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Thèse En vue de l'obtention d'un Diplôme de Doctorat

Domaine : SCIENCE DE LA NATURE ET DE LA VIE
Filière : SCIENCES BIOLOGIQUES
Spécialité : Ecophysiologie Animale

Intitulé

Etude des effets nocifs d'un solvant organique (toluène) sur la physiologie testiculaire, hépatique et hématologique et évaluation phyto-protectrice par une plante algérienne (*Punica granatum*) chez les rats Wistar

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FACULTY OF SCIENCES
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LABORATORY OF ANIMAL ECOPHYSIOLOGY

Thesis in view of obtaining a Doctorate Degree

Domaine: SCIENCE OF NATURE AND LIFE
Failure: BIOLOGICAL SCIENCES
Specialty: Animal Ecophysiology

Intitled

Study of the harmful effects of an organic solvent (toluene) on testicular, hepatic and hematological physiology and phytoprotective evaluation by an Algerian plant (*Punica granatum*) in Wistar rats.

Presented by: ARKOUB Fatma Zohra

Thesis Director : Pr. KHELILI Kamel, U. Badji Mokhtar - Annaba

In front of a jury composed of:

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بسم الله الرحمن الرحيم

{وَهُوَ الَّذِي أَنْزَلَ مِنَ السَّمَاءِ مَاءً فَأَخْرَجْنَا بِهِ نَبَاتَ كُلِّ شَيْءٍ فَأَخْرَجْنَا مِنْهُ خَضِرًا نُخْرِجُ مِنْهُ حَبًّا مُتَرَاكِبًا وَمِنَ النَّخْلِ مِنْ طَلْعِهَا قِنْوَانٌ دَانِيَةٌ وَجَنَّاتٍ مِنْ أَعْنَابٍ وَالزَّيْتُونَ وَالرُّمَّانَ مُشْتَبِهًا وَغَيْرَ مُتَشَابِهٍ انْظُرُوا إِلَى ثَمَرِهِ إِذَا أَثْمَرَ وَيَنْعِهِ إِنَّ فِي ذَلِكُمْ لَآيَاتٍ لِقَوْمٍ يُؤْمِنُونَ}

صدق الله العظيم

الآية 99 من سورة (الأنعام)

Abstract

The present-day lifestyle behaviors are major factors that adversely influence health and increase susceptibility to diseases. Therefore, Medicinal plants and their essential oils and extracts have been used to a large extent as possible strategies for modulating and reducing the disease risks associated with exposure to toxic chemicals. The current study was designed to investigate the adverse outcomes of subchronic exposure to toluene (Tol) on reproductive, hepatic, renal, and hematological function parameters, as well as the potential efficacy of *Punica granatum* juice (PJ) and peel aqueous extract (PAE) against the deleterious effects resulting from exposure to this solvent. Simultaneously, phytochemical analysis and assessment of the free radical scavenging activity (DPPH) were performed for the PJ and the PAE. The *in vivo* study was carried out using 70 male Wistar rats that were divided into 7 groups, each consisting of 10 rats. All groups were treated orally for 6 weeks as follows: Control (C), positive controls (CO: 1.25 mL/kg body weight [BW] of corn oil; PJ: 4 mL/kg BW; and PAE: 400 mg/kg BW), Tol (550 mg/kg BW diluted in corn oil), and a mixture each of PJ-Tol and PAE-Tol. At the end of the 45th day of the study, the sperm parameters, hepato-renal biochemical, and hematological markers were auto-analyzed, along with the measurement of testosterone concentration, oxidative stress markers, and the histopathological examination. The phytochemical analysis revealed that the DPPH scavenging activity and the total phenolic, flavonoid, and tannin contents were higher in the pomegranate peel extract versus the juice. The *in vivo* obtained results showed that Tol significantly decreased testes and epididymis weight, sperm concentration, motility, vitality, and plasma testosterone levels, with a significant elevation in dead sperm percentage. Notable changes in hepatic and renal markers were manifested by a significant increase in liver and kidney weight, enzymatic activities of AST, ALT, and ALP, total bilirubin, urea, and creatinine levels, whereas albumin and total proteins were significantly decreased. Toluene also induced a significant reduction in RBC, HGB, and HCT levels joined with an increase in WBC and LYM levels. Likewise, toluene led to increased levels of lipid peroxidation (MDA) and decreased antioxidant capacity (GSH and GPx). The histological observed changes confirm the aforementioned results. A significant improvement in almost all of the studied parameters was observed in the PAE-Tol group compared with the Tol and PJ-Tol groups. Based on the present findings, we can conclude that exposure to Tol can provoke testicular, hepatic, and renal dysfunction, oxidative damage, and hematological and histological profile alteration. In addition, PAE indicated greater effectiveness in attenuating Tol toxicity compared to PJ due to its antioxidant activity.

Keywords: Antioxidant activity, Oxidative damage, *Punica granatum*, Toluene, Rats

Résumé

Les comportements liés au mode de vie actuel sont des facteurs majeurs qui influencent négativement la santé et augmentent la susceptibilité aux maladies. Par conséquent, les plantes médicinales, leurs huiles essentielles et leurs extraits ont été largement utilisés comme stratégies possibles pour moduler et réduire les risques de maladie associés à l'exposition aux produits chimiques toxiques. La présente étude visait à étudier les effets néfastes d'une exposition subchronique au toluène (Tol) sur les paramètres des fonctions reproductive, hépatique, rénale et hématologique, ainsi que l'efficacité potentielle du jus de *Punica granatum* (PJ) et de l'extrait aqueux d'écorce (PAE) contre les effets délétères résultant de l'exposition à ce solvant. Simultanément, une analyse phytochimique et une évaluation de l'activité de piégeage des radicaux libres (DPPH) ont été réalisées pour le PJ et le PAE. L'étude *in vivo* a été réalisée sur 70 rats Wistar mâles, divisés en 7 groupes, chacun composé de dix rats. Tous les groupes ont été traités par voie orale pendant 6 semaines comme suit : Témoin (C), témoins positifs (CO : 1,25 mL/kg de poids corporel [PC] de huile de maïs; PJ : 4 mL/kg de PC ; et PAE : 400 mg/kg de PC), Tol (550 mg/kg de PC dilué dans l'huile de maïs), et un mélange de PJ-Tol et de PAE-Tol. À la fin du 45ème jour de l'étude, les paramètres du sperme, les marqueurs biochimiques hépato-rénaux et hématologiques ont été auto-analysés, ainsi que la mesure de la concentration de testostérone, les marqueurs du stress oxydatif et l'examen histopathologique. L'analyse phytochimique a révélé que l'activité de piégeage du DPPH et les teneurs totales en composés phénoliques, flavonoïdes et tanins étaient plus élevées dans l'extrait d'écorce de grenade que dans le jus. Les résultats obtenus in vivo ont montré que le Tol a significativement diminué le poids des testicules et de l'épididyme, la concentration, la motilité et la vitalité des spermatozoïdes, ainsi que la concentration de testostérone, avec une augmentation significative du pourcentage de spermatozoïdes morts. Des changements notables dans les marqueurs hépatiques et rénaux se sont manifestés par une augmentation significative du poids du foie et des reins, des activités enzymatiques d'ASAT, ALAT et PAL, des taux de bilirubine totale, d'urée et de créatinine, tandis que l'albumine et les protéines totales étaient significativement diminuées. Le toluène a également induit une augmentation des concentrations de peroxydation lipidique (MDA) et une baisse de la capacité antioxydante (GSH et GPx). De même, la toxicité du toluène a entraîné une réduction significative des nombres de GR, taux de HB et de HTC et a augmenté les nombres de GB et taux de LYM. Les changements histologiques observés confirment les résultats susmentionnés. Une amélioration significative dans presque tous les paramètres étudiés a été observée dans le groupe PAE-Tol par rapport aux groupes Tol et PJ-Tol. Sur la base des résultats actuels, nous pouvons conclure que l'exposition au Tol peut provoquer un dysfonctionnement testiculaire, hépatique et rénale, des dommages oxydatifs et une altération du

profil hématologique et histologique. En outre, Le PAE a indiqué une plus grande efficacité dans l'atténuation de la toxicité de Tol que le PJ en raison de son activité antioxydante.

Mots clés: Activité antioxydante, Dommage oxydatif, *Punica granatum*, Toluène, Rats.

الملخص

تعتبر سلوكيات نمط الحياة الحالية عوامل رئيسية تؤثر سلبًا على الصحة وتزيد من التعرض للأمراض. لذلك، تم استخدام النباتات الطبية وزيوتها ومستخلصاتها الأساسية إلى حد كبير كاستراتيجيات ممكنة لتعديل وتقليل مخاطر المرض المرتبطة بالتعرض للمواد الكيميائية السامة. تم تصميم الدراسة الحالية للتحقيق في النتائج الضارة للتعرض شبه المزمن للتولوين (Tol) على المؤشرات الوظيفة التناسلية، الكبدية، الكلوية، والدموية، بالإضافة إلى الفعالية المحتملة لعصير *Punica granatum* (PJ) ومستخلص القشر المائي (PAE) ضد الآثار الضارة الناتجة عن التعرض لهذا المذيب. بشكل متزامن، تم إجراء تحليل كيميائي نباتي وتقييم لنشاط كسح الجذور الحرة (DPPH) لكل من PJ وPAE. أجريت الدراسة في الجسم الحي باستخدام 70 من ذكور فئران ويستار التي تم تقسيمها إلى 7 مجموعات، تتكون كل منها من 10 فئران. عولجت جميع المجموعات عن طريق الفم لمدة 6 أسابيع على النحو التالي: مجموعة الشاهد (C)، الشواهد الإيجابية (CO: 1.25 مل/كلغ من وزن الجسم زيت الذرة ؛ PJ: 4 مل/كغ من وزن الجسم؛ و PAE: 400 ملغ/كلغ من وزن الجسم)، Tol (550 ملغ/كلغ من وزن الجسم مخفف في زيت الذرة)، ومزيج من كل من PJ-Tol و PAE-Tol. في نهاية اليوم 45 من الدراسة، تم تحليل مؤشرات الحيوانات المنوية، المؤشرات البيوكيميائية الكلوية والكبدية، مؤشرات الدم، بالإضافة إلى قياس تركيز هرمون التستوستيرون، مؤشرات الإجهاد التأكسدي، والفحص النسيجي. أظهر التحليل الكيميائي النباتي أن نشاط الكسح DPPH والمحتوى الكلي للفينول والفلافونويد والتانين كان أعلى في مستخلص قشر الرمان مقابل العصير. أظهرت النتائج التي تم الحصول عليها في الجسم الحي أن Tol أدى إلى انخفاض معنوي في عدد RBC، مستويات HGB وHCT مع زيادة في عدد WBC ومستويات LYM. تجلت التغيرات الملحوظة في مؤشرات الكبد والكلى من خلال زيادة معنوية في وزن الكبد والكلى، و زيادة النشاط الأنزيمي ل AST، ALT، و ALP، مستويات البيليروبين الكلي، اليوريا، والكرياتينين، بينما انخفض الألبومين والبروتينات الكلية بشكل ملحوظ. تسبب التولوين أيضًا في انخفاض معنوي في وزن الخصيتين والبربخ، تركيز، حركة، حيوية الحيوانات المنوية، ومستويات هرمون التستوستيرون، مع ارتفاع معنوي في نسبة الحيوانات المنوية الميتة. كذلك، أدت سمية التولوين إلى زيادة مستويات بيروكسيد الدهون (MDA) وانخفاض قدرة مضادات الأكسدة (GSH و GPx). تؤكد التغييرات النسيجية الملاحظة النتائج المذكورة أعلاه. لوحظ تحسن كبير في جميع المؤشرات المدروسة تقريبًا في مجموعة PAE-Tol مقارنة بمجموعتي Tol وPJ-Tol. بناءً على النتائج الحالية، يمكننا أن نستنتج أن التعرض لـ Tol يمكن أن يؤدي إلى اختلال وظيفي في الخصية والكبد والكلى، الضرر التأكسدي، تغيير المظهر الدموي والنسيجي. بالإضافة إلى ذلك، أشار PAE إلى فعالية أكبر في تخفيف سمية Tol مقارنة بـ PJ بسبب نشاطه المضاد للأكسدة.

الكلمات المفتاحية: النشاط المضاد للأكسدة، الضرر التأكسدي، *Punica granatum* ، التولوين ، الجرذان.

List of Abbreviations

ALDH-1: Aldéhyde déshydrogénase 1 de type H1, ou rétinaldéhyde déshydrogénase 1

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AMPK: AMP-activated protein kinase

AST: Aspartate transaminase

ATP: Adenosine triphosphate

AV: Atrialventricular

BK: Voltage-gated potassium channels

BP: Boiling point

cGMP : Cyclic guanosine monophosphate

CNS : Central nervous system

CYP 2EI: Cytochrome 2E a member of the cytochrome P450 family.

Da: Dalton (unit mass of atoms)

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EPO: Erythropoietin

FASD: Fetal alcohol spectrum disorder

fL: Femtolitre (Metric unit of volume equal to 10^{-15} litres)

GirK: G protein-coupled inwardly-rectifying potassium channels

GST: Glutathion-S-Transferase

H_2O_2: Hydrogen peroxide

Ha: Hectare

HGB: Hemoglobin

IC50: Half maximal inhibitory concentration

IL-1β: Interleukine 1 bêta

IL-6: Interleukine 6

iNOS: Nitric oxide synthases

LDH: Lactate dehydrogenase

LDH-C4: Lactate dehydrogenase isozyme of sperm

Log Ko/w: Octanol/water partition coefficient.

MCV: Mean corpuscular volume

MP: Melting point

NAD: Nicotinamide adenine dinucleotide

NADH: Reduced form of Nicotinamide adenine dinucleotide

NADPH: Reduced form of nicotinamide adenine dinucleotide phosphate

Nrf2: Nuclear factor erythroid 2-related factor 2

Ppm: Parts-per-million

Qx: Quintal (Old unit of mass measurement)

RBCs: Red Blood Cells

ROS: Radical Oxygen Species

ROS: Reactive Oxygen Species

SOD: SuperOxide Dismutase

TLV-TWA: Threshold limit value–Time-weighted average

VOCs: Volatile Organic Compounds

VSA: Volatile Substance Abuse

WBCs: White Blood Cells

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Introduction

From the point of conception and throughout life, humans experience a broad range of physical, biological, and chemical exposures. Lifestyle, as well as various environmental and occupational agents, may impair public health leading to a variety of adverse clinical outcomes affecting certain organic processes efficiency (**kahalerras *et al.*, 2021; Remita *et al.*, 2021; Kahalerras *et al.*, 2022).**

Although not as notorious as alcohol, tobacco, or marijuana, volatile substance abuse remains a common problem in the adolescent population worldwide **(Baydala, 2010).** In contrast to many other notorious drugs, inhalants are more easily available and cheaper, so few barriers exist to their consumption **(Filley, 2013).** Several investigators believe that VSA has quietly reached near-epidemic proportions as no consistent system exists for gathering data on the extent of inhalant-related toxicity in most countries **(Balster, 1996; D'abbs and MacLean, 2008; Garland and Howard, 2012).** Their acute toxicity ranges from alcohol intoxication, intense euphoria, and hallucinations to brain damage and death **(Bowen, 2006; Roberts *et al.*, 2015)**.

Toluene is the most consumed volatile substance with a high potential for different types of abuse **(Kim *et al.*, 2020; Real *et al.*, 2020).** It's a lipophilic substance that exhibits a great affinity for lipids and can diffuse readily into tissues (**Tas *et al.*, 2011).** Exposure to toluene is considered a predisposing factor for the occurrence of toxic effects mainly on the central nervous system. It may also lead to many other damaging repercussions, such as embryo and genotoxicity, teratogenicity, neurobehavioral changes, and renal and hepatic failure in experimental organisms **(Bowen, 2006; Moro *et al.*, 2012; Shaffie and Shabana, 2019).** High concentrations of toluene were detected in the brain and liver tissues of individuals who died from glue-sniffing **(Pero, 2010).** The toxic mechanisms of toluene in the various organs are poorly understood. However, some experimental studies have illustrated that the induction of oxidative stress via reactive oxygen species generation, enzyme inactivation, and antioxidant defense suppression is one of the main toxic effects of toluene **(kamel and Shehata, 2008; Moro *et al.*, 2010; Wang *et al.*, 2013).**

As a result of the oxidative stress adverse pathologies, several studies largely focused on vegetal substances to be essential elements in mitigating the side effects of modern synthetic treatments **(Bidie *et al.*, 2011; Bone and Mills, 2013).**

In recent years, interest in natural antioxidants has increased significantly. Scientific research in various specialties has been developed for the extraction, identification, and quantification of these compounds from medicinal plants **(Popovici *et al.*, 2009; Bourgou *et al.*, 2016).** Among the plants that have attracted great interest, is *Punica granatum*, known as the miracle tree, it's an important seasonal crop of the Punicaceae family that grows in countries bordering the Mediterranean Sea, Pakistan, India, and Iran (**Holland *et al.*, 2009)**. Different parts of pomegranate have been widely used in traditional medicine as a remedy for several diseases and symptoms. It is claimed to be a health-promoting food because of the great number of potentially active nutrients, which are found mainly in its juice and peel **(Russo *et al.*, 2019)**. This family of compounds has anti-oxidant activities that put pomegranate in a higher grade capacity **(Tapias *et al.*, 2014).** It is also attributed to this fruit a range of biological functions, such as anti-lipoperoxidation, anti-inflammatory, cardio-preventive, antidiarrheal, anticancer properties, as well as DNA repair activities **(Aviram *et al.*, 2002; Hong *et al.*, 2008; Dkhil *et al.*, 2013; Thangavelu *et al.*, 2017).**

Based on the facts stated above, the present study aimed to investigate the subchronic effects of oral administered toluene on Hematological, hepatic, renal, and reproductive markers in male Wistar rats. On the other hand, we tried to determine the phytochemical composition and antioxidant activity of *Punica granatum* juice and peel aqueous extract and their protective effect against the physiological deteriorations induced by this solvent.

It seemed appropriate to us to present our work according to the following plan:

-After a general introduction, the first part consists of a bibliographical study, which is quite extensive and includes studies carried out by renowned researchers whose results are references in the field.

-The second part constitutes the background of this study related to the analytical protocol, which is based on the methods used in this study.

-In the third part, we present the results.

-Finally, we discuss in detail the results obtained in this study, and we suggest some perspectives to this research work.

I. Bibliographic study

1. Toluene

1.1. History and definition

Toluene is the common name for methylbenzene. It was discovered in 1835 by Pelletier and Walter as a degradation product obtained from heating natural resin **(Soffritti *et al.*, 2004).** Its name derives from the older name toluöl which refers to tolu extract, an aromatic extract from a tropical tree Myroxylon balsamum: tolu balsam, named from a small town in Colombia, South America **(Deville, 1841).**

Toluene is a seven-carbon aromatic hydrocarbon compound that belongs to the family of volatile organic compounds **(US EPA, 2011).** It is a benzene homolog distinguished by having an additional methyl group (replaced hydrogen) on the benzene ring **(DOE, 1984).** At room temperature, it is a clear, colorless, volatile liquid with a distinctive sweet smell and properties very similar to those of benzene **(Balster *et al.*, 2009).** It is lighter than water in its liquid form, but three times heavier than air as a vapor **(Cruz *et al.*, 2014).**

1.2. Physicochemical properties

Toluene's structure and physicochemical properties are shown in Fig.1. Toluene (C_7H_8) has a molecular weight of 92.13 g/mol and a density of 0.876 g/mL **(Cruz *et al.*, 2014)**. It is practically insoluble in water (0.535 g/l at 25 °C), miscible with many organic solvents (acetone, diethyl ether, chloroform, ethanol...), soluble in glacial acetic acid. It is an excellent solvent for a large number of natural or synthetic substances (oils, fats, resins) **(US EPA, 2011).** Toluene has high affinity for lipids (log octanol/water partition coefficient = 2.73) and is flammable with a low flash point (the lowest temperature at which it can vaporize to form an ignitable mixture in air) of 4.4 °C **(Cruz *et al.*, 2014).**

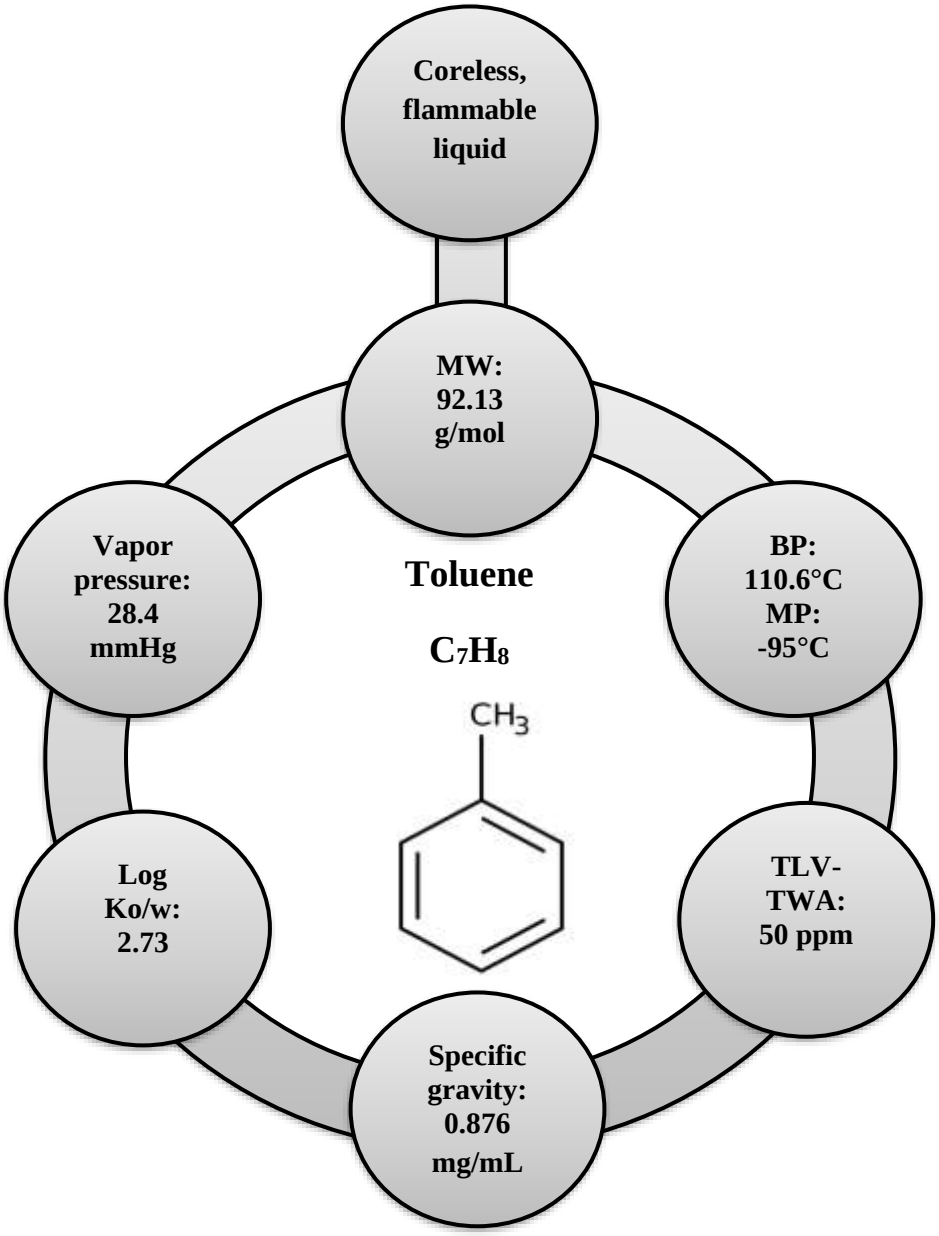


Figure 1: Chemical and physical properties of toluene (Cruz *et al.*, 2014).

1.3. Sources

Toluene occurs naturally in coal and crude oil. Forest fires (incomplete combustion of plant material), volcanic eruptions, and volatile emissions from vegetation are also natural sources of emissions **(Romero *et al.*, 2015)**. However, these sources are of minor importance compared to the anthropogenic emissions produced by various human processes.

The sources of anthropogenic emissions are the refining of crude oil and coal. It is found as a constituent of gasoline and aviation fuel at concentrations ranging from 5 to

20% by volume. It is also present in many naphthas and other petroleum products and as a by-product of styrene manufacturing **(Beller *et al.*, 1992).** Therefore, automobile emissions are the major anthropogenic source of toluene in ambient air.

Toluene can also be released into ambient air during its production, use in manufacturing, spray and paint applications, and the synthesis of organic chemicals **(Pierce *et al.*, 1998; Samoto *et al.*, 2006).** Furthermore, it is liberated from common household products (paints, paint thinners, adhesives, nail polish) and cigarette smoke **(Niu *et al.*, 2022).**

1.4. Uses

Toluene is primarily produced in the manufacturing of gasoline, and it is also a gasoline additive that can be used to improve octane ratings for fuel used in race cars and other automobiles **(ATSDR, 2000; Othmer, 2007).** It is also typically used as a solvent in the production of paints, ink, rubber, lacquers, glues, cosmetic products (particularly nail polish removers), perfumes, and adhesives because it can help dry, dissolve and thin other substances **(Aylward *et al.*, 2008; Malaguarnera, 2012)**. It is used as a synthesis intermediate in the production process to make other chemicals, including benzene, nylon, plastics, and polyurethane and in the synthesis of trinitrotoluene (TNT), benzoic acid, benzoyl chloride, toluene diisocyanate, and pharmaceutical products **(Malaguarnera, 2012; INERIS, 2016)**.

Surprisingly, toluene plays a big role in our ability to enjoy the popular beverage Coca-Cola. Toluene is involved in removing the cocaine from the coca leaves, which are used to develop the famous Coca-Cola syrup **(Sweere *et al.*, 2011).**

1.5. Environmental fate

Releases of toluene to the environment are mainly to air due to its volatile nature, with smaller amounts to water and soil. Water releases are due to spills and leaks of gasoline and other petroleum products and from the disposal of waste from paints, inks, and other products containing toluene. Because of the widespread use of petroleum products, releases occur nationwide **(Ware, 1988).** It can enter the soil from oil spills and from leaking underground tanks, but the extent of these sources is not known. It is also released into the soil from disposal site **(Johnson *et al.*, 1989; Lesage *et al.*, 1990).**

Toluene in the vapor phase will be degraded in the atmosphere by reaction with photochemically produced hydroxyl radicals; the half-life of this reaction in air is

estimated to be 2 days. Photolysis of toluene in the air, which also contains other pollutants such as nitrous oxides and ozone, may contribute to smog production **(US EPA, 2011)**.

Volatilization from water surfaces is expected to be the primary fate process based on the estimated Henry's law constant. The estimated volatilization half-lives for a model river and lake are 1 h to 4 days, respectively. Biodegradation is expected to occur rapidly in water, with reported half-lives of 4-56 days in aerobic and anaerobic water **(US EPA, 2011).**

In soil, toluene is expected to have high to moderate mobility in the subsurface. Volatilization of toluene from wet soil surfaces is expected to be an important fate process. Toluene can volatilize from dry soil surfaces due to moderate vapor pressure. The biodegradation half-life in various soils has been reported from several hours to 71 days **(HSDB, 2011).** Data indicates that the production and use of toluene do not adversely affect aquatic and terrestrial ecosystems. Photosynthesis and respiration by marine phytoplankton communities are inhibited by toluene at 34 mg/L. Toluene residues do not accumulate in fish or aquatic food chains **(WHO, 1986).**

1.6. Toxicokinetics

1.6.1. Exposure

General populations can be exposed to toluene by inhalation of indoor and outdoor air mainly when they use common household products and expose to vehicles gas**,** but those probable routes of exposure are less danger and do not present significant risk to human health. Occupational exposure in the workplace that occurs several hours a day is the typical route of exposure to toluene **(Cruz *et al.*, 2014).** In addition, toluene is deliberately inhaled by abusers, which are mostly adolescents and homeless **(Real *et al.*, 2020).** Abusers deliberately inhaling it via ‘huffing’ (flooding a cloth with the abused product and put it into the mouth to breathe fumes), ‘bagging’ (filling a plastic bag with the abused product and then held to the face and the fumes inhaled) or ‘spraying’ (spraying the abused product directly into the nose or mouth) **(Balster *et al.*, 2009; Johnston *et al.*, 2015; Roberts *et al.*, 2015).** In Australia, “chroming” is used as synonymous with inhaling paint sprays which contain toluene and propellant gases **(Takagi *et al.*, 2010).** Using any of these methods, inhalant effects appear very quickly, usually within seconds, and they last from 15 to 60 min. In order to level up the duration of effects, users repeat the exposure to keep the desired level of intoxication **(Cruz *et al.*, 2014)**.

