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Applied Biochemistry

Theme

Formulation of a topical anti-Inflammatory ointment using honey and pumpkin seeds powder "*Cucurbita Pepo*"

Presented by:

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Defended on: 03/07/2024

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Mémoire de Fin de Cycle
En vue de l'obtention du diplôme

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Biochimie Appliquée

**Formulation d'une pommade
anti-inflammatoire topique à base de miel
et de poudre des graines de citrouille
"Cucurbita Pepo"**

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AMMOUR HAMZA

Dedication

"I dedicate this work to the entirety of my existential journey",
encompassing all the diverse circumstances encountered in my life
both the favorable and the challenging."

If I were to deliver a message to you "Dear Reader"

Always strive to make the world a better place.

Invite everyone aboard the journey, make everyone aboard the train.

There is no purpose in a realm where one starves to eat while
another suffers excess!

"It is not about what you have, it is about the value you create from
what you have, there is no happiness greater than that which comes
from sharing it with those who need it". Do not blame the country or
the circumstances, as a small spark can ignite an unquenchable fire.

"Always keep the spark burning within you, for you, for the society
and for the humanity ".

AMMOUR HAMZA

Summary

List of abbreviation

List of figures

List of tables

Introduction.....1

Chapter I: Honey compounds, physico-chemical and biological properties

I. Definition and history.....3

II. Honey compounds.....3

II.1. Sugar 3

II.2. Ash content.....4

II.3. Organic acids..... 5

II.4. Vitamins.....5

II.5. Proteins, proline and amino acids.....6

II.6. Enzymes6

II.7. Carotenoids.....7

II.8. Phenolic compounds.....8

II.8.1.Phenolic Acids.....8

II.8.2. Flavonoids.....9

II.9.Pollen..... 10

III. Physico-chemical properties.....10

III.1. Water content (moisture)10

III.2. Color.....10

III.3. PH and free acidity.....11

III.4. Electrical conductivity (EC)11

III.5. Hydroxymethylfurfural (HMF).....12

IV. Biological properties of honey.....13

IV.1. Antioxidant Activity in honey13

| | |
|--|----|
| IV.2. Mechanism of Honey's Anti-inflammatory Activity..... | 13 |
| IV.3. Anti-microbial activity..... | 14 |

Chapitre II : Pumpkin seeds

| | |
|---|-----------|
| I. Definition..... | 16 |
| II. Botanical Description of Cucurbita pepo..... | 16 |
| III.. Development Cycle of Cucurbita pepo..... | 16 |
| VI. Pumpkins seeds (PS) compounds..... | 17 |
| VI.1. Carbohydrates..... | 17 |
| VI.2. Lipids..... | 17 |
| VI.3. Amino acids..... | 17 |
| VI.4. Minerals..... | 17 |
| VI.5. Phytosterols..... | 18 |
| VI.6. Tocopherols (Vitamin E)..... | 18 |
| VI.7. β -Carotene..... | 18 |
| VI.7. Phenolic compounds..... | 19 |
| VI. Biological properties of pumpkin seeds..... | 19 |
| VI.1. Antioxidant activity..... | 19 |
| VI.2. Antimicrobial Effects..... | 19 |

Part I: Material and Methods

| | |
|--|-----------|
| I.Honey samples..... | 21 |
| II. Physicochemical analysis | 21 |
| II.1. Refractory index, water content and brix degree..... | 21 |
| II.2. PH..... | 22 |
| II.3. Color..... | 22 |
| II.4. Hydroxymethylfurfural (HMF)..... | 23 |
| II.5. Electrical conductivity (EC)..... | 23 |

| | |
|---|-----------|
| II.6. Proteins dosage..... | 24 |
| II.7. Proline..... | 24 |
| II.8. Polyphenols..... | 25 |
| II.9. flavonoids..... | 25 |
| III. Biological activity..... | 26 |
| III.1. Radical scavenging activity | 26 |
| III.2. Reducing power..... | 26 |
| III.3. colorimetric ferrosin based assay..... | 27 |
| III.4. Anti-inflammatory activity..... | 27 |
| IV. Formulation of honey-PSP based ointment..... | 28 |
| IV.1. Pumpkin seeds sample..... | 28 |
| IV.2. Ointment formulation..... | 29 |
| IV.3. Extraction | 30 |
| IV.3.1. Pumpkin seeds powder extracts..... | 30 |
| IV.3.2. Ointments extracts..... | 30 |
| V. Physico-chemical properties..... | 31 |
| V.1. Color..... | 31 |
| V.2. Moisture content..... | 31 |
| V.3. Phenolic compounds..... | 31 |
| VI. Biological activity | 31 |
| VI.1. Antioxidant activity..... | 31 |
| VI.2. Anti-inflammatory | 31 |
| VI.3. Microbiological activity..... | 32 |
| VI.4. Peroxide Index (PI)..... | 32 |
| VI.5. TABARS TEST..... | 33 |
| VII. Skin patch test..... | 33 |
| VIII. Ointment sensory evaluation | 33 |
| IX. Statistical analysis..... | 34 |

Part II: Results and Discussion

| | |
|---|----|
| I. Honey | 35 |
| I.1. Physico-chemical analysis | 35 |
| I.1.1. Brix and Moisture content | 35 |
| I.1.2. PH..... | 36 |
| I.1.3. Color..... | 37 |
| I.1.4. Hydroxymethylfurfural (HMF)..... | 38 |
| I.1.5. Electric Conductivity (EC)..... | 39 |
| I.1.6. Proteins..... | 40 |
| I.1.7. Proline..... | 41 |
| I.1.8. Phenolic compounds..... | 42 |
| I.1.9. Flavonoïdes | 43 |
| I.2. Biological activities of honey | 44 |
| I.2.1. Antioxidant activity..... | 44 |
| I.2.1.1. DPPH radical Scavenging activity | 44 |
| I.2.1.2. Reduction power..... | 45 |
| I.2.1.3. Ferrosin..... | 46 |
| I.2.2. Anti-inflammatory..... | 46 |
| II. Pumpkin seeds powder | 48 |
| II.1. Total Phenolic Content..... | 48 |
| II.2. Total Flavonoids Content..... | 48 |
| II.3. Antioxidants activity: DPPH activity and reducing power..... | 49 |
| II.4. Anti-inflammatory..... | 50 |
| III. Ointment physicochemical, stability and biological activities | 51 |
| III.1. Physicochemical activity | 51 |

| | |
|---|-----------|
| III.1.1 Color..... | 51 |
| III.1.2. Moisture content | 52 |
| II.1.3. PH..... | 53 |
| III.1.4. Total phenolic compounds..... | 54 |
| III.1.5 Total Flavonoid Content..... | 55 |
| III.2. Biological activity of the formulations..... | 56 |
| III.2.1. Stability and antioxidant activity..... | 56 |
| III.2.2. Anti-inflammatory activity | 57 |
| III.2.3. TBARS and peroxide value..... | 58 |
| III.2.4. Microbiological activity: | 62 |
| IV. Skin patch test | 62 |
| V. Sensory characterization..... | 63 |
| V.1. Product Characterization..... | 63 |
| V.2. Discriminant Power by Descriptor | 63 |
| V.3. Model Coefficients | 64 |
| V.4.Product-adjusted averages | 66 |
| VI. External preference Mapping (PREFMAP)..... | 67 |
| VI.1. Principal component analysis (PCA)..... | 67 |
| VI.2.PREFMAP Mappenig (PCA)..... | 68 |
| VI.3. Pearson Correlation..... | 69 |
| Conclusion et perspectives..... | 72 |
| Bibiological References..... | 74 |

Liste of abbreviations

| | |
|---|---|
| Electrical conductivity (EC) | Hydroxymethylfurfural (HMF) |
| Reactive oxygen species (ROS) | Tumor necrosis factor (TNF- α) |
| Cyclooxygenase-2 (COX-2) | Interleukins (IL) |
| Hydrogen peroxide (H ₂ O ₂) | Nitric oxide synthase (iNOS) |
| Triacylglycerol (TAG) | Pumpkin seeds (PS) |
| Pumpkin seed oil (PSO) | Nitric oxides (NO) |
| Nitrogen dioxide (NO ₂) | Refractive index (RI) |
| Optical density (OD) | Ferric iron (Fe ³⁺) |
| Ferrous iron (Fe ²⁺) | Trichloroacetic acid (TCA) |
| Peroxide Index (PI) | Malondialdehyde (MDA) |
| Thiobarbituric acid (TBA) | Prostaglandins E ₂ (PGE ₂) |
| BSA (Bovine Serum Albumin) | 1,2-dimethylhydrazine (DMH) |
| Prostaglandins F ₂ α (PGF ₂ α) | 5-lipoxygenase (5-LOX) |
| | |
| Thiobarbituric acid reactive substances (TBARS) | |
| Intracellular adhesion molecule 1 (ICAM1) | |
| Thiobarbituric acid-reactive substance (TBARS) | |
| Serum glutamic oxaloacetic transaminase (SGOT) | |
| Vascular adhesion molecule 1 (VCAM1) | |

List of Figures

| N | Titles | Page | Page | | |
|-----------|--|-----------|-----------|--|-----------|
| 1 | Honey compounds. | 3 | 46 | Ferrosine activity in honey. | 18 |
| 2 | Phenolic acids in honey. | 8 | 47 | Anti-inflammatory activity in honey. | 19 |
| 3 | Flavonoids in honey. | 9 | 51 | The color of the formulations. | 20 |
| 4 | Honey samples. | 21 | 52 | Moisture content in the formulations. | 21 |
| 5 | Pumpkin seeds powder preparation. | 28 | 53 | pH of the ointments. | 22 |
| 6 | The formulated and Pharmaceutical ointments. | 30 | 54 | TPC in the ointments. | 23 |
| 7 | Brix and water content in honey. | 35 | 55 | TFC in the ointments. | 24 |
| 8 | PH value in honey. | 36 | 56 | DPPH radical scavenging activity in ointments. | 25 |
| 9 | Color of honey. | 37 | 57 | Reducing power activity in ointments. | 26 |
| 10 | HMF content in honey. | 38 | 58 | Anti-inflammatory activity in ointments. | 27 |
| 11 | Electric conductivity of honey. | 39 | 59 | TBA Lipid peroxidation in ointments. | 28 |
| 12 | Proteins content in honey. | 40 | 60 | Peroxide values in ointments. | 29 |
| 13 | Proline content in honey. | 41 | 64 | Discriminant Power by Descriptor. | 30 |
| 14 | Total phenolic content in honey. | 42 | 65 | Model Coefficients of the three Ointments. | 31 |
| 15 | Total flavonoids content in honey. | 43 | 67 | The PCA of the formulated ointments. | 32 |
| 16 | DPPH radical scavenging in honey. | 44 | 68 | PREFMAP Mapping. | 33 |
| 17 | Reducing power in honey. | 45 | 71 | Ointments PC Discriminant Power | 34 |

List of Tables

| | Page |
|--|-----------|
| Table I: Honey samples characterization. | 21 |
| Table II: The Ingredients of the formulation of ointments. | 29 |
| Table III: Discriminant Power by Descriptor. | 63 |
| Table IV: Product-adjusted averages. | 66 |
| Table V: Pearson correlation matrix between physico-chemical parameters, phenolic compounds, and antioxidant and anti-inflammatory activities of ointments. | 69 |

INTRODUCTION

In modern medicine, despite many advances, there is still a lack of effective treatments for wound healing. Demand for natural remedies is increasing in developing countries due to antibiotic resistance, which often exacerbates wound infections and makes them difficult to heal. Natural substances are considered effective, safe and affordable, particularly in low-income countries that suffer from chronic wounds due to factors such as poor hygiene, widespread disease and limited access to medical supplies. Consequently, there is growing interest in the use of natural remedies as affordable and effective alternatives to conventional treatments (**Builders et al., 2016; bardaa et al., 2016**).

Since ancient times, honey has been considered beneficial to health and is often used medicinally. Wound healing was probably its first medical use (as suggested by Sumerian writings dating from 2000 AD). The use of honey as medicine and ointment has continued to this day. Honey's primary role in wound care declined rapidly after the discovery of antibiotics. However, many antimicrobial agents became ineffective in the treatment of wounds due to the emergence of many drug-resistant bacteria and allergic reactions, delaying the healing process, allowing honey to regain its role as an antibacterial agent. In this context, the use of topical agents such as honey should be considered an interesting alternative to systemic antibiotics, when the latter are not deemed essential, given that honey has demonstrated no toxicity in human tissues and can be used both externally on intact skin and as a dressing on wounds. Recently, the use of honey as a medicine has been rediscovered both in vitro studies and in clinical trials (**Boekema et al., 2023**).

Honey-based dressings have demonstrated several therapeutic efficiencies, including anti-inflammatory, antibacterial and antioxidant activity, good biodegradability, biocompatibility, stimulated cell proliferation and re-epithelization, and accelerated wound healing. These properties have been attributed to a combination of different characteristics, hydrogen peroxide (H₂O₂), low pH and high osmolarity, volatile substances, phenols and organic acids. Which maintain a moist wound environment, stimulate autolytic debridement and supply nutrients to the wound bed by exerting antioxidant and anti-inflammatory effects that are considered optimal conditions for fibroblast and keratinocyte activities, such as collagen migration and organization, and promote re-epithelialization (epithelial-mesenchymal transition (EMT) (**Yasin et al., 2023**).

Plants and fruits possess bioactive compounds known for their health benefits for food and medicinal purposes. However, modern diets often prioritize quick and easily accessible food options, resulting in significant food waste. To solve this problem, current research is focusing on the valorization of rejected plant components and using food waste as a beneficial resource, thus promoting sustainability within the industry.

The biochemical constituents of pumpkin seeds and their oxidative stability would contribute to their valorization in the pharmaceutical, cosmetics and food industries. Pumpkin seeds are a rich source of bioactive compounds, including antioxidants, vitamins (notably vitamin E) and minerals such as zinc and magnesium. They are also rich in healthy fats, particularly omega-3 and omega-6 fatty acids. What's more, pumpkin seeds contain significant amounts of phytosterols, such as beta-sitosterol, which contribute to their benefits such as anti-diabetic, antibacterial, antioxidant, anti-inflammatory and cholesterol-lowering properties. These components collectively support heart health, immune function and potentially contribute to wound healing through their role in collagen synthesis and tissue repair (**Bardaa *et al.*, 2016**).

With this in mind, the aim of this study is to formulate a natural (organic) anti-inflammatory ointment incorporating honey and pumpkin seed powder, by evaluating the physico-chemical and biological properties of the three matrices examined: honey, pumpkin seed powder and the ointments produced.

This work consists of three distinct parts:

- The first part summarizes the different bibliographical knowledge on honey; pumpkin seeds.
- The second part describes the equipment and methods used to carry out this study.
- The third part presents a discussion of the results obtained, followed by a conclusion.

PART I
BIBLIOGRAPHIC SYNTHESIS

CHAPTER I
Honey Physico-chemical and
biological properties

I. Definition and history

Honey is a natural sweet substance manufactured by bees (*Apis mellifera* Family: *Apidae*) from both the nectar of plants (blossom honey) or from secretions of living parts of plants or from the excretions (honeydew honey) (**European Commission 2002**).

Honey has been utilized since ancient times (5500 years ago) by ancient cultures including Sumerian, Greeks, Chinese, Egyptians, Romans, and Babylonians, consumed honey both for nutritional aims and for its medicinal properties. Known as the only insect-derived natural product, hold nutritional, cosmetic, therapeutic, and industrial values (**Samarghandian et al., 2017**).

II. Honey compounds

Honey contains a variety of compounds as described in figure 1.

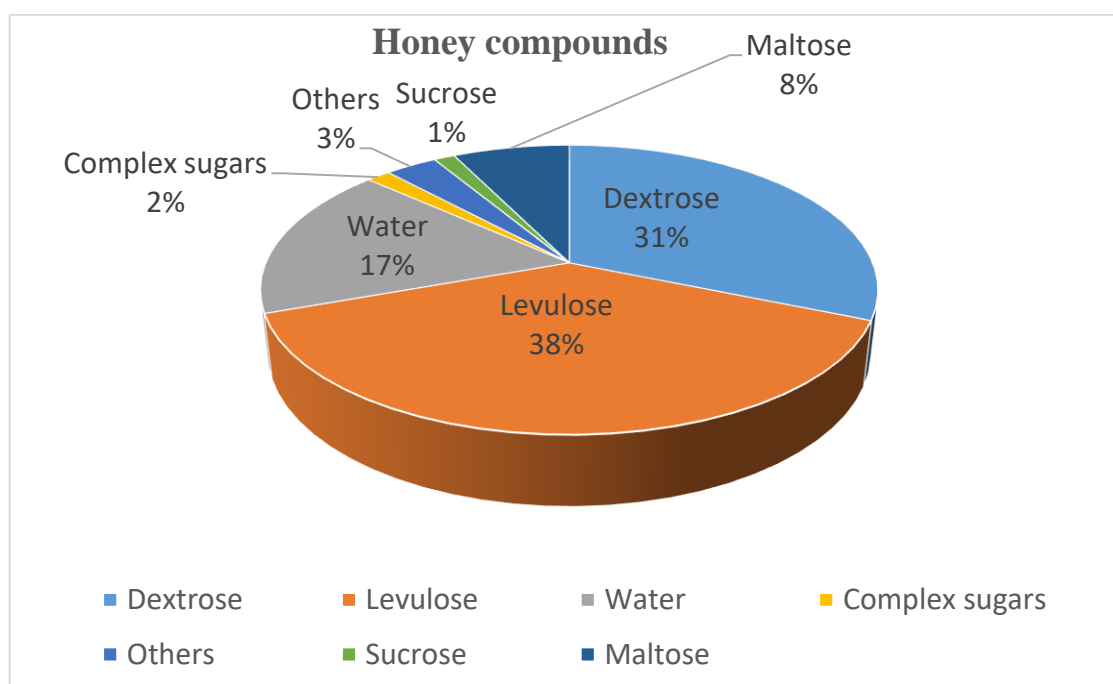


Figure 1: Honey compounds (**Ranneh et al., 2021**).

II.1. Sugar

Sugar are the major component of honey (80–83 %) (**Santos-Beluga and Gonzalez-Paramas, 2017**) representing 60 to 95 % of its dry matter (**Ranneh et al., 2021**). All depends on its botanical origin, which has been identified as the main factor in modulating the carbohydrates ratio, along with geographical origin, climate, processing and storage conditions.

These latter factors are implicated in the production of unwanted compounds generated by the reaction between carboxylic group on the reducing end of sugar and the free amino groups of amino acids and proteins, known as the Maillard reaction (**Escuredo *et al.*, 2014; Da Silva *et al.*, 2016**).

Sugar manifest mainly in the form of glucose and fructose (monosaccharides), which represent 75 % of the overall sugar content in honey (**Da Silva *et al.*, 2016; MiGuel *et al.*, 2017**). Moreover, the enzyme invertase, secreted from hypopharyngeal glands of bees, is involved in the hydrolysis of sugars from nectar, resulting in the production of monosaccharides (fructose and glucose) (**Santos-Beluga and Gonzalez-Paramas, 2017**). Fructose is the predominant sugar with (28-40 %), followed by glucose (20-35 %) (**Ranneh *et al.*, 2021**), (excluding some honey types where the glucose proportion may surpass fructose proportion), along with (10–15 %) disaccharides and small amounts of other sugars (**Da Silva *et al.*, 2016; Miguel *et al.*, 2017**).

Fructose and glucose ratio F/G is used as an indicator to identify monofloral honey. It has been used as an indicator to differentiate between honeydew and nectar honeys. Honeydew honeys generally represent the lowest concentration of glucose and fructose and the highest F/G ratio, except for some nectar honeys, which appear to have high F/G ratio, as is the case with Heather honey (**Santos-Beluga and Gonzalez-Paramas, 2017**).

Moreover; F/G ratio is used as an indicator of crystallization time, honey with a high F/G ratio larger than 1.33 takes a longer time to crystallize, Unlike honey with a lower F/G ratio under 1.11 where the crystallization takes less time (**Escuredo *et al.*, 2014; Santos-Beluga and Gonzalez-Paramas, 2017**).

II.2. Ash content

The mineral content in honey ranges from 0.04 % in light honeys to 0.2 % in dark honeys (**Alqarni *et al.*, 2014**). Potassium is the most abundant element, followed by other macroelements and microelements may be present in honey: magnesium, calcium, iron, phosphorus, sodium, manganese, iodine, zinc, lithium, cobalt, nickel, cadmium, copper, barium, chromium, selenium, arsenic, and silver (**Da Silva *et al.*, 2016; Miguel *et al.*, 2017**).

The content of trace elements present in honey depends on the type of soil and plant location, and may indicate the botanical origin of a specific honey (**Escuredo *et al.*, 2013; Alqarni *et al.*, 2014; Da Silva *et al.*, 2016**).

II.3. Organic acids

Organic acids represent a small fraction of the total components of honey used to discriminate the honeys based on botanical and/or geographical origin. Despite their low quantity, organic acids play an important role in shaping honey properties such as color, flavor, acidity, pH, electrical conductivity, antimicrobial and antioxidant activities (**Da Silva et al., 2016; Pauliuc et al., 2020**). More than 30 organic acids are produced from sugars by nectar transformation into honey mediated by different enzymes secreted by honeybees or directly from nectar (**Da Silva et al., 2016; Santos-Beluga and Gonzalez-Paramas, 2017**).

Organic acids can be used as indicators of aerobic or anaerobic fermentation in honey. High organic acids levels occur in honey during the fermentation process, considered as marker of fermentation, which affect the quality of honey. Many authors have reported the increase of acidity during fermentation and over time, resulting from yeasts activity through the transformation of honey sugars and alcohols into acids (**Cavia et al., 2007**), including gluconic (the most abundant), aspartic, butyric, citric, acetic, formic, fumaric, galacturonic, gluconic, glutamic, glutaric, butyric, glyoxylic, 2-hydroxybutyric and α -hydroxyglutaric acids (**Miguel et al., 2017; Boussaid et al., 2018**).

Gluconic acid is the predominant acid in honey originated from glucose oxidase which determine gluconic acid quantity produced by honeybees during ripening (**Karabagias et al., 2014**). Besides gluconic acid, citric acid is also present in honey, and the concentration of these two substances is used as a reliable parameter to differentiate floral honey from honeydew honey. The content of citric acid in floral honey is notably lower than honeydew honey (**Da Silva et al., 2016**).

II.4. Vitamins

Honey contains small amounts of vitamins, especially the vitamin B complex, which stems from pollen grains in suspension (**Ciulu et al., 2011**), including thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), biotin (B8 or H) and folic acid (B9) (**Miguel et al., 2017**).

These vitamins present in honey are preserved due to the low pH of honey (**Bonté and Desmoulière, 2013**). The removal of pollen from honey, and the oxidation of ascorbic acid by the hydrogen peroxide produced by glucose oxidase, leads to the loss of its vitamins content. (**Ciulu et al., 2011**).

Vitamin C was found in almost all types of honey and has been evaluated mainly due to its antioxidant effect, however the determination of vitamin C is an unstable indicator, due to its chemical and enzymatic oxidation vulnerability which makes it susceptible to change subjected by several factors such as light, oxygen or heat (**Da Silva *et al.*, 2016; Santos-Beluga and Gonzalez-Paramas, 2017**).

II.5. Proteins, proline and amino acids

Proteins concentration in honey stand between (0.2-0.7 %), with molecular weight ranging from 22 to 75 KDa, the concentration varies from one species of honeybee to another. Depends on the honey species, honeydew, nectar and pollen (**Santos-Beluga and Gonzalez-Paramas, 2017**).

The proteins and amino acids present in honey derive from both animal and plant sources, which include nectar, pollen, and rarely from the sap of plants. However, they frequently originate from the secretions of cephalic glands of bees during the enzymatic decomposition of pollen and nectar (**Da Silva *et al.*, 2016; Santos-Beluga and Gonzalez-Paramas, 2017**).

