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Final dissertation

To obtain a Master's degree in the Biological Sciences

Option: PHARMACOTOXICOLOGY

Theme

Histological and immunohistochemically study of liver in animals supplemented with food supplements.

Presented by:

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SUMMARY

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Résumé

Cette étude vise à évaluer les effets hépatiques et l'innocuité générale d'un complément alimentaire à base de noix de dattes chez des rats mâles suite à une administration orale subaiguë. Des rats Wistar mâles ont été répartis en deux groupes contrôle et traités. Le groupe traité a reçu un complément à base de noix de dattes pendant 14 jours. Les poids corporel et hépatique ont été enregistrés. Des analyses biochimiques ont été réalisées, incluant la glycémie, les taux d'ASAT et d'ALAT. L'examen histologique a été effectué avec les colorations H&E, Trichrome de Masson, PAS et réticuline. Une immunohistochimie a été réalisée pour l'alphafoetoprotéine, Ki-67, Bcl-2 et β-caténine afin d'évaluer les marqueurs de prolifération et d'apoptose. Aucune différence significative n'a été observée dans les poids corporel ou hépatique entre les groupes contrôle et traité. Les marqueurs biochimiques sont restés dans les plages normales, sans indication de cytolyse hépatique. L'analyse histologique a montré une architecture hépatique préservée, sans signe de fibrose, nécrose ou infiltration inflammatoire. La coloration immunohistochimique a révélé des profils d'expression normaux pour tous les marqueurs, suggérant une absence d'activité cellulaire anormale. L'administration subaiguë d'un complément à base de noix de dattes n'a pas induit de toxicité hépatique ni de dommage structurel chez les rats mâles. Ces résultats confirment la sécurité hépatique de ce supplément et suggèrent son potentiel en tant qu'agent nutritionnel ou thérapeutique sûr. Mots clés : noix de datte, complément alimentaire, analyses biochimiques, foie, histologie, immunohistochimie, rat

ملخص

هدفت هذه الدراسة إلى تقييم تأثيرات مكمل غذائي مستخلص من نوى التمر على الكبد والسالمة العامة لدى ذكور الجرذان تلقت المجموعة .بعد تناول فموي لمدة قصيرة. تم تقسيم ذكور جرذان إلى المجموعة المراقبة والمجموعة التجريبية التجريبية المكمل الغذائي المستخلص من نوى التمر لمدة 14 يو ًما. تم تسجيل أوزان الجسم والكبد. شملت التحليالت الكيميائية ُجري الفحص النسيجي وصبغة ماسون ثالثية ، (PAS) الحيوية قياس مستوى الجلوكوز في الدم وإنزيمي أ ، وصبغة ASAT وALAT. باستخدام تلوينات الريتكلين. كما تم Ki-67، Bcl-2و- β- الهيماتوكسيلين وإيزين كاتينين لتقييم (E&H)أللوان، ورد فعل حمض البيريوديك-شيف إجراء التحليل المناعي الكيميائي النسيجي للعالمات ألفا فيتو بروتين، مؤشرات التكاثر والموت المبرمج للخاليا. لم تُالحظ فروقات معنوية في أوزان الجسم أو الكبد بين المجموعتين. بقيت المؤشرات الكيميائية الحيوية ضمن المستويات الطبيعية، دون أي دليل على تكسير خاليا الكبد. أظهر التحليل النسيجي الحفاظ على بنية الكبد بدون عالمات تليف، نخر، أو تسلل التهابي. كشفت التلوينات المناعية الكيميائية عن أنماط تعبير طبيعية لجميع .العالمات، مما يشير إلى عدم وجود نشاط خلوي غير طبيعي لم تسبب المعالجة الفرعية لمكمل نوى التمر سمية كبدية أو أضرار هيكلية لدى ذكور الجرذان. تدعم هذه النتائج سالمة .

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INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a vital agricultural crop extensively cultivated in the Middle East and North Africa. It holds significant nutritional, economic, and cultural importance in these regions **(Al-Farsi & Lee, 2008).** Traditionally valued for its sweet fruit, the date palm also produces date seeds, a by-product often discarded or used as animal feed, which has recently attracted scientific interest for its health-promoting properties **(Al-Kaabi et al., 2013).**

Date seeds are rich in polyphenols, flavonoids, dietary fiber, and essential fatty acids, which impart potent antioxidant, anti-inflammatory, and hepatoprotective effects **(Al-Farsi et al., 2016).** Numerous studies have highlighted the potential of date seed extracts as natural agents for preventing or alleviating liver damage caused by oxidative stress and metabolic disorders such as diabetes **(Shakoor et al., 2020; Abdelaziz et al., 2015).** Given the liver's central role in metabolism, detoxification, and homeostasis, protecting hepatic function is critical for overall health **(Hall, 2016).**

The liver is especially vulnerable to injury from toxins, metabolic imbalances, and inflammation. Damage to liver cells can impair essential physiological processes, contributing to chronic liver diseases **(Hall, 2016).** Natural dietary supplements derived from plant sources like date seeds are promising candidates for hepatoprotection due to their bioactive compounds and low toxicity profiles **(Brown et al., 2020).** However, the formulation of effective and safe date seed-based dietary supplements requires rigorous scientific evaluation, particularly regarding their effects on liver structure and function.

Despite traditional use and preliminary findings on date seed bioactivity, there is a lack of comprehensive data on their hepatic safety and protective effects when administered as dietary supplements. It is important to investigate their impact through in vivo models to assess possible toxicity or benefits on liver tissue.

This study aims to evaluate the effect of a dietary supplement based on date seeds on liver health in male rats after subacute oral administration. The investigation includes biochemical assays and detailed histological, histochemical, and immunohistochemical analyses to explore changes in liver architecture and cellular markers.

Structure of the Document

- **Chapter 1 Literature Review** concise review divided into three sections • *Dietary supplements* • *Date palm and date seed* • *Liver physiology and pathology*

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- **Chapter 2 Materials and Methods:** Description of materials, experimental design, in vivo treatment protocols, and analytical techniques including biochemical and microscopic examinations.
- **Chapter 3: Results and Discussion:** Presentation and interpretation of experimental results compared to the existing literature.
- **Conclusion and Perspectives** Summary of the study's contributions and potential future research directions.

Chapter 1

1. Liver

1.1.Embryology of Liver

The liver originates from the foregut endoderm during gastrulation **(Zorn, 2008)**. In the third week, the liver diverticulum emerges as a ventral bud near the heart; its anterior part forms the liver and intrahepatic bile ducts, while the posterior part gives rise to the gallbladder and extrahepatic ducts **(Sadler & Langman, 2019).** Hepatoblasts invade the septum transversum to form the liver bud and biliary duct **(Zorn, 2008).** These cells differentiate into hepatocytes and biliary epithelial cells (BECs), while sinusoids arise from connections with the vitelline and umbilical veins **(Zhao, 2005) (Figure 1).**

The liver expands into the abdominal cavity, with the ventral mesogastrium (including the bare area) arising from surrounding mesoderm and anchoring to the future central tendon of the diaphragm. It also plays a major role in fetal hematopoiesis until the seventh month, after which bone marrow assumes this function and liver weight stabilizes at 5% of body mass **(Si-Tayeb & Lemaigre, 2010)**. By week 12, liver cells begin bile production, and the bile duct, formed from hepatic and cystic ducts, shifts to a posterior position behind the duodenum **(Zhao, 2005).**

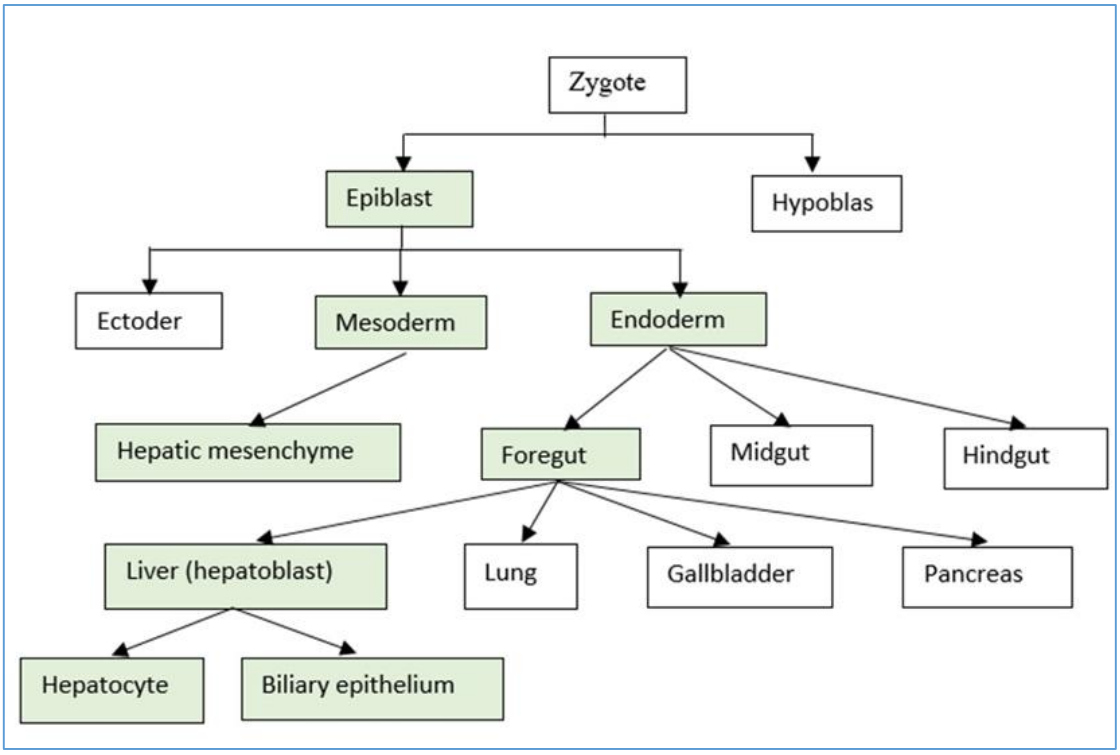

Figure 1: Liver development **(Zorn, 2008)**

1.2. Anatomy of the Liver

The liver is the largest smooth-surfaced organ in the human body, accounting for 2–3% of body weight (approximately 1800 g in males and 1400 g in females) **(Sibulesky, 2013).** Located in the right upper abdominal quadrant beneath the right hemidiaphragm, it is protected by the ribs and encased by the Glisson capsule, except at the diaphragmatic contact zone **(Abdel-Misih and Bloomston, 2010; Llewellyn and Fede, 2022).** The right lobe, larger than the left, and bordered by the gallbladder and umbilical fissure; the caudate lobe lies posterior to the hilum and is known as Spigel's lobe **(Bismuth et al., 1982).** The liver is divided into eight functional segments, each with its own portal pedicle (including hepatic ducts), portal veins, and hepatic artery branches **(Sibulesky, 2012) (Figure 2).**

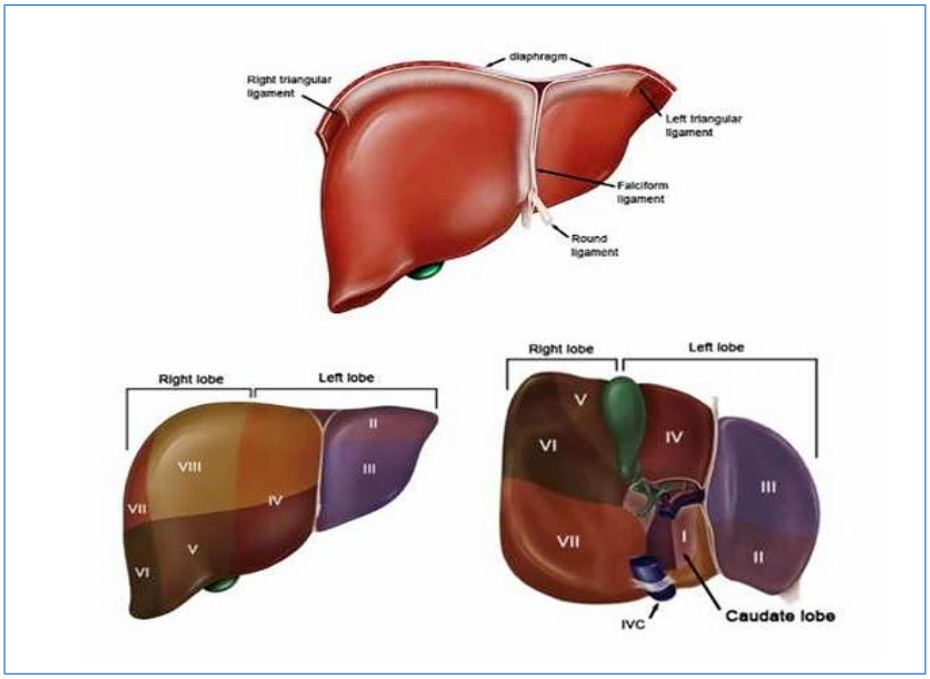

Figure 2: Anatomic Morphologic of the liver **(Abdel-Misih and Bloomston, 2010).**

The liver maintains homeostasis through the coordination of structural components, including the hepatic veins and peritoneal ligaments—such as the coronary and triangular ligaments—which anchor it to the diaphragm, while the falciform and teres ligaments connect it to the anterior abdominal wall; abdominal muscles also support its integrity **(Mahadevan, 2020).**

1.3.Histology of the Liver

The fundamental structural and functional unit of the liver is the hepatic lobule, which is about the size of a sesame seed and is distinguished by the particular arrangement of liver cells **(Figure 3).** The hepatic lobule is made up of a number of vital components, including hepatocytes, a portal triad, a central vein, liver sinusoids, Kupffer cells, which are macrophage-like cells, bile canaliculi, and the space of Disse, which is the narrow gap between the sinusoids and hepatocytes. The hepatic artery, portal vein, and bile duct are located in each corner of the hexagonal structure **(Trefts et *al*., 2017).** Cells in the lobule use oxygen and digest nutrients while also producing waste and metabolites during blood circulation. The blood undergoes deoxygenation, and the sinusoids are where metabolic waste is eliminated from the cells **(Juza et *al*.2014)**. Hepatocytes, sinusoidal endothelial cells, Kupffer cells, stellate cells, and cholangiocytes are the five different cell types that make up the liver **(Table 1)**.