Regulations exist to prevent physiological and behavioral adverse consequences and

although they vary among countries, safe exposure limits are usually in the range of 10 to 100 ppm. The immediately dangerous to life and health limit (IDLH) has been estimated at 500 ppm (**Cruz *et al.*, 2014)**. In spite of this, people who misuse toluene-based products are exposed to concentrations of several thousand ppm following an intermittent pattern of inhalation **(Marjot and McLeod, 1989; Sharp, 2008).**

1.6.2. Absorption

The main route of toluene absorption is inhalation because ingestion is an accidental or voluntary phenomenon. Absorption in humans and animals by the respiratory route is rapid; it appears in the blood after 10 to 15 minutes of exposure with an absorbed fraction of about 50% of inhaled toluene **(INERIS, 2005; Low *et al.*, 2008).** It is completely absorbed from the gastrointestinal tract with a slower rate of up to 2 to 3 hours **(INERIS, 2005; INRS, 2018; Shaffie and Shabana, 2019).** Absorption after skin contact with liquid toluene is limited, while skin contact with toluene vapors is practically non-existent **(Chapman *et al.*, 1990; Cok *et al*., 2003).**

1.6.3. Distribution

Toluene is distributed between red blood cells and bounded to hemoglobin and serum **(INRS, 2012).** The blood transports it to lipid-rich and highly vascularized tissues such as the brain, especially white matter, bone marrow, spinal cord, liver, and kidney **(Hannigan and Bowen, 2010; Bowen and Hannigan, 2013; Djurendic-Brenesel *et al.*, 2016).** Toluene concentrations in the brain are higher than in the blood; adipose tissue acts as a reservoir **(Hoet and Lison, 2008).** It readily passes the placental barrier with concentrations in the fetus of approximately 75% of that in maternal blood **(Hannigan and Bowen, 2010; Bowen and Hannigan, 2013).** It is also found in breast milk in humans and animals **(Fabietti *et al.*, 2004).**

1.6.4. Metabolism

The oxidative pathway (phase I) is considered the principal metabolic pathway (80%). In the liver, toluene is oxidized by cytochrome P450 monooxygenases mainly CYP 2EI to benzyl alcohol. It is then converted to benzyl aldehyde, which is in turn metabolized to benzoic acid, primarily by mitochondrial aldehyde dehydrogenase (ALDH)-2. In addition, benzoic acid is conjugated with glycine to form hippuric acid (83-94% of urinary metabolites) or with glucuronic acid to form benzylglucuronide (3-9% of urinary metabolites) **(US EPA, 2005; Waniusiow *et al.*, 2008)**. The conjugation pathway (phase

II) is considered a secondary metabolic pathway (10%). It is the one binding of benzyl alcohol to glutathione to form benzylglutathione c-stein-S conjugates and benzylmercapturic acids (BMA) **(Tassaneeyakul *et al.*, 1996).** There is also a minor pathway involving epoxidation by CYP IA2, CYP 2EI, and CYP 2B6 followed by conjugation leading to the formation of ortho-, meta-, and para-cresol **(ATSDR, 2001).**

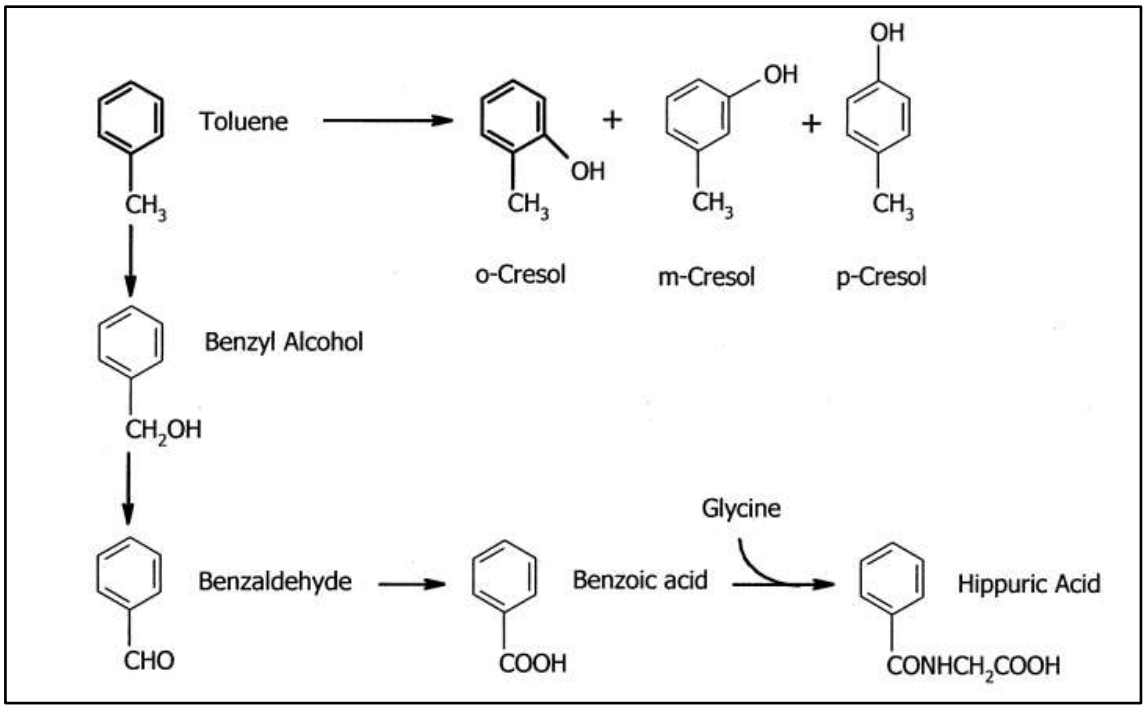


Figure 2: Pathways of toluene metabolism (Pierce *et al.*, 2002).

1.6.5. Elimination

The majority of toluene in the body is eliminated in the urine after 12 hours following exposure, mainly as metabolites **(ATSDR, 2000)**. A fraction between 5 to 20% of the absorbed toluene is excreted unchanged in the exhaled air. The biological half-life of toluene in alveolar air is about 20 hours. Pulmonary elimination is lower in women than in men. 60-80% of the absorbed dose is eliminated as hippuric acid which appears in the urine 30 minutes after the start of exposure for an airborne concentration of 100 ppm; hippuric acid is normally eliminated 24 hours after the end of a single exposure, but daily exposures of 8 hours may result in accumulation and greater excretion over the weekend. Free urinary toluene represents 0.06% of the absorbed toluene **(Baskerville *et al.*, 2001; Lauwerys and Hoet, 2001; Nadeau *et al.*, 2006)**. A small amount (<2%) of the absorbed dose is eliminated in the feces **(Lauwerys and Hoet, 2001; Nadeau *et al.*, 2006).**

1.7. Sites of action for toluene

Several studies *in vitro* and *in vivo* have investigated the molecular and cellular targets of abused inhalants, including toluene. Many of these studies have focused on defining the effects of toluene on ion channels that are critically involved in regulating neuronal excitability. As summarized below in Table 1, results from these studies indicate that both voltage-gated and ligand-gated ion channels are affected by concentrations of toluene associated with voluntary inhalation of these substances **(Cruz *et al.*, 2014)**.

Table 1: Summary of toluene effects on recombinant and native ion channels.

GABA	**α1/β1**	**increase**	**(Beckstead *et al.*, 2000)**
nAchR	α4/β2 α4/β4 α3/β2 α3/β4 α7	decease	**(Bale *et al.*, 2002; Bale *et al.*, 2005)** **(Bale *et al.*, 2002)** **(Bale *et al.*, 2002; Bale *et al.*, 2005)** **(Bale *et al.*, 2002)** **(Bale *et al.*, 2002; Bale *et al.*, 2005)**
Sodium Channels	Nav1.5 (cardiac) Nav1.4 (skeletal)	decease	**(Cruz *et al.*, 2003)** **(Gauthereau *et al.*, 2005)**
Ca^{++} Channels	Cav1/Cav2	decease	**(Tillar *et al.*, 2002; Shafer *et al.*, 2005)**
K^{+} Channels	mSlo Girk2 Girk1/2; Girk1/4	decease None	**(Del Re *et al.*, 2006)**
Gap Junction	Native (HEK cell)	decease	**(Del Re and Woodward, 2005)**

1.8. Physiological effects

1.8.1. Acute effects

Toluene has a low acute toxicity; its primary target is the central nervous system. The effects of inhalation of toluene vapors are primarily in the CNS **(ATSDR, 2000)**. Effects increase with the increasing of exposure concentration; symptoms include vertigo, fatigue, Somnolence, headaches and decrease in neurobehavioral performance **(US EPA, 2005)**. It can also irritate the eyes, nose, throat, and respiratory tract **(ATSDR, 2000)**.

The effects of acute oral exposure also appear in the CNS (the same as for inhalation), while skin contact produces irritation or dermatitis **(ATSDR, 2000)**.

Intoxicated patients may also experience tachycardia, tachypnea, muscle weakness, and vomiting **(Camara-Lemarroy *et al.*, 2015)**.

1.8.2. Chronic effects

Prolonged exposures to toluene cause primarily CNS damage, but also alter the other organism systems **(ATSDR, 2000).** Chronic CNS damage could result in memory and concentration difficulties, insomnia, a decrease in intellectual performance, and neurotransmitter levels perturbation **(Yucel *et al.*, 2008; Howard *et al.*, 2011).**

Studies have also linked toluene exposure to the development of neurosensory toxicity; symptoms include hearing loss, color discrimination and visual perception loss, alteration of pain perception, and reduction of olfactory sensitivity **(Chang *et al.*, 2006; Waniusiow *et al.*, 2008).**

Heavy long-term toluene abuse can also produce inflammation and degeneration of the nasal and respiratory epithelium, lung damage, as well as hepatic changes (increased liver weight and level of hepatic enzymes) cardiac arrhythmias, hypoxia, or a combination of these factors**(ATSDR, 2000)**.

Studies conducted on individuals have shown that toluene chronic exposure can lead to a syndrome that resembles renal tubular acidosis causing hypokalemia and kidney damage **(ATSDR, 2000; Bowen and Hannigan, 2006).**

1.8.3. Neurological effects

Neurotoxic effects are one of the primary concerns for short-term, acute exposures to VOCs, including toluene. Effects in both humans and animals include cognitive and motor coordination impairments **(Evans and Balster, 1991; Bushnell and Crofton, 1999).** Low levels of acute exposure may lead to increased reaction times, altered hearing and visual evoked potentials, and altered performance of cognitive and behavioral tasks **(Benignus, 1981; Benignus *et al.*, 2009).** Higher doses increase the degree of behavioral and motor impairments, leading to CNS depression, anesthesia, and unconsciousness **(Kenyon *et al.*, 2008)**.

Permanent effects on the central nervous system were observed in cases of abusive inhalation, for concentrations estimated to exceed 4000 ppm. These effects included ataxia, tremors, electroencephalogram changes, hallucinations, nystagmus, damage of the hippocampus, and alterations in language, hearing, and vision **(McWilliams et *al.*, 2000; Hodgson and Levin, 2003; ATSDR, 2007)**.

The repeated exposure to toluene can alter the activity of certain enzymes involved in the synthesis and/or degradation of certain neurotransmitters; it can also affect the concentrations of electrolytes, such as sodium, potassium and calcium **(Kanter, 2011)**.

Other effects reported are the altered brain weight and volume in rats, altered levels of glial fibrillary acidic protein (GFAP), and markers of oxidative stress **(ATSDR, 2000)**.

Exposure to toluene at a concentration below 50 ppm does not show any impairment of performance on neuropsychological or psychomotor tests **(Neubert *et al.*, 2001; Gericke *et al.*, 2001; Seeber *et al.*, 2004).** For exposures of the order of 70-100 ppm, the majority of worker studies show significant neurobehavioral and psychomotor alterations **(Foo *et al.*, 1990; Kang *et al.*, 2005).**

1.8.4. Immunological effects

Experiments with male mice at low levels of toluene resulted in induction of IL-2 production, activation of the transcription factors NF-B, signal transducers, activators of transcription (STAT) 5, and NF-AT in thymocytes **(Liu *et al.*, 2009).** On the other hand, occupational exposure to organic solvents may produce a decrease in the number of natural killer cells and an increase in the number of peripheral blood B-cells **(Tanigawa *et al.*, 2001).**

In vitro study demonstrated a significant decrease in thymus weight and a significant decrease in immune responses in all immunoassays after exposure to 105 mg/kg/day of toluene **(ATSDR, 2015)**.

1.8.5. Mutagenic and genotoxic effects

There are discrepancies in the findings related to the genotoxic effects of toluene in humans. It has been reviewed by the European Union but has not been classified toluene as a genotoxic compound **(ANSES, 2012).** Studies have shown no association between chronic toluene exposure and an increased incidence of chromosomal damage. No exposure differences were reported for DNA damage in leukocytes of factory workers exposed to factory air containing 96-412 mg/m3 toluene **(ATSDR, 2000).** However, A significant increase in the frequency of sister chromatid exchanges and chromosome breaks was recorded in lymphocytes from workers exposed to 750-1125 mg/m^3 (200-300 ppm) toluene **(ATSDR, 2000).** Another study indicates that toluene causes DNA single strand breaks (rat hepatocytes) but not DNA damage or repair (human fibroblasts), increases the rate of mutation but not the rate of morphological transformation; in the absence of a metabolic activator, it does not cause sister chromatid exchanges or chromosomal aberrations (Chinese hamster ovary cells or human lymphocytes) **(Zou *et al.*, 2006)**.

1.8.6. Cardiac effects

Toluene exposure is reported to cause sinus bradycardia **(Einav *et al.*, 1997; Turkoğlu *et al.*, 2010)**, AV blocks **(Tsao *et al.*, 2011)**, asystole, ventricular tachycardia and fibrillation, dilated cardiomyopathy **(Gunes *et al.*, 2013)**, heart failure, myocardial infarction **(Vural *et al.*, 2003)**, and sudden death **(Yasar *et al.*, 2016).**

Tachyarrhythmia is the classical manifestation of toluene cardiotoxicity. In contrast, anesthetized mice exposed to high doses of inhaled toluene develop bradycardia and atrialventricular block **(Taylor and Harris, 1970; Gordon *et al.*, 2007).**

Development of cardiomyopathy is also reported with toluene inhalation; it commonly occurs after chronic exposure to toluene **(Lisowska *et al.*, 2004).**

1.8.7. Hematological effects

Leukocytosis, decreased thrombocyte count, and bone marrow hypoplasia were observed in mice exposed to toluene by inhalation at 1000 ppm for 20 days and 4000 ppm for 8 weeks. Mild transient granulopenia followed by granulocytosis were also noticed in rabbits given toluene by gavage at 865 mg/kg for 6 days; however, some investigators related these effects to the presence of benzene as a contaminant in toluene **(NTP, 1990).**

1.8.8. Hepatic effects

In rats, toluene intoxication shows degeneration of the hepatocytes. Thus, intraperitoneally administered toluene causes hepatic effects in rats, which are reflected by swelling of the hepatocytes, focal necrosis, and vacuolization; accompanied by an increase in the enzymatic activity of the liver, in particular alanine aminotransferase (ALT), aspartate aminotransferase (AST), and an increase in the level of lipid peroxidase, along with a decrease in bilirubin level and liver weight **(Poon *et al.*, 1994; Esen and Uysal, 2018; Meydan *et al.*, 2019)**. While, an increase in liver weight is observed in rats inhaled 30 ppm of toluene for 4 weeks **(Poon *et al.*, 1994)**. Similarly, high-dose toluene administration can induce hepatocyte degeneration, pericentral fibrosis, hemorrhage, and mononuclear cell infiltration **(Tas *et al.*, 2011; Ayan *et al.*, 2013)**.

1.8.9. Renal effects

Occupational exposure to toluene has been reported to be associated with the development and progression of renal failure. Toluene inhalation is associated with a variety of severe metabolic alterations including renal tubular acidosis, hypokalemic paralysis, profound metabolic acidosis, kidney stone formation, and pyuria following

nephrotoxicity **(Kroege *et al.*, 1980; Streicher *et al.*, 1981; ATSDR, 2007; Camara-Lemarroy *et al.*, 2015).** Other potential types of kidney injury include proteinuria and hematuria **(Camara-Lemarroy *et al.*, 2015).**

Ketan et al. (2015) reported a significant decrease in SOD and CAT activities with a concomitant increase in lipid peroxidation in mice exposed to benzene, toluene, and xylene.

1.8.10. Reproductive and Developmental Effects

Toluene has been classified as having a possible risk to reproductive function. It has been listed under Proposition 65 as recognized by the State of California as a developmental toxicant **(OEHHA, 2014).**

It has been reported to have adverse effects on the female reproductive system through injury and disorders of the menstrual cycle or it affects fetal development causing malformations and decreased fetal body weight **(Saillenfait *et al.*, 2003; Lauwerys *et al.*, 2007; Hannigan and Bowen, 2010).**

Toluene is reported to cause a risk of late miscarriage at exposure levels below 100 ppm for early exposure during pregnancy. It is also considered a teratogen agent **(OEHHA, 2008).** Studies show that pregnant women who abuse toluene are likely to produce cases of the fetal solvent syndrome (FSS). FSS is a syndrome similar to that described in Fetal Alcohol Syndrome (FASD). It encompasses a wide range of conditions, such as neurobehavioral delay, dysmorphology, and perinatal growth. FSS includes more pronounced effects than FASD, such as ear abnormalities, a large fontanel, and abnormal scalp structure **(Bowen and Hannigan, 2006).**

Several occupational studies and case reports have indicated that toluene may have adverse effects on male reproductive functions. Testicular atrophy and reduced spermatogenesis were observed in a case of chronic toluene abuse, while caoutchoucs factory workers have high numbers of abnormal sperm **(Rendon *et al.*, 1994; ANSES, 2014).** Other animal studies suggest the possibility of decreased sperm volume, motility, vitality, and increased sperm abnormalities **(Kanter 2010; Djemil *et al.*, 2015).** It has also been suggested that toluene induces a reduction in antioxidant activity in the testes **(Kamel and Shehata 2008).**

On the other hand, toluene treatment was found to cause histopathological changes by decreasing seminiferous tubular diameter and dislocated germ cells, in addition to mitochondrial degeneration of Sertoli cells **(Kanter, 2010).**

Moreover, toluene inhalation has been shown to alter hormonal status, where it has been reported that men occupationally exposed to toluene had decreased plasma concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone **(Ono *et al.*, 1999; ATSDR, 2000).**

1.8.11. Toluene and oxidative stress

It is well known that the toxicity of toluene due to its ability to induce oxidative stress. The oxidative damage observed after exposure to toluene is caused essentially by a hyper-production of free radicals on the one hand, and a decrease in the efficiency of the anti-radical defense system on the other hand **(Mattia *et al.*, 1993)**. Several investigations have shown that exposure to toluene reduce the activity of several anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, and can also decrease the level of reduced glutathione **(Kamel and Shehata, 2008; Liu *et al.*, 2010).**

Other studies indicated that toluene (0.5–1.5 g/kg, via intraperitoneal injection) increased the generation of reactive oxygen species (ROS) in crude mitochondrial fractions from rat cerebellum, striatum, and hippocampus **(Mattia *et al.*, 1993).** Lipid peroxidation products and protein carbonyls increased in the hippocampus, cortex, and cerebellum of rats following weeks of toluene inhalation **(Baydas *et al.*, 2003; Kodavanti *et al.*, 2015).** Oxidative DNA damage has also been detected in the liver and kidney of rats after toluene inhalation for 7 days **(Tokunaga *et al.*, 2003)**.

2. Oxidative stress

2.1. Notion of stress

In biology, the term “stress” was first presented by Claude Bernard in 1868. According to him, the reactions triggered by stress were aimed at maintaining the balance of our organism. The sum of these internal reactions was named homeostasis by the American physiologist C.W. Bradford in 1915. The linking of these three concepts of stress-homeostasis-adaptation constitutes what is known as the biological approach to stress, which is an adaptation to the environment, within certain limits, and consequently maintaining life. At the cell level, stress is provoked by an environmental parameter variation leading to the implementation of homeostasis regulation mechanisms **(Bourouhou, 2016).**

2.2. Definition of oxidative stress

Oxygen, an essential molecule to life, development, and ability to adapt; is likely to cause damaging effects in the organism through free radicals and activated oxygen species formation and can lead to what is called "oxidative stress"**(Koechlin-Ramonatox, 2006).**

In biological systems, oxidative stress is defined as the imbalances of the metabolic balance of oxygen in the cell caused by an excess production of free reactive oxygen (ROS) or by a decrease of the antioxidant defense systems against these radicals (enzymatic and non-enzymatic), or by a combination of these two phenomena **(Persson *et al.*, 2014).** It is characterized by the reduced capacity of endogenous systems to fight against the oxidative attack directed toward target biomolecules, which is associated with several pathologies **(Cooke and Davidge, 2003; Davies, 2003).**

2.3. Active derivatives

2.3.1. Free radicals

A free radical is an atom or a molecule that has a single electron, not paired, which gives it great chemical reactivity **(Dasgupta and Klein, 2014).** Free radicals can be generated from many elements, but generally, those involving oxygen and nitrogen are the most important **(Burton and Jauniaux, 2011);** either by homolytic cleavage of a covalent bond or by heterolytic cleavage. And also, during a redox reaction with loss or gain of an electron from a non-radical compound **(Dasgupta and Klein, 2014; Halliwell and Gutteridge, 2015).**

There are three families of reactive species: reactive nitrogen species (RNS), reactive chlorine species (RCS), and the most common, ROS **(Berger, 2006; Pérez-Matute *et al.*, 2009).**

2.3.2. Reactive oxygen species (ROS)

ROS are chemical oxygen species resulting from physiological oxygen metabolism, which are capable of oxidizing other molecules **(Ozougwu, 2016).** They include free radicals of oxygen but also certain non-radical reactive derivatives **(Table 2).**

Table 2: Major reactive oxygen species generated in biological systems (Nimse and Pal, 2015; Dutta, 2016; Ozougwu, 2016).

symbol	Name
Free radical species	
O_2•-	Superoxide anion
•OH	Hydroxyl radical
ROO•	Peroxyl radical
RO•	Alkoxy radical
NO	Nitric oxide
CCl_3	Carbon tetrachloride radical
Non-radical species	
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
$^1O_2$	Singlet oxygen
ONOO	Peroxynitrite
O_3	Ozone

2.4. Sources of ROS

2.4.1. Mitochondrial source

Electron leakage at the level of complexes I and III during electrons transit in the mitochondrial respiratory chain is the essential source ROS production. This process leads to a reaction with molecular O_2, which conducts to the formation of superoxide anion, which will then be transformed into less reactive radicals such as H_2O_2 by the antioxidant systems **(Carrière *et* al., 2006).**

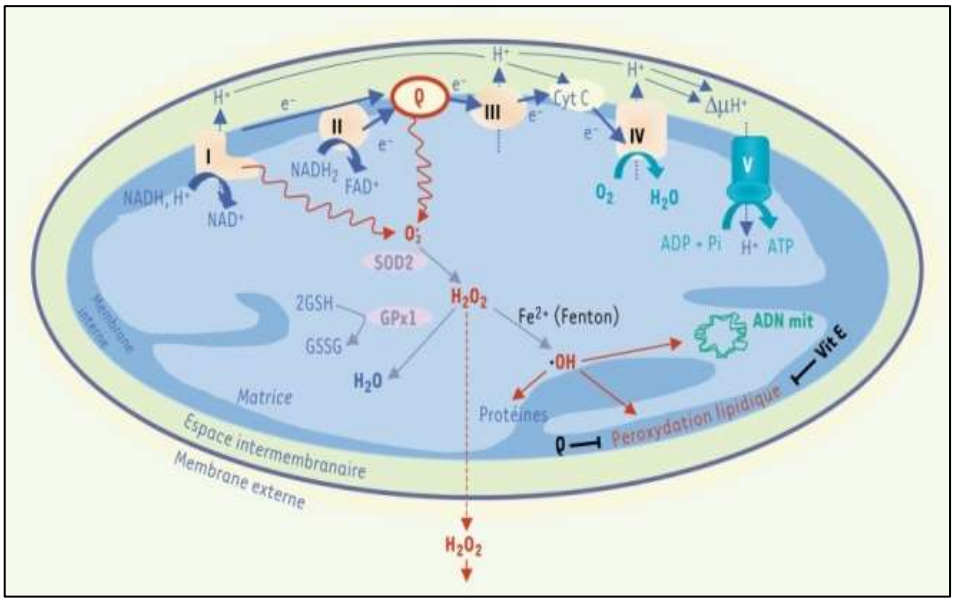


Figure 3: Mitochondrial production of ROS (Carrière *et al.*, 2006).

2.4.2. Non-mitochondrial source

a. Endogenous

- The peroxisome plays an important role in the intracellular production of H2O2 and contains numerous enzymes that generate H_2O_2 which is used as a substrate by peroxisomal catalase to enable peroxidation reactions of other substrates.
- The smooth endoplasmic reticulum contains enzymes that catalyze reactions to detoxify fat-soluble drugs and other toxic metabolic products. In particular, cytochrome P450 oxidizes unsaturated fatty acids, producing ROS and in particular superoxide anions $O_2^{\bullet -}$.
- In the plasma membrane, NADPH oxidase catalyzes the monoelectronic reduction of oxygen using NADPH or NADH as an electron donor, resulting in the formation of H_2O_2 and NADP+. This reaction is particularly involved in immune reactions **(Piotrowski and Marczak, 2000).**

b. Exogenous

Exogenous factors related to the environment or lifestyle are also a source of increase and accumulation of free radicals in the body.

These environmental factors, including non-genotoxic carcinogens, can be directly or indirectly involved in the generation of free radicals (xenobiotics, leukocyte activation, etc.) **(Hamma *et al.*, 2015)**.

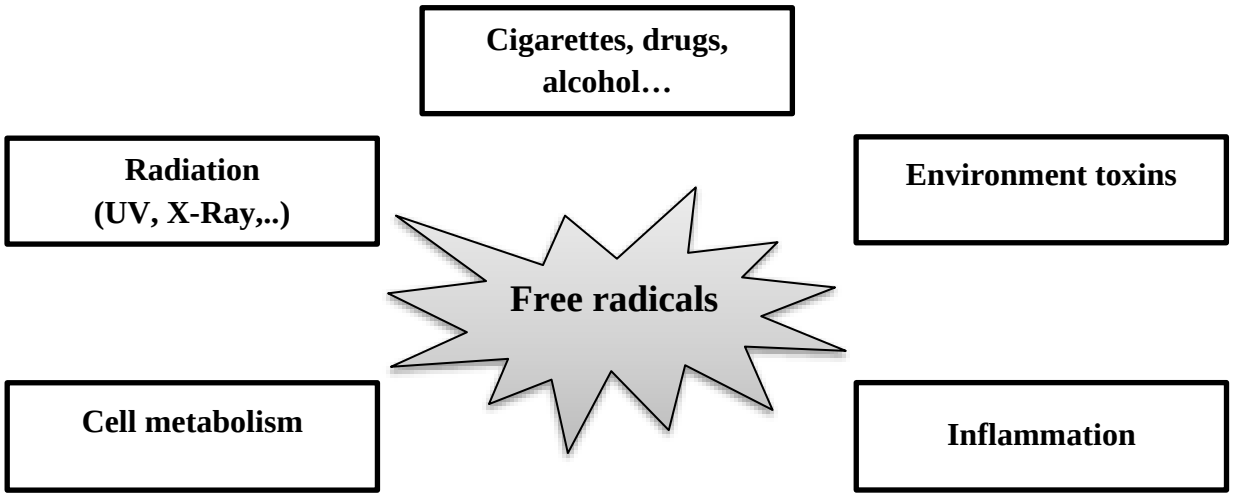


Figure 4: Exogenous and endogenous sources of free radicals (Hamma *et al.*, 2015).

2.5. Targets of reactive oxygen species

Reactive species are the inevitable consequence of the consumption of molecular oxygen by the body. Their production at low concentrations is determined, directed, and useful **(Afonso *et al.*, 2007)**. In fact, ROS in normal conditions participate in numerous functions: phagocytosis **(Goldsby *et al.*, 2001)**, cell signaling **(Afonso *et al.*, 2007; Buonocore *et al.*, 2010)**, regulation of metabolisms, and also modulation of gene expression **(Valko *et al.*, 2007)**. However, at high concentrations, their effects become deleterious to cells, tissues and various physiological functions, which can cause a cascade of reactions resulting in protein and DNA degradation and lipid peroxidation **(Hitchon and El-Gabalawy, 2004).**

2.5.1. Oxidation of lipid compounds

Polyunsaturated fatty acids and membrane phospholipids are the preferred targets of oxidative attacks. Membranes are more particularly targeted by the hydroxyl radical capable of abstracting hydrogen from the hydrocarbon side-chain of a fatty acid, they create a carbon-centred radical, C•. If oxygen is present, this may react to form a peroxyl radical (–C–O–O•), which in turn is capable of abstracting hydrogen from an adjacent fatty acid, so propagating the reaction **(Burton and Jauniaux, 2011)**. This process generates hydro peroxides which can continue to oxidize and fragment into aldehydes and alkanes (ethane, ethylene, pentane). The peroxyl radical can release various toxic aldehydes, malondialdehyde (MDA), or hydroxynonenal. Thus, a single oxidative event can alter numerous lipid molecules and induce an accumulation of hydroperoxides in the

membranes, which will reduce their fluidity as well as the activity of transmembrane proteins **(Valko *et al.*, 2006).**

2.5.2. Oxidation of protein compounds

Amino acids both free and in proteins, are a target for oxidative damage. The most reactive are histidine, proline, tryptophan, cysteine, and tyrosine **(Haleng *et al.*, 2007)**. Any free radical attack of an amino acid will cause oxidation of certain residues with, as consequences, the appearance of hydroxyl or carbonyl groups, cleavages of peptide chains and intra- and inter-chain bi-tyrosine bridges. Most of the damages are irreparable and can lead to important functional modifications (non recognition of a receptor by a ligand, loss of enzymatic activity) **(Baudin, 2006)**. Some oxidized proteins are poorly degraded and form aggregates that accumulate in the cells and in the extracellular compartment **(Atkin *et al.*, 2005)**.

2.5.3. Oxidation of DNA

Mitochondrial DNA is the primary target of ROS oxidation because of its low repair potential compared to nuclear DNA and its proximity to the mitochondrial respiratory chain, which is a major source of ROS production **(Servais, 2004).** It is attacked mainly by OH radicals where a variety of products can be generated through reactions with either the DNA bases or the deoxyribose sugars **(Halliwell and Gutteridge, 1999)**. The most common alterations are hydroxylation of purine and pyrimidine bases and the deoxyribose backbone causing strand cleavage and genetic mutations. These DNA damages seem to be strongly involved in either the arrest of the induction of transcription or transduction of signaling pathways or the involvement of replication errors or genomic instability, all of which are associated with carcinogenesis as well as DNA mutations and cell death **(Servais, 2004; Valko *et al.*, 2006)**.

