*., 2020). As a result, propolis from previously unstudied areas appears to be a promising source of novel bioactive compounds that can be tapped (Talla *et al.*; 2016; Rodriguez *et al*., 2023; Wang *et al*., 2022).

Propolis is a resinous material with a waxy appearance that is formed by bees (*Apis mellifera*) from various plant exudates (Chen *et al*., 2018). Bees cut off sections of plants using their mandibles to acquire the plant resin, which is then used to make propolis. Bees then use their forelegs to manipulate the resin before packing it into their hind legs (Mayer 1956). When the resin reaches the hive, it is combined with the saliva of the bees and partially digested by their enzymes (Zhang *et al*., 2011). This then gives Propolis.

According to available data, a poor water solubility affects more than 70% of newly developed medications which becomes a limiting factor in the drug's absorption after oral administration (Krishnaiah, 2010; Chen *et al*., 2021; Singh & Kumar, 2022). Poor solubility of the ingredient, poor solubility due to gastric and colonic acidity, poor metabolism by the effect of gut microflora, poor absorption across the intestinal wall, poor active efflux mechanism, and first-pass metabolic effects are among the factors that lead to the failure of clinical trials (Teeranachaideekul *et al*., 2007; Siddiqui and Sanna, 2016). Hence, propolis was formulated for topical delivery. Topical dosage forms are better preferred over other dosage forms as they provide local therapeutic effect when applied on the skin or mucous membranes (Garg *et al*., 2015). It therefore means that approaches to deliver certain drugs has become paramount to researchers for the treatment of skin diseases, injury as some systemic disorders (Murthy, 2020; Zhou *et al*., 2021; Oliveira *et al*., 2022).

2. MATERIALS AND METHODS

2.1 MATERIALS

The Materials Used Include; Propolis samples (from the scrapings of Bee hives in the garden of the Faculty of Agriculture, University of Benin).

Organisms Used:

Staphylococcus aureus, Streptococcus pyrogens, Pseudomonas aeruginosa, Escherichia coli and Staphylococcus epidermis obtained from the stock preparation of Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, Delta State University, Abraka. Reagents: Carbopol 934, Propylene glycol (Loba Chemie, Mumbai India), Ethanol (JHD, Guangdang Guanghua Chemical Factory Co. Ltd, Guandang, China), Triethanolamine (Molychem Mumbai, India), pH buffer solution, Distilled water, Deionized water, petri dishes, Mueller Hinton Agar (Titan Biotech India), Nutrient agar (Titan Biotech, India). All other reagents used were analytical grade.

Equipment:

Top loading balance (Shimadzu corporation, Shimadzu Philippines Manufacturing Inc. (SPM) model: 7x 420221 serial no: D0475700327), Weighing balance, UV-VIS spectrophotometer, Rotational viscometer, pH meter (JENWAY 3505), Incubator, Autoclave, Refrigerator, Magnetic stirrer, Colloid mill, Hot air oven, test tube & Pasteur pipette.

2.2 METHODS

2.1. Collection and Preliminary Processing of Propolis

Crude propolis was collected via scraping from apiaries located in the garden of the Faculty of Agriculture, University of Benin. The recovered material contained extraneous matter, including dead bees, dust, wood fragments, and beeswax. Purification was achieved by soaking the raw propolis in water for four days. During this period, lighter impurities floated to the surface, while the denser, purified propolis settled. The floating debris was decanted, and the settled propolis was collected for further processing.

2.2. Extraction of Propolis

The cleaned propolis was subjected to ethanolic extraction via maceration. The material was immersed in absolute ethanol for a period of two weeks, with daily agitation using a magnetic stirrer to enhance dissolution. Subsequently, the mixture was filtered through a glass filter to obtain a clear ethanolic extract. The filtrate was transferred into six porcelain dishes and concentrated by air drying in a dark room to yield the final propolis extract (Oroian *et al*., 2019; Almeida *et al*., 2020; Fernandez *et al*., 2022).

2.3. Phytochemical Screening

Qualitative phytochemical analysis of the propolis extract was performed using established protocols (Usunomena and Ngozi, 2016; Patel *et al*., 2021; Ahmad & Khan, 2020). The presence of key secondary metabolites was assessed as follows:

- Saponins: 0.5 g of extract was vigorously shaken with 5 ml distilled water. The formation of a stable, persistent froth, which produced an emulsion upon the addition of three drops of olive oil, indicated a positive result.

- Tannins: 0.5 g of extract was boiled in 10 ml water, filtered, and treated with a few drops of 0.1% ferric chloride solution. A brownish-green or blue-black coloration confirmed the presence of tannins.
- Reducing Sugars: 0.5 g of extract was dissolved in 5 ml distilled water, filtered, and hydrolyzed with dilute HCl. After neutralization with NaOH, the solution was heated with Fehling's A and B solutions. The appearance of a brick-red precipitate signified the presence of reducing sugars.
- Steroids: 0.5 g of extract was dissolved in 10 ml chloroform, and an equal volume of concentrated H_2SO_4 was carefully added along the test tube wall. A reddish upper layer and a yellowish lower layer exhibiting green fluorescence constituted a positive test.
- Flavonoids: An aqueous filtrate of the extract was treated with 5 ml dilute ammonia, followed by the addition of 1 ml concentrated H_2SO_4. A yellow coloration indicated the presence of flavonoids.
- Alkaloids: The extract was dissolved in dilute HCl, filtered, and the filtrate was treated with Mayer's reagent. The formation of a yellow precipitate confirmed the presence of alkaloids.

2.4. Formulation of Propolis-Loaded Carbopol Hydrogel

A Carbopol-based hydrogel incorporating propolis extract was formulated, with Carbopol 934® serving as the gelling polymer and propylene glycol as a humectant (Singh *et al*., 2021; Oliveira *et al*., 2023). The composition of the formulations is detailed in Table 1. In brief, Carbopol 934® (1 g or 2 g) was dispersed in 100 ml distilled water under continuous stirring. Ethanol (5 ml) was incorporated and mixed thoroughly into the forming gel. The mixture was allowed to cool, after which propylene glycol (2 ml) was added. Propolis extract was subsequently incorporated at concentrations of 2% v/v, 4% v/v, or 8% v/v. The formulation was stirred continuously using a magnetic stirrer, and the pH was adjusted to 6.86 by the drop-wise addition of triethanolamine. A control hydrogel, prepared using the same procedure but without the addition of propolis extract, served as a baseline for comparison.