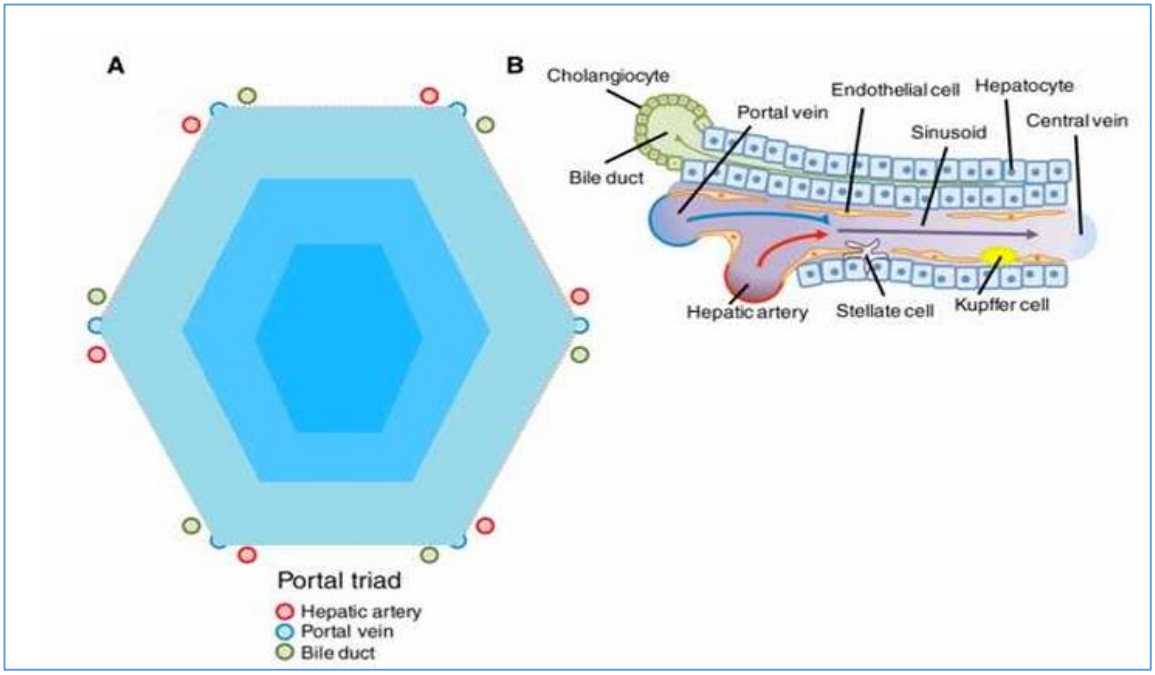

Figure3: Liver organization **(Trefts et *al*., 2017).**

Table 1: Liver cells and functions

Cells Type	Functions
Hepatocyte	The primary cellular component of the liver. The processes of synthesis, storage, degradation, metabolism, and portal substances' endocrine and exocrine functions
Sinusoidal endothelial cells	The fenestrated plexus facilitates the communication between portal blood and hepatocytes
Kupffer cells	Phagocytosis and cytokine release
Stellate cells	The role of function in the process of regeneration after injury, as well as its function as a precursor to myofibroblast formation and storage of vitamin A
Cholangiocyte	The functions of the gallbladder include the transportation of bile, the secretion of bicarbonate, and the secretion of water

Hepatocytes, the liver's main cell type, perform synthesis, storage, and blood filtration. Cholangiocytes, lining the bile ducts, are the second most common liver cells. Kupffer cells handle phagocytosis and immune defense. Stellate cells (Ito cells) contribute to hepatic fibrosis and store vitamin A when quiescent, though their exact role in this state is unclear. Hepatic sinusoidal endothelial cells, with 50–180 nm pores, enable the exchange of proteins and particles between plasma and liver cells **(Trefts, Gannon, & Wasserman, 2017; Juza & Pauli, 2014).**

1.4. Functions of Liver

The liver plays numerous vital roles in maintaining homeostasis, serving as a central organ in metabolism, detoxification, digestion, and nutrient storage:

- Bile is a vital physiological fluid produced by hepatocytes, helps remove toxins that the kidneys cannot excrete and supports fat digestion via bile salts and acids. It contains water, electrolytes, bile acids, pigments, bilirubin, cholesterol, and phospholipids. Bile travels through ducts to the duodenum or is stored in the gallbladder. Non-excreted components are recycled by intestinal bacteria into bile acids, reabsorbed in the ileum, and returned to the liver **(Kalra et *al*., 2023).**
- The liver stores or metabolizes fat-soluble vitamins. Vitamin E is delivered in the forms of alpha- and gamma-tocopherol. While the liver doesn't store or metabolize vitamin K, it is still essential for the activity of the liver enzyme gamma-glutamyl carboxylase.
- The heme oxidation process mostly occurs in the liver. Heme is changed into biliverdin during this process, which leads to the creation of unconjugated bilirubin. After being converted into bile, the majority of this bilirubin is later expelled from the body through feces. However, after being filtered, some of it enters the bloodstream and is ultimately eliminated by the kidneys **(Kalra and Yetiskul, 2023).**
- The liver's deiodination mechanism, which transforms thyroxine (T4) into triiodothyronine (T3), is essential to the thyroid hormones' overall operation.
- The majority of the plasma proteins in the human body, such as albumin, protein C, binding globulins, and protein S, are synthesized by the liver. With the exception of factor VIII, which is produced via both the intrinsic and extrinsic pathways, it also produces all clotting factors **(Kalra and Yetiskul, 2023).**

1.5.Mechanism of detoxification

The liver plays a crucial part in detoxification concerning alcohol, amphetamines, hormones, steroids, and barbiturates. Its primary function is to prevent the excessive buildup of these substances and mitigate any potential negative effects.

While metabolic detoxification is typically beneficial, there are instances where it can adversely affect hepatocytes. One illustration of the detrimental effects of alcohol abuse is the potential harm it can inflict upon hepatocytes, primarily due to the metabolic byproducts of alcohol, including acetaldehyde and hydrogen. The metabolic byproducts elicit an augmentation in adipose tissue deposition, which has the potential to impair hepatic functionality **(Ozougwu . 2017).**

Enzymes that are essential for detoxification are produced by the liver's primary functioning cells, called hepatocytes. Phase I and Phase II are the two stages into which this process can be separated.

- **Phase I** enzymes improve the hydrophilicity (water solubility) of lipophilic (fat-soluble) compounds by adding polar groups, such as hydroxyl (-OH) groups. This alteration aids in getting these molecules ready for additional processing.
- Enzymes conjugate the hydrophilic substances—such sugars or peptides like glutathione—to the polar groups that were introduced in Phase I during **Phase II.** The chemicals' solubility is further increased in this stage, which facilitates the body's removal of them **(Ozougwu, 2017).** A disturbance in Phase II enzyme activity may make the liver more vulnerable to harm. This is due to the possibility of accumulation of reactive compounds produced during Phase I metabolism, which could be harmful **(Liu et *al*., 2004).**

The liver is involved in metabolic detoxification, transforming both endogenous and exogenous substances, including medications and hormones, into metabolites that are less biologically active and less harmful. These metabolites are then eliminated from the body via the bowels or the kidneys **(Butura, 2008).**

Because of its vast network of blood veins, the liver has the amazing capacity to store a significant amount of blood. The liver can leak blood during a hemorrhage in order to help the circulatory system's overall blood volume. The liver is vulnerable to foreign particles or germs that enter through the portal vein because of its physical relationship to the gastrointestinal tract. The liver's kupffer cells have the ability to eradicate bacteria and serve as an infection-prevention barrier **(Ozougwu et *al*., 2017).**

2. Date palm "Phoenix dactylifera L."

Due to its association with the Phoenicians, the date palm, formally known as "Phoenix dactylifera L.," is a tree whose genus name, Phoenix, is taken from the name that the Greeks gave it. Its finger-shaped fruits are indicated by the species name, dactylifera. Cultivated in Mesopotamian oasis **(present-day Iraq and Syria)** for around 6,700 years, this tree is among the earliest fruit plants domesticated by mankind **(Benchelah et Maka, 2008)**.

Because of its resilience to dryness and extremely high temperatures, the date palm is a classic desert tree that has helped people live in hostile environments. Because of its economic, social, ecological, and nutritional advantages, it is the most prized fruit tree in oasis **(Tirichine, 2010)**.

2.1.Date palm production

Algeria produces 440,000 tons of dates annually, ranking seventh in the world. Over 10% of the over, 500,000 tons of dates produced in the nation in 2006 were soft dates. The main industrial application for these soft dates is the production of date paste, vinegar, and date juice **(Boudechiche et *al*., 2009).**

Although 30 countries produce dates, Egypt still produces the most (21%), followed by Iran (15%), Saudi Arabia (15%), Iraq (9%), Pakistan (7%), and Algeria (12%), the world's fourth-largest producer **(FAO, 2015)**. Since the beginning of time, dates have played a significant role in both human and animal diets **(Amellal, 2008)**.

The date palm produces a variety of byproducts, such as leaves, pedicels, trunks, and stones. They have several uses, especially for date kernels that are extracted from different date processing techniques such pitted dates, date paste, date syrup, and date juice **(Boussena and Khali, 2016)**.

2.2. Date kernels or Date seeds

Approximately 11–18% of the total fruit weight consists of date kernels (ND), which contain proteins, dietary fiber, lipids, ash, and carbohydrates. As by-products of the date fruit industry, these seeds are often discarded or partially used as animal feed. For this purpose, they are usually soaked and ground. The lauric acid content in date seeds varies between 0.56% and 5.4% **(Chitra and Mothil, 2016).**

The fruit, known as "date" or "Tamr" in Arabic, is a berry that can be elongated, oblon g, rounded, or sometimes spherical.The core is encircled by flesh, referred to as pulp **(Peyron, 2000).**

The pulp is composed of an endocarp with a lighter color and fibrous texture, which is occasionally reduced to a parchment membrane encircling the core; a mesocarp that is typically fleshy, varying in consistency depending on its sugar content, and deep in color; and a pericarp, or fine cellulosic envelope known as skin **(Figure 4) (Espiard, 2002).**

The dates' weights range from a few grams to fifty grams, and their lengths range from one to eight centimeters. Its hue shifts across all hues of yellow, from pale to brown to almost dark **(Peyron, 2000).**

2.2.1. Physicochemical composition of date kernels

2.2.2. Chemical composition

The climate, region, species, and geographic position all affect the percentage of date kernels components.

Water	Content varies between 7% and 19% **(Boudechiche et al., 2009).**
Minerals	calcium, magnesium, potassium, phosphorus, sodium, copper, iron, cobalt, cadmium, chloride, sulphur, boron, manganese, zinc, fluorine, but also aluminium, lead in various quantities **(Chandrasekaran et Bahkali, 2013).**
Lipids	Rich in saturated and unsaturated fatty acids, so the fat content ranges between 5% and 12% **(Lesheb, 2010)**. They contain several types of acids: myristic acid, lauric acid, oleic acid, and linoleic acid. However, the most abundant acids in dates of the various studied varieties are oleic acid and lauric acid **(Malika et al., 2019).** It can be used as a source of oleic acid because its content ranges from 41.1% to 58.8% **(Chandrasekaran et Bahkali, 2013).**
Proteins	It varies between 0.11% and 8.59% **(Khali et al., 2014).**
Fibres	Algerian date cultivars have a fiber level of 13.54% to 16.27% **(Khali et al., 2013).**
Sugars	Include sugars that are both lowering and non-reducing**(Munier, 1973; Chaira, 2007; Rahman et al., 2007; Lecheb, 2009).** Their sugar content ranges from 4.4% to 4.6%, depending on the species and type**(Lecheb, 2010).**
Ash	Very low and differs depending on the variety of date, in % DM between 0.01 and 1.08% **(Khali et al., 2014).**
Phytochemicals	Dates and kernel extracts have been found to contain phenolic compounds, volatile compounds, flavonoids (API Genin, Luteolin, and quercetin), phytosterols (β-sitosterol, ISO Fucosterol, Stigmasterol, Campe Stere), and carotenoids (lutein, Neo Xanthine, β-carotene, Rape Xanthine, and atheraxanthin) **(Hong et al., 2006; Saafi et al., 2009).**
Polyphenols	When ND is fresh, its polyphenol concentration ranges from 2.49 to 8.36 mg/100g **(Mansouri et al., 2005).**
Carbohydrate	Carbohydrate content in the core ranges from 60 to 86.89 percent **(Besbes, 2004a; Al-Farsi et al., 2007**).

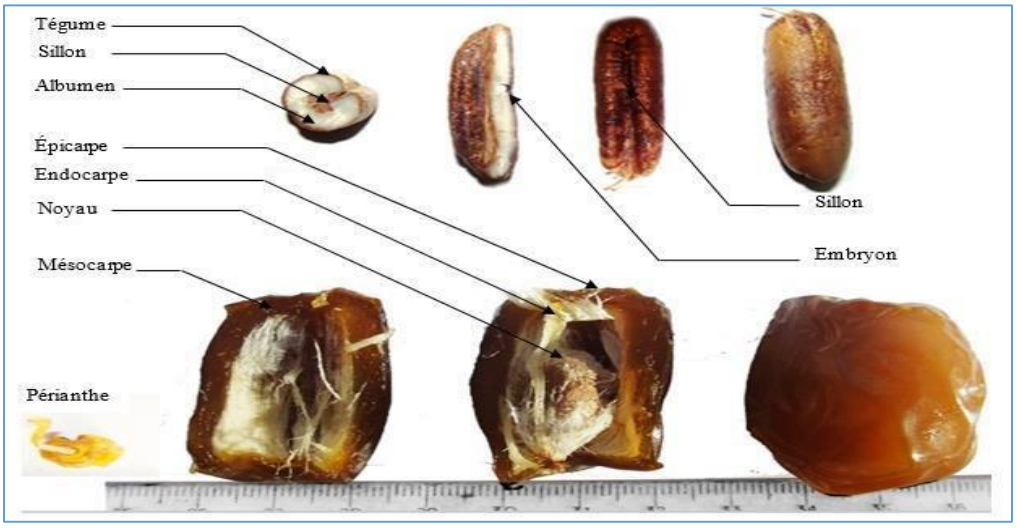

Figure 4: Date Fruit and Kernel of Date Palms **(Boulanouar, 2015)**

2.3.Positive effects of date seed on health

Date seed powder's (Figure 2) remarkable biological benefits to health are related to its therapeutic qualities, which enable it to function on multiple levels: DNA, diabetes, oxidative stress, viral infections, kidney disease, etc.