2.6. Antioxidants

Antioxidants are defined by **Halliwell (1999)** as "any substance that is capable, at relatively low concentration, of competing with other oxidizable substrates and thus preventing or slowing down the oxidation of this substrate. They can be classified according to their mode of action, their cellular location, and their origin.

2.6.1. Mode of action of antioxidants

In the body, there are several types of molecules with antioxidant activity, whose mechanisms differ. According to their mode of action, they are divided into two categories:

- Primary defense system, ex: catalase (CAT) and glutathione (GSH) act in prevention by limiting the initiation phase of oxidation reactions, thus blocking the production of ROS.
- Secondary defense system, ex: tocopherols blocking the propagation reactions by avoiding the passage from slightly reactive forms (O_2•-) to highly reactive ones (OH•). This is why it is called "chain-breaking molecules" **(Buettner, 1993; Pastre, 2005).**

2.6.2. Different cellular locations of antioxidants

It is possible to classify antioxidants as liposoluble or water-soluble molecules. Depending on their physical and chemical characteristics, they will have a preferential cellular location: extracellular for the water-soluble substances. They will be particularly effective on the free radicals present in each type of medium, respectively **(Opara, 2002)**.

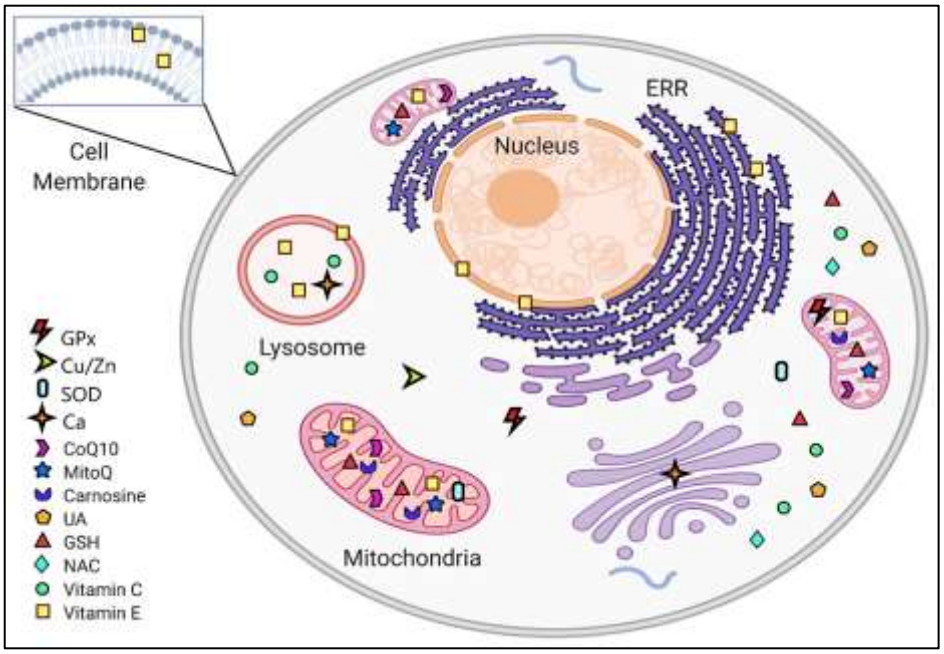


Figure 5: Sites of action of antioxidants (Opara, 2002; Duarte-Jurado *et al.*, 2021).

2.6.3. Enzymatic antioxidants

a. Superoxyde dismutase (SOD)

SODs represent an important family of metalloenzymes that are widely distributed within aerobic organisms to ensure their survival **(Blokhina, 2000).** This enzyme constitutes the first line of defense against oxidative stress by catalyzing the dismutation of the superoxide radical O_2•- into dioxygen and H_2O_2 according to the following reaction **(Kuciel and MazurKiewicz, 2004)**:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

In mammals, most of this activity occurs intracellularly in both cytosolic and mitochondrial compartments **(Kerher and Klotz, 2015).**

b. Catalase

Catalase is a hemoprotein expressed in all major organs of the body, particularly in liver, kidney, and erythrocytes **(Blondeau *et al.*, 1987)**. It is localized mainly in peroxisomes but functional catalase has also been detected in the cytoplasm, in mitochondria of rat cardiomyocytes and on the cytoplasmic membrane of human cancer cells **(Nishikawa *et al.*, 2009; Glorieux *et al.*, 2015)**. This enzyme catabolizes the end product of the dismutation reaction, H_2O_2, into water and molecular oxygen **(Kuciel and MazurKiewicz, 2004)**.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

c. Glutathion peroxydase (GPx)

These enzymes have in common a tetrameric structure with a selenium atom in its active site; therefore the enzymes of this family are Selenium (Se)-dependent. They expressed by most eukaryotes, this family has; eight GPxs enzymes in humans and mice (GPx1- GPx8) **(Brigelius-Flohe and Maiorino, 2013).** GPxs are present in the cytosol and play a significant role in regulating the intracellular physiological redox state **(Kalender *et al.*, 2013).** They catalyze the reduction of hydroperoxides (H_2O_2) and lipid peroxides in the presence of reduced glutathione (GSH) as an electron donor **(Ramming and Appenzeller-Herzog, 2013)**. The resulting glutathione disulfide (GSSG) is further reduced by glutathione reductase (GR), which uses NADPH as an electron donor for the regeneration of reduced glutathione **(Servais, 2004).**

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

$$GSSG + NADPH \longrightarrow 2GSH + NADP^+$$

d. Thioredoxins (Trx)

The thioredoxin system consists of an electron donor and two types of protein oxidoreductases: thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH as an electron donor **(Holmgren and Lu, 2010; Silva-Adaya *et al.*, 2014).** These enzymes have a structure close to that of glutathione reductase. They also consume NADPH in their function. Thioredoxins play a protective role against a wide variety of oxidative stresses due to its free radical scavenging properties **(Reichheld *et al.*, 2005).** They are involved in

the degradation of lipid peroxides and hydrogen peroxide and in the regeneration of the ascorbyl radical to ascorbic acid **(Hattori *et al.*, 2002; Bouguerne, 2012).**

In mammalian cells, thioredoxins and thioredoxin reductases are expressed as three isoforms: Trx1 and TrxR1 are cytosolic, Trx2 and TrxR2 are mitochondrial, and Sp-trx and TGR (or TrxR3) are expressed in the testis **(Arner, 2009; Holmgren and Lu, 2010)**.

2.6.4. Non-enzymatic antioxidants

These compounds are easily oxidized, relatively stable and lead to dismutations allowing the stopping of chain radical reactions. This protection system can be both membrane (vitamin E, A…..), cytosolic, and extracellular (glutathione, vitamin C, uric acid…..) **(Lacolley *et al.*, 2007).**

- **Liposoluble antioxidants**

a. Vitamin E

Vitamin E belongs to the tocopherol family with four isomers: α-tocopherol, which is the actual vitamin E, β-tocopherol, γ-tocopherol and δ-tocopherol. Alpha-tocopherol (α-TocH) is the most active form of the tocopherol class **(Guiga, 2019)**. It is a powerful antioxidant compound, particularly because of its lipophilic nature **(Gutowski and Kowalczyk, 2013)**. During the initiation of lipid peroxidation, following a radical attack, α-TocH, stops the lipid peroxidation reaction chain by capturing a lipid peroxyl radical. The vitamin E then becomes in turn a less reactive radical, which can be taken over by another antioxidant molecule **(Servais, 2004; Gutowski and Kowalczyk, 2013)**.

b. Carotenoids

Like vitamin E, carotenoids are lipophilic molecules located in the plasma membrane and therefore participate in the protection and maintenance of cellular integrity **(Fiedor and Burda, 2014)**. They form a large family of pigmentary conjugated polyenes (carotene family) which includes more than 600 molecules with an antioxidant capacity similar to that of tocopherols **(Landrum, 2010).** The most important and best known of the carotenoids is β-carotene **(Riccioni, 2009).** They are known to eliminate peroxyl radicals and singlet oxygen due to their long carbon chain, rich in double bonds **(Fiedor and Burda, 2014).**

- **Water-soluble antioxidants**

c. Glutathione

Glutathione is considered the main non-enzymatic intracellular antioxidant. It is a tripeptide formed by the condensation of glutamic acid, cysteine, and glycine **(Beaudeux and Durand, 2011).** It is the majority thiol at the intracellular level (cytosol, nucleus, mitochondria); either in its reduced form (GSH) or in its oxidized form (GSSG) **(Auberval, 2010; Poisson, 2013).** Its antioxidant capacity resides in the presence of a thiol group (–SH) present on the reduced cysteine. The thiol group allows glutathione to intervene in many reduction reactions (**Čolak and Žorić, 2019).** It participates in the scavenging of hydroxyl radical and singlet oxygen directly or detoxifying of hydrogen peroxide and lipid peroxides by the catalytic action of GPx **(Sen and Chakraborty, 2011)**, and also acts as a co-substrate of antioxidant enzymes such as glutathione peroxidase, glutathione reductase and transferase **(Lemaoui, 2011).** It is also the subject of synergistic interactions with certain compounds in their reduced form such as vitamin C, vitamin E and superoxide dismutases (**Sen and Chakraborty, 2011).**

d. Vitamin C

Vitamin C (or ascorbic acid) is a water-soluble antioxidant located in all cell membranes **(Gallie, 2013).** It can be synthesized by most plants and mammals *in vivo* from glucose, but humans are unable to produce it due to a mutation in the gene encoding L-gulono-gamma-lactone oxidase, the enzyme responsible for catalyzing the final step in the vitamin C biosynthetic pathway **(Lachapelle and Drouin, 2011; Telang, 2013)**. In situations of oxidative stress, its protective and detoxifying role results mainly from the capture of superoxide, hypochlorite, hydroxyl, and singlet oxygen anions **(Servais, 2004).** It can also reduce the α-tocopherol radical in cell membranes in combination with GSH and this during its oxidation to dehydroascorbic acid where it passes through an intermediate radical form (ascorbyl radical) which plays an essential role in the regeneration of oxidized vitamin E **(Garait, 2006; Nimse and Pal, 2015)**.

e. Uric acid

Uric acid is the end product of purine catabolism **(Stocker and Keaney, 2004)**. Under physiological conditions, uric acid is mostly ionized in its urate form (UrH2-) **(Haleng *et al.*, 2007)**. It is a powerful scavenger of •OH, ROO•, and NOO• radicals by producing the UrH•- radical **(Haleng *et al.*, 2007)**, which will in turn be reduced (notably by vitamin C) **(Stocker and Keaney, 2004)**.

f. Trace element

They are defined as a class of necessary nutrients that play an important role as enzyme cofactors, essential for the proper functioning of enzymes that exert a protective effect against oxidative stress **(Servais, 2004; Bonnefont-Rousselot, 2007).**

- **Zinc**

  Zinc plays an indirect antioxidant role by acting as a cofactor for many enzymes, in particular SOD **(Haleng *et al.*, 2007)**. It inhibits the production of ROS by transition metals **(Favier and Hininger-Favier, 2005)**, competing with iron and copper in the Fenton reaction. It protects the thiol groups (SH) of proteins against iron-induced oxidation by preventing the formation of intramolecular disulfide bridges. The antioxidant activity of zinc could also involve the induction of metallothioneins that can scavenge ROS **(Delattre *et al.*, 2005)**. The foods richest in zinc are meat and fish, whole grains and pulses; the recommended daily intake is about 20 mg **(Haleng *et al.*, 2007)**.

- **Selenium**

  Selenium is a component of glutathione peroxidase, an enzyme that plays an intracellular antioxidant role, similar to that of vitamin E. In food, we will mainly find organic selenium, it undergoes hepatic metabolism which leads to intermediates necessary for the synthesis of physiologically active derivatives such as GPx. The recommended daily dose is 50-70 µg/day **(Haleng *et al.*, 2007)**.

- **Copper**

  This trace element is also one of the essential cofactors of SOD. However, like iron, copper is considered a transition metal. The recommended daily intake is about 2.5 mg **(Haleng *et al.*, 2007).**

g. Polyphenols

Polyphenols are a family of organic molecules that are widespread in the plant world and in many aliments. The diet provides about 1g of polyphenols per day mainly through fruit and, to a lesser extent, in vegetables, and cereals **(Haleng *et al.*, 2007)**. They are characterized, as the name indicates, by the presence of several phenolic groups associated in more or less complex structures, generally of high molecular weight. This family includes a large group of more than 8000 molecules divided into about ten chemical classes. These molecules all have a common point: the existence of at least one aromatic ring with 6 carbons (phenol) itself carrying one or more hydroxyl functions (OH). There

are several families of molecules with relatively similar structures **(Watson, 2018)**. Phenolic acids, flavonoids, and tannins are the main phenolic compounds that are able to scavenge free radicals and inhibit lipid peroxidation by reducing hydroxyl, superoxide, and peroxyl radicals. They can also scavenge metal ions, as they have chelating properties **(Delattre *et al.*, 2005; Balasundram *et al.*, 2006)**. The phenolic hydroxyl groups (Ar-OH) provide hydrogens to the peroxide radicals L-OO• (very unstable) and neutralize them. The ArO• radical being quite stable and less reactive will break the chain **(Zitouni, 2017)**:

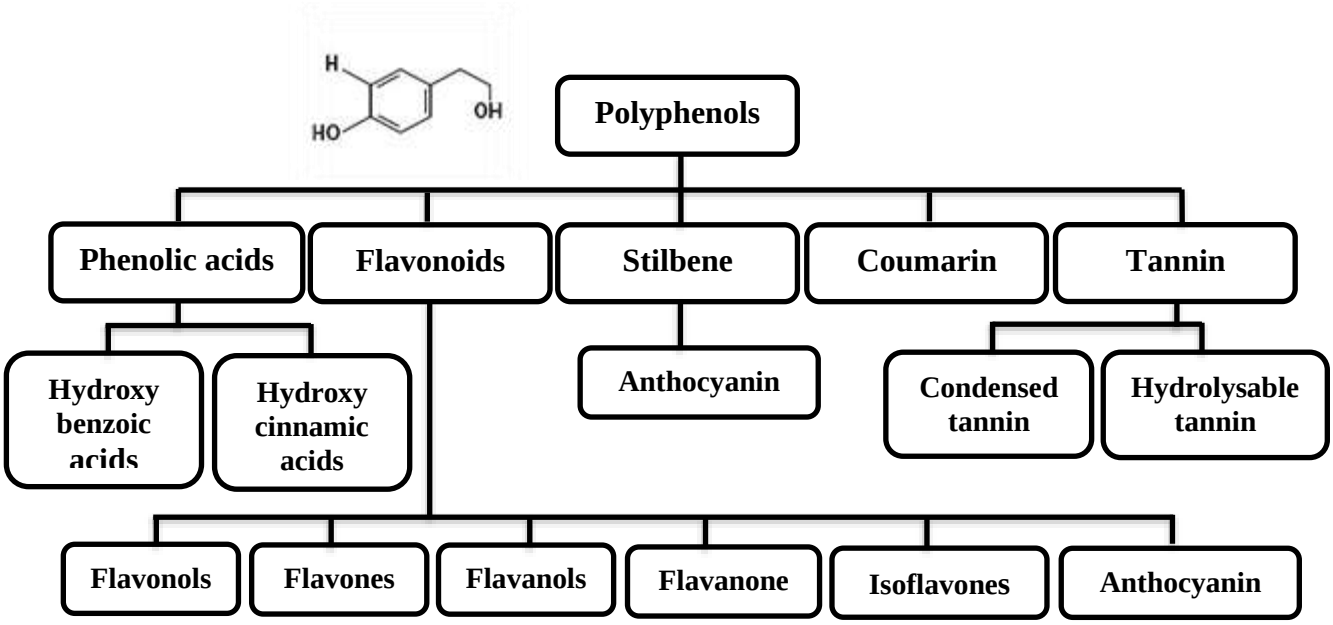


Figure 6: Classification of polyphenols (Watson, 2018).

- **Phenolic acids**

  A phenolic acid or acid-phenol is an organic compound with at least one carboxylic function and a phenolic hydroxyl **(Singla *et al.*, 2019)**. They are generally divided into two main groups: benzoic acids (protocatechuic, vanillic, ellagic gallic, syringic, salisylic and gentisic acid), containing seven carbon atoms (C6-C1) and cinnamic acids, containing nine carbon atoms and their basic molecules are caffeic, p-coumaric, ferulic acid and sinapic acid (C6-C3) **(Bijalwan *et al.*, 2016; Călinoiu and Vodnar, 2018).**

- **Flavonoids**

  Flavonoids are polyphenolic compounds with 15 carbon atoms forming a C6-C3-C6 structure, i.e. two aromatic rings linked by a 3-carbon bridge **(Abotaleb *et al.*, 2019)**. They are the most abundant compounds among all phenolic components,

which are responsible for the varied color of flowers, fruits, and leaves. They are divided into 6 classes: flavonone, flavanols or flavanols, flavanones, isoflavones, flavones, and anthocyanidins **(Chira *et al.*, 2008; Collin and Crouzet, 2011)**.

- **Tannins**

Tannins are natural polar polyphenolic substances, with a molecular mass between 500 and 3000 Da **(Han *et al.*, 2007)**. They are soluble in water, alcohols and acetone but insoluble in organic solvents **(Fettah, 2019)**; they are also characterized by their ability to combine with proteins and other organic polymers such as carbohydrates, nucleic acids, steroids, and alkaloids, to form stable complexes with them **(Malan *et al.*, 2001).** They constitute a large family of molecules characterized by the presence of at least one aromatic ring associated with one or more hydroxylated phenolic groups. Plant tannins are located in various organs. The highest concentrations are usually found in fruits, flowers, and leaves. Accumulation is more common in aged peels and tissues of pathological origin such as galls **(Borreani *et al.*, 2003).**

In general, they are subdivided into two groups based on their composition and their chemical reactivity **(Khababaee and Van Ree, 2001).**

-**Hydrolyzable tannins** are made up of simple phenolic molecules. They are oligo- or polyesters of sugar and a variable number of phenolic acid molecules **(Tehami, 2017)**.

-**Condensed tannins** are non-hydrolyzable compounds with a higher molecular weight. They are polymericflavanoids, which consist of flavan-3-ol units linked together by carbon-carbon bonds **(Tehami, 2017)**. This condensation gives them a structure similar to that of flavonoids **(Naumann *et al.*, 2017)**.

3. Pomegranate (*Punica granatum*)

3.1. History

The pomegranate originates back to the early Bronze Age (3500–2000 BC). Scientists put it in the top five positions in the list of oldest cultivated fruits, with olive, grape, date, and fig **(Aslanova and Magerramov, 2012)**, and this is evidenced by the Qur'an and the Bible **(da Silva *et al.*, 2013)**. In many religions and cultures, the pomegranate is considered a propitious symbol of life, good fortune, abundance, and fertility **(Gunjan *et al.*, 2012)**. The process of pomegranate domesticating took place during prehistoric times, when traders, sailors, and missionaries are said to have been responsible for its introduction to different regions **(Kandylis and Kokkinomagoulos, 2019)**.

3.2. Etymology

The species *Punica granatum* is better known as pomegranate. According to Pliny, the genus Punica was named by the Romans, making reference to the city of Cartago, in Tunis (Punic, Phoenician, Carthaginian), its name is derived from "Pomuni granatum," Pomum (apple) granatus (grainy), which translates to "seeded apple" **(Guerrero-Solano *et al.*, 2020)**.

Punica granatum's vernacular name varies from one language to another. Thus, the name of this tree will be **(Verbois, 2009)**:

In Arabic: الرمان

In French: Grenadier.

In German: Granatapfelbaum, Granatbaum, Gemeine Granat, Balluster.

In Spanish: Granado coltivado, Mangrano.

In Italian: Granato.

In Chinese: Ngan Che Lieou, Shi Liu.

3.3. Geographical Origin and Distribution

Pomegranate (*Punica granatum* L.) is one of the first domesticated fruits that have been cultivated since ancient times. The cultivation of pomegranate is considered domestication of a spontaneous species indigenous to the Asian region encompassing Georgia, Iran, Armenia, Azerbaijan, and Afghanistan where it has lived in the wild for over 4000 years, differing from cultivated varieties by its thorns and reduced fruit size. It is particularly abundant on the Caspian Sea coast **(Evreïnoff, 1957; Baum, 1988)**. It is also found on Egyptian bas-reliefs dating from 2500 B.C. and in the Thutmose III botanical garden created in 1450 B.C **(Baum, 1988; Reguieg Yssaad, 2019)**.

Pomegranate's air distribution includes Iran the homeland, and neighboring countries (Afghanistan, Baluchistan, and Northern India) **(Chauhan and Kanwar, 2012)**. It extends to central Asia regions to the Himalayas, Eyalet of Anatolia, and the Mediterranean area (Spain, Italy, Greece, Algeria, Tunisia, and Morocco). It also goes around Arizona and California and has been cultivated in South Asia, and the Middle East countries **(Behzadi Shahrbabaki, 1997; Chauhan and Kanwar, 2012; Shaygannia *et al.*, 2015).** Today, pomegranate is cultivated in most regions of the world; however, Iran has the highest production rates **(Al-Said *et al.*, 2009).**

3.4. Production of pomegranate in Algeria

According to **DSA (2018)**, the total pomegranate production in Algeria is 421136 Qx. It should be noted that the wilaya of Mostaganem recorded a large production with 186,261 Qx, followed by Djelfa 110,760 Qx and Reliz ane 90,565 Qx. The wilaya of M'sila ranks fourth in terms of pomegranate production, with 31,960 Qx.

3.5. Classification

The pomegranate (*Punica granatum*) was described by **Linnaeus (1753)** and introduced in his classification**,** which contains only two species: *P. granatum* L. and *P. protopunica* Balf. f. Where *Punica granatum* is the only species cultivated while *P. protopunica* is a wild species endemic to Socotra Island, Yemen **(Holland and Bar-Ya'akov, 2018)**. This classification was revised in 2003, giving rise to the APGII phylogenetic classification, which includes 457 families divided into 45 orders. Within this classification, the Punicaceae family no longer exists and is replaced by the Lythraceae family, a family with 30 genera and 600 species **(Spichiger *et al.*, 2004)**.

Table 3: Botanical classification of *Punica granatum* (Spichiger *et al.*, 2004).

	1753 classification	**2003 classification**
Branch	Spermatophytes	Angiosperms
Sub-branch	Angiosperms	Eudicots
Class	Magnoliopsida	Rosids
Order	Myrtales	Myrtales
Family	Punicaceae	Lythraceae
Genus	*Punica*	*Punica*
Species	*Punica granatum*	*Punica granatum*

3.6. Description

The pomegranate is a shrub or small tree that could grow up to 5 meters of height, but it may reach the morphology of a tree with more than 9 m in some cases **(Rana *et al.*, 2010)**. It may have more or less irregular and thorny branches with glossy, smooth, and hairless leaves (3–7 cm long and 2 cm broad) **(Holland *et al.*, 2009)**. The flowers (3 cm in diameter) are bright red and rarely yellow or white with tubular calyx consisting of three to seven petals that eventually become the fruit **(Bhandari, 2012; Shaygannia *et al.*, 2015)**. The bark of the tree turns gray as the tree ages **(Bhandari, 2012)**. The fruit ripens within 5–8 months after it has begun to form. During this process, the ripe fruit can be up to 5 inches with changes in skin color from yellow, green, or pink to fully red, pink, or deep purple **(Holland *et al.*, 2009; Bhandari, 2012).** Fruit peel, also called malicorium is a thick shell with hard consistency, bitter and astringent flavor that varies among cultivars grown in different regions **(Wald, 2009)**. Inside they present a spongy pericarp with membranes (endocarp) that separate the arils into compartments. In turn, each aril contains a membrane, pulp juice, and a seed **(Bhandari, 2012).**

Figure 7: *Punica granatum* fruit

3.7. Phytochemical composition

Chromatographic analysis of different varieties of pomegranate from different regions of the world shows both quantitative and qualitative diversity in its bioactive compounds (Table 4), with an abundance of the major polyphenolic classes, including, flavonoids, tannins, and anthocyanin pigments.

Anthocyanins from pomegranate have been sufficiently investigated and are included mainly glucosides of pelargonidin, delphinidin, and cyanidin forms **(Türkyilmaz, 2013;**

Kim *et al.*, 2016). These functional compounds are among the major contributors to the pharmacological properties and color qualities of pomegranate.

Flavonoids are another class of polyphenols and are among the most important secondary metabolites detected in pomegranate. Flavonoids from pomegranate are mainly represented by catechin, epicatechin, flavan-3-ol **(de Pascual-Teresa *et al.*, 2000)**, quercetin **(Gómez-Caravaca *et al.*, 2013)**, kaempferol, luteolin **(Van Elswijk *et al.*, 2004)**, naringin, pelargonidin, cyanidin **(Noda *et al.*, 2002)**, cyanidin 3-O-glucoside, cyanidin 3.5-di-O-glucoside, delphinidin 3-O-glucoside, delphinidin 3.5-di-O-glucoside **(Hernandez *et al.*, 1999)**, and punicaflavone **(Ali and Sharma, 2006)**.

Organic acids identified in pomegranate include mainly citric acid, oxalic acid, shikimic acid, acetic acid, maleic acid, fumaric acid, succinic acid, tartaric acid, and ascorbic acid **(Aarabi *et al.*, 2008)**.

Tannins are the most significant phenolic compounds present in pomegranate. These compounds are represented by glucosides, pomegranatate **(Wang *et al.*, 2006)**, punicalin **(Gil *et al.*, 2000)**, 4.4-di-O-methylellagic acid **(El-Toumy and Rauwald, 2003)**, 3-O-methyl-3.4-methylenedioxy **(El-Toumy and Rauwald, 2003)**, 3-O-methylellagic acid **(El-Toumy and Rauwald, 2003)**, punicafolin **(Nawwar *et al.*, 1994)**, punicacortein **(Tanaka *et al.*, 1986)**, gallic acid **(Huang *et al.*, 2005)**, ellagic acid **(Wang *et al.*, 2004)**, and punicalagin **(Gil *et al.*, 2000; Kulkarni *et al.*, 2004)**.

Table 4: Some phytochemical compounds of *Punica granatum* (Lansky *et al.*, 2005; Lansky and Newman, 2007).

Compound name	Chemical class	Location in the plant
Citric acid	Organic acid	Pulp (juice)
Malic acid		
Tartaric acid		
Gallic acid	Hydroxybenzoic acid	Pulp, peel, flower
Caffeic acid	Hydroxybenzoic acid	Pulp, peel
Quinic acid	Cyclitol carboxylic acid	
Catechin	Flavan-3-ols	Pulp, peel
Epicatechin		
Quercetin	Flavonol	Pulp, peel
Kaempferol		Peel
Rutin		Pulp, peel
Luteolin		Peel
Apigenin	Flavonol	Leaf
Naringin	Flavanone	Peel
Cyanidin	Anthocyanidin	Peel

Pelargonidin		
Cyanidin 3-O-glucoside Cyanidin 3,5-di-Oglucoside		Pulp
Delphinidin 3-O'Glucoside Delphinidin 3,5-di-O'Glucoside		
Pelargonidin 3-O'Glucoside Pelargonidin 3,5-di-O'Glucoside		
Ellagic acid	Ellagitannin	Pulp, peel, leaf
punicalagin		Peel, leaf, trunk
Punicalin		Root
punicic acid	Conjugated unsaturated fatty acid	Seed
Linoleic acid	Non-conjugated unsaturated fatty acid	
Oleic acid		
Palmitic acid	Saturated fatty acid	
Stearic acid		
Cholesterol	Sterol	
Campesterol		
Stigmasterol		
β-sitosterol		
17-α-estradiol	Sex steroid	
Estriol		
Estrone		
Tesrosterone		
γ-tocopherol	Tocopherol	
Coumestrol	Coumestan	
Pelletierine	Alkaloid	Trunk, root

3.8. Nutritional value

Pomegranate, like many other fruits, consists of sugars such as glucose, fructose, and sucrose. It also contains many minerals such as Fe which is relatively common, Ca, Ce, Cl, Co, Cr, Cs, Cu, K, Mg, Mn, Mo, Na, Rb, Sc, Se, Sn, Sr, and Zn and good amounts of vitamin C, B5, B6 and, B9. It is also one of the richest vegetable sources of estrogen derivatives **(Lansky and Newman, 2007).**

3.9. Health Benefits and properties

Several scientific studies have been conducted by researchers to evaluate the biological and pharmaceutical effects of pomegranate and its constituents. Such pharmaceutical and therapeutic properties can be attributed to the presence of different

bioactive constituents, especially punicalagin, and, to a lesser extent, to other metabolites, such as flavonols and anthocyanins **(Syed *et al.,* 2007)**. These compounds cover many properties, such as analgesic, anti-inflammatory, antidiabetic, anticancer, antioxidant, antimicrobial, immunomodulatory, cardiovascular supportive, gastroprotective, and hepatoprotective activities among others, through *in vivo* and *in vitro* studies **(Bhandari, 2012)**.

3.9.1. Antioxidant properties

The most commonly used assay to measure the antioxidant activity of different polyphenols is DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay **(Gülçin *et al.,* 2005)**. Interestingly, PJ has shown a strong free radical scavenging potential maybe by donating a proton to lipid peroxides (LOO•) and thus breaking free radical chain reactions **(Fraga *et al.,* 2010)**. Some authors reported that polyphenols may modulate the activities of glutathione peroxidase, catalase, glutathione S-transferase **(Vauzour *et al.,* 2010)**, and recycle antioxidant and reducing agents, such as vitamins E and C **(Fraga *et al.,* 2010)**. Punicalagin is one of the main polyphenols presented in pomegranate peel as evaluated by antioxidant power assay, the latter was found to be able to scavenge free radicals by donating an electron from their phenolic group to H_2O_2 and converting it to H_2O **(Aloqbi *et al.,* 2016)**. **Xu et al. (2014)** reported that punicalagin shows an antioxidant activity by enhancing the SOD1 mRNA expression and thereby inhibits reactive oxygen species generation and NO overproduction.

