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Study of antibacterial, antihemolytic and *in vivo* hepatoprotective effects of Eucalyptus honey

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Dedication

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N.RIHEM SOUHA

Abstract

The present work aims to study the antibacterial, antihemolytic and *in vivo* hepatoprotective effects of Eucalyptus honey collected from Khmis el-khachena Algiers, Algeria in 2023. The result of total phenolic content in Eucalyptus honey, showed that honey contained 241,75±00 µg galic acid equivalent (GAE)/mg of honey. Two different bacterial strains were used in the anti-bacterial studies *Escherichia coli (E. coli)* and *Staphylococcus aureus*. An inhibition of *Escherichia coli* growth was noted with different concentrations of honey 75%, and 100% with a diameter of inhibition zone of 30mm and 31mm (very sensitive bacteria). As for its anti-hemolytic activity, a direct correlation was observed between honey concentration and the percentage of hemolysis inhibition, reaching 21.53% at 600 ug/ml, indicating the ability of honey to inhibit hemolysis. To evaluate the *in vivo* study of hepatoprotective effect of Eucalyptus honey in N-acetyl-para-aminophenol (APAP) induced hepatotoxicity, three groups of albino wistar female rats were used: control group, APAP group and honey+APAP group. The last two groups were treated with a supratherapeutic dose of APAP 2g/kg on the 11th day of treatement but the honey+APAP group was pretreated with Eucalyptus honey 5g/kg. Biochemical and oxidative stress parameters analyses results have shown significant increase in the levels of ALAT, ASAT, LDH and MDA. And an increase in the levels of GSH, GST, SOD, and CAT by in the APAP group in relation to the control group. Eucalyptus honey+APAP group compared to APAP group showed a significant decrease in the transaminases by 87.8%(ALAT) and 68.1% (ASAT), LDH by 76.5% and MDA by 81.6%.There was also an increase in the levels of GSH, GST, SOD, and CAT by 65.3%, 74.09%,82.6%, and 76.75% respectively .The histological analyses results showed an alteration in the histoarchitecture with a severe dgree of hemorrhage in the hepatic tissue of the APAP group. However the pretreated group with Eucalyptus honey. Results showed that the hepatic lesions was less severe and maintained the architecture of the liver These results emphasize the clear ability of eucalyptus honey to in contrast the risk of oxidative stress and bacterial infection. In conclusion, Eucalyptus honey can be used as an alternative agent in the presence of *Escherichia coli* bacteria, anti-hemolytic and hepatoprotector product.

Key words: Eucalyptus honey, Antibacterial activity, Antihemolytic activity, N-acetyl-para-aminophenol (APAP), Hepatoprotective effect.

Resumé

Le présent travail vise à étudier les effets antibactériens, antihémolytiques et hépatoprotecteurs *in vivo* du miel d'eucalyptus récolté à Khmis el-khachena, Alger, Algérie, en 2023. Le résultat de la teneur phénolique totale du miel d'eucalyptus a montré que le miel contenait 241,75±00 µg d'équivalent d'acide galique (GAE)/mg de miel. Deux souches bactériennes différentes ont été utilisées dans les études antibactériennes : *Escherichia coli (E. coli)* et *Staphylococcus aureus*. Une inhibition de la croissance *d'Escherichia coli* a été notée avec différentes concentrations de miel 75%, et 100% avec un diamètre de la zone d'inhibition de 30mm et 31mm (bactéries très sensibles). Quant à son activité anti-hémolytique, une corrélation directe a été observée entre la concentration de miel et le pourcentage d'inhibition de l'hémolyse, atteignant 21,53% à 600 ug/ml, indiquant la capacité du miel à inhiber l'hémolyse. Pour évaluer l'étude *in vivo* de l'effet hépatoprotecteur du miel d'eucalyptus sur l'hépatotoxicité induite par N-acétyl-para-aminophénol (APAP), trois groupes de rats femelles wistars albinos ont été utilisés : groupe témoin, groupe APAP et groupe miel+APAP. Les deux derniers groupes ont été traités avec une dose suprathérapeutique d'APAP de 2g/kg au 11ème jour de traitement, mais le groupe miel+APAP a été prétraité avec du miel d'eucalyptus à raison de 5g/kg. Les résultats des analyses biochimiques et des paramètres de stress oxydatif ont montré une augmentation significative des niveaux d'ALAT, d'ASAT, de LDH et de MDA. Et une augmentation des niveaux de GSH, GST, SOD et CAT dans le groupe APAP par rapport au groupe témoin. Le groupe miel d'eucalyptus +APAP comparé au groupe APAP a montré une diminution significative des transaminases de 87,8 % (ALAT) et 68,1 % (ASAT), de la LDH de 76,5 % et de la MDA de 81,6 %, ainsi qu'une augmentation des niveaux de GSH, GST, SOD et CAT de 65. Les résultats des analyses histologiques ont montré une altération de l'histoarchitecture avec un degré sévère d'hémorragie dans le tissu hépatique du groupe APAP. Cependant, le groupe prétraité avec du miel d'eucalyptus. Ces résultats soulignent la capacité évidente du miel d'eucalyptus à contraster le risque de stress oxydatif et d'infection bactérienne. En conclusion, le miel d'eucalyptus peut être utilisé comme agent alternatif en présence de bactéries *Escherichia coli*, produit anti-hémolytique et hépatoprotecteur.

Mots clés : Miel d'Eucalyptus, Activité antibactérienne, Activité antihémolytique, N-acétyl-para-aminophénol (APAP), Effet hépatoprotecteur.

Abstract

ملخص

يهدف هذا البحث إلى دراسة تأثيرات عسل الكاليتوس المضادة للبكتيريا و التحلل الدموي والتأثير الوقائي الكبدي *In vivo*.أمكن الحصول على عينة العسل من منطقة خميس الخشنة بالجزائر العاصمة حصيلة عام 2023. تم تقدير المحتوى الفينولي الكلي للعسل واختبر النشاط المضاد لبكتيريا *(E. coli)* والمكورات العنقودية الذهبية*S aureus* وكذا النشاط المضاد للتحلل الدموي باستعمال عينة دموية سليمة .أما دراسة التأثير الوقائي الكبدي *In vivo* لعسل الكاليتوس فقد أنجزت باستخدام ثلاث مجموعات من إناث الجرذان : مجموعة الشاهدة ومجموعة N-acetyl-para-aminophenol (APAP) ومجموعة العسل +APAP. عولجت المجموعتان الثانية والثالثة بجرعة سامة قدرت بكمية 2 غ/كغ من APAP و ذلك بعد معاملة كل منهما بجرعة 5غ/كغ من العسل مدة 10أيام . أظهرت نتائج تقديرالمحتوى الفينولي الكلي لعسل الكاليبتوس كمية 241,75 ± 00 ميكروغرام المكافئ /ملغ من حمض الغاليك (GAE). ولوحظ نشاط تثبيطي لبكتيريا *(E. coli)* عكسه قطر منطقة التثبيط 30 ملم و31 ملم وذلك عند التركيزين 75% و100% على التوالي . كما بينت النتائج وجود علاقة طردية بين تركيز العسل ونسبة تثبيط الانحلال الدموي حيث بلغت 21.53% عند 600 ميكروغرام/مل مما يشير إلى قدرة العسل على تثبيط الانحلال الدموي. بالنسبة للتأثير الوقائي الكبدي *In vivo* لعسل الكاليتوس ، فقد أظهرت نتائج التحاليل البيوكيميائية واختبارات الإجهاد التأكسدي زيادة كبيرة في مستويات LDH,ASAT,ALAT و MDA وانخفاض في مستويات GSH، GST، SOD، وCAT وذلك لدى المجموعة المعالجة بمركب APAP مقارنة بالمجموعة الشاهدة . بينما لوحظ انخفاضًا في مستويات ALAT 87.8% و ASAT68.1 % ، و LDH بنسبة 76.5% و MDA بنسبة 81.6%. بالمقابل سجل زيادة في مستويات GSH و GST و SOD و CAT بنسبة 65.3٪ و 74.09٪ و 82.6٪ و 76.75٪ على التوالي وذلك لدى المجموعة المعاملة بعسل الكاليبتوس +APAP مقارنةً بمجموعة APAP . بالإضافة ،أظهرت نتائج الدراسة النسيجية تغيرا في البنية النسيجية للكبد مع وجود نزيف حاد بالأنسجة الكبدية لمجموعة APAP ، بينما لوحظ تراجع الاضطراب البنيوي الكبدي لدى المجموعة المعالجة مسبقاً بعسل الكاليتوس مما يؤكد كفاءة العسل الوقائية المضادة للبكتيريا الناتجة عن *(E. coli)* وكذا التحلل الدموي وتعديل الإجهاد التأكسدي .كخاتمة ،يمكن لعسل الكاليتوس أن يستعمل كعامل بديل لمعالجة العدوى البكتيرية الناتجة عن *(E. coli)* ومضاد للانحلال الدموي وكمادة واقية مضادة للإجهاد الكبدي الدوائي .

الكلمات المفتاحية: عسل الكاليتوس ، نشاط مضاد للبكتيريا، نشاط مضاد للانحلال، N-acetyl-para-aminophenol (APAP)، تأثير وقائي للكبد.

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Abreviations

- **AIF**: Apoptosis-Inducing Factor
- **ALAT**: Alanine Aminotransferase
- **ALD**: Alcoholic Liver Disease
- **ALF**: Acute Liver Failure
- **APAP**: N-acetyl-para-aminophenol
- **ASAT**: Aspartate Aminotransferase
- **ATP**: Adenosine Triphosphate
- **AW**: water activity
- **Ca^{2+}**: Calcium Ion
- **CAT**: Catalase
- **COX-2**: Cyclooxygenase-2
- **Cu Zn SOD**: Copper-Zinc Superoxide Dismutase
- **Cv:** Central vein
- **CYP**: Cytochrome P450
- **DNA**: Deoxyribonucleic Acid
- **EC**: Electrical Conductivity
- **ETC**: Electron Transport Chain
- **Fe^{2+}**: Ferrous Ion
- **Fe-SOD**: Iron Superoxide Dismutase
- **GAE**: Gallic Acid Equivalents
- **GPx**: Glutathione Peroxidase
- **GSH**: Glutathione
- **GST**: Glutathione S-Transferase
- **H_2O_2**: Hydrogen Peroxide
- **HMF**: 5-Hydroxymethylfurfural
- **Hp:** Hepatocyte
- **Hr :**Hemorrhage
- **LDH**: Lactate Dehydrogenase
- **MDA**: Malondialdehyde
- **MMP**: Matrix Metalloproteinase
- **MnSOD**: Manganese Superoxide Dismutase
- **MPT**: Mitochondrial Permeability Transition

Abreviations

- **NADPH**: Nicotinamide Adenine Dinucleotide Phosphate
- **NAPQI**: N-acetyl-p-benzoquinone imine
- **NO•**: Nitric Oxide
- **NOS_2**: Nitric Oxide Synthase 2
- **NOS_3**: Nitric Oxide Synthase 3
- **O_2^-**: Superoxide Anion
- **OD**: Optical Density
- **ONOO-**: Peroxynitrite
- **OS**: Oxidative Stress
- **PBS:** Phosphate-Buffered Saline
- **PH**: Potential of Hydrogen
- **RBC**: Red Blood Cells
- **RNS**: Reactive Nitrogen Species
- **ROS**: Reactive Oxygen Species
- **Sn:** Sinusoids
- **SOD**: Superoxide Dismutase
- **TBA**: 2-Thiobarbituric Acid
- **TCA**: Tricarboxylic Acid (also known as the Krebs Cycle or Citric Acid Cycle)
- **UDP**: UridineDiphosphate
- **UGT**: UDP-Glucuronosyltransferase

Introduction

Introduction

Oxidants serve a dual role as both toxic and beneficial compounds, as they can be either harmful or beneficial to the body. They are produced either from normal cell metabolisms in situ or from external sources such as pollution, cigarette smoke, radiation, and medication. When an overload of free radicals cannot gradually be destroyed, their accumulation in the cell generates a phenomenon called oxidative stress. This process plays a significant role in the development of chronic and degenerative illnesses such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular, and neurodegenerative diseases **(Pham-Huy *et al*., 2008).**

Red blood cells are exposed to both external and internal sources of oxidants that challenge their integrity and compromise their physiological function and supply of oxygen to tissues. Autoxidation of oxyhemoglobin is the main source of endogenous RBC oxidant production, producing superoxide radical and then hydrogen peroxide. In addition, potent oxidants from other blood cells activated by bacterial infection can reach the RBCs, such as neutrophils. Oxidative damage to the RBC plays a crucial role in the early removal of RBCs from circulation or provoking hemolysis **(Möller *et al.*, 2023).**

Hepatocytes, the main cell type in the liver, perform critical functions, including protein synthesis, and the production of biochemicals necessary for digestion and detoxification of some drug metabolites such as N-acetyl-p-benzoquinone imine (NAPQI) from paracetamol **(Casas-Grajales *et al.*, 2015).** NAPQI, a toxic metabolite produced during the metabolism of acetaminophen (APAP) **(Yan *et al*., 2018),** can deplete the antioxidant enzymes in these cells when present in high amounts, leading to oxidative stress.

Red blood cells are anucleated and lack a mechanism of DNA-inducible antioxidant response, relying on a complex and robust network of antioxidant systems **(Möller *et al.*, 2023).** The antioxidant defense of hepatocytes is often limited. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ or externally supplied through supplements and/or natural products**(Pham-Huy *et al*., 2008)** such as honey**(Cianciosi *et al.*, 2018)** and olive fruits **(Dekdouk *et al*., 2015).**

Honey is one of the most popular and valuable natural products introduced. Honey is obtained by bees (*Apis mellifera*; family: Apiidae) from the nectar of flowers **(Salim *et al.*, 2011).** It is the only natural product derived from insects that possess nutritional, cosmetic, therapeutic, and industrial properties **(Ghorab *et al*., 2021).**

Introduction

Honey contains numerous compounds, the most important of which are sugars (Monosaccharides represent about 75% of the sugars contained in honey, with 10 to 15% of disaccharides and small amounts of other sugars **(De-melo *et al.*, 2017),** proteins and amino acids (Amino acids represent 1% (w/w) **(Alaerjani *et al.*, 2022),** organic acids about 0.57% of organic acids **(Aurongzeb and Azim, 2011),** vitamins, minerals (The mineral content of honey varies from 0.04% in light honeys to 0.2% in dark honeys **(Titcomb and Tanumihardjo, 2019)**, phenolic compounds, volatile compounds, and water (A significant amount of water is present since the average water content is 17.2%, but this value can vary as honey is a biological product **(Cianciosi *et al.*, 2018)**.

Honey can exert several beneficial effects on health, including anti-inflammatory **(Saikaly and Khachemoune, 2017),** antimicrobial **(Mandal and Mandal, 2011),** anticancer **(Singer *et al.*, 2019),** cardiovascular **(Nwokorie *et al.*, 2022),** and antioxidant **(Ahmed *et al.*, 2018)** effects. The antioxidant effect is due to the presence of several active substances in honey such as polyphenols. These compounds interfers through many mecanisms such as: direct scavenging of ROS, the activation of antioxidant enzymes, the inhibition of oxidases, and metal-chelating activity **(Chen *et al.*, 2023).**

In the context of promoting traditional and natural medicines in Algeria, we have chosen to study the bacterial activity of local honey harvested from Khemis El Khachena, Algies, Algeria, we evaluated the antibacterial, anti-hemolytic, and hepatoprotective activity of monofloral eucalyptus honey *in vitro* and *in vivo.*

Bibliographic Part

Chapter 01

Honey Generalities

Chapter 01 : Honey generalities

1.1. The bee

1.1.1. Definition

The bee, or *Apismellifera*, is a living creature that is essential to maintaining the global environmental balance and serving as a pollinator for several plant species. Its interests also extend to the production of propolis, honey, royal jelly, and other products **(Giulia *et al*., 2022).**

1.1.2. Origin

The origin of *Apis mellifera*, the western honey bee, is suggested to be in the Middle East or Northeastern Africa. This indicates that the genetic evidence points towards the Middle East or Northeastern Africa as the likely geographic origin of *Apismellifera* **(Giulia *et al*.,2022).**

1.1.3. Anatomy of the bee

The bee's body is divided into multiple parts, the head, chest (Thorax) and abdomen **(Figure1)(Rodney *et al*.,2018).**

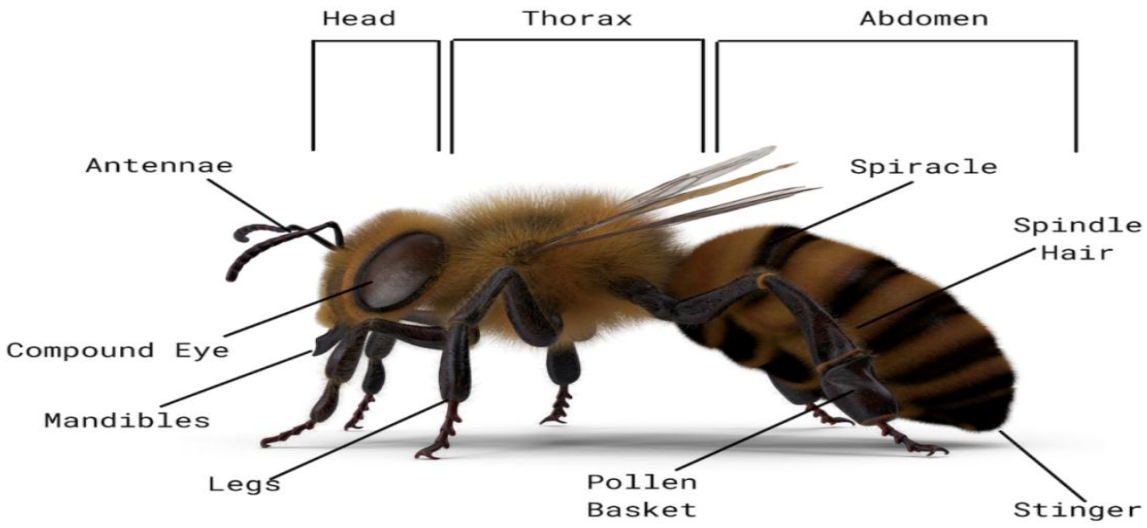

Figure 1: Structure of the bee's major parts
https://hbrc.ca/wp-content/uploads/2022/10/image-5-1024x737.png

1.1.3.1. The head

The head contains: the eyes, antennae, ocelli and mouthparts **(Figure2) (Table1)(Rodney *et al*.,2018).**

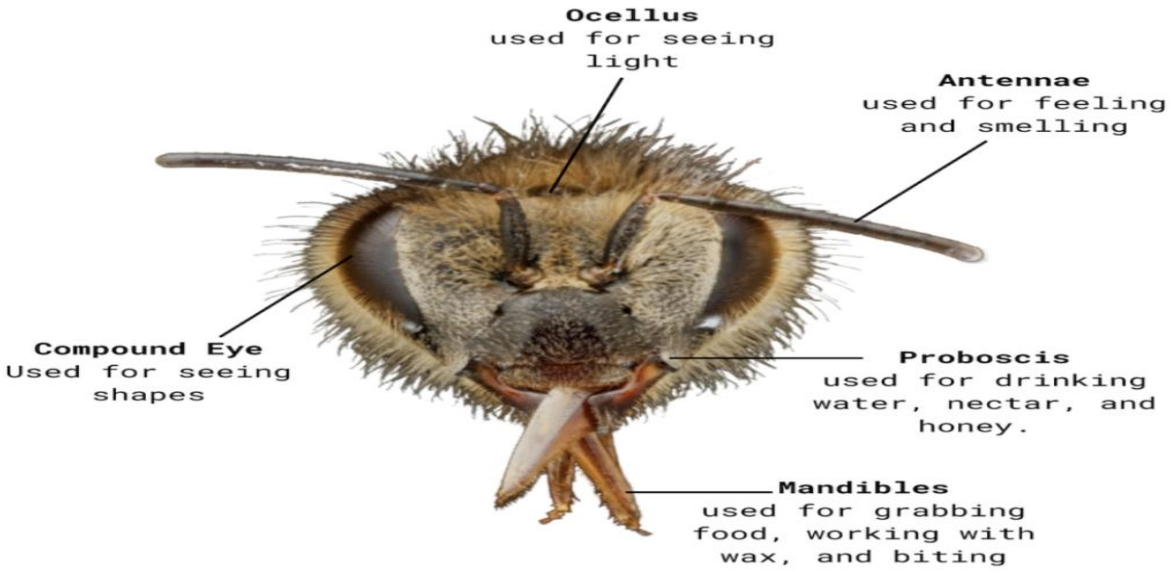

Figure 2: Structure of the bee's head

https://hbrc.ca/wp-content/uploads/2022/10/image-6-1024x926.png

Table 1: Parts of head**(Rodney *et al*.,2018)**

Part	Caracteristics	Function
Compound eyes	Large compound eyes made up of numerous hexagonal lenses.	-Allows it to see ultraviolet light and detect motion effectively.
Ocelli	Three small simple eyes called ocelli are located on the top of the bee's head. ocelli are sensitive to light intensity .	-Help bees orient themselves to the sun's position for navigation.

Antennae	Bees have a pair of segmented antennae that serve as sensory organs.	-Detecting odors, chemicals, humidity and temperature, communication, navigationand locating food sources.
Mouthparts		
Mandibles	The mandibles are attached to the head at each end of the labrum. The labrum is a short, wide flapalso known as strong jaw.	-Manipulating and shaping beeswax. -Constructing the hive. -Defending the colony, and handling food.
Proboscis	A long, tubular mouthpart. The proboscis can extend to reach deep into flowers for nectar collection.	-Feeding on nectar, water, and other liquids.

1.1.3.2. The Thorax

The thorax is the middle body segment of the honey bee. It Consists of three segments: prothorax,mesothorax and metathorax. Wings and legs are attached to the thorax, enabling flight and movement**(Figure3)(Rodney *et al*.,2018).**

Figure 3: Structure of the bee's thorax
https://hbrc.ca/wp-content/uploads/2022/10/image-8-1024x505.png

1.1.3.3. The abdomen

The honey bee's abdomen contains essential organs such as: wax glands, honey stomach, stinger, respiratory system**(Figure4) (Table2)(Rodney *et al*.,2018).**

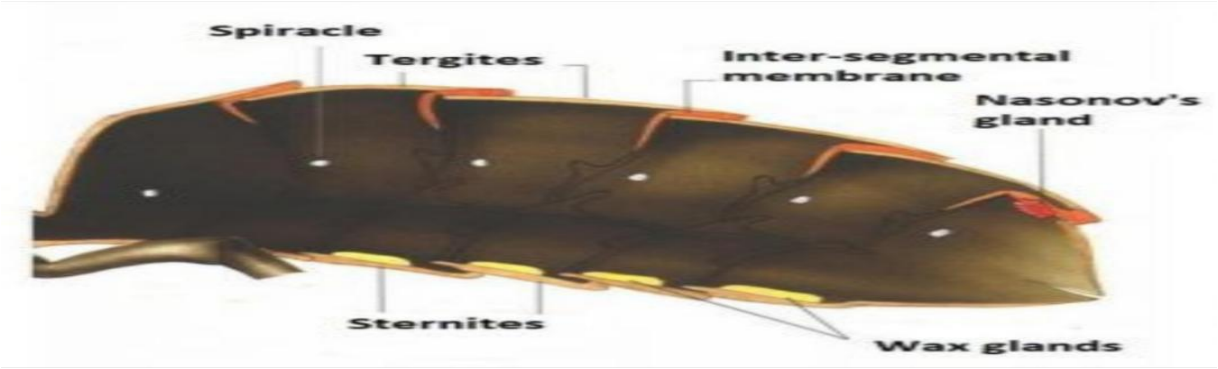

Figure 4: Structure of the bee's abdomen
https://abejas.org/wp-content/uploads/2018/06/external-8-1-300x288.jpg

Table 2: Honey bee's abdomen organs**(Rodney *et al*.,2018)**

Part	Caracteristics	Function
Wax glands	Specialized glands on the abdomen of worker bees	-Secrete wax that used to construct honeycomb cells for storing honey, pollen and developing brood.
Honeystomach	A specialized part of the digestive system.	-Nectar is stored in honey stomach temporarily before being transferred to other bees or stored in honeycomb cells.
Stinger	The stinger of a honey bee is made up of 3 parts and is attached to a venom sac. Only the workerbees and the queen have stingers.	-Honeybees use their stingers to defend their colony.