2.3.1. Antioxidants: Date seed can be utilized as a functional dietary element to increase metabolism because of its high antioxidant content, anti-free radical, and antioxidant qualities, which help the body lessen damage from oxidative stress **(Al-Farsi et *al*., 2007).**

2.3.2. Prevention of DNA damage: The body has a hepato-protective effect due to the antioxidant and anti-free radical action, which enables defense against oxidative DNA damage and chemically induced liver damage, hence avoiding liver poisoning **(Al-Farsi et *al*., 2007).**

2.3.3. Reduces blood sugar levels: It is possible to reduce blood sugar levels and treat blood sugar diseases, including diabetes and its consequences. Recent research has shown that date nut treatment may have preventive effects against early diabetes problems of the kidneys and liver **(Al-Farsi et *al*., 2007).**

2.3.4. Antiviral agents: Date nuts' antiviral properties allow for the prevention and treatment of a wide range of viral illnesses. Date extracts have a potent potential to totally stop bacterial lysis and boost the Pseudomonas phage's efficacy **(Al-Farsi et *al*., 2007).** .

2.3.5. Prevent kidney and liver damage: Date nuts can shield the liver and kidneys from harm because of their high pro-anthocyanidin content. This component's extract guards against hepatic and renal toxicity brought on by chemicals **(Al-Farsi et *al*., 2007)**.

3. Dietary Supplements

Dietary supplements, also known as food supplements, are products intended to complement the regular diet by providing essential nutrients such as vitamins, minerals, fatty acids, and amino acids. Their primary purposes include addressing nutritional deficiencies, supporting overall health, enhancing athletic performance, and meeting specific needs related to certain medical conditions. By compensating for nutrient gaps, these supplements may also help reduce the risk of developing chronic diseases **(Kerksick et *al*., 2018).**

According to the European Decree No. 2006-352 of March 20, 2006 **(Anonym 1),** many dietary supplements are formulated using plant extracts, vitamins, minerals, or concentrated substances for physiological and nutritional benefits (e.g., glucosamine, melatonin). They are available in various forms such as capsules, lozenges, tablets, pills, gummies, powder sachets, or liquid preparations like ampoules and droppers. These products are marketed to improve nutritional intake and offer benefits such as weight management, better digestion, healthier hair, and relief from menopause or pregnancy symptoms.

Food supplements can be composed of vitamins, minerals, plant extracts (excluding those used solely for pharmacological or therapeutic purposes), or other active compounds. They are widely used in areas such as nutrition, tonics, digestion, beauty, menopause, cardiovascular health, and weight control **(Karleskind et al., 2013).**

3.1.Definition

According to Algerian law, food supplements, including vitamins and mineral salts, are concentrated sources of these nutrients that can be purchased as capsules, tablets, powders, or solutions (Executive Decree No. 12-214 of 23 Jumada Ethania 1433, dated May 15, 2012). They are consumed in small amounts to compensate for inadequacies in the regular diet (Décret 2012).

3.2.Composition

Food supplements are composed of various ingredients that serve different nutritional, physiological, or technological purposes. These components can be broadly categorized into nutrients, substances with specific physiological roles, plant-based ingredients, authorized additives and flavorings, as well as other raw materials from animal sources. Each category plays a distinct role in supporting the supplement's intended health benefits **(Table1 and Figure 5).**

Table 1: Categories and Descriptions of Ingredients in Food Supplements **(Synadiet, 2020)**

Category	Description	Examples
Nutrients: Vitamins, Minerals, Trace Elements	Essential compounds not fully synthesized by the body and required for various biological functions, including metabolism and development.	Vitamins, iron, calcium, chromium, magnesium
Substances for Nutritional or Physiological Purposes	Chemically defined substances with nutritional or physiological qualities, excluding vitamins, minerals, and purely pharmacological substances.	Chitosan, glucosamine, lycopene
Plants or Preparations of Plants	Predominantly represented in supplements; include powders, dry extracts, aqueous extracts, and plant compounds, excluding those reserved for therapeutic use.	Plant powders, dry extracts, aqueous extracts
Additives, Flavors, and Technological Auxiliaries	Substances authorized for human food use to enhance flavor, color, prevent oxidation, and preserve the product.	Flavor enhancers, coloring agents, preservatives
Other Ingredients	Raw materials derived mainly from animals, used in various supplement formulations.	Shark cartilage, royal jelly

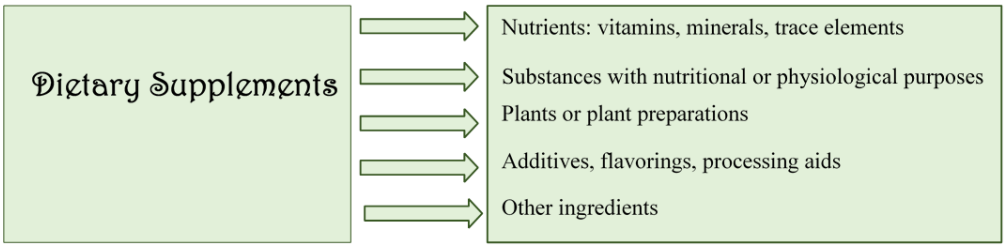

Figure 5: Categories of ingredients found in the Dietary supplements

3.3.Classification

Nutrition is fundamental to maintaining health and supporting the body's physiological functions. Nutrients are broadly categorized based on the quantities required and their roles within the body. Understanding this classification helps to appreciate the different functions nutrients perform, from providing energy to regulating vital biochemical processes.

3.3.1. Macronutrients

Macronutrients are large molecules formed by the bonding of smaller units, essential for cellular activity and providing energy for the body's functions. Energy intake is typically distributed as approximately 15% from proteins, 36% from fats, and 44% from carbohydrates **(Masson, 2021).**

a. Proteins: Proteins perform three vital roles in the body **(Théo, 2017):**

- **Energetic role:** Proteins provide necessary energy for bodily functions.
- **Functional role:** They support the body's defense mechanisms against diseases.
- **Constructive role:** Proteins are involved in building and repairing all living tissues.

Sources of protein: Animal proteins: milk, eggs, fish, and meat; Plant proteins: cereals and legumes **(Anses, 2013)**

b. Fats: Lipids serve two primary functions **(Anses, 2021)**

- **Energy storage:** Triglycerides stored in adipose tissue act as energy reserves.
- **Structural role:** Phospholipids form an essential part of cell membranes, ensuring their fluidity and integrity.

c. Carbohydrates: Carbohydrates are a diverse group of macronutrients divided into two main types **(Castelli, 2020):**

- **Simple carbohydrates:** These include glucose, fructose, and galactose, which combine to form disaccharides like lactose, maltose, and sucrose. These molecules typically consist of one or two sugar units and are found in foods such as milk and table sugar.
- **Complex carbohydrates:** These consist of starch and other long chains of sugars, digested more slowly than simple sugars. They are present in foods like bread, pasta, cereals, and potatoes, and are generally not sweet.

2. Micronutrients: Micronutrients are elements required in trace amounts but vital for various physiological functions. Important examples include calcium (Ca^{2+}), sodium (Na^{+}), potassium, and magnesium **(Anses, 2012).**

2.1 Minerals: example zinc

Zinc (Zn): Zinc is an essential trace mineral involved in many physiological processes. The daily requirement is about 10 to 15 mg for children and adults, increasing to approximately 20 mg during pregnancy and 25 mg during lactation. Major dietary sources of zinc include fish, seafood (especially oysters), and red meat **(Biomnis, 2013).**

2.2. Trace Elements: Trace elements, also known as semi-major elements, are minerals typically found in ionic form that play essential roles in maintaining cellular functions. They help regulate membrane potentials and osmotic balance, which are vital for the proper functioning of living organisms. Examples include potassium and sodium, among others **(Kienlen, 1977).**

2.3. Vitamins: Vitamins are organic compounds crucial for various metabolic processes and are often included in dietary supplements to prevent or treat deficiencies. Commonly used vitamins in supplementation include A, D, E, K, B1, B2, B6, B12, C, niacin, pantothenic acid,

folic acid, and biotin. For example, vitamin B6 is frequently available as a nutritional supplement to support physiological functions **(Caro et *al*., 2010).**

Table 2: List and maximum daily doses of minerals and vitamins that can be used in food supplements **(Valette, 2015)**

Minerals	**Daily doses**	**Vitamins**	**Daily doses**
Ca	800ug	A	800ug
Mg	300ug	D	5ug
Fe	14 mg	E	30 mg
CU	2000ug	K	25ug
I	150mg	B1	4,2mg
Zn	15mg	B2	4,8mg
Mn	3,5mg	B3	Nicotinamide : 54mg Acide nicotinique : 8mg
K	80mg	B5	18mg
Se	50ug	B6	2mg
Mb	150ug	B8	450ug
Cr	25ug	B12	3ug
P	450mg	B9	200ug
		C	180mg

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CHAPTER 2

MATERIALS AND METHODS

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CHAPTER 2
MATERIALS AND METHODES

The aim of this study is to evaluate the effects of date seed consumption as a nutritional supplement on the histological parameters of the liver in rats. The study primarily focuses on: Changes in body weight and food intake. Histological and immunohistochemical examination of the hepatic parenchyma in rats.

The experiments were conducted between April and June at the Following location:

- The experimental station of the university Saad Dahleb Blida 1, where we were able to carry out the experimentation, the sacrifices and the dissection of the animals as well The weighing of the organs.
- First of all, the main objective was the formulation of the capsules in the pharmacy laboratory of the SAAD DAHLEB Blida 1 university. contained date nut extract and PLCEBO capsules, contained only the rat food. This step aimed to prepare these capsules for the subsequent testing phases
- At the pathology laboratory of Mohamed Lamine Debaghine University Hospital (Bab El Oued), the adverse effects of date nuts were investigated through a series of histological analyses. These included topographic staining using Hematoxylin and Eosin (H&E) and Masson's Trichrome, histochemical staining with Periodic Acid-Schiff (PAS) and reticulin staining to identify reticulin fibers, and finally, immunohistochemical analysis to detect the expression of specific molecular markers.

1. Materials

1.1. Biological materials

1.1.1. Plant material

The plant material used in this study consisted of date seeds (*Phoenix dactylifera* L.) collected from Biskra, in the Sahara region, on March 17th, 2025. Following harvest, the seeds were thoroughly washed and air-dried in the shade at room temperature. Once dried, they were ground into a fine powder, which was then used to prepare the dietary supplement employed in this research.

Figure6: date seeds.

Figure 7: date seeds powder

1.1.2. Animals

The animal model used in this experiment consisted of healthy male albino Wistar rats, aged 2 months and weighing 338 ± 30 g at the start of the study. The animals were obtained from the animal breeding facility of USTHB University. They were randomly divided into two groups, with 5 rats in each group.

The rats were housed in plastic cages with stainless steel lids and were subjected to a 14-day adaptation period prior to the experiment. Housing conditions were maintained at a temperature of 20 ± 7°C, relative humidity of 60%, and a 12-hour light/dark cycle. The animals were fed a standard granulated diet and had *ad libitum* access to both food and water.

Figure 8: The rats housed in plastic cages with stainless steel lids lined with sawdust.

Figure 9: the weighing of animals

1.2. Non biological materials

The non-biological materials used in this study including glassware, reagents, equipment, and software are detailed in Appendices 1, 2, and 3.

2. Methods

2.1. Acclimatation period

Prior to the start of the experiment, the rats underwent a 7-day acclimatization period to allow them to stabilize in the new environment and adapt to human handling, thereby minimizing external factors that could influence the results. During this period, the rats were weighed daily to monitor weight progression, and their feed intake was also recorded on a daily basis.

2.2. Experimental design

Our study focused on assessing variations in body weight, feed intake, biochemical and the histopathological and immunohistochemical characteristics of the liver in rats following a 07-day administration of a dietary supplement based on date seed powder.

Fifteen healthy animals were divided into two groups:

• Group 1 (Control): Received a placebo (standard rat food).

• Group 2 (Treated): Received pills containing a mixture of date seed powder and placebo powder.

The treatment was administered daily over a period of 21 days.

Figure 10: Prepared capsules

2.2.1 Sacrifice, Dissection and Blood Collection

The rats were euthanized by placing them under a bell jar containing cotton soaked in chloroform. After 3 to 4 minutes of exposure, the animals lost consciousness due to chloroform inhalation. Prolonged inhalation induces acute respiratory failure, which rapidly results in death.

2.2.2. Rat Dissection

After the animal is sacrificed, the dissection is performed according to the following steps:

1. Blood collection is carried out by cardiac puncture, inserting a syringe needle into the left ventricle of the heart to obtain samples for the analysis of hepatic biochemical parameters, including glycemia, ASAT, and ALAT.
2. The animal is placed on a dissection board, with its dorsal side against the board.
3. The animal's legs are secured to the board using pins.
4. Using scissors, a ventral incision is made through the skin and muscles to expose the internal organs.

2.2.3 Histological technique

The histological technique was performed at the anatomopathology laboratory of the CHU Mohamed Lamine Debaghine, Bab el Oued. This technique can detect the presence or absence of liver injury. This is achieved by passing through a series of process that are:

- **Circulation :**

This step is done by means of a **circulatory automaton**, which consists of immersing the parts in a series of intermediate liquids in a predetermined order. It replaces the water present in the tissues with a matrix that is sufficiently stable to maintain the cellular structures. Circulation goes through 3 stages :

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- **Dehydration**

Consists of eliminating the water in the cytoplasmic compartment in order to drain the paraffin, which is a hydrophobic substance.

It is carried out progressively by passing through successive baths of alcohols of increasing concentration, 70°, 90° 96° and absolute alcohol 100°. These concentrations ensure that dehydration is gentle and not brutal, preserving the cells from distortion.

- **Clearing**

The cassettes are then immersed in 3 successive baths of xylene (an intermediary between alcohol and paraffin), 2 hours each, to replace the alcohol present in the tissues by the paraffin and prepare it.

- **Impregnation**

This is the final stage, which eliminates the xylene and impregnates the tissue with paraffin. The cassettes are placed in two baths of pure melted paraffin for 2 hours each. The baths are thermostated at 60˚C.

Embedding

This step is carried out using an embedding centre, where molten paraffin is poured into a metal mold that chosen according to the fragment size. The impregnated sample is placed in centre of the mold and a label cassette is placed on top of the mold topped up with more paraffin, then its placed on a refrigerated metal plate to solidify. which are detailed in **appendix 3.**

- **Sectioning**

Once the paraffin has solidified, the block is removed from the mold and placed on microtome to cut tissue section of 5 µm thick, then the ribbon floats on a warm water bath and picked up on labelled glass slide then they are dried in an oven set at 50°C for 15 minutes to help the section adhere. which are detailed in **appendix 3.**

- **Staining**

It is the process of coloring tissues by using dyes. It allows visualizing cells and extracellular matrix to be studied with light microscopes. In our study we used four types of dyes: Haematin and Eosin (HE), Masson's Trichrome, Reticulin, PAS (Periodic Acid Schiff reaction). The process of staining pass throws two stapeses:

- **Preparatory step**: includes

- **Dewaxing**: it's to remove the paraffin from the tissue so that the dyes can penetrate. Xylene is used for this; the slides are placed in tow xylene baths for 3 to 5 minutes each time (the 2nd bath must be pure).
- **Hydration**: in order to remove the xylene from the tissue and replace it with water. Ethanol is used in decreasing concentrations (absolute ethanol, 95%, 80% and 70% for 3 to 5 minutes each) and is finished off with a 3 to 5-minute treatment under running water.