Besides, flavonoids such as catechin, and some procyanidins interact with NADPH-oxidase leading to a decrease in superoxide anion production **(Fraga *et al.,* 2010)**. Studies have reported that pomegranate peel has total flavonoid content higher than that of juice extracts. Therefore, the peel has significant higher antioxidative efficacy than the pulp, seed, or juice extract **(Orak *et al.,* 2012; Orgil *et al.,* 2014)**, and has scavenging capacity against hydroxyl, superoxide anion, and peroxyl radicals, thus exhibiting protective effects against oxidative stress and lipid changes **(Doostan *et al.,* 2017)**.

3.9.2. Antioxidant properties of pomegranate juice compared to other fruits

Pomegranate juice revealed the greatest antioxidant potency, compared to other widely available polyphenol-rich beverages, such as blueberry juice, black cherry juice, açaí juice, cranberry juice, orange juice, red wine, green and black tea **(Seeram *et al.,* 2008)**. Despite the lower level of polyphenols in PJ compared to chokeberry juice, these two showed the same antioxidant properties **(Nowak *et al.,* 2017)**.

Table 5: Comparison between *Punica granatum* juice and other fruit juices (Seeram *et al.,* 2006).

Concentrated fruit juice	polyphenol concentration (mmoL/L)	Free radical scavenging ability (% reduction)
Pomegranate	5.0	95
Red plum	4.5	80
Grape	3.3	47
Cranberry	2.5	47
Kiwi	2.2	70
Orange	1.6	11
Grapefruit	1.5	16
Apple	1.4	55
Pineapple	1.1	27
Pear	1.1	5
peach	1.0	30

3.9.3. Anti-inflammatory properties

In vivo studies proved that punicalagin from pomegranate has been shown to significantly reduce inflammation through the inhibition of nuclear factor kappa B (NF-κB) activity and by the prevention of ERK-1 or ERK-2 (mitogen-activated protein kinase cascades) activation. Thus, it has the property of inhibiting IL-6 and IL-8 expression **(Thangavelu *et al.,* 2017)**. It is also reported that punicalagin can regulate the action of macrophages and T and B lymphocytes **(Gracious Ross *et al.,* 2001)**. Furthermore, pomegranate extracts may have the ability to inhibit prostaglandin E2 production, and interleukin-1beta (IL-1B)-induced tissue destruction in periodontitis by inhibiting nitric oxide (NO) production and nitric oxide synthase expression **(Lee *et al.,* 2010)**.

Preclinical and clinical research has revealed that pressed pomegranate seed oil inhibits cyclooxygenase and lipo-oxygenase. Cyclooxygenase, a key enzyme in the conversion of arachidonic acid to prostaglandins (the major mediators of inflammation), was inhibited by 37% by pressed seed oil extract **(Schubert *et al.,* 1999)**.

3.9.4. Anticarcinogenic properties

Pomegranate has also been considered to obtain positive effects against numerous cancer types. More specifically, Studies indicated that PJ suppressed TNFα-induced COX-2 protein expression and block nuclear factor kappa B (NF-κB) activity in a prostate cancer model and renal cell carcinoma *in vitro* **(Rettig *et al.,* 2008; An *et al.,* 2015)**. Studies have found that treatment with pomegranate extract may be used to manage patients with small, localized, incidentally identified renal tumors, thus the avoidance of nephrectomy.

Furthermore, anti-proliferative properties of Pomegranate peel extracts have shown growth arrest against prostate cancer cells via the induction of apoptosis **(Adaramoye *et al.*, 2017; Deng *et al.*, 2017)**. The anti-tumor potential of *Punica granatum* is not solely confined to the edible part of the fruit. For instance, punicalagin and ellagic acid isolated from the peel, as well as leaf extract, strongly attenuated proliferation of A549 and H1299 lung cancer cell lines and decreased H1299 cell migration and invasion, which may also suggest the potential of this extract in the prevention of cancer metastasis **(Li *et al.*, 2016)**. In recent studies, punicalagin has also been revealed to induce the cell death of papillary thyroid carcinoma cells **(Yao *et al.*, 2017)**, inhibit cell proliferation in a non-small lung carcinoma cell line **(Li *et al.*, 2016)**, and exert a strong antiproliferative activity against breast and cervical cancer cell lines **(Aqil *et al.*, 2012)**.

3.9.5. Hepatoprotective properties

Studies indicate that pomegranate flowers possess potent antioxidant and hepatoprotective properties against diabetes and obesity-associated fatty liver, at least in part, by activating the hepatic expression of genes responsible for fatty acid oxidation **(Xu *et al.*, 2009)**. Ferric nitrilotriacetate (Fe-NTA) is a potent hepatic tumor promoter and acts through the generation of oxidative stress. Pretreatment with pomegranate flower extract, at a dose regimen of 50-150 mg kg body weight, for a week, significantly and dose-dependently protected against ferric nitrilotriacetate (Fe-NTA)-induced oxidative stress, as well as a hepatic injury where it increased the GSH levels and activities of the antioxidant enzymes, namely, CAT, GPx, and GST. The extract may also alleviate the histopathological changes produced by Fe-NTA, such as ballooning degeneration, fatty changes, and necrosis, along with the modulation of the activities of hepatic enzymes (AST, ALT, and ALP) and levels of serum bilirubin and albumin **(Kaur *et al.*, 2006)**. Furthermore, pomegranate has shown to alleviate non-alcoholic steatohepatitis disease **(Al-Shaaibi *et al.*, 2016)**, while its extracts have been shown to protect against arsenic-induced inflammation and apoptosis in the liver cells of male Swiss albino mice **(Choudhury *et al.*, 2016)**.

3.9.6. Reproprotective properties

Pomegranate fruit is linked with fertility, birth, and eternal life, because of its many seeds. Several studies have shown that consumption of PJ may enhance the epididymal sperm concentration, sperm motility, spermatogenic cell density, diameter of seminiferous tubules, and germinal cell layer thickness **(Türk *et al.*, 2008; Zeweil *et al.*, 2013)**. It is also

suggested that PJ consumption decreased the abnormal sperm and improves the antioxidant activity (GSH, GPx, and Catalase) of rats **(Türk *et al.*, 2008)**. In addition, pomegranate's therapeutic effects contribute to the increase in testosterone production **(Suman and Bhatnagar, 2019)**.

3.9.7. Other health benefits

Fresh or dried root barks or ethanol extracts of pomegranate are used to remove intestinal parasites due to the alkaloid substances. Many researches have indicated that *Punica granatum* flower and peel extracts are used in traditional medicine to treat simple diarrhea, dysentery, and stomach disorders **(Shaygannia *et al.*, 2015)**. Pomegranate juice has been reported to inhibit aggregation and oxidation of atherosclerotic lesions and attenuate platelet activation **(Suman and Bhatnagar, 2019)**. It may also decrease the risk of heart attacks, strokes, and embolic diseases **(Thangavelu *et al.*, 2017)**. Moreover, the administration of crude powder of *Punica granatum* husk decreased the concentration of glucose, triglycerides, cholesterol, LDL cholesterol, and blood pressure and raised the level of HDL cholesterol. Compounds in pomegranate like punicalagin acid, ellagic, gallic, olaenolic, ursolic, and uallic acids have been recognized as having antidiabetic actions **(Suman and Bhatnagar, 2019)**; they have also been shown signs of neuroprotection **(Choi *et al.*, 2011; Subash *et al.*, 2015)**. According to **Mizrahi et al. (2014)**, punic acid, a derivative of pomegranate seed oil, shows neuroprotective activity by reducing lipid oxidation.

II. Materials and Methods

1. Materials

1.1. Chemical material

Toluene with a purity of 99.5% was purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). All other chemicals used in this experiment were analytical grade and supplied by Animal Ecophysiology Laboratory, Badji Mokhtar University Annaba-Algeria.

1.2. Biologic material

Albino male *Wistar* rats (230 ± 30g) provided by the Pasteur Institute of Algiers were used for the evaluation of the biological activity *in vivo*. Animals were kept under well-controlled conditions (temperature of 22±2°C and an 11h L/13h D photoperiodic cycle), given water *ad libitum* and fed with commercial pellets purchased from the agro-food complex (El-Kseur, Bejaia). The animals were left for 21 days in order to adapt to the animal house of the Biology Department.

1.3. Vegetal materiel

1.3.1. Presentation and situation of the harvesting area

The region of El Tarf is located along the coast and it is one of the most watered regions of northeastern Algerian. Their climate is of the humid Mediterranean type, characterized by two seasons of six months each **(Kherifi and Kherici-Bousnoubra, 2017).** The territory of El Tarf groups together 5 classes of use lands: vegetation, agriculture, sand, bodies of water and built-up space. The vegetation is composed mainly of forests and scrub, it occupies an area of 161,464 ha, or 56.03% of the total area. Agriculture with 36.47% comes in second position accounting for an area of 105,118 ha; it includes land for crops, arboriculture and pastures **(Arfa *et al.*, 2019)**.

The geographical location as well as the bioclimatic stage of the harvesting station is shown in Table 6.

Table 6: **Geographical and climatic characteristics of the harvesting site.**

Plant	Station	Latitude	Longitude	Bioclimatic floor
***Punica granatum* L**	Sidi Djemil Besbes	36.671028	36° 40' 15. 7" N 7° 46' 24.3" E	Humid

1.3.2. Plant

Mature *Punica granatum* L. fruits were collected in November 2018 from a local farm in the region of El-Tarf (Algeria). The whole fruit was washed, and then manually peeled without separating the seeds. Peels were kept, dried under shade, and finally grounded to a fine homogeneous powder and stored in amber glass bottles at ambient temperature. The ground material will constitute the dry matter which will be used for the preparation of the aqueous extract. Whereas, for the preparation of the juice, fresh seeds were used.

Figure 8: Different used constituents of the *Punica granatum* fruit.

2. Methods

2.1. Study of phytochemical activities *in vitro*

2.1.1. Preparation of the juice and the extract

Pomegranate juice was prepared daily using a commercial centrifugal blender (Sinbo, Turkey). The aqueous extract was prepared by macerating the powder with a volume of distilled water and combined at room temperature for 72h with occasional shaking **(Deore Leena *et al.*, 2016).** The peel extract was then filtered through cotton gauze; and finally, the filtrate was frozen in 30 mL aliquots, stored at 4°C and thawed as required.

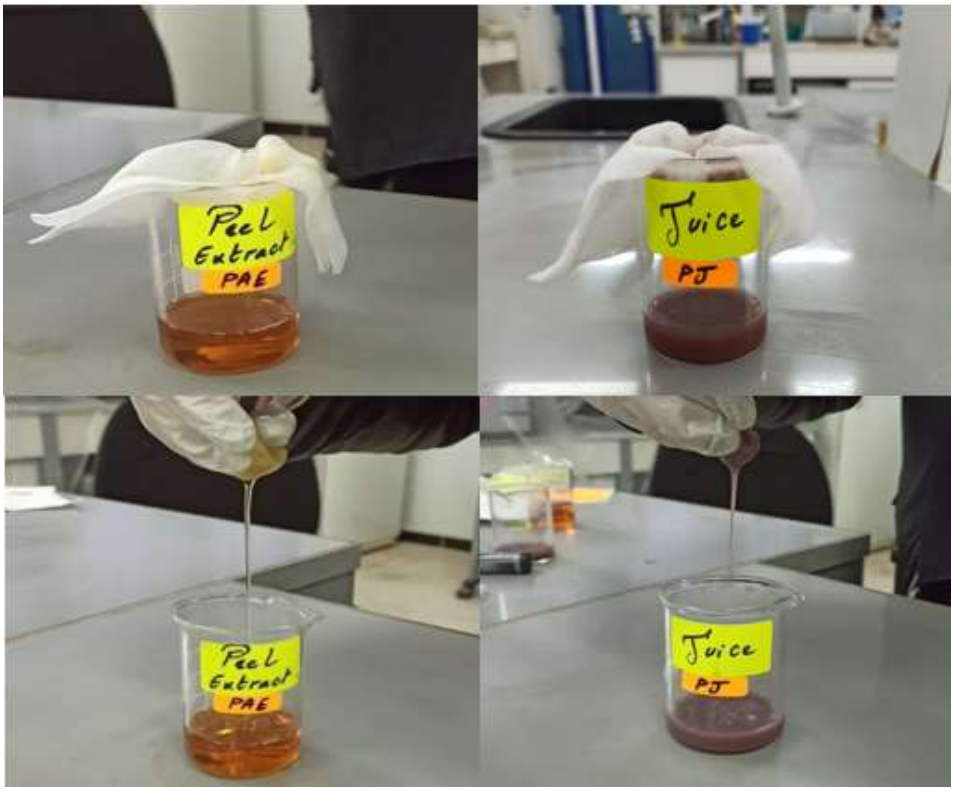


Figure 9: Preparation of *Punica granatum* juice and peel aqueous extract.

2.1.2. DPPH Radical Scavenging Assay

The free radical scavenging activity of the extracts was measured by the 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay **(Burits and Bucar, 2000)**. After dissolving the extracts in methanol, the solution of DPPH in methanol (0.04 mg/mL) was prepared and 1250 µL of this solution was added to 50 µL of fraction solution at different concentrations. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature, and then, the absorbance was measured at 517 nm where the BHT was used as standard. Radical scavenging activity was calculated using the following equation:

Radical scavenging activity (%) = (A blank –A sample / A blank) × 100

A blank: Absorbance of the control. A sample: Absorbance of the reagent with the extract.

2.1.3. Determination of total polyphenols content

The total polyphenols content was determined by the Folin-Ciocalteu method as described by **Li et al. (2007)**. 0.1 mL of extract was mixed with 0.5 mL of Folin-Ciocalteu reagent. After 4 min, 0.4 mL of 7.5 % sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 1h in dark at room temperature. The absorbance of the samples was measured at 760 nm and the results are expressed as microgram of gallic acid equivalents per milligram dried extract (mg GAE/g Extract).

2.1.4. Determination of total flavonoids content

Flavonoid determination was determined using the Aluminum trichloride ($AlCl_3$) method cited by **Mouffouk et al. (2018)**. Flavonoids have a free hydroxyl group (OH), which is likely to give in the presence of $AlCl_3$ a yellowish complex by chelation of Al^{3+} ion. The yellow coloration produced is proportional to the number of flavonoids present in the extract **(Basli *et al.*, 2012)**. 1 mL of extract was mixed with 1 mL of aluminum chloride ($AlCl_3$) solution (2%) and allowed to stand for 10 min. The absorbance of the mixture was then determined at 430 nm versus a blank equivalent to the prepared extract. Results were expressed as micrograms of quercetin equivalent per milligram dried extract (mg QE /mg Extract).

2.1.5. Determination of total tannins content

Total tannins were measured by the Folin-Denis's method **(Polshettiwar *et al.*, 2007)**. Tannin colorimetry was based on the measurement of the blue color formed by the reduction of phospho-tungsto-molybdic acid by tannins as a compound in an alkaline medium. 1 mL of extract and a standard solution of tannic acid (100-800 µg/mL) were brought to 7.5 mL with distilled water. Then, 0.5 mL Folin-Denis reagent (Sigma-Aldrich, USA) and 1 mL of Na_2CO_3 (Sigma- Aldrich, USA) solution were added. The volume was made up to 10 mL with distilled water. The absorbance was read at 700 nm. The total tannin content was expressed as mg of tannic acid equivalent (mg TAE)/ 100g extract (**Padma *et al.*, 2013).**

2.2. Study of biological activities *in vivo*

The biological explorations were based on an *in vivo* study including sub-chronic toxicity. The methodology and experimental protocol consist in treating male rats orally with the toluene and the *Punica granatum* juice and extract using a gastric tube, daily and for 6 weeks.

2.2.1. Preparation of the *Punica garanatum* juice and the peel extract Treatement

The methods for the preparation of the extracts were discussed in the first part. The juice and the extract used as treatments were prepared from the dose of 4 mL/kg BW and 400 mg/kg BW respectively.

2.2.2. Preparation of the toluene treatment

The dose of the toluene (Tol) used in the present study was 550 mg/kg BW. This dose was used as the toxicological dose and represented one tenth (1/10) of the lethal dose (LD50) **(ATSDR, 2000)**. Toluene was diluted in corn oil as a vehicle where each rat receives 0.3 mL.

2.2.3. Experimental design

After the period of adaptation, the animals were divided into 7 groups as follows:

- **Control (C):** received distilled water.
- **(CO):** received 1.25 mL/kg BW of corn oil.
- **(PJ):** given 4 mL/kg bw of pomegranate fresh juice.
- **(PAE):** treated with 400 mg/kg BW of pomegranate peel aqueous extract.
- **(Tol):** treated with 550 mg/kg BW of toluene (Tol) dissolved in corn oil.
- **(PJ-Tol):** treated with Tol (550 mg/kg BW), followed after 2 hours by PJ (4mL/kg BW).
- **(PAE-Tol):** treated with Tol (550 mg/kg BW), followed after 2 hours by PAE (400 mg/kg BW).

2.2.4. Animals sacrifice and samples preparation

At the end of the treatment period, the rats were sacrificed after a 12 hour fast. Semen and blood samples were immediately collected for sperm and haematological analysis. Serum and plasma were obtained by the centrifugation of the blood at 3000×g for 10 min, and were then stored at -20°C until biochemical and hormonal analysis. Organs were dissected and cleaned from adipose tissues, weighted and a fragment was stored at -20 °C until used to study the oxidative stress parameters. Another fragment was fixed in a 10% formalin solution in order to make histological sections.

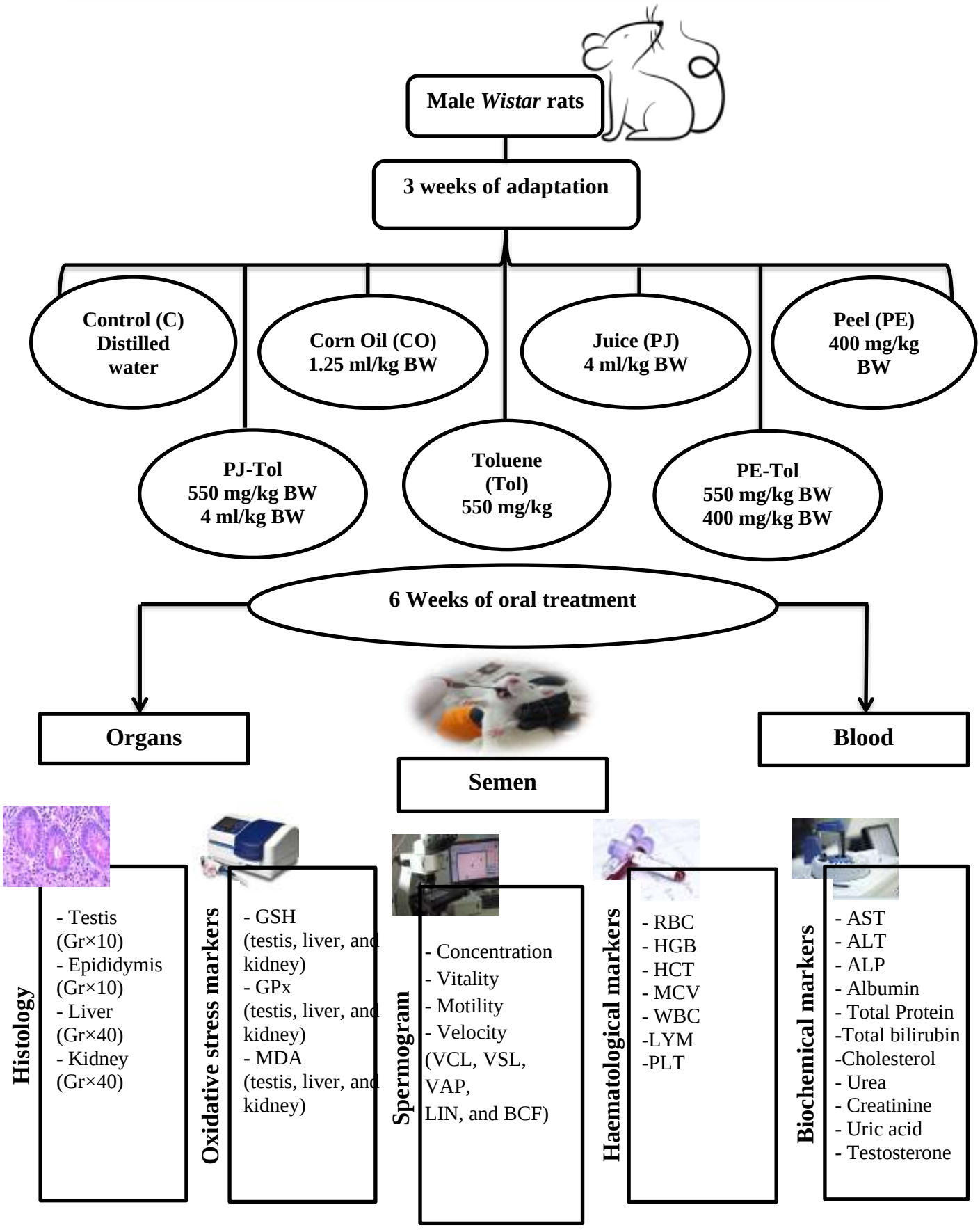


Figure 10: Summary diagram of the experimental protocol.

2.2.5. Determination of sperm biology

a. Determination of sperm concentration, motility, and velocity

To evaluate the characteristics of sperm quality we used computer-assisted sperm analysis (CASA system) (Barcelona, Spain). It is able to offer a variety of semen parameters i.e. sperm concentration, total motility, velocity, and percentage of linear motile, non-progressively motile and immotile sperm. The collected semen was obtained from epididymis cauda; analysis was performed immediately after 1:8 dilution of semen in 0.9% NaCl physiological solution. A drop of dilute semen was transferred with a micropipette to a GoldCyto 20-µm counting chamber. This preparation is examined under a microscope maintained at a temperature of 37 °C (Nikon-eclipse E200, Barcelona, Spain) and integrated into the microcomputer using (×4) negative-phase contrast combined with a phase condenser contrast.

b. Determination of sperm vitality (Hypo-osmotic Swelling Test)

The vitality of sperm was analysed using Hypo-osmotic Swelling Test, which was developed by **Jeyendran et al. (1984).** This test is based on semi-permeability of intact spermatozoid membrane. It provides additional information on the integrity and functionality of the plasma membrane at the level of the flagellum.

- **Solution used**

Dissolve 0.367g of sodium citrate ($Na_3\ C_6H_5O_7,\ 2H_2O$) and 0.675g of fructose in 50 mL of distilled water (store at -20°C).

- **Method**

For approximately 5 minutes, the mixture is heated to 37° C, and 1 mL of the solution is placed in a closed Eppendorf tube. Then, add 0.1 mL of diluted sperm and mix gently with a micropipette, and leave to incubate at 37.5°C for 30 minutes. After that, the sperm are observed under a microscope (Leica-Germany) at a magnification (x40). Swollen sperm are those with flagellum changes. The percentage of spermatozoa with flagellum changes is calculated out of a total of 100 spermatozoa counted **(WHO, 1993).**

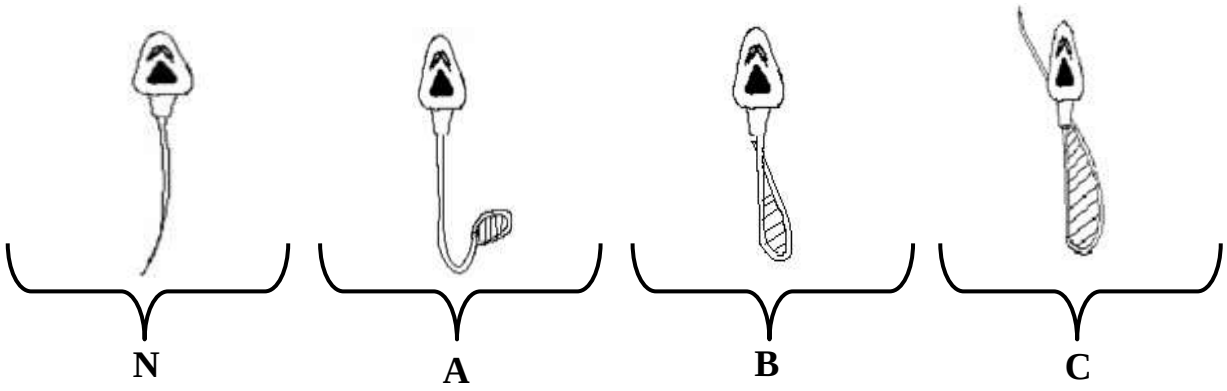


Figure (11): Schematic appearance of the hypo-osmotic swelling test

C: very significant modification of the flagellum

B: significant modification of the flagellum.

A: weak modification of the flagellum.

N: dead sperm.

2.2.6. Determination of testosterone level

Plasma testosterone level was estimated by using a chemiluminescence immunoassay-based commercial kit (Access testosterone 33560) and an Access immunoassay analyzer (California, USA).

2.2.7. Determination of haematological parameters

Assessment of haematological variables is a very common and primary diagnostic important routine that helps to explain the blood-related functions of consumed substances. Blood was collected using EDTA tubes to count the following haematological parameters: red blood cell (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), white blood cells (WBC), lymphocytes (LYM), Granulocyte (GRA), eosinophil (EOS), and other types of white blood cells Neutrophil (NEU) using Automated Hematologic Analyzer (Abacus 4).

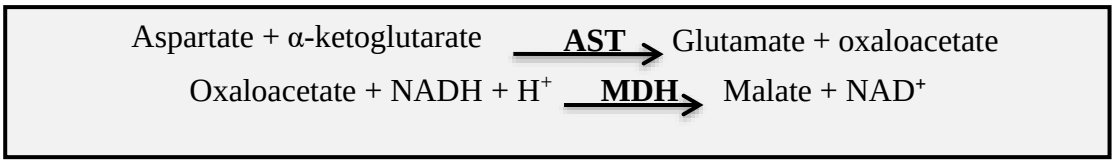
2.2.8. Determination of biochemical parameters

- **Exploration of hepatic function**

a. Aspartate aminotransferase (AST / GOT) measurement

Aspartate aminotransferase (AST) also called glutamate oxaloacetate (GOT) catalyzes the reversible transfer of an amino group from aspartate to α-ketoglutarate forming glutamate and oxaloacetate. Oxaloacetate is reduced to malate-by-malate dehydrogenase (MDH) and NADH, H+. The rate of decrease in NADH concentration is directly proportional to the

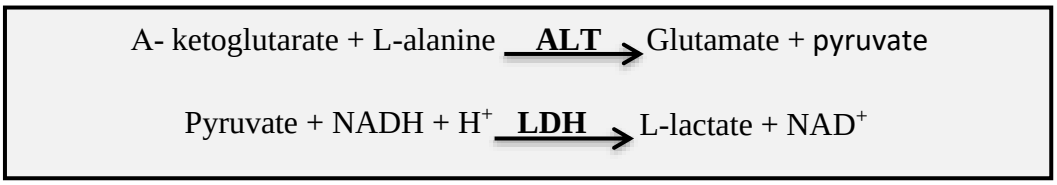
activity of aspartate aminotransferase in the sample (serum) **(Murray, 1984a).**



b. Alanine aminotransferase (ALT/GPT) measurement

The decrease in NADH concentration is directly proportional to the alanine aminotransferase enzyme activity in the sample (serum) **(Murray, 1984b).**

The principle is presented according to the following reaction scheme:



c. Alkaline phosphatase (ALP) measurement

Alkaline phosphatase (ALP) catalyzes the hydrolysis of p-nitrophenyl phosphate at pH 10.4 to give p-nitrophenol and phosphate. The formation of p-Nitrophenol is measured spectrophotometrically, where it is proportional to the catalytic activity of alkaline phosphatase in the sample (serum) (**Wenger *et al*., 1984**).

d. Total protein measurement

Proteins form an intensive blue-violet-colored complex with copper ions in an alkaline medium. The intensity of the color formed is proportional to the concentration of total protein in the sample (**Koller, 1984**; **Burtis *et al*., 2005**).

e. Albumin (ALB) measurement

Albumin reacts with bromocresol green (BCG) to form a colored complex. The pH of themedium is maintained at 4.2 by the buffer. After incubation, the absorbance of the mixture is measured at 628 nm (**Doumas, 1971**).

f. Total bilirubin measurement

Bilirubin is defined as the amount of serum pigment reacting with diazotized sulfanilic acid acid at acidic pH to produce azobilirubin quantifiable by spectrophotometry.

Sulfanilic acid + Sodium Nitrite → Diazotized sulfanilic acid

Diazotized sulfanilic acid + bilirubin **R° coupling** → Azobilirubin

The intensity of the color formed is proportional to the concentration of bilirubin in the sample (serum) **(Kaplan *et al.*, 1984a).**

- **Exploration of renal function**

g. Urea (Ur) measurement

The technique used for the determination of urea is the enzymatic method using urease according to the following reaction:

$Urea + H_2O \xrightarrow{\textbf{Urease}} (NH_4)_2 + CO_2$

$NH_4^+ + Salicylate + Na\ ClO \xrightarrow{\textbf{Nitroprussid}} Indophenol$

Ammonium ions can react with salicylate and sodium hypochlorite to form a green-colored complex, the color intensity is proportional to the concentration of urea present in the sample (serum) (**Fawcett and Scott, 1960**).

h. Creatinine (Cr) measurement

Creatinine reacts with alkaline picrate to give a colored complex, measured within a defined time interval and proportional to the creatinine concentration of the sample (serum) (**Murray, 1984c**).