Respiratory system	Bees have no lungs. Instead, they have small holes along the sides of their abdomen for air exchange called spiracles. The spiracles are connected to tubes (tracheae) that deliver the oxygen to the rest of the body.	-Air exchange and respiration of the bee.

1.2. Beekeeping

1.2.1. Definition

Beekeeping is the practice of caring for and managing honey bees to harvest products like: honey, beeswax and pollen, as well as to provide pollination services**(Sperandio*et al*., 2019).**

1.2.2. Importance of beekeeping

Beekeeping plays a vital role in pollinating crops, ensuring food security and maintaining biodiversity.It contributes to the global economy through the production of honey, beeswax, propolis and royal jelly.Additionally, bees are essential for the reproduction of many plant species, making them indispensable for a healthy ecosystem and sustainable agriculture**(Alleri, 2023).**

1.3. Honey

1.3.1. Definition

Honey is the natural sweet substance produced by *Apismellifera*from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants.Which the bees collect and transform by combining with specific substances of their own deposit, dehydrate, store and leave in honeycombs to ripen and mature **(Salim*et al*., 2011).**

1.3.2. Main melliferous resources and plants in Algeria

Over 4,000 different plant species can be found in Algeria. The most varied flora within this vast diversity is flowering plants, the majority of which are regarded as melliferous plants, or

those necessary for honeybees to make honey Some inventories of melliferous species have been carried out and published for different regions of Algeria, however, resources for honeybees remains poorly known compared to the plant biodiversity**(Ghorab*et al*.,2021).**

1.3.3. The different types of honey

1.3.3.1. Nectarhoney

Nectar is a sweet liquid, sometimes syrupy secreted and then excreted by specific glands, known as nectar glands found on many plants. Most of the liquid is made up of water and sucrose, but it also contains other sugars such as glucose and fructose and in very small quantities substances that help to give a honey its distinctive aroma and flavour, its "personality"**(Marchenay,1988).**

1.3.3.2. Honeydew honey

Honeydew is produced from the excretions of certain sap-sucking insects left on plants. These insects are homopteran hemipterans: aphids, but also mealybugs, cicadas and psyllids**(Koechler,2015).** It is therefore, a substance of both plant and animal origin which undergoes a double treatment: after passing through the aphid's digestive system, it passes through that of the bee. In other words, the biochemical transformations are numerous and complex **(Marchenay, 1988).**

1.3.3.3. Monofloral honey

Monofloral honeys are made from the nectar and/or honeydew of a single plant species, which means that the hives have to be set up close to the desired plant **(BontéandDesmolière,2013).**

Of course, it is almost impossible to certify that a honey is 100% unifloral, as other nectars from other plants may be mixed in, but only in very small quantities (**Bonté andDesmolière, 2013)**.

1.3.3.4. Polyfloral honey

Polyfloral honeys, or "thousand-flower" honeys are produced from the nectar and/or honeydew of several plant species without any particular predominance they are the result of foraging in an environment where several plant varieties are simultaneously producing nectar**(Koechler,2015).**

1.4. Properties of honey

1.4.1. Physicochemical properties

1.4.1.1. Water activity

The amount of water available to microorganisms is known as water activity (Aw). A portion of the water is captured by sugar, preventing the growth of microorganisms. (Aw) in honey varies between 0.49 and 0.65 while it can occasionally reach a value of 0.75.

The approximate water activity required for the formation of bacteria, yeast, and molds is 0.90, 0.80 and 0.70, respectively. (Aw) values less than 0.60 will prevent the osmophilic yeasts that ferment honey from growing **(De-melo*et al*., 2017).**

1.4.1.2. Electrical conductivity

A material's capacity to conduct an electric current is referred to as its electrical conductivity (EC). It is directly linked to the botanical origin, to the amount of minerals and inorganic ions and in some way to the organic acids, proteins and other elements that can function as electrolytes. Nectar must have an electrical conductivity of less than 0.8 mS/cm of EC, but honeydew honey must have an electrical conductivity greater than 0.8 mS/cm **(De-melo*et al* ., 2017).**

1.4.1.3. Density

Density is another characteristic of honey that is significantly influenced by its high sugar content**(De-melo*et al*.,2017)**. Honey has a relatively high density, varying between 1.40 and 1.45 g/cm3 This is very useful data that can be used to measure the water content of honeys. We can assume an average of 1.4225 at 20°C **(Chouia, 2014).**

1.4.1.4. pH

The main reason of honey's acidic pH is the presence of certain acids, primarily gluconic acid which is created when glucose oxidase breaks down glucose **(Thakur, 2022).**

The pH of honey varies between 3,2 and 5,5. It is generally below 4 in nectar honeys and above 5 in honeydew honeys. Honeys with a low pH break down more easily, so special care should be taken when storing them **(Chouia, 2014).**

1.4.1.5. Viscosity

When it comes to handling, processing, storing and sensory quality viscosity is crucial. The viscosity of honey decreases with increasing temperature and water content and decreases with decreasing carbohydrate concentration (**De-melo*et al*., 2017).**

1.4.1.6. Hygroscopicity

Because of its high sugar content primarily fructose, honey has a significant ability to absorb and retain moisture from its surroundings based on temperature, air moisture content, and relative humidity **(De-melo *et al*., 2017).**

1.4.1.7. Refractive index

An optical property called the refractive index ranges from 1.504 to 1.4815 increases with increasing solid content (or decreases with decreasing water content) and temperature **(De-melo*et al*., 2017).**

1.4.2. Organoleptic properties

1.4.2.1. Color

Honey's color which is an optical characteristic, can range from almost colorless to intense red including shades of yellow, amber and brown with a small amount of green to red. It is the most significant feature that improves the honey's appearance and therefore raises its market worth**(Table3)(Thakur, 2022).**

Table 3: Color classifications for honey quality standard**(White, 1975)**

USDA colorstandard designation	Range (Absorbance at 560nm)
Water White	0 - 0.094
Extra White	0.094 - 0.189

White	0.189 - 0.378
Extra Light Amber	0.378 - 0.595
Light Amber	0.595 - 1.389
Amber	1.389 - 3.008
Dark Amber	>3.008

1.4.2.2. Odor and taste

The smell of honey varies.The aroma, taste and color of honey depend on the plants from which the bees have collected the nectar. Sunflowers, for example give a golden-yellow honey, clover gives a sweet white honey. Dark honey generally has a stronger taste and a high mineral content, light honey has a more delicate flavor **(Chouia ,2014).**

1.4.3. Nutritional properties

Honey was the only commonly accessible sweetener and a significant source of carbs throughout a considerable portion of human history. Due to its high nutritional content (303 kcal/100g) and quick absorption of carbohydrates, honey is a food that is appropriate for people of all ages. Simply put, when taken orally its carbohydrates are quickly and easily absorbed into the bloodstream, where they are then used by the body for its energy needs. For this reason, honey is especially advised for athletes and kids as it can help increase the effectiveness of the elderly's and disabled systems **(Manyi-Loh*et al*.,2010).**

1.5. Composition of honey

Honey is a complex combination of biochemicals .The origin of the plants that are foraged by bees affects its content, which is determined by a multiple stages production procedure that influences the product's chemical composition **(De-Melo*et al*.,2017).**

1.5.1. Inorganic biochemicals

1.5.1.1. Water

The water content of honey is related to different factors such as the botanical and geographical origin of nectar, soil and climatic conditions, season of harvesting, intensity of

Nectar flux, degree of maturation, manipulation by beekeepers during period of harvest, as well as extraction, processing and storage conditions**(Sainz-Lain and Gomez-Ferreras, 2000).**

The amount of water that bacteria may access for a given food is known as water activity (aw) **(De-Melo*et al*.,2017).**

1.5.1.1.1. Mineral salts

Flower honey contains 0.1 to 0.35 g of mineral salts and trace elements /100 g of honey, but honey has up to 1 g/100 g and more.The main mineral substance is potassium. Currently, much more is sought for the electrical conductivity of honey which is more easily measurable and is used mainly for the characterization of monofloral honey. Depending on the geographical and botanical origin of the honey, the mineral content and conductivity will be different **(Table 4)(Ajibola*et al*., 2012).**

Table 4: Amount of minerals in 1 cup of honey **(339 g)**
https://www.nutritionvalue.org/Honey_nutritional_value.html

Minerals		
Nutrient	**Amount**	**Percentage (%)**
Calcium	20.34 mg	2
Magnesium	6.78 mg	2
Manganese	0.271 mg	12
Phosphorus	13.56 mg	1
Potassium	176.28 mg	4
Sodium	13.56 mg	1

1.5.2. Organic biochemicals

1.5.2.1. Sugar

Sugars account for up to 95% of the solid matter in honey, making it essentially a product made of carbohydrates **(Bogdanov*et al*.,2008).**

The main sugars in honey are fructose (32–44%) and glucose (23–38%), with small amounts of other monosaccharides like galactose also present. The composition of sugars in honey can vary depending on the botanical source, with different types of honey showing different sugaratios. Honey also contains disaccharides like maltose and sucrose, with sucrose levels influenced by factors such as botanical origin, honey maturity, nectar flow, and artificial bee feeding **(De-Melo*et al*.,2017).**

Glucose, fructose, sucrose, maltose, and dextrin were proven to be present in the early publications on sugar separation **by Mayloth (1951) and Vavruch (1952).**

1.5.2.2. Organic acids

Another major category of chemicals found in honey is organic acids. The primary acid in honey is gluconic acid, which is present in a varying equilibrium with the matching glucono-lactone. Other acids found in honey include succinic, oxalic, maleic, lactic, citric, butyric, formic, and maleic **(Ball, 2007,Bogdanov*et al*., 2004 , Ahmed*et al*., 2018).**

Honey's acidity is a result of its organic acids, which also greatly influence its distinct flavor **(AurongzebandAzim , 2011).**

1.5.2.3. Vitamins

Researchers found small amounts of vitamins in honey. However, these amounts were deemed insignificant for human nutrition. This suggests that honey cannot be relied upon as a significant source of vitamins(**Titcomb and Tanumihardjo, 2019).**

The vitamins found in honey include thiamine, riboflavin, pantothenic acid, nicotinic acid, pyridoxine, ascorbic acid, vitamin K, folic acid, and biotin **(Figure 5).**

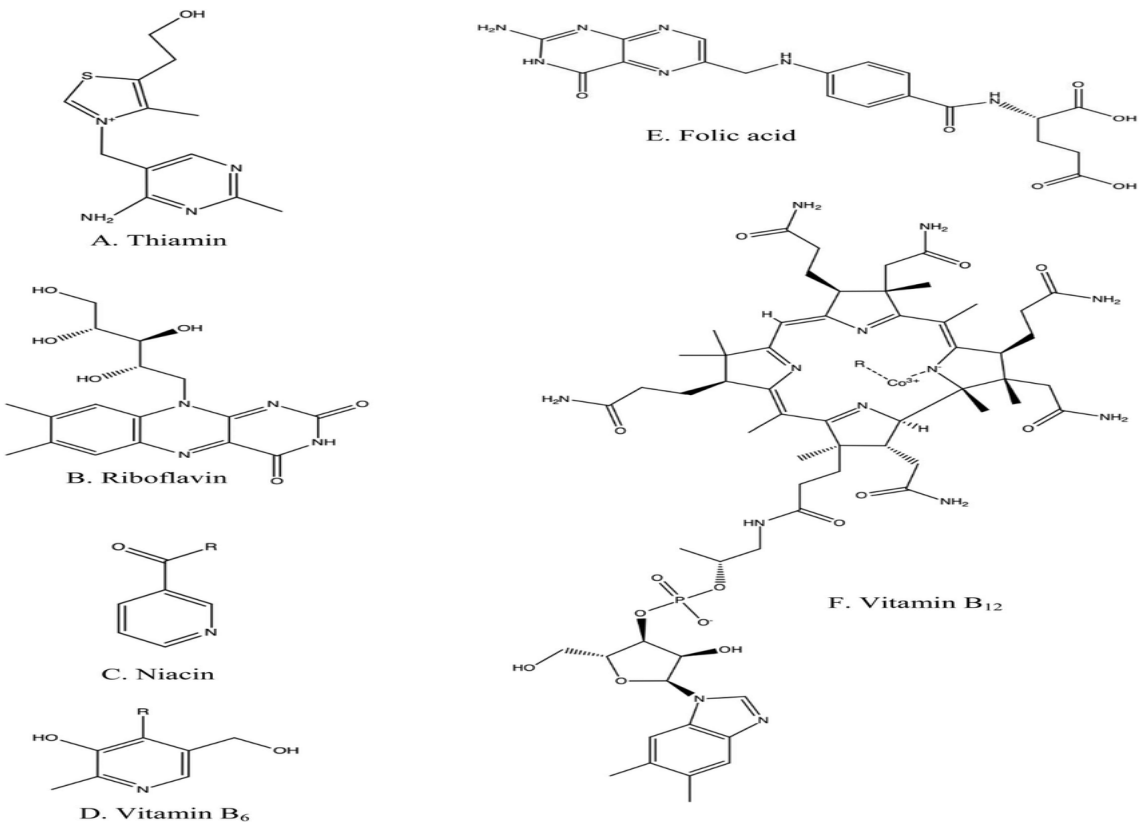

Figure 5:vitamins structure**(Titcomb and Tanumihardjo, 2019)**

While present, the quantities of these vitamins are too small to provide significant nutritional benefits **(Table 5).**

The controversy surrounding vitamin C in honey is discussed briefly. Early animal assays were negative, but some researchers reported the presence of a substance similar to vitamin C. The extent and significance of vitamin C in honey remain uncertain**(Ajibola*et al*., 2012).**

Table 5: table presents the amount of vitamins in 1 cup of honey (339 g)

https://www.nutritionvalue.org/Honey_nutritional_value.html

Vitamin B2	0.129 mg	10 %
Vitamin B3	0.410 mg	3 %
Vitamin B5	0.231 mg	5 %
Vitamin B6	0.081 mg	5 %
Vitamin B9	6.78 mcg	2 %
Ascorbicacid	1.7 mg	2 %

1.5.2.4. Enzymes

Honey composed of various enzymes. Enzymatic and non-enzymatic reactions play a crucial role in determining its quality and biological activities.

Role of enzymatic reactions

- Production and degradation of Hydrogen Peroxide

Hydrogen peroxide is produced from glucose by glucose oxidase or non-enzymatically by polyphenols. It is degraded by enzymes and non-enzymatic reactions like vitamin C and the Fenton reaction **(Figure6)**. The colloidal structure of honey influences hydrogen peroxide generation, which contributes to its antibacterial activity**(Alaerjani*et al.*, 2022).**

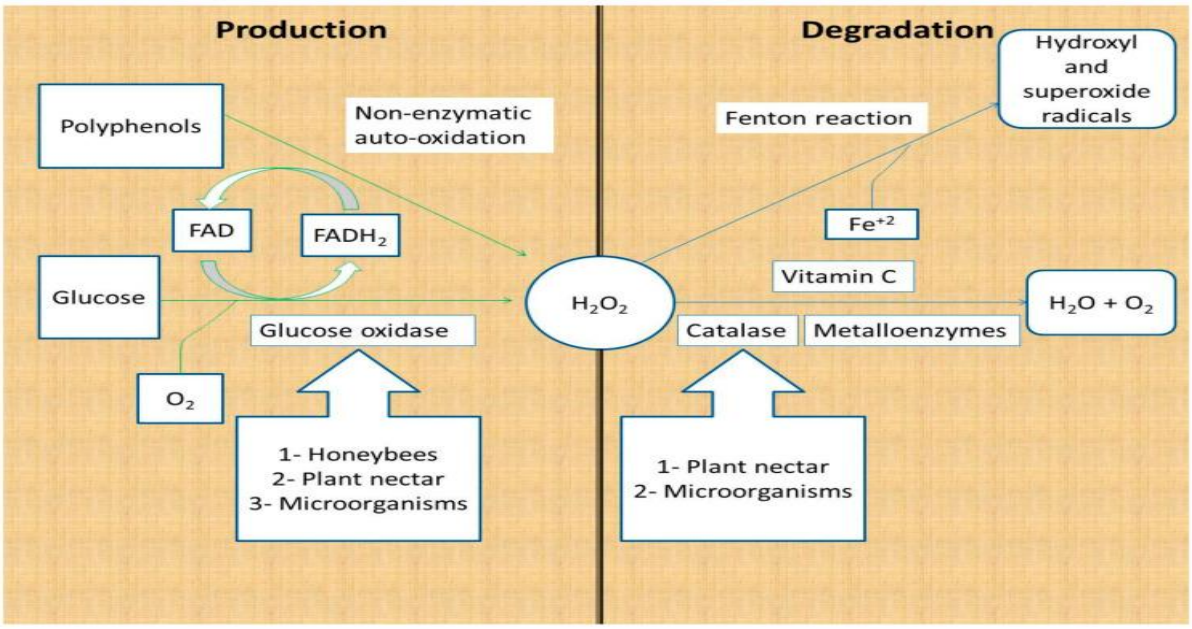

Figure 6: Production and degradation of hydrogen peroxide in honey

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Table 6: Proteases in honey**(Alaerjani*et al*., 2022)**

Enzyme	Role
Serineproteases	Responsible for breaking down proteins into short peptides. This process contributes to the quality and biological activities of honey.
Trypsin,chymotrypsin, and elastase	Cleaving peptide bonds. This diversity in proteases adds to the functional properties of honey.

Short peptides produced by honey proteases have various beneficial effects, including antioxidant, antitumor, antimicrobial and weight loss properties. These peptides are derived from specific amino acids and their sequences**(Figure7)(Alaerjani*et al*., 2022).**

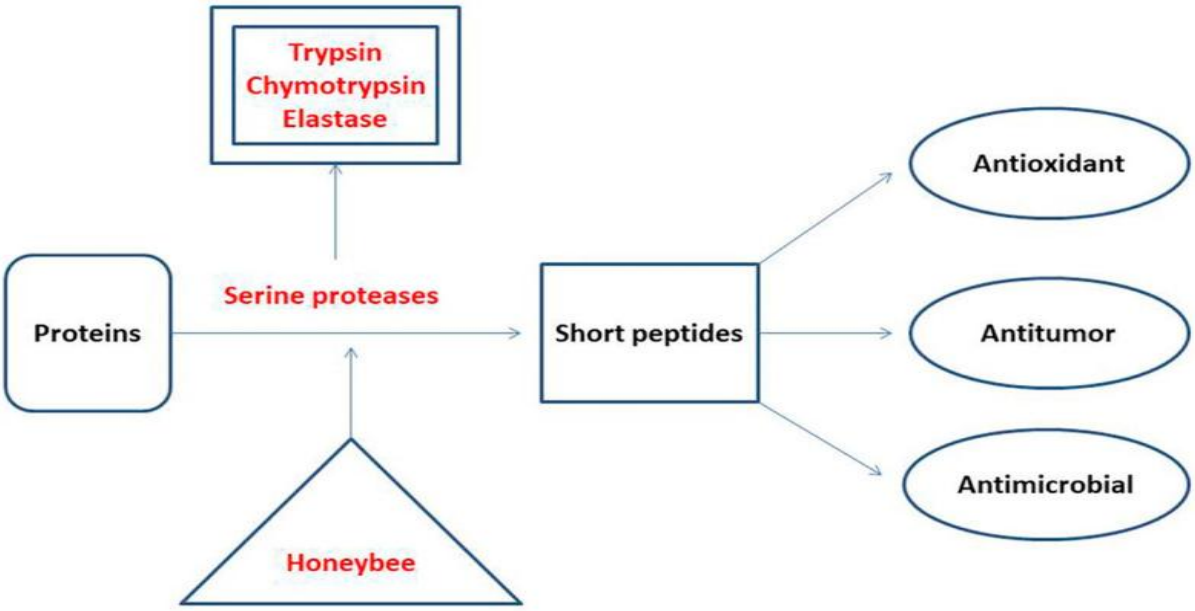


Figure 7: The proteases in honey

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9331712/bin/molecules-27-04719-g002.jpg

- Diastase and invertase

Diastase and invertase are hydrolytic enzymes secreted by honeybees to ripen nectar into honey. **(Alaerjani*et al*., 2022)**

- Diastase converts nectar polysaccharides to glucose which contributes to the sweetness and texture of honey, while invertase converts sucrose to fructose and glucose:

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O_6$$

Saccharose + water → Glucose + Fructose

- Non-Enzymatic reactions

a) Production and degradation of HMF

HMF is a compound produced from the breakdown of glucose or fructose, and its formation in honey is influenced by factors like pH, acidity, minerals, and sugars.

Storage and processing conditions affect HMF production, with high temperatures and long durations increasing it. However, low temperatures do not lead to excessive HMF production. HMF can degrade to formic and levulinic acids, as well as soluble polymers and insoluble humans **(Figure 8).**

Contrary to some studies, HMF concentrations are not necessarily indicative of honey storage or treatment. HMF can have both negative and positive effects on human health, with potential toxicity and health benefits. The toxic level of HMF for humans is still unclear, but honey is generally safe for consumption **(Alaerjani*et al*., 2022).**

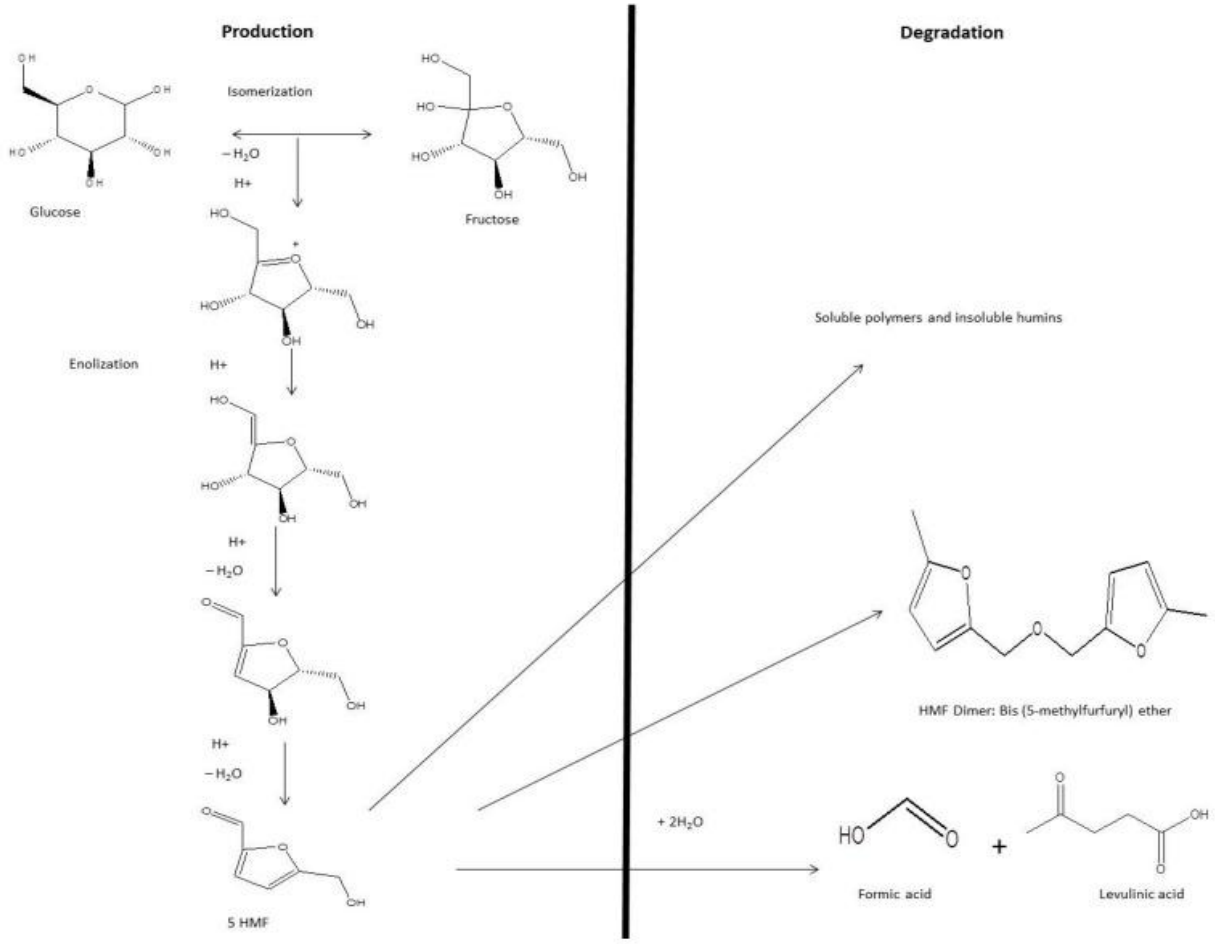


Figure 8: Production and degradation of HMF in honey
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9331712/bin/molecules-27-04719-g005.jpg