Table 1: Formulation Table for the preparation of Propolis Hydrogel

Formulation	**P_0**	**P_1**	**P_2**	**P_3**	**P_4**	**P_5**
Propolis (%v/v)	-	2	4	8	4	4
Carbopol 934® (%w/v)	1	1	1	1	2	2
Glycerol (%v/v)	2	2	2	2	2	2
Ethanol (%v/v)	5	5	5	5	5	5
Triethanolamine	q.s	q.s	q.s	q.s	q.s	q.s
Deionized water to	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml

*QS: Quantum satis; quantum sufficient

2.5. Evaluation of Hydrogel Consistency and Stability

The prepared hydrogels were evaluated for spreadability, pH, viscosity, and stability, including monitoring changes in pH, viscosity, and physical appearance over time (Thorat and Rane, 2010; Badmaer *et al*., 1999; Liu *et al*., 2022; Sharma *et al*., 2021).

2.5.1. Spreadability

The spread ability of the hydrogel was determined in triplicate. One gram (1 g) of the formulation was placed between two standard glass plates (10 cm x 20 cm). A twenty-five-gram (25 g) weight was placed on the top plate for one minute. The spreadability factor (Sf) was calculated using the formula Sf = A/W, where A is the total area covered (mm^2) and W is the total weight applied (g) (Arhewoh *et al*., 2022; Khan *et al*., 2020; Fernandes *et al*., 2023).

2.5.2. pH Determination

The pH of the formulations was measured in triplicate. Five grams (5 g) of each hydrogel was diluted to 50 ml with de-ionized water in a volumetric flask. The pH of the resulting solution was recorded using a calibrated pH meter (Gehan *et al*., 2014).

2.5.3. Viscosity and Rheological Behavior

The rheological behavior was assessed by measuring viscosity in centipoise (cP) using a CAP-2000 Brookfield viscometer, following a modified method (Akanksha *et al*., 2009). A sample was weighed into a clean, dry 250 ml beaker. Viscosity was determined using spindle No. 5 at 50 rpm. All measurements were conducted at 27 ± 1°C.

2.6. Preparation of Nutrient Broth and Inoculation

Nutrient broth was prepared by dissolving 5.423 g of Nutrient Agar (NA) in 150 ml of distilled water, divided into two portions (2.7113 g in 75 ml each). The solutions were sterilized by autoclaving at 121°C for 20 minutes at 1 atmosphere of pressure. After sterilization and cooling, the broth was aseptically transferred into five test tubes. A strain of *Staphylococcus aureus* was inoculated into one tube. This inoculation procedure was repeated separately for the remaining four test microorganisms. All inoculated

broths were incubated for 24 hours at 37°C (Chidinma, 2019; Khan *et al*., 2020; Fernandes *et al*., 2023).

2.7. Antimicrobial Sensitivity Testing

The antimicrobial activity was evaluated using the agar well diffusion method.

For the Crude Ethanolic Extract:

Freshly prepared Mueller Hinton Agar (MHA; 15.6 g/300 ml) was sterilized (121°C, 15 minutes, 1 atm), cooled, and aseptically poured into 10 plates. After solidification, the surface of each agar plate was inoculated by spreading one of the five test microorganisms (*Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa*) and labelled. Six wells (6 mm diameter) were bored using a sterile cork borer. Into these wells, various concentrations of the crude extract (1000 mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml) were added. The central well was filled with Cicatrin (500 mg/10ml) as a positive control. The plates were left for 15 minutes for diffusion and then incubated at 37°C for 24 hours. The test was performed in duplicate.

For the Propolis Hydrogel Formulations:

Freshly prepared MHA (7.85 g/150 ml) was sterilized, cooled, and poured into 10 plates. The plates were inoculated with the five test organisms as described above and labelled. Six wells were bored for the various hydrogel batches: P_1 (2% v/v propolis), P_2 (4% v/v), P_3 (4% v/v), P_4 (2% v/v), and P_5 (0% v/v control gel). The central well received absolute ethanol to account for its potential antimicrobial effect, as the gels contained 5 ml of ethanol. Each hydrogel batch was dissolved in water, heated to melt, and added to the designated wells using a sterile Pasteur pipette. The plates were left for 15 minutes and incubated at 37°C for 24 hours. This test was also conducted in duplicate.

2.8. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the ethanolic propolis extract was determined by the agar dilution method. Freshly prepared Mueller Hinton Agar was sterilized (121°C, 15 minutes, 1 atm) and allowed to cool. A two-fold serial dilution of the extract was prepared (1000 mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml). One milliliter of each dilution was mixed with 19 ml of sterile molten agar and aseptically poured into labeled petri dishes, which were rocked gently to ensure proper mixing. Each agar plate was divided into five sections. The five test microorganisms were streaked onto their respective sections of every plate. The plates were inverted and incubated at 37°C for 24 hours. The MIC was recorded as the lowest concentration of the extract that completely inhibited the visible growth of a microorganism after the incubation period.

2.9 Quantitative Analysis of Propolis using Ultraviolet/Visible (UV/VIS) Spectrophotometer:

2.9.1. Determination of Total Flavonoid Content

The total flavonoid content was determined following the method described by Bohm and Kocipai-Abyazan (1994). Briefly, 10 g of the propolis extract was repeatedly extracted with 100 ml of 80% aqueous methanol at room temperature. The combined extract was filtered through Whatman No. 42 filter paper (125 mm). The filtrate was transferred to a pre-weighed crucible and evaporated to dryness over a water bath. The crucible was weighed to a constant weight to determine the mass of the flavonoid-rich residue. The total flavonoid content was calculated as a percentage of the starting material.

2.9.2. Determination of Total Saponin Content

Total saponin content was quantified using the gravimetric method of Obdoni and Ochuko (2001). Twenty grams (20 g) of the pulverized propolis sample was mixed with 100 ml of 20% aqueous ethanol in a conical flask. The mixture was heated at 55°C in a water bath for 4 hours with continuous stirring. The mixture was filtered, and the residue was re-extracted with an additional 200 ml of 20% ethanol. The combined filtrates were concentrated to 40 ml at approximately 90°C. The concentrate was transferred to a 250 ml separatory funnel, and 20 ml of diethyl ether was added. The mixture was shaken vigorously, and the ether layer (containing impurities) was discarded. This purification step was repeated. The aqueous layer was then extracted twice with 60 ml of n-butanol. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The washed n-butanol fraction was heated in a water bath to evaporate the solvent. The resulting residue was dried in an oven to a constant weight. The percentage saponin content was calculated based on the weight of the dried residue.

2.9.3. Determination of Total Phenolic Content

Total phenolic content was estimated using a colorimetric method based on the Folin-Ciocalteu principle with modifications involving ferric chloride. A standard solution was prepared by dissolving 0.2 g of ferric chloride ($FeCl_3$) in 50 ml of distilled water, made up to 100 ml to obtain a 0.2% w/v solution. A standard calibration curve was constructed by plotting the concentration of this standard solution against its absorbance at a wavelength of 202 nm using a double-beam spectrophotometer. For the sample analysis, the propolis extract was reacted with ferric chloride to form a characteristic blue-black complex. A serial dilution of the sample (1.0%, 0.5%, 0.25%, 0.125%, and 0.0625%) was prepared. The absorbance of each dilution was measured at 202 nm. The concentration of phenolics in the sample was determined by extrapolating the absorbance values from the Beer-Lambert plot of the standard.