Creatinine + picrate alkaline **PH alkaline** → Complexes creatinine-picrate

i. Uric Acid (UA) measurement

The uric acid present in the sample gives according to the reaction described above a colored complex, quantifiable by spectrophotometry:

Uric Acid + 2 **Uricase** → Allantoine + CO_2 + 2

2 H_2O_2 + Amino phenazone + **Peroxidase** → Quinonimine + 4

The intensity of the color formed is proportional to the concentration of uric acid in the

sample (serum) (**Schultz, 1984**).

2.2.9. Determination of oxidative stress parameters

a. Reduced glutathione assay (GSH)

-Preparation of the homogenate

Glutathione (GSH) was measured in the testis, liver, and kidneys. 200 mg of the tissue was cold-milled using an ultrasonic homogenizer in the presence of 8 ml of a 0.02 M EDTA acid to obtain a homogenate.

-Principle

The GSH assay was carried out according to the method of **Weckbecker and Cory (1988).** The principle of this assay is based on measuring the optical absorbance of 2-nitro-5-mercapturic acid (JENWAY 6300). The latter results from the reduction of DTNB acid (Ellman's reagent) by the (-SH) groups of glutathione (Figure 13). For this, deproteinization of the homogenate is essential in order to keep only the thiol groups specific to glutathione.

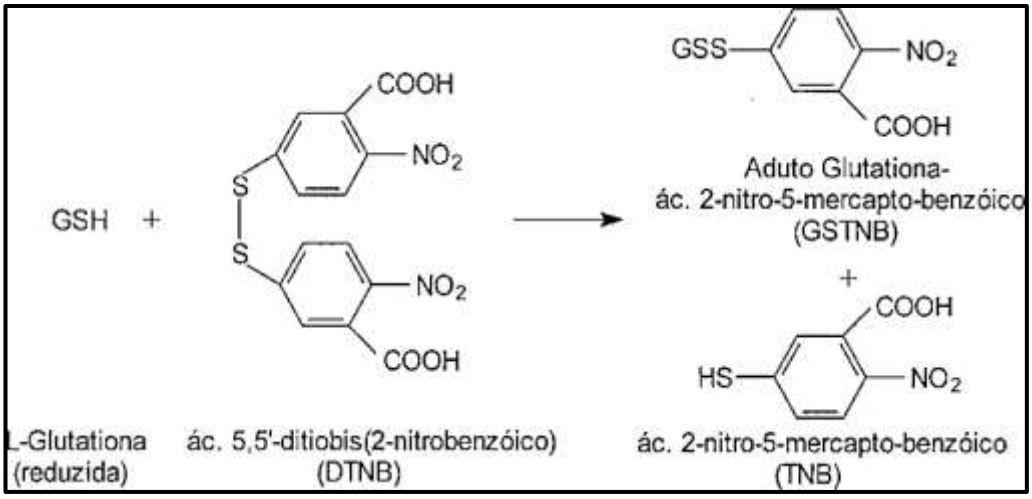


Figure 12: Principle of glutathione assay.

-Operating mode

- ✓ Take 0.8 ml of the homogenate.
- ✓ Deproteinize by adding 0.2 ml of a 0.25% SSA acid solution.
- ✓ Shake the mixture and leave for 15 minutes in an ice bath.
- ✓ Centrifuge at 1000 rpm for 5 min.
- ✓ Take 0.5 ml of the supernatant.
- ✓ Add 1 ml of Tris + EDTA buffer (0.02 M EDTA), pH 9.6.
- ✓ Mix and add 0.025 ml of 0.01 M DTNB (dissolved in absolute methanol).

- ✓ Leave for 5 min at room temperature for color stabilization which develops instantly.
- ✓ Read optical densities at 412 nm against the blank.

-Calculation of the concentration

The concentration of glutathione is obtained by the following formula:

$$\mathbf{GSH} = \frac{\mathbf{DO} \times \mathbf{L} \times \mathbf{10.525}}{\mathbf{13.1} \times \mathbf{0.8} \times \mathbf{0.5} \times \mathbf{mg\ protiens}}$$

OD: Optical Density.

L: Total volume of solutions used in deproteinization (0.8 mL homogenate 0.2 mL ofsalicylic acid).

1.525: Total volume of solutions used in the determination of GSH in the supernatant (0.5 mL supernatant +1 mL Tris + 0.025 mL DTNB).

13.1: Coefficient of absorbance of the -SH group at 412 nm.

0.8: Volume of the homogenate.

0.5: Volume of supernatant.

b. Glutathione peroxidase assay (GPx)

-Preparation of the homogenate

Glutathione peroxidase (GPx) was measured in the testis, liver, and kidneys. 200 mg of tissue were cold-milled using an ultrasonic homogenizer in the presence of 5 mL of a TBS solution (Tris 50 mmol, NaCl 150 mmol, pH 7.4) to obtain a homogenate.

-Principle

The determination of GPx activity was carried out according tothe method of **Flohé and Günzler** (**1984**). This method is based on the reduction of hydrogenperoxide (H2O2) in the presence of reduced glutathione (GSH); the latter is transformed into(GSSG) under the influence of GPx according to the following reaction:

$$\mathbf{H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O}$$

-Operating mode

- ✓ Take 0.2 ml of the homogenate.
- ✓ Add 0.4 ml of GSH (0.1 mM).
- ✓ Add 0.2 ml of TBS buffer solution (pH 7.4).
- ✓ Incubate in a water bath at 25°C for 5 min.
- ✓ Add 0.2ml of H2O2 (1.3 mM) to initiate the reaction (leave for 10 minutes).

- ✓ Add 1 ml of TCA (1%) to stop the reaction.
- ✓ Put the mixture in ice for 30 minutes.
- ✓ Centrifuge for 10 minutes at 3000×g.
- ✓ Take 0.48 ml of the supernatant.
- ✓ Add 2.2 ml of the TBS buffer solution.
- ✓ Add 0.32 ml of DTNB (1 mM).
- ✓ Mix and after 5 minutes read the absorbance at 412 nm against the blank.

-Calculation of the concentration

The determination of GPx enzymatic activity is done using the following formula:

$$\mathbf{Q} = \frac{(\mathbf{OD\ sample} - \mathbf{DO\ standard}) \times \mathbf{0.04}}{\mathbf{OD\ standard}}$$

Q: Quantity of GSH disappeared (oxidized).

OD sample: Optical density of the sample.

OD standard: Optical density of standard

0.04: Substrate concentration (GSH).

$$\textbf{GPx activity (M GSH / min / mg proteins)} = \frac{\mathbf{Q}}{\mathbf{mg\ of\ proteins}}$$

c. Malondialdehyde assay (MDA)

-Preparation of the homogenate

200 mg of tissue from the different groups are were cold-milled using an ultrasonic homogenizer in the presence of 5 ml of a phosphate buffer solution (0.1 M, pH 7.4) to obtain a homogenate.

-Principle

Malondialdehyde (MDA) content, which indicates the tissue lipid peroxidation, which is formed during the attack of polyunsaturated lipids by ROS, generated by certain contaminants, was determined by the reaction between TBA and MDA extractable by organic solvents such as butanol according to the method of **Ohkawa et al.** (**1979**). The assay is based on the formation in a hot and acidic medium (100°C) between MDA and TBA of a colored pigment absorbing at 530 nm, extractable by organic solvents such as butanol (Figure 14).

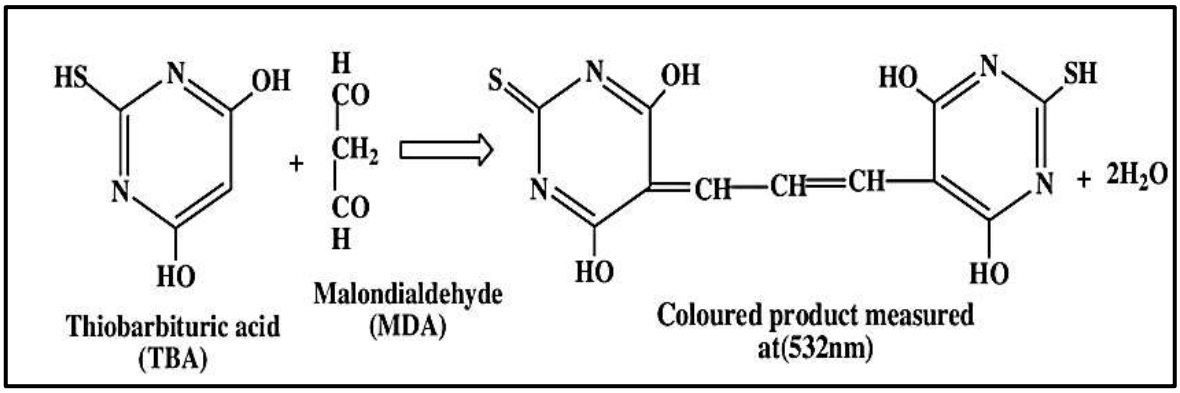


Figure 13: **Principle of the malondialdehyde assay.**

-Operating mode

- ✓ Take 0.5 ml of the homogenate.
- ✓ Add 0.5 ml of 20% TCA acid.
- ✓ Add 1 ml of 0.67% TBA acid.
- ✓ Mix and incubate in a water bath at a temperature of 100°C for 15 min.
- ✓ Cool and add 4 ml of n-butanol.
- ✓ Centrifuge for 15 minutes at 3000×g.
- ✓ Collect the supernatant, and read the optical density at 530 nm against the blank.

-Calculation of the concentration

The amount of MDA in the sample is expressed in nmol/gram of tissue. It is obtained from a standard curve performed with 1,1,3,3 tetraethoxypropane under the same conditions.

d. Tissue protein assay

Tissue proteins were quantified according to the colorimetric method of **Bradford (1976)** which uses Coomassie Brilliant Blue G250 (BBC) as reagent and bovine serum albumin (BSA) as standard. The BBC reacts with the groups.

All measurements were realized by a UV/visible spectrophotometer (JENWAY-6300).

2.2.10. Histological study

The histological study was made according to the hematoxylin & eosin (H&E) method of **Hould (1984)** following these steps:

a. Fixation

The purpose of the fixation is to preserve the cellular structure of the tissue. Fixation is necessary to protect and harden the tissue against the deleterious effects of later procedures,

which, otherwise, would disrupt cellular structure beyond recognition. Furthermore, fixation minimizes a process called autolysis. Autolysis is the degradation of the cellular structure which results from the release of degradative enzymes from the excised tissue itself. The fixation process must be started as quickly as possible after removal of the sample.

b. Dehydration

Dehydration is done through a circulator automaton where the samples were immersed in baths of ethanol at increasing concentrations (70, 95 and 100%) and in baths of xylene which is a lightning agent giving the tissue more transparency. Then, an oven of xylene evaporates the anatomical pieces. The purpose of this step is to eliminate the intracellular water, in order to be able to make a fine section afterwards without losing the initial cellular structure.

c. Inclusion

The samples are immersed in molten liquid paraffin at 60 ° C and placed in molds called Leuckart bars, and then it filled with paraffin. This operation uses devices called “inclusion” Chilled to achieve rapid solidification (10 to 15 min) of the paraffin block containing the tissue.

d. Cuts and staining

The paraffin blocks obtained are placed in a microtome one by one in order to produce sections 5 µm thick arranged in regular series in the form of a strip. The making of histological sections then involves 3 steps:

- ✓ The obtained paraffin tapes are placed on glass slides coated with a solution of poly-L-lysine (sigma).
- ✓ Gluing: the glass slides are placed on a hot plate, set at a temperature of 35°C. The heat allows the spreading of the ribbons of slices.
- ✓ Drying the preparation: by tilting the slides and drying them with absorbent blotting paper.

The purpose of the staining is to accentuate the contrasts in order to differentiate the different tissue constituents (nucleus, plasma membrane and cytoplasm). The slides are placed in baths of solvents and stains as shown in the following table:

Table 7: The different coloring products.

Products	Times
Xylene I	5 min
Xylene II	5 min
Ethanol 100%	5 min
Ethanol 96%	5 min
Ethanol 70%	5 min
Distilled water	5 min
Haematoxylin	5 min
Tap water	Rinsing
Eosin	20 sec
Ethanol 70%	5 min
Ethanol 96%	5 min
Ethanol 100%	5 min
Xylene III	5 min
Xylene IV	5min

e. Assembly and microscopic observation

The assembly consists of fixing a glass coverslip on the histological preparations after staining with a drop of Eukitt. Finally, we proceed to the observation under an optical microscope after drying the slides.

2.2.11. Statistical analysis

Statistical significance was determined using a one-way analysis of variance with Tukey's post-hoc test. Data are reported as the mean ± standard error of the mean (Mean ± SEM) using GraphPad Prism 7 (GraphPad Software) and Statistical SPSS for Windows (version 26.0), with significance set at $P<0.05$.

III. Results

1. Phytochemical activities

1.1. DPPH Scavenging Activity

The assessment of antioxidant activity in *Punica granatum* juice and peel aqueous extract, are shown in Figure 14. The results which are related to DPPH scavenging activity suggest that the PAE with have higher activity than juice.

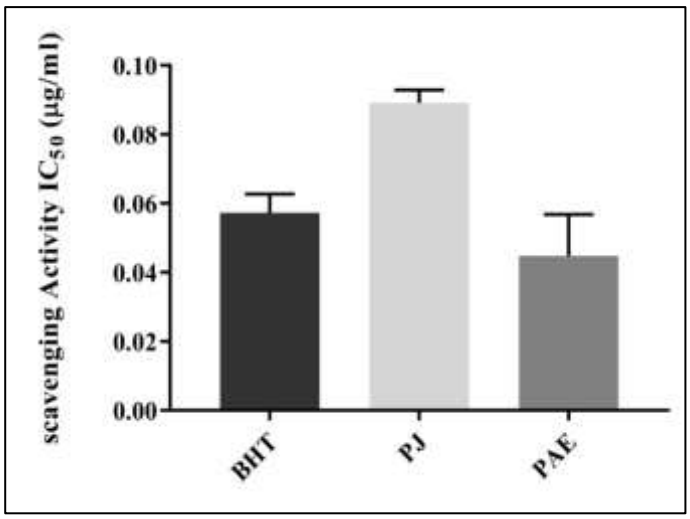


Figure 14: DPPH scavenging activity of *Punica granatum* juice and peel aqueous extract (mean ± SEM). (**DPPH:** 2,2-Diphenyl-1-picrylhydrazyl, **BHT:** Butylated Hydroxytoluene, **IC50:** Inhibitory concentration, **PJ:** Pomegranate Juice, **PAE:** Peel Aqueous Extract).

1.2. Total phenolic, Flavonoid, and Tannin Contents

Total phenolic, flavonoid, and tannin contents in both peel aqueous extract and juice of *Punica granatum* are reported in Fig 15, 16, and 17. According to the results, the amounts of total phenolic, flavonoid, and tannin were much greater in PAE (0.78±0.01mg GAE/g, 0.49±0.002 mg EQ/ g, and 0.74±0.033 mg TAE/100g of Pomegranate respectively) than in the PJ (0.52±0.005 mg GAE/g, 0.22±0.029 mg EQ/g of flavonoids, and 0.44±0.042 mg TAE/100g of Pomegranate).

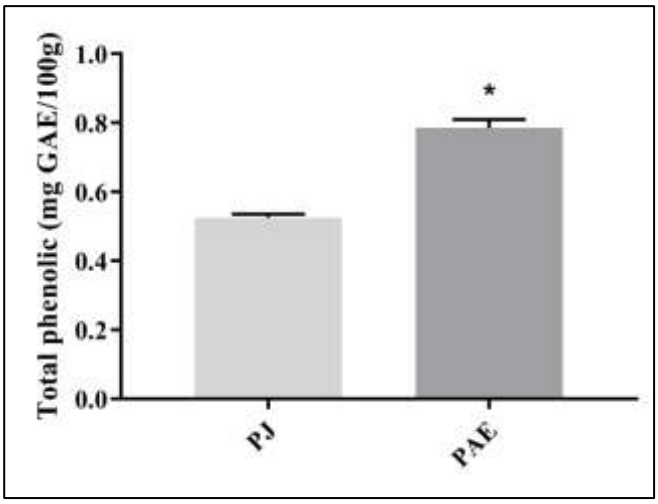


Figure 15: Total phenolic content of *Punica granatum* juice and peel aqueous extract (mean±SEM). (GAE: Gallic Acid Equivalent**).**

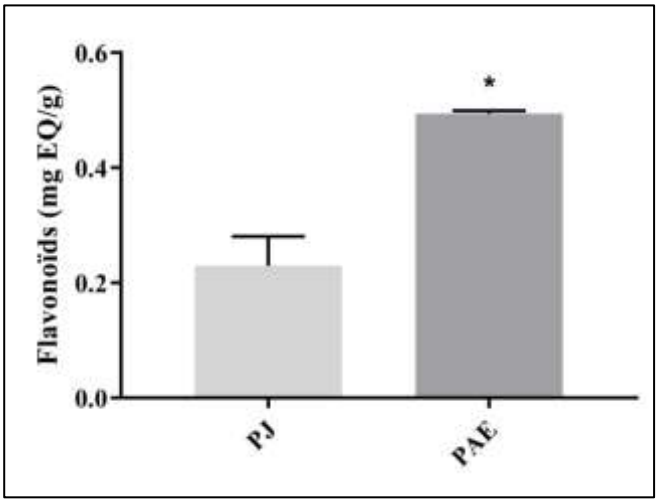


Figure 16: Flavonoïd content of *Punica granatum* juice and peel aqueous extract (mean±SEM). (EQ: Equivalent Quercetin**).**

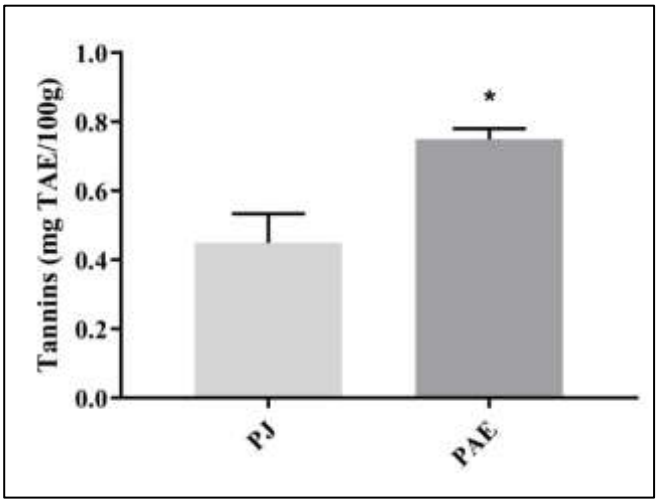


Figure 17: Tannin content of *Punica granatum* juice and peel aqueous extract (mean±SEM). (TAE: Tannic Acid Equivalent**).**

2. Biological activities

2.1. Hematologic profile

2.1.1. Red blood cells count

According to Fig 18, we found a significant decrease in red blood cell counts in Tol-treated animals compared with controls. In contrast, red blood cell counts recovered significantly in the PJ-Tol and PAE-Tol groups compared to the Tol group.

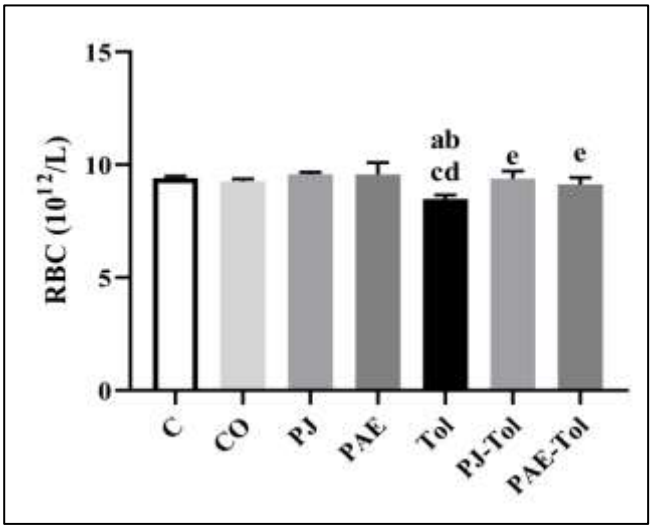


Figure 18: Red Blood Cells (RBC) count in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.2. Hemoglobin levels

Our results revealed that the treatment of animals with the toluene caused a significant reduction in hemoglobin levels compared with animals of the controls (Figure 19). However, we recorded a significant elevation in the levels of hemoglobin in the PJ-Tol and PAE-Tol rats compared to the Tol group.

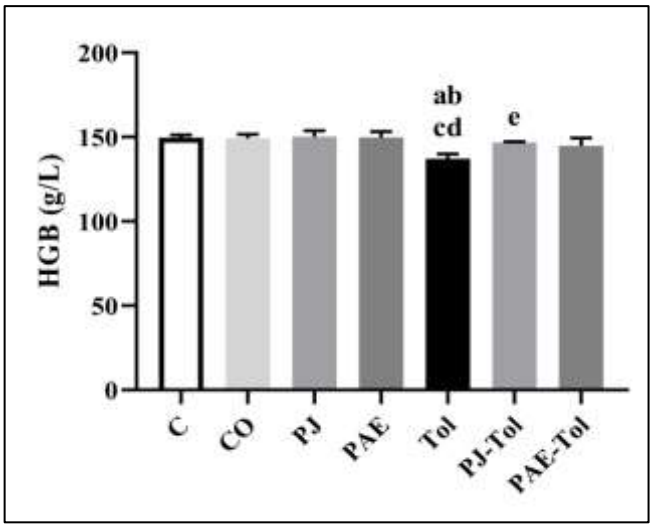


Figure 19: Hemoglobin (HGB) levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.3. Hematocrit levels

The results of our study showed a statistically significant decrease in levels of hematocrit in the Tol group compared with the control and positive controls groups. In contrast, the treatment of rats with PJ-Tol and PAE-Tol resulted in an improvement represented by a significant increase in hematocrit levels compared with the Tol group (Figure 20).

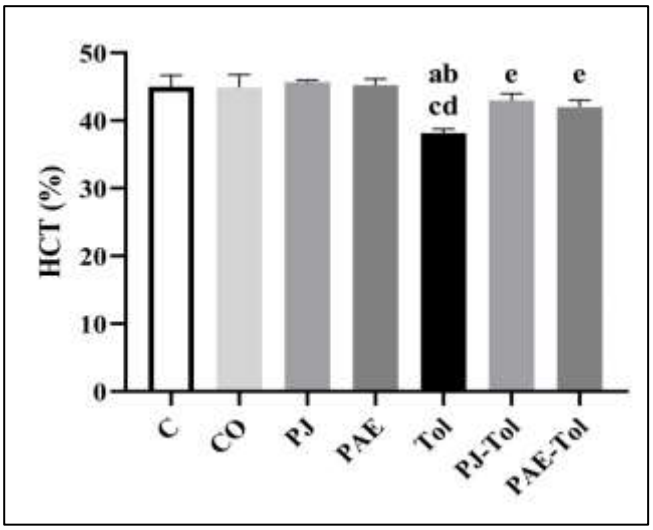


Figure 20: Hematocrit (HCT) levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.4. Mean corpuscular volume

There were no significant differences in mean corpuscular volume (MCV) between rats in the different treated groups (Figure 21).

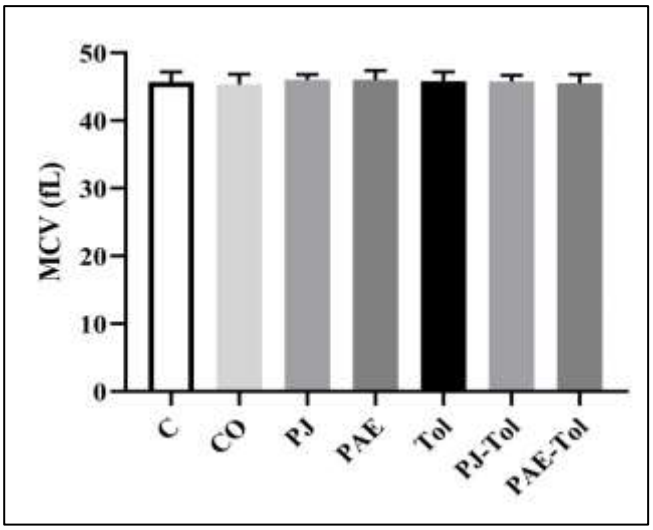


Figure 21: Mean Corpuscular Volume (MCV) in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.5. White blood cells count

Compared to the control and positive control groups, treatment of rats with Tol induced a significant elevation in white blood cell counts (Figure 22). However, treatment of rats with PAE and Tol induced a significant decrease in white blood cell counts compared to Tol-treated group.

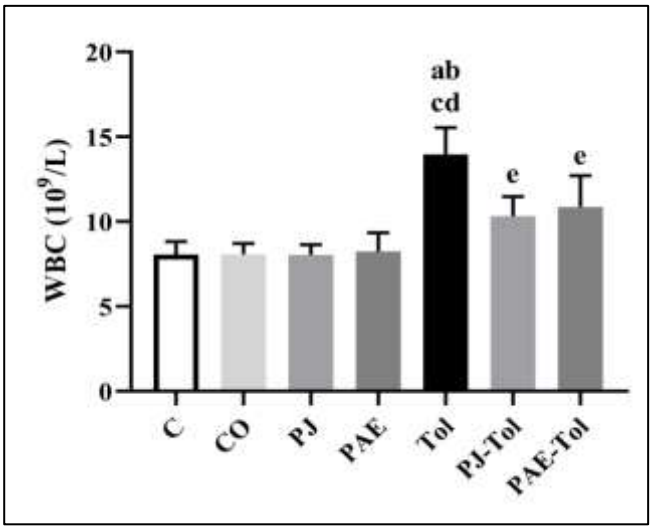


Figure 22: White Blood Cells (WBC) count in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.6. Lymphocytes levels

Fig 23 showed a significant increase in the lymphocytes levels in Tol compared with the controls. Simultaneously, they showed a significant decrease in the PAE-Tol group compared with the Tol group.

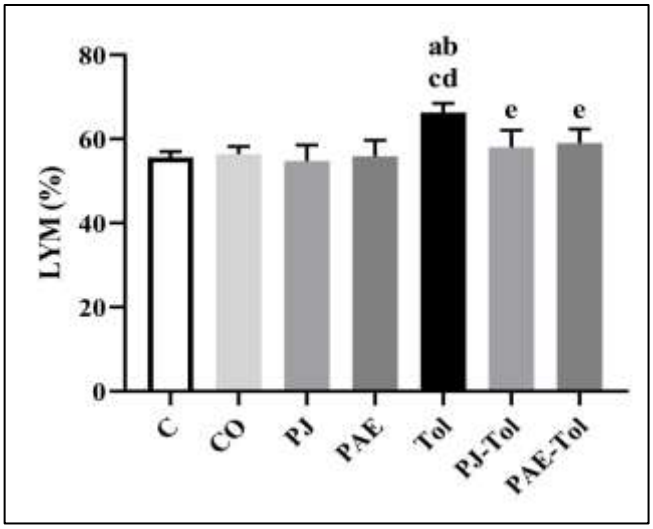


Figure 23: Lymphocytes (LYM) levels in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.1.7. Platelets levels

No significant change was noted in platelets levels in animals treated with Tol alone compared to all other experimental groups (Figure 24).

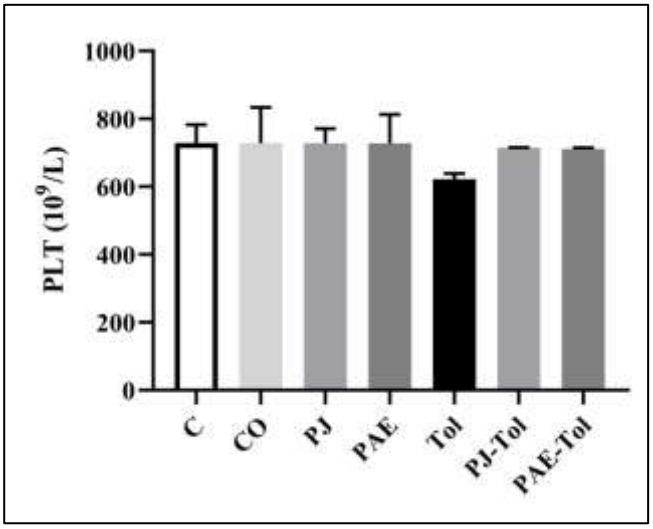


Figure 24: Platelets (PLT) levels in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different

different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2. Hepatic profile

2.2.1. Liver weight

The data analysis indicates a significant increase in the liver absolute weight in the Tol-treated group compared with the control and positive control groups. Simultaneously, no significant changes were recorded in the PJ-PAE and PAE-Tol-treated groups compared with the Tol group (Figure 25).

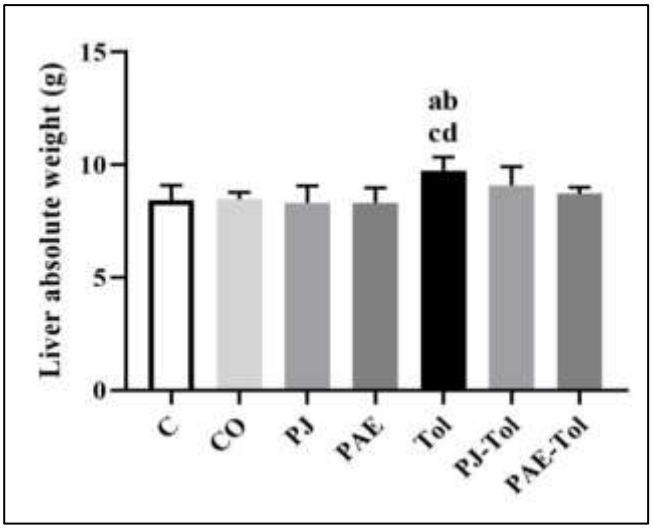


Figure 25: Liver absolute weight of rats in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.2. Transaminases Activity

Compared to the control (C) and positive control (CO, PJ, and PAE) groups, the treatment of rats with Tol induced a significant increase in the enzymatic activity of transaminases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Interestingly, *P. granatum* supplementation significantly improved the activity of enzymes in the PJ-Tol and PAE-Tol group compared with the Tol group (Figures 26 and 27).