1.5.2.5. Phenolic compounds in honey

Phenolic compounds are molecules that have phenol (hydroxybenzene) bound to either an aliphatic (branched structures that do not form rings) or aromatic (unsaturated cyclical) structure **(Becerril-Sánchez *et al* ., 2021).**

Phenolic substances contained in food are mainly divided into simple phenols, coumarins, quinones, betacyanidins, lignans and lignins, acid phenols, flavonoids and tannins; these last three are the main phenolic compounds in food**(Figure9)(Becerril-Sánchez *et al* ., 2021).**

These substances are considered to be the main substances with antioxidant activity in honey, primarily related to its ability to scavenge free radicals, by forming more stable and less toxic molecules(**Cianciosi *et al* ., 2018).**

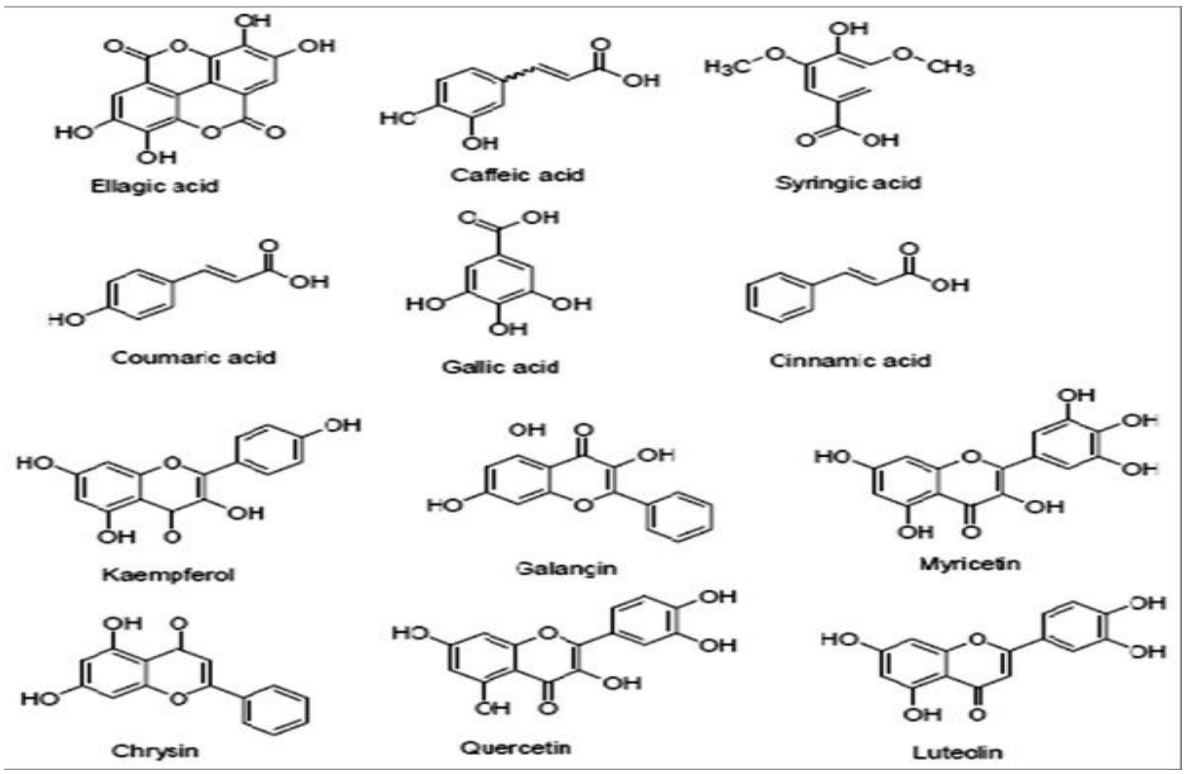


Figure 9: Chemical structures of the flavonoids, organic acids and, phenolic ingredients**(Samarghandian*et al* ., 2017)**

1.5.2.5.1. Phenolic acids

Phenolic acids (phenolic carboxylic acids) contain a phenolic ring and at least one organic carboxylic acid function; according to structure, it can be divided into: C6-C3 (such as p-coumaric acid, ferulicacid,caffeic acid…etc), C6-C2 structures (such as acetophenone and phenylacetic acid) and C6-C1 structures (such as syringes, vanillin and gallic acid).

Typically, most of these compounds are combined with the following structural components of plant (cellulose, lignin), but also other types of organic molecules such as glucose, other sugars, or flavonoids**(Figure 10)(Cianciosi *et al*., 2018).**

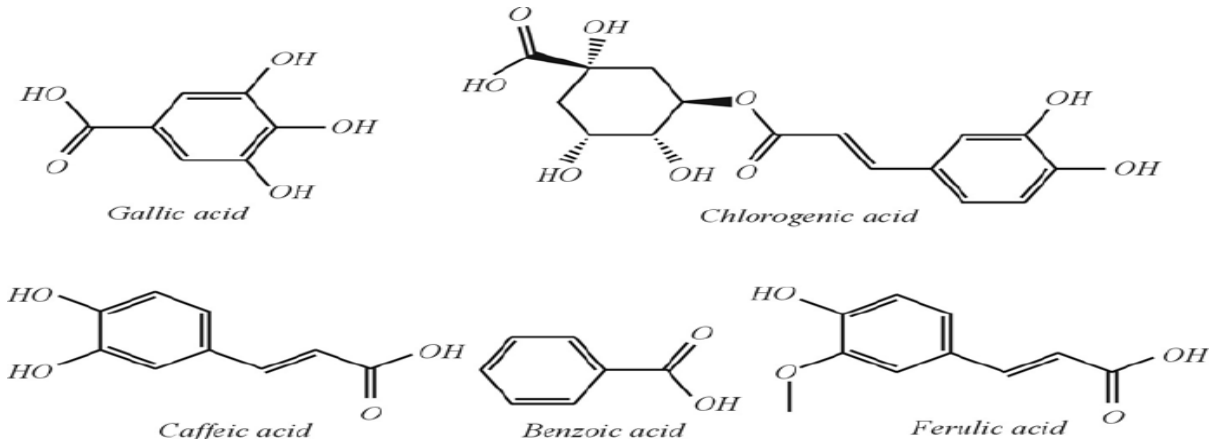


Figure 10: Structure of phenolic acids identified in the honey samples

https://www.researchgate.net/profile/AudriusMaruska/publication/228494420/figure/fig3/AS:301937951035394@1448999025730/Structure-of-phenolic-acids-identified-in-the-honey-samples.png

1.5.2.5.2. Flavonoids

Flavonoids are low molecular weight natural compounds, mainly Water soluble. They consist of two benzene rings alternating with a linear chain of three atoms From carbon (C6-C3-C6); this structure often rearranges itself to form three rings of 15 carbon atoms Called A, B, and C **(Figure11)**.

Generally, these compounds have at least two phenolic (OH) groups, Usually associated with sugars (glycosides), mainly glucose but also xylose, galactose, rhamnose, arabinose, rutin and glucorhamnose; if flavonoids are not related to sugar They are called aglycones. Flavonoids are then classified into flavanols, flavones, dihydroflavonols, flavonols, flavanones, isoflavones, anthocyanins and anthocyanins according to the degree of oxidation of the C ring. The most common substances in honey are flavones, flavanols, and flavonols**(Cianciosi** flavonols**(Cianciosi *et al* ., 2018).**

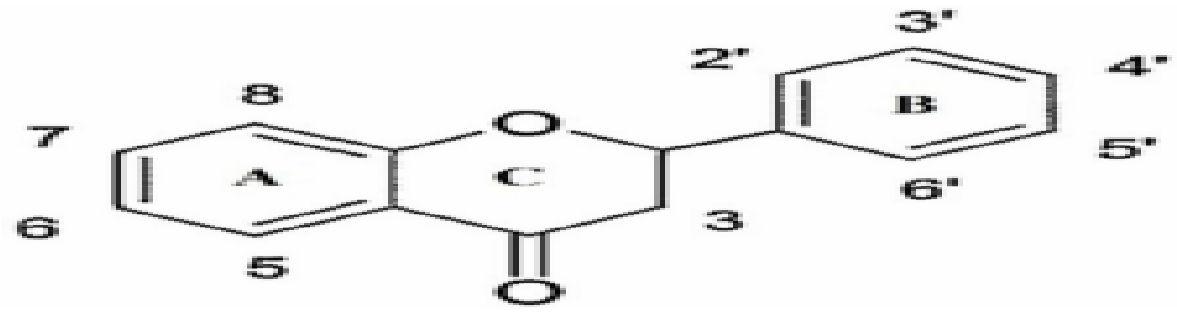


Figure 11: Basic structure of flavonoids**(Cianciosi *et al* ., 2018)**

1.5.2.5.3. Tannins

Tannins are a common phenolic antioxidant found in a variety of fruits, vegetables, barks, and leaves. They have been suggested to have therapeutic and medical uses.One such magical molecule with strong antioxidant properties is tannic acid.There are two classes of tannins. Gallic acid or other similar compounds partially or totally esterify the hydroxyl groups in hydrolyzable polyhydric alcohol, which makes up one group. Tannins, which are created when phenolic chemicals condense, make up the other category. Condensed tannins are the name for such non-hydrolyzable tannins (**Figure 12)(Ghosh , 2015).**

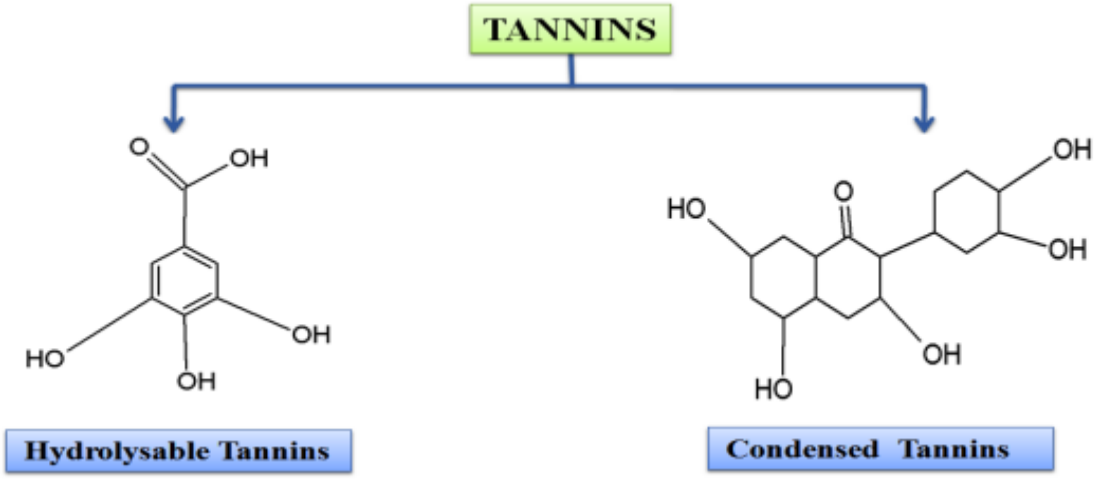


Figure 12: Types of tannins and their basic structures**(Ghosh , 2015)**

1.6. Bioavailability and metabolites of honey

In terms of nutrition, bioavailability is the percentage of a nutrient that is found in food that is taken up, stored, and utilized for regular bodily processes. Determined by research on both humans and animals that phenolic chemicals taken in through food survive digestion in the upper digestive tract and travel to various sections of the proximal and distal intestine in significant amounts.

Phenolics are conjugated (often methylated, sulfated, and glucuronidated) in the small intestine and subsequently in the liver during the absorption process. process of metabolic detoxification that aids in the biliary and urine elimination systems. The colonic epithelium comes into touch with both parent and degraded phenolic compounds. The colonic microbiota metabolizes these compounds to simpler forms, which are subsequently found in urine, feces, blood, and tissue**(Figure 13)(Afrin *et al.*, 2019).**

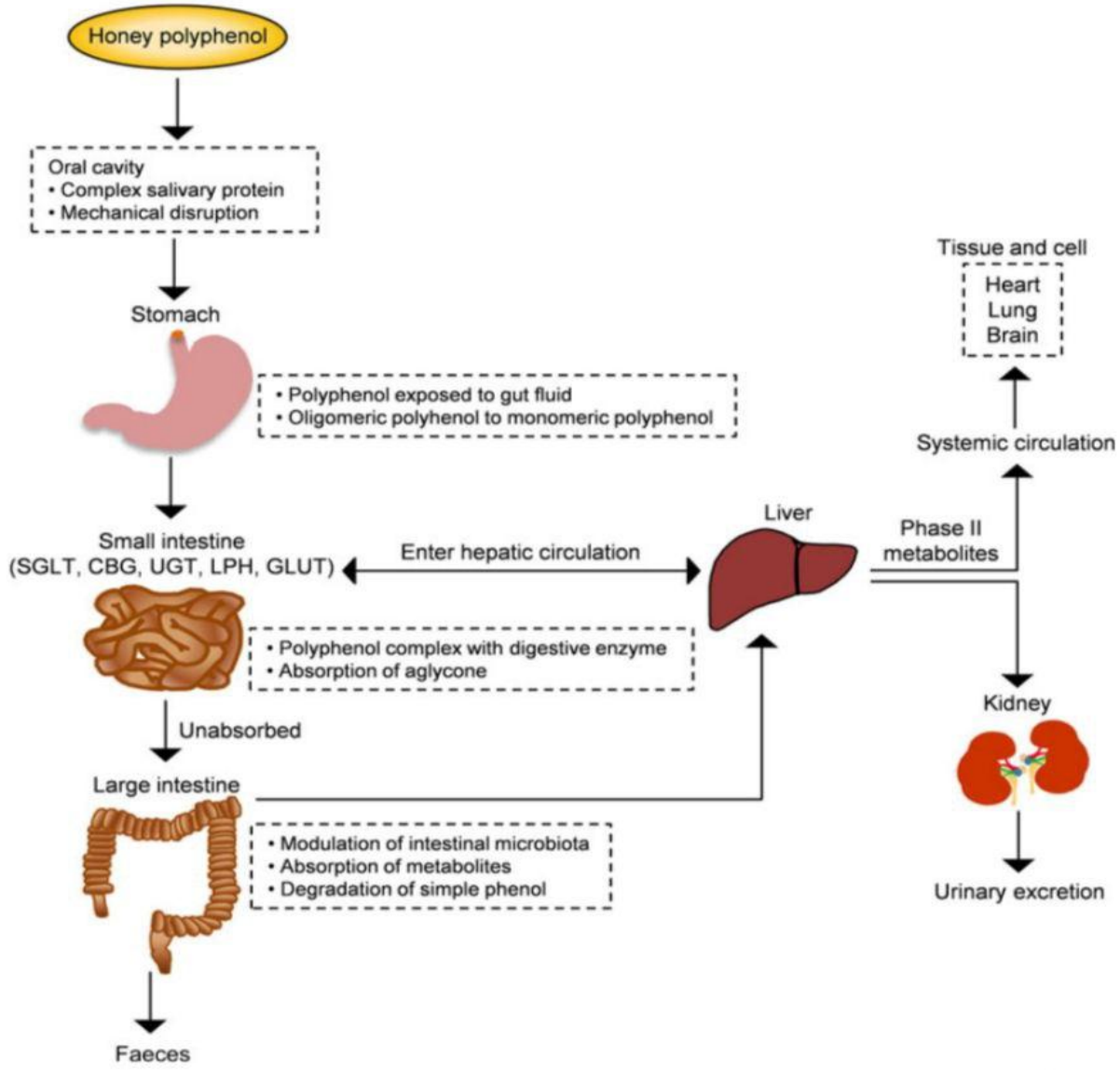


Figure 13: Schematic depiction of the absorption and metabolism of honey polyphenols in the human**(Afrin*et al.*, 2019)**

Chapter 02

Antioxidant Effect Of Honey On The Liver And Erythrocytes

Chapter 02: Antioxidant effect of honey on the liver and erythrocytes

2.1 Antioxydanteffect of honey on the liver

2.1.1 Generalities on liver

The liver is a remarkable organ with a lot of unique anatomical and functional characteristics **(Mahadevan, 2014).** It is the biggestand heaviest solid glandular organ in the body with 1,2 to 1,6 (Kg) **(Marchesini *et al*., 2008).** It receives its large blood supply from an unusual combination of two sources: venous and arterial **(Mahadevan, 2014).**

Most of the right hypochondriac region is occupied by the liver, which is located high up in the abdominal cavity. It expands into the left hypochondrium's medial region as well as the epigastric area. The lower edge of the rib cage encircles the liver for the most part, protecting it. The liver is surrounded by a thin layer of connective tissue called Gleason's capsule **(Mahadevan, 2014).**

It is the primary organ responsible for xenobiotic and endogenous molecule metabolism and the preservation of the organism's metabolic homeostasis **(Casas-Grajales *et al*., 2015).**

2.1.1.1 Structure

The liver lobule is the primary functional unit of the liver. A single lobule is roughly hexagonal in form and around the size of a sesame seed, outlined the following as the main components of a hepatic lobule:

Hepatocyte plates which make up the majority of the lobule, portal triads at each corner of hexagon, central vein, liver sinusoids that run from the central vein to the portal triads, hepatic macrophages (Kupffer cells), bile canaliculi (little canals), space of Disse which is a small space between the sinusoids and the hepatocytes **(Figure14) (Ozougwu*et al*., 2017).**

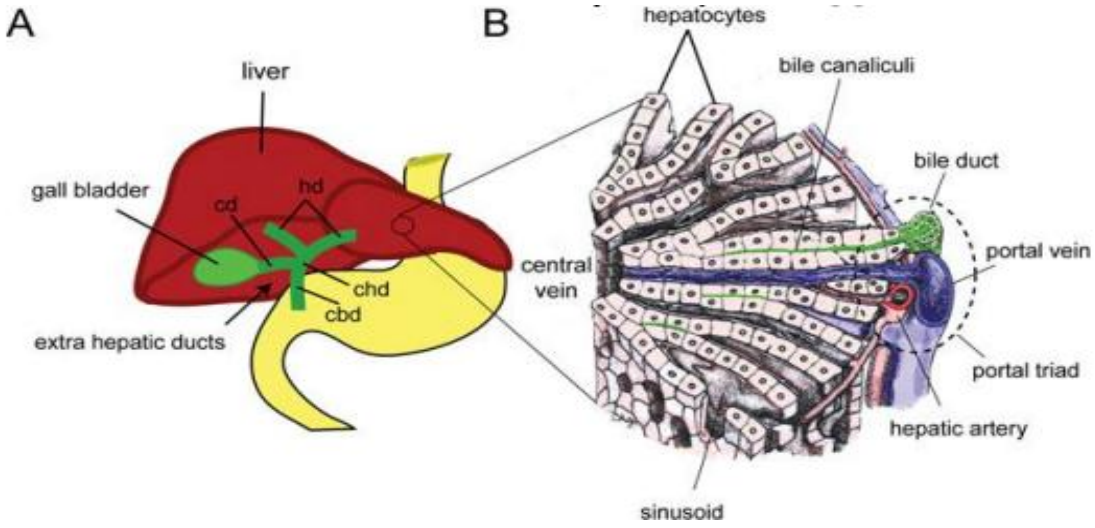


Figure 14: Liver's cellular structure **(Ozougwu *et al*., 2017)**

Organized into cords, hepatocytes encircle a complex, specialized capillary bed that is covered in fenestrated endothelial cells **(Figure 14)**. The portal vein and the hepatic artery, which are branches of the input vascular supply, can run unidirectionally through the sinusoids and into the central veins, which then combine to form the hepatic vein and link to the inferior vena cava. This is how the vascular network is structured. Known as the "portal triad," the portal vein, hepatic artery, and collecting bile duct are located in a cluster at the corners of an approximately hexagon-shaped structure called the "hepatic lobule"**(Figure 14)** that is found repeatedly in the liver tissue. Three broad zones can be distinguished inside the lobule: transitional (zone 2), pericentral (zone 3), and periportal (zone 1). Zones 1 through 3 expose the hepatocytes to progressively higher amounts of processed xenobiotics/toxins and progressively lower amounts of oxygen**(Figure15) (Kang *et al*., 2012).**

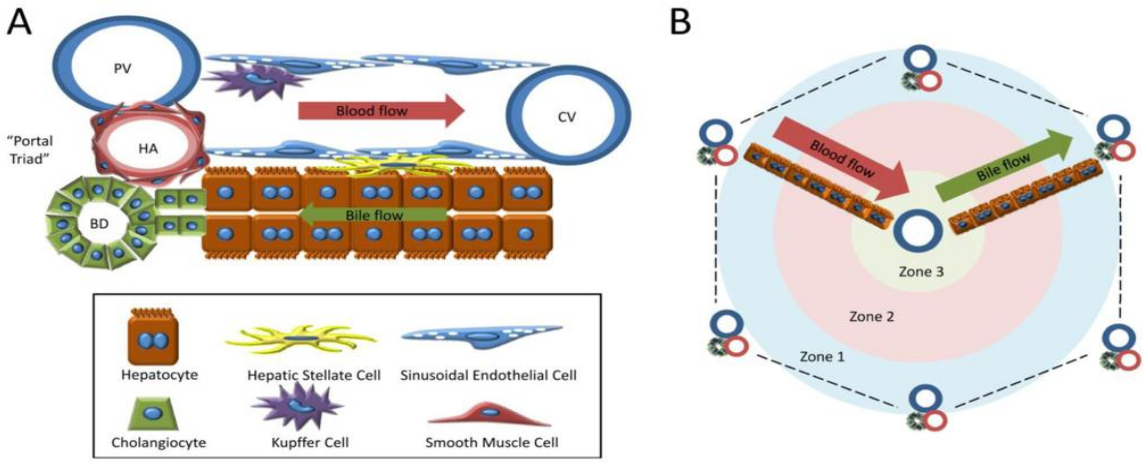


Figure 15: liver's architecture**(Kang *et al*., 2012)**

2.1.1.2 Function

The liver undertakes a lot of functions, the most well-defined of which are bile secretion, bilirubin metabolism, vascular and hematologic activities, food metabolism, metabolic detoxification, and mineral and vitamin storage **(Ozougwu*et al*., 2017).**

2.1.1.2.1 Bile secretion

Bile is a yellowish green, alkaline, bitter fluid that comprises conjugated bile acids, or bile salts, cholesterol, water, electrolytes, and the pigment bilirubin. Hepatocytes produce it, which is then secreted into the canaliculi. Conjugated bile acids, or bile salts, are necessary for the intestinal emulsification and absorption of lipids **(Ozougwu*et al.*, 2017).**

2.1.1.2.2 Metabolism of nutrients

- **Lipid and cholesterol metabolism**

Lipid and lipoprotein uptake, synthesis, packing, and secretion are all carried out by the liver, which is essential for digested absorption. The biliary synthesis and secretion system of the liver facilitates the effective absorption of lipids from food **(Treftse*t al.*, 2017).**

- **Protein and amino acid metabolism**

The liver, an organ that synthesizes proteins, is in charge of 85–90% of the amount of circulating proteins **(Treftse*t al*., 2017),**it produces the albumin and globulins (apart from gammaglobulin) that make up plasma proteins. Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and several other non-essential amino acids are also produced by the liver **(Ozougwu*et al.*, 2017).**

- **Glucose metabolism**

Blood glucose levels are stabilized in part by the liver, which releases glucose when blood sugar levels are hypoglycemic (low) and absorbs glucose when blood sugar levels are high. It then stores the glucose as glycogen (glyconeogenesis) or converts it to fat. The liver has the ability to convert amino acids and glycerol to glucose if all glycogen stores have been depleted **(Ozougwu*et al.*, 2017).**

2.1.1.2.3 Metabolic detoxification

The liver is in the role of detoxifying and metabolizing a wide range of endogenous and exogenous substances, making them more hydrophilic, which frequently changes their potency and level.Phase Iand phase IIare the two primary classes of enzymes that are generated in hepatocytes and are principally responsible for the actions**(Figure16) (Ozougwu*et al.*, 2017).**