3. RESULTS AND DISCUSSION

This section presents the analysis and findings of the study, beginning with the yield and characterization of the crude propolis extract, followed by an evaluation of the physical and chemical properties of the formulated hydrogels. The detailed results for yield, phytochemical content, physical properties, and stability parameters are presented and discussed in the following subsections.

3.1 Percentage Yield of the Crude Extract of Nigerian Propolis

Ethanol extraction of Nigerian propolis gave an excellent percentage yield of 50.5% by the use of maceration technique. This is in line with comparative research carried out on various extraction methods of Propolis by Bankova *et al.,* (2021); Oliveira *et al*., (2022) and Smith & Jones, (2021) in which maceration technique gave a good yield of Propolis.

Table 2: Percentage yield of the crude extract of Nigerian Propolis

Plant Extract	Weight of Dried Plant material (g)	Extraction medium	Actual Percentage Yield (g)	Yield (%)
Ethanolic extract of Propolis (bee hive)	40.67	Ethanol	20.52	50.5

Percentage yield = (Actual yield / Weight of dried plant material) X 100

3.2 Qualitative and Quantitative Assessment

Phytochemicals are compounds with biological action, that are mostly produced by plants. Plants and agricultural wastes serve as a primary source for many active substances in the pharmaceutical industry. They display pharmacological properties that can be used to treat bacterial and fungi infections, as well as chronic degenerative disorders including cancer and diabetes (Mendoza and Silva, 2018; Chen *et al*., 2020; Li *et al*., 2021).

Table 3: Qualitative and Quantitative evaluations of Nigerian Propolis Extract

Metabolite	Present/Absent	Quantity (mg/g)
Alkaloid	Present	351.6
Flavonoids	Present	300
Phenolics	Present	50
Tannin	Present	10.9
Saponin	Present	0.199

The qualitative evaluations indicated the presence of alkaloids, saponins, phenolics, tannins and flavonoids using the methods described by Evans (1996); Silva *et al.,* (1998); Patel *et al*., (2020) and Kumar & Singh, (2022). It has been shown that the solvent used in the extraction of Phyto-constituents from propolis (due to varying polarity), the extraction time, time of collection, variability across geographical locations are factors that influence the type of metabolites present in that particular sample and its corresponding in-vitro activity (Arhewoh *et al.,* 2022). Various studies had identified different metabolites present in Nigerian propolis. However, the time of collection and seasonal differences has been the deciding factor. Studies by Alaribe *et al.,* (2018) on south-east and south-west Nigerian propolis revealed the presence of flavonoids, saponins, phenols, tannins and flavonoids which is consistent with the results of this present study. As stated in earlier studies, flavonoids are the major compounds found in propolis (Piccinelli *et al.,* 2005). Quantitative study revealed the presence of more alkaloids and flavonoids. Phenols, saponins and tannins were the least in that order. Studies by Jin and Chang, (2018); Gomes *et al*., (2022) and Li *et al*., (2021) reported a total phenol content of 38.37mg/g and a total flavonoid content of 15.28mg/g similar to the findings of this research.

3.3 Results for the Physical Properties of the Formulated Hydrogel

The different batches of hydrogel had different colour based on the concentration of the extract added. P_o had no extract added, and so was white. P_1 had 2%$^v/_v$ of the extract added, and appeared light brown. P_2 had 4%$^v/_v$ of the extract added, and appeared dark-brown. P_3 had 8%$^v/_v$ and as such appeared dark-brown. P_4 had 4%$^v/_v$ of the extract added and appeared dark-brown. P_5 had 4%$^v/_v$ of the extract added, and appeared dark-brown. The pH of the different batches of cream ranged from $6.73 \pm 0.09 - 6.86 \pm 0$ which corresponded to the pH of the stratum corneum of the skin, and as such will not irritate the skin. This pH is sufficient to prevent the growth of fungi or bacteria in the formulated gel.

Table 4: Physical properties of hydrogel formulated with the ethanolic extract of propolis

Formulation	pH	Spread ability (cm^2)	Depth of penetration (cm)	Colour
P_0	6.80 ± 0.04	23.62 ± 0.41	18.67 ± 0.47	White
P_1	6.73 ± 0.09	23.80 ± 1.69	17.33 ± 0.47	Light brown

P_2	6.86 ± 0	35.62 ± 2.90	17.10 ± 0.94	Dark brown
P_3	6.86 ± 0	43.29 ± 3.73	16.9 ± 0.25	Dark brown
P_4	6.73 ± 0.09	16.26 ± 2.22	15.57 ± 0.12	Light brown
P_5	6.86 ± 0	17.86 ± 0.42	14.83 ± 0.26	Dark brown

A decrease in pH over time was observed from the result which may result in acidic formulations. Increase in pH increases dehydrative effect, irritability (Baranda *et al.,* 2002). The pH of the formulations was evaluated over 90 days at three temperatures (refrigerator 4–8°C, room temperature 28°C, and incubator 50°C). Formulation P_o and P_1 had the most optimized pH profile (6.86 – 4.48 and 6.86 – 5.04 respectively at the three temperatures).

Spreadability of semisolid formulations is a crucial aspect to consider in administering topical preparations. The spreadability values relays the spreadability characteristics of a formulation when shear is applied. Batches P_4 and P_5 had lower spreadability due to the concentration of the polymer in the formulation i.e 2%$^{w}/_{v}$. However, across the formulation there was a slight increase in the spreadability of the formulation stored in the refrigerator and a decrease in the spreadability of the formulation stored at 50°C and those stored at room temperature were just in-between.

3.4 Results for the Physical Evaluation of Nigerian Propolis

The physical properties of the propolis extract were evaluated. It was observed for it colour, which was observed to be dark-brown in colour. This finding is in line with the study of Alaribe *et al.,* (2018) in which propolis was evaluated for its texture and found to be smooth. The evaluation of the organoleptic properties of the crude extract was similar with that of the formulated hydrogel.

Table 5: Physical properties of Propolis Extract stabilized in 1% tween 80.

Organoleptic Properties	Propolis Extract	Propolis Hydrogel
Colour	Dark brown	Dark brown
Texture	Smooth	Smooth
Odour	Characteristic	Characteristic

3.5 Results for the physicochemical Evaluation of Nigerian Propolis

Batch P_4 and P_5 had lower spreadability and lower depth of penetration due to their increased viscosity. The results revealed that the degree of spread is a contributory function of the polymer concentration and the percent content of propolis. Increase in polymer concentration and the concentration of propolis led to decrease in spread and depth. This shows that there was gradual decline in pH, spreadability and depth from P_0 to P_5.

