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Evaluation of the healing activity of propolis hydrogel in an excision-based model.

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Abstract

Propolis hydrogel made of carbopol has been shown to provide better contact with the wounded site and reduce wound healing time. This study explored the physicochemical properties of Nigerian propolis hydrogel and its wound healing activity. Propolis was obtained from bee hives using standard techniques and macerated in absolute ethanol at 40 ^{o}C for 2 weeks in a dark room at room temperature. The preparation was filtered, concentrated and stabilized in 20 ml 5 %v/v tween 80. The hydrogel was prepared at different concentrations of propolis and carbopol. The physicochemical properties of the formulation were evaluated and statistically analyzed. The animals were grouped into six; group 1 was treated with the control (cikatrin), group 2 was treated with the blank gel, group 3, 4, 5 and 6 were treated with formulation P_1, P_2, P_3 and P_4 respectively. The wound healing activity was evaluated on days 7, 14, and 21 after inducing dermal injury. The quantitative study of the extract indicated the presence of alkaloid (351 mg/g), flavonoid (300 mg/g), phenolic (50 mg/g), tannin (10.9 mg/g), and saponin (19.9 mg/g). The pH of the formulation was 6.73 - 6.6 (p = 0.04); spreadability was 4.72 - 3.17 (p = 0.64); depth was 18.67 - 14.83 (p < 0.001); viscosity was highest in formulations of 2 % carbopol (72,802-74,122 mPas.s). The stability studies revealed that preparations stored in the refrigerator had better profiles, (pH of 6.86-4.51; spreadability of 3.25-5.57; viscosity of 7997-72,802 mPas.s) over 90 days period. The histoarchitectural features were characterized by ulceration, granulation tissue formation, and various shades of wound healing. This study has shown that propolis formulated as hydrogel can be used to induce wound healing, and thus indicated the need for standardizing propolis content in wound dressings and other topical delivery systems to obtain optimum activity with good physicochemical profile.

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Introduction

Propolis or blue glue is a resinous mixture that honey bees produce while mixing saliva and bees wax with exudates gathered from tree buds sap, or other botanical sources. Propolis hardens the cell wall and contributes to an aseptic internal environment. It is also used by honey bees to prevent decomposition of the carcass of the intruder in the hives and to maintain internal hive temperature (Moreira *et al.,* 2008). The precise composition of propolis varies with the source, and over 300 chemical components including flavonoids, terpenes, and phenolic acids had been identified. Moreover, its chemical composition varies with geographical location, botanical origin and bee species (Drescher *et al.,* 2019). Various pharmacological effects have been reported (Sforcin, 2016; Toreti *et al.,* 2013) and it is gradually becoming important in the area of modern medicine, veterinary medicine, pharmacology as well as cosmetics, where it can be formulated as capsules, lozenges, creams, gels, emulsions, and ointments. The anti-inflammatory and antimicrobial properties of propolis may be of value in promoting wound healing (McLennan *et al.,* 2008; Pillai *et al.,* 2010). The wound healing process includes anti-inflammation, tissue formation, and tissue remodeling (Eming *et al.,* 2007). The undesirable odour, sticky nature, light sensitivity, and lipophilicity are some drawbacks that limit its use on the skin or injured tissues (Działo *et al.,* 2016). Thus, the formulation of propolis into a topical dosage form such as hydrogel would guarantee its use in wound healing.

Carbopol is a biocompatible polymer composed of polyacrylic acid and has been incorporated in wound dressings, topical, and transdermal drug delivery systems (Chirani *et al.,* 2015). Its high viscosity, bioadhesiveness, thermal stability, and compatibility makes it ideal for developing topical formulations such as hydrogel (Sasutjarit *et al.,* 2005). Kaler *et al.,* (2014) suggested that a hydrogel made of carbopol would guarantee a favourable environment within the wound site and as a result reduce wound healing time. This corroborates earlier studies that the water retention ability of hydrogels composed of carbopol and its bioadhesiveness would keep the drug or the active ingredient in close contact with the wound site (Sasutjarit *et al.,* 2005). Thus, the study explored the physicochemical properties, stability profile and wound-healing activity of propolis hydrogel.