➢ **Staining step:**

- **Hematoxylin and Eosin (H&E)**

We use Harris Haematin, which is a basic dye that stains acidic structures (nuclei) in purple, and eosin, which is an acidic stain that stains basic cytoplasmic structures in pink. We pass the sections through Harris Haematin for 15 minutes (Haematoxylin + ethanol + potassium alum + distilled water + mercuric oxide) then in acid alcohol, in ammonia water and finally stain them with eosin solution (15 secs to 2min). After each reagent we wash the slides in tap water.

- **Masson Trichrome**

It's a special stain that highlights collagen fibers. This technique involves 3 successive staining: nuclear staining by Haematoxylin, cytoplasmic staining by a mixture of acid dyes "Fuchsin Acid Ponceau of Xylidine" and selective staining of collagen by another acid dye "Light Green". In first step, the slides are staining by Haematoxylin for 5min, after washing with tap water they are placed in Fuchsin Ponceau for 5 min then in 1% aqueous phosphomolybdic acid solution for 1 min and without washing they are immersed in light green for 1 to 5 min, after that in 1% acetic water and then in 100 % alcohol.

The final results are: blue nuclei, pink to red cytoplasm, bright red blood cells and keratin, green collagen and mucus and pink elastin.

- **Reticulin**

It's a special stain that highlights reticulin fibers, it rely on argyrophilic reaction, which based on the property of reticular fibres to pick up silver salts that have already been reduced. The sections are first oxidised with 1% potassium permanganate then immersed in an oxalic acid solution for 2 min, secondly they are sensitised with iron alum for 1 min and impregnated in the nitrate complex, the silver salts are then reduced

with formalin solution, rinsed with gold chloride for 5 min and finely fixed with sodium thiosulphate. after each stage it's necessary to rinse the sections with tap water. At the end, we obtain: reticulin fibre: black, grey nuclei and brick-red collagen.

- **PAS (Periodic acid Schiff reaction)**

It's a specific reagent for substances having released aldehyde groups after oxidation by periodic acid. these aldehydes are visualized by the Schiff reagent (basic fuscin decolorized by sulfurous acid) which forms with them a red condensation product. The slides are placed in an aqueous solution of 1%periodic acid and immersed in Schiff's reagent for 15 min, then stain by Harris hematoxylin for 3min. After each step the slides are rinsed with water. As results we obtain a reactive substance colored purplish red.

- **Mounting:**

After staining process, the slides must be dehydrated with alcohol and cleared with xylene to fix the thin glass cover to the glass slide using Eukitt adhesive.

2.2.4 Immunohistochemical technique

The principal of this method is to localizing proteins in the cells of a paraffin-embedded tissue section, by antigen detection using a primary antibody specific to the antigen sought, which is itself recognized by a secondary antibody coupled to an enzyme (peroxidase or alkaline phosphatase). The Ag-Ab/enzyme complex reacts with a chromogen substrate to produce a colored reaction that is easy to visualize under the microscope.

The aim of this step is to demonstrate the expression of two immunomarkers in the thyroid of the three batches. This procedure looks for the apoptosis marker p53 and tumor marker alpha fetoprotein.

Samples are first fixed in 10% formalin solution, then embedded in paraffin. Sections of around 5µm are made and mounted on charged slides for maximum tissue adhesion. The slides then are dried overnight in an oven at 40°C to remove any excess paraffin.

All the steps below were preformed using an automated slide stainer (BenchMark ULTRA, Ventana Medical System, Tucson, AZ).

Figure11: IHC automaton (BenchMark ULTRA, Ventana Medical System, Tucson, AZ).

Pre-treatment

- **Dewaxing**: to remove any remaining traces of paraffin.
- **Antigenic restoration**: this is a key stage which involves unmasking the antigenic sites to break the methylene bridges formed during fixation under the effect of heat.
- **Inhibition of endogenous molecules**: to block peroxidase sites and avoid false positivity (non-specific marking) to reduce the background signal.

- **Immunohistochemical reaction IHC**

It mainly includes

- Incubation of a primary Ab directed specifically against the antigenic sites sought, for this study the Antibodys are: anti-alpha fetoprotein and anti-p53 were identified. (these are ready-to-use preparations).
- Colorimetric detection of the primary Ab using a secondary Ab (known as indirect IHC) conjugated to the HRP (Horse Radish Peroxidase) enzyme, which is an oxidoreductase that catalyses the conversion of the chromogenic substrate DAB (3-3diaminobenzidine), which forms a brown precipitate that can be visualised under the light microscope. This secondary Ab is used non-specifically because it binds to the constant part of the primary Abs, and its incubation time varies according to the protocol developed.
- Counter-stain with Haematoxylin to stain nuclei blue.

Microscopic observation: At the end of the process of the tow techniques (histology and immunohistochemistry), the slides are then examined under the microscope and interpreted.

2.2.5. Analysis of biochemical parameters

- **Asparate aminotransferase**

Aspartate aminotransferase (ASAT/GOT) catalyzes the transfer of an amino group from aspartate to α-ketoglutarate, producing glutamate and oxaloacetate. Oxaloacetate is then reduced to malate-by-malate dehydrogenase (MDH) in the presence of reduced nicotinamide

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adenine dinucleotide (NADH). This reaction is monitored kinetically by measuring the decrease in absorbance at 340 nm, corresponding to the oxidation of NADH to NAD^+. The rate of absorbance decrease is directly proportional to the ASAT enzymatic activity in the sample.L-Asparate + 2-Oxoglutarate L-Glutamate + Oxalacetate

Oxalacetate + NADH + H+ L-Malate + NAD+

- **Alamine aminotransferase**

Alanine aminotransferase (ALAT/GPT): catalyzes the transfer of an amino group from alanine to α-ketoglutarate, producing glutamate and pyruvate. The pyruvate is then reduced to lactate-by-lactate dehydrogenase (LDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH). This reaction is monitored kinetically by measuring the decrease in absorbance at 340 nm, which corresponds to the oxidation of NADH to NAD^+. The rate of this decrease is directly proportional to the ALAT enzymatic activity in the sample.L-Alamine + 2-Oxoglutarate L-Glutamate + Pyruvate

Pyruvate + NADH + H+

Lactate + NAD+

- **Glucose**

Glucose is oxidized by glucose oxidase (GOD) to gluconic acid and hydrogen peroxide (H_2O_2). In the presence of peroxidase (POD), the H_2O_2 reacts with 4-chlorophenol and phenol aminophenazone (PAP) to form a red quinoneimine dye. The intensity of the colored complex, which is directly proportional to the glucose concentration in the sample, is measured spectrophotometrically at 500 nm **(Landari et al., 2019).**

2.2.6.Statistical analysis

- All data was statistically performed using Statistica version 10.0 (soft Inc., Tulsa, Oklahoma, USA). The values were presented as mean ± standard error of mean (SEM). The statistical significance was used a linear mixed model for repeated measures such as body weight, food intake and one-ANOVA. A p-value < 0.05 was considered statistically significant.

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CHAPTER 3
Results and Discussion

RESULTS

The effects of the date seed-based dietary supplement were evaluated through biochemical, histological, immunohistochemical, and statistical analyses. The following sections highlight the impact of the treatment on body and liver weights, glycemic, liver enzyme levels ASAT and ALAT, and liver tissue histological architecture. In addition, immunohistochemical analysis was performed targeting specific markers: ki67 (cell proliferation), beta-catenin (cell signaling and adhesion), and alpha-fetoprotein (afp), a marker associated with hepatic regeneration or transformation.

Together, these results provide a comprehensive overview of the therapeutic potential of this supplement in modulating metabolic and hepatic parameters in diabetic rats.

1. Body and liver weights

1.1. Body weight gain

Figure 13 presents a histogram of body weight gain in male rats following subacute exposure to the date seed-based dietary supplement, showing a slightly reduced increase in the supplemented group compared to controls.

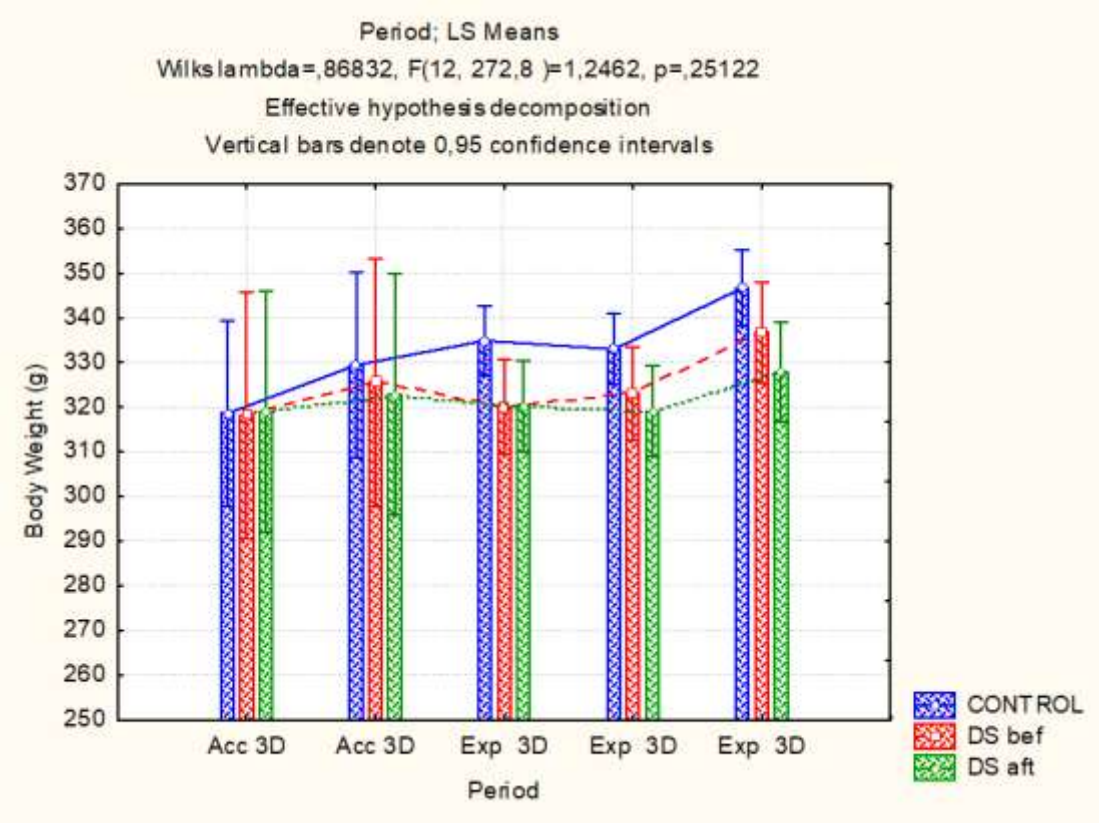


Figure 12: Evolution of body weight during acclimatization and experimentation periods in control and supplemented date seed ($p \leq 0.05$)

DS before: body weight of rat supplemented with date seed before administration.
DS one hour after administration.

The results shown in Figure X indicate a normal progression and a consistent increase in body weight in both groups of rats during the acclimatization period. By the end of this period, the treated group reached an average body weight of 329.4 ± 11.15 g, while the control group averaged 325.6 ± 12.3 g, demonstrating comparable growth patterns between the two groups.

1.2.Liver absolute weight

Figure 14 shows no significant change in absolute liver weight in male rats following exposure to the date seed-based dietary supplement, although the supplemented group exhibited a slightly lower value compared to controls. This suggests that the treatment had no meaningful effect on liver weight.

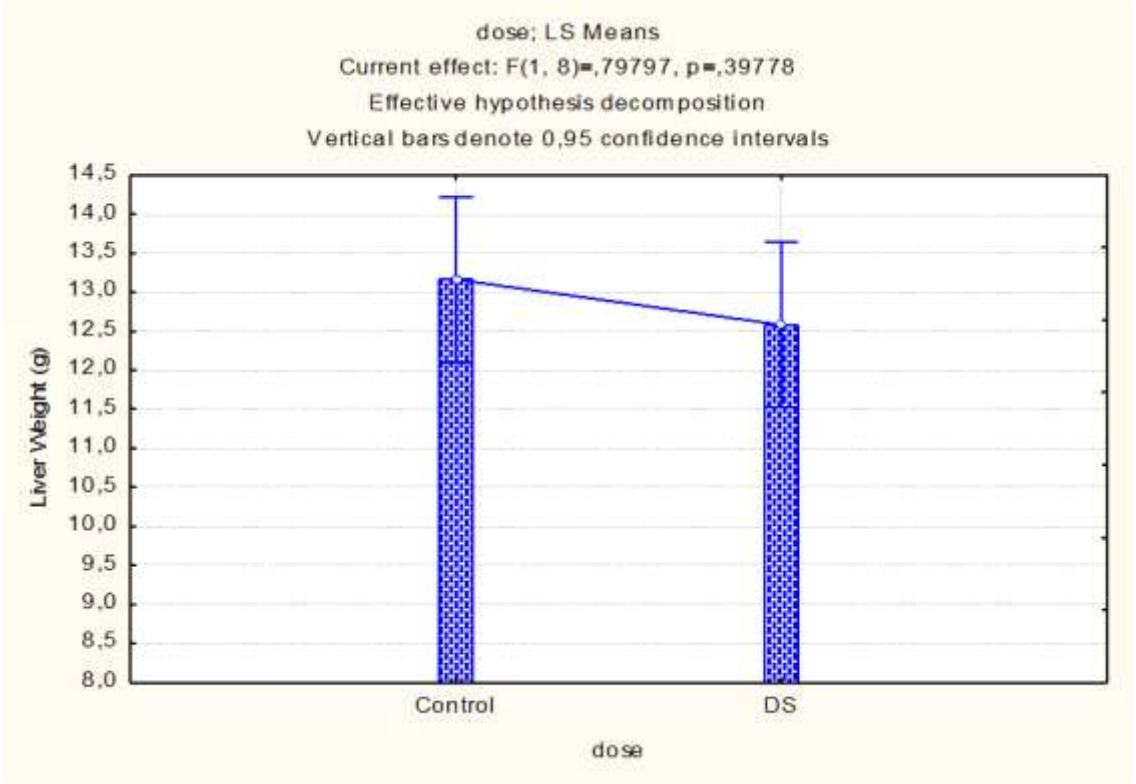


Figure 13: Evolution of liver weight after treatment in control and supplemented date seed ($p \leq 0.05$). DS: date seed.