Table 6: Physicochemical Properties of Hydrogel

Formulation	pH	Spreadability (mm^2/g)	Depth of penetration (mm)
P_0	6.80 ± 0.04	4.72 ± 0.08	18.67 ± 0.47
P_1	6.73 ± 0.09	4.76 ± 0.34	17.33 ± 0.47
P_2	6.86 ± 0	4.67 ± 0.17	17.10 ± 0.94
P_3	6.86 ± 0	4.59 ± 0.14	16.9 ± 0.25
P_4	6.73 ± 0.09	3.25 ± 0.44	15.57 ± 0.12
P_5	6.86 ± 0	3.17 ± 0.05	14.83 ± 0.26
p-value	0.04	0.22	<0.001

**Significance level: p-value <0.05*

3.6 Stability of Propolis Hydrogel Formulations

The pH, spreadability, and viscosity of the hydrogel formulations were evaluated over a 90-day period under three storage conditions: room temperature, refrigeration (4–8°C), and incubation at 50°C.

3.6.1 pH of the Hydrogel

The pH of the preparations was evaluated and the results revealed that formulation P_1 (1%$^{w}/_{v}$ polymer concentration and 2%$^{v}/_{v}$ propolis) had the most stable pH profile over the wide range of temperature evaluated. Thus, the plot of pH for formulation P_1 was flat with minimal deviations from the 6.86 (Figure 1a – Figure 1f).

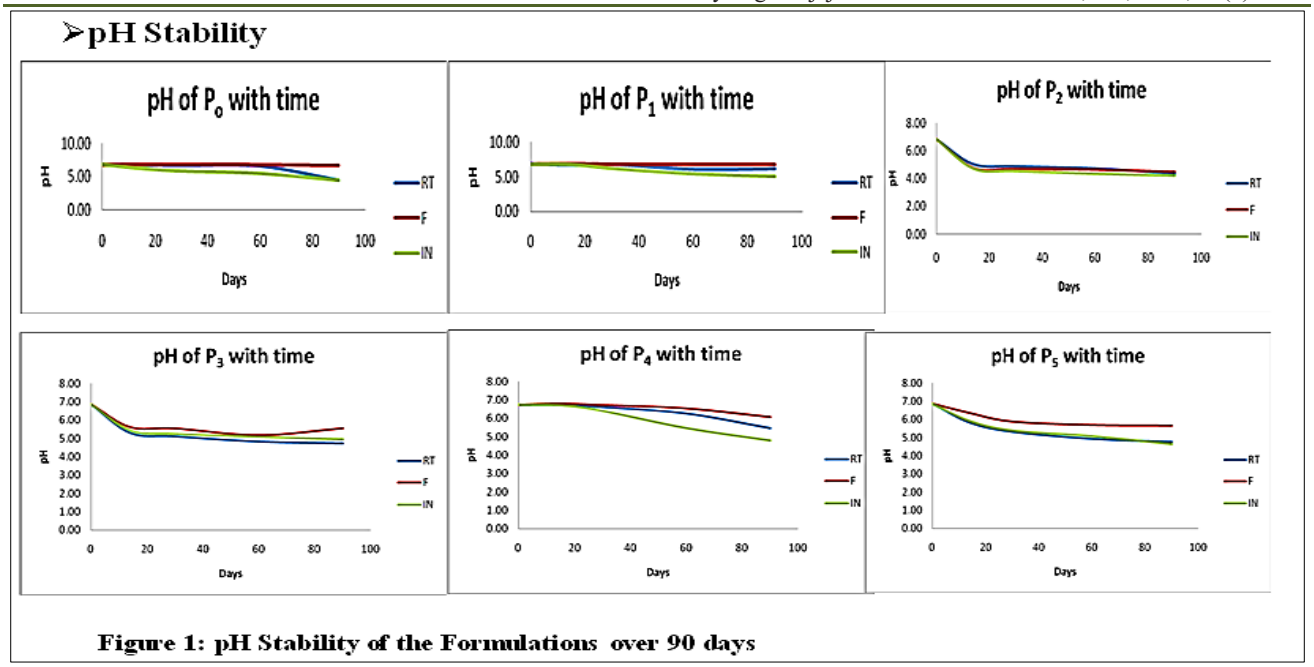

Figure 1: pH Stability of the Formulations over 90 days

3.6.2 Spreadability of the Formulations

The spreadability results are presented in Figure 2a to Figure 2f. Formulations stored at 50°C showed a marked decrease in spreadability over the 90-day period, which may be attributed to solvent loss through evaporation. In contrast, formulations stored under refrigeration displayed an increase in spreadability, likely due to moisture absorption by the polymer matrix.