Materials and methods

Reagents and Equipment

Carbopol 934, glycerol (Loba Chemie, Mumbai India), ethanol (JHD, Guangdang Guanghua Chemical Factory Co. Ltd, Guandang, China), triethanolamine (Molychem Mumbai, India) tween 80 (Sigma-Aldrich), pH buffer solution, distilled water, sterile gauze, pH meter, Brookfield viscometer, Petri dishes, measuring cylinders, scissors, scalpels, histological kit, UV-VIS spectrophotometer. All other reagents used were analytical grade.

Sample Collection and Extraction

The crude propolis was obtained from bee hives in Edo State, Nigeria following standard techniques in February, 2021. The sample was extracted by maceration with ethanol at 40°C for two (2) weeks in a dark room. It was stirred daily with a magnetic stirrer and filtered using a glass filter to obtain a clean filtrate. The filtrate was then transferred into six (6) porcelain dishes and allowed to concentrate by air drying in a dark room. The concentrate was then stabilized in 20 ml 5 %v/v tween 80, packaged, and kept in the refrigerator at 4 - 8 °C prior to use (Oroian et al., 2019).

The yield of the Sample

The yield of the sample after extracting was determined by dividing the final weight with the initial weight and expressing the resulting figure as a percentage as presented in equation 1.

$$\text{Yield} = \frac{\textit{Final weight}}{\textit{initial weight}} \times 100\% \quad \text{- Equation 1}$$

Qualitative and Quantitative Study

The various constituents (alkaloids, flavonoids, phenolics, tannins and saponins) were identified and quantified using established methods of Trease and Evans (1996).

Laboratory Animals

Albino rats (18) weighing between (100 - 150 g) were used for the study. They were kept in a room and fed for two weeks (14 days) to acclimatize at ambient temperature 27 ^{o}C and relative humidity of 60 % and a 12 h light-dark cycle in the animal house of the Faculty of Basic Medical Sciences, Delta State University, Abraka. All animals were cared for in compliance with the internationally accepted guide for the care and use of laboratory animals, published by the US National Institutes of Health (2010).

Formulation of Hydrogel

Propolis hydrogel was prepared with carbopol 934® (as the polymer) and glycerol (emollient) as presented in Table 1. Carbopol 934® (1 g, 2 g) was dispersed in 100 ml of distilled water with continuous stirring. Five milliliters of ethanol was mixed properly with the polymer gel. The solution was cooled and 2 ml of glycerol was added. Propolis (2 %v/v, 4 %v/v, and 8 %v/v) was measured and transferred into the polymer gel. The entire solution was stirred continuously using a magnetic stirrer. Triethanolamine was added drop-wise to the formulation to adjust the pH to 6.86. Same method was followed for the preparation of the control sample without propolis (Jin and Chang, 2018).

Evaluation of Organoleptic Properties

The organoleptic properties which includes colour, odour, and texture were evaluated by three independent researchers in formulation science (Alalor *et al.,* 2021).

Evaluation of Physicochemical and Stability Profile

The hydrogel was evaluated for spreadability, pH, viscosity (at 6 rpm, room temperature, and spindle 4), and stability (pH and viscosity changes) (Arhewoh *et al.,* 2022; Akanksha *et al.,* 2009).

Ethical Clearance

Ethical clearance for the study was gotten from the Research and Bioethics Committee of the Faculty of Basic Medical Science, Delta State University with reference number: REC/FBMS/DELSU/22/144.

Experimental Design

The animals were divided into six (6) groups comprising three animals (3) in each group. Group 1: Excision wound-induced rats were treated with a standard drug combination Cikatrin (containing neomycin and bacitracin sulphate) which is used for cuts and burns designated as the control group. Group 2: Excision wound-induced rats were treated with the blank hydrogel. Group 3: Excision wound-induced rats were treated with a formulation P_1 (2 %v/v propolis and 1 % polymer solution). Group 4: Excision wound-induced rats were treated with a formulation P_2 (4 %v/v propolis and 1 % polymer solution). Group 5: Excision wound-induced rats were treated with a formulation P_3 (8 %v/v propolis and 1 % polymer solution). Group 6: Excision wound-induced rats were treated with a formulation P_4 (2 %v/v propolis and 2 % polymer solution). The treatment schedule was done on alternate days with the topical formulation as well as a standard drug (cikatrin powder). Changes in wound areas were described histologically by harvesting the healing tissue on days 7, 14, and 21 and carrying out the H&E staining procedure.