This process leads to the formation of proline (honey contains 50-85 % of proline from the total amino acids composition), which manifests as the predominant amino acid in Honey and pollen (considered the major source of proteins), followed by glutamic acid, alanine, phenylalanine, tyrosine, leucine and isoleucine. Proline is often utilized as a key indicator to assess the maturity of honey and to detect possible adulteration with sugar; the established threshold for pure honey is a minimum of 180 mg/kg of proline (**Escuredo *et al.*, 2013**).

II.6. Enzymes

The enzyme content in honey depends on the physiological period of the colony, the time and amount of nectar collection, and pollen consumption (**Yapici *et al.*, 2023**). Only small fractions of the proteins present in honey are enzymes, but this does not exempt it from being an important element; they play a significant role in honey properties and nutritional value. Enzymes catalyze the conversion of nectar into honey, considered an important criterion in evaluating honey quality.

Honey contains four main enzymes: diastase (amylase), invertase (sucrase), glucose oxidase, catalase and acid phosphatase, originated from different sources including the honeybees, nectar, pollen grains and microorganisms, its Biochemical reactions can be divided into two types: enzyme-catalyzed and non-enzymatic reactions (**Alshareef *et al.*, 2022; Yapici *et al.*, 2023**)

Enzyme-catalyzed reactions in honey are known to affect its quality and biological activities, including the conversion of sucrose and maltose to glucose and fructose by diastase and invertase. Glucose conversion to gluconic acid and hydrogen peroxide by glucose oxidase.

Moreover, hydrogen peroxide breaks down to water and oxygen by catalase enzymes. Honey samples that exhibit low catalase activity and high glucose activity are recognized to have a high hydrogen peroxide concentration, reflecting its efficacy in wound healing.

Furthermore, other enzymes in honey and honeybee products are characterized by proteases, glucosylceramidase and acid phosphatase enzymatic reaction.

Classic non-enzymatic reactions in honey include the production of HMF from glucose or fructose diacarbonyl molecules such as methylglyoxal from the dihydroxyacetone of nectar, transglycosylation and the formation of amino sugars (Maillard reaction) (**Alaerjani *et al.*, 2022**).

II.7. Carotenoids

Carotenoids are a class of natural pigments classified under tetraterpenoids, containing 40 carbon atoms and characterized by their long polyene chains with alternating conjugated double bonds. Carotenoids are broadly divided into two categories: hydrocarbon carotenes and oxygenated carotenoids, also known as xanthophylls.

Among the approximately 600 naturally occurring carotenoids, the major provitamin A forms are the α , β , and γ isomers.

Carotenoids have garnered significant scientific interest due to their notable properties, providing significant added value to foods associated with its antioxidant anti-cancer activity, photoprotection, cardiovascular disease protection and anti-inflammatory activity(**Anand *et al.*, 2022**).

II.8. Phenolic compounds

Phenolic compounds are a chemically heterogeneous group, comprising approximately 10,000 compounds (Da Silva *et al.*, 2016), based on carbon chain classification there are 16 classes of phenolic compounds (Zawawi *et al.*, 2021). Phenolic compounds are plant-derived secondary metabolites produced by plants to provide protection against stress and oxidative damage; they are transferred into honey through nectar. Phenolic compounds in honey are divided into two distinguished classes non-flavonoids (phenolic acid) and flavonoids (flavones, flavonols, flavanones, flavanols, anthocyanidin, isoflavones and chalcones) (Da Silva *et al.*, 2016; Ciulu *et al.*, 2016). The contribution of phenolic compounds to the antioxidant activity of honey varies among honey types and geographical origins (Zawawi *et al.*, 2021), and they have been used as phytochemical markers for the floral origin (Ranneh *et al.*, 2021).

II.8.1. Phenolic Acids

Phenolic acids are organic compounds that possess at least one carboxylic function and one hydroxyl group (Pandey and Rizvi, 2009). Two sub-groups can be distinguished: hydroxybenzoic acids, such as salicylic acid, and hydroxycinnamic acids, like caffeic acid (Bruneton, 2008). These compounds exhibit antibacterial, antifungal, and antioxidant properties (Figure 2). Caffeic, abscisic, syringic, benzoic, vanillic, and gallic acids have been identified in honey (Tahir *et al.*, 2017).

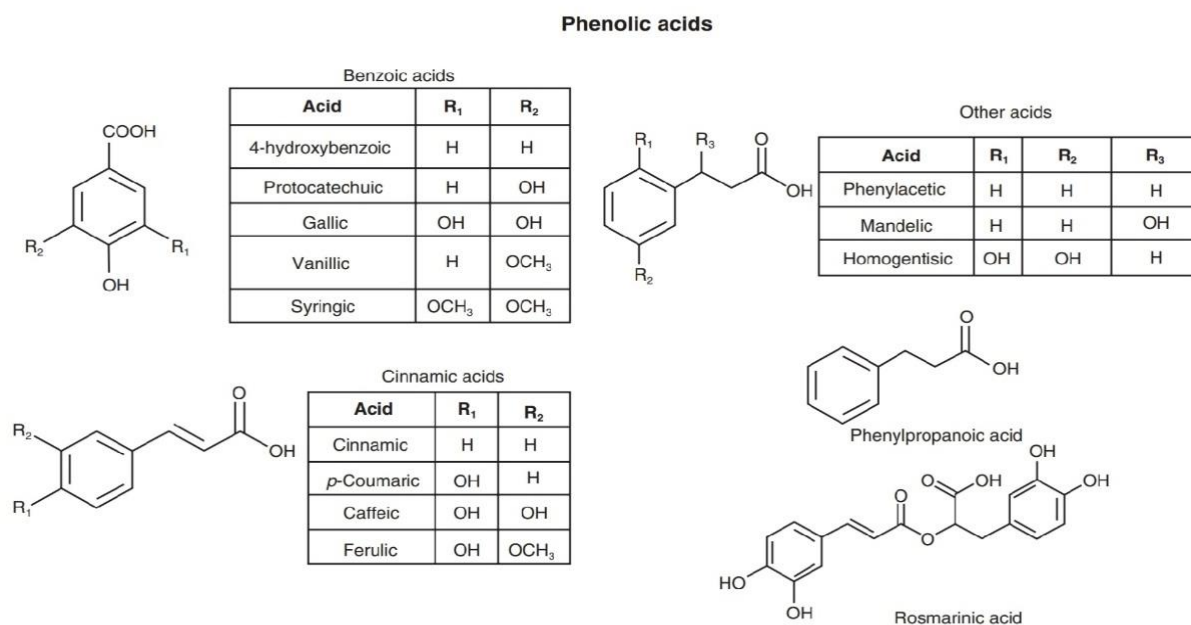


Figure 2: Phenolic acids in honey (Santos-Beluga and Gonzalez-Paramas, 2017).

II.8.2. Flavonoids

Flavonoids are pigments widely distributed in the plant kingdom and contribute to the coloration of honey. They possess very interesting antioxidant properties, helping the neutralize of free radicals in the body. The quantity and type of flavonoid vary depending on the floral source. Generally, the darker the honey (such as those from sunflower and honeydew), the richer it is in flavonoids (**Rossant, 2011**). Among the flavonoids identified in honey are catechin, quercetin, chrysin, galangin, kaempferol, and apigenin (**Figure 3**) (**Tahir et al., 2017**).

Flavonoids are the main active functional components in honey, contributing to its total antioxidant activity and bringing beneficial effects for human health (**Alvarez-Suarez et al., 2012**), the capacity of flavonoids depends mostly on the number and position of hydroxyl groups and other substituents, as well as the glycosylation of flavonoid molecules (**Ranneh et al., 2021**).

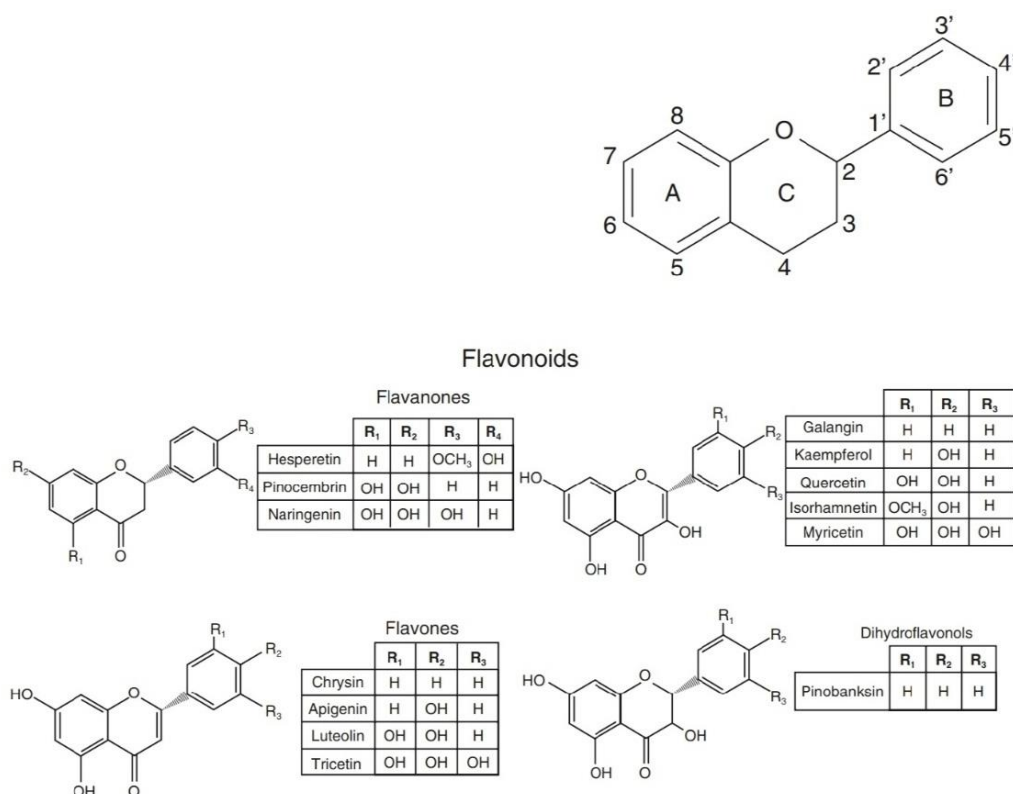


Figure 3: Flavonoids in honey (Santos-Beluga and Gonzalez-Paramas, 2017).

II.9. Pollen

In seed plants, pollen is the mobile male fertilizing element produced by the flower. These are tiny grains (20 to 55 μm in diameter) with highly variable shapes depending on their floral origin, initially contained in the anther at the tip of the stamens. Pollen has a high protein content (20 to 40 %). It contains approximately 36 % carbohydrates, 11 % water, 5 % lipids, and 3 % mineral salts (K, Mg, Ca, Fe, etc.). It also contains pigments such as carotenoids and some vitamins (B, C, D, E, and A) (Le Conte, 2002).

III. Physico-chemical properties

III.1. Water content (moisture)

Honey contain an average of 17 % water. The variation of honey's moisture content has been attributed to factors such as the botanical/geographical origin of nectar, soil and climatic conditions, extraction, processing, storage conditions, season of harvesting, the nectar flux intensity, maturation degree and manipulation during period of harvest (Patruica *et al.*, 2022). Furthermore, water content playing a significant role in honey physical properties such as maturation viscosity and crystallization (Patruica *et al.*, 2022).

The **Codex Alimentarius standard** set the moisture content of honey to be below 20%, the high water content generate a suitable environment for fermentation, yield to yeast and bacterial proliferation caused by low osmotic pressure.

Furthermore high sugar concentration alleviates free water content by lowering the water activity. Free water value considered a better parameter than honey moisture to estimate the preservation of honey quality. Conversely, very low water content can make the honey difficult to handle and process (Bogdanov, 2011; De-Melo *et al.*, 2018)

III.2. Color

The color of honey varied between white (rape honey), extra light amber (sunflower honey, thyme and polyfloral), and light amber (mint and raspberry honey). (Bogdanov, 2011; Pauliuc *et al.*, 2020).

Color is one of the honey features that influences consumer choice, often related to consumption patterns and used to judge its quality (Pita-Calvo and Vázquez, 2017; Pascual-Maté *et al.*, 2018; Pauliuc *et al.*, 2020).

The color of honey is a sensory parameter that varies from one honey to another due to variation on its components such as mineral content, phenolic compounds (mainly flavonoids content), storage, thermal processing, botanical origin, climate and soil conditions. Some authors have reported that pollen, sugars-related products, carotenoids, xanthophylls, anthocyanins, amino acids, enzymatic reactions, and crystallization contribute to honey's color (De-Melo *et al.*, 2018; Pauliuc *et al.*, 2020).

Color changes of honey may occur slowly during storage or rapidly during thermal treatment, due to the Maillard reaction (Sakač *et al.*, 2019). Many researchers have found that honey with darker color possess a higher mineral content, dextrin, acidity and polyphenol contents than light honey. Granulated honey tends to appear lighter in color than liquid honey, where finer crystals always give a paler appearance. (De-Melo *et al.*, 2018).

III.3. pH and free acidity

The pH is related to its stability and shelf life, serving as a useful indicator for possible microbial contamination. Typically, the pH values of honey range between (3.5 and 5.5), influenced by the presence of organic acids, particularly gluconic acid, and inorganic ions such as phosphate and chloride. Processing and storage conditions can alter honey's pH, affecting its texture, stability and shelf life (Pauliuc *et al.*, 2020; Polak-Sliwinska and Tanska, 2021).

The Codex Alimentarius Committee on Sugars (2001) permits a maximum value of 50.00 meq/kg of free acidity in honey. Higher values may indicate fermentation of sugars into organic acids, especially in honey with higher water content (Polak-Sliwinska and Tanska, 2021). However, the presence of various organic acids, geographical origin, and harvest season can influence the acidity of honey (Da Silva *et al.*, 2016).

III.4. Electrical conductivity (EC)

Electrical conductivity (EC) represents the capacity of honey to carry the flow of an electric current. The EC of honey ranges from 0.038 to 0.641 mS/cm (Codex Alimentarius Standard for Honey, 2001). It is directly related to the botanical origin and correlated with mineral content, inorganic ions, pH, proteins, sugars, polyols, pollen grains and somehow to organic acids (De-Melo *et al.*, 2018), EC is used as parameter to differentiate between blossom honey and honeydews (Guerzou *et al.*, 2021).

According to the current European regulation, the EC of honeydew honey and chestnut honey must be higher than 0,8 mS/cm, while nectar honey must be less than 0,8 mS/cm (De-Melo *et al.*, 2018).

III.5. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) is a six-carbon heterocyclic organic compound containing both aldehyde and alcohol (hydroxymethyl) functional groups (Shapla *et al.*, 2018). The HMF content is a chemical parameter that can be used to study the degree of freshness of honey and consequently its degree of deterioration. (De-Melo *et al.*, 2018), HMF is formed during two reactions; Acid-catalyzed degradation of hexose and decomposition of 3-deoxyosone in the Maillard reaction (Shapla *et al.*, 2018).

HMF is easily formulated at low temperatures under acidic conditions. However high heat processing temperature, prolonged storage, adulteration with commercial sugars, use of metallic containers, pH, bee species, high acidity, moisture content, sugars (mainly fructose), amino acids (such as alanine) and minerals (such as magnesium, manganese, iron and zinc) increase HMF production to a large extent (De-Melo *et al.*, 2018).

Commercial honey is subjected to heat processing to eliminate microorganisms, such as osmophilic (sugar-tolerant) yeasts, thereby preventing spoilage, minimizing the risk of fermentation during storage, reducing the honey's moisture content to a safe limit, and delaying crystallization. However, this processing leads to the formation of HMF besides other alteration, including a reducing in the enzymatic activity of diastase as side effect (Scepankova *et al.*, 2024). Honey samples had an HMF content varied from minimum of 8.26 mg/kg (sunflower honey) and a maximum of 50.8 mg/kg (thyme honey) (Pauliuc *et al.*, 2020).

The Codex Alimentarius Standard commission has set the maximum HMF content in honey at 40 mg/kg (with a higher limit of 80 mg/kg for honeys originating from tropical regions). In most previous studies, HMF has been reported to have negative effects on human health. However, in more recent extensive studies, HMF has been proved to have a wide range of positive effects, such as antioxidative, anti-allergic, anti-inflammatory, anti-hypoxic and anti-hyperuricemic effects, it has been reported that humans may ingest between 30 and 150 mg HMF daily via various food products, although safe levels of HMF consumption are not well clarified. (Pita-Calvo and Vázquez, 2016).

IV. Biological properties of honey

Honey is a source of several bioactive components, possessing a wide range of biological activities such as antioxidant, anti-inflammatory and anti-microbial activities.

IV.1. Antioxidant Activity in honey

Honey is an anti-oxidant with different compounded especially phenolic compounds, such as quercetin, hesperetin, chrysin, and the Maillard products called melanoidins, which have the capacity to reverse the oxidation affect. The phenol quercetin directly inhibit cellular transcription factors' activities surpassing the phosphorylation and activation process minimizing the production of free radicals (Tafere, 2021).

IV.2. Mechanism of Anti-inflammatory Activity in honey

Honey is used to treat infected wounds and inflammations while promoting the regeneration of dead tissues that provide a conducive environment for microorganism growth (Song and Salcido, 2011; Benhanifia *et al.*, 2011). Glucose oxidase is a key component of honey that contributes to its anti-inflammatory activity. Added by bees during honey production, this enzyme is responsible for producing hydrogen peroxide (H₂O₂) and converting glucose into gluconic acid, making honey very acidic and thus reducing the risk of infection (Weston, 2002; Bang *et al.*, 2003; Adams *et al.*, 2008).

Honey inhibits the synthesis of prostaglandins responsible for the heat and pain associated with inflammation (Kassim *et al.*, 2010). It also inhibits COX-2 and the formation of (ROS), thereby promoting healing. Additionally, honey prevents the expression of inducible nitric oxide synthase (iNOS) and the production of matrix metalloproteinase-9 in keratinocytes (Zare *et al.*, 2007; Leong *et al.*, 2012; Hussein *et al.*, 2012; Majtan *et al.*, 2013).

Woo *et al.* (2005) and Palmieri *et al.* (2012) demonstrated that chrysin in honey plays an effective anti-inflammatory role. This compound reduces COX-2 expression by inhibiting the synthesis of the nuclear factor of this pro-inflammatory enzyme.

IV.3. Anti-microbial activity

Antibiotics are known medications that attack bacteria directly or indirectly by using energy inside the cell. Honey's anti-microbial activity characterized by four pathways; Dehydration of germs by osmotic pressure from high sugar content and low water activity, high acidity (low PH), organic acid and volatiles compounds, low protein content, high carbon to nitrogen ratio, low redox potential due to the high level of reducing sugars, a viscosity that limits dissolved oxygen and other chemical (**Samarghandian *et al.*, 2017; Edo *et al.*, 2023**)

These factors enhance the anti-bacterial activity of hydrogen peroxide, glucose oxidase, and catalase, preventing germs proliferation (**Edo *et al.*, 2023**).

CHAPTER II

Pumpkin seeds “*Cucurbita pepo*”

I. Definition

The *Cucurbita*, is an important plant widely cultivated as a commercial crop around the world. It is known for its high nutritional value and health protective properties, which have led to increased interest in recent years. The *Cucurbita* genus includes five cultivated species: *C. maxima*, *C. moschata*, *C. pepo*, *C. ficifolia*, and *C. mixta* (Salehi *et al.*, 2019). Among the 50 common varieties of *Cucurbita* worldwide, there are two general categories: pumpkins and squashes, which are economically important species cultivated globally. The species *C. pepo* including gourd, pumpkin, squash, acorn squash, marrow, and summer squash, has the greatest economic value (Naik *et al.*, 2015). Known for its edible fruits and seeds (Rajasree *et al.*, 2016).

II. Botanical description of *Cucurbita pepo*

The term "pumpkin" derives from "citrus," indicating a yellow color. *C. pepo* is divided into six different varieties based on the color of the fruit (Pitrat and Foury, 2015). *C. pepo* is popularly known as "Kraa" (Afshari *et al.*, 2020). This annual herbaceous plant has very long creeping stems, a shallow branched root system, and is characterized by branched tendrils and large (5 to 10 cm) pentamerous, unisexual, gamopetalous, yellow flowers. The leaves, with five more or less distinct lobes, are hairy, long-petiole, and covered with stiff hairs. The fruit is a large, fleshy berry containing numerous seeds within a spongy pulp. The seed is flattened and whitish, narrowing to an oblique neck at one end and bordered by a rounded ridge (Ratnam *et al.*, 2017).

IV. Development Cycle of *Cucurbita pepo*

The seeds germinate 5–7 days after sowing. The plants develop an extensive fibrous root system. Flowering begins 35–60 days after emergence and is more or less continuous. Pumpkin fruits are picked when they are nearly or fully mature, 4 to 6 weeks after flowering, and are harvested in several rounds until the harvest ends, 90–180 days after planting. The pumpkins reach maturity in approximately 90 to 120 days. Harvesting occurs four months after sowing. Pumpkins are harvested from September to October. Sometimes, harvesting can begin from mid-August to early September, requiring good handling and storage of the pumpkins (Ahmad and Khan, 2019).

V. Pumpkins seeds (PS) compounds

V.1. Carbohydrates

Pumpkin seeds (PS) contain variable amount of carbohydrates, which makes it a valuable constituent in food formulations. Carbohydrate content varied from one variety to another, ranging from 18% to 25%. PS can be used in formulation of various bakery products like cake, cookies, bread, etc. (Singh and Kumar, 2024).

V.2. Lipids

Lipids are the predominant compounds in PS (35%- 50%), including essential and non-essential fatty acid. The composition of triacylglycerol (TAG) species within the pumpkin seed oil (PSO) is crucial for understanding their physical and functional properties (Sing and Kumar, 2024). Unsaturated fatty acid composed 80.65 % of total fatty acids beside 19.35 % of saturated fatty acids (Gutierrez, 2016). *C.pepo* species are rich in palmitic, stearic, oleic and linoleic acid, belonging to the omega w-6 and omega w-3 family, which exert remarkable nutritional value and play important roles in many metabolic pathways (Lestari and Meiyanto, 2018).

V.3. Amino acids

PS's proteins content varies from one variety to another, ranging between (25–37 %). Amino acids play a pivotal role as protein-building units. PS contain all the 9 essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine along with other non-essential ones, which act as intermediates in the metabolism process and are essential for physiological functions of the human body growth, and repair of damaged cells (Singh and Kumar, 2024).

V.4. Minerals

Pumpkin seeds contain 3–5 % ash, varying slightly depending on pumpkin variety and climate. PS contain diverse important minerals : P, Mg, K, Zn, Fe, Ca, Na, Mn, Se and Cu, which exhibit different biological functions, acting as cofactors in enzymes activities which making PS nutritionally superior and playing an important role in neurological, muscles and cardiovascular system functions. Minerals are also required in minor amounts for regulating. (Singh and Kumar, 2024).

V.5. Phytosterols

Plant sterols, or phytosterols, are naturally occurring compounds in plants that have a structure similar to cholesterol. These substances are not produced in mammals and must be obtained entirely through dietary sources. The most prevalent phytosterols are β -sitosterol, campesterol, and stigmasterol.

β -Sitosterol is the most abundant plant sterol in the human diet. It is also the major component of several traditional medicines effective in prostate therapy, anti-cancer, analgesic properties, decrease blood cholesterol, anti-inflammatory, hepatoprotective, anti-tumor, antioxidant, anti-diabetic, cardioprotective activities, analgesic properties, decrease blood cholesterol and regulate numerous cell-signaling pathways, all while exhibiting minimal toxicity. (Loizou *et al.*, 2009; Lomenick *et al.*, 2015)

V.6. Tocopherols (Vitamin E)

Tocopherol is substantial active substance in seeds and oils that helps maintain normal metabolism in the body, as well as vascular and central nervous systems functions. PS contain high amount of tocopherol portion (α , β , γ and δ), which provides stability to seeds and oil, making them a typical source for high-quality edible oil. α -Tocopherol plays an important role in regulating gene transcription and helps regulate cells proliferation, differentiation, cell adhesion, immune response etc. It possesses anti-oxidative, anti-inflammatory properties and play role in preventing DNA damage. (Singh and Kumar, 2024).

In a study conducted by Ryan *et al.* (2007) reported that Pumpkin seeds have the greatest content of tocopherols more than all others food that have been tested in that study.