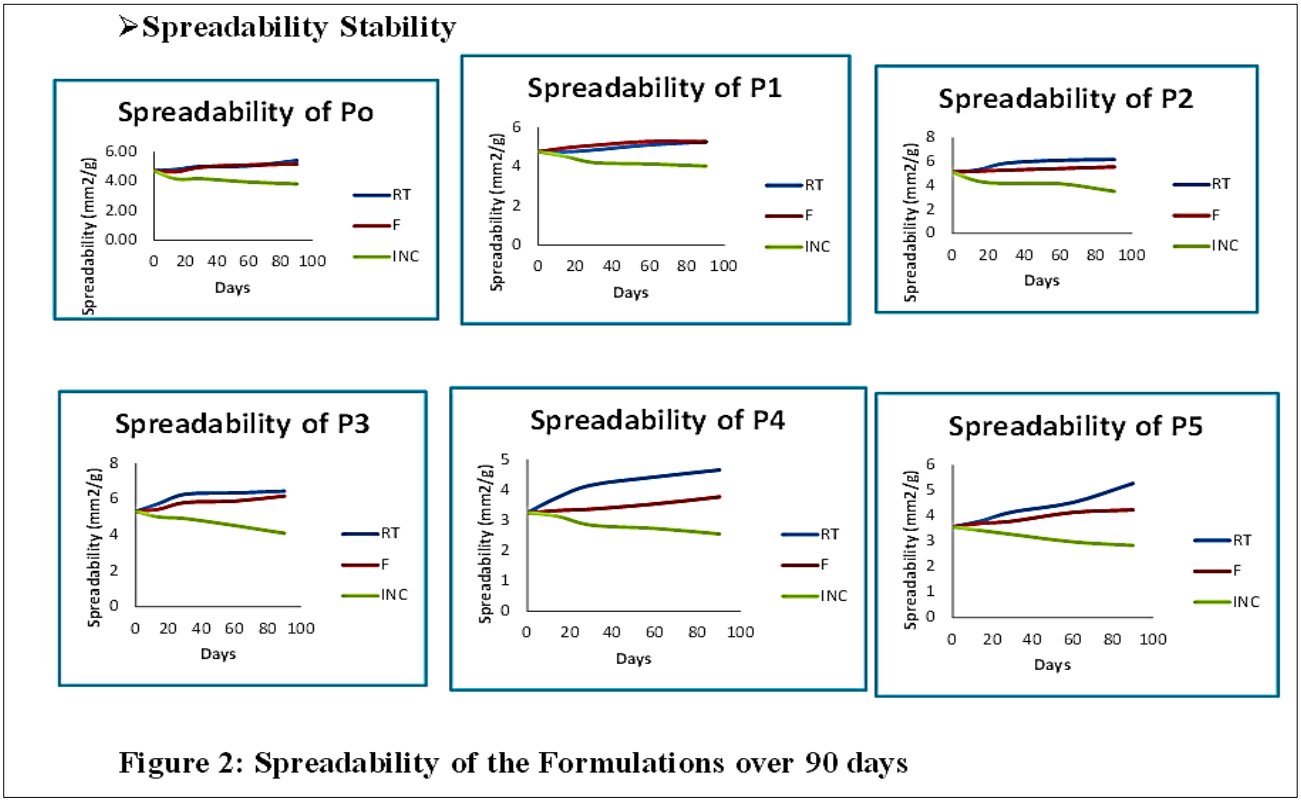

Figure 2: Spreadability of the Formulations over 90 days

3.6.3 Viscosity of the Formulations

The viscosity profiles of the formulations are presented in Figure 3a to Figure 3h. The results revealed that formulation P0 (the blank preparation without propolis) exhibited the lowest viscosity. In contrast, preparations P4 and P5 demonstrated the highest viscosities, attributable to their increased polymer concentration.

Formulations P0, P1, and P2 were observed within the lower viscosity range. An increase in either the concentration of propolis or the polymer solution was found to correspondingly increase the viscosity of the preparations. Additionally, a general reduction in viscosity was observed over time for formulations stored under refrigeration (4–8°C), as illustrated in Figures 3a to 3d.