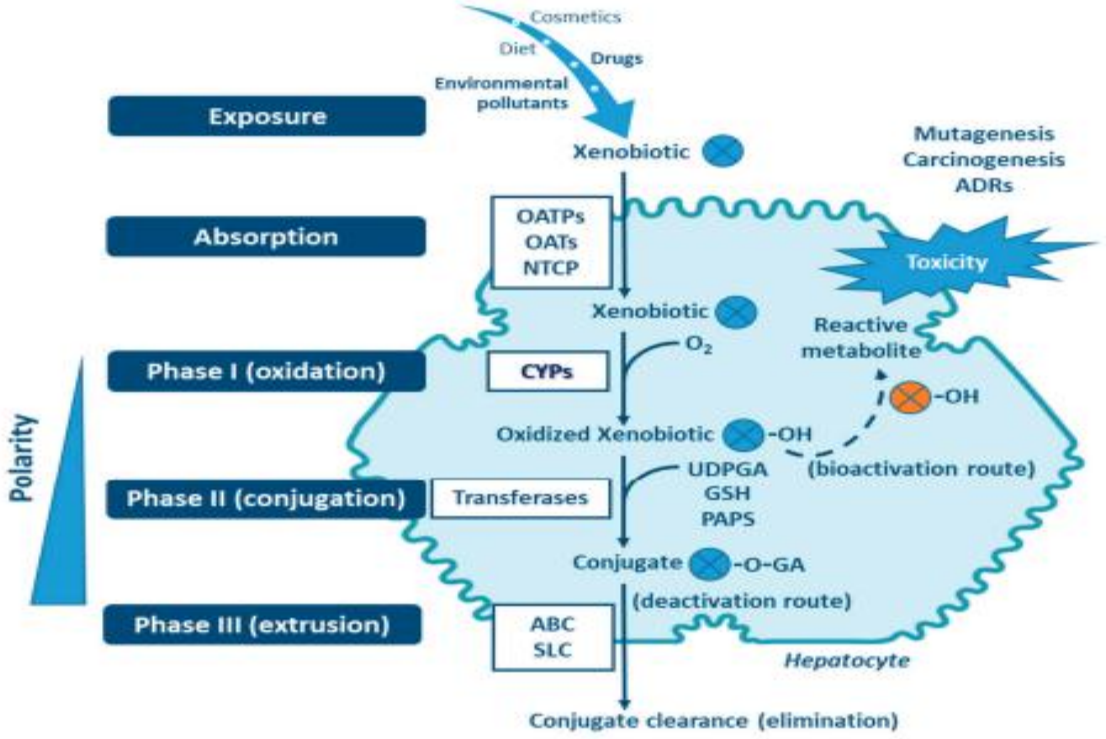


Figure 16: Xenobiotic metabolism in the hepatocyte**(Esteves*et al.*, 2017)**

Phase I

The cytochrome P450 supergene family of enzymes, which makes up the majority of the Phase Ⅰ detoxification system, is typically the first line of defense enzymatically against foreign substances. Phase I biotransformation is the method used to metabolize the majority of medications. A cytochrome P450 enzyme (CypP450) adds a reactive group, like a hydroxyl radical, to a normal Phase I process by using oxygen and NADH as a cofactor. Reactive compounds are created as a result of this detoxification phase, and these molecules may be more dangerous than the original molecule. These reactive compounds have the potential to harm the cell's proteins, RNA, and DNA if Phase II conjugation is not used to further metabolize them **(Liska, 1998).**

Phase II

Usually, Phase I activation is followed by Phase II conjugation processes, which produce a xenobiotic that has changed into a water-soluble substance that may be eliminated by the urine or bile. The body undergoes a variety of conjugation reactions, such as glutathione and amino acid conjugation, glucuronidation, and sulfation (**Table7)**.Cofactors are needed for these processes, and they must be refilled through diet **(Liska, 1998).**

Table 7: Major phase II detoxification activities in human liver **(Liska, 1998)**

Reaction	Enzyme	Localization	Substrates
H_2O	Epoxide hydrolase	Microsomes Cytosol	Epoxides
Glutathione	Glutathione transferases	Microsomes	Electrophiles
Glucuronic acid (UDPGA)	Glucuronyltransferases	Microsomes	Phenols, thiols, amines, Carboxylic acids
Sulfuric acid (PAPS)	Sulfotransferase	Cytosol	Phenols, thiols, amines
Methyl group (SAM)	N- and O- methyl transferases	Cytosol Microsomes	Phenols, amines
Acetic acid (Acetyl-CoA)	N-acetyl transferases	Cytosol	Amine
Aminoacids (Acetyl-CoA, taurine, glycine)	Amino acid transferases	Microsomes	Carboxylic acids

2.1.2 Oxidative stress in the liver

2.1.2.1 Definition

Oxidative stress is usually referred to as an imbalance between prooxidants and antioxidants **(Jones, 2006)**.There will be a graduated response to oxidative stress, as the idea of a balance

highlights. Therefore, whereas more significant perturbations may result in irreversible damage and cell death, tiny perturbations in the balance are likely to cause homeostatic adjustments in response to changes in the immediate environment. Therefore, it is inevitable that the line separating pathological insults from normal physiological changes will blur **(Burton and Jauniaux, 2011).**

2.1.2.2 Reactive oxygen species

Both free radicals and the non-radical intermediates of these two types of molecules are referred to as reactive oxygen species. Species having one or more unpaired electrons are known as free radicals, and their increased reactivity is attributed to this incomplete electron shell. Although a variety of components can produce free radicals, the most significant ones in biological systems are those that involve oxygen and nitrogen**(Figure17) (Burton and Jauniaux, 2011).**

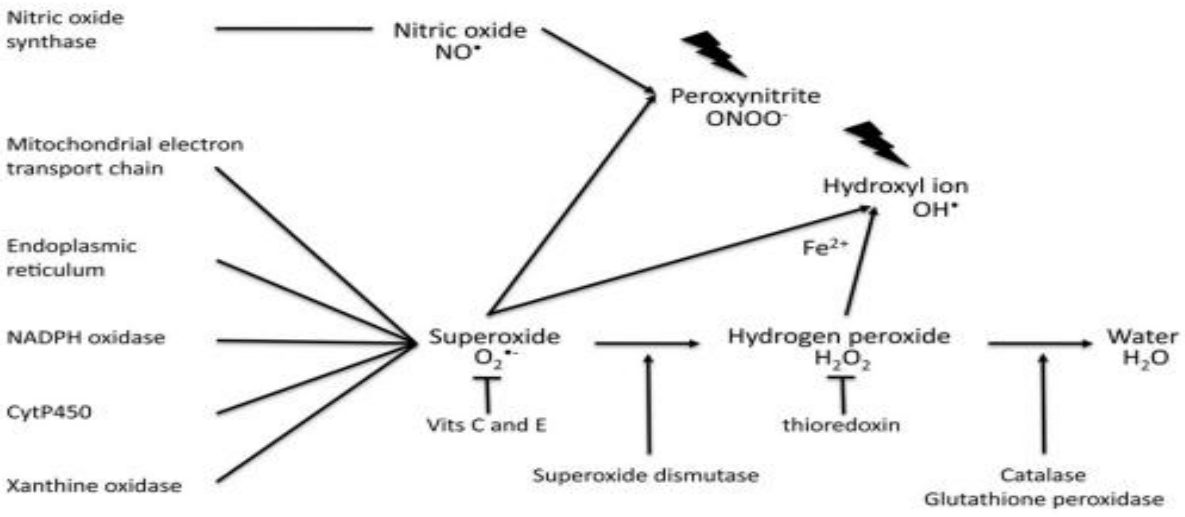


Figure 17: The main reactive oxygen species, possible sources, and detoxification processes. Nicotinamide adenine dinucleotide phosphate is known as NADPH**(Burton and Jauniaux, 2011)**

2.1.3 Causes of oxidative stress in the liver

Since previous studies and trials on liver diseases have demonstrated evidence of liver diseases, damage, and injury under an oxidative stress environment, ROS play a critical role in the development of numerous diseases **(Sadasivam*et al*., 2022).**

The primary causes of oxidative stress mechanisms are described in **(Figure18)**

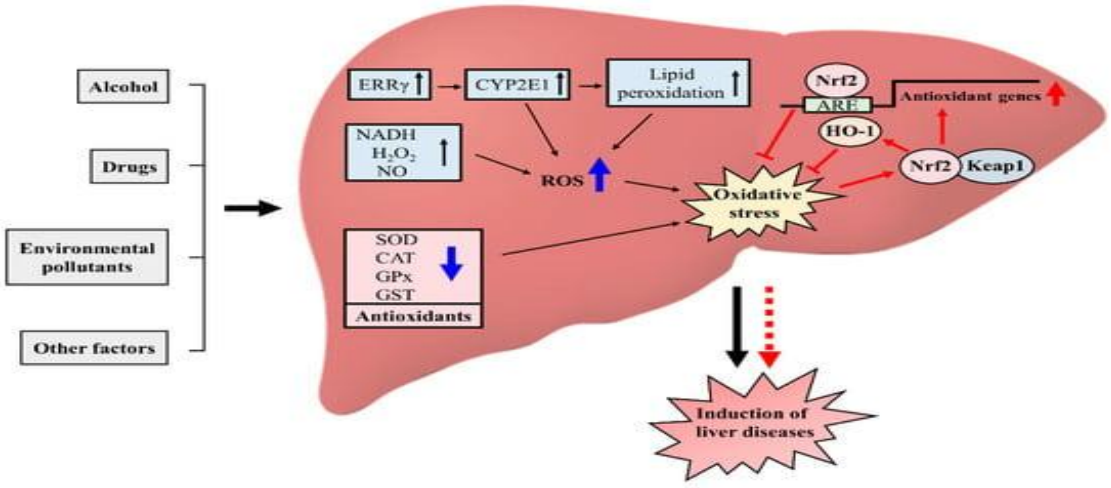


Figure 18: Several elements that lead to oxidative stress, redox imbalance, and the development of liver disorders **(Sadasivam*et al.*, 2022)**

2.1.3.1 Oxidative stress caused by alcohol

One of the leading causes of liver-related mortality is alcoholic liver disease (ALD), which is linked to longer and higher doses of alcohol consumption. From steatosis, ALD can develop into more serious liver illnesses such cirrhosis, fibrosis, and hepatitis **(Li *et al.*, 2015).**

The exact cause of ALD is unknown, although it appears that ethanol metabolism directly contributes to the generation of reactive oxygen species (ROS), mitochondrial damage, and steatosisall of which are common outcomes of both acute and long-term alcohol consumption. It is well demonstrated that the ethanol oxidation process involves at least three different enzyme pathways **(Figure 19) (Li *et al.*, 2015).**

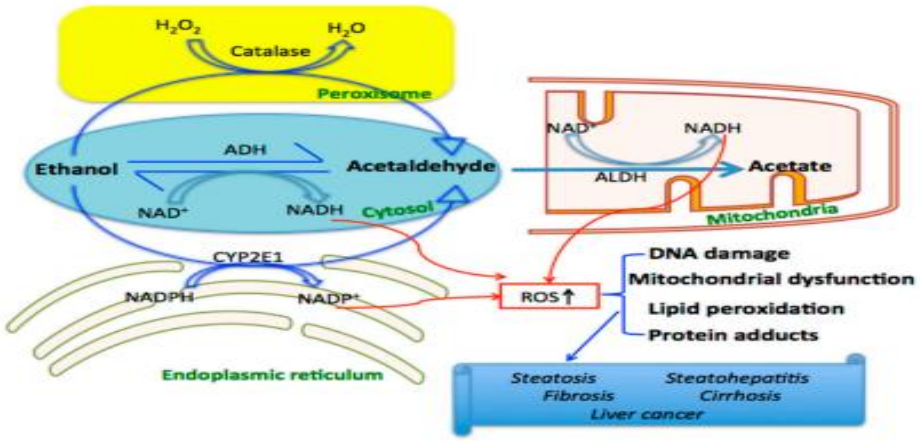


Figure 19: Hepatocyte's ethanol metabolism and the production of reactive oxygen species**(Li *et al.*, 2015)**

2.1.3.2 Oxidative stress caused by drugs

When it comes to medication toxicity, the liver is the organ that is most commonly targeted **(Li*et al*., 2015).**

2.1.3.2.1 Acetaminophen (APAP)

Because of its analgesic and antipyretic qualities, acetaminophen (APAP) is one of the most commonly used medications. When taken as directed, it is safe and effective; nevertheless, an overdose can cause hepatotoxicity and acute liver failure (ALF**) (Yan, 2018).**

- **Structure of acetaminophen**

Chemically, paracetamol is known as 1-hydroxy-4-acetamido-benzene **(Figure20).**The pharmacological activity is reduced or eliminated by changing the position of the two substituents -OH and -NH-CO-CH3 on the benzene ring, by esterification of the phenol function or by introducing other substituents on the ring**(Le Garrec*et al.*, 1994).**

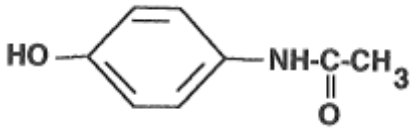


Figure 20: Structure1-hydroxy-4-acetamido-benzene **(Le Garrec*et al*., 1994)**

- **Pharmacokinetics**
- **Absorption**

With a high oral bioavailability of 88%, acetaminophen is quickly absorbed and reaches its peak blood concentration in about 90 minutes. At the prescribed dosages, APAP has a 1.5–2.5 hour plasma half-life and is not frequently attached to plasma proteins. On the other hand, metabolism is disrupted and the half-life is extended to 4-6 hours following an overdose, and this is directly correlated with the severity of liver damage **(Mazaleuskaya*et al*., 2015).**

- **Metabolism**

Microscopic APAP metabolism takes place in liver microsomes. The three stages of APAP metabolism are as follows:

- **90%** of APAP is directed toward phase (II) metabolic pathways, where UDP-glucuronosyl-transferases (UGT) and sulfo-transferases (SULT) catalyze APAP

- ❖ conjugation. This results in the conversion of APAP to glucuronidated and sulfated metabolites, which the body excretes in urine **(Figure 21).**
- ❖ Urine excretes a little, detectable amount of APAP **(2%)** that has not been metabolized.
- ❖ Hepatic cytochrome CYP 2E1 (and to a lesser extent CYP 1A2 and 3A4) excretes **10%** of the remaining APAP, which is then converted to phase I oxidation, which results in the formation of the highly reactive poisonous metabolite N-acetyl-para-benzo-quinone imine (NAPQI) **(Yoon*et al*., 2016).**

In most cases, NAPQI is conjugated and rapidly detoxified by glutathione GSH **(Yan *et al*., 2018).**

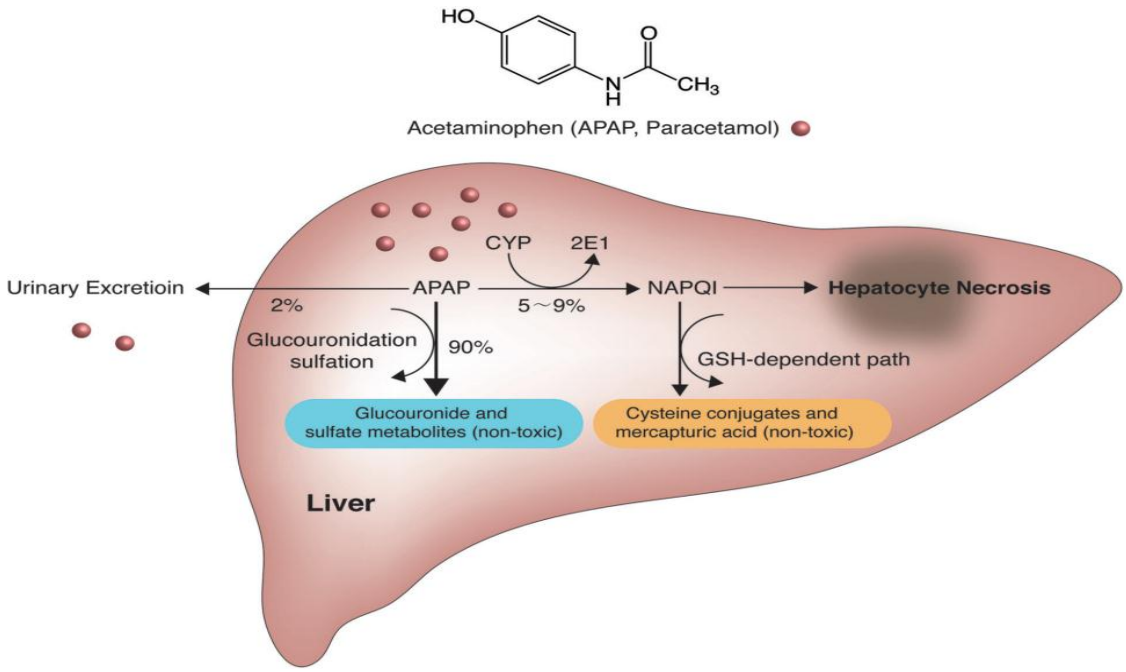


Figure 21: Metabolic pathways of APAP**(Yoon *et al*., 2016)**

- **Elimnation**

A dose of 1g orally is completely eliminated between 15 and 20 hours. Excretion of metabolites is essentially renal, and appears to be independent of urinary output and urinary pH **(LeGarrec*et al*., 1994).**

2.1.3.2.2 Acetaminophen (APAP) hepatotoxicity

Excessive NAPQI depletes GSH in APAP overdose, which causes sulfhydryl groups in cellular proteins particularly mitochondrial proteins to bind covalently. This causes oxidative

stress and malfunction in the mitochondria, which leads to the necrosis of the hepatocytes **(Figure 22) (Yan *et al*., 2018).**

In contrast to total protein-binding in hepatocytes, NAPQI primarily targets mitochondrial proteins. It also interferes with complex I/II of the mitochondrial electron transport chain (ETC), causing electrons to leak from the ETC to oxygen and form superoxide radicals through the action of manganese superoxide dismutase (MnSOD), which then produces hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), or reacts with endogenous nitric oxide (NO) to produce peroxynitrite ($ONOO^-$). Then, GSH directly detoxifies H_2O_2.As a result of these abundant free radicals, GSH is reduced **(Yan *et al*., 2018).**

This results in ATP depletion, malfunction of the TCA cycle and β-oxidation, opening of the MPT pore, and the translocation of mitochondrial proteins to the nucleus, including AIF and Endo G. Nuclear DNA breaks as a result, which leads to necrotic cell death **(Figure22) (Yan *et al*., 2018).**

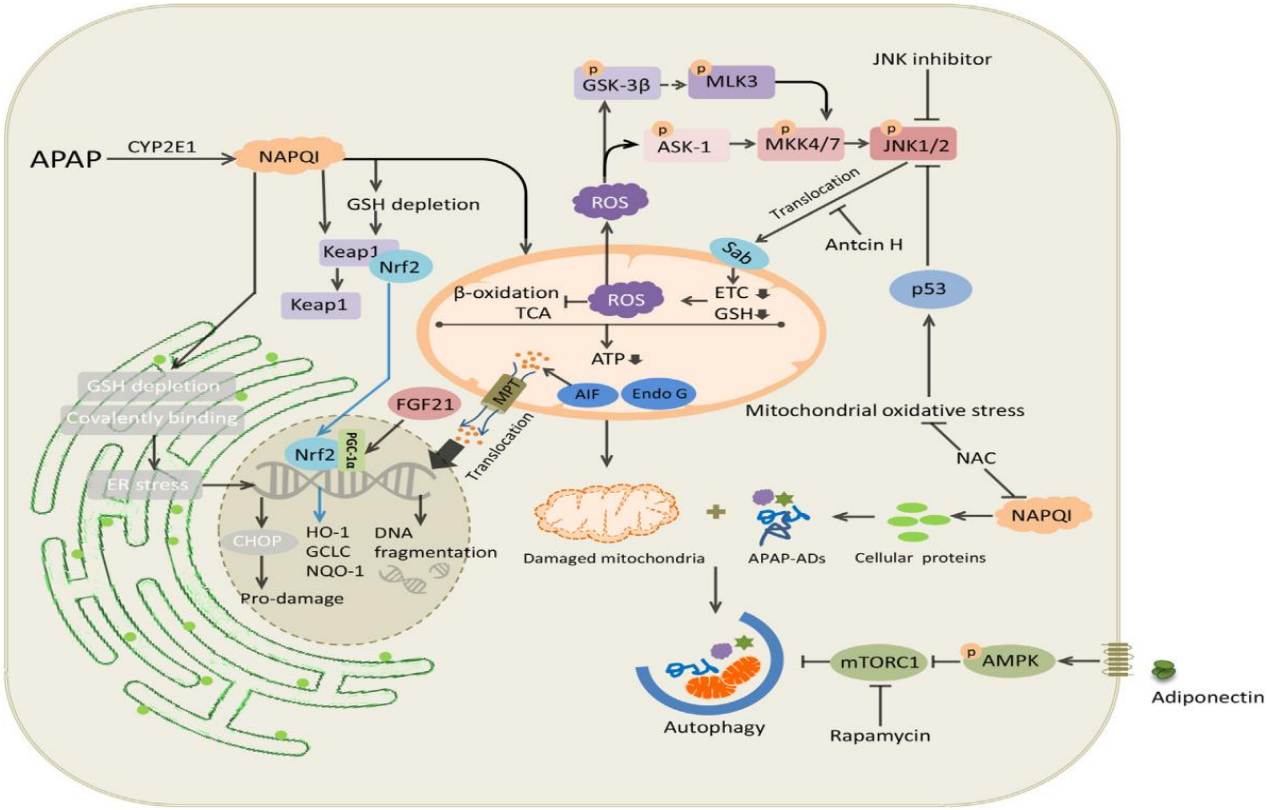


Figure 22: Intracellular signaling events in acetaminophen hepatotoxicity**(Yan *et al*., 2018)**

2.1.3.3 Other factors inducing oxidative stress

Additional elements that might cause oxidative stress include temperature, radiation, and environmental contaminants. Research has been done to investigate how radiation functions and how it relates to oxidative stress **(Sadasivam*et al*., 2022).**

2.1.4 Antioxidants

2.1.4.1 Definition

Antioxidants are substances that have the ability to counteract free radicals and protect cells from damages. By giving the free radical an electron, antioxidants stabilize them and protect other molecules from the damaging effects of reactive oxygen species (ROS) **(Kumar and Kumar, 2023).**

There are two main classes of antioxidants **(Figure23):**

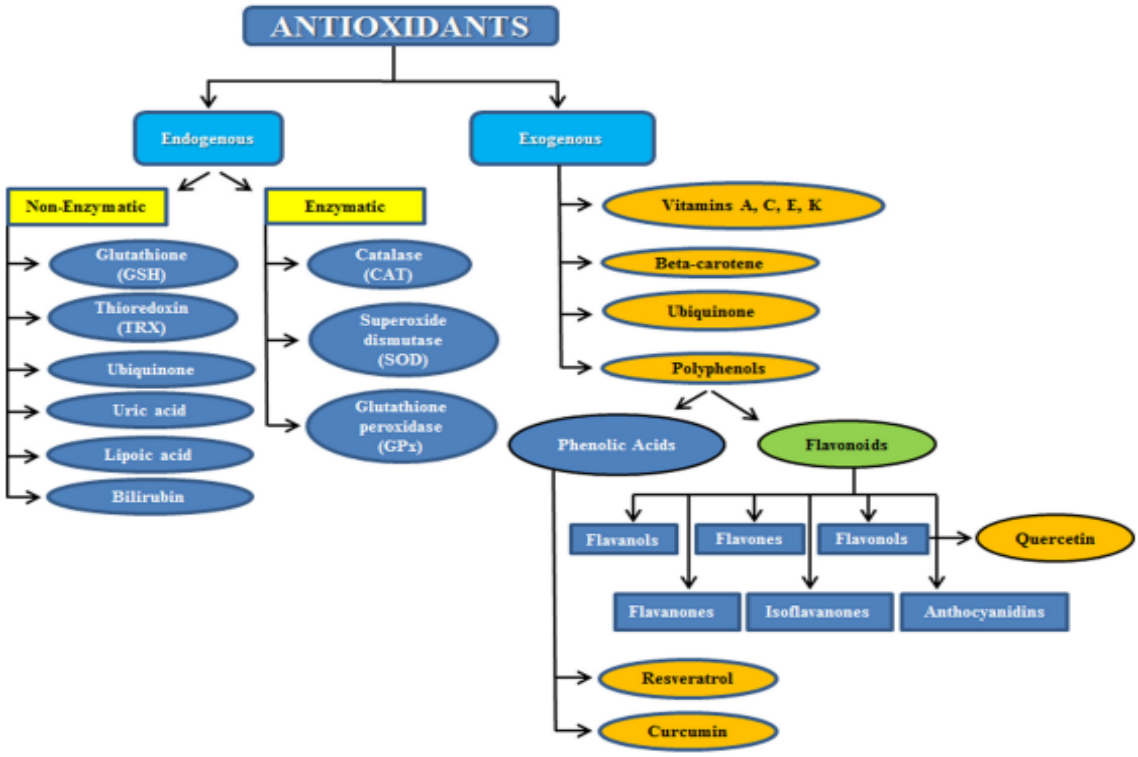


Figure 23: Endogenous antioxidants and exogenous antioxidants **(Simioni*et al*., 2018)**

2.1.4.2 Liver antioxidants and mechanism of action

Superoxide dismutase (SOD): The highly reactive and toxic superoxide anion (O_2^-) is changed into the less dangerous hydrogen peroxide (H_2O_2) by this enzyme.