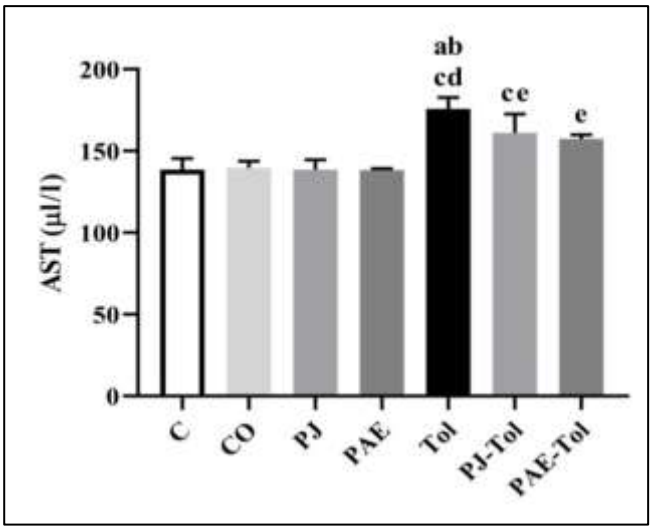


Figure 26: Aspartate aminotransferase activity (AST) in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

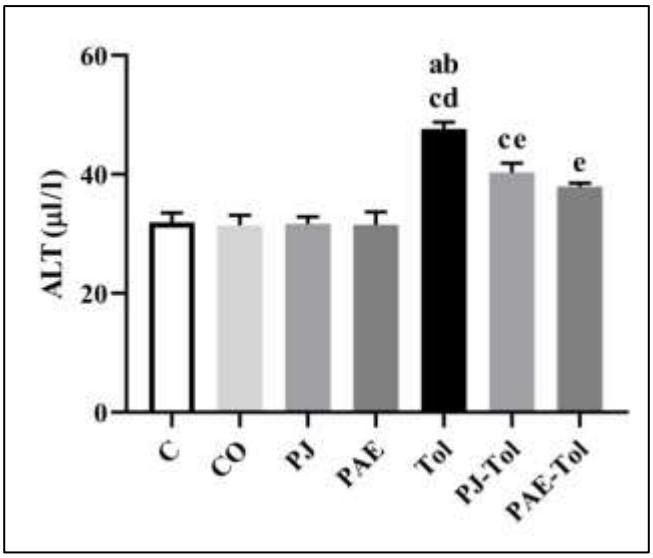


Figure 27: Alanine aminotransferase (ALT) in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.3. Alkaline phosphatase (ALP) activity

The Alkaline phosphatase (ALP) activity is summarized in Fig 28. Compared with the controls, a statistically significant increase in the ALP activity was recorded in the Tol group. In contrast, there was a recovery with a significant decrease in ALP activity in the PAE-Tol group compared to the Tol group.

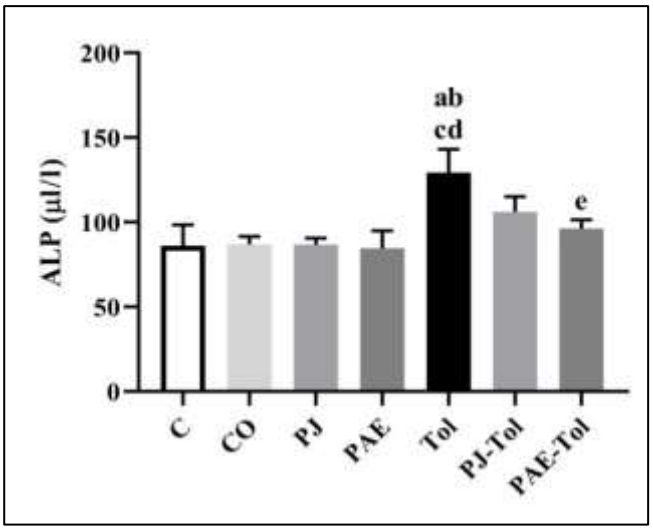


Figure 28: Alkaline phosphatase (ALP) activity in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.4. Total bilirubin levels

Results presented in Fig 29 showed a significant increase in the concentrations of total bilirubin in Tol-treated group when compared to control and positive control groups. This elevation was none significantly changed in PJ-Tol and PAE-Tol treated groups, when compared with the Tol group.

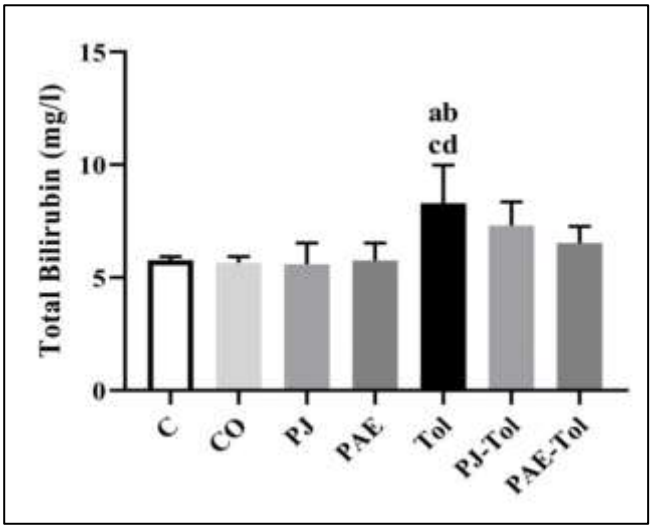


Figure 29: Total Bilirubin levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.5. Albumin levels

Albumin levels were decreased in a significant manner in the Tol-treated rats compared with the controls. These levels were increased significantly in the PAE-Tol animals compared with those of the Tol group. Whereas, no significant change was noted in animals of the PJ-Tol group compared with the Tol group (Figure 30).

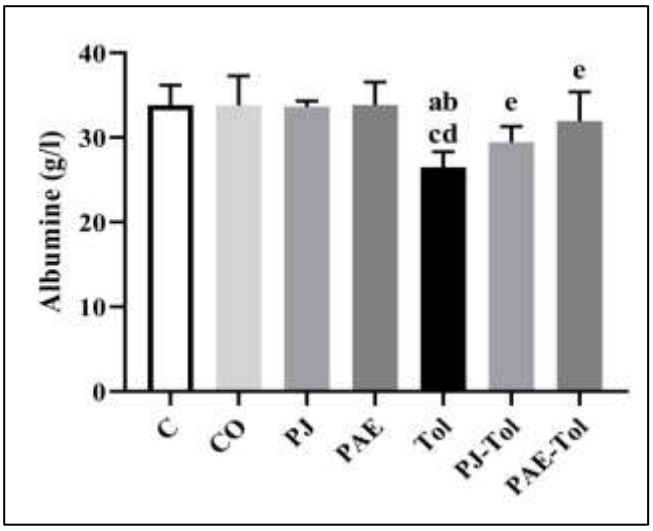


Figure 30: Albumin levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.6. Total proteins levels

Treatment with Tol produced a significant decrease in the levels of total proteins compared with the control and positive control groups. Nevertheless, this decrease was significantly restored (elevated) after PAE co-administration compared with the rats treated with Tol (Figure 31). PJ co-administration slightly increased the total proteins levels.

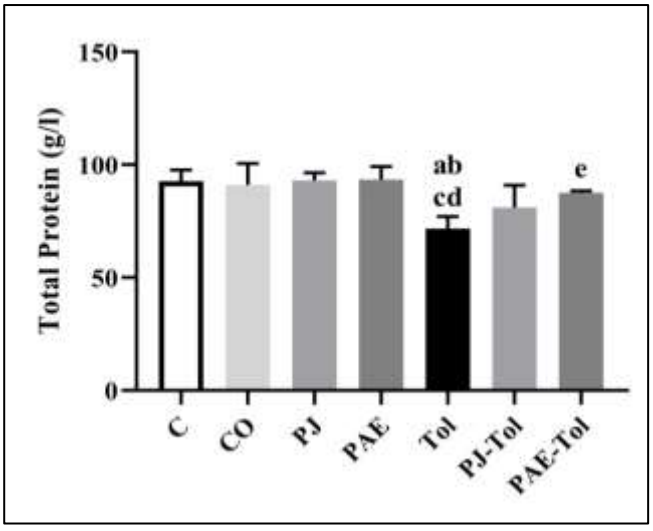
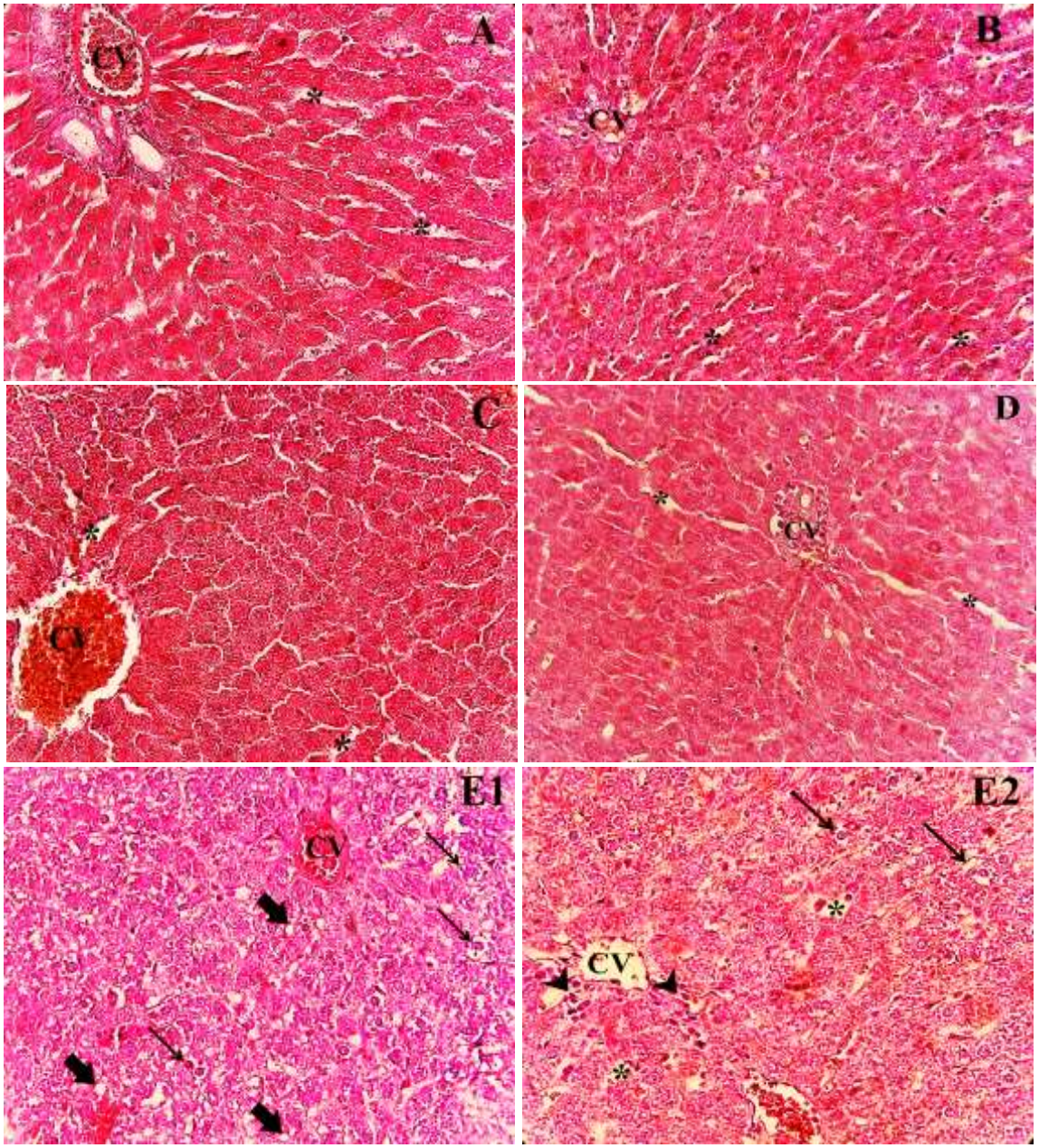


Figure 31: Total Protein levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically

different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.2.7. Histological study of liver

As shown in Fig 32, liver sections showed the normal histological structure of the central vein and surrounding hepatocytes in the control group (A). Sections of liver from rats, treated with CO (B), PJ (C), and PAE (D), showed to be similar to control. In the toluene-treated group, infiltration of inflammatory cells, ballooning degeneration of hepatocytes, and steatosis were recorded ((E1)–(E2)). However, PJ-Tol and PAE-Tol-treated groups showed moderate to mild injury respectively, where the appearance was close to that of the control in the PAE-Tol group.



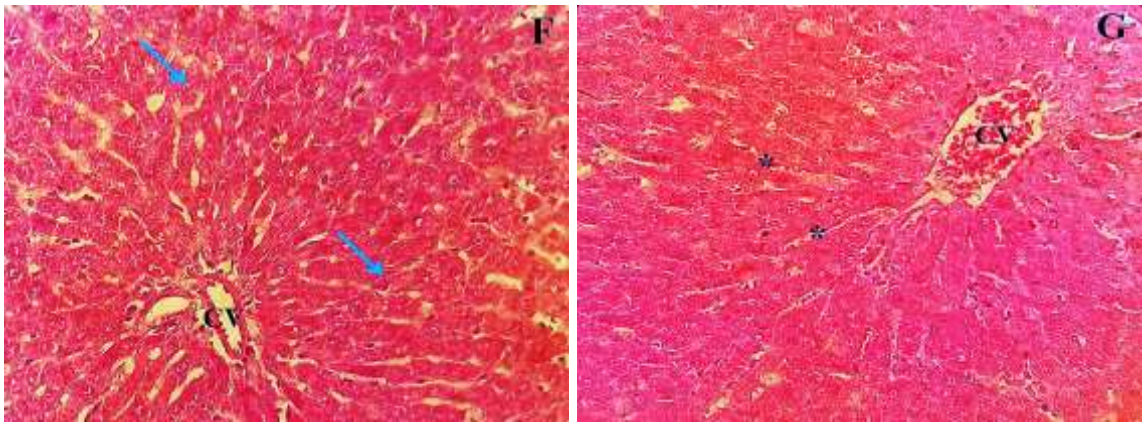


Figure 32: Histopathology light microphotographs of the liver from different experimental groups (×40). Control (A), CO (B), PJ (C), and PAE (D) showing the normal histological structure of the central vein (CV) and surrounded by blood sinusoids (stars) (×40). Tol group showing (E1) ballooning degeneration of hepatocytes (black arrow) with steatosis (thick arrow), (E2) inflammatory cells infiltration (arrowhead) in the portal area (×40). PJ-Tol (F) and PAE-Tol (G) groups showing a few degenerative changes and diffuse kupffer cells proliferation (blue arrow) in between the hepatocytes (×40). (**CV:** Central vein, **(*):** surrounded by blood sinusoids, **black arrow:** ballooning degeneration of hepatocytes, **thick arrow:** steatosis, **arrowhead:** cells infiltration, **blue arrow kupffer:** cells proliferation). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

2.3. Renal profile

2.3.1. Kidney weight

Compared to the control and positive control groups, a highly significant increase in kidneys absolute weight was observed in rats exposed to toluene. On the other hand, the co-treatment of toluene with pomegranate juice or peel aqueous extract reduced insignificantly the toxic effect compared to rats in the Tol group (Figure 33).

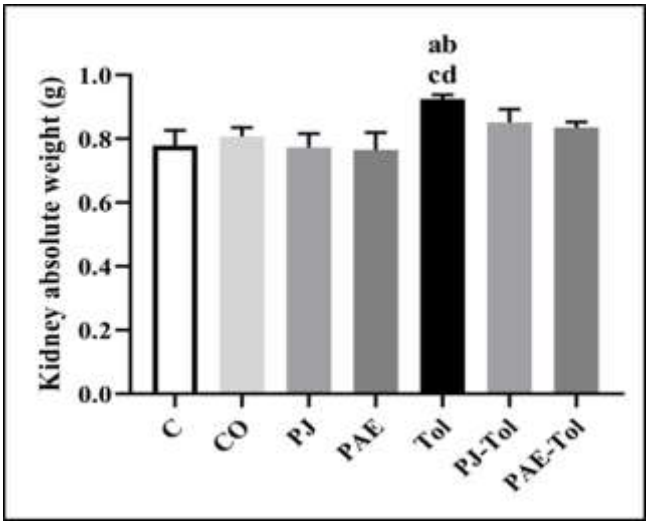


Figure 33: Kidney absolute weight of rats in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**:

statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.3.2. Urea levels

The obtained results reveal that the treatment of rats with toluene caused a significant increase in urea concentration compared to the control and positive control groups (Figure 34). This concentration decreased significantly in PAE-Tol-treated animals compared with Tol-intoxicated animals. No significant differences were noted in the PJ-Tol animals compared to the Tol-treated animals.

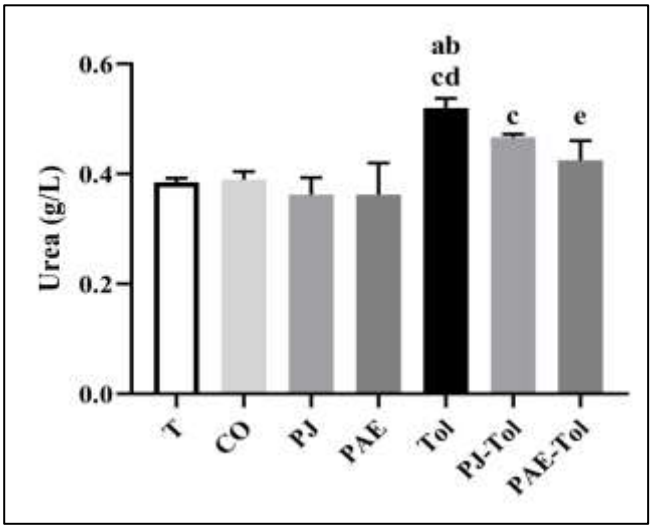


Figure 34: Urea levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.3.3. Creatinine levels

Compared to the controls, treatment of rats with Tol induced a significant increase in the levels of creatinine (Figure 35). Simultaneously, there was a significant decrease in creatinine levels in the PAE-Tol group compared to the Tol group. No significant variation in these levels was noted in animals of the PJ-Tol group compared with animals of the Tol group.

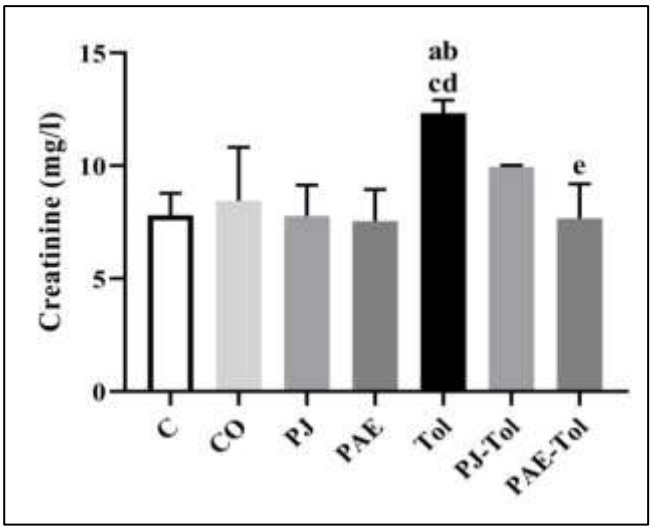


Figure 35: Creatinine levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.3.4. Uric acid levels

Regarding uric acid levels, we found that the results remained insignificant in all experimental groups (Figure 36).

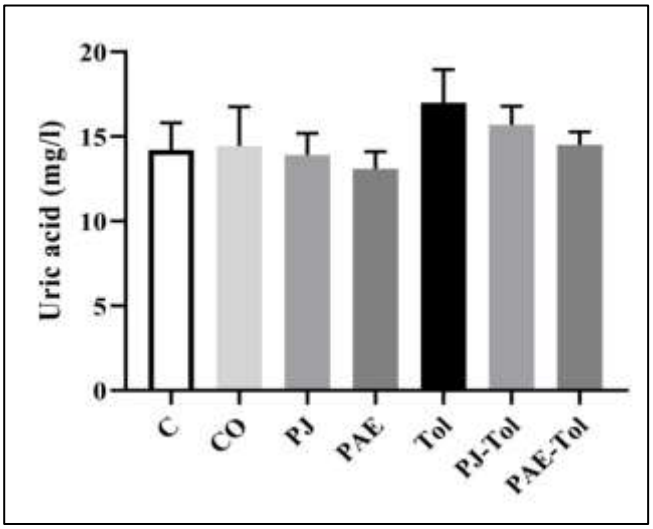
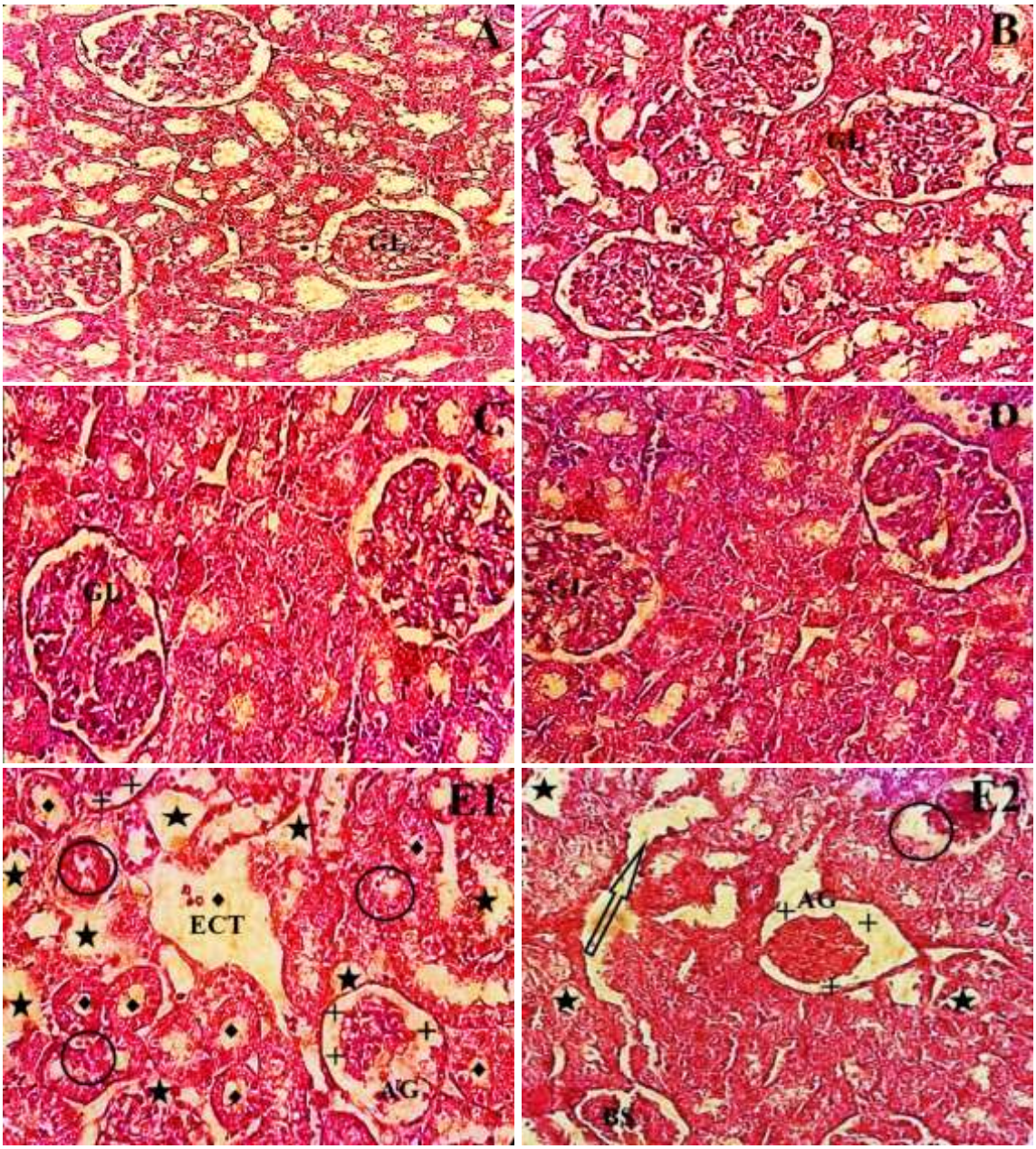


Figure 36: Uric Acid levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.3.5. Histological study of kidney

Renal microscopic observation of the control (A) and positive control CO (B), PJ (C), and PAE (D) rats reveal the presence of renal parenchyma with normal architecture, consisting of the glomerulus (GL) surrounded by a narrow and clear space. The administration of Tol (E1 and E2) causes renal parenchyma changes and disorganization, characterized by extensive vacuolization, dilatation of bowman's space, degeneration of the renal tubular epithelium, and

glomerulus atrophy. Whereas renal tissue of rats in PJ-Tol (F) and PAE-Tol (G) groups was significantly improved compared with the Tol group (Figure 37).



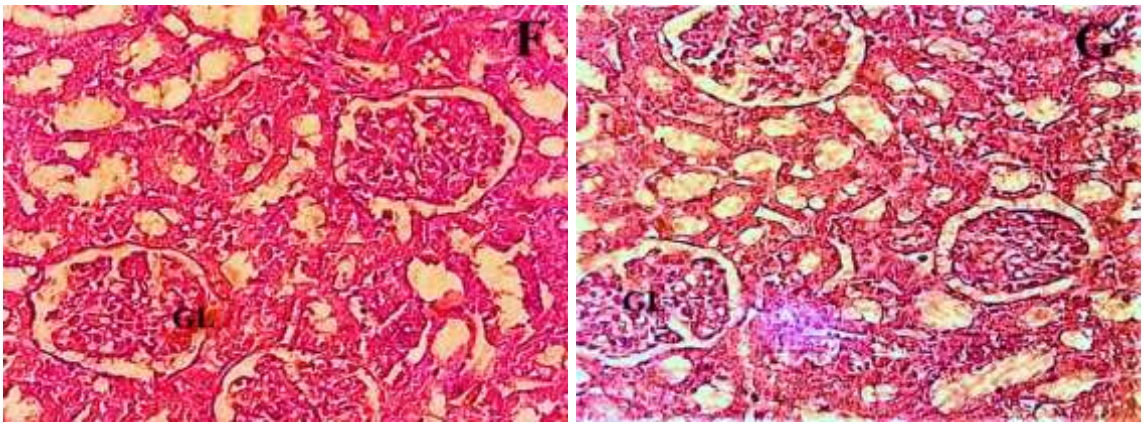


Figure 37: Histopathology light microphotographs of the kidney from different experimental groups (×40). Control (A), CO (B), PJ (C), and PAE (D) revealed a normal renal glomerulus (GL) and tubular epithelium (×10). Tol group (E1) (E2) showed a deleterious morphological change versus those of the control groups, which was illustrated by glomerular atrophy (GA) with dilatation of Bowman's space (BS) and degeneration of the renal tubular epithelium (stars, circles and arrows). Yet, PJ-Tol (F) and PAE-Tol (G) groups displayed a better-preserved epithelium and morphology with a normal architecture nearly like those of controls.
(**GL**: glomerulus, **BS**: Bowman's Space, **AG**: atrophy of the glomerulus with widening of Bowman's capsule, **stars**: dilatation of the convoluted tubule, **(+):** dilatation of Bowman's space, **arrow**: tubular dilatation and degeneration of the renal tubular, **ECT**: empty convoluted tubule). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

2.4. Reproductive profile

2.4.1. Testes and epididymis weight

The reproductive organs absolute weight (testes, epididymis) is shown in Fig 38 and 39. Exposure to toluene (Tol) significantly decreased testes weight compared with the control and positive control groups. It also appears through our results observation that epididymis weight was significantly reduced when compared with positive control groups (PJ) (PAE). On the other hand, the PJ-Tol and PAE-Tol showed an insignificant increase in these organ weights compared to the Tol group.