V.7. β -Carotene

β -Carotene (C₄₀H₅₆) is an deoxygenated isoprene-containing compound, reflecting an distinctive color provided by double bonds. β -Carotene possess cyclic rings in both extremities (Anand *et al.*, 2022).

Carotenoids undergo cis \leftrightarrow Trans isomerization in polar environments yet during isolation, cis-isomers are typically the predominant form. β -Carotene is acknowledged as a functional food component due to its numerous therapeutic and preventive benefits (Anand *et al.*, 2022). β -Carotene is the precursor of vitamin A, It holds vital role in cell differentiation, immune response and photosynthesis, β -Carotene helps in maintaining the metabolic functions of glucose and play protective role against diabetes (Singh and Kumar, 2024).

V.7. Phenolic compounds

Flavonoid and phenolic compounds displays antioxidant properties and boost the immune system. Phenolic compounds like kaempferol, *p*-coumaric acid, ferulic acid, apigenin, quercetin, vanillic acid, *p*-hydroxybenzoic acid vanillin, tyrosol, luteolin and sinapic acid, were found in PS (Syed *et al.*, 2019; Singh and Kumar, 2024).

VI. Biological properties of pumpkin seeds

VI.1. Antioxidant activity

PSO has been proven to contain high antioxidant vitamins like tocopherol and carotenoid exerting an oxidative stability (Abou Seif, 2014).

Yasir *et al.* (2016) reported that extract of PS exhibited antioxidant and genoprotective effects.

Gupta *et al.* (2018) reported that γ -tocopherol is more efficient against damage related to nitrogen oxides exposure. Nitric oxides (NO) can be oxidized to relatively more reactive species like nitrogen dioxide (NO₂), peroxy nitrite radical at an accelerated rate within the cell membrane, playing a role against toxic substances and enter in the process of free radicals neutralization.

EL-Boghdady, (2011) reported in rats that pumpkin seeds protects the small bowel from destruction induced by methotrexate due to anti-inflammatory and anti-oxidative activities.

Flavonoids and phenolic compounds in PS play an important role as antioxidants, by scavenging the free radicals and eliminating (ROS), β -Sitosterol acts physically as membrane stabilizers and chemically as a potent radical scavenger which inhibit the cell signaling pathways and act as anti-cancerous agents (Singh and Kumar, 2024)

VI.2. Antimicrobial Effects

The major cause of deaths of many people is the bacteria, parasites, viruses and fungi that develop many diseases and lead to death despites of the hygienic environment and foods. The pumpkin seed oil contains antimicrobial components inhibit the growth of *Aeromonas veronii*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella Typhimurium*, and *Staphylococcus aureus* (Syed *et al.*, 2019).

PART II
Experimental part

PART II-1
Material and Methods

This study aims to establish a comparison between two honey samples properties and biological activity, followed by a comparison between mixtures of each honey's sample incorporated with pumpkin seeds powder to form honey-pumpkin seeds based ointment. The study aims to set a comparison between two pharmacological medicine and honey-pumpkin seeds based, to investigate its potential as a candidate for wounds healing.

I. Honey samples

The current study was conducted in the University of bejaia, on two honey's samples of the year 2023, which has been harvested from Boussada and Bejaia, respectively (Figure 4 and table I). Room temperature, low humidity, darkness (away of lights) are conditions that have been respected during samples storage.

Table I: Honey samples characterization.

| Sample | Consistency | Harvest region | Harvest period | color |
|--------|----------------|----------------|----------------|--------|
| H1 | Liquid/viscous | Boussada | March 2023 | Brown |
| H2 | Crystallized | Bejaia | November 2023 | Yellow |

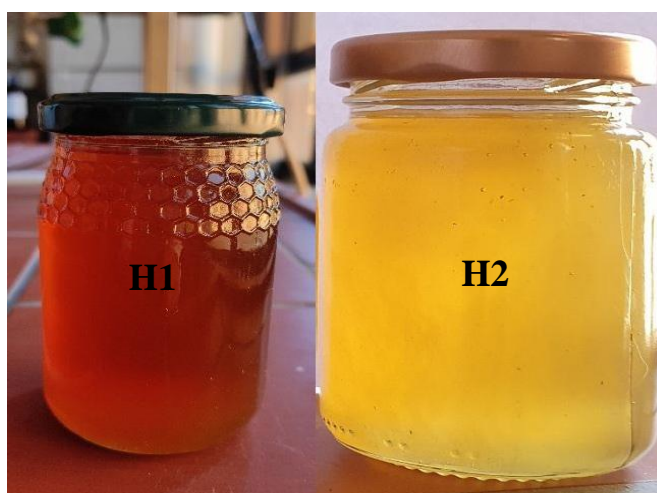


Figure 4: Honey samples.

II. Physico-chemical analysis

II.1. Refractive index (RI), water content and Brix degree

The refractive index (RI) is an optical property that varies between 1.504 and 1.4815, increasing when solid content is high or water content is low. The (RI) expressed the percentage in weight of dissolved solids in a sugar solution at 20 °C. (De-Melo *et al.*, 2018).

Brix refractometry method was conducted to measure the sugar and water content of each honey sample (**Shiddiq et al., 2018**), using a standard model Abbe type refractometer at 20 °C, measuring directly the brix percentage correspond to water content and refractive index (RI), water content (%) was then obtained from the Chataway table (**Baloš et al., 2020**).

The determination was conducted using the refractometer method outlined by **Bogdanov et al. (1997)**. The measurement was conducted using, an Abbe refractometer after calibration using distilled water. Subsequently, a thin layer of liquid honey was spread over the prism of the device. After adjustment, the reading was taken through the refractometer's eyepiece.

The boundary separating the two clear and dark zones appeared within the field of vision. This line intersected two vertically graduated scales, indicating the percentage of brix and the refractive index, respectively.

II.2. pH

The pH measurement was conducted using a pH meter at 25 °C, to indicate the acidity of honey. pH meter consists of a probe containing two electrodes: a reference electrode and a glass electrode that indicated pH of the solution. The pH was measured according to the method described by **Bogdanov et al. (1997)**. The pH meter electrode was first rinsed with distilled water and then immersed in a 10 % (w/v) prepared honey solution. The pH value was then read on the device's screen.

II.3. Color

The color of honey is relatively linked to its richness in pigments, including flavonoids and carotenoids. The lighter the honey, the fewer pigments it contains, and vice versa (**Blanc, 2009**). The color intensity of honey was measured as follows: 1g of honey was dissolved in 4 mL of distilled water and homogenized using a magnetic stirrer. The absorbance was measured at 450 nm using a spectrophotometer to identify the optical density (OD) (**Bogdanov et al., 1997**).

II.4. Hydroxymethylfurfural (HMF)

(HMF) is a natural degradation product of sugars in an acidic medium. Its level increases with rising temperatures. It serves as an indicator of honey's freshness and overheating. A honey solution (20 %, w/v) was mixed with 1 mL of Carrez I solution (potassium hexacyanoferrate 15 %, w/v) and 1 mL of Carrez II solution (zinc acetate 30 %, w/v). The mixture was adjusted to 50 mL with distilled water. The solution was filtered through a Whatman paper, discarding the first tens of milliliters of filtrate. A volume of 5 mL of filtrate was added to 5 mL of distilled water (sample tube); the same volume of filtrate was added to 5 mL of 0.2 % sodium bisulfite for the sample blank. The absorbance was measured at 286 nm and 336 nm using a UV spectrophotometer. If the absorbance exceeded 0.6, dilution was recommended (**Bogdanov et al., 1997**).

The HMF content was expressed in mg/Kg and was calculated using the following the equation:

$$\text{HMF (mg/Kg)} = (\text{A284}-\text{A336}) \times 149.7 \times 5/\text{W}$$

- **A284 and A336** : Absorbance at 284 nm et 336 nm.
- **49,7** : Constant. **W**: Honey sample weight. **5**: Sample weigh

II.5. Electric Conductivity

According to the method described by **Bogdanov et al. (1997)**, the (EC) of honey was measured using a conductivity meter. The principle involves immersing the device's probe in an aqueous solution of dry matter. This method helps differentiate between honeydew honeys (> 0.8 mS/cm) and nectar honeys (< 0.8 mS/cm), indicating their mineral content. A quantity X g of honey is dissolved in 25 mL of distilled water, where (X = (MS is the dry matter of the honey sample). The measuring cell of the conductivity meter was introduced into the prepared solution containing 20 % dry matter at 20 °C, and the value expressed in mS/cm, was read on the device (**Bogdanov et al., 1997**). The electrical conductivity was calculated using the following equation:

$$\text{EC (mS/cm)} = \text{measured value} - \text{A}$$

- **A**: Measured value x 0.032 x 20 **Correction facture**: 0.032 **T°**: 20 °C

II.6. Proteins dosage

Protein content is determined using the method described by **Azeredo *et al.* (2003)**.

In test tubes, 0.1 mL of honey solution (50 %, v/v) is mixed with 5 mL of Bradford reagent (the blank contains 100 µL of distilled water instead of 100 µL of honey solution) and incubated for 2 min. The absorbance measured at 595 nm. The results are expressed in mg equivalent of bovine serum albumin (BSA) per 100 g of honey, referring to the BSA calibration curve.

II.7. Proline

The proline amino acid assay is based on the action of ninhydrin on proline in an acidic medium, resulting in a pink coloration. The intensity of the coloration is related to the amount of proline present and indicates the maturity and compliance of the honey. Its level decreases significantly when honey is adulterated by the addition of sugars (**Lokossou *et al.*, 2017**).

The proline content of the honeys was determined by the method of **Bogdanov *et al.* (1997)**. This colorimetric method involved preparing three tubes, each containing 500 µL of the aqueous honey solution (5 %, w/v). Additionally, three reference tubes contained 500 µL of proline solution, and one blank tube contained 500 µL of distilled water. To each test tube, 1 mL of formic acid and 1 mL of ethanolic ninhydrin (3 %, w/v) were added. After 15 min of agitation and heating at 100 °C in a water bath, the prepared solutions were transferred to 70 °C for 10 min. A volume of 5 mL of 2-propanol (50 %, v/v) was added to all tubes. After incubation for 45 min, the absorbances were measured at 510 nm. The proline concentrations were determined using the following equation:

$$\text{Proline (mg / kg)} = (\text{As} \times \text{MP} \times 80) / (\text{AP} \times \text{Ms})$$

- **As**: Honey sample absorption. **MP**: proline (Mg) for standard solution.
- **AP**: proline standard solution absorbance. **Ms**: Quantity taken of honey in kg. **80**: Dilution facture.

II.8. Polyphenols

The Folin-Ciocalteu reagent is a mixture of phosphotungstic acid (H₃ PW₁₂ O₄₀) and phosphomolybdic acid (H₃ P Mo₁₂ O₄₀). This reagent is reduced during the oxidation of phenols into a mixture of blue tungsten and molybdenum oxides. The resulting coloration is proportional to the amount of polyphenols present in the honey (**Boizot and Charpentier, 2006**).

The polyphenol content is determined according to the method described by **Naithani et al. (2006)**. The reaction mixture contains a mixture of 100 µL of honey solution (0.1 g/mL), 100 µL of Folin Ciocalteu reagent (50 %, v/v), and 2 mL of sodium carbonate solution (2 %, w/v), in the blank, the 100 µL of honey solution is replaced with 100 µL of distilled water. After 30 min of incubation in the dark, the absorbance was read at 750 nm. The results are expressed in mg equivalent of gallic acid per 100 g of honey sample (mg EAG/100 g) using the standard gallic acid curve.

II.9. Flavonoids

The assay for flavonoids present in honey is based on the formation of the flavonoid-aluminum complex, which is responsible for the yellow coloration with a maximum absorbance at 510 nm (**Djeridane et al., 2006**).

The flavonoid content of the honey samples was determined based on the colorimetric method described by **Al et al. (2009)**. A volume of 1 mL of honey solution (50 %, w/v) was mixed with 300 µL of sodium nitrite (5 %, w/v) and 4 mL of distilled water. After 5 min, 300 µL of aluminum chloride (10 %, w/v) was added, 2 mL of sodium hydroxide (1 M) was added to the mixture. After 6 min of incubation, the absorbance was measured at a wavelength of 510 nm. The results are expressed in mg equivalent of quercetin per 100g of honey sample (mg EQ/100 g) using the standard quercetin curve.

III. Biological activities

III.1. Radical scavenging activity (DPPH)

The chemical compound 2, 2-diphenyl-1-picrylhydrazyl is a stable purple-colored free radical. In the presence of antioxidants, proton transfer, leading to a decrease in color intensity (Chaabi, 2008), reduces it. The scavenging activity was determined using the method described by Meda *et al.* (2005). A volume of 500 μL of each aqueous honey solution (2.5 %, w/v) was added to 1 mL of an ethanolic solution of DPPH (6×10^{-5} M). Two tubes, one for the blank and another for the control, were prepared. The blank contained 1 mL of ethanol, while the control contained 1 mL of the DPPH solution. The mixture was incubated for 15 min, and the absorbance was measured at 517 nm. The percentage of reduction was calculated using the following formula:

$$\text{Scavenging activity (\%)} = [(\text{Ac} - \text{As}) / \text{AC}] \times 100$$

- Ac: Absorbance of the control. (1 ml solution DPPH). As Absorbance of the sample.

III.2. Reducing power

The reducing power assay involves the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants in an acidic medium using trichloroacetic acid (TCA) and in the presence of a chromogenic agent, potassium hexacyanoferrate. This reduction produces a green coloration, the intensity of which is proportional to the reducing power (Gülcin *et al.*, 2005).

The reducing power was determined according to the method described by Beretta *et al.* (2005). A volume of 500 μL of the honey solution (2.5 %, w/v) was added and homogenized with a volume of 500 μL of phosphate buffer (0.2 M; pH = 6.6) and 500 μL of potassium hexacyanoferrate (1 %, w/v). The mixture was incubated in a water bath at 50°C for 20 min, then 500 μL of trichloroacetic acid (10 %, w/v) was added to stop the reaction. A volume of 100 μL of ferric chloride (1 %, w/v) was added to a volume of 500 μL taken from this mixture and then diluted with 800 μL of distilled water. After 10 min, the absorbance was measured at 700 nm. The results are expressed in mg equivalent of gallic acid per 100 g of honey sample (mg GAE/100 g) referring to the standard gallic acid curve.

III.3. Colorimetric ferrosin-based assay

The Ferrosin assay, as described by **Ebrahimzadeh *et al.* (2007)**, is a method used to evaluate the chelating power of honey samples. Chelating agents inhibit the formation of the ferrosin-Fe²⁺ complex, which was indicated by a decrease in pink coloration. Following the procedure outlined by **Wang *et al.* (2008)**, a volume of 500 µL of honey (2.5 %, w/v) is mixed with 50 µL of iron (II) chloride (0.6 mM) and 900 µL of methanol. After 5 min, 100 µL of ferrosin solution (10 mM) was added to the mixture. The absorbance was then measured at 562 nm after 10 min of incubation. The expression of the chelating activity of the honey sample is determined using the following formula:

$$\text{Chelating activity (\%)} = \left(1 - \frac{[A1 - A2]}{A0}\right) \times 100$$

- **A0**: Absorbance of the control (1 ml of ferrosin solution). **A1**: Absorbance of the sample.
- **A2**: Absorbance without ferrosin solution.

III.4. Anti-inflammatory activity

in vitro anti-inflammatory activity of honey samples, based on the inhibition of heat-induced denaturation of bovine serum albumin (BSA). Following the method described by **Kandikattu *et al.* (2013)**, three different concentrations of honey solutions (0.5, 1, and 2 mg/mL) are prepared. Each concentration was then mixed with an equal volume (500 µL) of a BSA solution prepared in Tris-HCl buffer. After incubation at 37 °C for 15 min followed by heating at 75 °C for 5 min, the mixture was cooled, and the absorbance is measured at 660 nm. A blank is prepared for each concentration. The results are calculated using the following formula:

$$\text{Anti - inflammatory activity (\%)} = \left(\frac{[As - Ab]}{Ac}\right) \times 100$$

- **As**: Absorbance of the sample. **Ab**: Absorbance of the blank.
- **Ac**: Absorbance of the control (1 ml of BSA solution with 1 ml of Tris-HCl solution).

VI. Formulation of a Honey-PSP-based ointment

VI.1 Pumpkin seeds sample

Mature pumpkin fruits (*Cucurbita pepo*) was collected from a local farm in Bejaia (Figure 5). The seeds were retrieved after being separated from pumpkin using knife, then was dried in hot-air oven at 60 °C for several hours after being dried away from light for 24 h in dry space, pumpkin seeds were then grounded into a fine powder using a grinder following the method described by **Hussain et al. (2021)**.

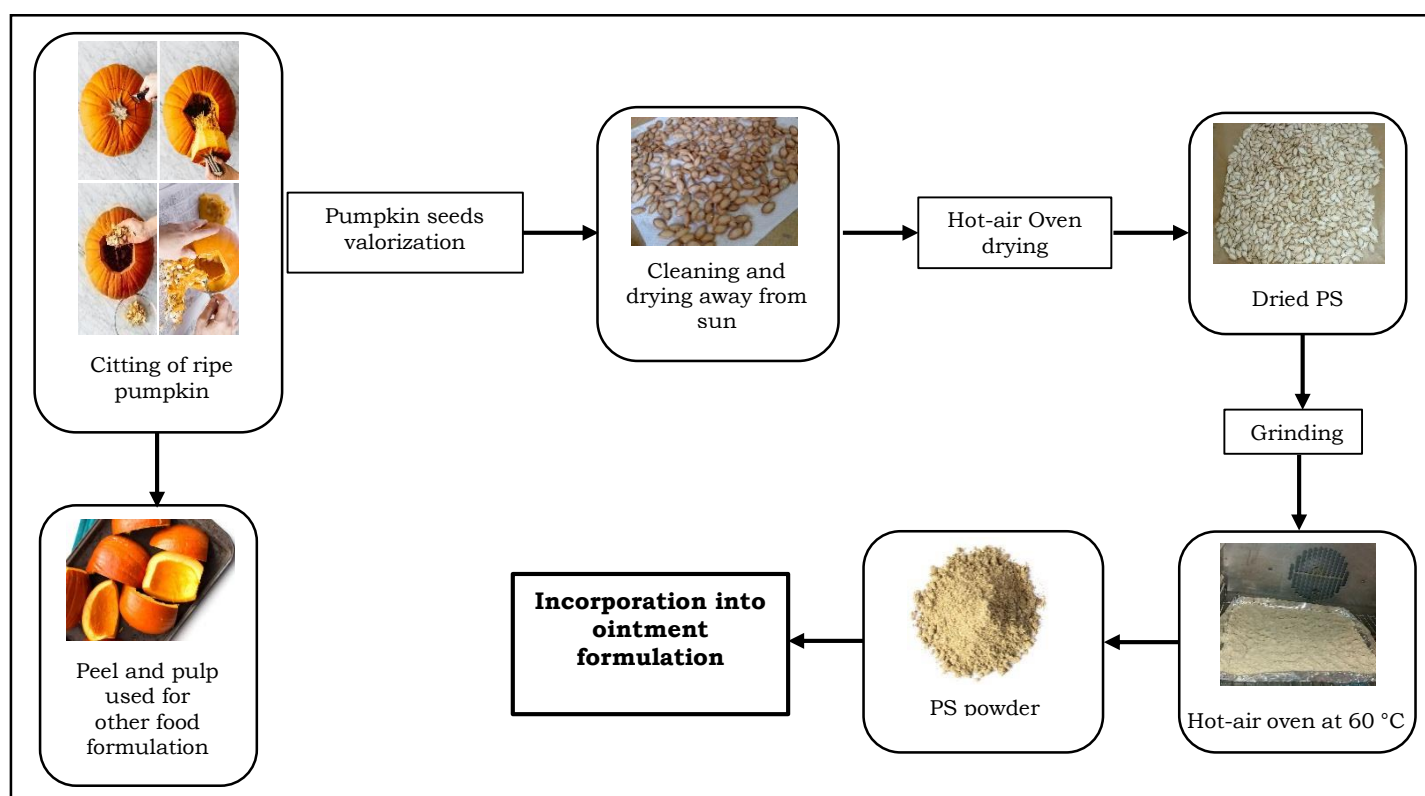


Figure 5: Pumpkin seeds powder preparation.

VI.2. Ointment formulation

The ingredients used in the formulation ointments are shown in the table II.

Table II: The Ingredients of the formulation of ointments

| Ingredients | C- | O1 | O2 |
|--------------------------|----|----|----|
| Paraffin oil (g) | 75 | 60 | 60 |
| Sesame oil (g) | 5 | 5 | 5 |
| Coconut oil (g) | 5 | 5 | 5 |
| Vegetal glycerin (g) | 5 | 5 | 5 |
| Beeswax (g) | 10 | 10 | 10 |
| Honey 01 (g) | - | 10 | - |
| Honey 02 (g) | - | - | 10 |
| Pumpkin seeds powder (g) | - | 5 | 5 |

C-: control. O1: H1-PS based-ointment. O2: H2-PS based-ointment

According to the method described by **Maru and lahoti, (2019)** with modifications

❖ Melting the Beeswax

In a double boiler, beeswax was heated and melted at approximately 70°C and until liquefied.

❖ Incorporation of Oils

Once the beeswax was melted, paraffin, sesame, coconut oil and vegetable glycerin were added gradually alongside with constant stirring until homogenization was fully attended.

❖ Incorporation of Honey and PSP

The mixture was removed from heat, once the mixture has cooled further, and then the honey was added followed by PSP under stirring process to ensure the incorporation.

The formulated ointment as well as the two ointment (MEBO® and NEWBO®), purchased from a pharmacy in the wilaya of Bejaia, are illustrated in the (Figure 6).

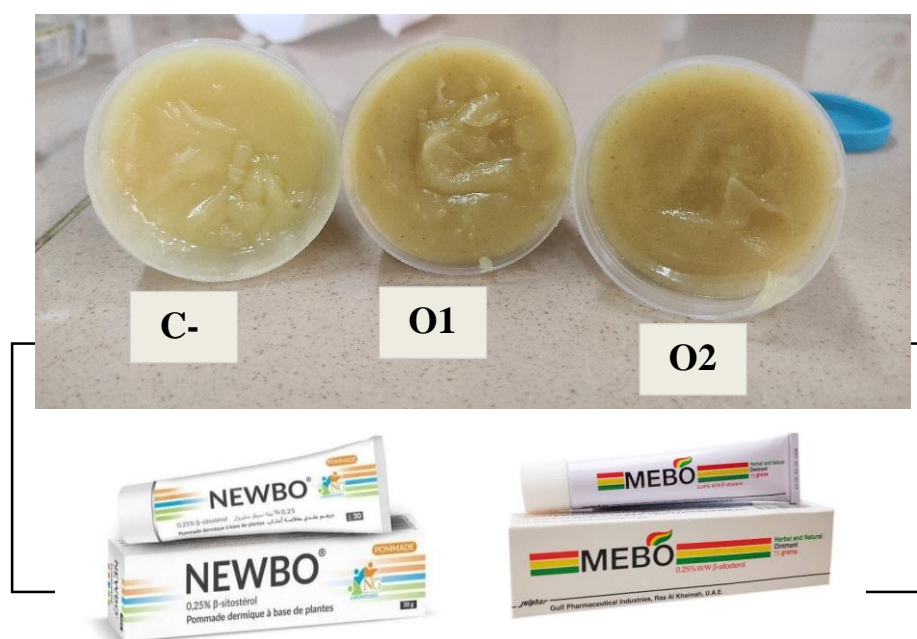


Figure 6: The formulated and pharmaceutical ointments.

VI.3. Extractions

❖ Pumpkin seeds powder extracts

Pumpkin seeds powder extraction was conducted following **Asif *et al.* (2017)** with some modification. 8 mL of 80 % methanol was added to 0.5 g of powder, and sonication was performed for 10 min, after centrifugation at 5000 rpm. The supernatant was retrieved while the was underwent a second centrifugation, then the supernatant was retrieve and mixed with the former. Filtration was carried out using Whatman filter paper, then the extracted sample was stored at 4 °C temperature for later analysis.