Histoarchitectural Evaluation of the Wound Healing Activity

The wound healing activity of the formulation was carried out according to the method of Kokane *et al.,* (2009) and Dash and Murthy, (2011) with slight modifications. These modifications were necessary to enable the researcher carry out a non-invasive anesthetic procedure. On a wounding day, the animals were anesthetized using chloroform at a dose of 0.1 ml/kg. The wound area was prepared with 70% alcohol, afterwards, the dorsal fur of the animals was shaved with a shaving machine and the anticipated area of the wound to be created was outlined on the back of the animals along the dorsal thoracic region 1 cm away from vertebral column. Full-thickness circular excision wounds sized about 1 cm^2 were created along the markings using toothed forceps, scalpel, and scissors. Hemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. Treatments of the infected wounds

commenced on the 2nd day to allow the establishment of infection on the wound. The wounding day was considered day 0. Cikatrin® powder (the control), propolis hydrogel, and the hydrogel without propolis were topically applied to the respective groups till the wound was completely healed. The healing tissue was then harvested and H and E staining was carried out to describe histomorphological changes. Throughout the experiment, presence or absences of phlogistic characteristics (infiltration, edema/localized swelling, abscess, or lesion and exudates) was monitored every 24 hours (Maria *et al.,* 2011).

Results

Yield of the Sample

The yield of the sample after extracting was 20.52 g (50.5 %) and this weight was stabilized in 5 % tween 80. The initial weight used was 40.67 %.

Organoleptic properties of the Crude Extract and Hydrogel

The colour, texture, and odour of the extract and hydrogel were judged by three independent panelists and found to be brown in colour, and smooth with a characteristic odour respectively.

Table 1. Formulation Table

Formulation	P_0	P_1	P_2	P_3	P_4	P_5
Propolis (%v/v)	-	2	4	8	2	4
Carbopol 934® (%w/v)	1	1	1	1	2	2
Glycerol (%v/v)	1.45	1.45	1.45	1.45	1.45	1.45
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

*q.s: quantity sufficient to pH 6.86

Qualitative and Quantitative Evaluation

The quantitative evaluation revealed that a large amount of alkaloid and flavonoid was present per gram of the propolis extract (Table 2).

Physicochemical Properties of the Hydrogel

The physicochemical properties of the hydrogel are represented in Table 3. Batch P_4 and P_5 had lower spreadability and lower depth of penetration due to their high viscosity. Differences in pH, depth, and viscosity were statistically significant ($p < 0.05$).

Table 2. Quantitative study of Metabolites present

Metabolites	Quantity (mg/g)
Total Alkaloid Content	351.6
Total flavonoid content	300
Total phenolic content	50
Total tannin	10.9
Total saponin	19.9

Rheological Behaviour

The rheological behavior of the hydrogels revealed that the formulations exhibited pseudoplastic behavior with shear thinning (Fig. 1 and 2). Po and P_1 had the least viscosity.

pH Profile

The pH profile of the formulations ranged from 6.86 (at day 0) to about 4.51 at day 90 (for P_2 only). Batches P_0, P_1 and P_4 had better stable pH profile followed by P_3 and P_5 (Fig. 3 and 4). Preparations stored in the refrigerator had better pH profile compared to those stored at room temperature.

Histopathological Assessment of Excised Skin Sections

The histoarchitectural features were characterized by ulceration, granulation tissue formation, and other shades of wound healing activity which include acute and chronic wound healing, re-epithelization, and extensive network of fibro-connective tissue which resembles that of a normal skin. Groups treated with propolis exhibited better healing activity than the control groups (Fig. 5-16).