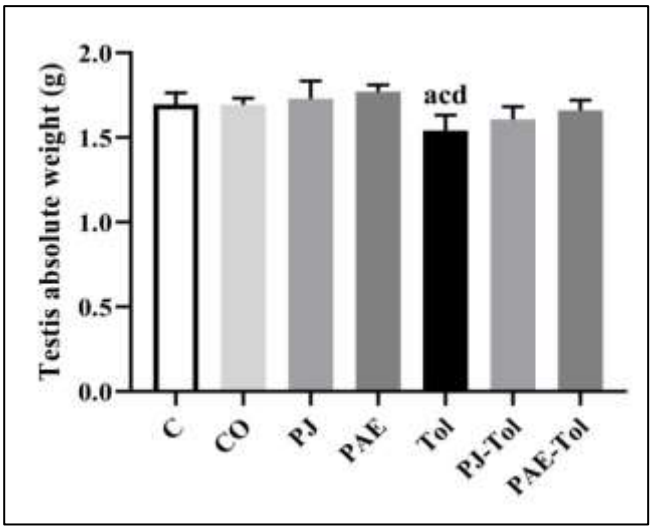


Figure 38: Testis absolute weight of rats in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

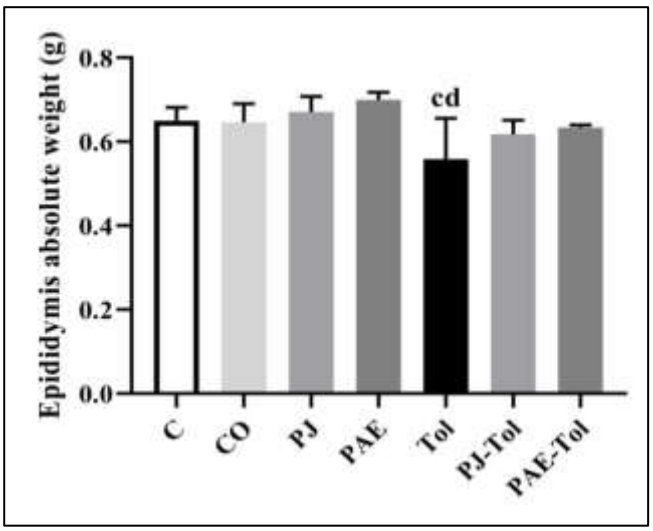


Figure 39: Epididymis absolute weight of rats in different experimental groups (Mean±SEM). **a**: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.2. Sperm Concentration

Sperm concentration exhibited significantly lower in the Tol-treated rats than in those of the control and positive control groups. This concentration was significantly increased in the PAE-Tol group compared with the Tol group. While no significant variation was noted in the animals that received PJ supplementation compared with the Tol group (Figure 40).

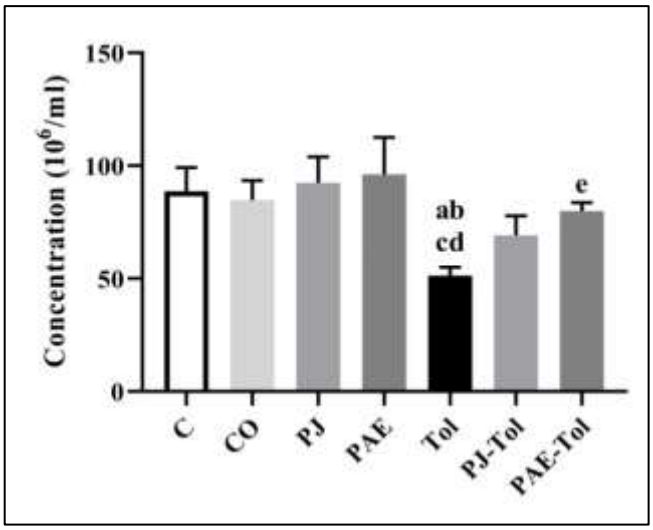


Figure 40: Sperm concentration in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.3. Sperm Vitality

The percentage of live sperm of the Tol group was significantly reduced; meanwhile, the percentage of dead sperm was significantly elevated compared with the control and positive controls. Contrary, there was a marked significant increase in sperm vitality with a significant decrease in death sperm in the PJ-Tol and PAE-Tol groups compared with the Tol group (Figure 41).

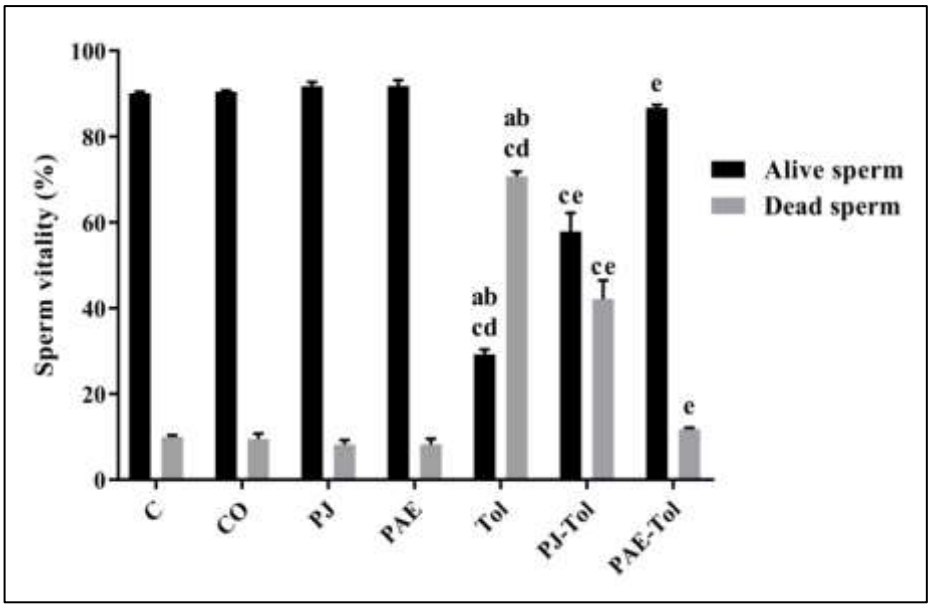


Figure 41: Sperm vitality in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.4. Sperm motility

A significant decline was found in the percentage of total, progressive, and non-progressive sperm motility, whereas the percentage of immotile sperm was significantly increased in rats of the Tol group compared with the control and the positive groups. However, the administration of PAE with Tol significantly increased sperm motility and decreased the percentage of immotile sperm compared with the tol-treated group (Figures 42 and 43).

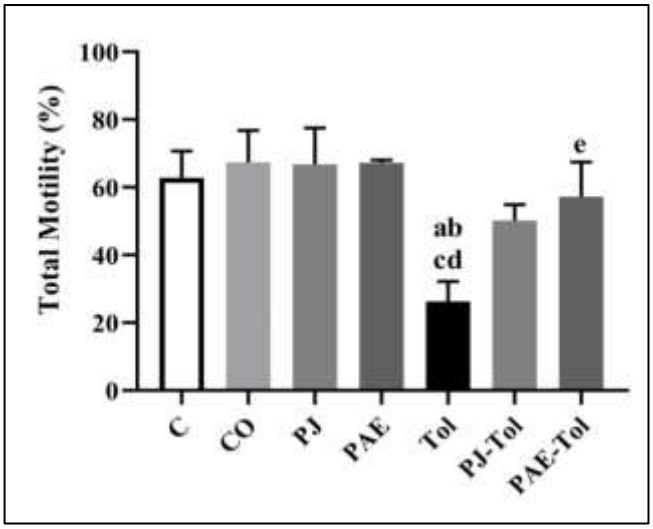


Figure 42: Sperm total motility in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

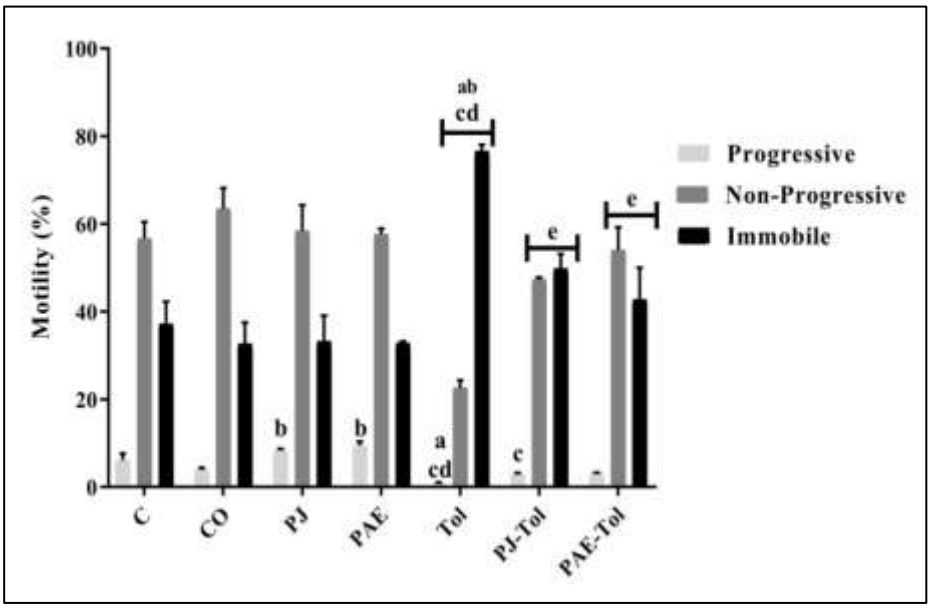


Figure 43: progressive and non-progressive motility and immotile sperm in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.5. Sperm kinematic parameters

Rats exposed to toluene showed a significant decrease in sperm in parameters reflecting velocity indicators (VSL, VAP, BCF and LIN) compared with the other groups (Figures 44, 45, and 46). On the other hand, Tol affected VCL insignificantly. The supplementation with PJ and PAE significantly increased VSL values compared with the Tol group.

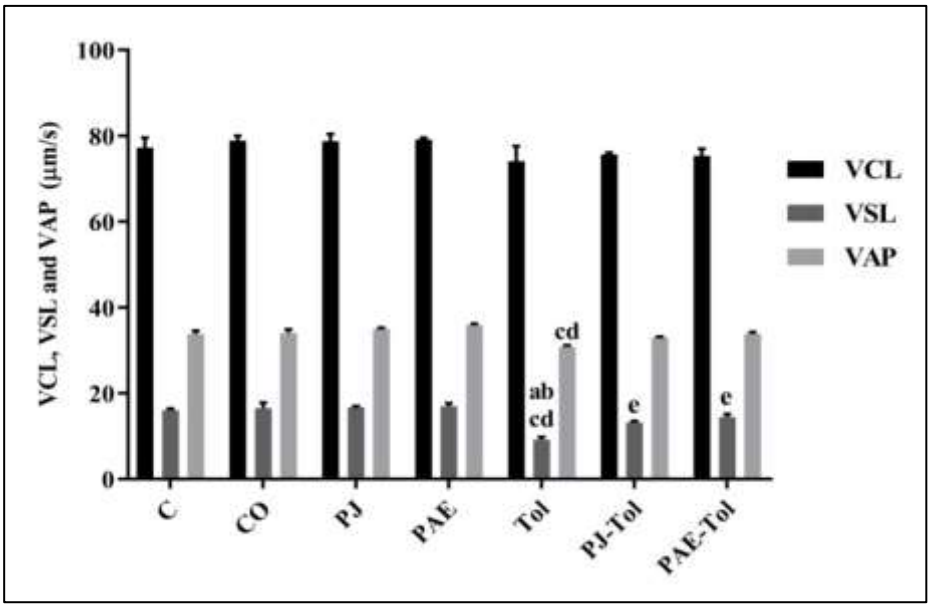


Figure 44: Spem curvilinear (VCL), straight line (VSL), Average path (VAP) velocity in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

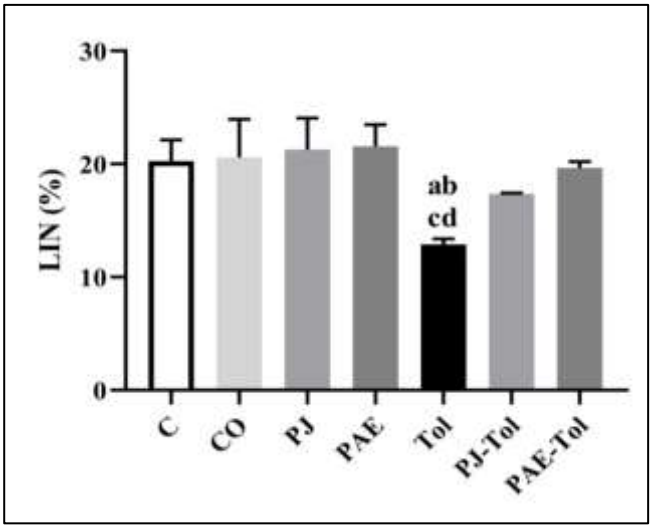


Figure 45: Sperm linearity of the curvilinear trajectory (LIN) in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

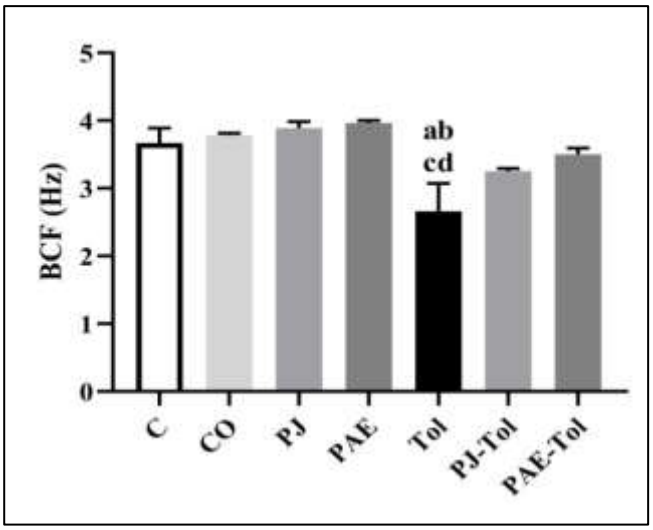


Figure 46: Sperm beat cross frequency (BCF) in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.6. Testosterone levels

The testosterone levels showed a significant reduction in the plasma of Tol-treated animals compared with the control and positive control groups. On the other hand, PAE co-administration significantly attenuated this decrease in co-treated rats compared with the Tol-treated rats. No significant difference was noted in the PJ-Tol animals compared with those of the Tol group (Figure 47).

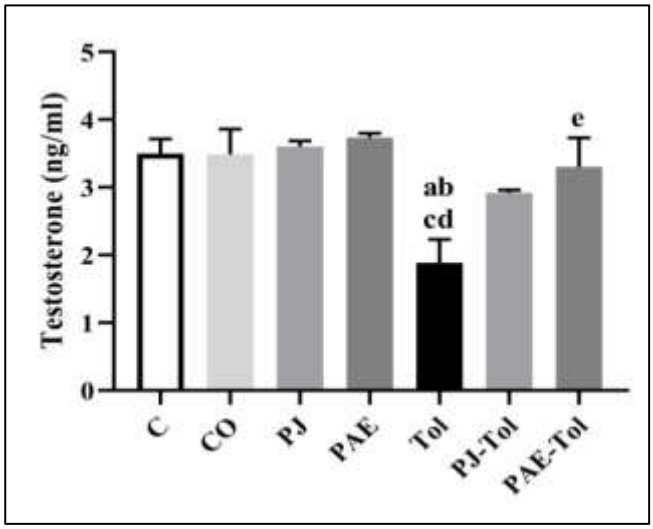


Figure 47: Testosterone levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.7. Histological study of testes and epididymis

The histological examinations of the different studied tissues after 6 weeks of treatment are laid out in Fig 48. Histological appearances of testicular tissue showed a normal arrangement of the germinal epithelium at different stages of spermatogenesis, with a regular feature of the seminiferous tubules, and interstitial cells in the control (A) and positive control CO (B), PJ (C), and PAE (D) groups, whereas Tol exposure resulted in mild central degeneration of the seminiferous tubules accompanied by distortion of the basement membrane and reduction in the diameter of these tubules, a decrease in lumen sperm count, and depletion of the germinal layer. Also, sections were severely affected by edema of the interstitial spaces with wide separation between adjacent seminiferous tubules. PJ co-treatment insignificantly slightly improved Tol-changes, although there were tubules with a low sperm count were seen in the lumen with enlarged interstitial spaces, but this improvement in PAE-co-treated animals was more distinctive, the appearance was close to that of the control group.

The sections of the cauda epididymis also revealed that Tol exposure (E1 and E2) resulted in epididymal ducts atrophy, interstitial edema, an irregular membrane basement, and nearly empty lumen with only a limited number of sperms when compared with control and positive control rats. Regarding the rats treated with the mixture of Tol and pomegranate, PJ-Tol co-administration (F) demonstrated mild interstitial edema with an improvement in sperm count; however, PAE-Tol co-administration (G) significantly attenuated the severity of Tol-induced histopathological changes. Epididymis of rats from the control (A) and positive control CO (B), PJ (C), and PAE (D) groups showed a normal histological structure with normal sperm count (Figure 49).

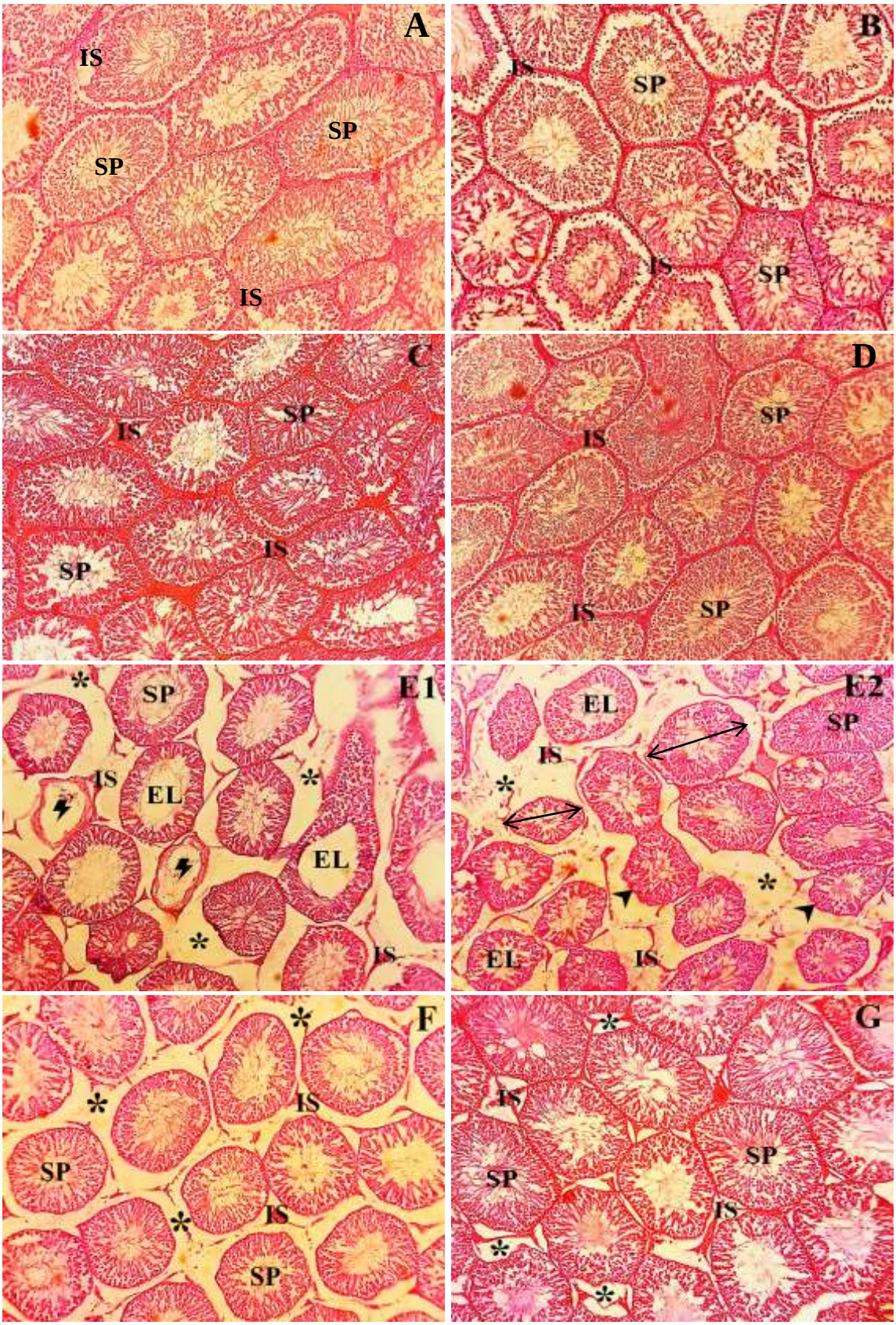
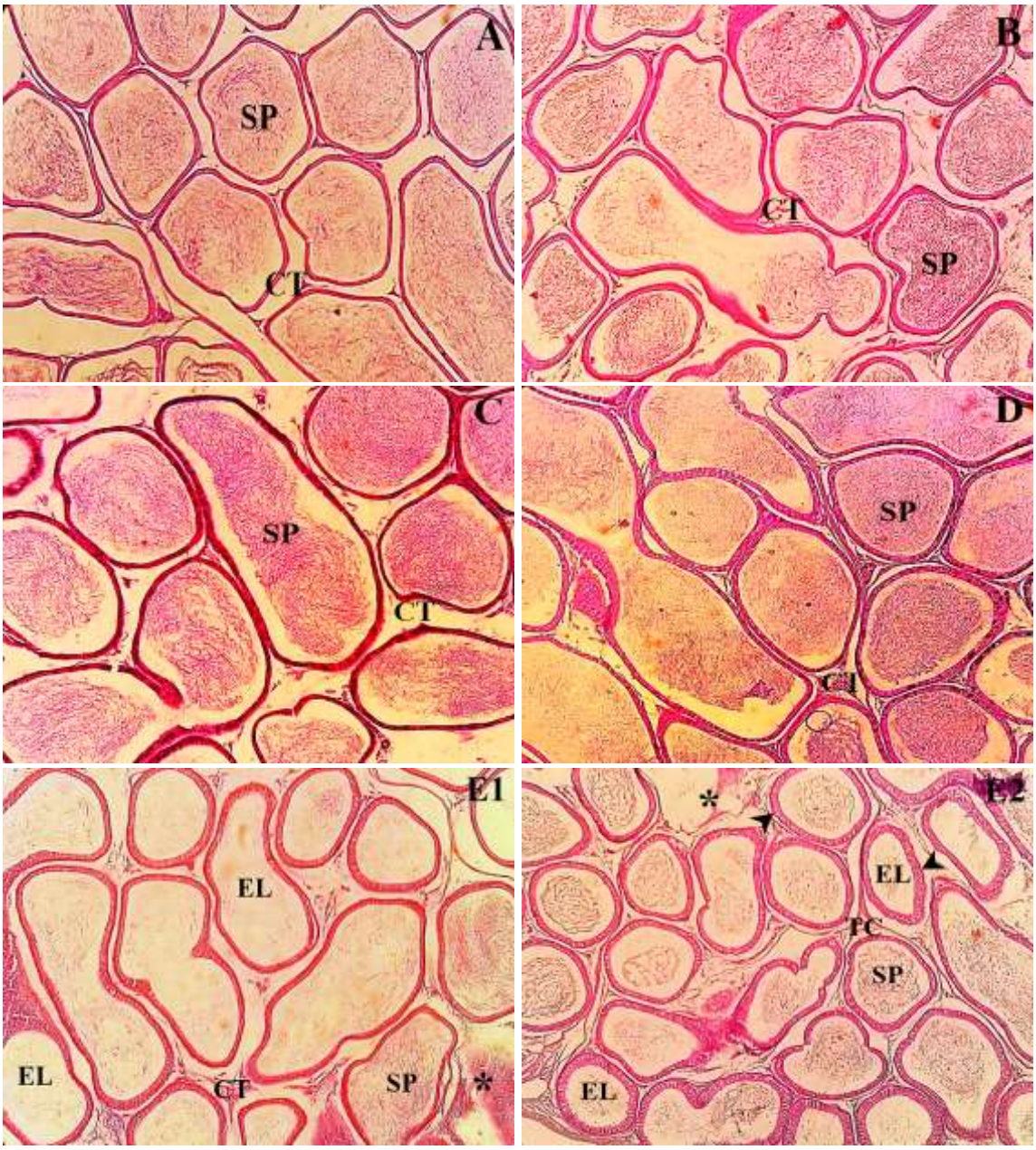


Figure 48: Histopathology light microphotographs of the testes from different experimental groups (×10). Controls (A), CO (B), PJ (C), and PAE (D) revealed a normal architecture of seminiferous tubules with a lumen containing spermatozoa (SP) and interstitial connective tissue (IS) (×10). Tol group (E1) (E2) showed a number of histopathological

changes, such as degeneration in some seminiferous tubules (🖋) with a distortion of their basement membrane (arrowhead), a decrease in sperm count (EL) and severe edema in intertubular areas (stars). PJ-Tol (F) and PAE-Tol (G) groups showed a better-preserved epithelium in some seminiferous tubules and a slight decrease in sperm count, with moderate to mild edema in intertubular areas respectively (×10). (**SP**: Spermatozoa, **IS:** Interstitial Space, **EL:** Empty Lemen, **(*):** interstitial edema, (🖋): tubular degeneration, **arrowhead:** disrupted membrane basement).



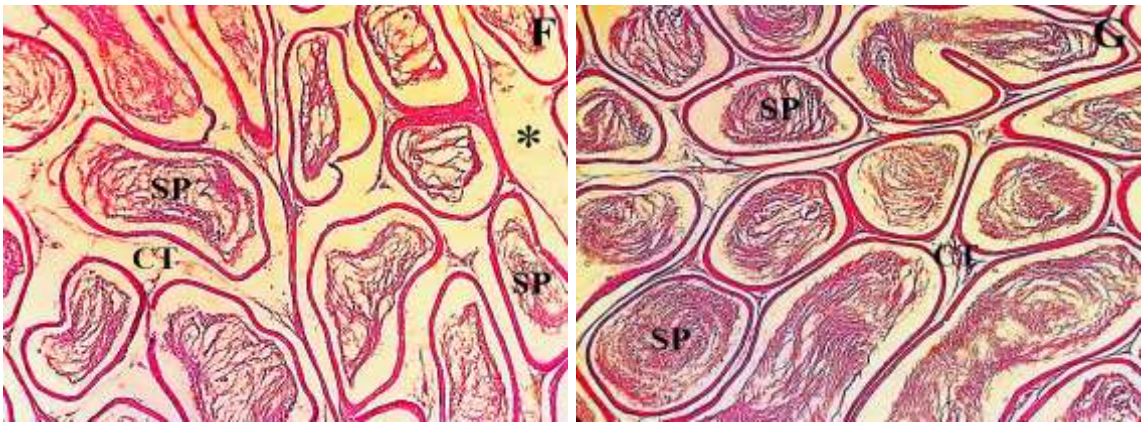


Figure 49: Histopathology light microphotographs of the cauda epididymis from different experimental groups (×10). Control (A), CO (B), PJ (C), and PAE (D) revealed a normal architecture of Epididymal ducts with a lumen containing spermatozoa (SP) and connective tissue (CT) (×10). Tol group (E1) (E2) showed slight changes, such as distortion of ducts basement membrane (arrowhead), a decrease in sperm count (EL) and mild interstitial edema (stars). PJ-Tol (F) and PAE-Tol (G) groups showed a better-preserved in sperm count, with mild edema in intertubular areas (×10). (**SP:** Spermatozoa, **CT:** Connective Tissue, **EL:** Empty Lemen, **(*):** interstitial edema. (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

2.5. Oxidative stress markers

2.5.1. Reduced Glutathione (GSH) content

According to the obtained results, cellular GSH content was reduced significantly in testes, liver, and kidneys in rats of the Tol group compared with rats of the controls (Figures 50, 51, and 52). However, the administration of *P. granatum* PAE-Tol restored the GSH content compared with the Tol-exposed group. The groups supplemented with PJ-Tol did not show significant differences compared with the Tol group.

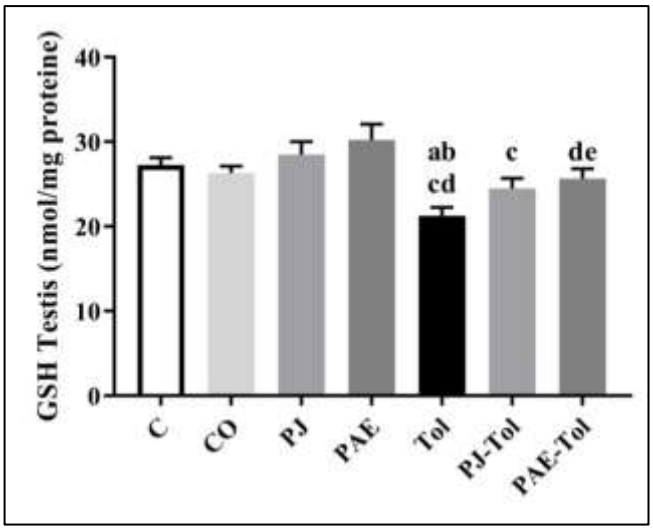


Figure 50: Testicular GSH content in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different

different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

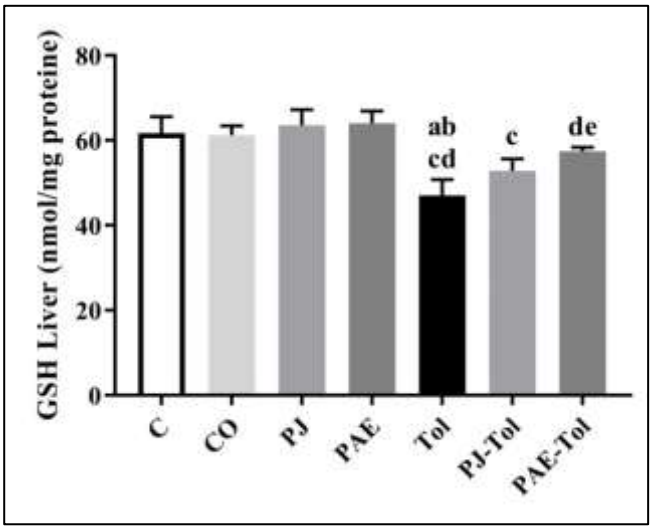


Figure 51: Hepatic GSH content in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

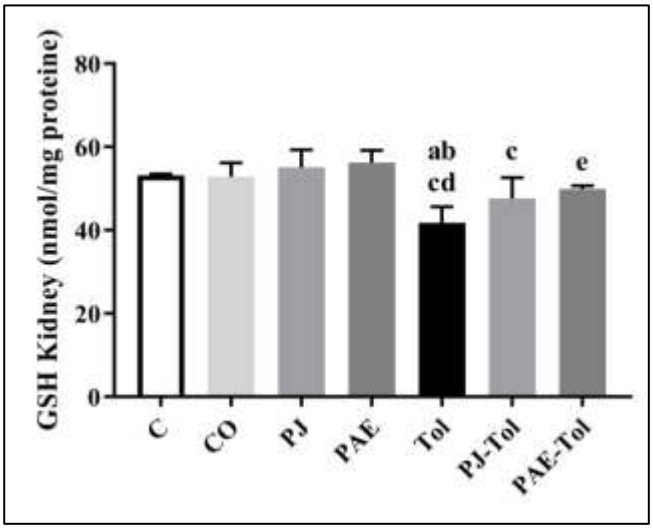


Figure 52: Renal GSH content in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.5.2. Peroxidase Glutathione (GPx) activity

As shown in Fig 53, 54, and 55 exposure of rats to Tol significantly depleted the testicular, hepatic, and renal GPx activity compared with the control and positive control groups. This considerable drop was significantly improved in the PAE-Tol-treated animals compared with the Tol group animals. No significant differences were recorded in PJ-Tol group animals compared with those of the Tol group.