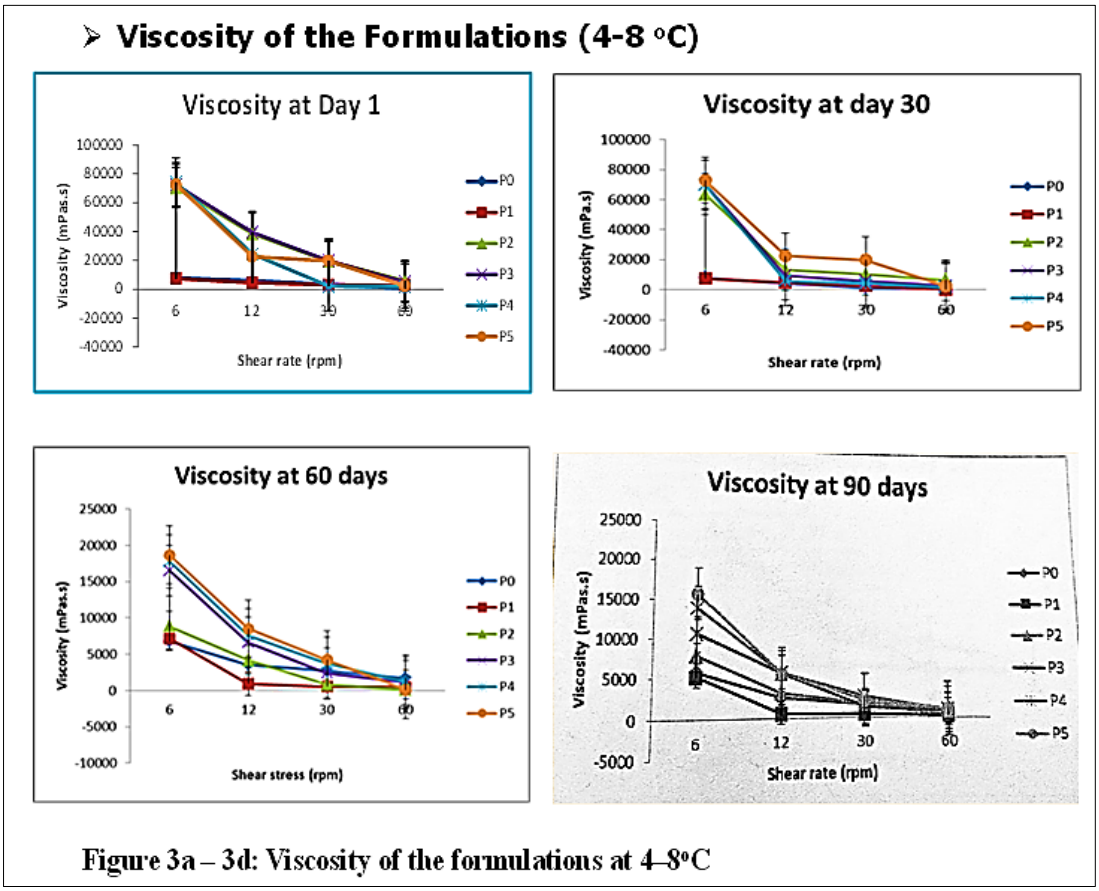

Figure 3a – 3d: Viscosity of the formulations at 4–8°C

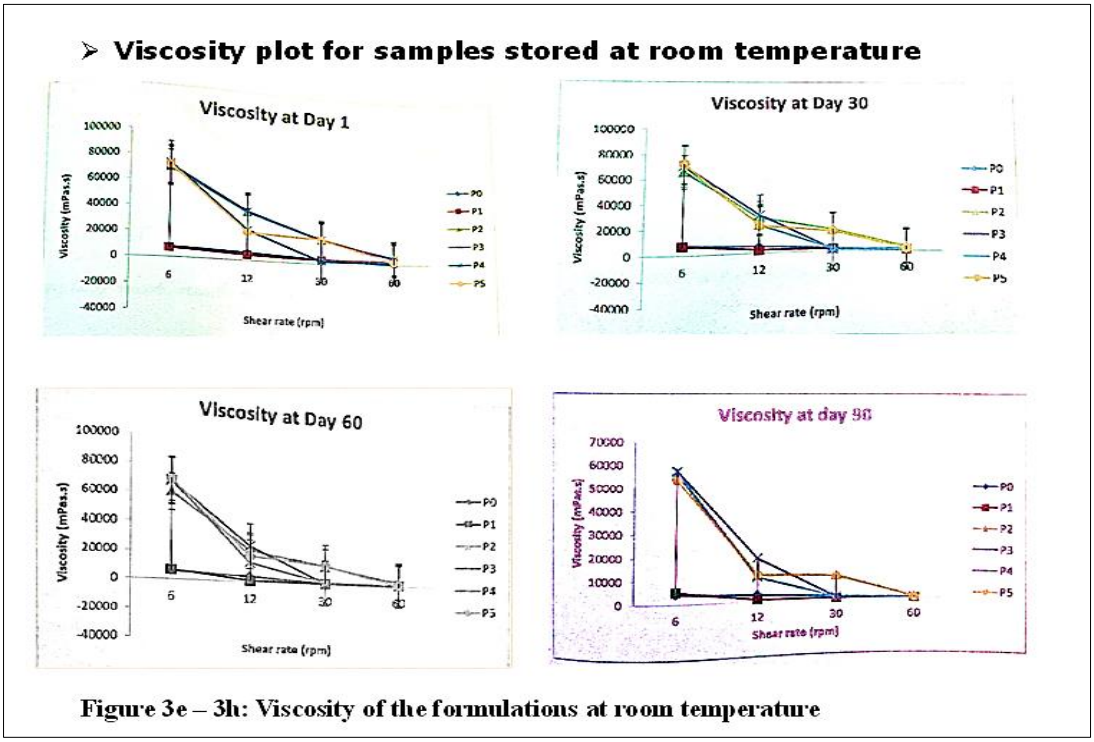

Figure 3e – 3h: Viscosity of the formulations at room temperature

3.6.4. Depth of Penetration

The depth of penetration for the hydrogel formulations is detailed in Figure 4a through 4f. The results indicate that preparations stored at room temperature and under refrigeration exhibited a progressive increase in penetration depth over the observation period. Conversely, formulations stored at 50°C demonstrated a progressively decreasing depth of penetration.

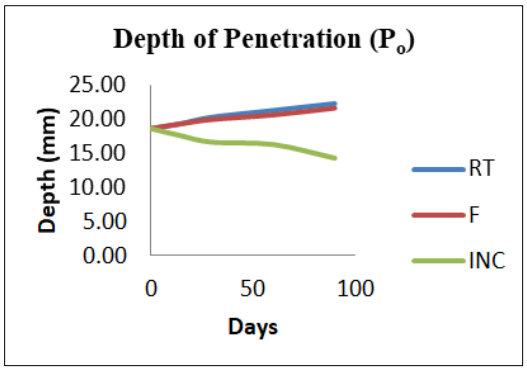

Figure 4a: 1% Carbopol solution

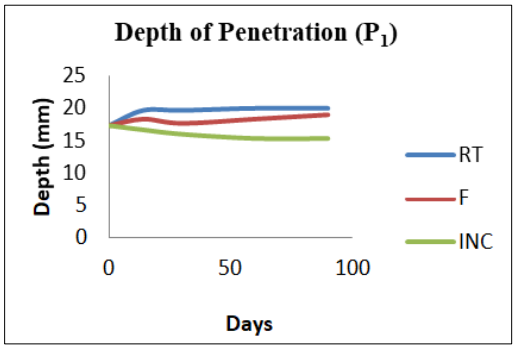

Figure 4b: 2%v/v propolis, 1% Carbopol solution

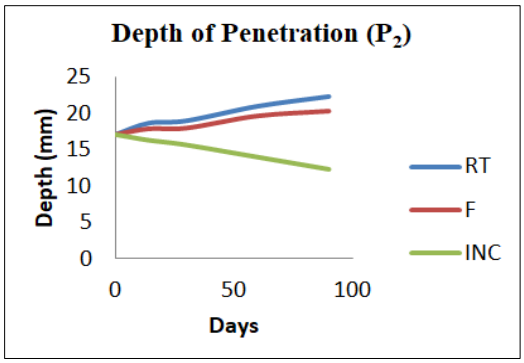

Figure 4c: 4%v/v propolis, 1% Carbopol solution

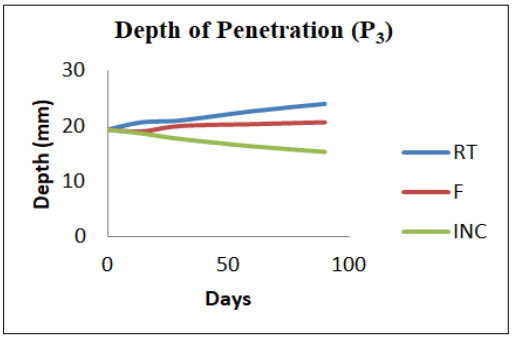

Figure 4d: 8%v/v propolis, 1% Carbopol solution

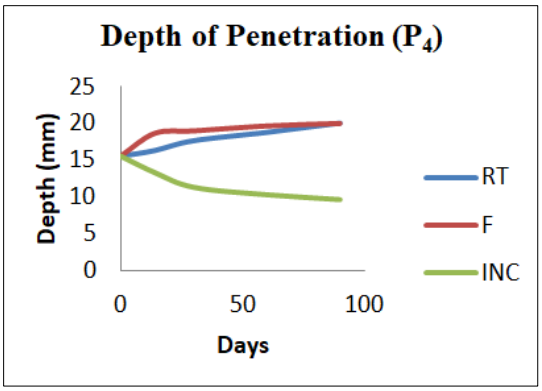

Figure 4e: 2%v/v propolis, 2% Carbopol solution

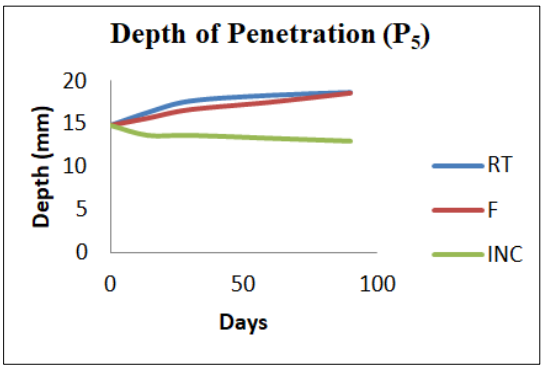

Figure 4f: 4%v/v propolis, 2% Carbopol solution

3.7 Microbial Sensitivity Pattern of the Ethanolic Extract of Nigerian Propolis

Microbial sensitivity testing, or susceptibility testing, determines the effectiveness of an antimicrobial agent against specific bacteria. The evaluation of Nigerian propolis for its antibacterial properties yielded significant results. The ethanolic extract demonstrated a broad-spectrum antibacterial effect against the selected clinical isolates. Notably, the activity against *Staphylococcus epidermidis* was minimal, even at higher concentrations. The results further established a clear concentration-dependent antimicrobial response. At a concentration of 62.5 mg/ml, the extract exhibited pronounced potency against Escherichia coli and Staphylococcus aureus.