Discussion

Propolis, which is well tolerated with rare incidents of allergy and no toxicity, is an excellent candidate for burn management, enhancing skin cell proliferation, activation, and growth capacity (Sehn *et al.,* 2009). This study explored the wound healing activity of propolis hydrogel by histologically assessing cut skin sections of the wounded epidermis. This study was a build up to the study of Jin and Chang, (2018) on the wound healing activity of Korean propolis hydrogel, however with lots of inputs and modifications. Furthermore, Nigerian propolis was used in this research at different concentrations as well as polymer solution to evaluate its stability and histologically assess the wound healing effect of the resultant formulation (hydrogel). The histoarchitectural features of the excised skin sections were carried out to assess the extent of wound healing at varying concentrations of propolis and time.

Table 3. Physicochemical Properties of Propolis Hydrogel.

Formulation	pH	Spreadability (mm^2/g)	Depth of penetration (mm)	Viscosity mPas.s
P_0	6.80 ± 0.04	4.72 ± 0.08	18.67 ± 0.47	7997
P_1	6.73 ± 0.09	4.76 ± 0.34	17.33 ± 0.47	7406
P_2	6.86 ± 0	4.67 ± 0.17	17.10 ± 0.94	70,526
P_3	6.86 ± 0	4.59 ± 0.14	16.9 ± 0.25	72,313
P_4	6.73 ± 0.09	3.25 ± 0.44	15.57 ± 0.12	74,122
P_5	6.86 ± 0	3.17 ± 0.05	14.83 ± 0.26	72,802
p-value	0.04	0.22	< 0.001	<0.001

**Significance level: p-value < 0.05.*

Generally, ethanol has been shown to be one of the best solvents for propolis preparation while other solvents such as ethyl ether, water, methanol and chloroform may be used for extraction and identification of propolis compounds (Szliszka *et al.,* 2013). The crude extract was dark brown, smooth in texture with a characteristic odour. These characteristics have been the most common organoleptic properties of propolis (Arhewoh *et al.,* 2022). Its colour varies from green to brown to red, depending on its botanical source. It has been shown by Bankova *et al,* (2000) that propolis has a characteristic and pleasant aromatic smell with variability in colour depending on the biological source and age.

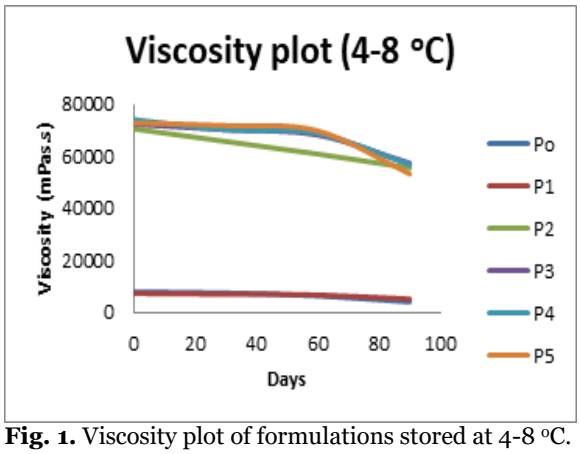

Fig. 1. Viscosity plot of formulations stored at 4-8 °C.

This has initiated the classification of propolis based on colour and thus, green brown and red propolis have been identified (Zhang *et al.*, 2016). The botanical source of Nigerian brown propolis has been identified by Omar *et al.* (2016, and 2017) to belong to *Dalbergia spp* and *Macaranga spp.*

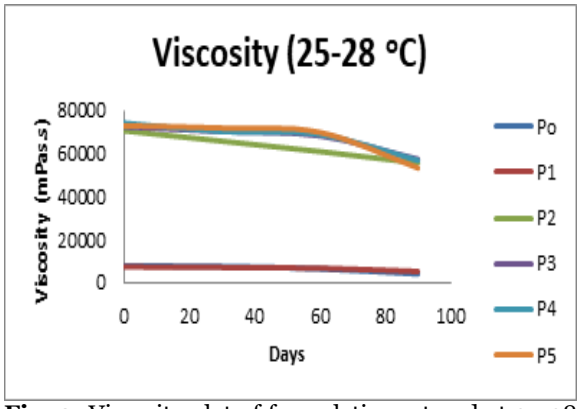

Fig. 2. Viscosity plot of formulations stored at 25-28 °C (room temperature).