❖ Ointments extracts

Five ointments have submitted to extraction according to the method described by **Asif *et al.* (2017)**, where 9 mL of methanol (80 %) was added to 1 g of each ointment, and sonication was performed for 10 min, centrifugation at 5000 rpm/10 min was conducted after stirring. Next, the supernatant was retrieved while the pellet underwent a second centrifugation, then the two supernatant were mixed. The resulting supernatant was used to estimate the content of polyphenols and flavonoids, as well as antioxidant and anti-inflammatory activities.

V. Physicochemical properties

V.1. Color

The color evaluation was conducted using the method described by **Bardaa et al. (2016)** a colorimeter or spectrophotometer is a precision instrument used to quantitatively measure the color of various samples. It operates by emitting light onto the sample, detecting and measuring a specific wavelengths of light absorbed or reflected by the sample.

The Cie Lab coordinates (L^* , a^* , b^*) were directly monitored by a spectrophotocolorimeter. In this coordinate system, the L^* value is a measure of lightness, ranging from 0 (black) to 100 (white), the a^* value ranges from -100 (greenness) to $+100$ (redness) and the b^* value varies from -100 (being blue) to $+100$ (yellowness).

V.2. Moisture content

The evaluation was conducted using gravimetric method as described by **Majhi and Sarkar, (2019)**.

Initially, the ointment samples were prepared and collected using sterile tools to avoid contamination. The fresh ointment samples were immediately weighed to obtain their initial weight (fresh weight). Subsequently, the samples were placed in an oven set at 100°C and dried for 4 hours to ensure complete evaporation of moisture. After drying, the samples were weighed again to obtain their dry weight. The water content of the ointments was then determined using the following formula:

$$\text{Moisture Content (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

- W_1 : initial weight of the cup and sample before drying. W_2 : Final weight of the cup and sample after drying.

V.3. Phenolic compounds

The dosage of polyphenols and flavonoids of the extracts, ointments obtained and pharmaceutical ointments was estimated using the same method presented in the paragraphs **II.8** and **II.9**.

VI. Biological activities

VI.1. Anti-oxidant activity

The dosage of DPPH and reducing power tests of the extracts obtained are evaluated using the same methods described in the paragraphs **III.1** and **III.2**.

VI.2. Anti-inflammatory activity

The ointment's anti-inflammatory activity was determined using the same method presented in the paragraphs **III.4**.

VI.3. Microbiological Analysis

To estimate the microbial load of the prepared ointments, a count of the total aerobic mesophilic flora (TAMF), which reflects the general microbiological quality, was performed. Using the method described by **Adjlane et al., (2014)**. A stock solution (10^{-1}) was prepared by suspending 1g of each ointment (O₀, O₁, O₂) in a test tube containing 9 mL of sterile physiological water. After homogenization, 100 μ L of the (10^1) dilution was inoculated by streaking with a swab onto 3 plates of potato dextrose agar (PDA) and 3 plates of plate count agar (PCA). The plates were then incubated at 30 °C for 72 hours. The number of colonies was calculated according to the following equation:

$$N = \frac{\Sigma C}{(n_1 + 0.1n_2) d}$$

- **N**: Number of colonies. **ΣC** : Sum of colonies counted on the selected plates (between 20 and 300 colonies). **n_1** : Number of plates selected at the first dilution.
- **n_2** : Number of plates selected at the second dilution. **d** : Dilution rate corresponding to the first selected dilution.

VI.4. Peroxide Index (PI)

The PI is determined according to the **ISO 3960 4th edition (2017)** method. In a flask, 5 g of the ointment were mixed with 12 mL of chloroform, 18 mL of acetic acid, and 1 mL of potassium iodide (0.5 g/mL); the mixture is incubated in the dark for 1 minute before adding 75 mL of distilled water and a few drops of starch paste (0.005 g/mL). Titration was performed with a 0.01 N sodium thiosulfate solution until the solution is completely decolorized. A blank test (without the ointment) was conducted in parallel. The results, expressed in meq O₂/kg, were calculated using the following equation:

$$IP (meq O_2 kg) = N \times (V_1 - V_0) \times 1000/p$$

- **IP**: Peroxide index. **V_0** : Volume of the sodium thiosulfate solution for the blank test in mL.
- **V_1** : Volume of the sodium thiosulfate solution used in mL.
- **N**: Normality of the sodium thiosulfate solution, 0.01 N. **P**: Sample weight in grams.

VI.5. TBARS Test

The measurement of thiobarbituric acid reactive substances (TBARS) was performed by homogenizing 2 g of the ointment in 16 mL of 20 % trichloroacetic acid (TCA) (w/v). The mixture was then centrifuged at 4000 rpm/min for 15 min. 2 mL of 20 mM TBA solution was added to 2 mL of the obtained supernatant (the extracted malondialdehyde (MDA)) and mixed for 5 seconds using a vortex. The mixture was then placed in a water bath at 95 °C for 10 min. After cooling, the absorbance was measured at a wavelength of 532 nm (Djenane *et al.*, 2012). The amount of TBARS was calculated based on the molar extinction coefficient $\epsilon=155 \text{ Mm}^1.\text{cm}^{-1}$, using the following equation:

$$\text{M equivalent MDA/Kg} = (\text{Abs} \times V_{\text{TCA}} \times 2 \times M \times 10^{-2}) / 1.56 \times m$$

- V_{TCA} : Volume of TCA used for the extraction (16 mL). M : Molecular weight of MDA (72 g/mol).
 m : Mass of the sample analyzed (g)

VII. Skin Patch Tests

Fifteen voluntary participants (including 4 teachers, 5 doctoral students, and 6 Master's students), aged between 23 and 50 years, applied the ointments on a surface of 4 cm² area on the backs of their hands and arms for 48 hours. The objective of this test is to detect the presence or absence of skin reactions.

VIII. Ointments sensory evaluation

A sensory analysis by an expert panel was conducted with a group of 12 individuals (9 women and 3 men, aged 25 to 50 years) from the University of Bejaia, including teachers, staff, and doctoral students, who were previously trained and practiced in sensory evaluation. The jury evaluated the three ointments (O0, O1, and O2) in a single session. The ointments were presented at 20 ± 2 °C in small transparent plastic pots.

The sensory descriptors evaluated included color, odor, consistency, shine, texture, and spreadability on the skin, rated on a scale of 1 to 5. Additionally, the acceptability of the ointments formulated at the laboratory scale was assessed using a global preference test, which involved assigning a score from 1 to 9 for the ointments. A score of 1 indicated "Not at all liked," and 9 indicated "Extremely liked" (Balthazar *et al.*, 2015).

Each expert was asked to fill out a questionnaire (see appendix) containing all the information related to the evaluation parameters of the coded ointment samples.

IX. Statistical Analyses

The analyses were conducted in triplicate, with means calculated using Microsoft Office Excel2016. The results for the honey samples were evaluated using the Student's t-test, while the results for the ointment samples were assessed using analysis of variance (ANOVA) with the software STATISTICA 7.1.2 (Statsoft Co., Tulsa, OK, USA).

A correlation matrix was applied to the presented data of the ointments using $p < 0.05$, $p < 0.01$, and $p < 0.001$ to indicate the highest significance levels. The PCA of the ointments was also performed using STATISTICA 7.1.2 to study the data in a reduced-dimensional space while minimizing information loss, examining the similarities among different ointment samples, and determining the relationships between variables.

The sensory data for the formulated ointments were processed using XLSTAT 2024.1.0 (1418).

PART II-2
Results and discussion

I. Honey

I.1. Physico-chemical analysis

I.1.1. Brix and Moisture content

The sugar content in honey is expressed by the Brix degree, which indicates the amount of dry matter (g) present in 100 g of honey at 20°C (Albu et al., 2021).

The total soluble solids (sugar content) assessed by brix ranged from 83.53 % (H1) to 77.81 % (H2), while water content was 14.91 % in H1 and 21.24 % in H2, respectively (Figure 7).

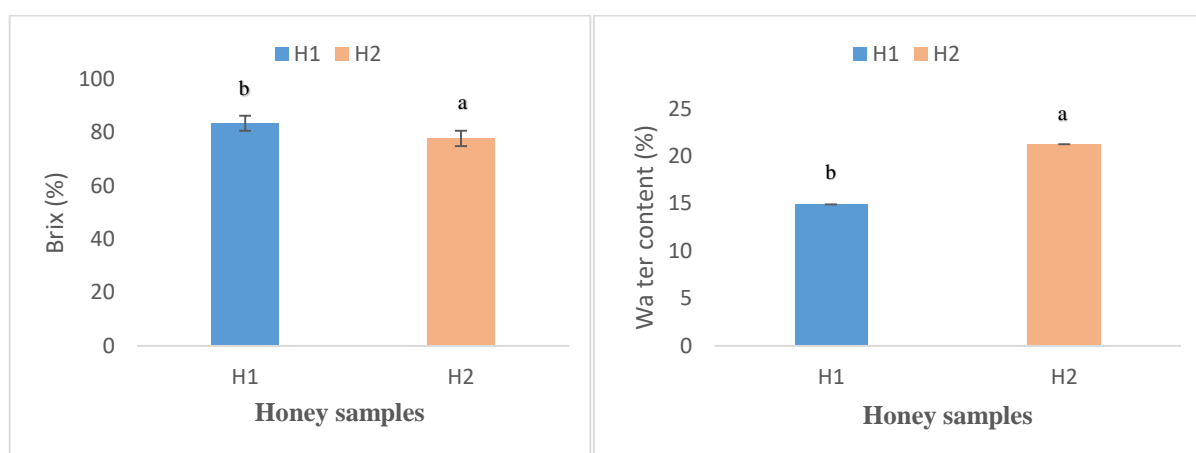


Figure 7: Brix and water content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations. Values with the same letters are not statistically different. (a>b>c>d)

These results are close to those reported by *Popescu et al. (2016)* in Romanian honey (78.70 to 82.30 %), and lower than those reported by *Nyau et al. (2013)* (83.6 to 85.7 %) and by *Anguebes et al. (2016)* in Mexico (82.44 to 86.06 %). The reported water content was similar to those previously reported in the republic of Serbia by *Sakač et al. (2019)*, (13.2 to 21.3 %). Higher results were reported by *Terrab et al. (2003)* honeys (14 to 24.1%) in northwest Moroccan, and by *Stihi et al. (2016)* in Romania (16.70 to 22.80 %). Lower results obtained by *Mohammed et al. (2017)* in Saudi Arabia (8.80 to 13.85 %) and *Otmani et al. (2021)* in Algeria (14 to 15 %).

H1 values are consistent with the standards set by *Codex Alimentarius*, while H2 exceeded the allowed limit (20 %).

This variation has been attributed to the botanical/geographical origin of nectar, soil and climatic conditions, extraction, processing, storage conditions, season of harvesting, flux intensity of nectar and degree of maturation. Furthermore, water content plays a significant role in honey's physical properties such as maturation, viscosity and crystallization (*Patruica et al., 2022*).

The high sugar content in H2 may be attributed to storage condition or honey processing, which creates an adequate environment for fermentation, yielding yeast and bacterial proliferation due to low osmotic pressure. Furthermore, high sugar concentration alleviate the free water content by decreasing the water activity, thereby playing an antimicrobial role in protecting honey along with low moisture content (*Bogdanov, 2011; De-Melo et al., 2017*).

The student's t-test on the Brix degree and water content of the two honeys studied reveals a significant difference between H1 and H2.

I.1.2. pH

The pH value is related to the stability and the shelf life of honey, serving as a useful indicator for possible microbial contamination (*Pita-Calvo and Vázquez, 2017*).

The pH value of honey typically ranged between (3.5 to 5.5). Due to the presence of organic acids (*Pita-Calvo and Vázquez, 2017; Pauliuc et al., 2020*), the obtained results demonstrated a PH value 4.1 and 4.62 in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 8)

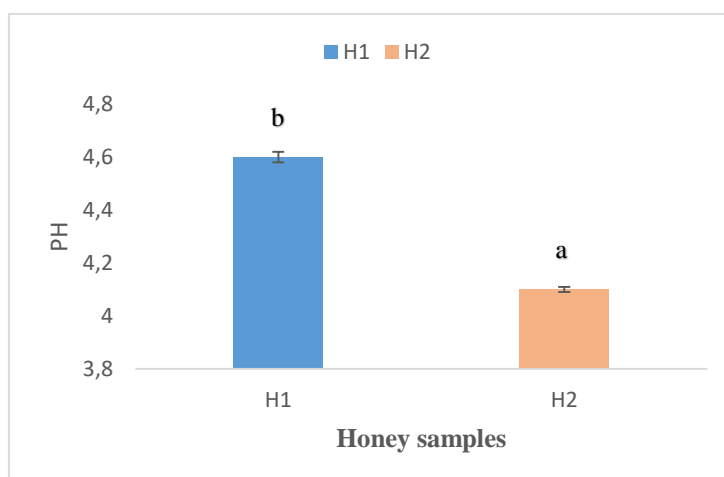


Figure 8: PH value in honey

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations. Values with the same letters are not statistically different. (a>b>c>d).

These results consistent with those reported by **El Sohaimy et al. (2015)** (4.11 to 4.64), who examined 4 honeys from different regions (Egyptien, Yemen, Saudi and Kashmir), similar to those reported by **Mouhoubi et al. (2018)** (3,97 to 4,5) and higher than those reported by **Ávila et al. (2019)** in Brazil (3,21 to 3,62), while lower than those obtained by **Al-Farsi et al. (2018)** in Omani Sidr honey (4.71 to 7.51). The PH tested values measured confirm that the two honeys tested are acidic within the standard limit set by **codex alimentation, 2001**.

I.1.3. Color

The color of honey is one of its most important physical characteristics as it is related to its floral origin and composition (pollen, pigments, and minerals) (**Pauliuc et al., 2022**).

The results obtained show that the color intensity of the honey samples analyzed was 0.21 and 0.52 OD in H2 and H1 respectively, with color spanning from yellow H2 to brown H1. H1 and H2 are significantly different for this parameter. (Figure 9).

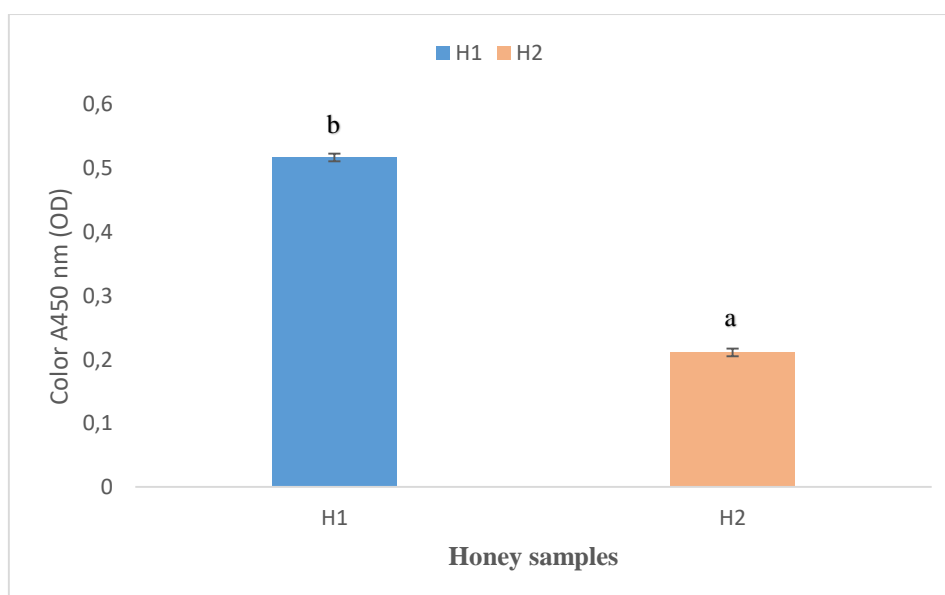


Figure 9: Color of honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations. Values with the same letters are not statistically different. (a>b>c>d).

These values are close to those reported by **Ayad et al. (2021)** in Algerian honeys (0.27 to 0.81 OD), but differ significantly from those reported by **Imtara et al. (2018)** in Palestinian honeys, which characterized by a relatively lighter color (0.07 to 0.23 OD).

Color serves as an indicator of the honey's chemical composition variability, with darker honey known to possess higher concentration of phenolic acids, flavonoids, carotenoids, and minerals compared to lighter honey (Pauliuc et al., 2022). Furthermore, aging, processing, light, and Storage temperature can also affect the color of honey (Moniruzzaman et al., 2013).

I.1.4. Hydroxymethylfurfural (HMF)

The obtained results demonstrated a value of 23.7 and 11.63 mg/kg in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 10).

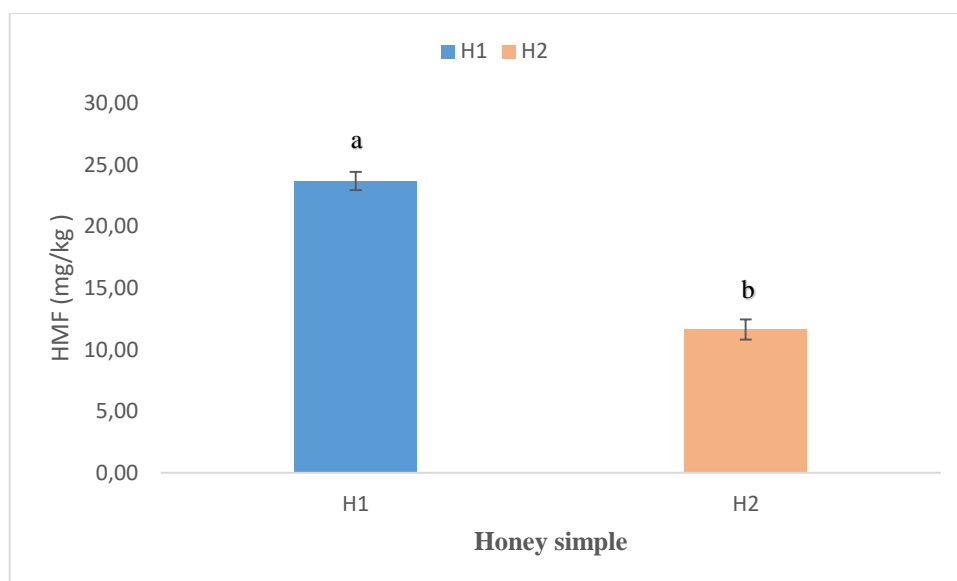


Figure 10: HMF content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

The results obtained fall within the range reported by Amessis-Ouchemoukh *et al.* (2021) (1 to 34 mg/kg), and higher than those reported by Bouhala *et al.* (2020), in jjjel (2 to 9 mg/kg), while lower than those reported by Qamer *et al.* (2008) in Nepal (30.36 to 56.10 mg/kg).

High heat processing temperature, long storage duration, tampering with commercial sugars, pH, bee species, high acidity, moisture content, sugars (mainly fructose), amino acids and minerals, largely increase the HMF production (De-Melo *et al.*, 2018).

I.1.5. Electric Conductivity (EC)

According to **Codex Alimentarius Standard for Honey, (2001)**, EC represents the capacity of honey to carry the flow of an electric current.

EC is an indicator often used to assess honey quality and distinguish floral honey from honeydew honey (**Karabagias et al., 2014; Alqarni et al., 2014**).

The obtained results demonstrated a value of 0.68 and 1.10 mS/cm in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 11).

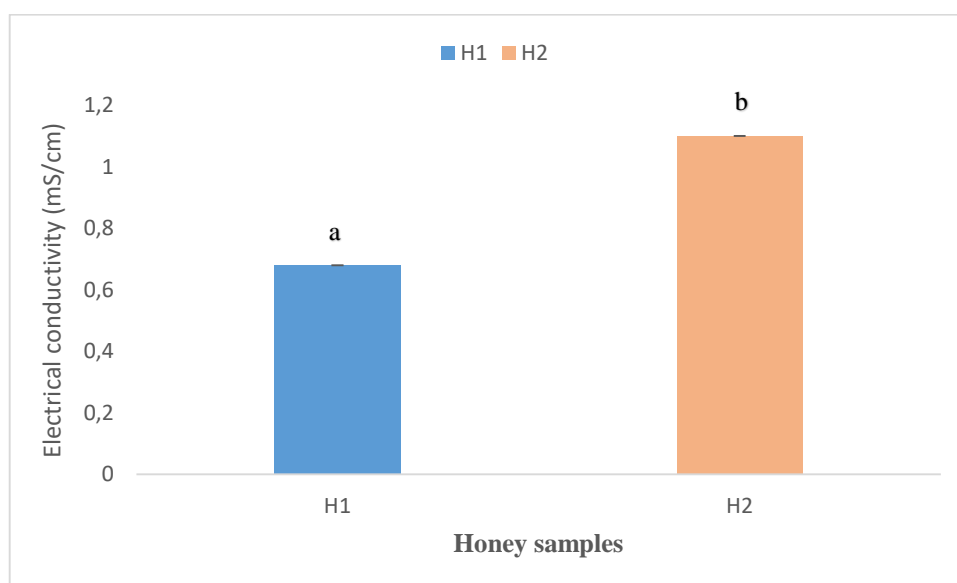


Figure 11: Electric conductivity of honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results are in accordance with those found by **Alqarni et al. (2014)** (0.21 to 3.13 mS/cm) and by **Bouhala et al. (2020)** in Algerian honey (0.29 to 1.13 mS/cm). **Guerzou et al. (2021)** reported lower results (0.083 to 0.641 mS/cm).

The **Codex Alimentarius Standard** set the honeydew's honey conductivity to be EC >0.80 mS cm, while EC <0.80 mS for nectar's honey. H2 value fall within the range obtained by **Oroian et al. (2017)** in Honeydew (0.91 to 1.10 mS/cm), therefore H2 stems from Honeydew honey.

The honeydew honey EC exhibited higher values, due to the high minerals content and acidity, revealing the presence of ions, organic acids and proteins (**Yücel and Sultanoglu, 2013**); thus, the higher their content, the higher the resulting conductivity.

Alqarni et al. (2014) has reported that dark-colored honey exhibits higher EC compared to light-colored honey, which contrast with the obtained results , where the light-colored honey H2 exhibit higher electric conductivity than dark-colored honey H1.

I.1.6. Proteins

Proteins in honey have predominantly been investigated for botanical and geographical origin, assessing the freshness, authenticity, and maturation of honey, as well as detecting potential adulteration (**Pascual-Maté et al., 2018**).

The proteins content values obtained in this study were 654.12 and 320.78 mg EBSA/ 100 g in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 12)

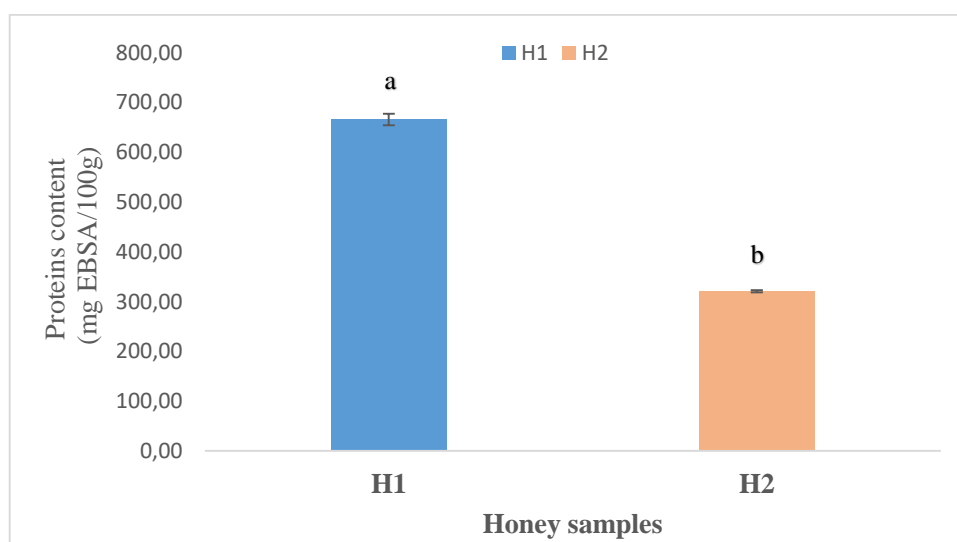


Figure 12: Proteins content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results are superior to those obtained by **Tsavea et al. (2022)** in Greece (30,1 to 82,7 mg EBSA/ 100g) and by **Amessis- Ouchemoukh et al. (2021)** in Algeria (35 to 137 mg EBSA/100 g) respectively, while lower than those obtained by **Zhang et al. (2021)** (670 to 880 mg EBSA/ 100 g).