Based on the metal cofactors they contain, SOD enzymes can be categorized into three groups: Fe-SOD, Mn-SOD, and Cu/Zn-SOD. Cu/Zn-SOD is found in the cytosol and extracellular space, Mn-SOD is found in the mitochondria, and Fe-SOD is found in the chloroplasts **(Kumar and Kumar, 2023).**

Catalase (CAT): This enzyme changes the toxic reactive oxygen species hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Though it can also be found in the cytosol and mitochondria, peroxisomes are where CAT is primarily found in cells **(Kumar and Kumar, 2023).**

Glutathione peroxidase (GPx): This enzyme catalyzes the reduction of lipid hydroperoxides and hydrogen peroxide (H_2O_2) to their corresponding alcohols and water (H_2O), respectively, using reduced glutathione (GSH) as a cofactor. Several cellular compartments, including the cytosol, mitochondria, and peroxisomes, contain GPx**(Kumar and Kumar, 2023).**

2.1.5 Antioxidant activity of honey

The precise antioxidant mechanism of honey is uncertain, several theories have been put forth, including superoxide radical activities, hydrogen donation, flavonoid substrate action for hydroxyl, and free radical sequestration**(Figure 24)(Ahmed *et al*., 2018).**

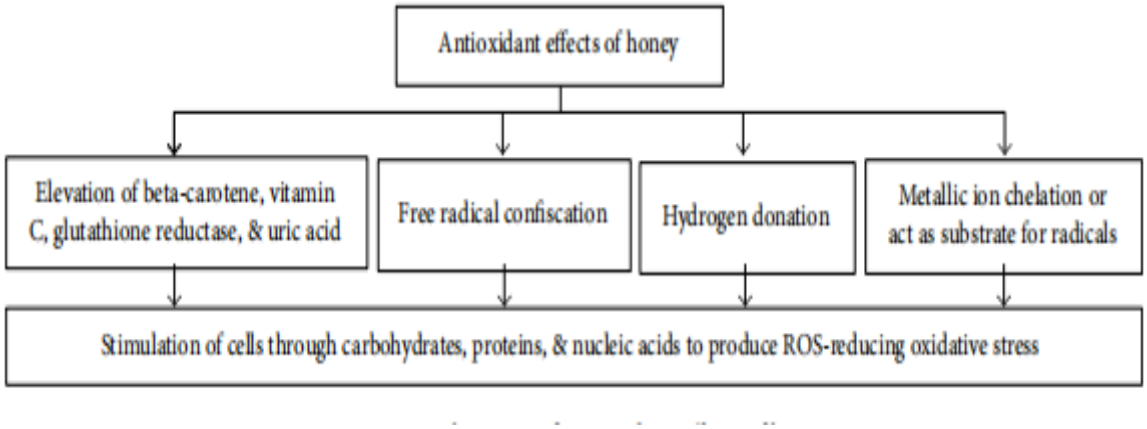


Figure 24: Mechanisms of antioxidant effects of honey**(Ahmed *et al*., 2018)**

There are also other antioxidant mechanisms of honey due to flavonoids **(Figure 25).**

2.1.5.1 The activation of antioxidant enzymes

Flavonoids stimulate phase II detoxifying enzymes and other antioxidant enzymes.(e.g., NAD(P)H-quinoneoxidoreductase, glutathione S-transferase, and UDP-glucuronosyltransferase) **(Chen *et al*., 2023)**.

2.1.5.2 The inhibition of oxidases

By blocking the actions of protein kinase C and xanthine oxidase (XO), which catalyze the generation of superoxide anion **(Chen *et al*., 2023)**.

2.1.5.3 Metal-chelating activity

Hydroxyflavones have the ability to form complexes with metal cations, and the amount and location of the hydroxyl substituents greatly affects the chelating capabilities of these compounds **(Chen *et al*., 2023)**.

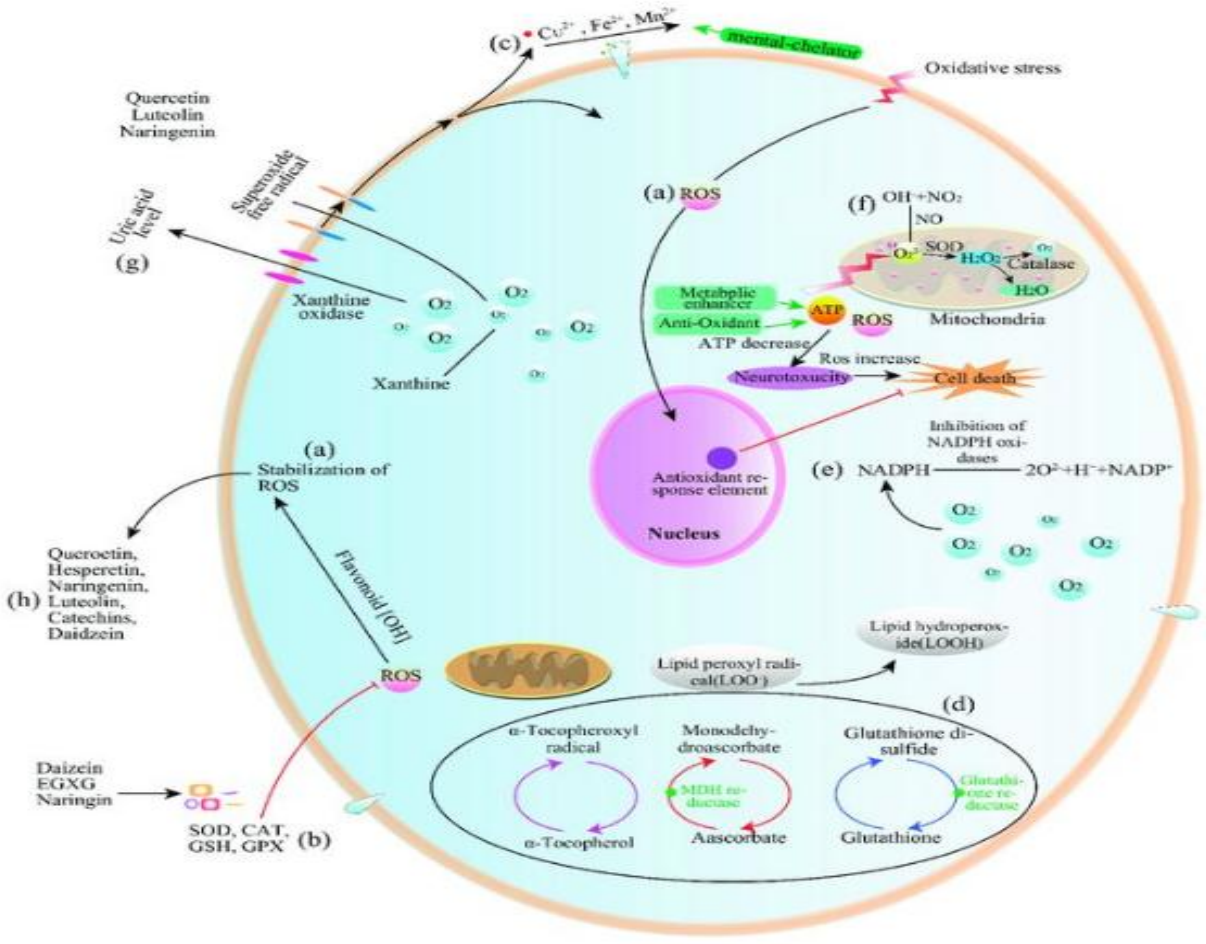


Figure 25: Mechanisms of flavonoid antioxidant activity**(Chen *et al*., 2023)**

2.2 Antioxidant effect of honey on red blood cell

2.2.1 Red Blood Cell

2.2.1.1 Structure of erythrocyte

The mature erythrocyte is anucleated and has a discoid, biconcave form. This design provides the flexibility required to move through the circulatory system as well as a larger surface area which allows proper exchange of gases and the cell's ability to function.

This special cell is surrounded in its structure by a double layer of phospholipid membrane, which is kept in place by the cytoskeleton, a network of proteins. This cytoskeleton, which allows both cellular structural integrity and flexibility, is made up of spectrin, actin, band 3, protein 4.1, and ankyrin**(Figure26)**.

These compounds work together to support a flexible yet structurally sound structure**(Smith, 1987) (Kuhn, 2017).**

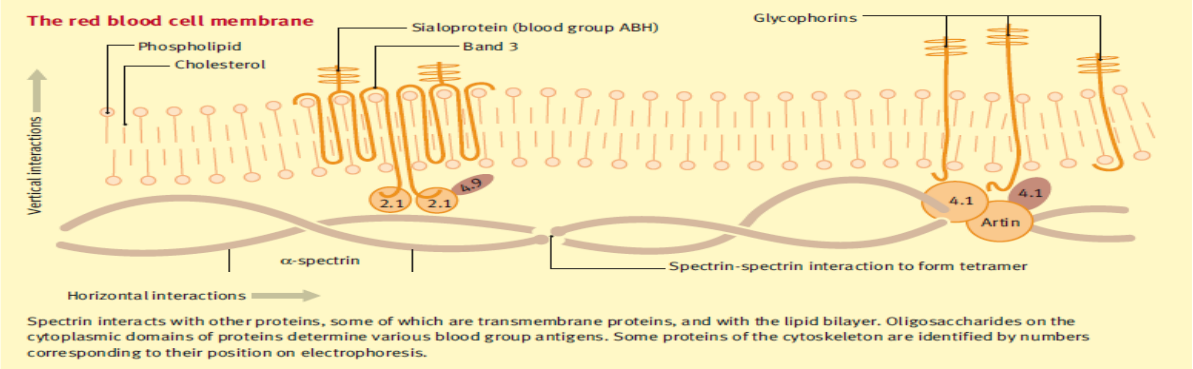


Figure 26: Diagram of the erythrocyte membrane**(Ted, 2009)**

Heme is a crucial component of hemoglobin, the protein responsible for oxygen transport in red blood cells.

- **Structure of heme:** it is a complex organic molecule that consists of an iron ion (Fe^{2+}) coordinated in the center of a porphyrin ring. This iron ion is where oxygen binds reversibly for transport in the blood.

In hemoglobin, the heme groups are associated with globin chains. Hemoglobin is a tetramer composed of two alpha**(**α) globin chains and two non-alpha (β) globin chains. It provides the structural framework for the heme groups to bind to the iron ions and facilitate oxygen

transport in red blood cells. The specific arrangement of the globin chains and heme groups in hemoglobin is crucial for its function in oxygen binding and release **(Figure27) (Ted, 2009).**

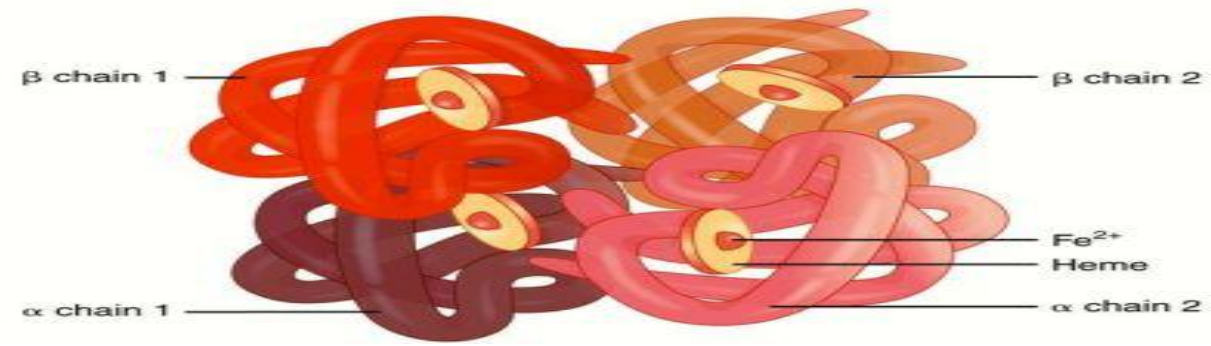


Figure 27: Structural representation of hemoglobin molecule
https://i2.wp.com/www.pathologystudent.com/wp-content/uploads/2016/11/heme-1.jpg?resize=400%2C255&ssl=1

- **Function of heme:** each heme group binds to one oxygen molecule, allowing for efficient oxygen transport from the lungs to tissues and the release of carbon dioxide back to the lungs**(Ted, 2009).**

2.2.1.2 Function of erythrocyte

Red blood cells play crucial roles in the body, primarily related to the transport of oxygen **(Figure 28)** and carbon dioxide**(Table 8)(Hamasakiand Yamamoto, 2000).**

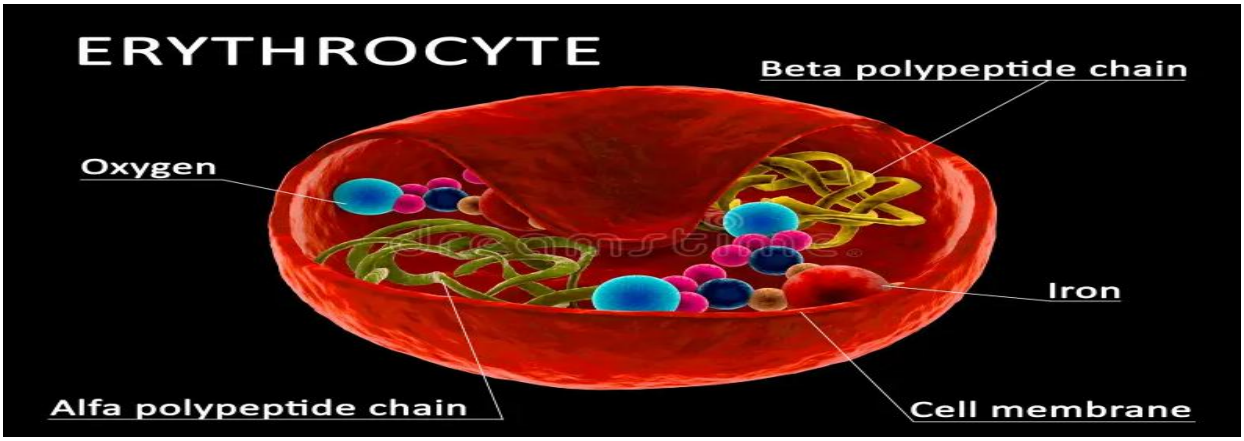


Figure 28: The Intricate Journey of Oxygen in Erythrocytes
https://th.bing.com/th/id/OIP.hIPs1NLaZMvgUOg7UICy7AAAAA?rs=1&pid=ImgDetMain

Table 8: key functions of red blood **(Hamasaki and Yamamoto, 2000)**

key functions of red blood	Mechanism
-Oxygen transport	-Red blood cells contain hemoglobin, a protein that binds to oxygen in the lungs and carries it to tissues throughout the body **(Figure28).**
-Carbon dioxide transport	-Red blood cells also help transport carbon dioxide, a waste product of metabolism, from the tissues back to the lungs to be exhaled **(Figure29).**
-Maintaining acid-base balance	-Red blood cells contain carbonic anhydrase, an enzyme that helps regulate the pH balance in the blood by convertingcarbon dioxide into bicarbonate ions.
-Energy production	-Red blood cells rely on glycolysis (Embden-Meyerhof pathway) for energy production since they lack mitochondria for aerobic respiration.
-Recognition of metabolically active cells	-Through the chloride shift mediated by band 3 protein, red blood cells can recognize metabolically active cells and supply them with the necessary oxygen.

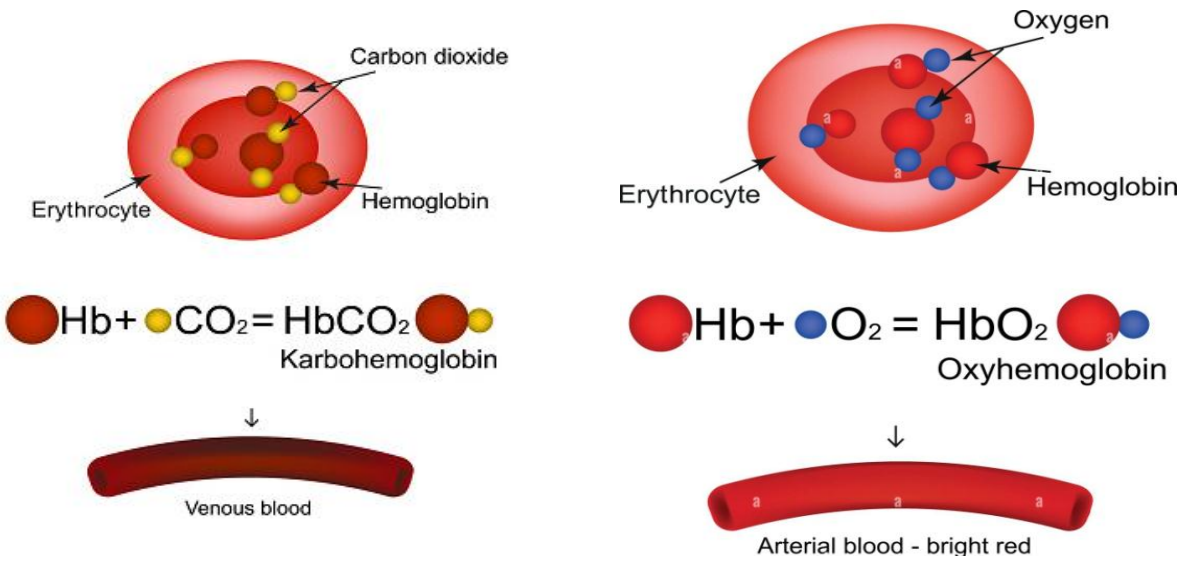


Figure 29: Hemoglobin'srole in carbon dioxide and oxygen transport

https://c8.alamy.com/compes/2bga7fx/oxihemoglobina-la-hemoglobina-transporta-oxigeno-infografias-ilustracion-vectorial-2bga7fx.jpg

2.2.2 Effect of oxidative stress on the red blood cell

The oxidative stress (OS) on erythrocytes, leads to the oxidation of hemoglobin and damage to proteins and lipids of the erythrocyte membrane. OS depletes ATP and adenine nucleotides, with membrane protein denaturation preceding lipid peroxidation.

Ca^{2+} entry and membrane fluidity alterations are proposed as signals for apoptosis induction in erythrocytes. OS can alter cell membrane structure, affecting membrane fluidity and cell function (**Figure 30).** Erythrocyte membrane rigidity is influenced by oxygen levels and can serve as an indirect marker of OS. Iron release via the Fenton reaction and hydroxyl radical production play a key role in red cell damage under OS conditions**(Perrone*et al.*, 2012).**

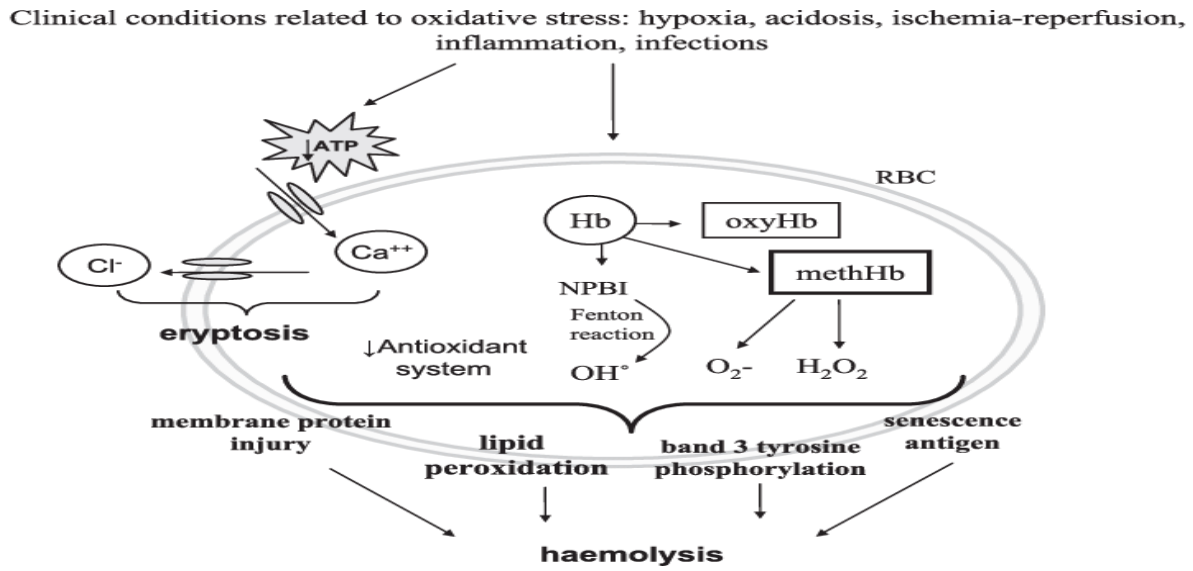


Figure 30: schema presents the effect of oxidative stress on the red blood cell **https://www.researchgate.net/profile/Giuseppe_Buonocore/publication/232714636/figure/fig1/AS:601634837131315@1520452331792/Concise-mechanisms-involved-in-red-blood-cells-haemolysis-induced-by-oxidative-stress.png**

RBCs will also be exposed to oxidants derived from endothelial and immune system cells, which generate nitric oxide ($NO^{\bullet}$), superoxide, peroxynitrite ($ONOO^{-}$), H_2O_2, and hypochlorous acid (HOCl). Nitric oxide is produced by the endothelial enzyme nitric oxide synthase (NOS_3) as a signal molecule to induce vasodilation and by immune system cells at larger amounts by inducible NOS_2, that result in the formation of more potent oxidizing species that can kill invading pathogens **(Figure31)(Tuehrand, 2019).**

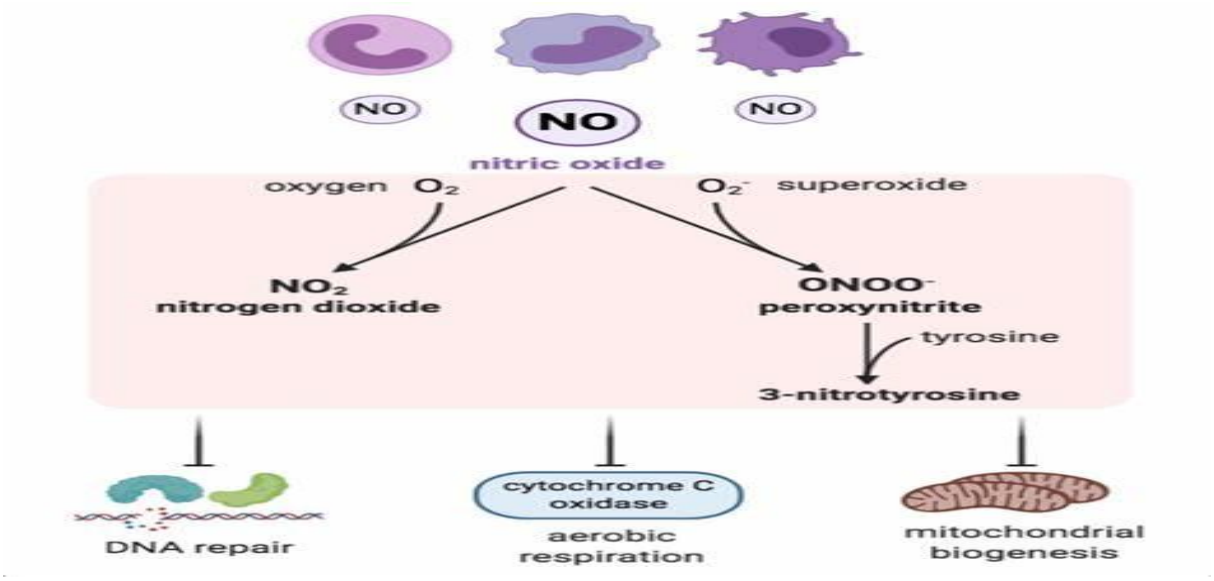


Figure 31: Role of nitric oxide in cellular biochemical processes**https://th.bing.com/th/id/OIP.X5rvIToWKkcYXlLq-jMSJgHaI6?rs=1&pid=ImgDetMain**

2.2.2.1 Hemolysis

As mentioned above, both proteins and lipids can be damaged by oxidants in RBCs. The ultimate result of oxidative damage to RBCs is hemolysis, the loss of membrane integrity, and the release of hemoglobin and other intracellular proteins **(Figure 32)**.