The sample of propolis was harvested from bee hives in Edo State, Nigeria in February, 2021. Alaribe *et al.,* (2018) worked on propolis samples from the south east and south west region of Nigeria and found out that the phyto-geographic differences amongst propolis samples in Nigeria have no qualitative influence on the physical properties of the extracts. He further revealed that the extracts had aromatic smell, were waxy or gummy with different shades of brown colour which is consistent with the findings of this index study.

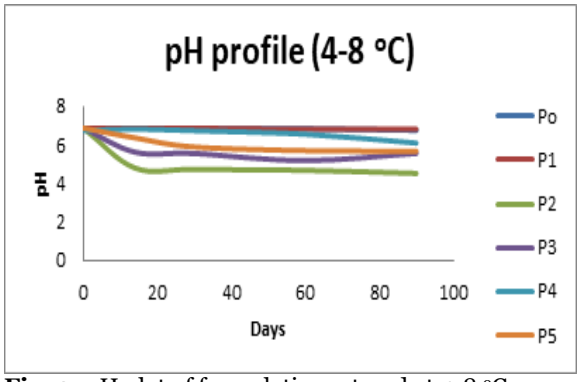

Fig. 3. pH plot of formulations stored at 4-8 °C.

The qualitative evaluations indicated the presence of alkaloids, saponins, phenolics, tannins and flavonoids using the methods described by Trease and Evans (1996) and Silva *et al.,* (1998). The solvent used in the extraction of constituents from propolis (due to varying polarity), the extraction time, time of collection, and variability across geographical locations are factors that influence the type of metabolites present in that particular sample and its corresponding *in vivo and 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 have been the influencing factors.

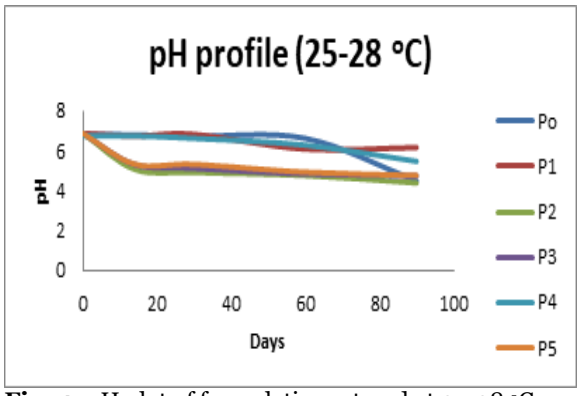

Fig. 4. pH plot of formulations stored at 25-28 °C.

Studies by Alaribe *et al* (2018) on the southeast and southwest 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). This 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) reported a total phenol content of 38.37 mg/g and a total flavonoid content of 15.28 mg/g which are lower than the findings of this research.

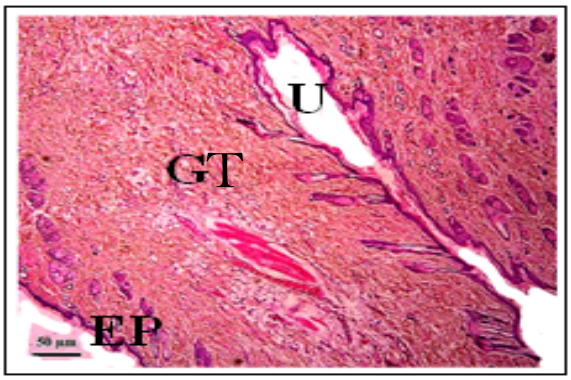

Fig. 5. Histoarchitectural features of excised skin sections at day 7 (Control) x 40.

The homogenous and appealing appearance of the formulations indicated no signs of phase separation, and physical or chemical instability (Arhewoh *et al.,* 2022).

Fig. 6. Histoarchitectural features of excised skin sections at day 7 (P_1) x 40.