2. Liver biochemical analyses

2.1.Glycemia

Figure 15 shows variations in glycemia levels in male rats following subacute exposure to the date seed-based dietary supplement, with the supplemented group (1, 5 ± 0.1) exhibiting a slightly higher increase compared to controls (1, 4 ± 0, 09).

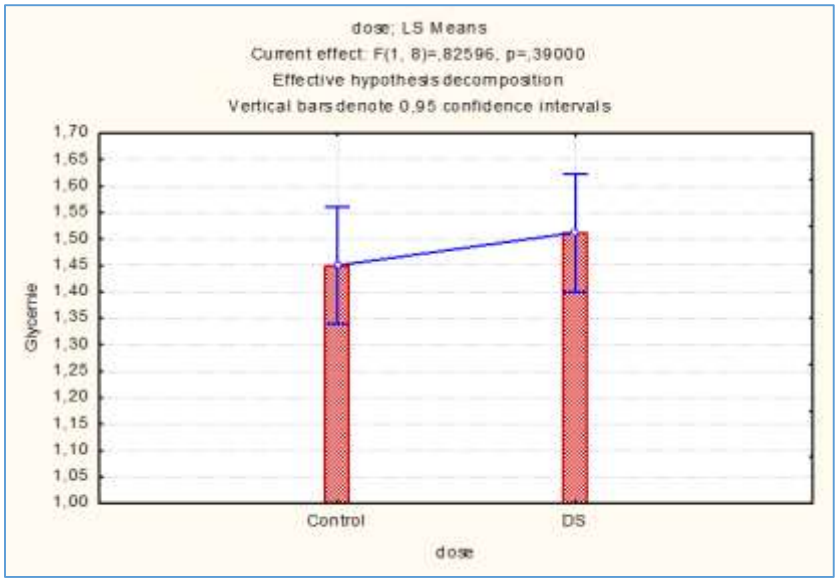


Figure 14: Variations in glycemia levels in the supplemented and control groups (p≤0.05).
DS: date seed.

2.1.1. Asparate aminotransferase

Figure 16 shows variations in Aspartate aminotransferase (ASAT) levels in male rats following subacute exposure to the date seed-based dietary supplement, with the supplemented group (112,7 ± 0,6) exhibiting a slightly higher increase compared to controls (112,8 ± 3,6).

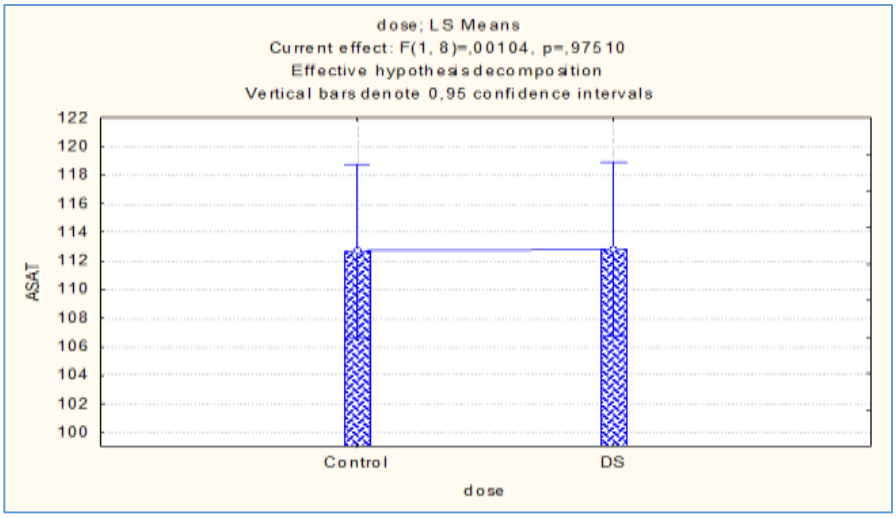


Figure 15: Variations in ASAT levels in the supplemented and control groups (p≤0.05).
DS: date seed.

- **Alamine Aminotransferase**

Figure 17 shows variations in alanine aminotransferase (ALAT) levels in male rats following subacute exposure to the date seed-based dietary supplement, with the supplemented group (174,4 ± 0,9) exhibiting a slightly higher increase compared to controls (137,8 ± 21,1).

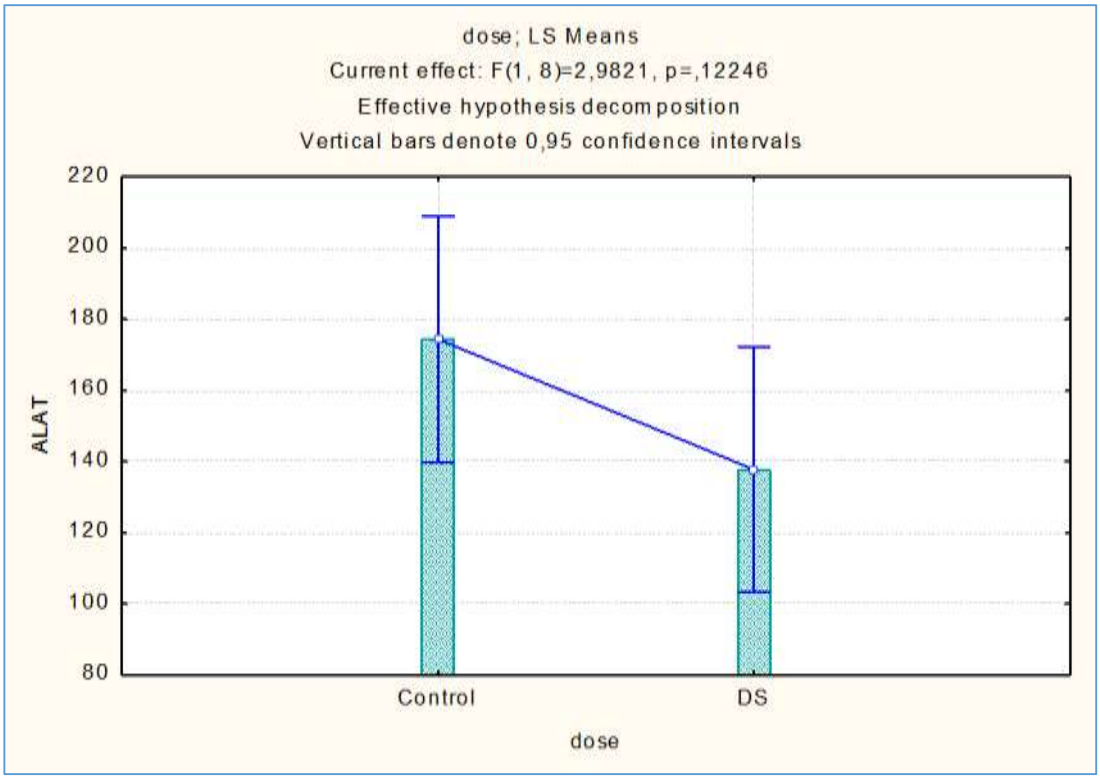


Figure 16: Variations in ALAT levels in the supplemented and control groups (p≤0.05).
DS: date seed.

The results presented in the figure indicate that blood glucose levels remained stable between the control and treated groups (1.4 g/L vs. 1.5 g/L), as revealed by the biochemical analysis. Similarly, ASAT and ALAT levels showed no significant variation (P≤0.05), suggesting the absence of notable hepatic cytolysis.

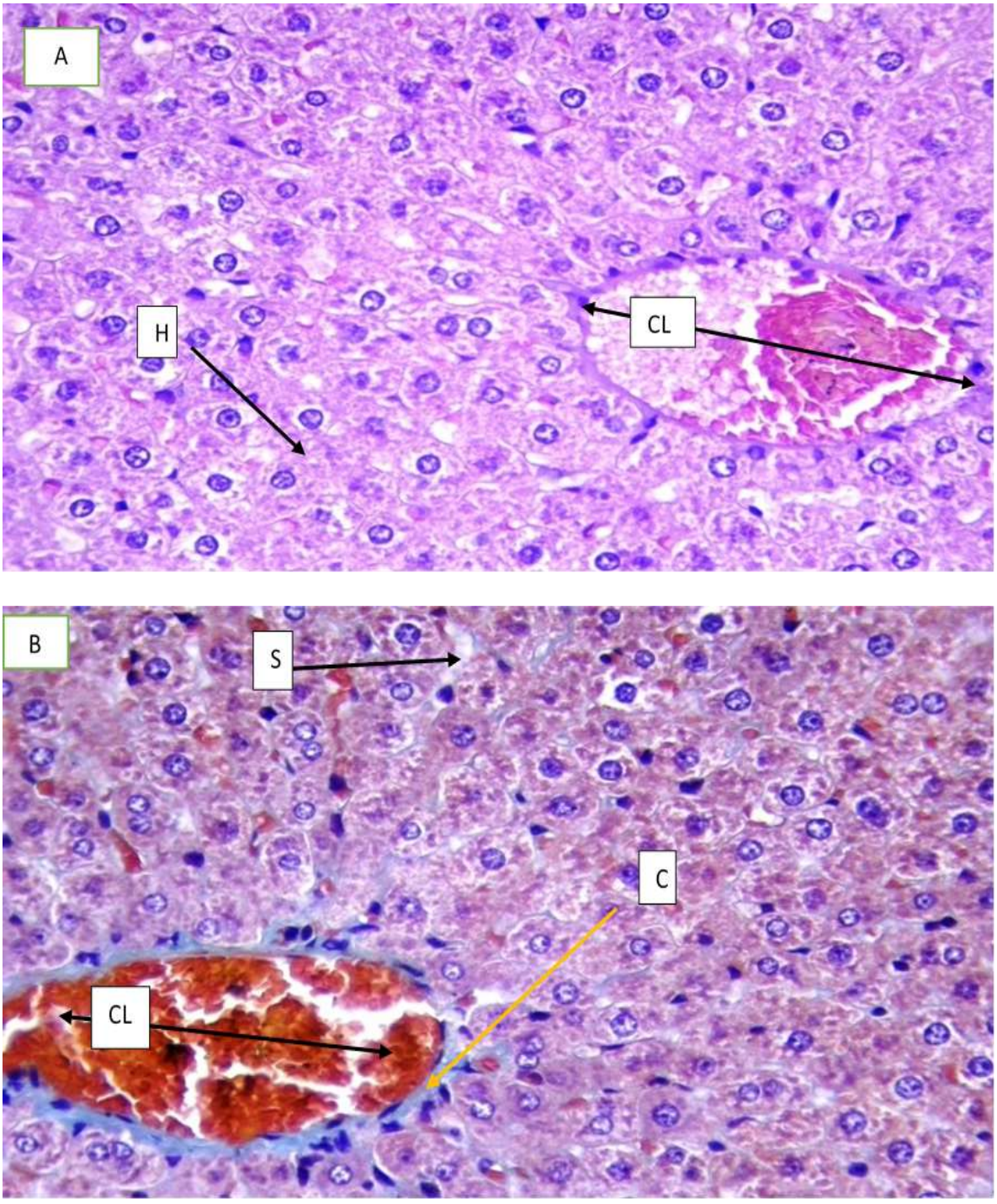
These findings should be interpreted in conjunction with histological and complementary data for a comprehensive assessment.

3. Histological results

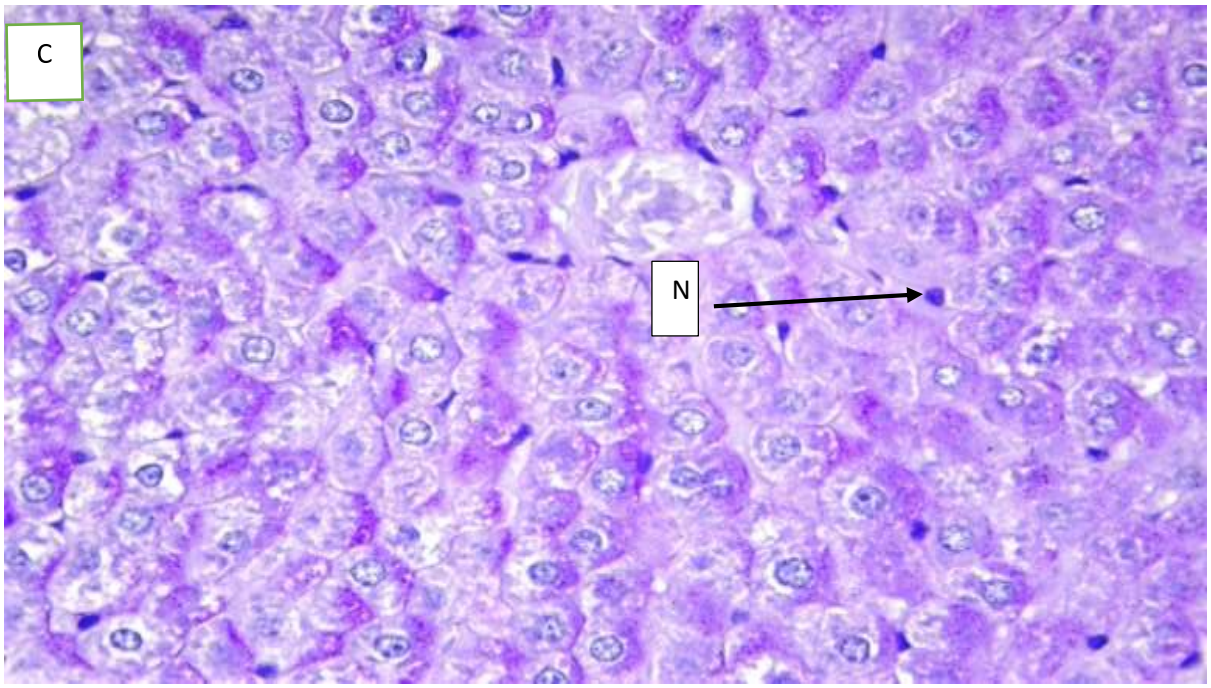
To assess the effect of the date seed-based dietary supplement on hepatic architecture, a detailed histological study of the liver was conducted. The staining techniques employed included topographical stains such as Hematoxylin and Eosin (H&E) and Masson's Trichrome; histochemical stains such as the Periodic Acid-Schiff (PAS) reaction and Reticulin; and Immunohistochemical markers including alpha-fetoprotein, Ki-67, Bcl-2, and β-catenin.