I.1.7. Proline

Proline is often utilized as a key indicator to assess the maturity of honey and to detect possible adulteration with sugar. The proline established threshold of pure honey is at minimum of 180 mg/kg.

The values obtained in this study were 194.05 and 206.9 mg/Kg for H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 13).

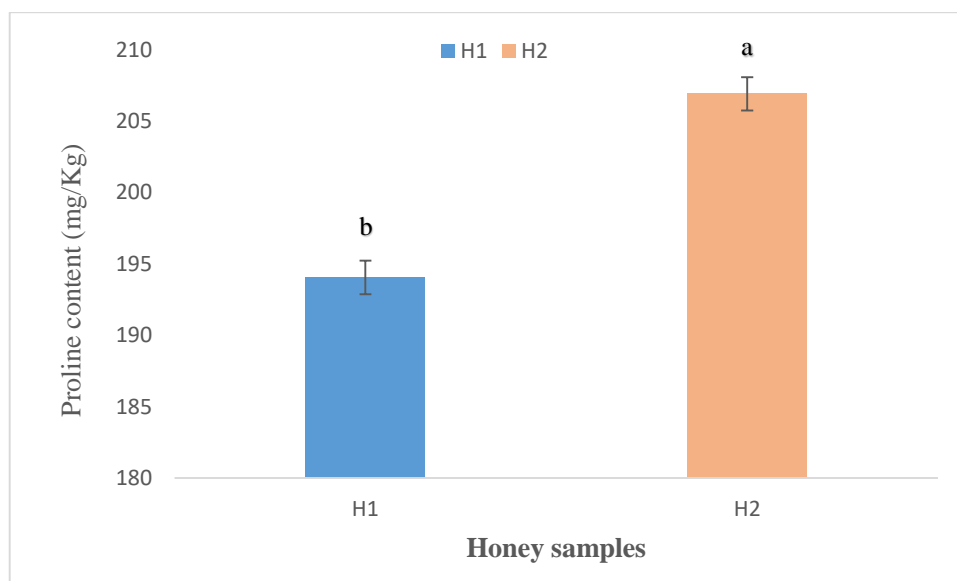


Figure 13: Proline content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

The values obtained in the present study are higher than the those reported by **Boussaid et al. (2018)** in Tunisian honey (59 to 102 mg/kg) and lower than those reported by **Ouchemoukh et al. (2007)** in Algerian honey (202 to 680 mg/kg).

The values obtained was above the minimum threshold value 180 mg/kg, which indicate the maturity and purity of the honeys examined from proline criteria.

I.1.8. Phenolic compounds

Polyphenols are powerful antioxidants that can reach more than 0.8 % (by weight) in bee products and used as criterion to evaluate the quality of honey.

The correlations between antioxidant activity and total concentration of phenols were confirmed in several study **Rosa et al. (2011); al et al., (2009)**. Other study has been shown that polyphenols are responsible for the antimicrobial activity in honey **Estevinho et al. (2008)**.

The obtained results demonstrated a value of 65.44 and 3.95 Gallic Acid Equivalent (GEA)/100g in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 14).

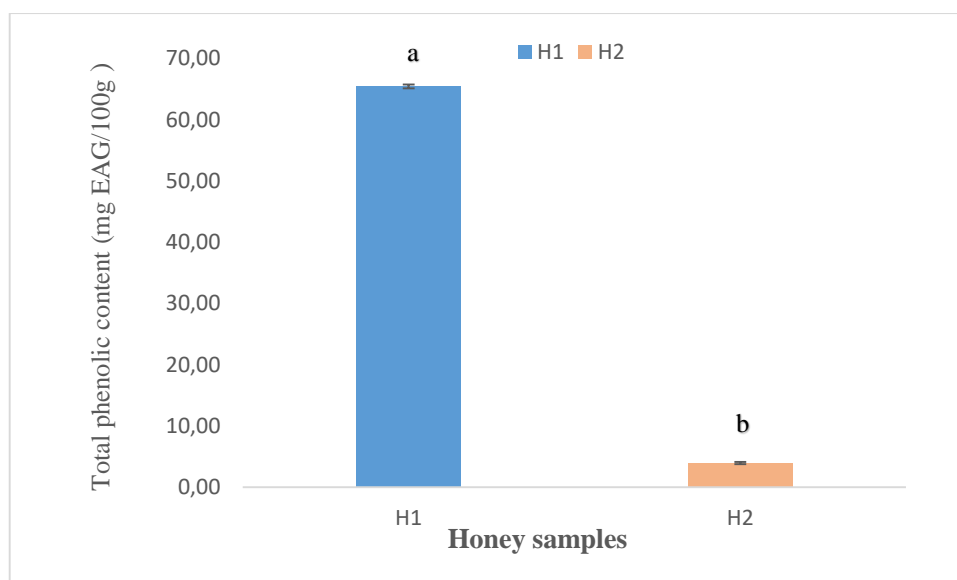


Figure 14: Total phenolic content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results are close to those obtained by **Spilioti et al. (2014)** in Greece (11 to 50 mg GAE/kg), lower than those reported by **Ouchemoukh et al. (2017)** in Algerian honey (90 to 318 mg EAG/100 g), and higher than those obtained by **Azad and al. (2016)** in china (8.70 to 1.20 mg GAE/100 g).

Total phenolic compounds (TPC) contribute to the antioxidant activity of honey, varying among honey's types and geographical origins, and have been used as phytochemical markers for the floral origin. (**Ranneh et al., 2021; Zawawi et al., 2021**).

I.1.9.Flavonoids

Flavonoids are phenolic compounds with low molecular weight, representing up to 50% of TPC (*Wali et al., 2020*)

The obtained results demonstrated a value of 5.19 and 1.15 mg QE/100 g in H1 and H2. H1 and H2 are significantly different for this parameter. (Figure 15).

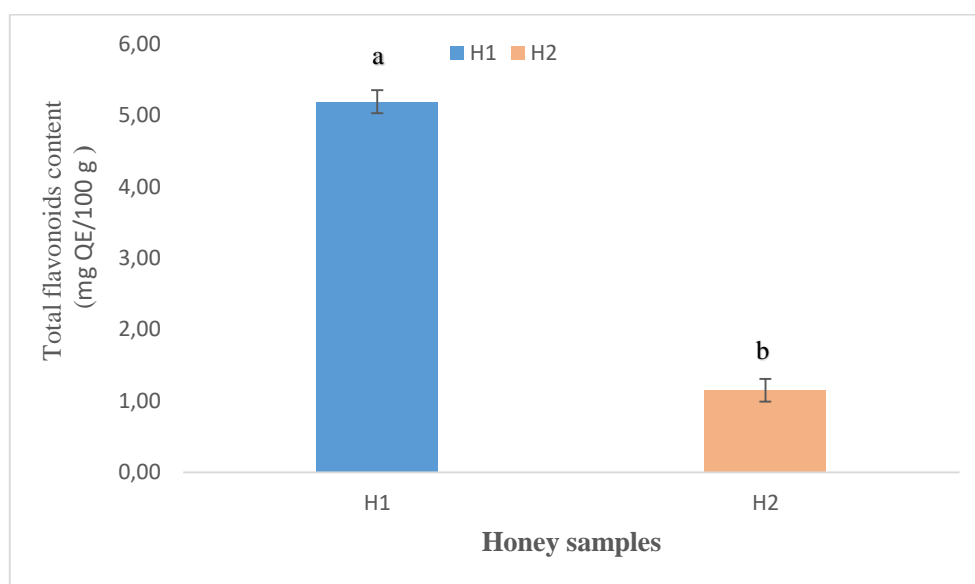


Figure 15: Total flavonoids content in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results are close to those obtained by **Shamsudin et al. (2019)** in Malaysia (2.38 to 6.53 mg QE/100 g), within the range obtained by **Jara-Palacios et al. (2019)** in Spain (1.81 to 25.22 mg CAE/100 g), higher than those obtained by **Muñoz et al. (2014)** in Peru (1.41 to 1.63 mg QE/100 g), and lower than those reported by **Ayad et al. (2021)** in Algerian honey (7 to 20 mg EC /100 g).

Total Flavonoid content (TFC) vary depending on the floral source. Generally, the dark-colored honeys (sunflower and honeydew honey) exhibit higher flavonoids content than light colored honey. (**Rossant. 2011**), this aligns with the present results, as H1 showed higher flavonoids content than H2.

Among the flavonoids identified in honey are catechin, quercetin, chrysin, galangin, kaempferol, and apigenin (**Tahir et al., 2017**).

I.2. Biological activities of honey

I.2.1. Antioxidant activity

I.2.1.1. DPPH radical Scavenging activity

DPPH activity evaluates the anti-oxidant potential of substances, by serving as free radical scavengers or the hydrogen providers. DPPH varied based on the botanical origin of honey.

The obtained results demonstrated a value of 53.79 and 46.77 % in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 16).

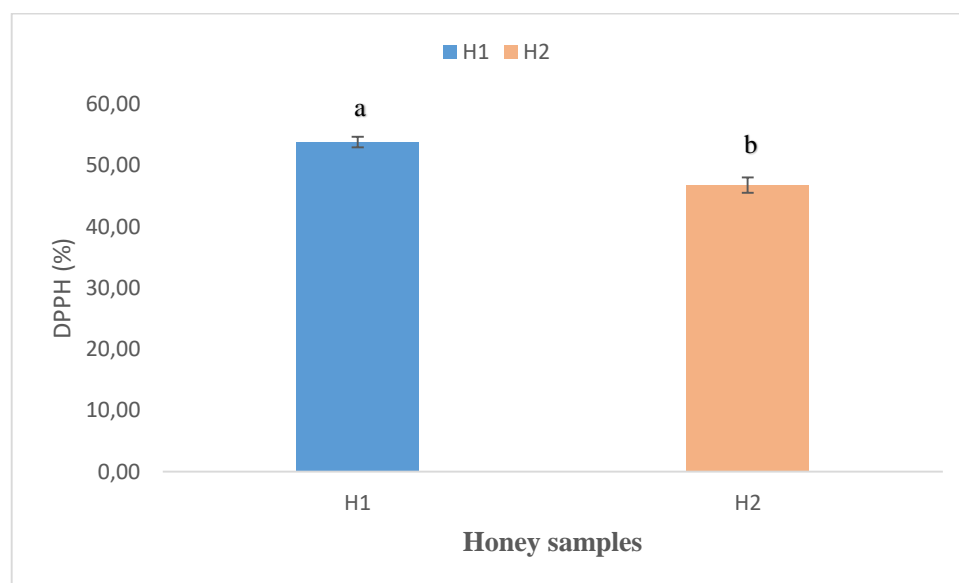


Figure 16: DPPH radical scavenging activity in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results align with those reported by **Patruica et al. (2022)** in acacia honey, wild cherryhoney and rapeseed honey (41.89 to 59.81 %), lower than those reported by **Mracevic et al. (2020)** in Serbia (75.89 to 79.1%) and higher than those reported by **Sousa et al. (2016)** (11 to 46 %).

Various studies have demonstrated a correlation between antioxidant activity, (TPC) and (TFC), which vary based on the botanical origin. (**Patruica et al., 2022**).

I.2.1.2. Reduction power

The reducing power activity obtained showed a value of 22.95 and 10.41 mg EAG/100 g in H1 and H2 respectively. H1 and H2 are significantly different for this parameter. (Figure 17).

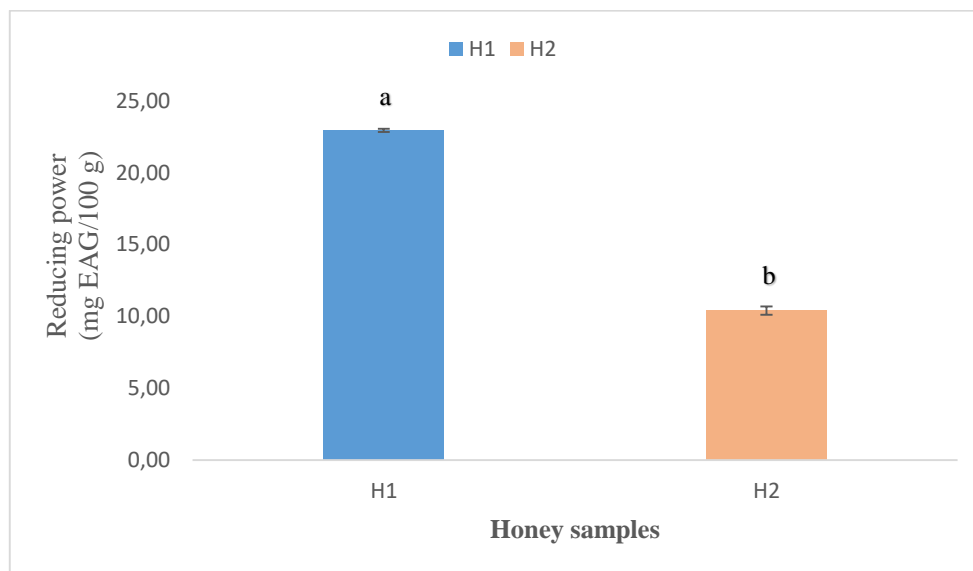


Figure 17: Reducing power in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

These results are close to those obtained by **Ayad et al. (2021)** (12.54 to 24.97 mg EAG/100 g), higher than those reported by **Ouchemoukh. (2012)** (0.17 to 0.71 mg EAG/100 g) in Algerian honey and lower than those reported by **Zhang et al. (2021)** (83 to 118 mg EAG /100 g).

The variation in honey's antioxidants activity is attributed to the difference in pollen and nectars collected by bees, however, the antioxidant activity of honey has been attributed to the presence of phenolic acids, flavonoids, carotenoids, ascorbic acid, peroxidase, catalase as well as Maillard reaction products (**Pauliuc et al., 2020**).

I.2.1.3. Ferrosin

The obtained results demonstrated a ferrosin value of 78.35 and 73.99 % in H1 and H2 respectively, these results fall within the range reported by **Tahir et al. (2017)** (52 to 88%). Higher than those obtained by **Temizer et al. (2018)** (36 to 37%). H1 and H2 are significantly different for this parameter. (Figure 18).

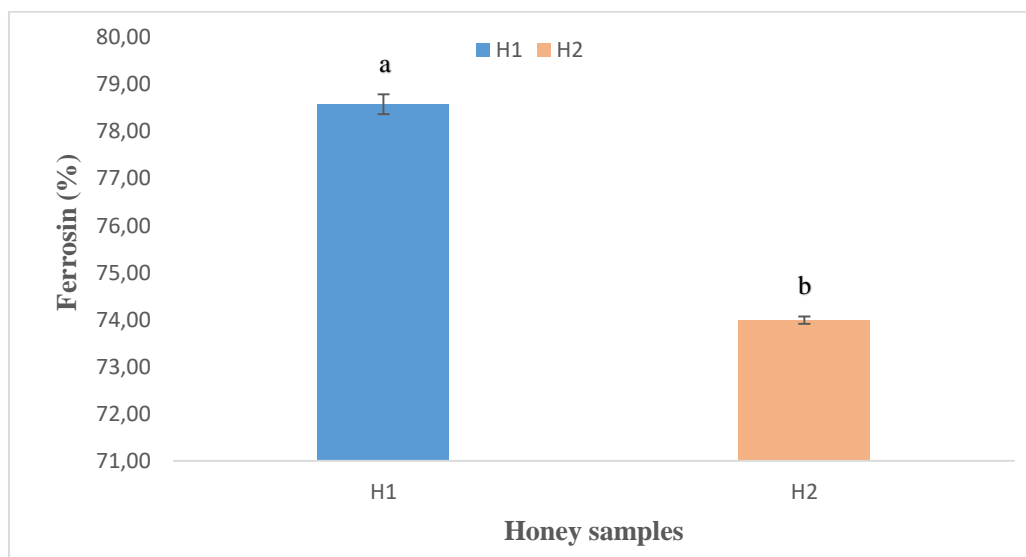


Figure 18: Ferrosin activity in honey.

The results are presented as the mean \pm standard deviation. The vertical bars represent the standard deviations.

Values with the same letters are not statistically different. (a>b>c>d).

The variability in the chelating power of the analyzed honeys reflects the distinct nature of the chelating compounds present and their differing affinities for iron, which compete with ferrozine. Among the phenolic compounds in honey known for their chelating ability are ferulic and caffeic acids, quercetin, kaempferol, and naringenin (**Zduńska et al., 2018**). Several studies have indicated that phenolic compounds can sophisticate and stabilize transition metal ions, likely due to the strong nucleophilic nature of their aromatic rings (**Amessis-Ouchemoukh, 2014**).

I.2.2. Anti-inflammatory activity

The anti-inflammatory activity of honey includes the inhibition of protein denaturation, such as that of BSA (Bovine Serum Albumin), which occurs during inflammation. The inhibition of this heat-induced denaturation is part of the anti-inflammatory mechanism of honey (**Otmani et al., 2021**).

The obtained results demonstrated a value of 49.78 and 67.53 % in H1 and H2 respectively, these results fall within the range reported by **Zaidi et al. (2019)** for Algerian honeys (2 to 85%). H1 and H2 are significantly different for this parameter. (Figure 19).

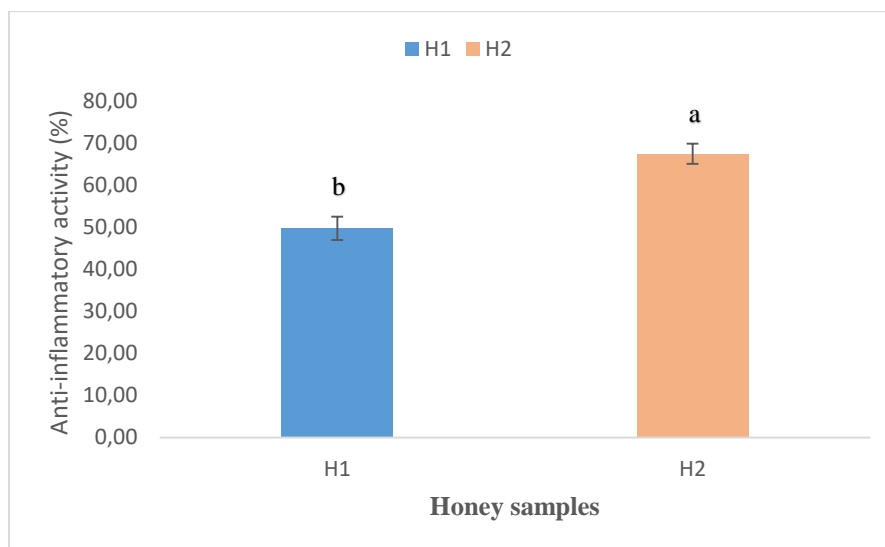


Figure 19: Anti-inflammatory activity in honey.

The variability in the protective activity of honey against BSA (bovine serum albumin) can be explained by distinct contents of bioactive compounds such as (TPC) and (TFC).

Despite the high (TPC) and (TFC) in H1 compared with H2, the latter shows higher anti-inflammatory activity.

According to studies conducted by **Takzaree et al. (2016)** reported that the application of honey and propolis combined accelerated wounds healing process, reduced inflammation phase and increased tissues granulation, angiogenesis , and reported that peroxide hydrogen peroxide with its insulin-like effect influence the cells involved in the healing process and cause angiogenesis as cells proliferate in wound bed. collagen emerge and as results fast tissue epithelialization appeared ,while acidic PH and its osmotic pressure stimulates the activity of phagocyte and lymphocyte in the wound and increases the other antibacterial components and proline with antioxidant activity prevents the production of free radicals and play a role in inflammatory effect in honey.

According to **Kassim et al. (2010)**, honey leads to a significant decrease in two inflammation mediators (COX-2 and TNF- α) by blocking the translocation of NF-kB into the nucleus, thereby reducing the concentration of prostaglandins E2 (PGE2), prostaglandins F2 α (PGF2 α), and thromboxanes B2 responsible for inflammation symptoms.

II. Pumpkin seeds powder

II.1. Total Phenolic Content

PS are a loaded source of many phenolic compounds, like, quercetin, kaempferol, vanillic acid, *p*-coumaric acid, apigenin, ferulic acid, *p*-hydroxybenzoic acid etc. In this study, PS exhibit a value of 29.92 mg GAE/g of (TPC), which fall within the range reported by **Singh and Kumar. (2024)** (30 to 40 mg GAE/g) in *C. moschata Duch*, **Xanthopoulou et al. (2009)** reported values ranging from (27.89 to 41.66 µmol GAE/g) in methanolic extraction of (PS) seeds *Cucurbita spp*, this variation may be attributed to the geographical original and processing methods.

Badu et al., (2020) reported value of 160.74 mg GAE/g, in ethanolic extraction of the *cucurbita pepo* seeds, higher than the results obtained in the present study, **Kulaitienė et al. (2018)** obtained values varied from (37.0 to 60.6 mg EAG/kg) in cold pressed oil .

This variation may be attributed to the extraction solvent, methods of extraction, drying method and pumpkin origin.

II.2. Total Flavonoids Content

Flavonoids contributed significantly to the overall antioxidant activity in (PS), playing a crucial role in removing (ROS) and maintaining the body balance (**Singh and Kumar, 2024**).

In this study, flavonoids demonstrated a value of 17.05 mg GAE/g, which is close to the range reported by **Singh and Kumar. (2024)** who worked on *C. moschata Duch* variety (21.26 to 40 mg GAE/g) and higher than those obtained by **Jahan et al. (2023)** in *maxima Duchesne* (8.27 mg GAE/g), **Badu et al. (2020)** reported higher results in *Cucurbita pepo* seeds 44.04 mg GAE/g using ethanolic extraction. This variation may be attributed to the difference in extraction solvent, drying method and pumpkin origin.

II.3. Antioxidants activity: DPPH activity and reducing power

DPPH activity measures the anti-oxidant potential of substances, which serves as free radical scavengers or the hydrogen providers. In the present study DPPH activity demonstrated a value of 11.14 %, which fall within the range reported by **Saavedra et al. (2015)** in *Squash* Pumpkin seeds (4.0 to 18.9 %). **Singh and Kumar, (2024)** demonstrated higher results in *maxima Duchesne* seeds (52.33 %). **Jahan et al. (2023)** reported low antioxidant activity (0.11%) in the seeds of *maxima Duchesne* of Bangladesh.

Yasir et al. (2016) reported that the extracts obtained from pumpkin seeds exhibited antioxidant as well as geno-protective effects. Thereby, the variation in antioxidant activity may be attributed to climatic conditions, pumpkin type, origin, extraction and processing methods.

Boujemaa et al. (2020) worked on pumpkin seeds oil were extracted using Soxhlet extractor. *Cucurbita maxima* (CMA) showed the highest tocopherol , TPC (27.52) , PUFA value ,these parameters showed positive correlation with higher antioxidant activity found in (CMA), similarly to other studies **Latif and Anwar. (2011)** and **Zhang et al. (2012)** which demonstrated a proportional relation between high antioxidant activity and high PUFA, tocopherols and TPC.

In contrast we reported a slightly higher TPC (29.92 6 mg EAG/kg) compared to **Boujemaa and al. (2020)**, but significantly lower antioxidant activity. We suggest that the lower antioxidants activity in pumpkin seeds utilized in the present study may due to the absence of variation or the poor synergistic effect between its phenolic compounds. Lower amount of PUFA, tocopherols, carotenoids, β -Sitosterol, and as these compounds play crucial role in the antioxidant activity of PS as mentioned in several study.

Thompson and Cooney. (2020) reported that γ -tocopherol is highly efficient in reducing the damage provided by nitrogen oxides.

β -Sitosterol acts physically as membrane stabilizers and chemically as a potent radical scavenger, which inhibit the cell signaling pathways. (**Singh and Kumar, 2024**).