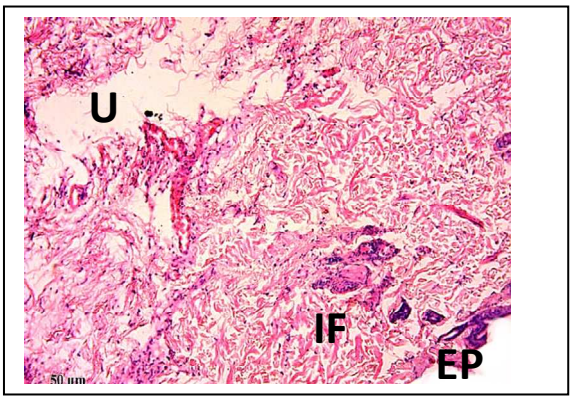

Fig. 7. Histoarchitectural features of excised skin sections at day 7 (P_3) x 40.

The pH of the skin normally ranges from 4 to 6. The acceptable pH range for topical preparations is 6.8 - 7.5 at 25 ^{o}C and this depends on the type of formulation used (Baranda *et al.,* 2000). The chemical stability of pharmaceutical preparations is a criterion for assessing the rate and extent of drug degradation. The spreadability of semisolid formulations, which is the ability of the semisolid preparation to evenly spread on the skin, is an important aspect to consider in administering topical preparations.

The spreadability values refer to the extent to which the formulations readily spread on the application surface by applying a small amount of shear (Vijay *et al.,* 2013). Striking differences in spreadability exist from one batch to the other. Batches P_4 and P_5 had lower spreadability due to the concentration of the polymer in the formulation (2 % w/v). The rheological behavior of the hydrogels revealed that the formulations exhibited pseudoplastic behavior with shear thinning. Formulation P_4 and P_5 had the highest viscosity, lowest spreadability and depth. This is because the preparation was formulated with 2 %w/v polymer solution.

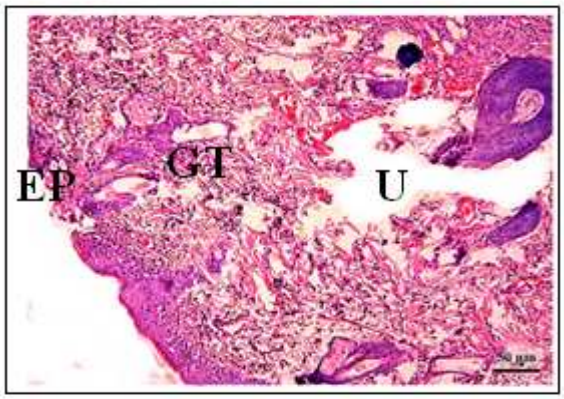

Fig. 8. Histoarchitectural features of excised skin sections at day 7 (P_4) x 40.

Key: EP (epidermis), U (area of ulceration), GT (granulation tissue), A (adnexia), MT (muscle tissue) IF (inflammation infiltrate), EP (epidermis), D (dermis), CT (connective tissue), DP (dermal papillae), OC (oedematous changes), RD (reticular dermis), V (blood vessels), HF (hair follicle), IP (injured epidermis), FT (fibrous tissue).

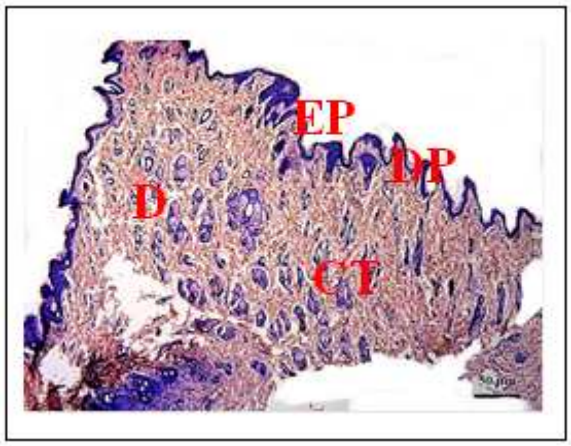

Fig. 9. Histoarchitectural features of excised skin sections at day 14 (Control) x 40.