Free hemoglobin is particularly toxic, and this is evident in several RBCs diseases **(Tacey*et al*., 2009).**

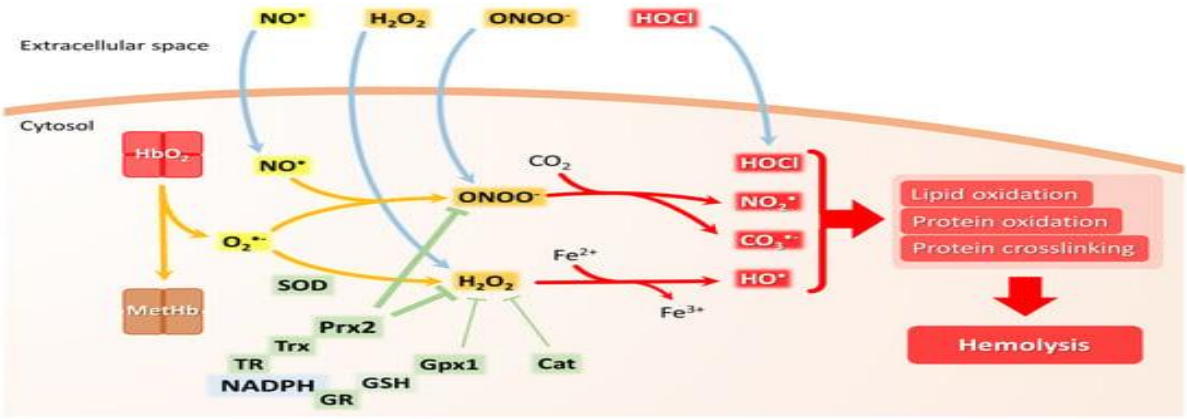


Figure 32: Main endogenous and exogenous sources of ROS and RNS in the RBCs as well as the principal antioxidant actors**(Orrico*et al.*, 2023)**

The most noticeable type of oxidative damage to red blood cells is hemoglobin oxidation, which has been linked to numerous cases of oxidative damage. Hemoglobin oxidation, resulting in the creation of hemichrome a misfolded form of hemoglobin and its precipitation to form Heinz bodies, which are visible under a microscope, are the two ways that certain medications, including phenylhydrazine, can cause hemolytic anemia **(Blanc *et al* .,2015).**

2.2.3 Antioxidant of red blood cell

Red blood cells (RBCs) have developed various mechanisms to protect themselves from oxidative stress caused by reactive oxygen species (ROS)**(Table 9) (Barodka*et al*., 2013).**

Table 9: Mechanisms of the RBC against the ROS**(Perrone *et al* ., 2012)**

-Antioxidant enzymes	RBCs contain enzymatic antioxidants such as superoxide dismutase, catalase,glutathione peroxidase, and peroxiredoxin-2, which help neutralize ROS and reduce oxidative damage**(Figure 33).**
-Non-enzymatic antioxidants	-RBCs also utilize non-enzymatic low molecular weight antioxidants like , ascorbic acid **,**Gluthathion, α-tocopherol (Vitamin E), Flavonoidsto counteract oxidative stress.
-Iron regulation	-Red blood cells regulate iron levels to prevent excessive release of reactive iron, which can lead to oxidative damage and hemolysis **(Perrone*et al* ., 2012).**
-Regulation of membrane fluidity	RBC regulate their membrane fluidity to maintain optimal function and protect against oxidative damage **(Perrone*et al* ., 2012).**

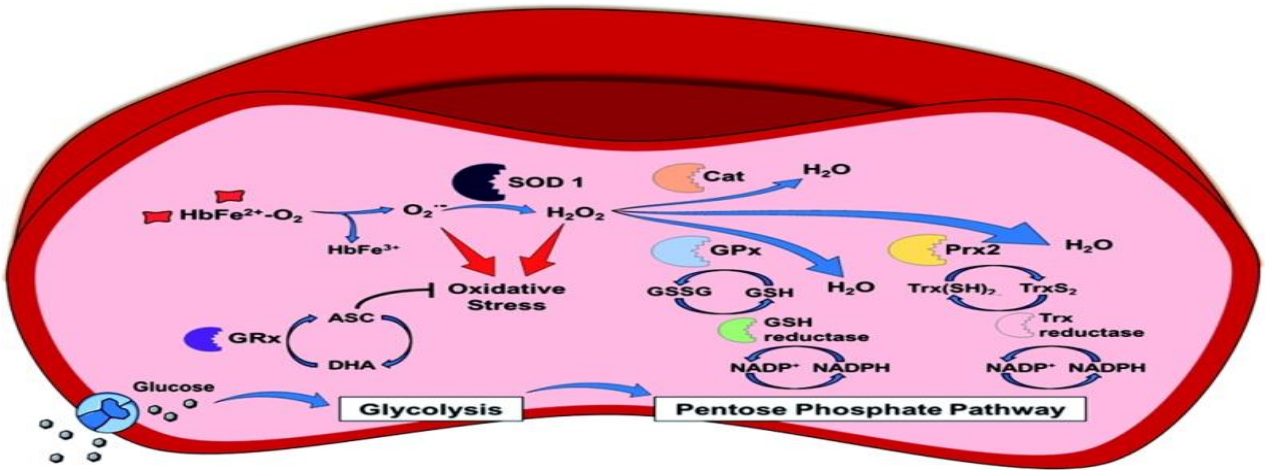


Figure 33: Diagram of different antioxidant pathways in the RBC**(Kuhn *et al.*, 2017)**

2.2.4 Effect of honey on the RBC damage

Honey have an anti-hemolytic effect on red blood cells due to flavonoidswhicharethe main functional components of honey and also an important element in the honey's overall

antioxidant activity **(Alvarez-Suarez*et al*.,2012**).Gluthathion, tocopherol (Vitamin E), Flavonoids.

Flavonoids protect the red blood cell membrane through several mechanisms.

1. Firstly, they inhibit lipid peroxidation,by actively scavenging free radicals, such as reactive oxygen species (ROS), that initiate the peroxidation process**(Figure 34)**. These compounds contain aromatic hydroxyl groupsthat can neutralize free radicals, preventing them from damaging the lipids in the membrane. By preventing this damage, flavonoids help maintain the structural integrity of the membrane.
2. Secondly, flavonoids interact with membrane proteins, particularly near tryptophan residues, which are important for protein function. This interaction can enhance membrane stability and function.
3. Additionally, flavonoids can affect membrane microviscosity, which is crucial for maintaining the fluidity of the membrane. Overall, these actions of flavonoids contribute to protecting the red blood cell membrane from oxidative stress and damage**(Chaudhuri, 2007).**

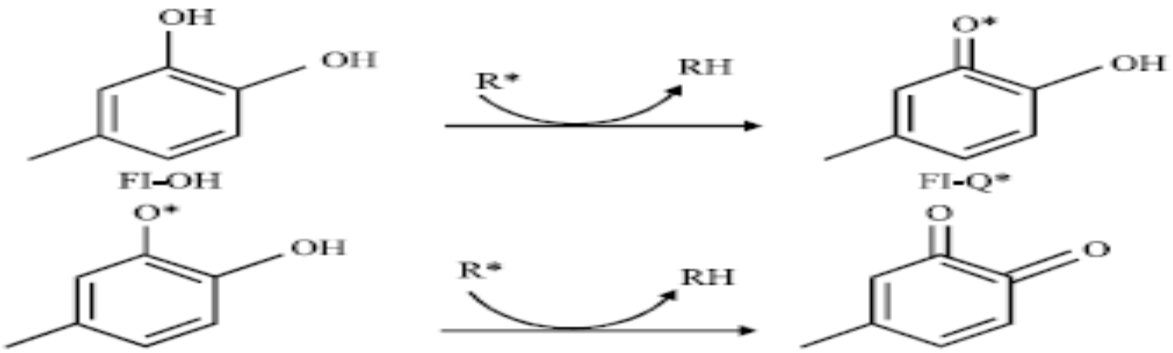


Figure 34: Scavenging of ROS (R*) by flavonoids**(Vivek Kumar Gupta *et al*., 2010)**

2.3 Antibacterial activity of honey

Honey possesses antibacterial properties through various mechanisms, making it an effective natural antibacterial agent **(Table10).**

Table 10: Factors explains antibacterial activity**(Mandal and Mandal, 2011)**

Factor	Mechanism
-High sugar	The high sugar content and low moisture content of honey create an osmotic effect. This high osmolarity draws moisture out of bacterial cells, causing dehydration and ultimately leading to bacterial cell death.
-Gluconic acid	Honey contains gluconic acid, which contributes to its acidic nature. The acidic environment created by gluconic acid inhibits the growth and survival of many bacteria, as they thrive in neutral pH environments
-Enzymes	Enzymes in honey produce hydrogen peroxide, a well-known antiseptic with Strong antibacterial properties. This hydrogen peroxide production contributes to the overall antibacterial activity of honey.
-Phenoliccompounds	-Honey contains phenolic compounds that have been shown to possess antimicrobial properties. These compounds can inhibit the growth of bacteria and contribute to the overall antibacterial effect of honey.
-Antimicrobial peptides	Some types of honey, such as manuka honey, contain antimicrobial peptides like bee defensin-1. These peptides have bactericidal effects and play a role in the antibacterial activity of honey.

-Non-peroxide effect	Certain types of honey, like manuka honey, exhibit significant antibacterial effects even when the hydrogen peroxide activity is blocked. Thisnon-peroxide effect is attributed to factors such as low pH levels and high sugar content, which hinder microbial growth.

By combining these mechanisms, honey acts as a potent antibacterial agent that can effectively inhibit the growth of a wide range of bacteria, including antibiotic-resistant strains. Its multifaceted approach to combating bacteria makes honey a valuable natural remedy for various infections and wounds, highlighting its potential as an alternative or complementary antibacterial treatment option **(Mandal and Mandal, 2011).**

2.4 Other pharmacological effects of honey

Studies have shown that honey has other pharmacological effects, including :**(Table11)**

Table 11: Other pharmacological effects of honey

Effect	Mechanism
Anti-viral effect	Honey limit the growth of viruses by affecting their transcription and reproduction **(Al-Hatamleh *et al.*, 2020).**
Anti-inflamatory effect	-Inhibition of inflammatory mediators (COX-2) -Icrease NO and decrase of prostaglandins -Activation of monocyte (immune cells) involved in inflammatory response **(Saikaly and Khachemoune, 2017).**

Anti-cancer effect	-Flavoinds and phenolic acids in honey inhibits proliferation of cancer cells (melanoma cells) **(Singer *et al*., 2019).**
Effect on cardio vascular system	-Honey reduce blood pressure which prevent the risk of some cardiovascular disises. -Polyphenols in honey enhace endothelial function of cardio cells **(Nwokorie*et al*.,2022).**

Practical part

CHAPTER I

Materials And Methods

CHAPTER I MATERIALS AND METHODS

3.1 Materials

3.1.1 Honey sample

For the purpose of this study, a Eucalyptus unifloralhoney sample was obtained from Co-perative of Setif collected from Khemis El-khechna , Algiers, in 2023. Honey was kept in proper conditions and at room temperature.

Table 12: Bacterial strains and Their characteristics

Sample	Floral Origin	Provenance	Plant classification
Eucalyptus	Monoflorale	Khemis El-khechna , Algiers	**Kingdom**: Plantae **Division**: Magnoliophyta **Class**: Magnoliopsida. **Order**: Myrtales. **Family**: Myrtaceae **Genera**: Eucalyptus **(Nicolle, 2024).**

Figure 35: Eucalyptus honey flower
https://triggplants.com.au/wp-content/uploads/2018/10/EUCALYPTUS-GRACILIS-1.jpg

3.1.2 Strains of bacteria

In this study,we have used 2 different strains of bacteriaobtained from the research centre of biotechnology (Constantine, Algeria).

Table13: Bacterial strains and their characteristis

Bacterial Strain	Gram (+/-)	Caused Infection	Treating Antibiotic(s)	Refrances
-***Escherichia coli***	**(-)**	-urinary tract infections -pelvic inflammatory disease	Fosfomycine	**(De Mouy *et al.*, 2007)**
-***Staphylococcus aureus***is	**(+)**	-Pneumonia -bone infections	-Doxycycline	**(Méric *et al*., 2015)**

3.1.3 Animals

Adult female Albino Wistar rats weights 140- 225 (g),aged 8weekswere provided by the animal facility of University of Mentouri 1 (Constantine, Algeria) .The animals were kept under suitable Laboratory conditions throughout the period of investigation and had free access to food and water.

3.2 Methods

3.2.1 Evaluation of antibacterial activity of honey

Well diffusion method

3.2.1.1 Preparation of inoculum

A fresh suspension of each bacterial strain was prepared. The inoculum were prepared by picking one or two well-isolated colonies of the tested bacteria using a sterile platinum loop and suspending them in a sterile normal saline solution. The density of the suspension was determined by comparison with the opacity standard of the 0.5 McFarland barium sulfate solutions. A sterile swab was dipped into the isolate's suspension, pressed against the side of the tube to remove excess liquid.

3.2.1.2 The empirical principle

The evaluation of the antibacterial activity of honey was determined by the agar diffusion technique described by **Hegazi *et al.*, 2021**. In a sterile hood, the petri dish was placed. Mueller-Hinton agar was inoculated with the bacterial inoculum using a sterile swab. It was allowed to dry before proceeding to the next step.

Each tested bacterium was uniformly inoculated into the surface of Mueller-Hinton agar. Using a sterile punch, wells were created in the agar. Care was taken to ensure that the wells were evenly spaced and not too close to the edges of the plate (6 mm diameter, 4 mm depth and approximately 2 cm interval) **(Figure 37).**

Figure 36: Application of diluted honey in the well

The agar well diffusion assay is extensively applied to assess the antimicrobial activity of natural products **(Valgas*et al*., 2007)**. This assay is based on the measurement of the size of a growth inhibition zone around the sample, which may be placed into a well cut into the agar **(Magaldi*et al.*,2004) .**

Apply the diluted honey to the prepared wells on the agar, 50µL of each honey concentration were pipetted into individual wells. Incubate the plate at 37°C for 24 hours**(Figure38).**

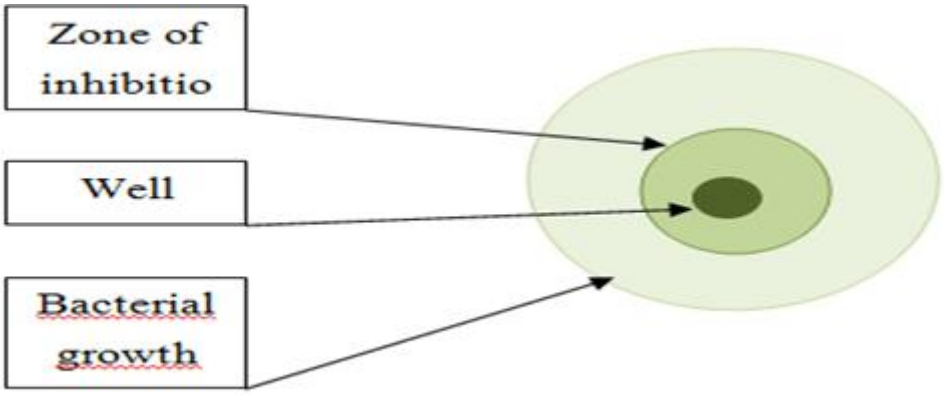


Figure 37: Agar diffusion method

Two antibiotics, clindamycin and amoxicillin, were used as positive controls, whereas distilled water was used as a negative control**(Figure39).**

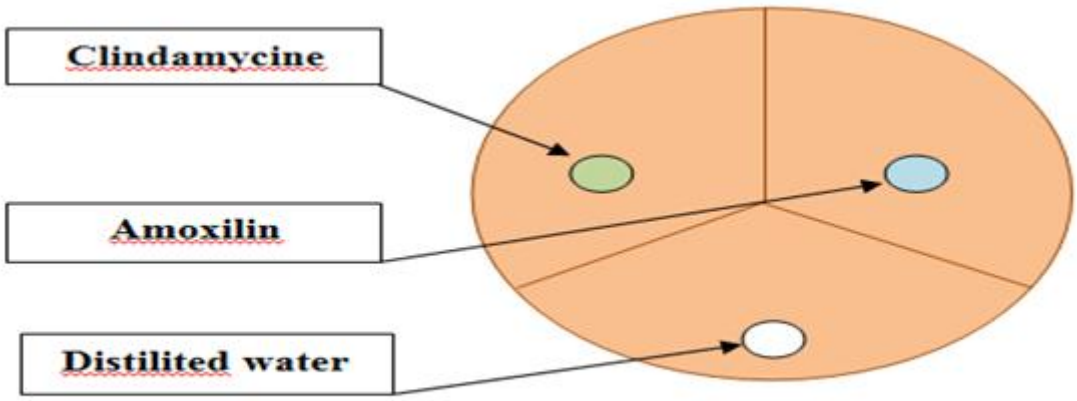


Figure 38: Positive and negative control in this stud

The antibacterial activity is assessed by measuring the diameters of the inhibition zones (in millimeters) formed around the wells. The sensitivity of the target bacteria to different concentrations is classified according to the diameters of the inhibition zones (Ø):

Bacteria is not sensitive ⟶ (Ø< 8 mm).

Bacteria is sensitive ⟶ (9 <Ø< 14).

Bacteria is very sensitive ⟶ (15 <Ø< 19).

Bacteria is extremely sensitive ⟶ (20 <Ø).

3.2.2 Evaluation of the anti-hemolytic activity of honey

The anti-hemolytic activity tests of honey were carried out *in vitro* on a suspension of female blood, aged 21 yearin good health conditions.

In this study, we used a biological test based on erythrocyte lysis induced by pro-oxydant in the blood.

Lipid peroxidation of the red blood cell membrane through a pro-oxidant H_2O_2 causes membrane damage and subsequently hemolysis **(Nabavi *et al.*, 2010).**

3.2.2.1 Principle

This test allows monitoring the positive or negative evolution of an antioxidant on an individual's defenses against radical species. It involves subjecting a suspension of red blood cells to oxidative stress. The red blood cells will therefore use their antioxidant equipment to fight against the aggression until the membrane is modified and the cell releases its content. Thus, the evaluation of anti-hemolytic activity is done by spectrophotometric measurement of hemoglobin at 540 nm. In this test, erythrocyte lysis is induced by H_2O_2 .

3.2.2.2 Operating mode

Preparation of erythrocytes

The anti-hemolytic activity of honey was determined according to the protocol described by **(Alinezhad*et al.*, 2012, Ebrahimzadeh*et al.*, 2010, Chehtit-Hacid 2016)**

Briefly, the blood was collected on heparin and then centrifuged at 3000 rpm for 10 min. The supernatant, which consisted of plasma, platelets, and the white blood cell layer, was gently removed using a micropipette. The erythrocytes contained in the pellet were taken up in Phosphate-Buffered Saline (PBS) (at 0.2 M and pH 7.4). After mixing by inversion, the samples were again centrifuged at 3000 rpm for 10 min and the supernatants were removed using a micropipette. The pellet was washed 3 times under the same conditions. The washed erythrocytes were resuspended in PBS (1/1), (v/v) and were kept at 4° C within the limits of 6 hours of their preparation.

Preparation of honey concentrations

The different concentrations of honey was prepared in PBS at pH 7.4. using these concentrations (100µg/ml; 200µg/ml; 400µg/ml and 600 µg/ml).

Anti-hemolytic test

Anti-hemolytic effect 10% erythrocyte suspension was prepared in PBS at pH 7.4. 250 µl of this suspension were added to 0.5 ml of each honey to be tested at different concentrations already prepared in PBS. The obtained mixture was incubated for 30 min at 37º C. 0.5 ml of H_2O_2 at 100mM prepared in PBS were added to induce the peroxidation of the lipid membranes of the erythrocytes.

The reaction mixture was incubated for 2h in a water bath at 37° C. Then, the medium was diluted by completing it with PBS until having 4.5 ml in each test tube. Then, the samples underwent centrifugation at 3000 rpm for 10 min.

Finally, the supernatants obtained in each tube were aspirated and the OD readings were performed at 540 nm using a spectrophotometer. A control containing 250 µl of a 10% erythrocyte suspension were added to 0.5 ml of PBS. The obtained mixture was incubated for 30 min at 37º C. 0.5 ml of H_2O_2 at 100mM prepared were added to induce the peroxidation of the lipid membrane of the erythrocytes of this suspension. The percentage of inhibition of hemolysis was calculated according to the formula described below:

% of hemolysis inhibition = (Ac - Ae / Ac) *100 Where:

- Ac: it’s the absorbance obtained in the absence of honey (Control).

- Ae: it's the absorbance obtained in the presence of honey. For each test, we carried out 3 trials.

3.2.3 Evaluation of hepatoprotective effect of honey

3.2.3.1 Animals treatment

18 females of *Albino Wistar* rats were used in this experiment. Three groups of six rats were formed.

All groups were orally treated for 10 successive days as follow:

- **Control group:** Received NaCl (0, 9%).
- **APAP group :** received NaCl (0,9%) as the control group
- **APAP + Honey group:** were given eucalyptus honey diluted in NaCl (0, 9%) in dose of (5g/kg) **(Gabl RM *et al*., 2011).**
- The groups of (APAP) and (APAP + honey) were treated with a single dose of Paracetamol (2g/Kg) on the 10th day after 1h of receiving their normal dose **(Galal RM *et al*., 2012)**

all animals of all groups were dissected on the 11th day

- Blood sampling.

Blood samples were collected from retro- orbital plescus for the control group and from the Hepatic portal vein for the groups of (APAP) and (APAP + honey)the blood samples were accumulated in heparinised tubes, then were centrifuged and the recovered plasma was used for biochemical essays.

- Liver sampling

livers were isolated part of it preserved in formalin for histological excoriation and other 3g of the liver were homogenized by using 30 ml d PBS for oxidative stress analyses.

3.2.3.2 Analysis of biochemical parameter

The plasma samples were taken to the university hospital Abd El Hamid Ben Badis. Where the following analysis were performed: ASAT ALAT and LDH using Automated analyzer.

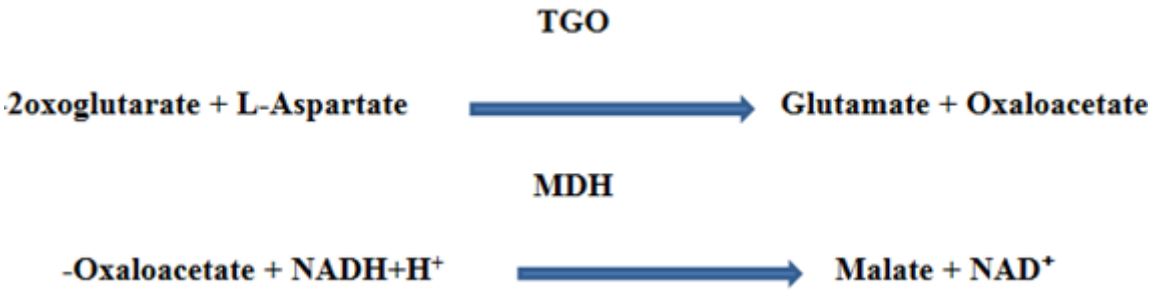
- Alanine amino transferase

For the determination of alanine amino transferase activity: the reaction is initiated by adding the patient's sample to the reagent. The reaction scheme is as follows:

The rate of decrease in NADH concentration is directly proportional to the alanine transferase activity in the sample.

- Aspartame aminotransferase

Determination of aspartate aminotransferase activity, the reaction is initiated by adding the patient's sample to the reagent, the reaction scheme is as follows:



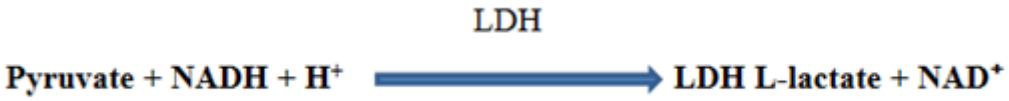
The rate of decrease in NADH concentration is directly proportional to

aspartate amino transferase activity in the sample.