Moreover, polyphenols and carotenoids restrain the damage effects induced by free radicals by enhancing the antioxidant defense mechanism and other diseases (**Kulczyński and Gramza-Michałowska, 2019**).

II.4. Anti-inflammatory activity

PS extract showed an anti-inflammatory activity of 36.57 %, this result were lower than those obtained by **Basdogan et al. (2021)**, where the cold pressed PS oil showed higher anti-inflammatory activity (79 to 59%), compared to hot pressed oil (58 to 49%). This study indicated the effect of high temperature in bioactive compounds content; therefore, the difference in PS anti-inflammatory activity depends significantly on the extraction method and processing.

Findings from several observational studies reported a direct association between tocopherol and inflammatory biomarkers (**Abdulla et al., 2018; Saboori et al., 2015**), this direct association may be attributed to a response to nitrogen radical-mediated damage.

Moreover, recent discoveries suggest a specific anti-inflammatory pathway independent of its antioxidant activity, at physiological concentrations the γ -tocopherol inhibits (COX-2), 5-lipoxygenase (5-LOX)-mediated eicosanoids, consequently down regulating major pro-inflammatory mediators. (**Thompson and Cooney, 2020**).

Nutrients in PS like, zinc, selenium and fatty acid especially linoleic acid (Omega 6) exhibit anti-inflammatory activity features contributing to the overall anti-inflammatory activity (**Hussein et al., 2020**).

In study provided by **Loizou et al. (2010)**, β -sitosterol exhibit an anti-inflammatory activity by inhibiting vascular adhesion molecule 1 (VCAM1) and intracellular adhesion molecule 1 (ICAM1) expression in TNF- α -stimulated human aortic endothelial cells (HAECs).

III. Ointment physicochemical, stability and biological activities

III.1. Physicochemical activity

III.1.1 Color

In this coordinate system, the L^* value is a measure of lightness, ranging from 0 (black) to 100 (white). The a^* value ranges from -100 (greenness) to $+100$ (redness), and the b^* value varies from -100 (blue) to $+100$ (yellowness).

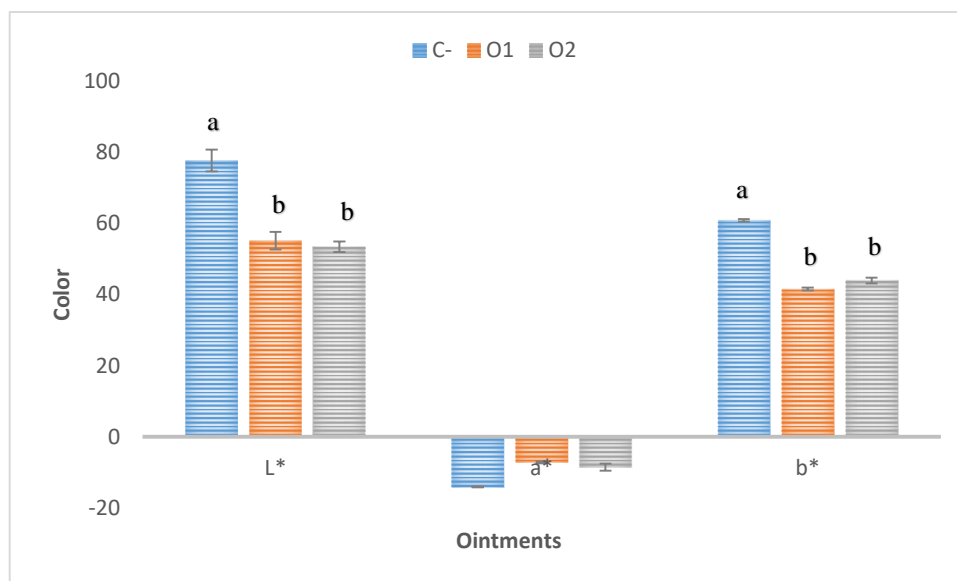


Figure 20: The color of the formulations.

The color of the three formulations C-, O1, and O2 was measured. In the coordinates (L^* , a^* , b^*) for color determination, C- exhibited a value of 77.73 for L^* , while there was no significant difference between (O1) 55.14 and (O2) 53.42, showing the negligible effect of dark honey (H1) compared to light honey (H2). The incorporated formulation showed lower lightness compared to C-, which attributed to PSP. (Figure 20)

The a^* value, as well as the L^* value, showed no significant difference between (O1) -7.23 and (O2) -8.53, while C- showed a more green color with a value of -14.1.

The b^* value, similar to L^* and a^* , showed no significant difference between (O1) 41.54 and (O2) 43.96, with C- exhibiting a higher yellowness value of -14.1. Overall, the change in color between the control formulation and the incorporated ointment was mostly attributed to PSP, while the effect of dark-colored honey was negligible.

III.1.2. Moisture content

The ointments formulated showed low moisture content: 2.11, 2.27, 2.6, 2.92 and 2.41% in C-, O1, O2, NEWBO, and MEBO, respectively. After 45 days of preservation at 25°C, the water content was slightly increased in C-, NEWBO, and MEBO to 2.28, 3.01, and 2.53 %, respectively. O1 showed the highest increase, reaching 3.24%, followed by (O2) 3.17%.(Figure 21).

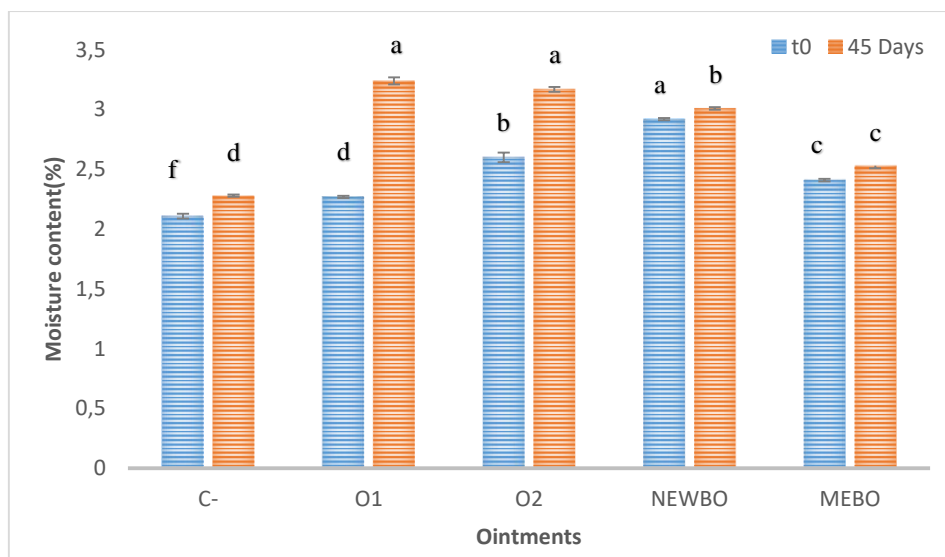


Figure 21: Moisture content in the formulations.

All the formulations classified as “Ointments” due to their low water content. Formulations with water content >20% are classified as creams.

This increase in moisture content in the incorporated formulations may be attributed to the hygroscopic property of honey, which by definition the capacity to absorb, retain and draw moisture out of the environment and dehydrate bacteria. This property is largely due to the high sugar content combined with low water content, which create low water activity and high osmotic pressure (**Mandal,M and Mandal,S., 2011**). This explains the higher water content in O1 compared to O2 after storage. O1 showed high sugar content 83.53% and low water content 14.91%, compared to O2 , which exhibited lower sugar content 77.81% and higher water content 21.24%.

The ANOVA test indicates the homogeneity of all formulations, with no significant difference.

II.1.3. pH

The ointments formulated showed pH varied between (6.14 to 6.32), pharmaceutical ointment showed lower PH 5.79 and 5.88 for NEWBO and MEBO respectively. After 45 days of preservation at 25°C, the pH slightly decreased in all ointments, reaching 6.3, 6.11, 6.18, 5.76, and 5.86 in C-, O1, O2, NEWBO, and MEBO, respectively. The statistical analysis revealed two homogenous groups O1 with O2 and NEWBO with MEBO. (Figure 22).

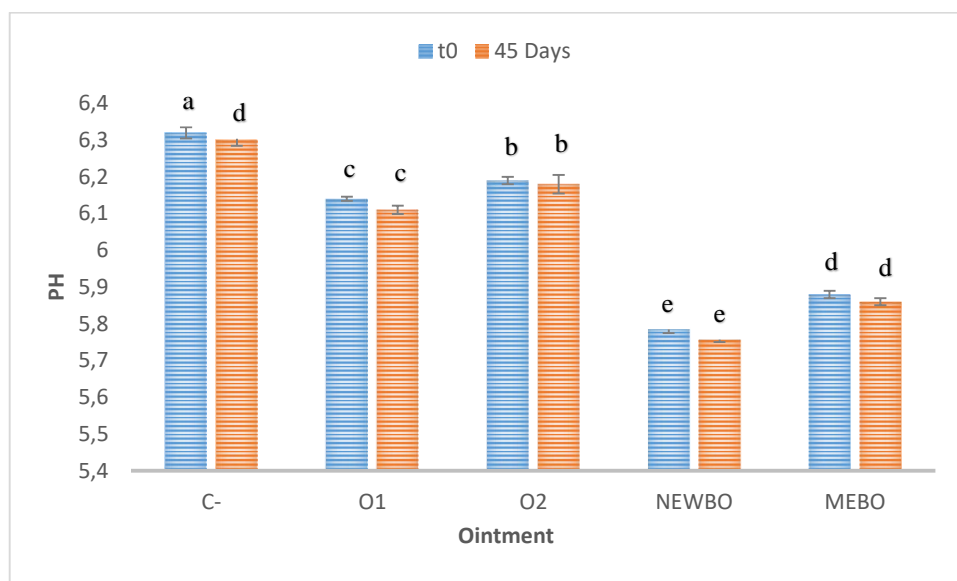


Figure 22: pH of the ointments.

The pH reported in this study was within the range reported by **Kawarkhe et al. (2016)** in 6 creams (5.8 to 6.9), lower than the pH reported by **Hovanet et al. (2022)** in lipophilic ointment (6.80 to 7.02), and by **Maru and lahoti, (2019)** (5.41 to 5.42).

The increase in pH reported in O1 and O2 compared to C- , may be attributed the honey's acidic pH .

The pH of the ointments meet the standard acidic skin pH (4.5 to 6.5), the acidic PH < 4 can cause skin irritation while alkaline pH > 6.5 can cause dry and scaly skin (**Sihotang, 2021**). Furthermore, skin pH play an important role in the communication pathways between keratinocytes, immune cells and Microbes, maintaining skin barrier permeability and homeostasis under both health and stress conditions .(**Lukic et al., 2021**)

III.1.4. Total phenolic compounds

The (TPC) ranged from (36.03 to 45.63 GAE mg/100 ml) in formulated ointments, lower than the two PO, which varied from (48.8 to 68.21 GAE mg/100 ml).

Those results are in the range found by **Mapoung and al. (2021)** in 23 creams (0.46 to 47.92 mg GAE/30), lower than those obtained by **Bernatoniene et al. (2011)** in 3 creams of *Calendula* extract with different concentration (50 to 92 GAE mg/100 ml).

In another study by **Abbood. (2018)** among cosmetic cream samples in The Syrian, reported lower values than those obtained in this study (2.9 to 29.8 mg GAE/g). O1 and O2 presented no significant difference while the other honey samples show differences for this parameter.

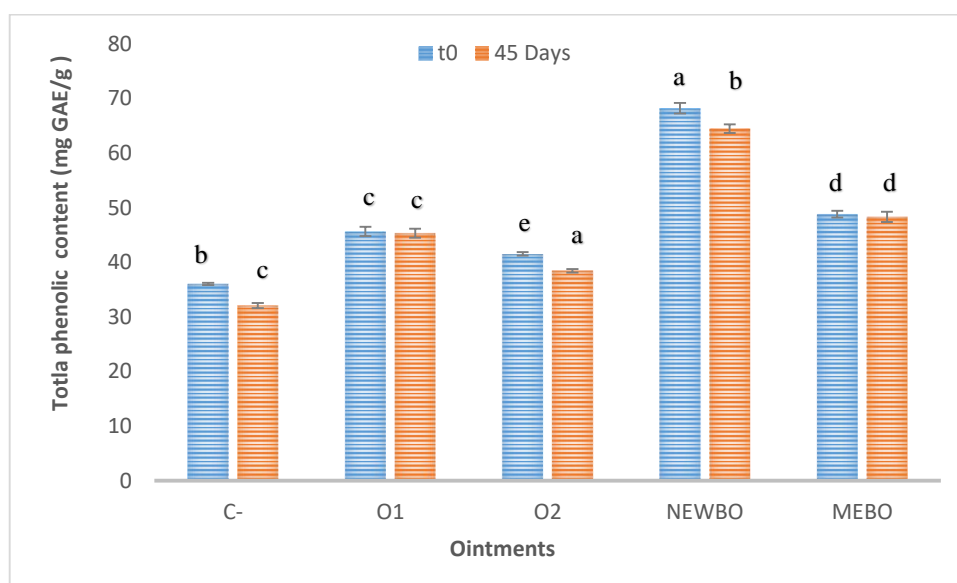


Figure 15: TPC in the ointments.

Ointment incorporated with honey and PSP exhibit higher (TPC) 45.63 and 41.52 GAE mg/100 ml in O1 and O2 respectively compared with (C-) (36.03 GAE mg/100 ml). Moreover, when compared with pharmacological ointment, MEBO (48.8 GAE mg/100 ml) and NEWBO (68.21 GAE mg/100 ml). The incorporated formulation demonstrated lower (TPC). Hence, honey and PSP boosted the (TPC) compounds in the formulations; this increase may due to the various phenolic compounds within honey and pumpkin seeds. (Figure 23).

After 45 days of preservation at 25°C, the polyphenol content was slightly decreased in O1 and MEBO to 45.32 and 48.31 GAE mg/100 ml, respectively, with a higher decrease in O2 38.45 GAE mg/100 ml. The most significant decrease was observed in NEWBO and C- ointments, reaching 64.47 and 32.06 GAE mg/100 ml, respectively.

III.1.5 Total Flavonoid Content

The (TFC) ranged from (16.32 to 24.96 QE mg/100g) in formulated ointments, higher than the two pharmacological ointment, which varied from (19.08 to 22.48 QE mg/100g). There was no significant difference between C- and NEWBO as well as between O1 and O2.

Imam et al. (2015), reported lower results than those obtained in this Study (3.66 to 5.32 QE mg/100g) depends on the anti-aging cream concentration. Another pharmaceutical preparations formed by **Shaghghi et al. (2009)**, in Dermatol cream and Calendula ointment, showed a value of 20.4 and 47.6 QE mg/100g respectively.

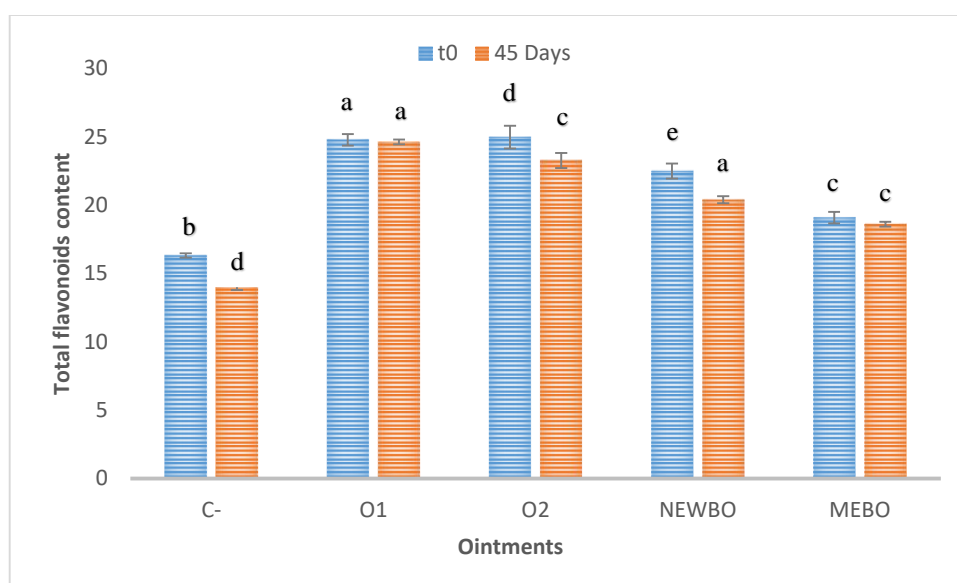


Figure 24: TFC in the ointments.

Ointment incorporated with honey and PSP demonstrated higher flavonoid content O1 (24.77 QE mg/100g), (O2) 24.96 QE mg/100g) compared with (C-) 16.32 QE mg/100g and lower concentrations than those obtained in pharmacological ointment NEWBO and MEBO (68.21 and 48.8 and 22.48 QE mg/100g) respectively. (Figure 24).

The incorporated formulation showed an increase in flavonoids content; this might be attributed to the various flavonoid compounds contain in honey and PS.

After 45 days of preservation at 25°C, the flavonoid content slightly decreased in O1 to 24.61 GAE mg/100 ml, followed by NEWBO and O2 at 18.6 and 23.26 GAE mg/100 ml, respectively. The most significant decrease was observed in the C- ointment, reaching 14.17 GAE mg/100 ml.

III.2. Biological activity of the formulations

III.2.1. Stability and antioxidant activity

The antioxidant activity of ointment formulations was evaluated using DPPH scavenging and reducing power activity.

DPPH scavenging activity in the incorporate formulations was varied 33.74 to 27.6 %, with 32.92 and 27.73 % for NEWBO and MEBO respectively. No significant difference between C- and O1 as well as between O2 and MEBO. (Figure 25).

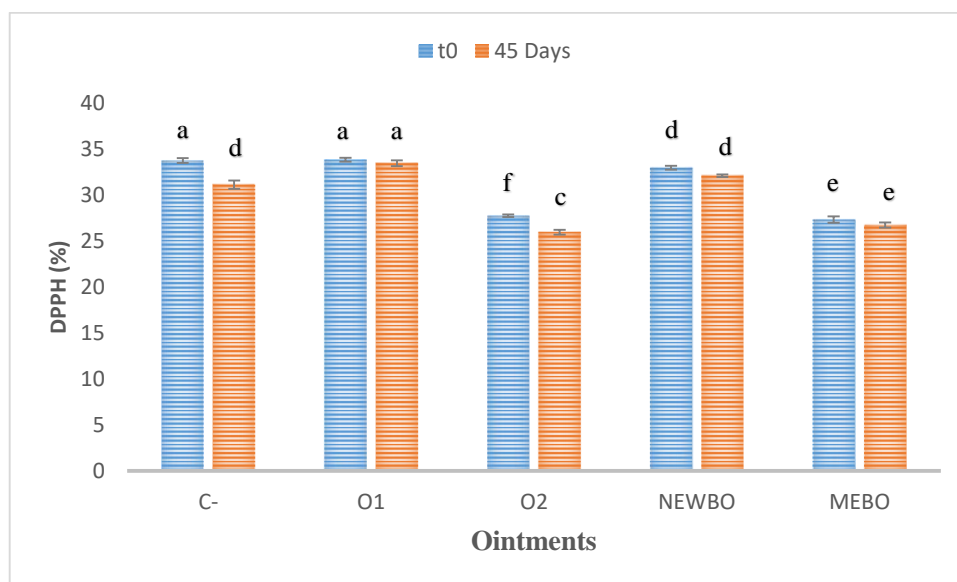


Figure 25: DPPH radical scavenging activity in ointments.

The DPPH radical scavenging capacity found was in the range reported by **Campanini et al. (2014)**, in 6 topical formulation (3.4 to 74.8 %) and close to those obtained by **Di Mambro and Fonseca, (2005)** in 2 lipid formulations (48.23 to 45.63 %). **Casagrande et al. (2007)** reported values ranged from (37.80 to 38.50 %) in 2 formulations containing quercetin.

O2 reported lower DPPH activity 27.73 % compared with O1 and C-, which exhibited no difference in the scavenging activity, the incorporated formulation exhibit no effect in DPPH activity, O2 imposed a negative effect in DPPH activity that may due to the low phenolic compounds in H2 compared with H1.

Moreover, O1 showed higher reducing power 9.60 mg EAG/ 100 g comparing with (C-) 1.76 mg EAG/ 100 g and (O2) 3.87 mg EAG/ 100 g. NEWBO and MEBO showed a value of 3.06 and 2.32 mg EAG/ 100 g respectively. No significant difference between O2 and NEWBO.

The incorporated formulations showed an increase in DPPH scavenging compared to C-, boosting the overall antioxidant activity, thus preventing oxidative stress.

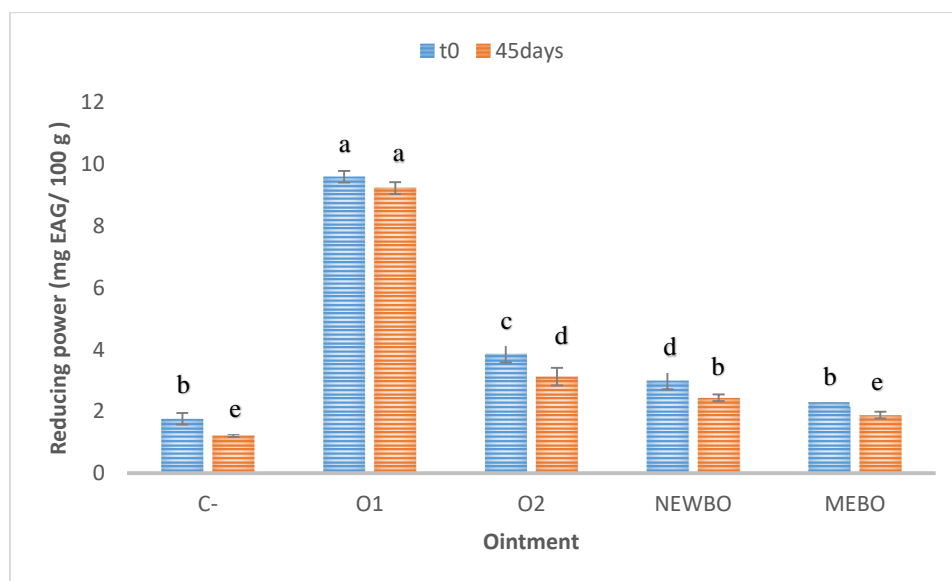


Figure 26: reducing power activity in ointments.

After 45 days of preservation at 25°C, the DPPH scavenging activity slightly decreased in O1 to 33.44 %, followed by NEWBO, O2, and MEBO at 26.73, 25.95, and 32.1 % respectively. The most significant decrease was observed in the C- ointment, reaching 31.11 %. (Figure 26).

After 45 days of preservation at 25°C, the reducing power activity slightly decreased in O1 to 9.23 %, followed by MEBO, NEWBO, and O2, with 1.88, 2.44, and 3.13 % respectively. The C- formulation showed the highest decrease, reaching 1.22 %.

III.2.2. Anti-inflammatory activity

In-vitro anti-inflammatory activity of the formulations varied from (57.28 to 86.4 %), the incorporated formulation showed the higher activity 83.50 and 86.41 % for (O1) and (O2) respectively, compared with (C-) 57.28 % and pharmacological ointments NEWBO and MEBO, which showed an activity of 71.84 and 81.55 % respectively. O2 and MEBO showed no significant difference. (Figure 27).