Table 7: Microbial sensitivity pattern of ethanolic extract of Nigerian Propolis (bee hive)

Concentration (mg/ml)	Zone of inhibition (mm)				
	S/A	S/P	S/E	E/C	P/A
1000	21.0 ± 1.0	19.5 ± 0.5	7.5 ± 1.5	18.0 ± 1.0	20 ± 1.0
500	17.0 ± 1.0	18.5 ± 0.5	6.0 ± 0	17.5 ±0.5	19.0 ± 2.0
250	18.5 ± 0.5	18.5 ± 0.5	2.0 ± 2.0	15.0 ± 1.0	18.0 ± 1.0
125	18.5 ± 0.5	17.0 ± 0	NI	14.5 ± 0.5	17.0 ± 0
62.5	15.5 ± 1.5	16.5 ± 0.5	NI	15.0 ± 1.0	16.5 ± 0.5
PC (Cicatrin)	17.0 ± 1.5	21.0 ± 1.0	NI	8.0 ± 1.0	7.0 ± 0

Keys:
NI: No Inhibition
PC: Positive control (Cicatrin 500mg/ml)
S/A: Staphylococcus aureus
S/P: Streptococcus pyrogen
S/E: Staphylococcus epidermis
E/C: Escherichia coli
P/A: Pseudomonas aeruginosa

It was observed that the higher the concentration of propolis; the larger the diameter zone of inhibition. Of all the microorganisms used *Staphylococcus aureus* was more susceptible to propolis, followed by *Pseudomonas aeruginosa, Streptococcus*

pyrogenes, Escherichia coli and *Staphylococcus epidermis*.

The antibacterial activity of the extract (propolis) against *Staphylococcus aureus* at 500mg/ml in comparism with the positive control (Cicatrin) at 500mg/ml was equal with a diameter zone of inhibition of 17.0 mm (Patel *et al*., 2023; Ahmed *et al*., 2022). In comparison against *Staphylococcus epidermidis*; the positive control had a slightly better activity with a diameter zone of inhibition of 21.0 ± 1.0 mm, while that of the extract was found to be 18.5 ± 0.5mm. At 500mg/ml concentration of the extract, there was a greater activity against *Staphylococcus epidermidis* with a diameter zone of inhibition of 6.0 ± 0 mm, and no inhibition was observed with Cicatrin at the same concentration. The activity of propolis was then determined against *Escherichia coli* which gave a greater diameter zone of inhibition of 17.5 ± 0.5mm than Cicatrin which had a diameter zone of inhibition of 8.0 ± 1.0mm. Propolis also had a greater activity *Pseudomonas aeruginosa* with a diameter zone of inhibition of 19.0 ± 2.0mm than Cicatrin, which had a diameter of 7.0 ± 0mm. These results are in line with the findings of Ahuja, (2011); DeCastro, (2001); Wagh, (2013) who also recognized the activity of propolis against gram-positive and gram-negative organisms proliferated on wounded surface.

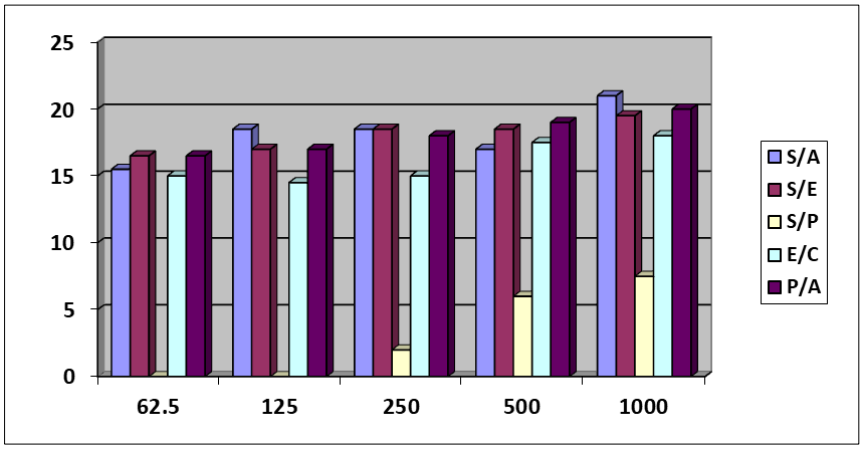

Figure 5: A plot of increased concentration of propolis extract against mean inhibition zone diameter (IZD)

Key:
S/A: *Staphylococcus aureus*
S/E: *Staphylococcus epidermis*
S/P: *Streptococcus pyrogenes*
E/C: *Escherichia coli*
P/A: *Pseudomonas aeruginosa*

Figure 5 revealed that as the concentration of crude propolis was increased, a higher mean diameter inhibition zone was obtained.

The highest mean inhibition zone diameter (IZD) against *Staphylococcus aureus* was observed at 1000mg/ml of the crude extract, similar results occurred against the different organism with increased concentration. It is worthy to note that organisms which were at first resistant became susceptible as the concentration of crude extract was increased (Li *et al*., 2023; Nguyen *et al*., 2021). This result is in line with the findings of Ahuja, (2011); and Wagh (2013) who found out that the activity of propolis is concentration dependent.

3.8 Microbial Sensitivity Pattern of the Batches of Hydrogel

Microbial sensitivity analysis, also called susceptibility testing is a test that determines the sensitivity of bacteria to an antibiotic. Propolis was formulated into a hydrogel, and its antibacterial property evaluated. Similar findings with the extract occurred with the formulated hydrogel. The polymer enhanced the release of the active ingredient (Propolis) to elicit its antibacterial activity. P_0 which had no concentration of propolis showed slight antibacterial activity. Po which had no concentration of propolis showed slight anti-bacterial activity. This is because ethanol has some anti-bacterial activity itself. It showed no activity against *Staphylococcus epidermidis* and *Escherichia coli* and this serves as a control.

Table 8: Microbial sensitivity pattern of the various batches of Hydrogel formulated using ethanolic extract of Propolis

Concentration of Extract in hydrogel (%$^{v}/_{v}$)	Zone of inhibition (mm)				
	S/A	S/P	S/E	E/C	P/A
P_0(0)	5	8	NI	NI	6

P_1(2)	12	11	NI	NI	11
P_2(4)	12	11	NI	7	14
P_3(8)	11	12	NI	6	13
P_4(4)	12	11	NI	1	10
P5(4)	12	11	NI	1	10

Key

NI: No inhibition

P_0- 0% extract concentration in hydrogel
P_1- 2% extract concentration in hydrogel
P_2- 4% extract concentration in hydrogel
P_3- 8% extract concentration in hydrogel
P_4- 4% extract concentration in hydrogel
P_5- 4% extract concentration in hydrogel

Batch P_1 had relative activity against *Pseudomonas aeruginosa, Staphylococcus* aureus and Streptococcus pyrogenes. The mean inhibition zone diameter (IZD) observed by the crude extract against S*taphylococcus aureus* was 12mm, this was more than the control which has a mean inhibition zone diameter (IZD) of 6mm. Also, the mean inhibition zone observed by the extract against Streptococcus pyrogenes was 11mm, this was also more than the control. No activity was observed against Staphylococcus epidermis and Escherichia coli.