- Lactate dehydrogenase (LDH)

The following reaction indicates that lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate by NADH

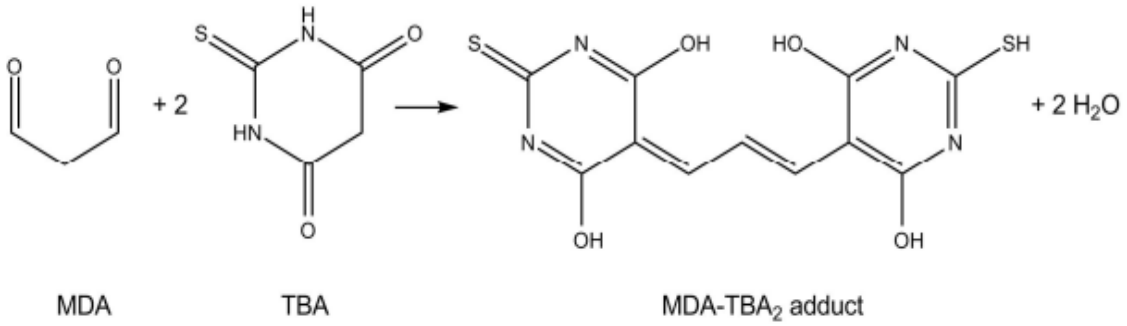
The photometrically observed rate of decrease in NADPH concentration is directly proportional to the catalytic concentration of LDH in the sample.



3.2.3.3 Analysis of hepatic oxidative stress

- **Rate of lipid peroxidation**

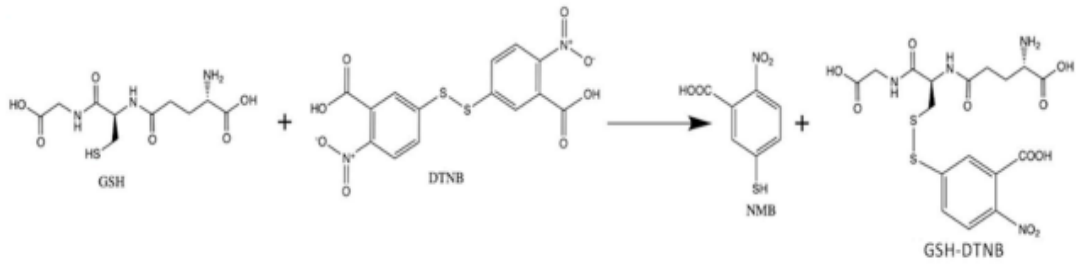
The measurement of MDA using TBA according to **Okhawa *et al* ., (1979**). In this reaction a molecule of MDA is condensed with two molecules of thiobarbituric acid (TBA) to form a pink-colored complex (MDA-TBA) according to the following reaction:



For this assay, 0,5ml of liver homogenate was placed in the presence of a mixture of TBA (1ml) and trichloroacetic acid (TCA at 0.5ml, 25%) then placed for 45 minutes in a water bath at 100°C. After stopping the reaction on ice and adding 4ml of butanol. After centrifugation at 30000 rpm for 10 minutes, the absorbance was read at 535 nm using a spectrophotometer.

- **Reduced glutathione**

The determination of reduced glutathione (GSH) is based on the colorimetric method of **Ellman (1959).** The principle is based on the oxidation of GSH by 5,5'- Dithiobis 2-nitrobenzoic acid (DTNB), releasing 2-nitro-5-mercaptobenzoic acid (NMB), as follow :

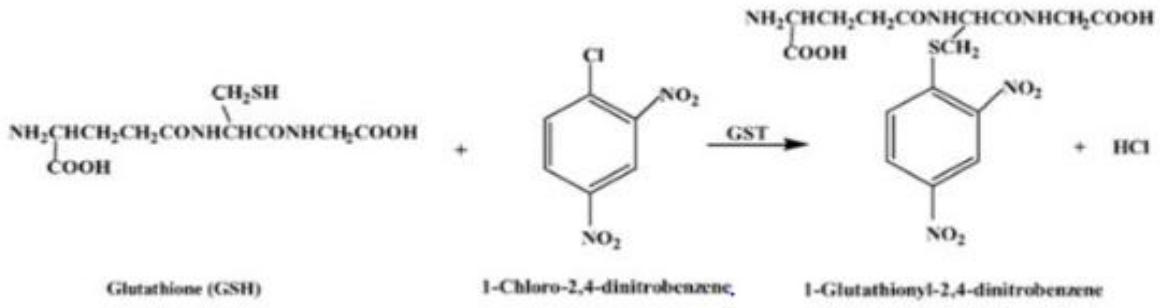


For this assay, 0.5ml of homogenate is treated with 0.5ml of TCA (10%) then centrifuged at 10000 rpm for 10 minutes. The assay was then continued in a reaction environment consisting of 100µl of the supernatant obtained, DTNB (100µl) and PBS (1.7ml, pH 8).

The absorbance was read at 412 nm against a blank prepared under the same conditions.

- **Glutathione S-transferase**

The conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) is used to measure the reaction.



For this essay, 25µl of the supernatant of liver homogenate was placed with 100µl of (CDNB), 100µl of GSH (30mM) and 2.8ml of PBS (pH 6.5)

The absorbance was read at 340 nm for 3 minutes against a blank prepared under the same conditions.

- **Catalase**

The enzymatic activity of catalase is measured using the method of **Chance and Maehly (1955).**The principle of this method is to monitor the decomposition of hydrogen peroxide (H_2O_2) into molecular oxygen and water by catalase.

Briefly, the reaction mixture containing 2,9 ml of phosphate buffer (0,1 M, pH 7) and 10µl of sample. The reaction was initiated by the addition of 0,1 ml H_2O_2 (30mM, pH 7.0) and the change in absorbance was recorded for 3 minutes at 240 nm. Specific activity is expressed as Units /mg protein.

- **Superoxide dismutase (SOD)**

The measurement of Superoxide dismutase (SOD)according to the method of flohe. The principle is based on the inhibition of pyrogallolautooxidation by SOD.

- **Histological study**

The liver samples that were preserved in formalin for histological studies were carried out in the Pathological Anatomy Department ofthe university hospital Abdel Hamid Ben Badis.

3.2.4 Total phenolic content (TPC) in eucalyptus honey

3.2.4.1 Principle of the reaction

The total phenolic content is determined using the Folin-Ciocalteureagent**(Singleton and Rossi, 1965)** according to a microplate assay method described by **Muller *et al*., (2010)**. The FCR reagent, consisting of a mixture of phosphotungstic acid (H3PW12040) and phosphomolybdic acid (H3PMo12040), is reduced during the oxidation of phenols to a mixture of tungsten oxides (W8023) and molybdenumoxides (Mo8023).

The coloration produced is proportional to the total phenolic content and has a maximum absorption around 750-765 nm**.**

3.2.4.2 Procedure

- 20 µl of honey + 100 µl of diluted FCR (1:10) + 75 µl of sodium carbonate (7.5%) and we incubate the mixture in darkness for 2 hours.
- Using a microplate reader read at 765 nm.
- A blank is prepared in the same way by replacing thehoney with the solvent used (Methanol).

CHAPTER 02

Results And Discussion

Chapter II : Results and disscussion

4.1 Total phenolic content (TPC) in Eucalyptus honey

The Folin-Ciocalteu method is very sensitive but unfortunately not very specific because of many non-phenolic reducing compounds can interfere, such as carotenoids and vitamin (E) **(Kahkonen *et al*., 1999),**sugars and proteins. However, it remains the most widely used method. The contents of Eucalyptus honey were expressed in gallic acid equivalent from the calibration curve carried out under the same conditions. The first reading of the results shows that the total phenolic content of eucalyptus honey was 241,75±00µggalic acid equivalent (GAE)/mg of honey .

4.2 Evaluation of Antibacterial activity

After completing the 24-hour incubation, we observed the plates for zones of inhibition around the wells. These zones indicate areas where bacterial growth has been suppressed by eucalyptus honey**(Figure41)**.

Sensitivity is observed in *Escherichia coli*, with inhibition zones ranging from 21mm to 31mm in response to varying concentrations of 25% (v/v), 50% (v/v), 75% (v/v), and 100% (v/v) **(Figure40).**

These results are similar to those obtained by **(Omafuvbe and Akanbi, 2009);(Laallam*et al*., 2015).**In fact, antibacterial activity is pronounced in the absence of *Staphylococcus*

aureus. These values are in agreement with those given by **(Nayaka *et al*., 2020)**and in line with the results obtained by**(Sajid *et al*., 2020); (Mandal and Mandal, 2011).**

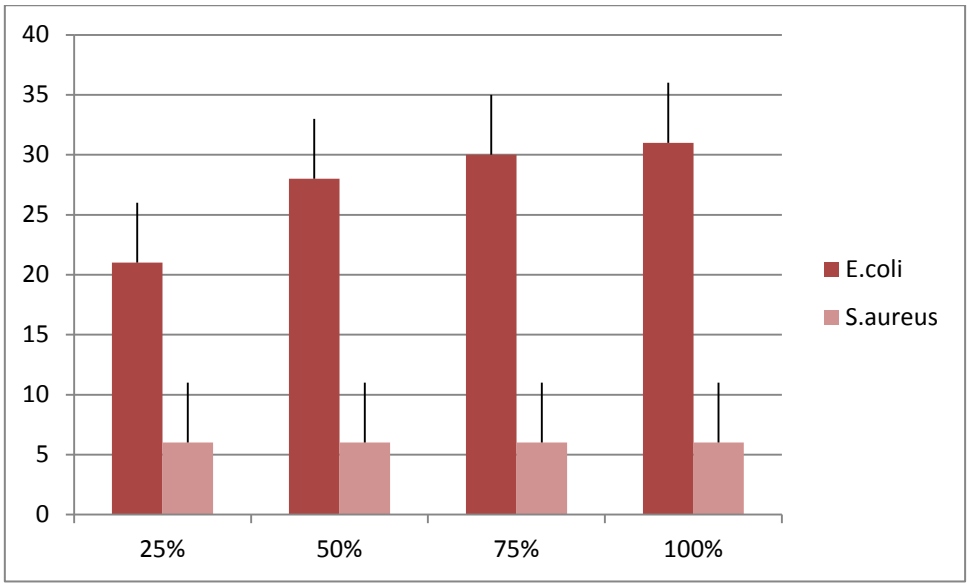


Figure 39: Antibacterial activity of Eucalyptus honey expressed in Ø(mm)

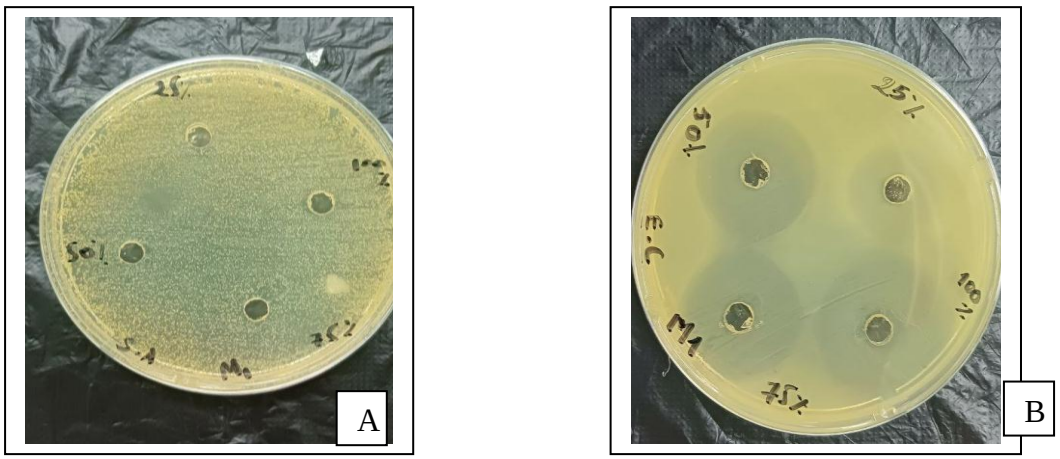


Figure 40: inhibitor effect of ***Staphylococcus aureus (A), Escherichia coli (B)***

It is important to note that the ***Staphylococcus aureus*** bacteria showed a strong sensitivity to amoxicillin with an inhibition diameter of 54mm, and *Escherichia coli* presented an inhibition diameter of 35mm **(Table 15) (Figure43).**

Table 14: Measures of the diameters of the inhibition zones in the control dishes

Bacterial strains / **controls**	***Staphylococcus aureus***	***Escherichia coli***	**Sensivity**
Amoxiciline	54 mm	35 mm	Extremely sensitive
Clindamycine	36 mm	6 mm	Extremely sensitive to *S.aureus* and non sensitive to *E.coli*

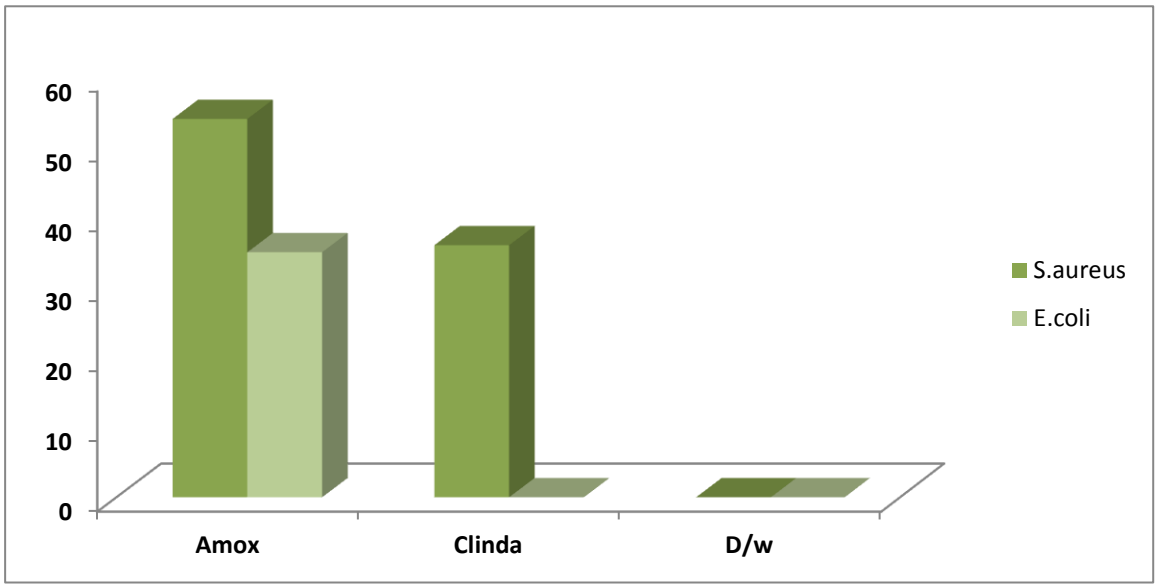


Figure 41: Antibacterial activity of antibiotics expressed in Ø (mm)

Figure 42: Inhibitory effect of amoxicillin and clindamycin against *E.coli*

We observe the presence of inhibition zones in the first positive control (Amoxiline) in both bacterial strains: *Escherichia coli*and *Staphylococcusaureus***(Table 15).**

The efficacy of eucalyptus honey has been demonstrated to be significantly higher compared to clindamycin (0mm) when tested against the *Escherichia coli* bacteria. This is evidenced by the inhibition diameters (21mm, 28mm, 30mm, and 31mm) in response to concentrations of 25%, 50%, 75%, and 100%successively.

Based on the results, we conclude that:*Staphylococcus aureus*is resistant to eucalyptus honey.*Escherichia coli*isextremely sensitiveto Eucalyptus honey**.**The Clindamycine has only reacted to *Staphylococcus aureus* **,** with no effect on *Escherichia coli,*which means they are sentitive to this antibiotic.

According to **Nayaka *et al*., (2020)** and **Laallam *et al*., (2015),** they showed that Gram-positive bacteria are more resistant compared to Gram-negative bacteria like *Escherichia coli*, which reacts with high values in front of honeys. This sensitivity is due to the nature of the bacterial wall, which has lipoproteins, while the presence of lypo-polysaccharides are considered barriers that slow the penetration of the antibacterial agent **(Nedji and Loucif-Ayad, 2014).**

The antibacterial effect of honey can partially be explained by its significant content of enzymes like glucose oxidase secreted by the bee during the transformation of nectar into honey. This enzyme allows the production of gluconic acid and hydrogen peroxide **(Mandal and Mandal, 2011).**

When honey is diluted, glucose oxidase will be activated, thus producing more H_2O_2.Hydrogen peroxide is the main contributor to the antibacterial activity of honey, and the different concentrations of this compound in different honeys favor variable antibacterial effects **(Moussa *et al*., 2012).**

Indeed, **Laallam *et al*., (2015)** explained that hydrogen peroxide reacts with other very reactive oxidizing molecules capable of attacking the cell membrane by inhibiting the entry of nutrients and preventing the elimination of waste, which gradually triggers the death of bacteria.

4.3 Evaluation of Anti hemolytic activity of Eucalyptus honey

The results of the anti-hemolytic study revealed the following: **(Table 16).**

Table 15: Percentage of inhibition in relation to the concentration of honey

Concentration (ug/ml)	Percentage of inhibition (%)
100	6,15
400	9,23
600	21,53

We observe an increase in the percentage of inhibition as the concentration ofhoney increases(**Figure 44).**

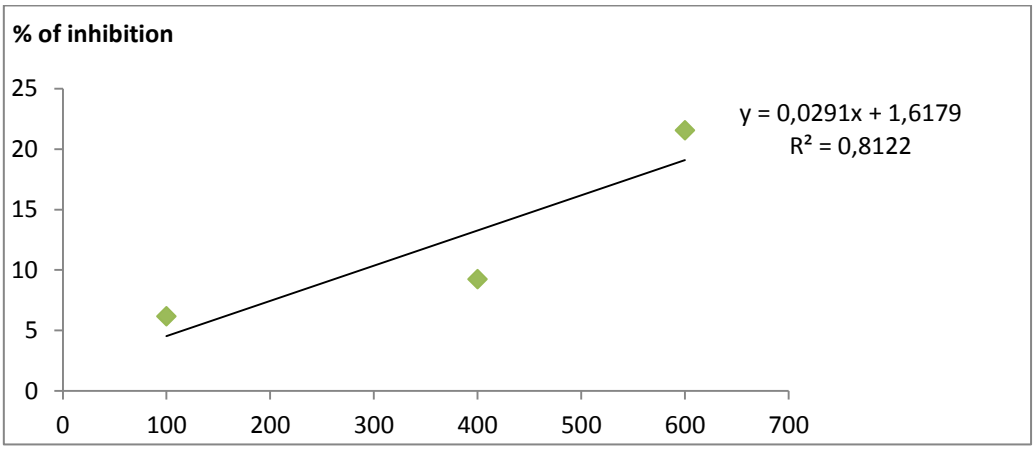


Figure 43: Inhibitory effect of Eucalyptus honey on hemolysis

Eucalyptus honey, due to its antioxidant properties, can protect red blood cells from oxidative damage that can lead to their breakdown, a process known as hemolysis. The effectiveness of this protective action increases proportionally with the concentration of honey (100, 400 and 600ug/ml).

Eucalyptus Honey contains polyphenols, which are powerful antioxidants. These antioxidants can neutralize the harmful effects of free radicals, including those produced by H_2O_2. This neutralization process involves the donation of an electron from the antioxidant to the free

radical, which stabilizes the free radical and prevents it from causing further damage**(Preti and Tarola, 2022).**

The flavonoids in honey can bind to the cell membrane, creating a protective barrier that prevents the interaction of H_2O_2 with the cell membrane and preventing the cell from lipide peroxidation**(Stefanis *et al*., 2023).**

4.4 Evaluation of hepatoprotective effect of honey

4.4.1 Effects of treatments with acetaminophen and eucalyptus honey on changes in biochemical parameters

The effect of Acetaminophen (APAP) and eucalyptus honey on liver enzymes (ASAT, ALAT) and the total glycerides TGL is presented in **(Figure 45).** The results showed significant increase ($p < 0,05$) in the enzymatic activity of ASAT, ALAT and TGL in the group treated with APAP(2g/kg) compared to the control group. However the results of APAP+honey group showed that eucalyptus honey (5g/kg) reversed the incrased levels of these markers compared to the APAP group.

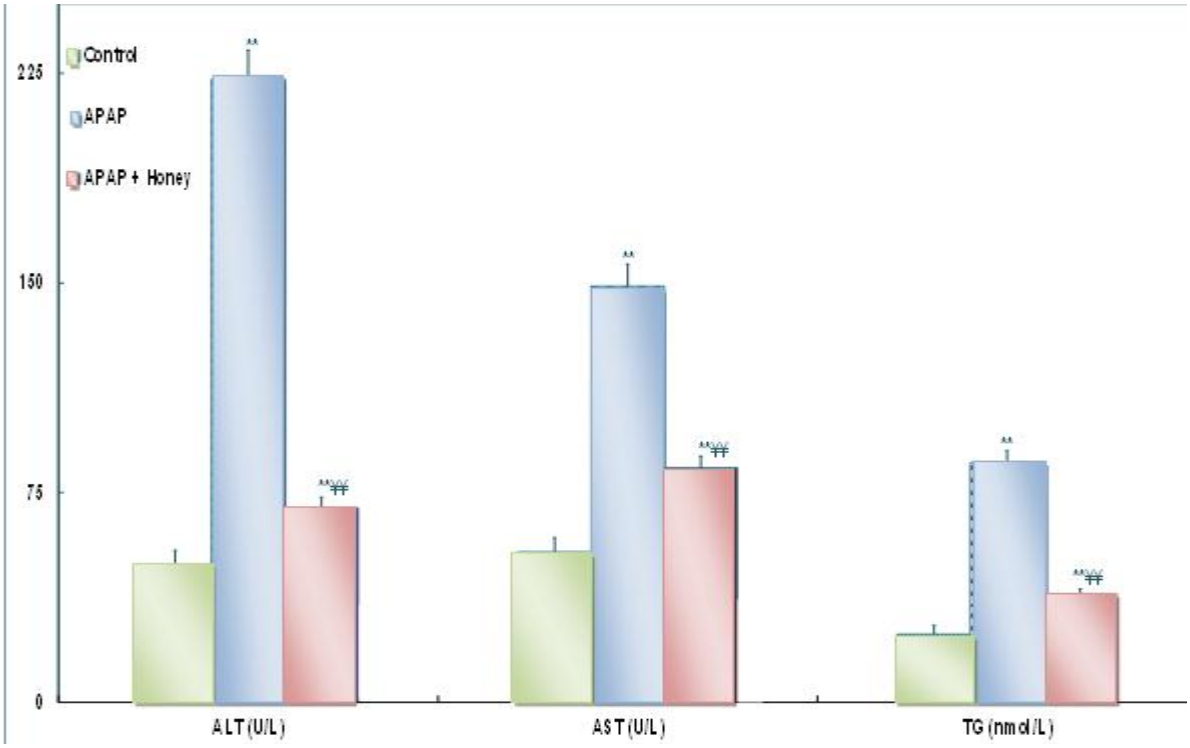


Figure 44: Effect of paracetamol and eucalyptus honey on the levels of transaminases and LDH

4.4.2 Effects of treatments on changes in the rate of lipid peroxidation (MDA)

Acetaminophen (APAP) produced lipid peroxidation manifested by an increase in MDA levels 2-fold higher than the control. On the other hand the pretreatment with eucalyptus honey (5g/kg) in APAP+honey group was manifested by the inhibition of lipid peroxidation by a rate of 81 %**(Figure46).**

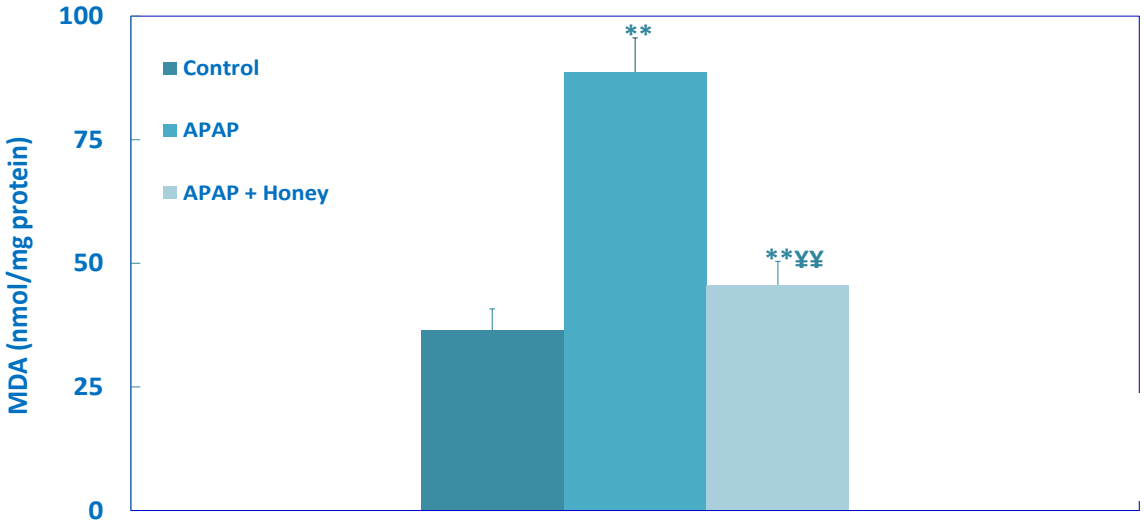


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Figure 45: Effect of treatment with APAP and eucalyptus honey on the rate of lipid peroxidation

4.4.3 Effects of treatments on changes in the rate of reduced glutathione (GSH)

The treatment with acetaminophen in APAP group marked a depletion of hepatic GSH stores in relation to the control group. The pretreatment with eucalyptus honey 5g/kg protected the APAP+honey group against GSH depletion by 65,3% compared to the APAP group **(Figure 47)**

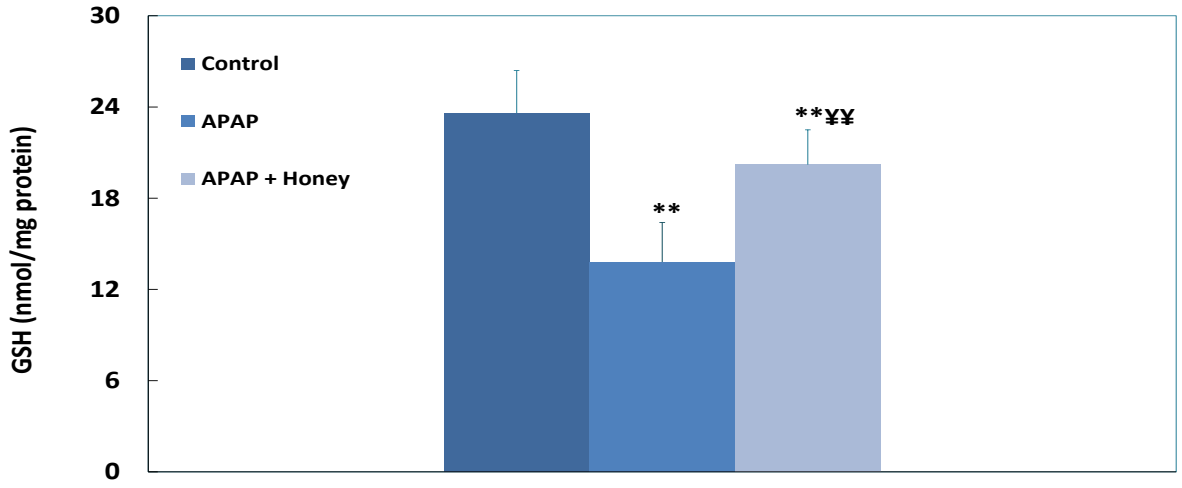

Figure 46: Impact of acetaminophen and eucalyptus honey on the levels of GSH

4.4.4 Effects of treatments on changes in the rate of SOD , Catalase, GST

According to the results shown in **(Figure 48), (Figure 49)** and **(Figure 50).**There is a significant decrease in the enzymatic activity of SOD,catalase, and GST in the APAP group in relation to the control group . The pretreatment with eucalyptus honey (5g/kg) have significantly restored this reduction in SOD, Catalase and GST by 82.6 %,76.75% and 74.09% respectively.