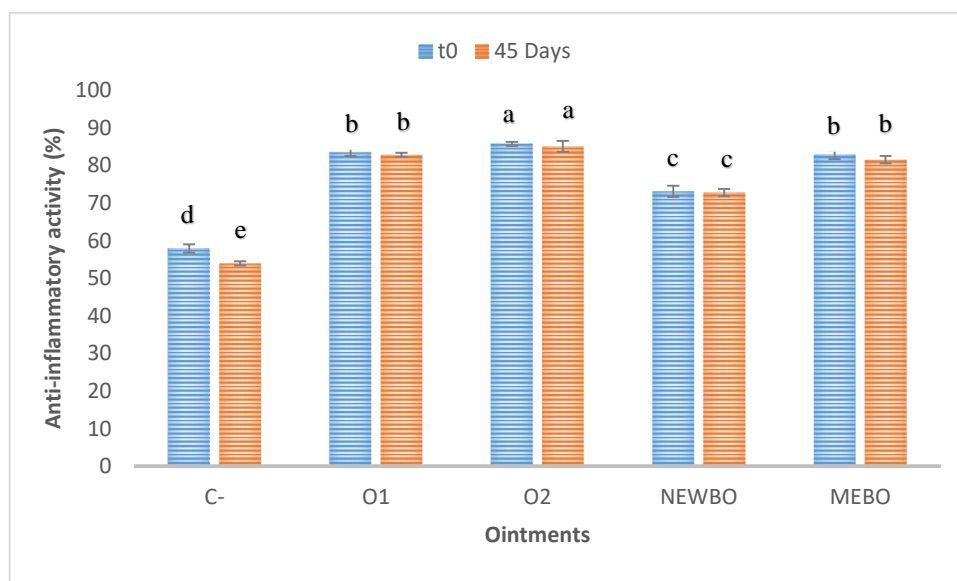


Figure 27: Anti-inflammatory activity in ointments.

The results obtained were slightly close to those reported by **Safia et al. (2019)** in rosewater formulation of 50g concentration (52.2 to 80.6 %) and higher than those of 30 g concentration (41.4 to 65.2 %), the high anti-inflammatory effects observed in incorporated formulation compared to the control formation can be attributed mainly to the polyphenol compounds present in honey, PS and the synergetic effect that might occur between the different components found in honey and PS as well as the other formulation ingredients.

After 45 days of preservation at 25°C, the anti-inflammatory activity remained almost stable in all ointments O1, O2, NEWBO, and MEBO, except for a decrease in (C-), which reached 53%.

III.2.3. TBARS and peroxide value

Thiobarbituric acid-reactive substance (TBARS) assay is used to assess the lipid peroxidation of poly-unsaturated fatty acids to MDA, as MDA reacts with thiobarbituric acid (TBA), which is used as a reagent to give a red color pigment which is considered as a sign of oxidation and can be measured spectrophotometrically. (**Singh et al., 2022; Marin et al., 2018**).

The TBARS value was evaluated at (t0) and (t45days) of conservation under 25° temperature, TBARS values showed a slightly increase in oxidation in all formulations tested from 0,001 mg/kg (t0) to attend (0.0012 to 0.0019 mg/kg) (t45days).

The standard showed a higher increase rate (0.0019 mg/kg) compared to the (O1) and (O2) 0.0012 and 0.0014 mg/kg, which showed a significantly lower value at the end of storage period. After storage The 3, formulations showed a difference for this parameter. (Figure 28).

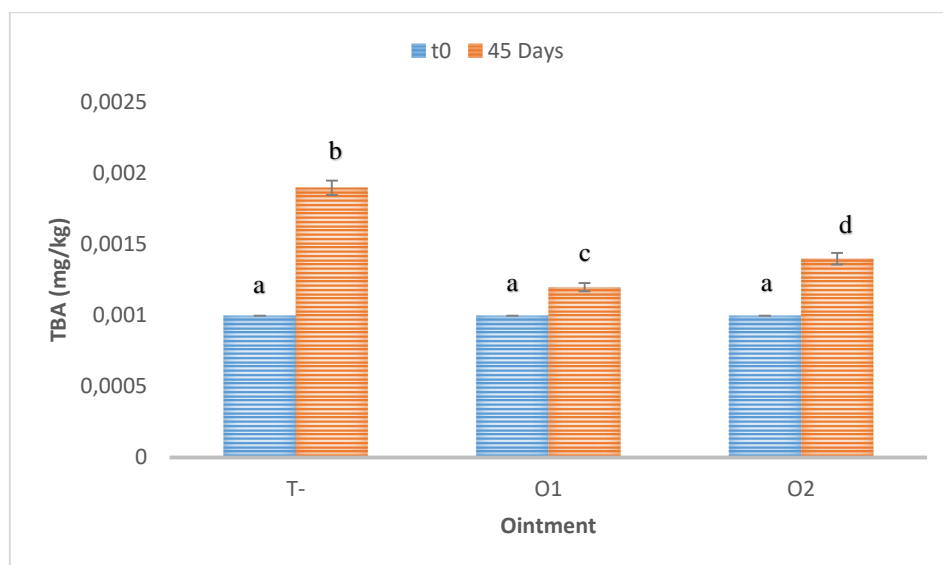


Figure 27: TBARS Lipid peroxidation in ointments.

The TABRS is parameters used to assess the oxidative stability of food or products, the lower TABRS value related to higher oxidative stability. TABARS values reported in this study reflect the oxidative stability of the different formulations examined.

Furthermore, O1 and O2 exhibit higher oxidative stability compared to C-, which can be directly attributed the effect caused by the honey and PSP.

Peroxide value measures the development of peroxides in unsaturated fats, which occurs when double bonds break, forming short-chain volatile products that contribute to a rancid odor (Canneddu et al., 2016). This parameter is widely used to assess oil quality. The peroxide value in edible oils indicates their oxidative state and susceptibility to rancidity. Oils with peroxide values above 10 meq O₂/kg are considered unstable and prone to rancidity, while those with values below 10 meq O₂/kg are deemed stable and resistant to oxidation (Buthelezi et al., 2019).

Peroxide values showed a slightly increase from 0.5 meq O₂/kg at (t₀) in O1 and O2 to reach 0.7 and 0.9 meq O₂/kg respectively at (t_{45days}), the highest peroxide alleviation was reported in C-, increasing from 0.6 meq O₂/kg at (t₀) to attend 1.2 meq O₂/kg at (t_{45days}), Thus the incorporated formulation improved the stability compared to C-. After storage The 3 formulations showed a difference for this parameter. (Figure 29).

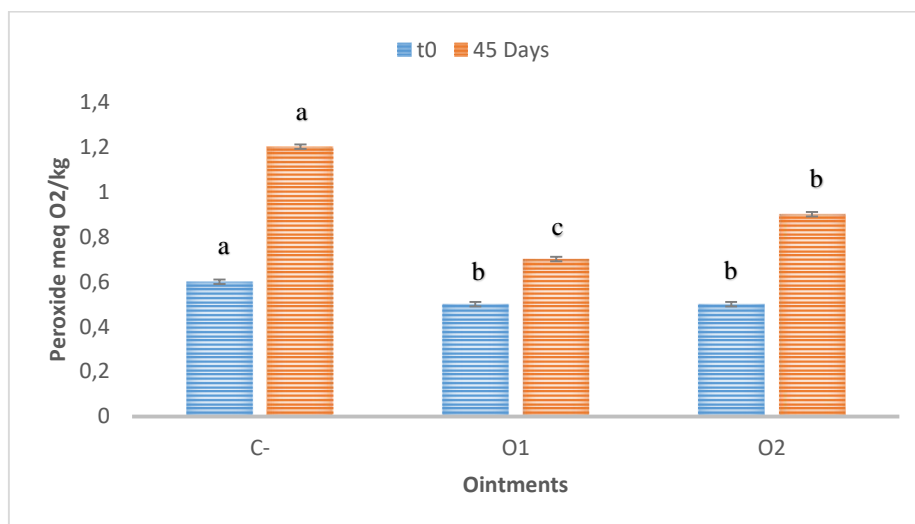


Figure 29: peroxide values in ointments.

Bioactive compounds, such as phenols and Flavonoids that are present in PSP and honey, tocopherol and β -sitosterol in PS contributed to the total antioxidant effect and prevented lipid oxidation and in the incorporated ointments; as a result, less TBARS and peroxide values were reported.

O1 showed lower degradation in bioactive compounds (polyphenols and flavonoids) and a lower increase in biological activities (DPPH scavenging, reducing power), as well as lower lipid peroxidation and peroxide value compared to C-. These factors are considered relevant indicators of formulation stability. This stability may be attributed to the diverse bioactive compounds present in H1 compared to H2 and C-, which provide a preservative effect, as well as the effect attributed to PS various compound, thereby enhancing the ointment's stability and extending its shelf life.

Antioxidant capacity of honey is well established, although the precise mechanisms of action are not yet fully understood. Mechanisms such as radical scavenging, hydrogen donation, metallic ion chelation, flavonoids substrate action for hydroxyl and superoxide radical actions are considered as possible antioxidant mechanisms in honey (Patouna et al., 2023).

Monofloral honey is associated with numerous health benefits, including wound healing, antioxidant, antimicrobial, anticancer, and anti-inflammatory properties. Those properties contribute to the observed decreases in MDA and TBARS levels (**Mărgăoan et al., 2021**).

Omotayo et al. (2010) demonstrated that the consumption of Tualang honey significantly reduced the elevated levels of TBARS in diabetic rats induced with streptozotocin, indicating a reduction in lipid oxidative damage in the honey-treated diabetic rats.

Bardaa et al. (2016) evaluated the inhibition of lipid peroxidation, in compared to the standard antioxidant BHT. The results showed that PSO had a higher lipid peroxide inhibitory effect compared to BHT. This significant antioxidant activity of the cold-pressed PSO is primarily attributed to its high tocopherol content, as demonstrated by several studies.

In vivo study by **Paul et al. (2020)** demonstrated that mice pretreated with PSO extract showed a significant reduction in formaldehyde-induced elevation of serum glutamic oxaloacetic transaminase (SGOT). Phytochemical analyses conducted in vitro revealed that PSO extract exhibits free radical scavenging and total antioxidant activities, attributed to its phenolic and flavonoid content. Consequently, PSO extract provides substantial protective effects against FA-induced organ toxicities by mitigating oxidative stress and inhibiting lipid peroxidation.

Upon initiation of lipid peroxidation following radical attack, α -tocopherol is Known as the sole fat-soluble antioxidant inhibit lipid propagation, by donates a hydrogen atom to reduce the RO₂ radical allows the breaking of destructive chain reactions. That antioxidant activity arises from tocopherols ability to scavenge peroxy radicals, converting them into tocopheroxyl radicals. **Bardaa et al. (2016)**

In a study by **Baskar et al. (2012)**, β -sitosterol supplementation significantly reduced the elevated levels of liver lipid peroxides induced by 1,2-dimethylhydrazine (DMH). Additionally, β -sitosterol provided protective effects against DMH-induced depletion of antioxidants, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, and reduced glutathione, in the colonic and hepatic tissues of experimental animals. The supplementation also restored levels of nonenzymatic antioxidants, including vitamin C, vitamin E, and glutathione. Histopathological changes observed in DMH-induced animals were nearly normalized in rats treated with β -sitosterol.

III.2.4. Microbiological activity:

The findings shown that all the 3 formulations showed excellent microbiological stability, for both the estimated aerobic count as well as yeast count, no colony was detected in all the plates for all the formulations including the control analyzed, which complied with the degree of microbiological contamination limit set by the **FDA** and the **United States Pharmacopoeia (USP)** (non-eye area less than 1000 cfu/g and for eye area less than 102 cfu/g).

The low moisture content and water activity in the three formulation generate an unsuitable environment for and bacterial fermentation yield yeast and proliferation (**Xie et al., 2021**).

PS and honey may play a synergistic effects enhancing the effect caused by the vegetable oil pre-exist , In a study conducted by **Leichtweis et al. (2022)**, the extracts obtained from pumpkin seeds were capable of inhibiting the growth of some bacterial strains and one of the two fungal strains assessed, all of the samples from Portugal exhibited inhibition capacity against “*Yersinia enterocolitica*”, while the ones from Algeria inhibited “*Staphylococcus aureus*” In terms of food preservation.

Honey phenolic compound, high acidity, low PH, low water activity, hydrogen peroxide, glyoxal, 3-deoxyglucosulose, and MGO may play role in the overall antimicrobial activity (**Angioi et al., 2021 ; Tashkandi., 2021**).

IV. Skin patch test

The skin patch test conducted showed no signs of irritation after 48h. The absence of skin irritation after the exposure to the formulation indicates its potential safety for topical application, this finding suggests that the may be well-tolerated by the skin supporting its suitability for therapeutic use.

V. Sensory characterization

Sensory characterization of the control ointments and those incorporated with honey and an expert panel composed of 12 qualified juries carries out pumpkin seed powder produced at a laboratory scale.

V.1. Product characterization

The aim of this test is to identify key characteristics as well as descriptors or sensory attributes, which differentiate the products evaluated by the jury. When the results are represented by green, it means that the descriptor coefficient is positive (appreciated); in red, it indicates a significantly negative coefficient (not appreciated). In white, it indicates that the characteristics are not detected (Husson and Cadoret, 2008).

V.2. Discriminant power by descriptor

The objective of this test is to represent the sensory attributes of the products evaluated by the expert jury, ranked according to their discriminating power, from strongest to weakest, based on the p-value. The results are presented in both figure 30 and table III.

Table III: Discriminant Power by Descriptor

| Descriptor | Value test | p-values |
|----------------------------------|-------------------|-----------------|
| Color | 6,640 | 0,000 |
| Texture | 2,646 | 0,004 |
| Spreadability on the skin | 2,029 | 0,021 |
| Brightness | 0,645 | 0,260 |
| Odor | 0,381 | 0,351 |
| Consistance | -1,035 | 0,850 |

The table collects the descriptors ordered from the most discriminant to the least discriminant on the control ointments and those incorporated with honey and PSP. The obtained values reveal that color is the most discriminant descriptor, and the associated p-values all show a significant effect of the descriptor.

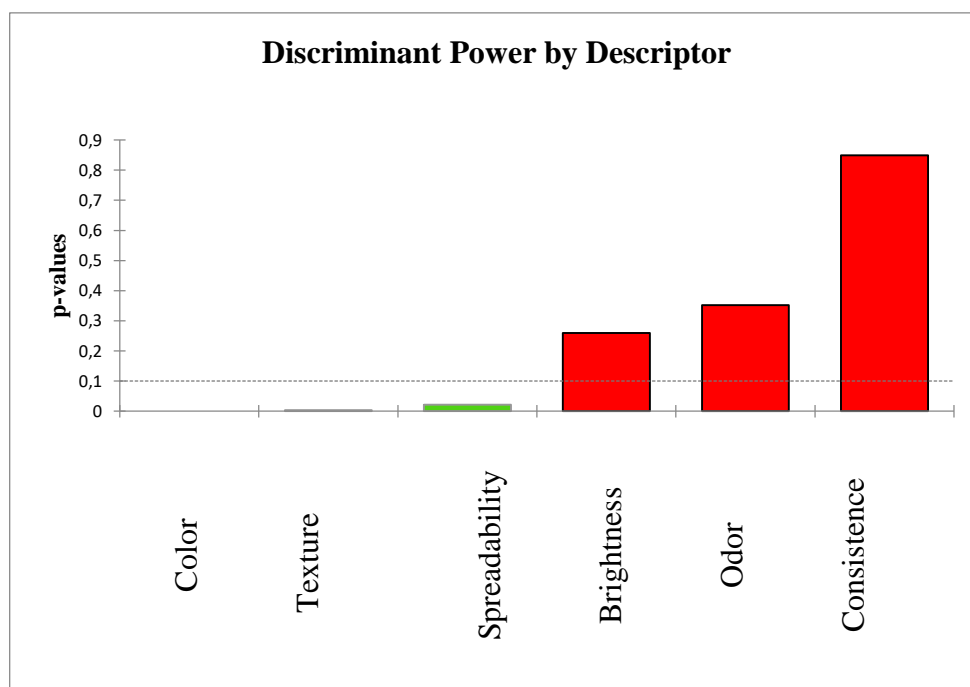


Figure 30: Discriminant Power by Descriptor

The figure also illustrates the descriptors ordered from the most discriminant to the least discriminant for the control ointments and those incorporated with honey and PSP. Color, texture, and spread ability on the skin are the descriptors with the strongest discriminant power among the three elaborated ointments, with color > texture > spreadability on the skin, indicating that the experts noted sensory differences between the three ointments for these three descriptors.

The following descriptors: brightness, odor, and consistency, have a weak discriminant power, which means that the experts did not observe differences between the descriptors of the ointment samples. Figure 30.

In conclusion, generally speaking, the three formulated ointments have different descriptors that distinguish them from each other.

V.3. Model Coefficients

The coefficients of the selected model are displayed for each descriptor and each product. This allows evaluating the overall performance of the expert panel based on two factors (product and judge) for each descriptor (**Pagès and Husson, 2005**). For each product, a graphical representation of the coefficients was associated with the different descriptors. The results of the model coefficients are presented in the Figure 31.

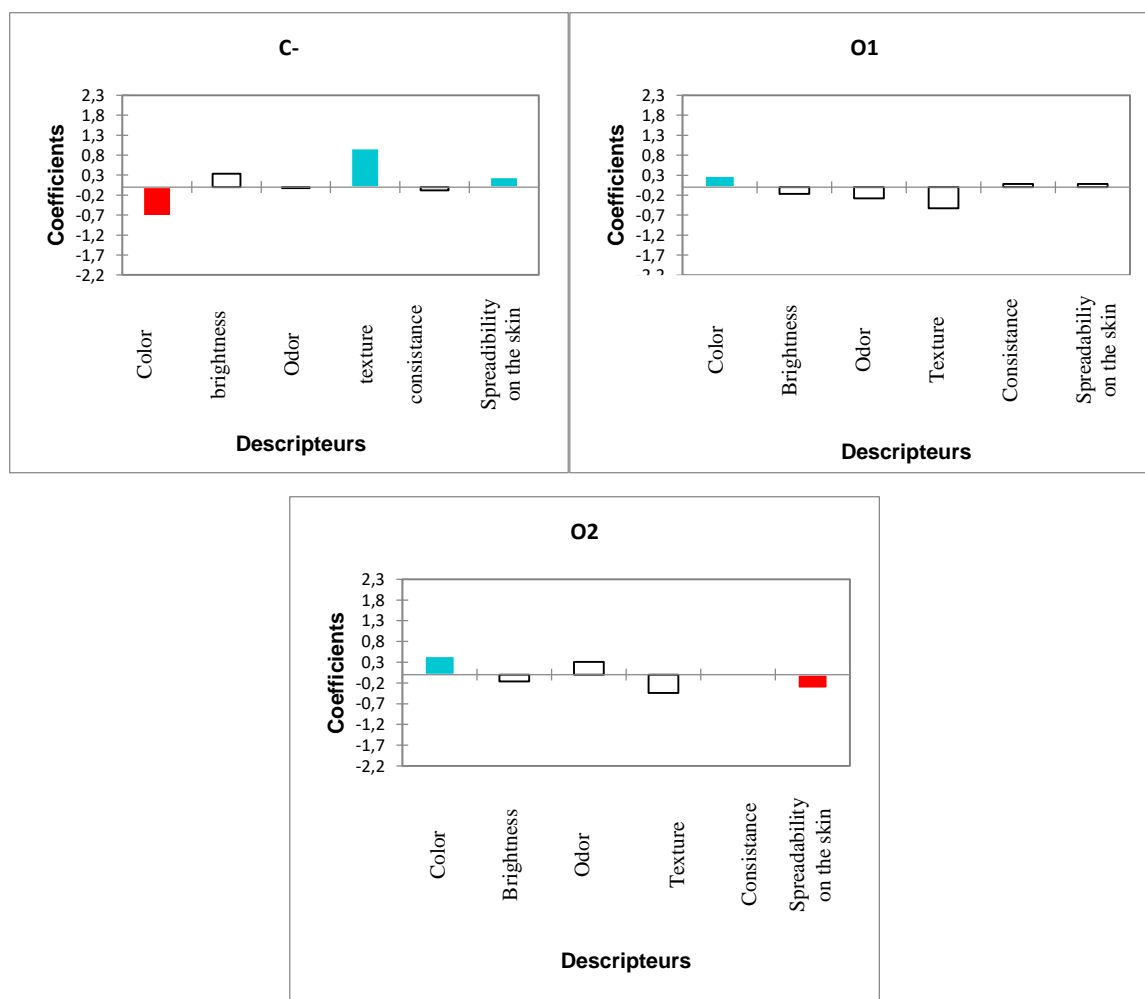


Figure 31: Model Coefficients of the Three Ointments: Negative Control (C-) and those Supplemented with Honey and PS (O1 and O2).

The graphs presented in the figure allow for visualizing and determining whether the descriptors of the different ointment samples are appreciated or not by the expert juries. For the C-, the color characteristic is presented in red, indicating that this characteristic is not appreciated by the experts, while the two characteristics, texture and spreadability on the skin, are illustrated in blue, meaning the juries appreciate these characteristics.

However, for the two ointments supplemented with honey and pumpkin seed powder O1 and O2, the color characteristic is appreciated by all the experts and is shown in blue, whereas characteristics that the juries could not detect are shown in white. Additionally, for ointment O2, the spreadability on the skin characteristic, shown in red, is not appreciated by the juries.

The obtained results indicate that the ointments supplemented with honey and pumpkin seed powder are characterized by a good color that is appreciated by the juries.

The Descriptors displayed in white are not significant, indicating that the jury members could not detect these characteristics in the products.

V.4. Product-adjusted averages

The main objective of this test is to define the adjusted means calculated from the model for each descriptor-product combination. The results of the adjusted means by product are presented in the Table IV.

Table IV: Product-adjusted averages

| | Color | Consistency | Odor | Spreadability | Texture | Brightness |
|----|-------|-------------|-------|---------------|---------|------------|
| O2 | 4,083 | 2,083 | 3,333 | 4,250 | 2,750 | 3,083 |
| O1 | 3,917 | 2,167 | 2,750 | 4,667 | 2,667 | 3,083 |
| C- | 2,917 | 2,000 | 3,000 | 4,833 | 4,167 | 3,583 |

The table of adjusted means by product highlights the means when different products and characteristics (sensory attributes) are cross-referenced. Blue cells indicate that the descriptors have a significantly positive discriminant effect on the product, red cells correspond to descriptors having a significantly negative discriminant effect on the product, and white cells indicate that the descriptors have no discriminant effect on the products.

For the two ointments, O1 and O2, the color descriptor has a significantly positive discriminant effect on the product, while the descriptors texture, odor, consistency, and shine have no significant discriminant effect on the product, meaning these descriptors are not appreciated. The spreadability on the skin descriptor shows a significantly negative discriminant effect on O2.

However, the spreadability on the skin and texture descriptors of the control ointment C- have a significantly positive discriminant effect on the product, while color has a significantly negative discriminant effect on the product.

The descriptors brightness, consistency, and odor have no significant discriminant effect on the ointments, indicating that the juries could not differentiate between the ointments in terms of these descriptors.

VI. External Preference Mapping (PREFMAP)

VI.1. Principal Component Analysis (PCA)

The map presented in the Figure illustrates the correlations between the variables present in the observations and the factors through PCA.

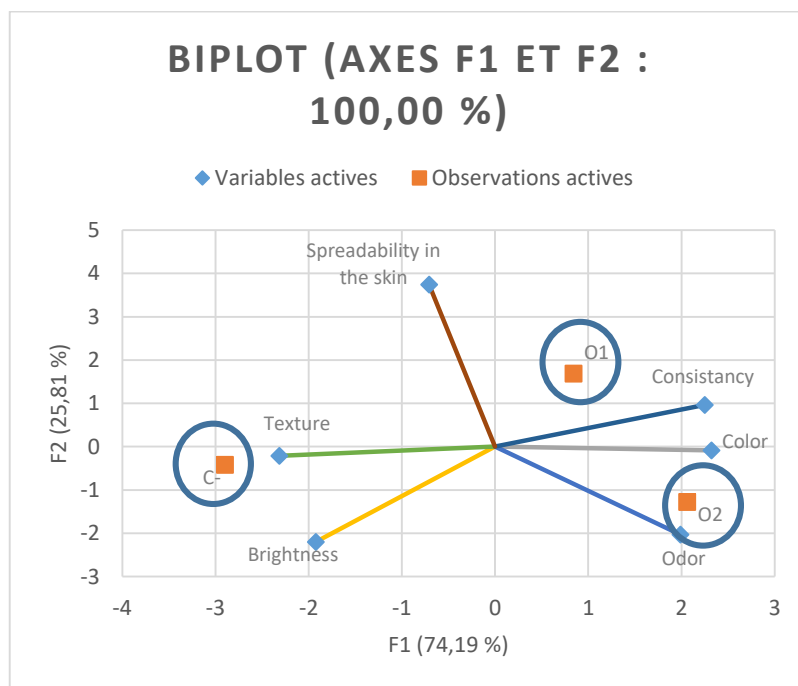


Figure 32: The PCA of the formulated ointments

The PCA reveals two principal components, PC-1 and PC-2, which together represent 100.00% of the total variation. PC-1, representing 74.19 % of the data, separates the reformulated ointments into three main groups.