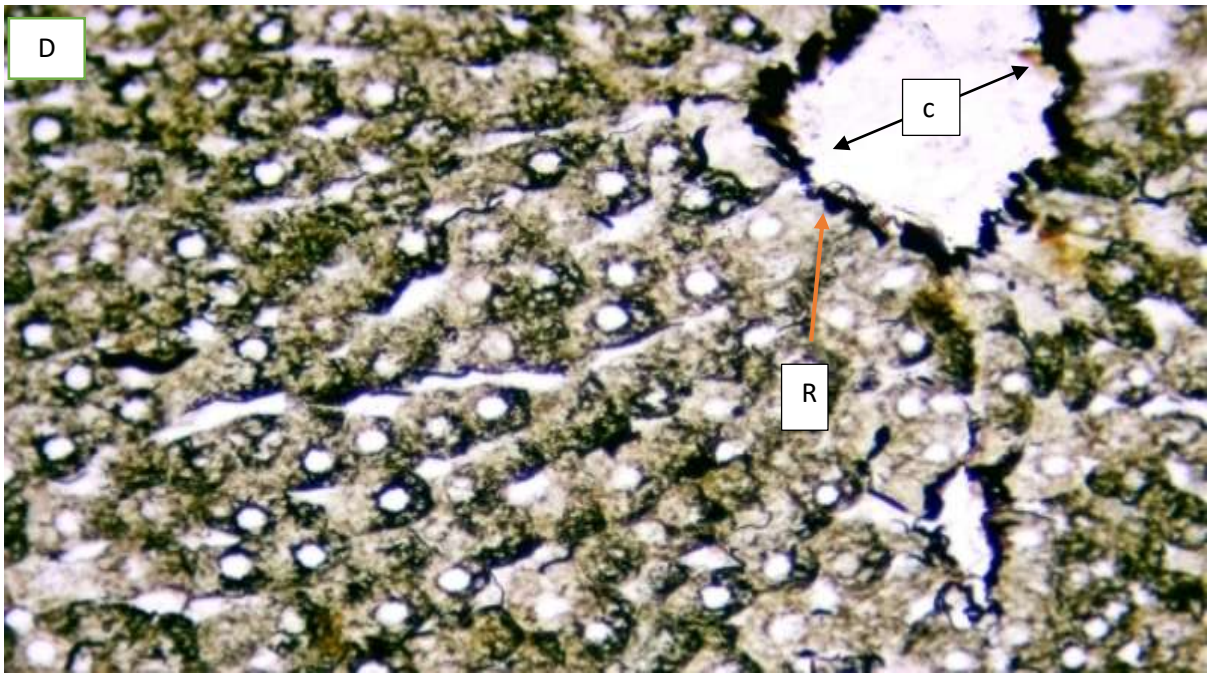
Through the examination of histological sections at various magnification levels, we evaluated the structural organization of liver tissue and assessed the potential effects of the treatment administered to the animals.

Control group Hematoxylin and Eosin (H&E) and Masson's Trichrome



Plank 1: Image (A) shows a histological section of rat liver from the control group, stained with Hematoxylin and Eosin (H&E). This preparation reveals the hepatic architecture, characterized by numerous polyhedral hepatocytes (H). Each hepatocyte has a central basophilic nucleus. A central vein (CL) is clearly visible, identifiable by its wide lumen filled with red blood cells. (Magnification ×40). **Image (B)** shows the liver section stained with Masson's Trichrome, highlighting nuclei in blue and collagen fibers (C) in blue around the vessel. (Magnification ×40)

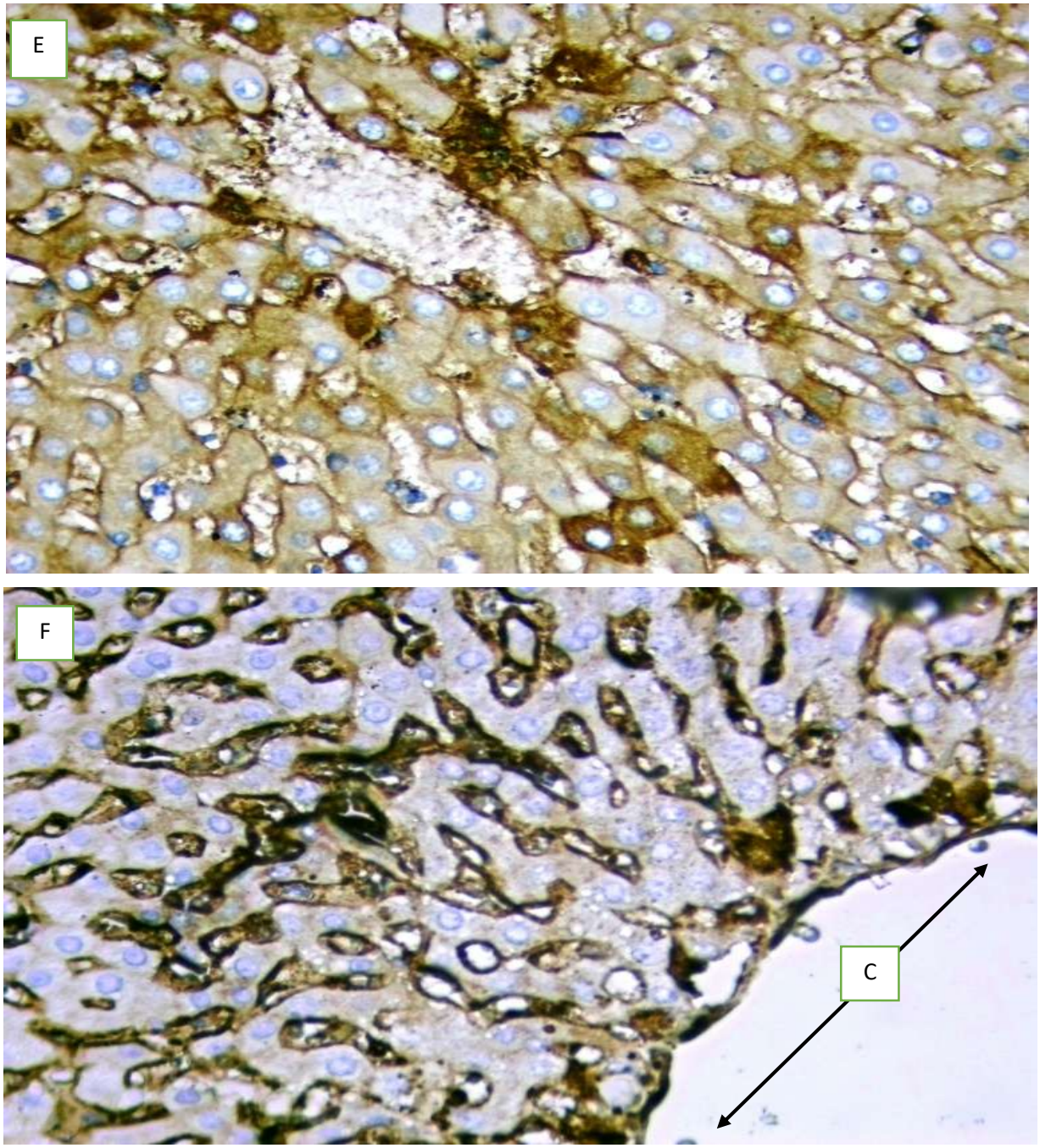




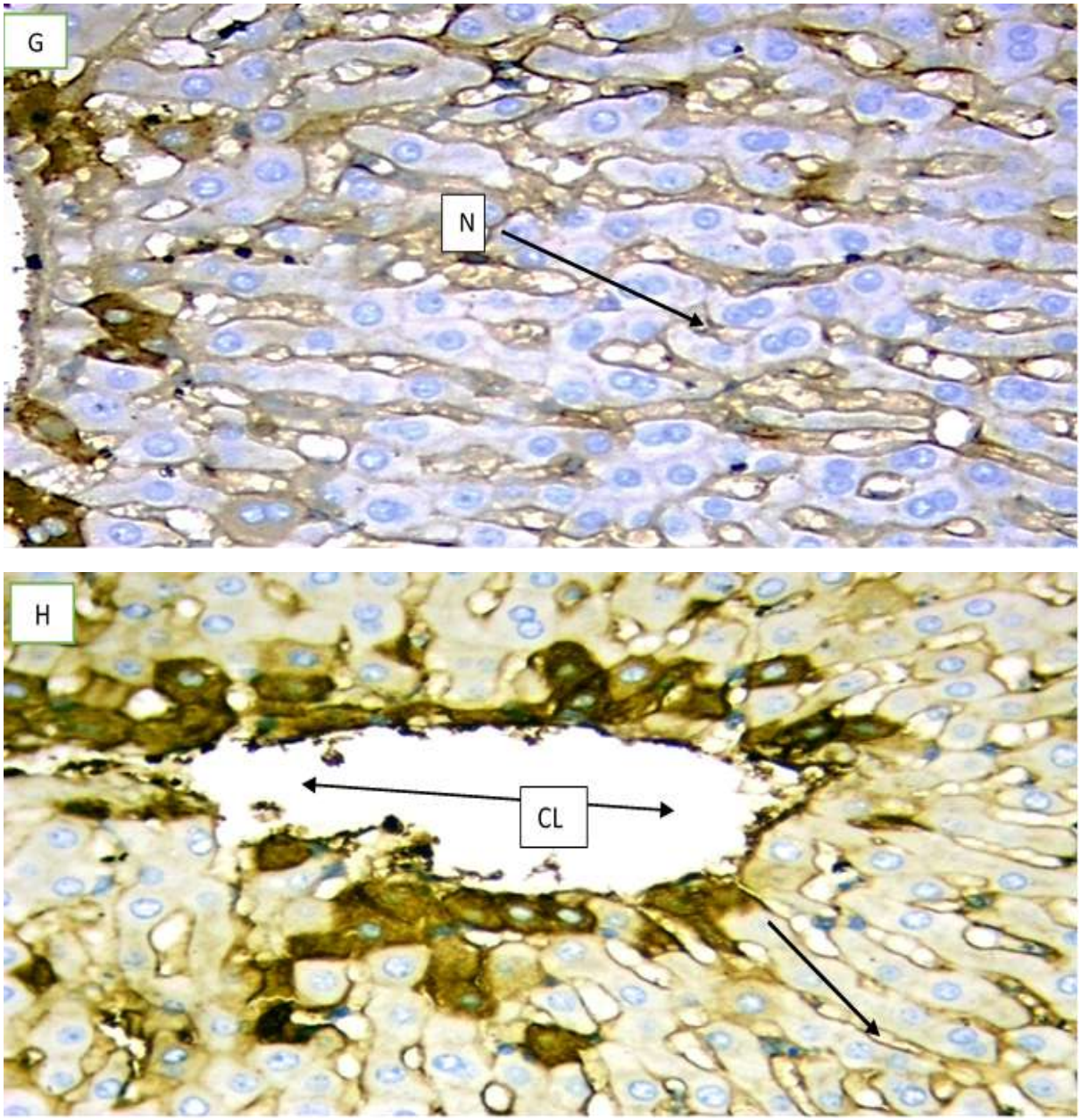
plank 2: Image (C) shows a histological section of rat liver from the control group, stained with Periodic Acid-Schiff (PAS). This technique clearly reveals the presence of glycogen in the cytoplasm of hepatocytes, which appears as a diffuse purple staining. The homogeneous and widespread distribution of this staining indicates that the hepatocytes are rich in glycogen, a storage form of glucose, which is a normal functional characteristic of the liver. Hépatocytes with their blue-stained nuclei are also visible.

Image (D) represents a specific staining technique for reticuline fibers **(R)** of the hepatic tissue, clearly demonstrating the reticulin fiber network in black as previously shown in the image

Immunohistochemistry study α-Fetoprotein, KI67; Bcat, BCL2



plank 3: Histological section of rat liver (control group) stained immunohistochemically for alpha-fetoprotein (AFP) and ki67. A negative immunoreaction is observed in hepatocytes, highlighted by blue-stained nuclei, indicating the absence of expression. A slight positive immunoreaction is present, reflecting normal cell proliferation in the healthy adult liver. (G x40)

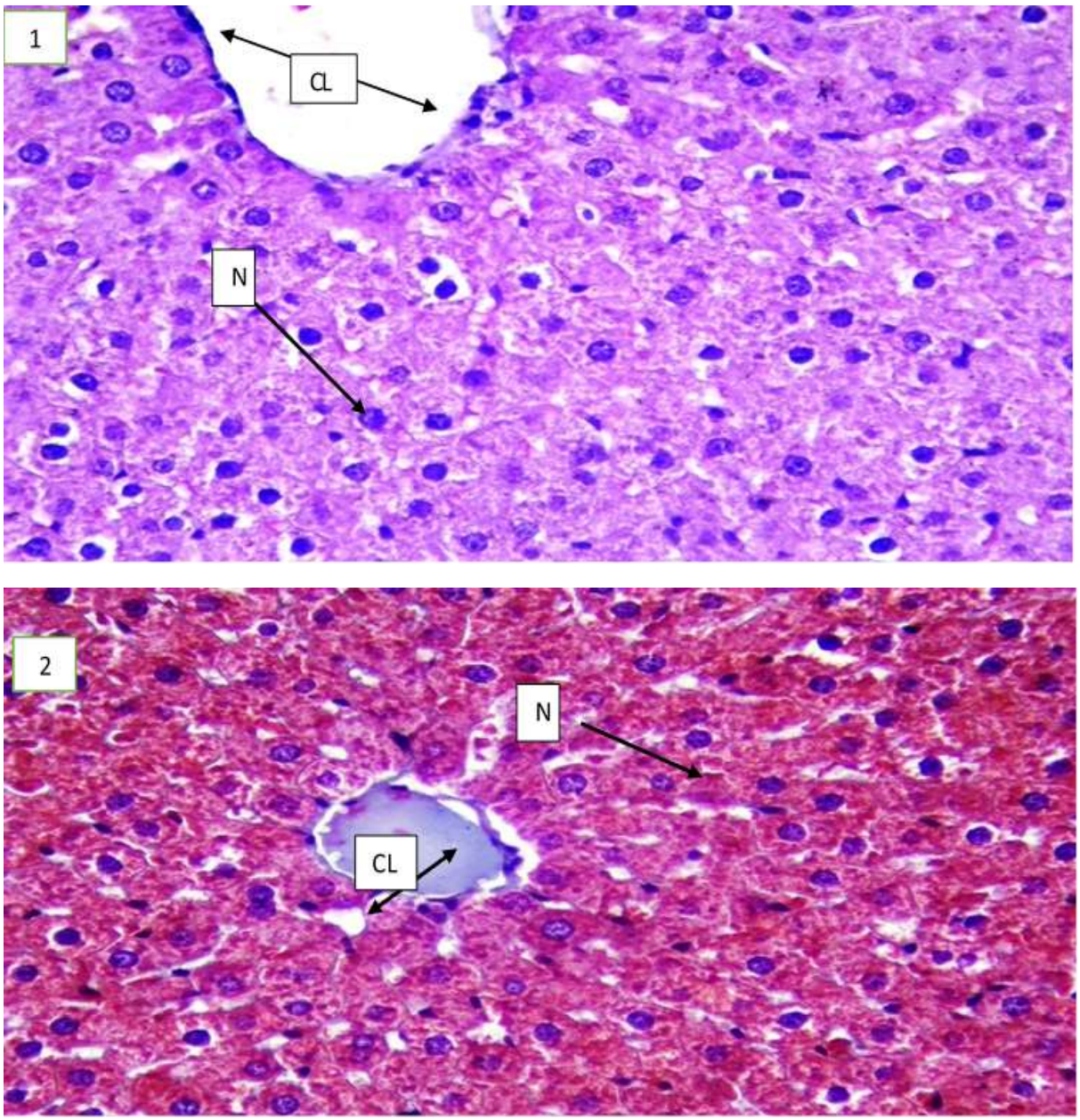


plank 4: Section of rat liver (control group) stained for β-catenin. **In image (G),** the staining is normally localized at the cell membrane (associated with adherens junctions). The image shows a homogeneous membranous staining pattern, with no abnormal nuclear or cytoplasmic accumulation.