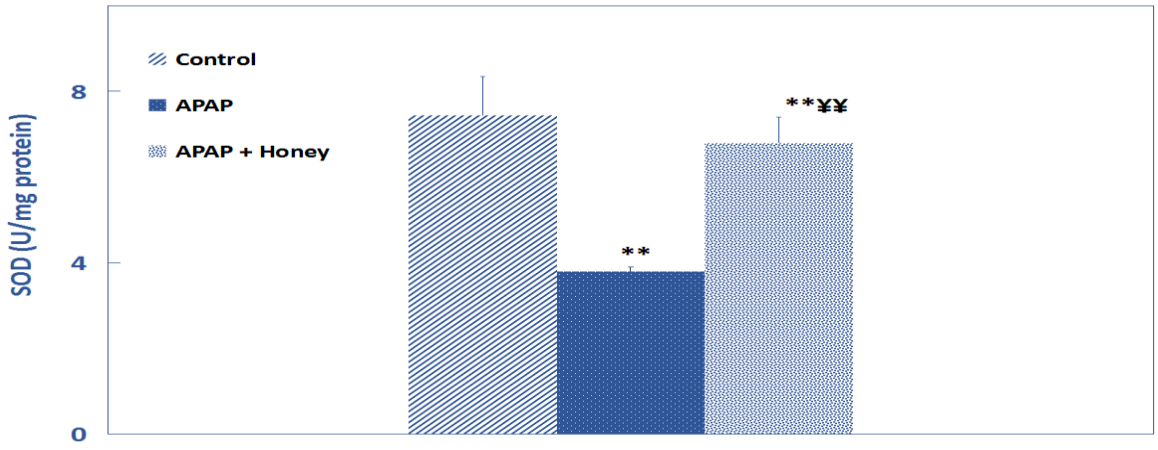

Figure 47: Impact of acetaminophen and eucalyptus honey on the levels of SOD

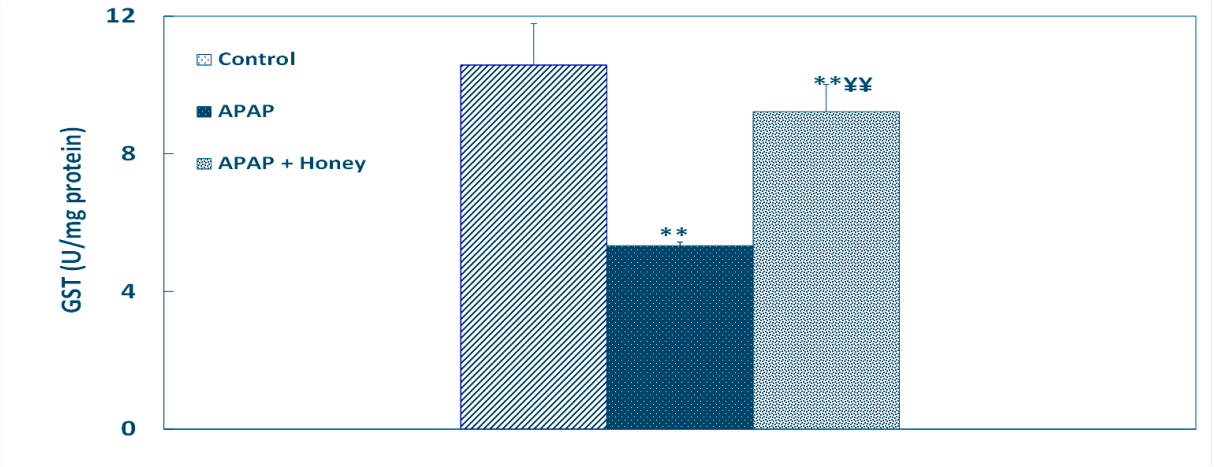

Figure 48: Effect of treatment with APAP and Eucalyptus honey on the rate of GST

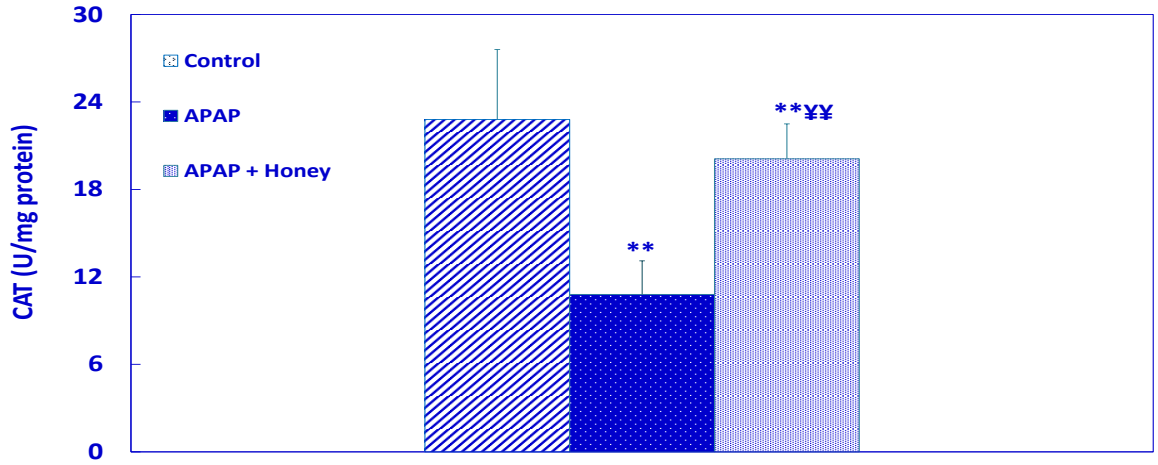

Figure 49: Effect of paracetamol and Eucalyptus honey on the levels of Catalase

4.4.5 Results of histopathological examination of hepatic tissue

As recorded in photomicrographs, control rats showed normal liver architecture with radial arrangement of hepatic cords, normal central vein and sinosoidal area **(figure51).**

The histopathological profile of the liver of APAP -treated rats revealed drastic alterations in histoarchitecture: disrupted liver architecture ,loss of radial arrangement of hepatic cords, parenchymal cell damage, dilated congested sinusoids (dS), mixed inflammatory cell infiltration and centrilobular necrosis **(Figure52).**Associated with severe degree of hemorrhage (hr) **(Figure52).**In the contracts, the liver lesions from pretreated rats with honey was less sever and retained hepatic architecture quite close of the liver of the control group of rat **(figure 53).**

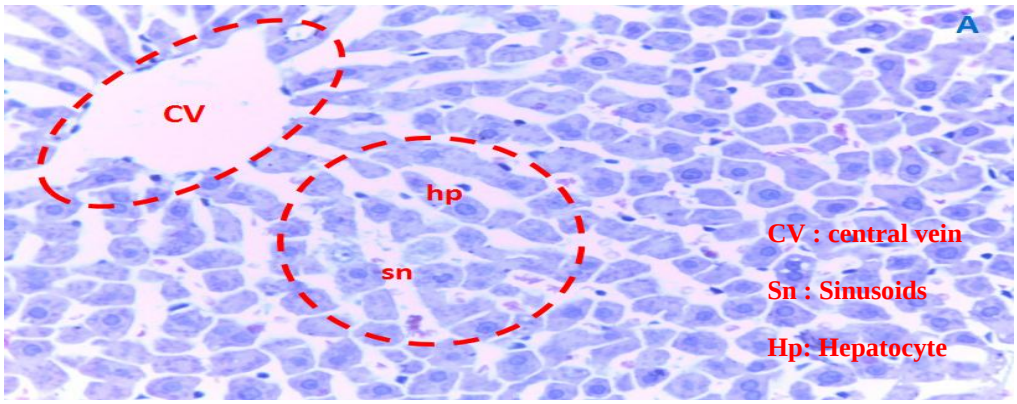

Figure 50: Photomicrograph of liver section of control rats

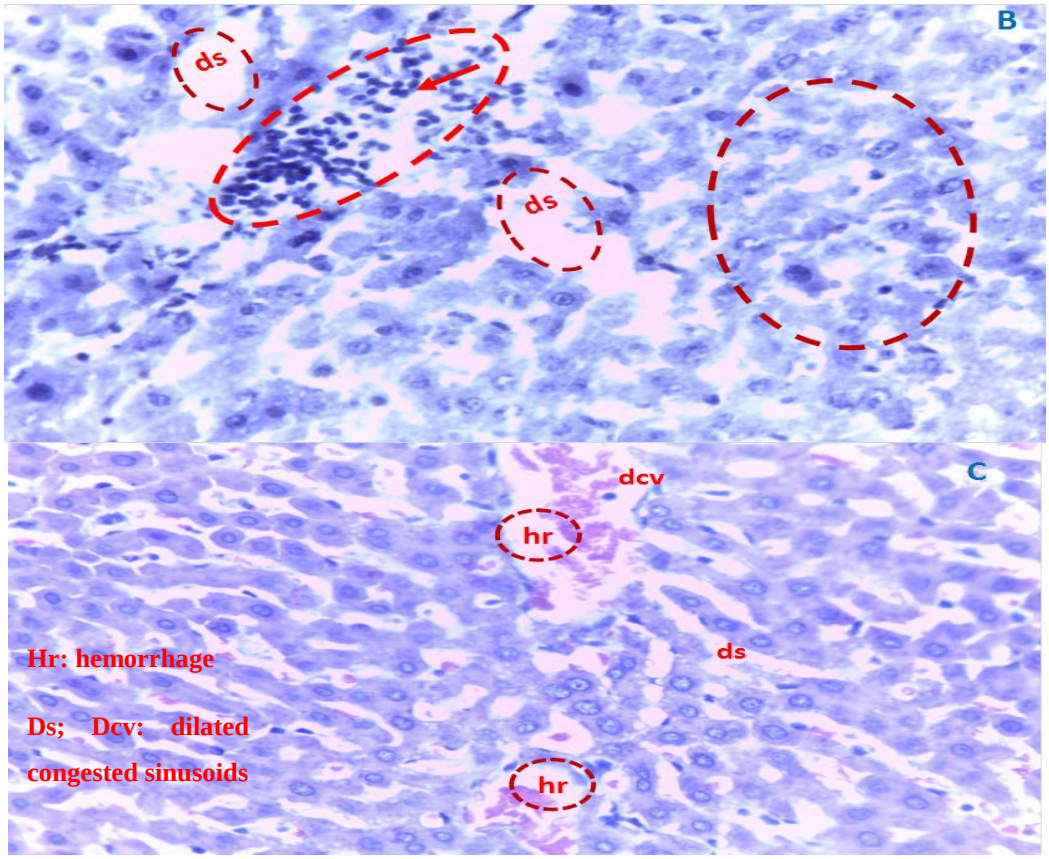

Figure 51: Photomicrograph of liver section of APAP treated rats

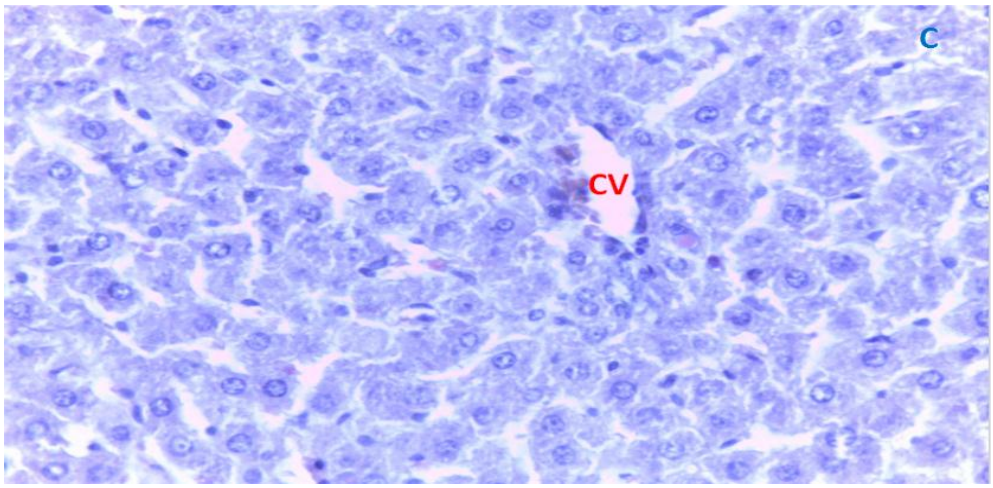

Figure 52: Photomicrograph of liver section of pretreated rats with eucalyptus honey

The supratheraputic dose of 2g/kg that was administrated for the APAP group lead to the saturation of acetaminophen metabolic pathways and the accumulation of the toxic metabolite NAPQI. These extreme amounts of NAPQI targeted the mitochondrial proteins and interfered with the mitochondrial electron transport chain (ETC) more specifically with the complexes 1 and 2 resulting with an alteration in ETC, leackage of electrons and forming superoxide radicals, one of the mains is hydrogen peroxide which is detoxified directly by GSH leading to its depletion. This mitochondrial alteration leads finally to necrotic cell death **(Yan *et al.*, 2018).**

This hepatotoxicity that was induced by APAP is manifested in the high concentration of transaminases and LDH in the plasmasamples of rats, which are sensitive indicators of necrotic lesions within the liver.This liberation from the hepatocyte cytoplasm indicates severe damage to hepatic tissue membranes during acetaminophen intoxication.

In this present study we also observed a decrease in the levels of GSH for the APAP group which means that GSH was depleted by the excessive amounts of the metabolite NAPQI. This also indicates the increase in the levels of MDA which is a marker of lipid peroxidation and ROS formation. As another evidence for the ROS formation we noted a decrease in the levels of SOD, CAT, and GST which are other endogenous liver antioxidants.

Histological analyses of liver sections from rats exposed to hepatotoxic doses of paracetamol indicate an alteration in the hepatocytes and centrilobular necrosis. These changes confirm the observed biochemical and oxidative stress analyses results.

For the pretreated group with eucalyptus honey 5g/kg we observed a decrease in the levels of transaminases activities, LDH, and MDA, and an increase in the levels of GSH, SOD, CAT, and GST. These results indicate the antioxidant effect and the important role of eucalyptus honey in the mechanism of its hepatoprotective effects. Due to the presence of several active substances in eucalyptus honey such as polyphenols.These compoundsinterfers through many mecanisms such as: direct scavenging of ROS, the activation of antioxidant enzymes, the inhibition of oxidases, and metal-chelating activity**(Chen *et al.*, 2023)**. Demonstrating thehepatoprotective effects of eucalyptus honey against paracetamol hepatotoxicity.

Also the histological results of APAP+honey group are well correlated with what we noted. In which we observed that the pretreatment with eucalyptus honey made the hepatic lesions that was caused by the administred overdose of APAP less severe due to its antioxidant activity and the protaction of hepatocytes membranes.

Conclusion and perspectives

Conclusion

Honey, is an extremely complex and diverse biological compound, has numerous properties both nutritionally and therapeutically. The present work aims to study the antibacterial, antihemolytic and *in vivo* hepatoprotective effects of Eucalyptus honey .The present work allowed the dosage of polyphenols, the evaluation of the antibacterial effect of Eucalyptus honey against two bacterial strains *Escherichia coli* and *Staphylococcus aureus* ,the anti-hemolytic activity with respect to the blood of a healthy volunteer aged 21 years and the evaluation of *in vivo* hepatoprotective effect of Eucalyptus honey in APAP induced hepatotoxicity. Results showed that the Total phenolic content (TPC) in Eucalyptus honey reached 241,75±00 µg galic acid equivalent (GAE)/mg of honey. In the anti-bacterial study we used Two different bacterial strains *Escherichia coli (E. coli)* and *Staphylococcus aureus.* Results showed that *Escherichia coli (E. coli)* was extremely sensitive to Eucalyptus honey. This sensitivity was noted with inhibition zone diameters (30 -31mm) at concentrations of (75%-100%) respectively compared with the antibiotics amoxicillin and clindamycin. Eucalyptus honey could act as an alternative to *Escherichia coli* bacteria. For the anti-hemolytic test ,Eucalyptus honey showed an effective protection action against red blood cell hemolysis .This action increased proportionally with the concentration of honey (100, 400 and 600ug/ml). The highest percentage of inhibition was at 600 ug/ml with 21.53%. For the evaluation of hepato-protective effect of Eucalyptus honey in APAP induced hepatotoxicity. 18 Albino Wistar female rats weights 140-225g, aged 8 weeks were used and devided into 3 groups: control group, APAP group, and APAP+honey group. The last group was pretreated with Eucalyptus honey 5g/kg for 10 days. Both APAP and APAP+honey groups were treated with a toxic overdose of 2g/kg of acetaminophen on the 11th day. Biochemical and oxidative stress parameters analyses results have shown significant increase in the levels of ALAT, ASAT, LDH and MDA. And a decrease in the levels of GSH, GST, SOD, and CAT in the APAP group in relation to the control group. APAP+Eucalyptus honey group compared to APAP group showed asignificant decrease in the transaminases by 87.8%(ALAT) and 68.1% (ASAT), LDH by 76.5% and MDA by 81.6%.There was also an increase in the levels of GSH, GST, SOD, and CAT by 65.3%, 74.09%, 82.6%, and 76.75%respectively. The histological analyses results showed an alteration in the histoarchitecture with a severe dgree of hemorrhage in the hepatic tissue of the APAP group.

In contrast the pretreated group with Eucalyptus honey Results showed that the hepatic lesions was less severe and maintained the architecture of the live .These results emphasize the clear ability of Eucalyptus honey to countrast the risk of oxidative stress and bacterial infection. In conclusion, Eucalyptus honey can be used as an alternative agent in the presence of *Escherichia coli* bacteria , an anti-hemolytic and hepatoprotector product.

Prespectives

In the light of these results, a number of perspectives should be explored:

- To deepen the phytochemical study in order to determine qualitatively and quantitatively the bioactive molecules present in Eucalyptus honey
- To deepen the study of antibacterial activity on other pathogenic bacterial species and other microorganisms, in particular fungi and yeasts.
- To evaluate the mechanisms of the anti-hemolytic action of Eucalyptus honey.

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Annexe

Annexe

Preparation of McFarland

. McFarland's original standards were developed by mixing specified quantities of barium chloride and sulfuric acid. The mixture of the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A McFarland 0.5 standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2$ -$2H_2O$) with 9.95 ml of 1% sulfuric acid (H_2SO_4) **(McFarland,1907).**

Preparation of Phosphate-Buffered Saline (PBS)

Prepare 450 ml distilled water in a suitable container.

Add 4 g sodium chloride to the solution.

Add 0.1 g potassium chloride to the solution.

Add 0.72 g dibasic sodium phosphate to solution.

Add 0.1225 g monobasic potassium phosphate to solution.

Adjust solution to desired pH ≈ 7.4 or other pH.

Add distilled water until the volume is 500 ml (Gibco™)

Academic year : 2023-2024	**Presented by :** HORCHI Wala Soundous NADIR Rihem Souha

Study of anti-bacterial, anti-hemolytic and in vivo hepatoprotective effect of Eucalyptus honey

Dissertation To Get a Diploma of Master Degree Toxicology

The present work aims to study the antibacterial, antihemolytic and *in vivo* hepatoprotective effects of Eucalyptus honey collected from Khmis el-khachena Algiers, Algeria in 2023. The result of total phenolic content in Eucalyptus honey, showed that honey contained 241,75±00 µg galic acid equivalent (GAE)/mg of honey. Two different bacterial strains were used in the anti-bacterial studies *Escherichia coli (E. coli)* and *Staphylococcus aureus*. An inhibition of *Escherichia coli* growth was noted with different concentrations of honey 75%, and 100% with a diameter of inhibition zone of 30mm and 31mm (very sensitive bacteria). As for its anti-hemolytic activity, a direct correlation was observed between honey concentration and the percentage of hemolysis inhibition, reaching 21.53% at 600 ug/ml, indicating the ability of honey to inhibit hemolysis. To evaluate the *in vivo* study of hepatoprotective effect of Eucalyptus honey in N-acetyl-para-aminophenol (APAP) induced hepatotoxicity, three groups of albino wistar female rats were used: control group, APAP group and honey+APAP group. The last two groups were treated with a supratherapeutic dose of APAP 2g/kg on the 11th day of treatement but the honey+APAP group was pretreated with Eucalyptus honey 5g/kg. Biochemical and oxidative stress parameters analyses results have shown significant increase in the levels of ALAT, ASAT, LDH and MDA. And an increase in the levels of GSH, GST, SOD, and CAT by in the APAP group in relation to the control group. Eucalyptus honey+APAP group compared to APAP group showed a significant decrease in the transaminases by 87.8%(ALAT) and 68.1% (ASAT), LDH by 76.5% and MDA by 81.6%.There was also an increase in the levels of GSH, GST, SOD, and CAT by 65.3%, 74.09%,82.6%, and 76.75% respectively .The histological analyses results showed an alteration in the histoarchitecture with a severe dgree of hemorrhage in the hepatic tissue of the APAP group. However the pretreated group with Eucalyptus honey. Results showed that the hepatic lesions was less severe and maintained the architecture of the liver These results emphasize the clear ability of eucalyptus honey to in contrast the risk of oxidative stress and bacterial infection. In conclusion, Eucalyptus honey can be used as an alternative agent in the presence of *Escherichia coli* bacteria, anti-hemolytic and hepatoprotector product.

Key words: Eucalyptus honey, Antibacterial activity, Antihemolytic activity, N-acetyl-para-aminophenol (APAP), Hepatoprotective effect..

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