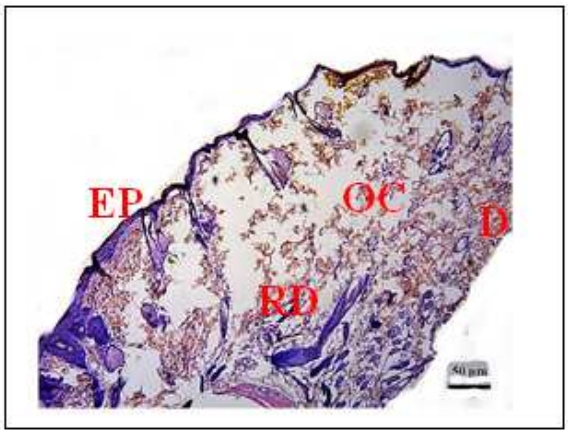

Fig. 10. Histoarchitectural features of excised skin sections at day 14 (P1) x 40.

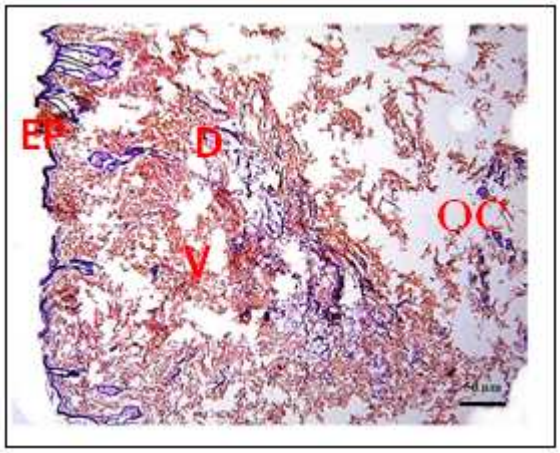

Fig. 11. Histoarchitectural features of excised skin sections at day 14 (P_3) x 40.

The dermal injury was inflicted by cutting off the epidermis with a pair of scissors and forceps and thereafter treated from the second day. Only four propolis (control, P_1, P_3, P_4) formulations were evaluated for their wound healing activity. This is because the activity of P_5 can be explained by what exists among P_1, P_2 and P_3. Healing tissues were harvested on day 7, 14 and 21. Results revealed that the propolis hydrogel induced granulation tissue formation, scar tissue formation, new vascularization, as well as the formation of an extensive network of connective tissue and fibrous tissue stroma. This was similar to the findings of Olczyk *et al.,* (2013) which posited that propolis induces re-epithelization, stimulation of the wound bed matrix modeling and proposed that such activity may be connected with the ability of its flavonoid compounds to reduce lipid peroxidation and to prevent necrosis of cells.

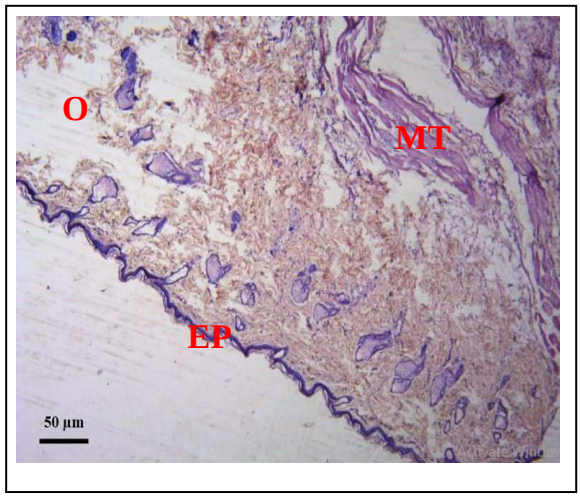

Fig. 12. Histoarchitectural features of excised skin sections at day 14 (P4) x 40.

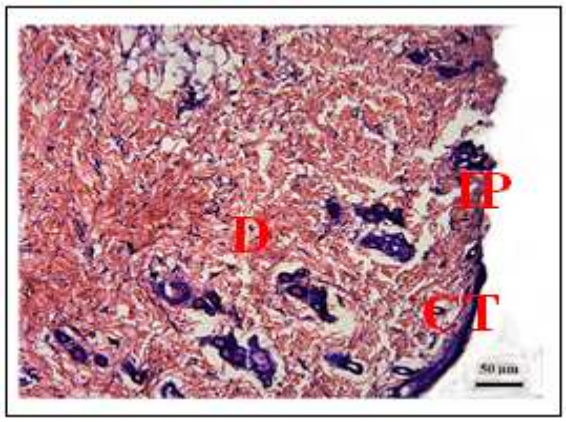

Fig. 13. Histoarchitectural features of excised skin sections at day 21 (Control) x 40.