In **image (H),** staining for Ki67, a marker of cellular proliferation, reveals few or no positively stained nuclei (absence of brown staining). This indicates a low mitotic activity, which is consistent with a quiescent liver, without pathological proliferation. (G x40).

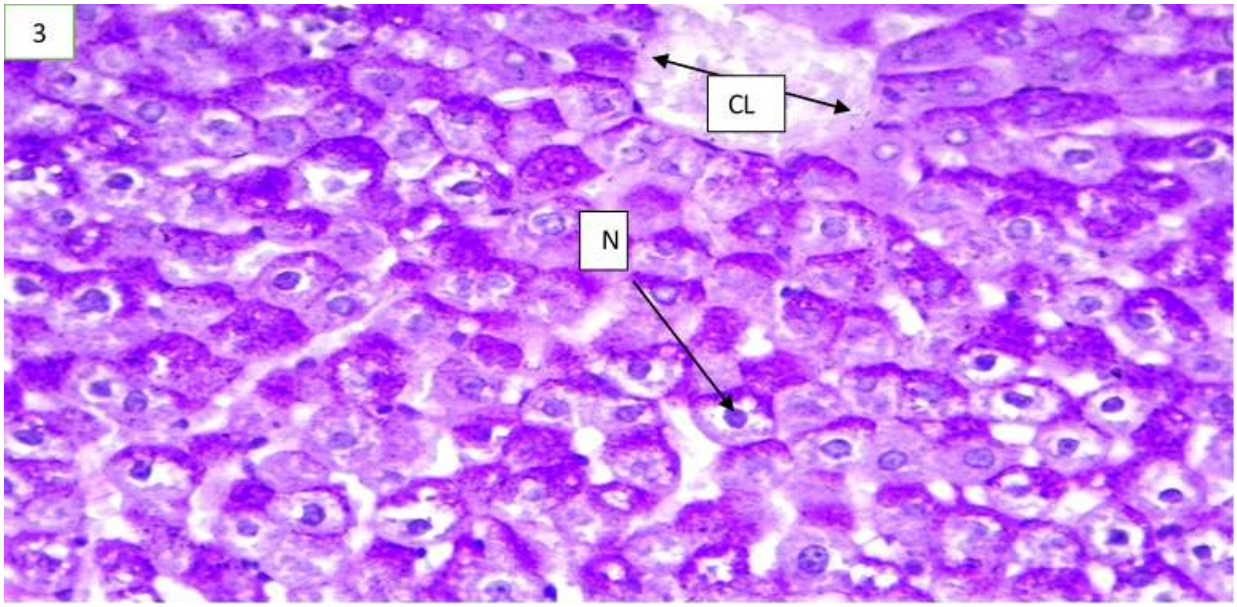
Supplemented group

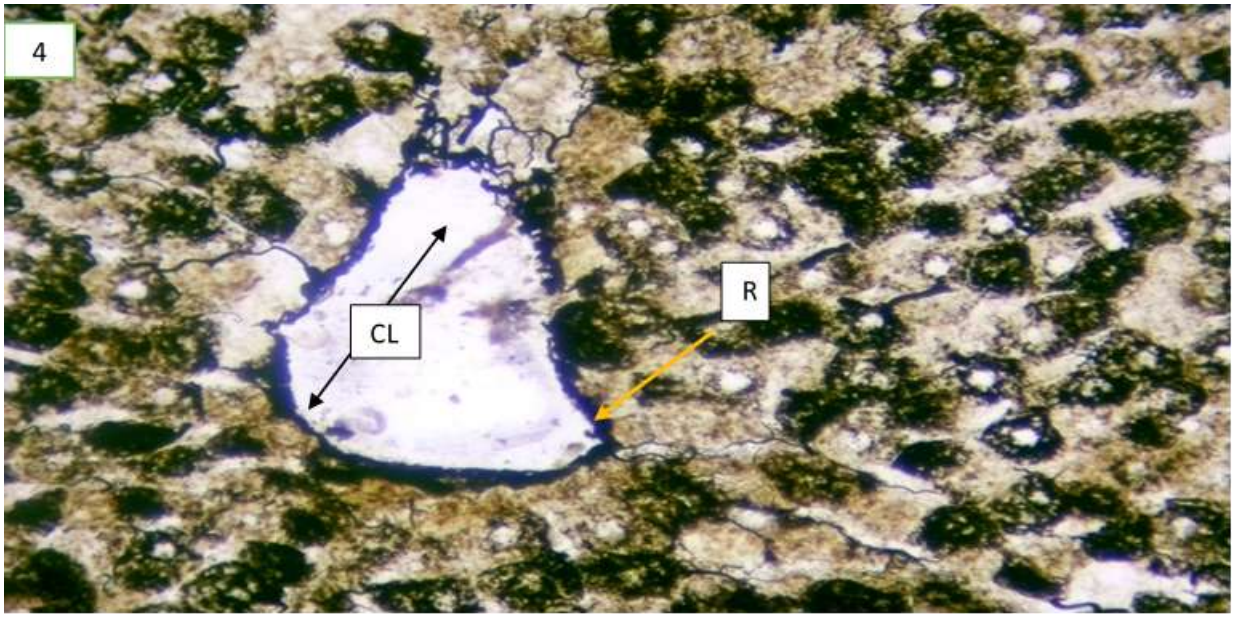
Topographical histological stains: Hematoxylin and Eosin (H&E) and Masson's Trichrome and histochemical stain: Periodic acid Schiff reaction (PAS) and Reticuline.



plank 1: **Image 1** Histological section of treated rat liver, Hematoxylin-Eosin (H&E) staining, shows a well-preserved lobular architecture, characterized by trabeculae of hepatocytes radiating around the centrilobular vein **(CL).** Hepatocytes appear with homogeneous cytoplasm and rounded nuclei, sometimes bi-nucleated or mono-nucleated stained blue **(N)** and abundant cytoplasm stained pink. (Gx40).

Image 2 histological section of treated rat liver, Masson's Trichrome staining. The staining highlights a normal distribution of collagen fibers (in blue) around the centrilobular vein. No excessive proliferation of connective tissue is observed. (G x40).

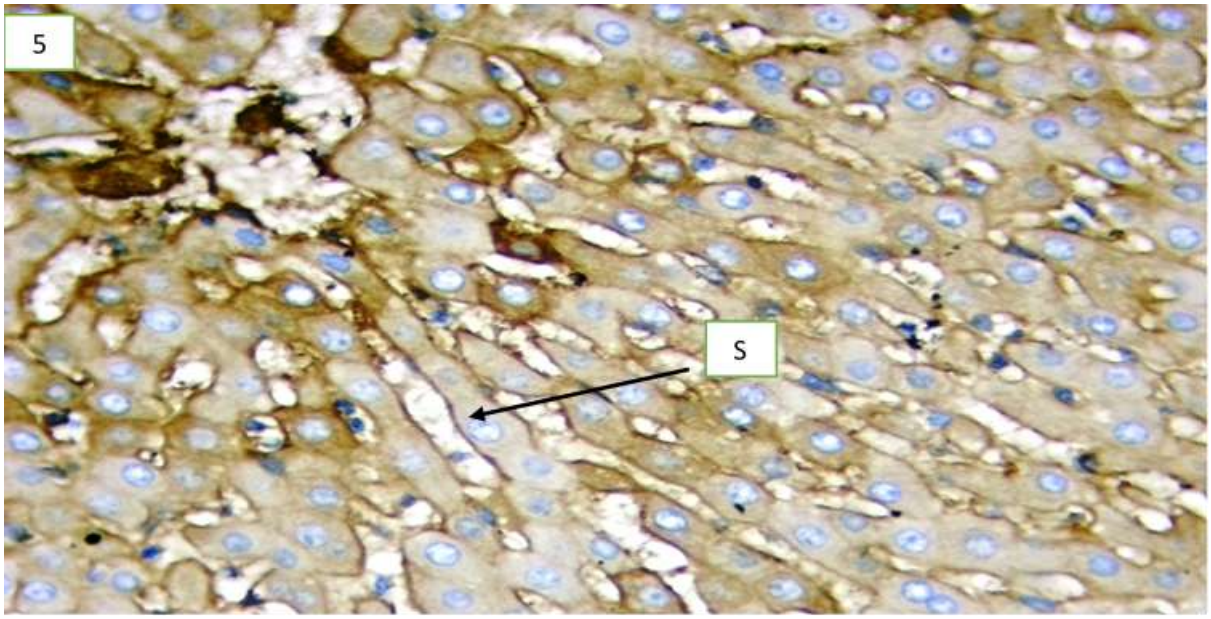


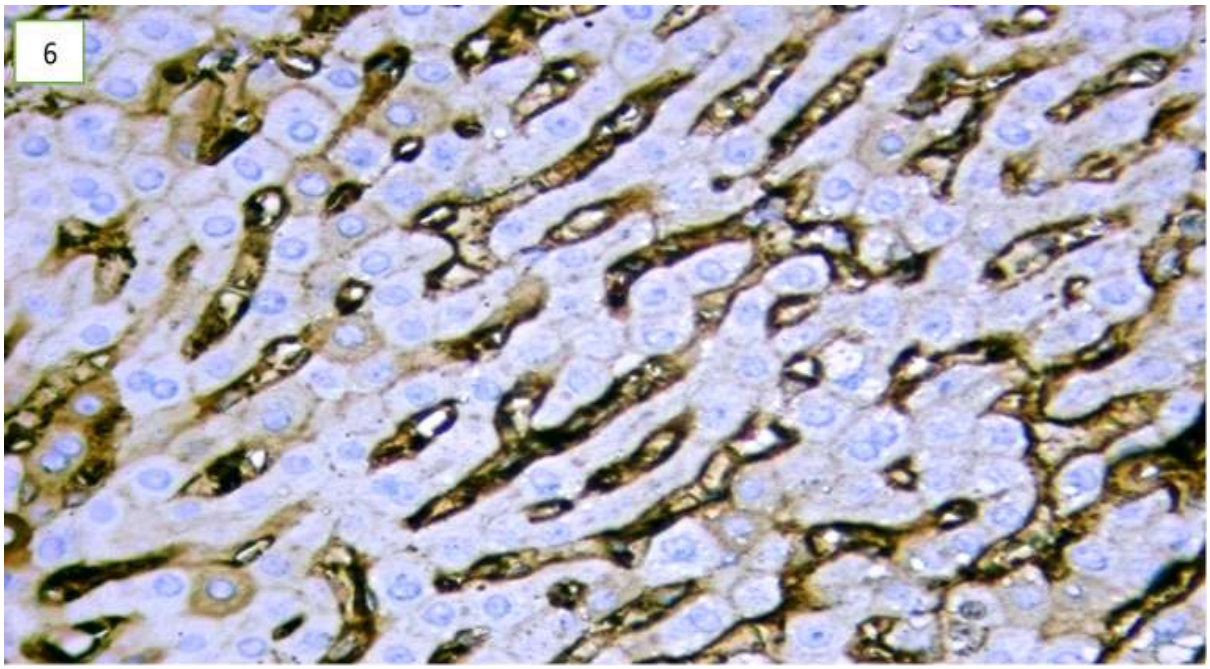


Plank 2 Image 3 highlights a PAS-stained liver tissue section. Intense, diffuse purple/magenta staining of the cytoplasm of the majority of hepatocytes is observed. This staining is characteristic of the abundant presence of glycogen, the storage form of glucose in the liver. The regularity and intensity of this staining indicate a normal and efficient carbohydrate storage metabolic function of the hepatocytes in this field, which is typical of healthy liver tissue.

Image 4 shows a liver section stained specifically for reticulin fibers. A fine, delicate, black network of reticulin fibers is visible, clearly demarcating individual hepatocytes and sinusoids. The presence of a centrilobular vein, the wall of which is also outlined by these fibers, is visible.