Two groups consist of ointments O1 and O2 located in the positive part of the map. O1 is characterized by its good color and remarkable, attractive odor, while O2 is noted not only for its good consistency but also for its appealing and appreciated color. The last group consists of C-, situated in the lower left quadrant, which is appreciated by the juries due to its remarkable texture and brightness. Figure 32.

Additionally, this figure shows that the descriptors odor, color, and consistency are significantly positively correlated as they are closely clustered together, as are texture and brightness.

The results obtained indicate that the sensory variables of the ointment (color, odor, texture, and spreadability on the skin) determine the quality of the ointment, its acceptance, and its commercialization.

VI.2. PREFMAP Mapping

The PREFMAP method is applied using the coordinates of the ointments in the two-dimensional factorial space and the evaluations provided by the experts. The juries differentiated the ointments elaborated with 100.00% of variability explained by the two principal components, CP-1 and CP-2 (Figure 33). CP1 is characterized by all descriptors. The PREFMAP mapping illustrated in the figure shows the preference percentage of ointments enriched with honey in a contour plot graph.

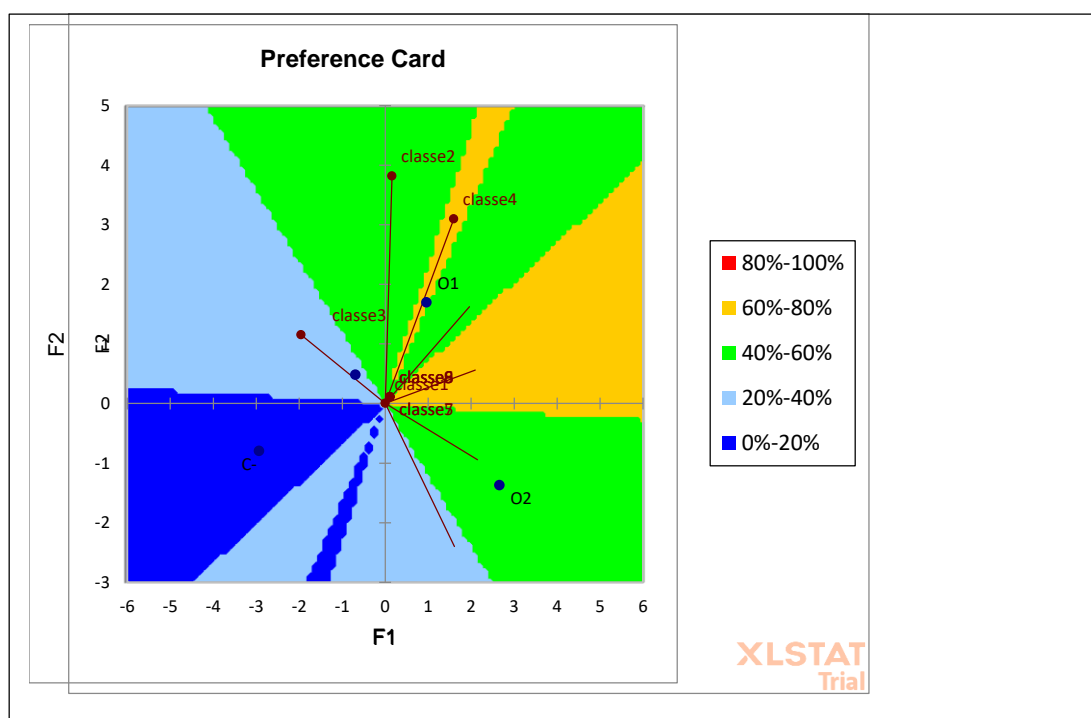


Figure 33: PREFMAP Mapping

Based on the figure, O1 falls within the range of 60 % to 80 % preference among the juries, with a satisfaction percentage of 70 %. This indicates that this ointment formulation is the most liked and preferred by the juries.

On the other hand, O2 and C- are positioned on the green and dark blue sides of the graph, respectively. They represent preference percentages between 40 to 60 % and between 0 to 20 %, with satisfaction percentages of 47 and 14 %, respectively. This suggests that these ointments are the least favored by all juries.

VI.3. Pearson Correlations

Table V: Pearson correlation matrix between physico-chemical parameters, phenolic compounds, and antioxidant and anti-inflammatory activities of ointments

| | L* | a* | b* | Moisture | PH | TPC | TFC | DPPH | RP | Anti-Inflammatory Activity |
|----------------------------|----------|------------------|-------|----------|------------------|-------|-------------|--------|----------|----------------------------|
| L* | 1,00 | | | | | | | | | |
| a* | -0,78*** | 1,00 | | | | | | | | |
| b* | 0,18 | -0,53* | 1,00 | | | | | | | |
| Moisture | -1,00*** | 0,80** * | -0,21 | 1,00 | | | | | | |
| PH | 0,92*** | - 0,80** * | 0,53 | -0,93*** | 1,00 | | | | | |
| TPC | -0,81*** | 0,76** | -0,50 | 0,81*** | - 0,90* ** | 1,00 | | | | |
| TFC | -0,11 | 0,68** | -0,40 | 0,14 | -0,14 | 0,27 | 1,00 | | | |
| DPPH | 0,02 | -0,24 | 0,64* | -0,05 | 0,22 | 0,11 | -0,11 | 1,00 | | |
| RP | -0,11 | 0,37 | 0,38 | 0,12 | 0,15 | -0,05 | 0,68 ** | 0,34 | 1,0 0 | |
| Anti-Inflammatory Activity | -0,31 | 0,75** | -0,44 | 0,35 | -0,32 | 0,15 | 0,77 *** | -0,60* | 0,4 9 | 1,00 |

TPC: total polyphenol content, **TFC:** total flavonoids. **RP:** reducing power, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Interpretation:

The Pearson correlation analysis illustrated in the table reveals several highly significant positive correlations. Specifically:

- ❖ **Color a* (redness) and Moisture:** There is a very high significant positive correlation ($r = 0.80$, $p < 0.001$) between color a* and moisture content. This indicates that as the redness of the ointment increases (higher a* value), so does its moisture content.
- ❖ **Color a* and Bioactive Compounds:** There are other highly significant positive correlations of color a* with polyphenols ($r = 0.76$, $p < 0.01$), flavonoids ($r = 0.68$, $p < 0.01$), and anti-inflammatory activity ($r = 0.75$, $p < 0.01$). This suggests that ointments with higher redness (a* value) tend to have higher levels of these bioactive compounds and stronger anti-inflammatory properties.

- ❖ **Color L* (lightness) and Other Parameters:** Color L* shows a perfect negative correlation with moisture content ($r = -1.00$, $p < 0.001$), indicating that as the ointment becomes lighter (higher L* value), its moisture content decreases completely. Additionally, there is a very high positive correlation with pH ($r = 0.92$, $p < 0.001$) and a strong negative correlation with polyphenols ($r = -0.81$, $p < 0.001$).
- ❖ **Color b* (yellowness) and DPPH Test:** Color b* exhibits a significant positive correlation with the DPPH antioxidant activity test ($r = 0.64$, $p < 0.05$). This suggests that ointments with higher yellowness (b* value) have greater antioxidant capacity as measured by the DPPH assay.
- ❖ **Bioactive Compounds (Polyphenols and Flavonoids):** Polyphenols show a very high positive correlation with moisture content ($r = 0.81$, $p < 0.001$) and a very high negative correlation with pH ($r = -0.90$, $p < 0.001$). Flavonoids also exhibit highly significant positive correlations with reducing power ($r = 0.68$, $p < 0.01$) and anti-inflammatory activity ($r = 0.77$, $p < 0.001$), suggesting that the anti-inflammatory properties of the ointment are largely due to its richness in flavonoids.

Overall, these correlations indicate that specific sensory attributes of the ointment, such as color parameters (a^* , L^* , b^*), are closely associated with its chemical composition (moisture, polyphenols, flavonoids) and functional properties (antioxidant and anti-inflammatory activities). These insights can guide the formulation and quality assessment of ointments based on their sensory and bioactive characteristics.

VI.4. Ointments PC

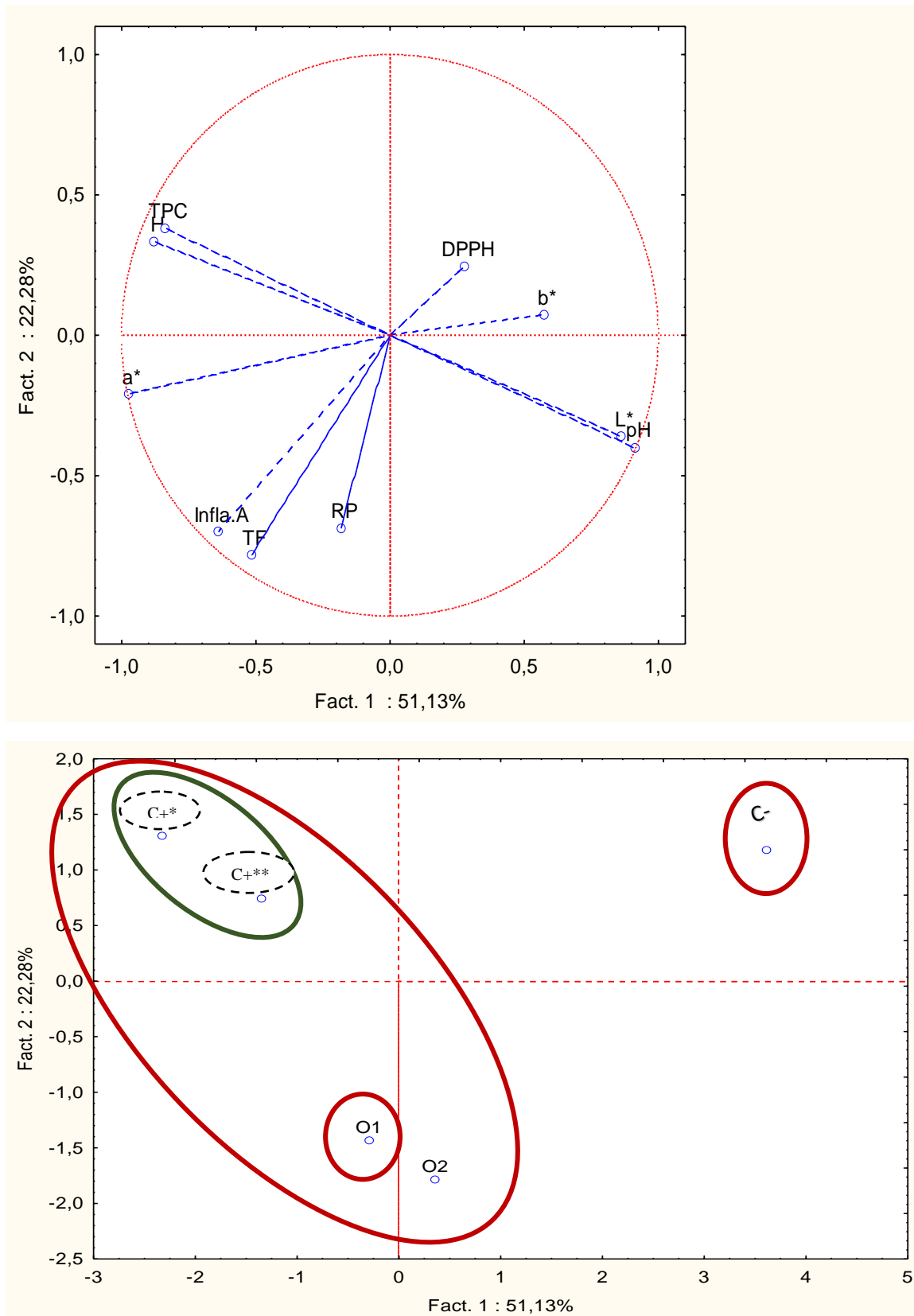


Figure 34: Ointments PC Discriminant Power

Interpretation:

Principal Component Analysis (PCA), a statistical method, was used to analyze and identify similarities among five ointment samples. It explains 73.41% of the variance, with the first principal component (PC-1; 51.13%) showing distinct patterns:

In the positive part of PC-1, characteristics such as DPPH antioxidant activity, moisture content, pH, color L*, and color b* are clustered. Conversely, in the negative part of PC-1, there are high concentrations of polyphenols and flavonoids, along with reducing power and color a*, and anti-inflammatory activity.

The second principal component (PC-2; 22.28%) illustrates a different set of features. It highlights DPPH antioxidant activity, moisture content, polyphenol content, and color b* in the positive quadrant, while flavonoid content, color a*, color L*, pH, reducing power, and anti-inflammatory activity are prominent in the negative quadrant.

The discrimination by PC-1 reveals three main groups and a subgroup of ointment samples:

- ❖ **Negative Control Group (C-):** Located in the upper positive quadrant, this group shows low results across all analyses.
- ❖ **Ointment O1:** Positioned in the lower negative quadrant, indicating higher levels of flavonoids, reducing power, and anti-inflammatory activity.
- ❖ **Central Group (O2, NEWBO, MEBO):** This group centrally clusters ointment O2 and two commercial ointments NEWBO and MEBO, indicating similarity in phenolic compounds, moisture content, color a*, and biological activities. Within this group, there is a subgroup represented by the two commercial ointments NEWBO and MEBO, which share very similar characteristics in polyphenol content and moisture.

Additionally, the correlation circle obtained confirms and supports the correlations found through Pearson analysis, validating the relationships between the various measured parameters and their interdependencies within the dataset.

Conclusion

Conclusion

The study aims to evaluate the physicochemical characterization and biological activities (antioxidant and anti-inflammatory) of two types of honey samples, pumpkin seed powder and formulated ointments. Both honey samples studied were of suitable quality, exhibiting representative physicochemical parameters and biological activities in accordance with the standards cited by the Codex Alimentarius, with the exception of the water content of H2 honey, which exceeded the standard <20%.

Overall, H1 honey has a higher polyphenol and flavonoid content and higher antioxidant activity than H2 honey, which shows higher anti-inflammatory activity. Pumpkin seed powder has a higher flavonoids and polyphenols content, as well as notable antioxidant and anti-inflammatory activities.

The study demonstrated that incorporating honey and pumpkin seed powder into an ointment formulation significantly improves its physicochemical parameters and biological activities. Overall, the H1 honey-incorporated ointment demonstrated superior activity compared with control and pharmaceutical ointments. This superiority is attributed to the higher levels of polyphenols and flavonoids in H1 compared with H2, which is in line with existing literature indicating that dark-colored honey H1 generally has higher polyphenol and flavonoid contents than light-colored honey H2.

The formulation incorporated with honey and PS showed increased levels of polyphenols and flavonoids, leading to better antioxidant and anti-inflammatory activities. In addition, the formulated ointment reported a higher water content after several days' storage compared to the control, confirming the honey-induced osmotic pressure and water retention, maintaining a moist environment in the wound bed and attracting immune cells, eliminating necrotic tissue, which facilitates tissue regeneration. The spreadability and viscosity of the ointment enabled the effective formation of a dressing that protects the skin and isolates it from external environmental factors, preventing infections and other triggers.

The incorporation of these natural ingredients improved the formulation's stability, maintaining its integrity and efficacy over time compared with the control and pharmaceutical ointments, confirming the antimicrobial and preservative effects induced by honey, which are in line with the literature, as well as the activating effects attributed to honey and pumpkin seeds, according to the results obtained.

These results suggest that the formulated ointment has substantial therapeutic potential in skin care and inflammatory conditions. What's more, the incorporation of natural ingredients not only enhances therapeutic efficacy by avoiding the negative effects induced by chemicals such as antibiotics, but also promotes the valorization of natural waste products. This points to a promising approach to developing a natural, stable and cost-effective topical treatment option for effective wound healing, particularly in low- and middle-income countries.

In addition to the physicochemical and biological evaluations carried out, the present study could benefit from further investigations in several key perspectives:

- Assessing the long-term shelf-life of the ointment under different temperature conditions would elucidate its stability and shelf-life, essential considerations for practical application and storage.
- Antimicrobial activity testing would involve exposing the ointment to various pathogens to comprehensively assess its antimicrobial efficacy.
- Exploring different extraction methods for pumpkin seed powder (extract and oil) and comparing their effectiveness in recovering bioactive compounds by HPLC analysis would optimize the formulation's bioactivity potential.
- Formulating ointment using PSO, honey, beeswax while excluding other oils to mitigate potential confounding effect caused by other oils or their synergistic effect and facilitate the attribution of specific biological activity to those key components (PSO, honey, beeswax).
- Expanding our study to include *in vitro* and *in vivo* models would provide a more comprehensive assessment of the ointment's efficiency in wound healing, reflecting its potential clinical application.

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ABSTRACT

Honey and pumpkin seeds (PS) are prized for their nutritional and medicinal benefits. With antibiotic resistance being an ongoing issue, honey serves as an alternative to antibiotics to address their overuse problem. PS are often considered industrial waste and discarded; their valorization would be beneficial both economically and medically as a cost-effective treatment, particularly in low-income countries. From this standpoint, the aim of this study is to evaluate the effect of incorporating honey and PS into a formulation. The incorporation of honey and PS into the ointment formulation significantly enhanced physicochemical and biological activity. PS powder-honey 1 ointments (O1) and PS powder-honey 2 ointments (O2) showed higher reducing power, 9.60 and 3.87 mg EAG/100 g respectively, compared to (C-) 1.76 mg EAG/100 g. O1 and O2 reported higher anti-inflammatory activity, 83.5 % and 86.41% respectively, compared to (C-) 57.28%. The incorporated ointment reported higher water content after 45 days of storage compared to C- and pharmaceutical ointment (PO) (NEWBO and MEBO), which may be due to the osmotic pressure and water retention induced by honey. O1 improved the formulation's stability, bioactive components content, and biological activities over 45 days, with lower TABRS values in O1 and O2 with 0.0012 and 0.0014 mg/kg respectively, compared to (C-) 0.0019 mg/kg, and lower peroxide values in O1 and O2 at 0.7 and 0.9 Meq/kg respectively, compared to (C-) 1.2 Meq/kg. This may be due to the antimicrobial and preservative effects induced by honey as well as the bioactive components of PS. These findings suggest that the honey-PSP based ointment is a potential candidate for a promising approach for developing a stable, cost-effective and natural topical treatment option for wound healing.

Keywords; Honey, Pumpkin seeds, Ointment, Antioxydants activity, Anti-inflammatory activity, Oxidative Stability, wound healing

المخلص

العسل وبذور اليقطين معروفين بفوائدهما الغذائية والطبية. مع استمرار مشكلة مقاومة المضادات الحيوية، يُعد العسل بديلاً للمضادات الحيوية لمعالجة مشكلة إساءة استخدامها. تعتبر بذور اليقطين غالباً نفايات صناعية يتم التخلص منها؛ الاستفادة منها سيكون مفيداً اقتصادياً وطبياً كعلاج فعال منخفض التكلفة، خاصة في البلدان ذات الدخل المنخفض. من هذا المنظر، تهدف هذه الدراسة إلى تقييم تأثير دمج العسل وبذور اليقطين في تركيبة مرهم. أدى دمج العسل وبذور اليقطين في تركيبة المرهم إلى تعزيز نشاطها الفيزيوكيميائي والبيولوجي بشكل ملحوظ. أظهر المرهم المدمج بمسحوق بذور اليقطين-عسل 1 (م1) والمرهم المدمج بمسحوق بذور اليقطين-عسل 2 (م2) قوة اختزالية عالية 9.60 و 3.87 ملغ مقابل حمض الغاليك/100 غ على التوالي، مقارنة بالمرهم الشاهد السلبى (ش-) 1.76 ملغ مقابل حمض الغاليك/100 غ. (م1) و(م2) أظهرتا نشاطاً عالياً مضاداً للالتهاب بنسبة 83.5% و 86.41% على التوالي مقارنة بـ (ش-) 57.28%. أظهر المرهم المدمج محتوى مائياً أعلى بعد 45 يوماً من التخزين مقارنة بـ (ش-) والمرهم الصيدلانية (م ص) (نيويو وميبو)، مما قد يكون ناتجاً عن الضغط التناضحي والقدرة على الاحتفاظ بالماء الناتج من العسل. لقد ساهم (م1) في تحسين استقرار التركيبة، ومحتوى المكونات البيولوجية، والأنشطة البيولوجية على مدار 45 يوماً، مع قيم المواد المتفاعلة مع حمض الثيوباربيتوريك أقل في (م1) و(م2) بنسبة 0.0012 و 0.0014 ملغ/كغ على التوالي مقارنة بـ (ش-) 0.0019 ملغ/كغ، وقيم بروكسيد أقل في (م1) و(م2) بنسبة 0.7 و 0.9 مكون/كغ على التوالي مقارنة بـ (ش-) 1.2 مكون/كغ. قد يعزى ذلك إلى التأثيرات المضادة للميكروبات والحفاظة المحفزة بواسطة العسل، فضلاً عن المكونات البيولوجية لبذور اليقطين. تشير هذه النتائج إلى أن المرهم القائم على العسل وبذور اليقطين هو مرشح محتمل لنهج واعد في تطوير خيار علاجي طبيعي وفعال واقتصادي للعلاج الموضعي للجروح.

الكلمات المفتاحية ; عسل، بذور اليقطين، مرهم، نشاط مضاد للاكسدة، نشاط مضاد للالتهابات، الثبات التأكسدي، شفاء الجروح .

Résumé

Le miel et les graines de citrouille (GC) sont prisés pour leurs bienfaits nutritionnels et médicinaux. Avec la résistance aux antibiotiques étant un problème persistant, le miel sert d'alternative aux antibiotiques pour résoudre leur problème de surutilisation. Les PS sont souvent considérées comme des déchets industriels et jetées ; leur valorisation serait bénéfique tant sur le plan économique que médical en tant que traitement rentable, en particulier dans les pays à faible revenu. Dans cette optique, l'objectif de cette étude est d'évaluer l'effet de l'incorporation de miel et de GC dans une formulation. L'incorporation de miel et de GC dans la formulation a considérablement amélioré l'activité physicochimique et biologique. La pommade à base de poudre de graines de citrouille et de miel 1 (P1) et La pommade à base de poudre de graines de citrouille et de miel 2 (P2) ont montré un pouvoir réducteur plus élevé, 9,60 et 3,87 mg EAG/100 g respectivement, par rapport à (C-) 1,76 mg EAG/100 g. P1 et P2 ont rapporté une activité anti-inflammatoire plus élevée, 83,5% et 86,41% respectivement, par rapport à (C-) 57,28%. La pommade incorporée a rapporté une teneur en eau plus élevée après 45 jours de stockage par rapport au contrôle, ce qui peut être dû à la pression osmotique et à la rétention d'eau induites par le miel. P1 a amélioré la stabilité de la formulation, les composants bioactifs et les activités biologiques sur 45 jours, avec des valeurs TABRS plus faibles dans O1 et O2 à 0,0012 et 0,0014 mg/kg respectivement, contre (C-) 0,0019 mg/kg, et des valeurs de peroxyde plus faibles dans O1 et O2 à 0,7 et 0,9 Meq/kg respectivement, contre (C-) 1,2 Meq/kg. Cela peut être dû aux effets antimicrobiens et conservateurs induits par le miel ainsi qu'aux capacités des GC. Ces résultats suggèrent que la pommade à base de miel et de graines de citrouille est un potentiel candidat pour une approche prometteuse pour le développement d'un traitement topique naturelle, stable et économique pour la cicatrisation des plaies.

Les mots clé ; Miel, Graines de citrouille, Pommade, Activité antioxydant, Activité anti-inflammatoire, Stabilité oxydative, Cicatrisation des plaies.