The anti-inflammatory activity taking place at day 7 prior to healing was cellular response to injury. This is the first response that takes place when there is injury. Macrophages, platelets, lymphocytes and blood cells infiltrate the wounded site as first line response to injury (Chin *et al.,* 2005). This activity begins within 24 hours, and lasts for 2 weeks. While several other cells are implicated in the healing process, the key players are neutrophils, macrophages, and T-lymphocytes (Chin *et al.,* 2005). This accounts for the cellular infiltration and blood vessels observed at day 14. The oedematous changes present at day 14 (symptom of inflammation) were covered up by an extensive network of fibrous tissue stroma at day 21.

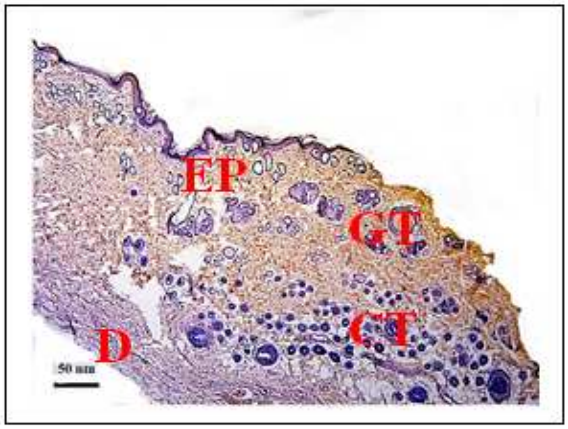

Fig. 14. Histoarchitectural features of excised skin sections at day 21 (P_1) x 40.

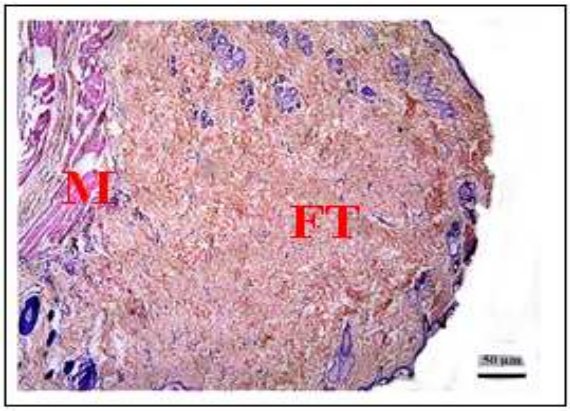

Fig. 15. Histoarchitectural features of excised skin sections at day 21 (P_3).

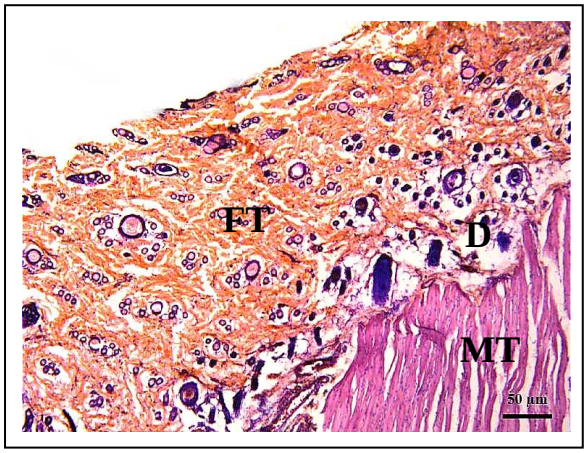

Fig. 16. Histoarchitectural features of excised skin sections at day 21 (P_4) x 40.

Conclusion

The hydrogel had better physicochemical profile when stored in the refrigerator. The anti-inflammatory activity taking place at day 7 was cellular response to injury. The oedematous changes present on day 14 (a symptom of inflammation) were covered up by an extensive network of fibrous tissue stroma at day 21. This study has shown that propolis formulated as hydrogel can be used to induce wound healing, and thus it becomes imperative for further studies to standardize propolis in wound dressings and other topical delivery systems to obtain optimum activity with good physicochemical profile.

Declaration of interest

The authors declare no conflict of interest

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