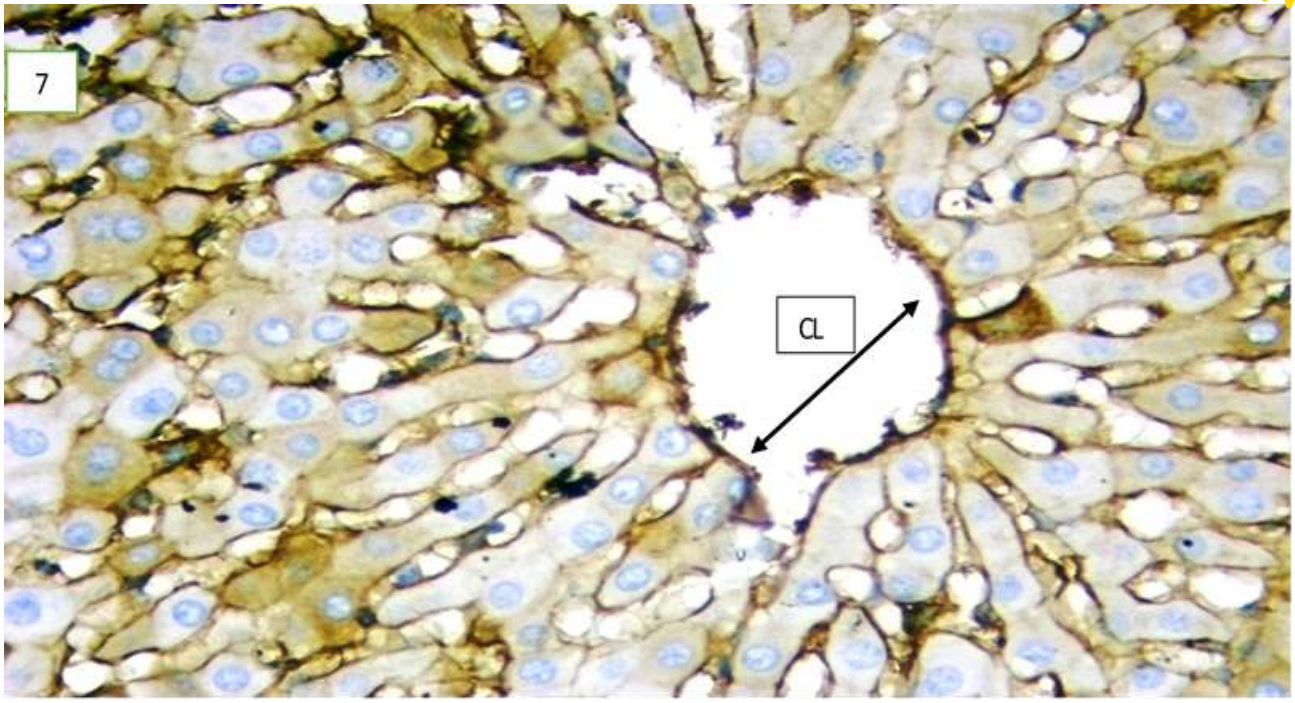
Immunohistochemistry study α-Fetoprotein, KI67; Bcat, BCL2

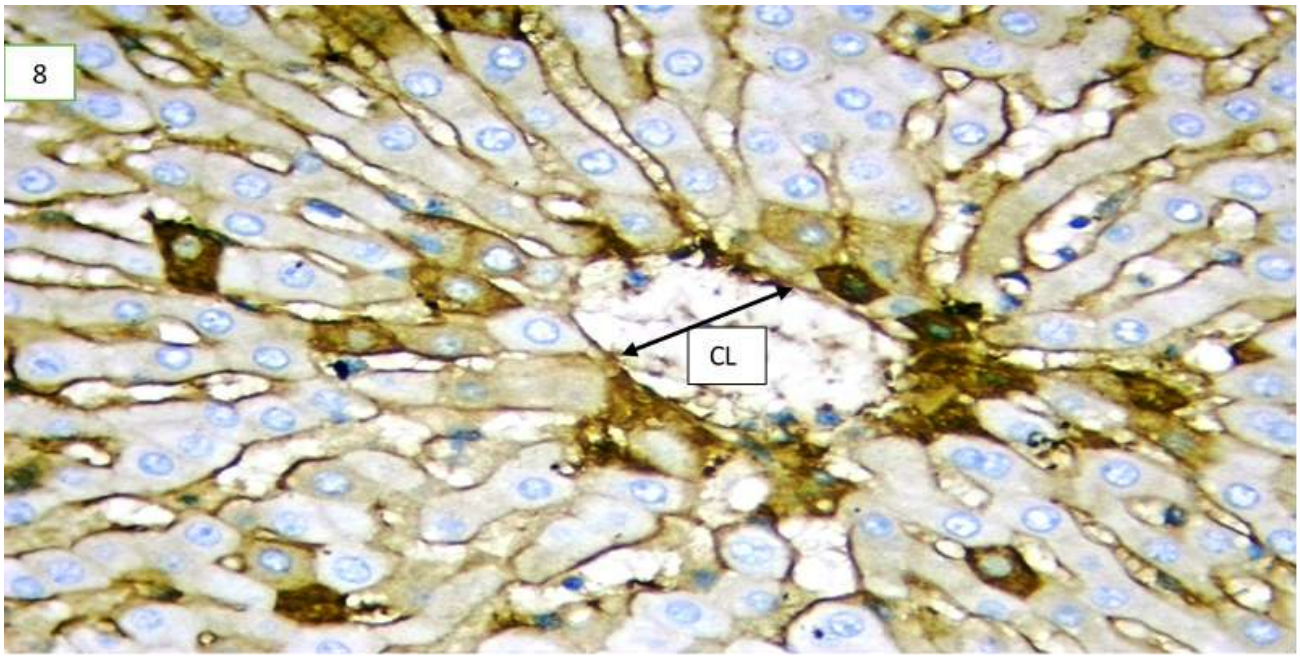




Plank 3 the **image 5** shows a section of the treated rat liver stained with immunostaining for α-fetoprotein which shows an absence of significant positive reaction in hepatocytes. Nuclei are visible in counterstaining (blue).

Image 6 Immunohistochemical staining for Ki-67 reveals few or no positive nuclei in liver tissue. Brown nuclear staining is absent or very weakly expressed. Ki-67 is a specific marker of cells in the proliferative phase. Low or no expression indicates very low mitotic activity.





Plank 4 image 7 shows β-catenin labeling is localized to the plasma membrane of hepatocytes, without accumulation in the cytoplasm or nucleus. β-catenin is involved in cell adhesion (membrane) and in Wnt signaling (abnormal nuclear translocation in pathology). The isolated membrane localization observed here reflects a physiological and not a pathological distribution of the protein. This indicates that the Wnt/β-catenin pathway is not activated in these tissues.

Image 8 shows Immunostaining of the Bcl-2 protein, it reveals a very weak or absent expression in hepatocytes. No significant brown cytoplasmic accumulation is observed in liver cells. The tissue retains a normal architecture, Bcl-2 (B-cell lymphoma 2) is an anti-apoptotic protein that plays a key role in the regulation of cell survival. Its expression is generally low in normal adult liver, and it can be overexpressed in cases of cellular stress, toxic insult or tumor processes.

Discussion

In this study, we evaluated the hepatic safety and histo-architectural integrity of male rats following subacute administration of a date seed–based dietary supplement. The assessment included biochemical analysis, organ weight measurement, and histological and immunohistochemical evaluations.

During the acclimatization and treatment periods, body weight increased normally in both the control and supplemented groups, with no statistically significant differences between them. Similarly, absolute liver weights remained stable, and no significant changes were observed between treated and control animals. These results suggest that the supplement had no adverse effect on general growth or liver mass, indicating systemic and hepatic safety at the administered dose.

These findings were consistent with the biochemical results, where glucose levels, ASAT, and ALAT remained within normal ranges in both groups, confirming the absence of hepatic cytolysis or metabolic stress.

Supporting this, **Abdelaziz et *al*. (2015)** demonstrated that aqueous suspensions of *Phoenix dactylifera* seeds led to a 51% reduction in blood glucose in streptozotocin-induced diabetic rats, along with normalization of liver and kidney function markers (AST, ALT, urea, creatinine). This suggests a protective effect against diabetes-related hepatic and renal complications.

Further, **Shakoor et *al*. (2020)** reported that DS significantly inhibited α-amylase and α-glucosidase activity, enzymes responsible for starch digestion, and enhanced glucose uptake in HepG2 cells. These mechanisms contribute to DS's hypoglycemic and insulin-mimetic effects.

Histological examination using H&E, Masson's Trichrome, PAS, Reticulin, and immunohistochemical markers (alpha-fetoprotein, Ki-67, Bcl-2, β-catenin) revealed no evidence of hepatocellular damage, fibrosis, apoptosis, or abnormal proliferation.

These results align with previous studies highlighting the hepatoprotective and antioxidant potential of date seed extracts (DS). Components such as polyphenols, flavonoids,

and proanthocyanidins have been shown to improve liver function and preserve tissue architecture in various models of hepatic injury. For example, proanthocyanidin-rich DS significantly reduced liver enzyme levels and protected against histological damage in rats exposed to hepatotoxins such as carbon tetrachloride **(Al-Qarawi et *al*., 2008)** and paracetamol **(El Abed et *al*., 2016).**

Type 2 diabetes (T2D) is a chronic disease that accounts for more than 95% of diabetes cases worldwide. In traditional medicine, medicinal plants, including date seed derivatives, have been used as complementary strategies for glycemic control. Date seed powder (DSP) supplementation has shown promising results in improving blood glucose levels and oxidative stress in patients with T2D **(Mohamadizadeh et *al*., 2024).**

Collectively, our findings support the safety profile of date seed supplementation, particularly regarding liver morphology and function. The lack of change in body and liver weights, combined with preserved liver histology and stable biochemical markers, indicates that the supplement does not induce hepatic stress or toxicity. While our study did not involve diabetic models, the results are consistent with existing literature suggesting the beneficial metabolic and hepatoprotective effects of date seed derivatives.

Further studies involving diabetic models and long-term administration will help better define the therapeutic potential of date seed–based supplements in metabolic and liver-related disorders.

CONCLUSION

This study investigated the hepatic safety and biological effects of a date seed–based dietary supplement following subacute administration in male Wistar rats. Our findings revealed no significant alterations in either body or absolute liver weights, indicating that the supplement did not negatively affect general growth or liver development during the exposure period.

Histological evaluations using topographical (H&E, Masson's Trichrome), histochemical (PAS, Reticulin), and immunohistochemical (alpha-fetoprotein, Ki-67, Bcl-2, and β-catenin) staining techniques confirmed the preservation of normal hepatic architecture. The liver sections of supplemented animals showed intact hepatic cords, central veins, and sinusoidal organization, with no evidence of fibrosis, inflammatory infiltration, or necrosis. Immunohistochemical analysis further supported the absence of abnormal hepatocyte proliferation, apoptosis, or pre-neoplastic transformation.

Biochemical analyses corroborated these histological findings. Blood glucose levels remained stable between control and treated groups, and liver enzyme activities (ASAT and ALAT) did not show significant elevation, suggesting an absence of hepatic cytolysis or metabolic disturbance. These results align with previous studies reporting the hepatoprotective and antioxidant effects of date seed constituents, particularly polyphenols, flavonoids, and proanthocyanidins.

Moreover, the known antidiabetic and enzyme-inhibitory properties of date seed extracts such as the inhibition of α-amylase and α-glucosidase, and enhanced glucose uptake indicate that such supplements may not only be safe but potentially beneficial in metabolic regulation. While this study focused on healthy rats, the preserved liver function and structure under supplementation conditions are promising for potential therapeutic use, especially in metabolic disorders like type 2 diabetes.

In conclusion, the date seed based supplement appears to be hepatically safe in the short term and does not induce toxicological or structural liver changes. These findings lay the groundwork for future research on its long-term safety, efficacy in diabetic models, and possible use as a functional food or adjunct therapy in metabolic disease management.

Perspectives

This study provides a basis for further exploration of date seed–based supplements as safe and potentially beneficial agents for liver and metabolic health. Future research should:

- Investigate their efficacy in disease models such as diabetes and liver injury.
- Conduct long-term and dose-response studies to confirm safety and effectiveness.
- Explore underlying mechanisms at the molecular level.
- Initiate clinical trials to assess their therapeutic value in humans.
- Develop functional food applications, promoting sustainable use of date seed by-products.

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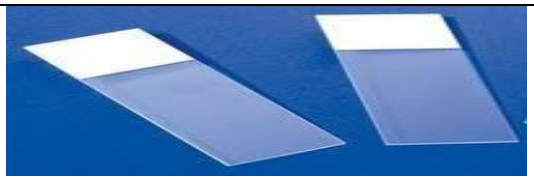
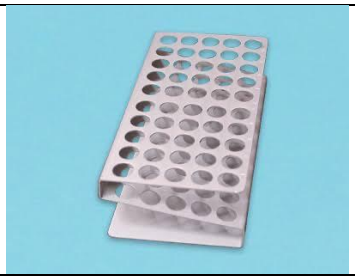
Appendices

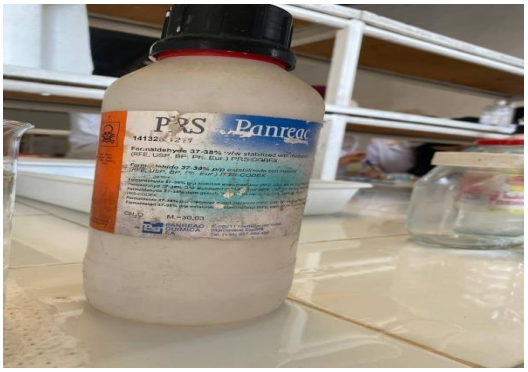
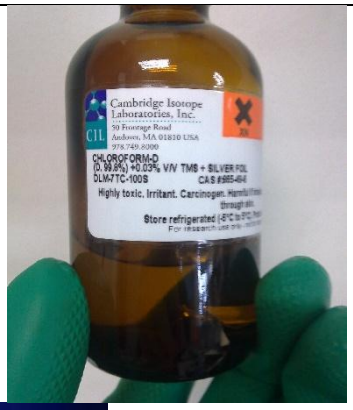
Appendices Appendix

1: Small materials and laboratory glassware

Beacher	
Lame : normal and signalise	
Syringue 5ml	
Syringue 10ml	
Heparin tube	
Balance	
Cassettes	
Biopsy containers	

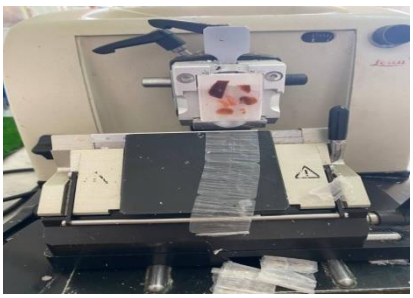
Dissection kit	
Aluminum foil	
Tube rack	
Pins	
Cotten	

Appendix 2 : Reagents and drugs

chloroform	
Formalin	 

Appendix 3 : Equipment used in this study and software

APPENDICES

Equipement	Brand	Picture (original)
Circulatoire automate	Leica	
Inclusion automate	Leica EG1150 H	
Bain-marie	Thermo scientific tissue flotation bath	
Microtome automate	Leica	
Immunohistochemical automate	Ventana BenchMark Ultra	

Statistica	Version 10.0 , soft Inc, Tulsa, Oklahoma, USA	
Anova		

PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH SAAD DAHLAB UNIVERSITY - BLIDA 1

Faculty of Natural and Life Sciences

Biology Department

Final dissertation

To obtain a Master's degree in the Biological Sciences

Option: PHARMACOTOXICOLOGY

Theme

Histological and immunohistochemically study of liver in animals supplemented with food supplements.

Presented by:

BENHAGOUGA RAYAN ISSAADI RAMI DEMANE ABDELKADER

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