Batch P_2 was more active than batch P_1 against Escherichia coli and Pseudomonas aeruginosa, with a mean inhibition zone diameter (IZD) of 7mm against Escherichia coli which was greater than the control. Staphylococcus aureus was resistant against batch P2.

Batch P_3 which had the greatest concentration of propolis also had activity against *Escherichia coli* with a mean inhibition zone diameter (IZD) of 6mm. It also exhibited activity against *Pseudomonas aeruginosa* with a mean inhibition zone diameter (IZD) of 13mm. *Staphylococcus epidermis* was resistant batch P3.

Batch P_4 had slight activity against *Escherichia coli* with a mean inhibition zone diameter (IZD) of 1mm. It had activity against *Pseudomonas aeruginosa* with a mean inhibition zone diameter (IZD) of 10 mm. Further activity was seen as against *Staphylococcus aureus* with a mean zone of inhibition of 12 mm, and with *Streptococcus pyrogenes* with a mean inhibition zone diameter (IZD) of 11mm. *Staphylococcus epidermis* was also resistant to batch P4.

Batch P5 exhibit similar activity with batch P4.

3.9 Minimum Inhibitory Concentration of the Ethanolic Extract of Propolis

Minimum inhibitory concentration (MIC) is the lowest drug concentration that prevents visible microorganism growth after overnight incubation. An MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. More often because a lower MIC value indicates that less of the drug is required in order to inhibit growth of the organism. In conclusion, drugs with lower MIC are more effective antimicrobial agents.

Table 9: Results of the determination of minimum inhibitory concentration of the ethanolic extract of propolis (bee hive)

Microorganism	Concentration (mg/ml)					
	1000	500	250	125	62.5	31.25
S/A	-	-	-	-	-	+
S/E	-	-	+	+	+	+
S/P	-	-	-	-	+	+
E/C	-	-	+	+	+	+
P/A	-	-	-	-	-	+

Key:

+ : Indicates growth
- : Indicates no growth

From **Table 9,** the Minimum inhibitory concentration (MIC) ranges from 62.5 to 500mg/ml. When determined against *Staphylococcus aureus*, there was inhibition at 1000mg/ml, 500mg/ml down till 62.5mg/ml of the crude extract, but at 31.25mg/ml there was no inhibition i.e growth occurred. From the result the MIC of the crude extract against *Staphylococcus aureus* is at 62.5mg/ml of the crude extract (Propolis). This implies that if the gel or a product is formulated with propolis at a concentration lower than 62.5mg/ml concentration of propolis there will be no antibacterial inhibitory effect against *Staphylococcus aureus* (Fernandez *et al*., 2022; Wang *et al*., 2021).

Similarly, when determined against *Staphylococcus epidermis*, there was distinctive inhibition at 1000mg/ml, and down till 500mg/ml of the crude extract, but at 250, 125, 62.5, and at 31.25mg/ml there was no inhibition i.e growth occurred. From the result the MIC of the crude extract against *Staphylococcus epidermis* is at 500mg/ml of the crude extract (Propolis). This implies that if the gel or a product is formulated with propolis at a concentration lower than 500mg/ml concentration of propolis there will be no antibacterial inhibitory effect against *Staphylococcus epidermis.*

Similarly, when determined against *Streptococcus pyrogenes*, there was prominent inhibition at 1000mg/ml, 500mg/ml, and down till 62.5mg/ml of the crude extract, below 62.5mg/ml there was no inhibition i.e growth occurred.

From the result the MIC of the crude extract against *Streptococcus pyrogenes* is at 62.5mg/ml of the crude extract (Propolis). This implies that if the gel or a product is formulated with propolis at a concentration lower than 62.5mg/ml concentration of propolis there will be no antibacterial inhibitory effect against *Streptococcus pyrogenes.*

For the determination of the MIC of propolis against *Escherichia coli*, at a concentration of 1000mg/ml of the crude extract there was no growth. Similar inhibition was seen at 500mg/ml, but at 250; 125; 62.5 and 31.25mg/ml of the crude extract there was no inhibition of the crude extract. Therefore, the minimum inhibitory concentration of the crude extract against *Escherichia coli* is at 500mg/ml. This implies that if the gel or a product is formulated with propolis at a concentration lower than 500mg/ml concentration of propolis there will be no antibacterial inhibitory effect against *Escherichia coli*. Similarly, when determined against *Pseudomonas aeruginosa*, there was inhibition at 1000mg/ml, down till 500mg/ml of the crude extract, but below 500mg/ml there was no inhibition i.e growth occurred. From the result the MIC of the crude extract against *Pseudomonas aeruginosa* is at 500mg/ml of the crude extract (Propolis). This implies that if the gel or a product is formulated with propolis at a concentration lower than 500mg/ml concentration of propolis there will be no antibacterial inhibitory effect against *Pseudomonas aeruginosa.*

Figure 6: Plates showing the Minimum inhibitory concentration (MIC) of the crude extract of Nigerian propolis at different concentration against *Staphylococcus aureus, Streptococcus pyrogenes, Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa.*

4. CONCLUSION AND RECOMMENDATION

4.1 CONCLUSION

The study demonstrates that Nigerian propolis is a viable source of bioactive compounds, yielding 50.5% (w/w) of crude extract via ethanolic maceration. Qualitative and quantitative analysis revealed a rich phytochemical profile, with high concentrations of alkaloids (351.6 mg/g), flavonoids (300 mg/g), phenols (50 mg/g), tannins (10.9 mg/g), and saponins (0.199 mg/g). Formulation stability studies indicate that propolis-loaded Carbopol hydrogel maintains optimal physicochemical properties when stored under refrigeration (4–8°C), although room temperature storage also proved acceptable with minimal parameter fluctuations. Microbiological assessment confirmed the extract's broad-spectrum antibacterial activity, with a concentration of at least 500 mg/mL recommended for effective prevention of bacterial infection and support of wound healing.

4.2 RECOMMENDATION

Based on the findings, it is recommended that the maceration extraction protocol be adopted as a standard for the preliminary recovery of bioactive compounds from Nigerian propolis. To further the pharmaceutical application of this resource, subsequent research should aim to isolate and characterize the specific alkaloid and flavonoid constituents to delineate their individual pharmacological roles. Additionally, studies should be conducted to ascertain the precise mechanism of propolis's antibacterial effect and to standardize its optimal concentration in wound dressings for treating bacterial infections, thereby ensuring better antimicrobial activity alongside optimized physicochemical properties.

For product development, refrigeration should be prioritized as the primary storage condition to ensure the long-term stability of propolis-based topical hydrogels. Finally, in vivo studies are essential to clinically validate the wound-healing efficacy and safety of hydrogel formulations containing propolis at the recommended minimum concentration of 500 mg/mL.

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