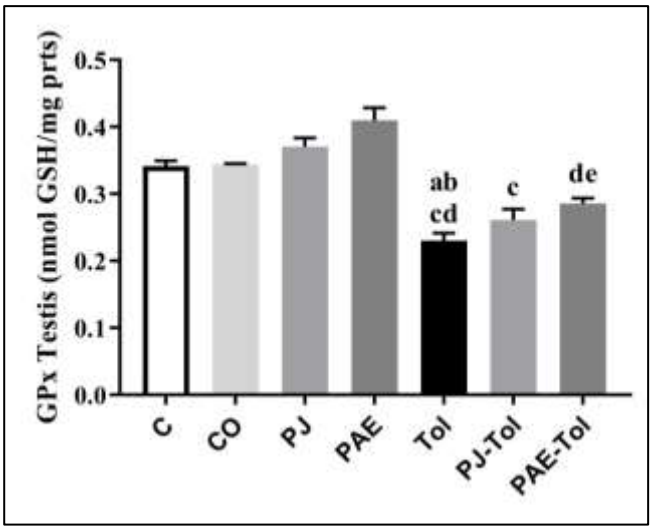


Figure 53: Testicular GPx activity in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

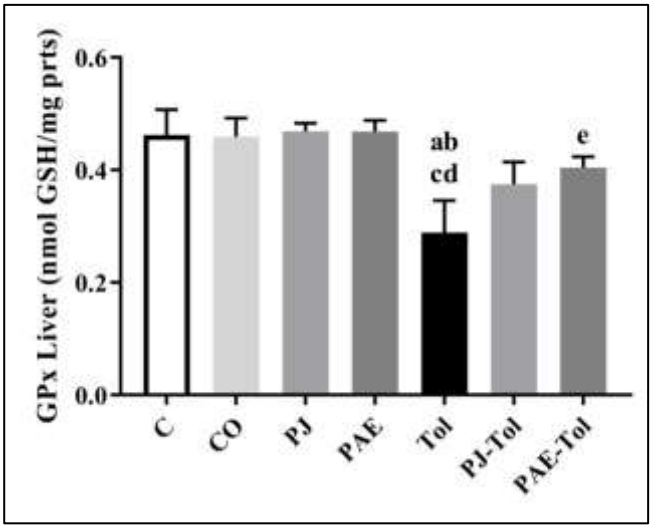


Figure 54: Hepatic GPx activity in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

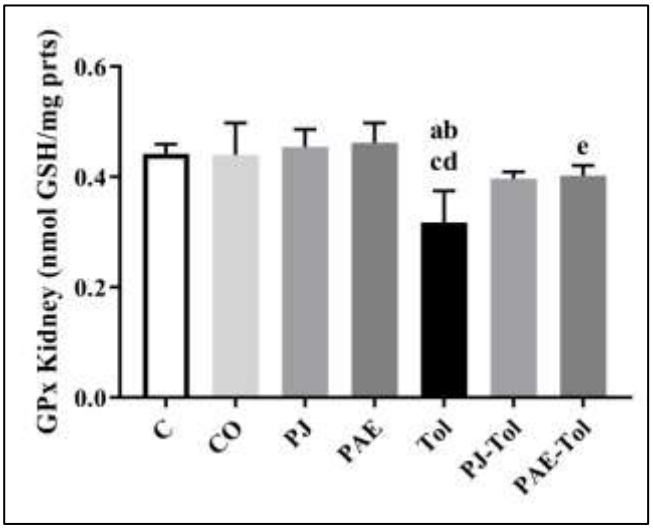


Figure 55: Renal GPx activity in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different

Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

2.4.3. Malondialdehyde (MDA) levels

Fig 56, 57, and 58 shows that the testes, liver, and kidneys MDA levels were significantly increased in the Tol-treated group compared with the control and positive control groups. The MDA levels of PAE supplemented rats were lower than those the groups treated with Tol. There was a slight decrease in MDA levels in the Tol-PJ group compared with the Tol group.

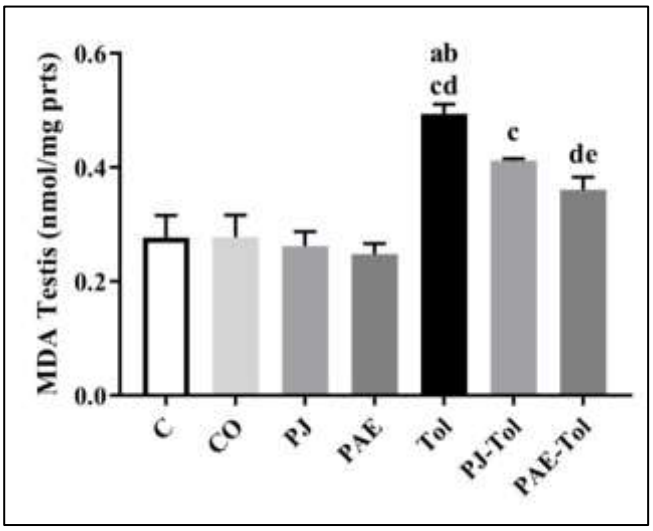


Figure 56: Testicular MDA levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

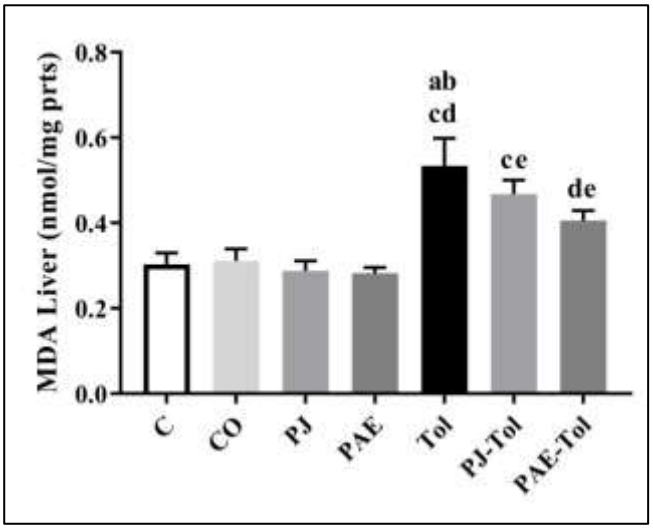


Figure 57: Hepatic MDA levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

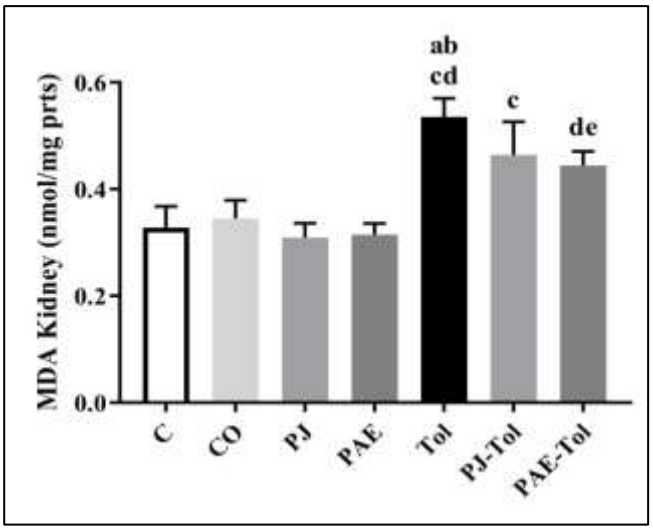


Figure 58: Renal MDA levels in different experimental groups (Mean±SEM). a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group. **e**: statistically different Vs Tol group.

VI. Discussion

Nowadays, the return to natural products has become one of the significant directions of several research projects. Plant substances and extracts, as well as their essential oils, seeds, and peel powders have been proven to possess excellent bioactive constituents, notably, polyphenols, which have been demonstrated to play basic roles in the prevention or attenuation of the physiological disruptions stemming from exposure to different chemicals by the regulation of cellular signals (transcription of pro-and anti-apoptotic and inflammatory genes or genes coding for the expression of antioxidant enzymes) **(Kiełczykowska and Musik, 2020).** In this context, the present study was planned in the first place to evaluate the antioxidant activity and the bioactive components contained in the *Punica granatum* juice (PJ) and peel aqueous extract (PAE).

Bodies of the literature suggest that pomegranate has a protective effect due to its high content of phenolic compounds, phenolic acids, ellagic tannins (punicalin, punicalagin, gallagic, and ellagic acid), flavonoids (anthocyanins, catechins, rutin, epigallocatechin-3-gallate), and anthocyanins (delphinidin, cyaniding, and pelargonidin), which possess many prominent capacities (**Su *et al.*, 2010; Li and Gu, 2011)**. The quantitative estimation performed here proved that the aqueous extract of peel displayed the highest amount of total phenolic compounds, flavonoids, and tannins. These results are in harmony with the previous reports in the same field indicating that pomegranate peels have a higher total phenolic compound content and antioxidant activity than PJ **(Elfalleh *et al.*, 2011; Gozlekc *et al.*, 2011)**. Moreover, pomegranate peel was reported to have the highest antioxidant activity compared to other 28 species of fruits peels **(Li *et al.*, 2006).**

The antioxidant capacity of these compounds, as determined by DPPH radical scavenging activity assay, can neutralize the free radicals to give stable diamagnetic molecules **(Abd El-Rehem and Ali, 2013).** According to this study, the PAE showed the highest ability in DPPH scavenging activity compared to PJ which was measured by the lowest IC50. It is well documented that the most responsible constituent for pomegranate antioxidant capacity is hydrolysable tannins **(Elfalleh *et al.*, 2011)**.

In light of what was aforementioned, determining the antioxidant capacity can take two directions. *In vitro* study, to assess the antioxidant potential by the relative amount of antioxidant compounds contained in the extracts. The second is *in vivo*, to determine the antioxidant properties of plant extracts in the biological systems. Hence, an experimental

study was also conducted to evaluate the effects of toluene (Tol) alone and co-administrated with PJ and PAE on reproductive, hepatic, renal, and hematological indices in adult male rats.

It was proven that the toxicity of toluene seems to be related to its biotransformation, resulting in particular production of epoxides of toluene which are species with high reactivity that can induce damage to various body systems **(Kamel and Shehata, 2008).**

Blood is the crossroad of all toluene pathways in the body; the latter is distributed between red blood cells where it is attached to hemoglobin, and serum with a 1:1 partition in humans **(INRS, 2012),** which complicates the interpretation of the rate of different blood cells. Our study indicates that administration of Tol caused abnormal changes in some of the investigated hematological parameters. These disturbances are marked by a significant decrease in red blood cells, hemoglobin, and hematocrit. Our findings might be consistent with the reports obtained by **Abou-Hadeed et al. (2021).** Different published reports proven that hemoglobin is a major source of radical production when it interacts with redox xenobiotics giving rise to superoxide radicals, hydrogen peroxide and in certain cases peroxy radicals **(Fibach and Rachmilewitz, 2018).** Therefore, exposure to certain xenobiotics makes red blood cells very sensitive to oxidizing agents, which lead to their structural and functional integrity membrane loss, thus, blood hemolysis **(Allagui *et al.*, 2014).** Additionally, this decrease may be due to the reduced synthesis of testosterone, which is involved in the hematopoiesis process, stimulating the production of erythropoietin. Without enough EPO, the hematopoietic marrow does not receive enough stimuli needed for RBCs production and growth **(Bachman *et al.*, 2014).**

In contrast, a notable increment in white blood cells and lymphocytes was recorded in animals intoxicated with Tol. In toxicity studies, excess level of weight blood cells indicates the impact of chemicals in inducing the immune response of treated animals. Thus, WBCs and their differential count including neutrophils, lymphocytes, eosinophils, monocytes, and basophils play important role in the immune system to combat infections where the overproduction of leukocytes can be an important biomarker for inflammation and stress-related disorders **(Ross, 1999).** Numerous experimental studies have supported the role of oxidative stress upon interaction with cellular components, in causing activation of pro-inflammatory signals, and expression of inflammatory cytokines, which means an obvious inflammatory response in the spleen **(Zhou *et al.*, 2019).**

In terms of the investigation of *Punica granatum*'s role, PAE and PJ showed the ability to protect the hematopoietic cells from the damaging effects of exposure to Tol. Pomegranate juice has been proven to have excellent beneficial elements. It contains proteins, carbohydrates, fats, fiber, and sugars. Most importantly, it contains many trace elements and minerals, such as potassium, phosphorus, calcium, magnesium, iron, zinc, and copper. This latter is necessary for the formation of hemoglobin and it is a component of many enzymes **(Melgarejo *et al.*, 2000; Lansky *et al.*, 2007; Wald, 2009).**

The reduced HGB levels in pomegranate peel groups may be associated with the lack of iron by the antinutritional factors of tannins **(Delimont *et al.*, 2017).** However, tannins and phenolic compounds of pomegranate peel are related to immunomodulatory properties, which are proved by the stimulation of lymphocyte growth **(Arıca and Ürkmez, 2016).**

It has been also suggested that pomegranate regulates the inflammatory responses of macrophages and T lymphocytes. Previous studies have shown that most anti-inflammatory drugs act as antioxidants and eliminate free radicals generated during inflammatory processes (**Baret *et al.*, 1984).** In the same context, many research works advocate that plant parts, which contain tannins, alkaloids, flavonoids and phenolic acids, possess analgesic and anti-inflammatory effects **(Mills and Bone, 2000; Morteza-Semnani *et al.*, 2006).**

The liver is a remarkable organ that acts as the chief player in the metabolism and biotransformation of many toxic compounds such as toluene through various chemical reactions. Thus, it targets many types of impairment that produce hepatotoxicity and cause health complications **(Mossa *et al.*, 2013)**.

Again, exposure to Tol at 550 mg/kg BW daily for 45 days yielded a significant increase in the liver absolute weight. This could be attributed to an increase in the activity of inflammatory agents that could have resulted in the inflammation of liver tissues. It has recently been shown that exposure to gasoline containing toluene provoked an elevation of the levels of pro-inflammatory cytokines, such as interleukin IL-1β, IL-6, tumor necrosis factor-α, and interferon-γ **(Moro *et al.*, 2019)**.

Since serum AST, ALT, and ALP have a predominantly hepatic origin in the body, they are often considered to be the most sensitive indicators of liver injury. The activities of hepatic enzymes recorded in this study were much higher in the Tol-treated group. Such

results are comparable to previously reported work **(Tas *et al.*, 2011),** which documented a clear increase in hepatic enzymes in response to toluene treatment. This increase might be attributed to an elevation in the oxidative metabolism of the liver, resulting in membrane degeneration, as well as cholestatic damage, leading to the leakage of enzymes into the blood serum **(Nsonwu-Anyanwu *et al.*, 2021).** However, the elevated bilirubin levels observed could indicate decreased hepatic conjugation or overproduction of bilirubin caused by hemolysis **(El-Demerdash, 2004; Lukyanenko *et al.*, 2013)**.

Consistently, toluene brings a decline in albumin and total protein levels, which might be linked to the obstruction of protein biosynthesis and free amino acid metabolism in the liver **(Gaskill *et al.*, 2005)**. It was established that exposure to Tol could promote cell apoptosis by elevating pro-apoptotic protein (Bax) levels and caspase-3 activity in the liver **(Kamel and Shehata, 2008; Ayan *et al.*, 2013)**. The AMPK has also been associated with the mechanism involved in cell apoptosis in response to ROS, such as H_2O_2 **(Hardie *et al.*, 2012)**.

The majority of toluene in the body is eliminated in the urine after 12 hours following exposure, mainly as metabolites. The kidneys play a pivotal role in this elimination; as a result, they are highly vulnerable to toxic damage because they are exposed directly to blood plasma through their open fenestrae **(Abdel-Moneim *et al.*, 2015)**. Taking our results into account, we found that Tol caused a significant increase in the absolute weight of kidneys. This observation was confirmed in rabbits (*Oryctolagus cuniculus*) exposed to 150 ppm of toluene, where a drastic increase in kidney weight was noticed **(Djemil, 2017).** This increase could be due to swelling of the renal tubular cells, also their congestion and disorganization **(Afravy *et al.*, 2017).**

As markers, uric acid, urea, and creatinine are among the essential parameters for assessing renal function and glomerular filtration **(Brunet, 2005).** The nephrotoxicity of Tol was clearly revealed by the increased levels of these parameters, which was in tandem with the results of **Meydan et al. (2013)** and **Salihu et al. (2022)**. It has been indicated that the increase in serum urea and creatinine levels are caused by glomerular functional disturbances **(Erseçkin *et al.*, 2020).** Urea and creatinine are nitrogenous end products of metabolism. Urea is the major metabolite derived from dietary protein and tissue protein turnover. Creatinine is the product of muscle creatine catabolism; and is usually produced at a fairly constant rate by the body based on muscle mass**.** Both urea and creatinine are

filtered from the blood by glomeruli and are partially reabsorbed by water **(Corbett, 2008).** The high blood urea may be due to dehydration which is indeed recorded during our experiment, low blood volume, troubled urea excretion, increased urea enzymes activity, and decreased serum proteins **(Garba *et al.*, 2007).** The latter may be correlated with increased protein catabolism into amino acids and then into urea and creatinine. Thus, these amino acids formed can be transformed by the action of plasma transaminases into carboxylic compounds **(Forêt, 2012),** which could explain the high enzymatic activity of AST and ALT in Tol-treated rats.

Toluene is commonly eliminated by the kidney as hippuric acid. If renal elimination of hippuric acid is impaired, the acid can accumulate in the blood to bring out an anion gap, renal tubular acidosis, hypokalemic paralysis, and profound metabolic acidosis **(Cruz *et al.*, 2014).** In this respect, **Neghab et al. (2015)** reported that occupational exposure to toluene may be associated with the development and progression of renal failure and renal tissue alterations.

Uric acid is an intermediate product of the purine degradation pathway in the cell **(Sautin and Johnson, 2008).** Since that uric acid exhibits strong free radical-scavenging activity in humans **(El-Demerdash, 2004; Yousef, 2004).** It protects cells, particularly by quenching hydroxyl, superoxide, and peroxynitrite radicals, and also preventing lipid peroxidation that correlates with hypertension, and cardiovascular disease conditions **(Squadrito *et al.*, 2000; Sautin and Johnson, 2008).** Considering the association of organic solvents with high blood pressure and heart disease **(Kim *et al.*, 2012),** we hypothesized that the insignificant modification in the concentration of uric acid may be interpreted as a protective response against ROS production. Several major epidemiological studies have identified low uric acid levels as a translation to the greater oxidative stress generation **(Ames *et al.*, 1981)**. Alternatively, a decrease in uric acid level may reflect malnutrition, as long as it is largely stand-on the diet **(Hsu *et al.*, 2004)**.

Previous findings registered by **Abdel Moneim and El-Khadragy (2012),** and **Hassanen et al. (2019)** indicated that the administration of pomegranate peel ethanolic extract and juice significantly attenuated the damaging impact on the liver and kidney. Similarly, we found that PJ and practically PAE were efficient in modulating the observed variations in hepatic and renal biochemical parameters maybe by reversing the progression of the pathological lesions due to its strong antioxidant capacity and the presence of a high level

of bioactive compounds such as gallic acid, this phytoconstituent was proved to be very effective against oxidation by restoring damaged tissues in rats **(Li *et al.,* 2006; Jadeja *et al.,* 2010).**

Despite the thought that reproductive issues are commonly viewed as a woman's concern, infertility occurs in equal amounts for females and males. Data indicate that substances and drug abuse create a high incidence in the global decline in male fertility, which emerges as a critical health issue **(Taha *et al.,* 2020).**

In the present study, our outcomes demonstrated that the Tol has induced a momentous reduction in testicular absolute weight. These results corroborate with previous study reported by **Djemil et al. (2015)** and disaccorded with another one by **Nakai et al. (2003).** The testicular weight is basically dependent on the mass of the differentiated spermatogenic cells and steroid biosynthesis by Leydig cells **(Rehnberg *et al.,* 1989; Ihsan *et al.,* 2011).** Accordingly, the decrease in testicular weight observed in this study is a pointer to toluene-induced alteration on the germ cells differentiation and gonadotropins release **(Nakai *et al.,* 2003)**.

Sperm parameters including concentration and vitality are important functional indicators to assess sperm fertilizing capacity. Epididymal sperm concentration was notably dropped in toluene-treated animals, according to our findings. Furthermore, within the same group, the hypo-osmotic swelling test indicated a clear decline in sperm vitality. Such results are consistent with those who reported a decrease in sperm concentration and vitality in male rabbits after 150 ppm toluene oral administration (**Djemil *et al.,* 2015).** Previously it was established that toluene provokes disturbance in the meiotic process of spermatogenesis, which may ultimately elicit sperm concentration lacking **(Ishigami *et al.,* 2005). Homma-Takeda et al. (2002)** revealed that 2,4,6-Trinitrotoluene injection has prevented germ cell development. Further, this drop could be linked to the inadequate supply of androgens, which in turn interfere with the differentiation of germ cells and the spermatogenic cycle **(Elmasry *et al.,* 2018).** Other authors have postulated that alteration of testicular germ epithelium DNA could be the origin of sperm concentration regression **(Schardein, 1993; Murata *et al.,* 1999).** Regarding the drop witnessed in sperm vitality the cause perhaps was associated with a decrease in the Follicle Stimulating Hormone (FSH) concentration that is a mediator in the activation of growth factors and production of nutrients by Sertoli cells, which maintain the maturation and normalization of the micro-

environment around sperm and germ cells **(Svensson *et al.*, 1992; Menegazzo *et al.*, 2011).** Polyunsaturated lipids oxidation of sperm can also alter sperm vitality by changing membranes' fluidity and permeability **(Çeribaşi *et al.*, 2010).**

Consistently, toluene provokes a decline in sperm motility and other kinematic parameters (VSL, VAP, LIN, and BCF). Spermatic motility with all its parameters such as VCL, VAP, VSL, BCF, LIN, and STR was reported to be essential elements to estimate fertility rate **(Moore and Akhondi, 1996)**. Thus, changes in these indicators play a main role in the pathogenesis of toluene reproductive toxicity. Proper ionic balance plays a crucial role in regulating sperm flagellar motility. In fact, there is abundant experimental evidence stating that the psychoactive drugs were demonstrated to alter sperm motility through a variety of mechanisms including regulation of calcium signalling **(Srivastava and Coutinho, 2010).** It is equally known that exposure to toluene interact with a variety of ion channels as it can cause voltage-sensitive calcium channels inhibition and produces noticeable changes in different systems **(Tillar *et al.*, 2002**). Therefore, it can be speculated that sperm motility diminution returns to voltage-dependent calcium channel arrest ''CatSper'' located in the mid and principal piece of the flagellum; as a result, obstruction of calcic flux that is the principal mediator embroiled the AMPc pathway to regulate the activity of active phosphorylation/dephosphorylation systems on axonemal proteins, and thus flagellar curvature **(Darszon *et al.*, 2005; Turner, 2006).** In the same vein, high-level toluene brings an inhibition to the function of potassium channels (BK and GirK) and the voltage-sensitive sodium channels (Na_v) **(Del Re *et al.*, 2006; Scior *et al.*, 2009**), the important regulators for cellular processes including sperm regulation function such as maturation, motility and viability **(Publicover *et al.*, 2008; Yi *et al.*, 2011; Cejudo-Roman *et al.*, 2013).** Thus, it is hypothesized that the observed deterioration in sperm quality under these conditions suggests that toluene can deeply disrupt ion channels in sperm, thereby affecting male fertility.

Further, According to **Xiao et al. (2001),** Exposure to toluene by inhalation decreased the relative activity of LDH-C4, which is considered the key enzyme for sperm motility. This process needs high levels of ATP and requires as a final step, the conversion of pyruvate to lactate joined by oxidation of NADH to NAD+ and catalyzing by LDH. On the other hand, there are several hypotheses indicating that VOCs including toluene influence chemical susceptibility by increasing nitric oxide (NO) levels **(Kanter, 2010),** which lead to sperm

motility diminution, probably by mitochondrial membrane potential modification as well as intracellular cGMP up-regulation **(Weinberg *et al.*, 1995).**

Steroid hormones are believed to be a crucial upstream factor in the stimulation and control of the maintenance and development of male reproductive functions. In this experiment, similar to **Nakai et al. (2003)** toluene exhibited a remarkable drop in plasma testosterone concentration in male rats. In this case, toluene may have impaired Gonadotropin-Releasing Hormone (GnRH) neurons in the hypothalamus-hypophyseal-gonadal axis resulting in the reduction of luteinizing hormone (LH), which induce signals for the synthesis of testosterone **(Yilmaz *et al.*, 2001)**. The inhibition of these signals results in a reduction in serum testosterone levels, as has been observed in men exposed to low concentrations of toluene where their LH level was affected (**Luderer *et al.*, 1999).**

As well, it has been suggested that ROS generated by toluene through biotransformation processes can increase the expression of Cyclooxygenase (COX-II) **(Kamel and Shehata, 2008),** which might impact androgen synthesis via the production of a large amount of inflammatory prostaglandins **(Fouad and Jresat, 2014).**

Pomegranate peel aqueous extract appears to be more useful than pomegranate juice in moderating the effect of toluene on spermatic parameters. In parallel, pomegranate peel extract was demonstrated to boost sperm count, motility, and viability, as well as increase seminiferous tubule diameter and epithelium thickness due to the antioxidant activity of its phenolic compounds **(Zeweil *et al.*, 2013; Tapias *et al.*, 2014; Utomo *et al.*, 2019)**. It has been documented that these metabolites such as catechin and quercetin ring exhibit the capacity to improve sperm quality and reduce lipid peroxidation **(Taepongsorat *et al.*, 2008; Boonsorn *et al.*, 2010).** In addition, Flavonols such as gallocatechin are good antioxidants proven twice as effective as vitamin E **(Plumb *et al.*, 2002).** The latter at a daily dose of 300 mg has elevated sperm motility in infertile men **(Suleiman *et al.*, 1996)**. The presence of anthocyanin in pomegranate has been suggested to stimulate reproductive hormonal regulation, as a consequence, it affects by modulating the development and the function of the testicular germ line and Sertoli cells **(Utomo *et al.*, 2019).**

Several lines of evidence obtained from the current experiment suggest that ROS causes the toxic effect of Tol. The ratio of GSH and GPx in liver, kidneys, and testes was markedly decreased in rats exposed to Tol, which was accompanied by an increase in MDA levels. These results agreed with the literature reporting that inhalation of Tol raised

MDA concentration and diminished GPx activity **(Kamel and Shehata, 2008; Abouee-Mehrizi *et al.*, 2020)**. It is known that the toluene metabolism is a major intracellular ROS generator in the tissues **(Murata *et al.*, 1999)**, where it occurs through NADPH oxidation by the mitochondria electron transport chain **(Nsonwu-Anyanwu *et al.*, 2021)**. The results obtained here were possibly linked to the Tol-induced increase in ROS generation, leading to lipid peroxidation and antioxidants depletion, which, in turn, contributes to the occurrence of cellular apoptosis **(Ochsendorf, 1999).** It can be supposed that the marked decreases in GSH concentration were related not only to the overproduction of ROS but could also result from impaired regeneration by GSH reductase. Considering the fact that cells are rich in mitochondria (1000-2000 pieces in hepatocytes and approximately 50–75 pieces in sperm midpiece) **(Wiesner *et al.*, 1992; Ankel-Simons and Cummins, 1996),** they are targets of drug toxicity mainly by oxidative stress. In response to this stress, the permeability of the mitochondrial membrane may increase, which may induce mitochondrial dysfunction and declined metabolic regeneration of reducing equivalents, resulting in decreased regeneration of GSH **(Monks *et al.*, 1999)**. Many investigators have postulated that toluene produces high levels of H_2O_2 and NO, which override enzymatic activity, thus inducing lipid peroxidation, DNA oxidation, and oxidation or nitrosylation of thiols **(Wink *et al.*, 1999; Kamel and Shehata, 2008)**.
In the same circumstance, the higher concentration of MDA is also a direct evidence of the generation of free radicals and the alteration of the cellular lipidic membranes (**Bayil *et al.*, 2008),** especially in the testes where there is a large amount of polyunsaturated fatty acids, which makes them sensitive to oxidative stress by producing a loss of membrane integrity and could subsequently alter the cellular function **(Wathes *et al.*, 2007).**

All disruptions through oxidative stress injuries outlined here were affirmed by the histological outcomes in rats treated with Tol. The histological analysis of both liver and kidney sections support the biochemical findings. The main hepatic histopathological features included infiltration of leukocytes, steatosis, degenerative changes, and other manifestations. Toluene was also reported to cause slight pericentral fibrosis and ballooning degeneration **(Tas *et al.*, 2011)**, as well as sinusoid dilation, hemorrhage, inflammatory cell infiltration, vacuolization, and necrosis (**Meydan *et al.*, 2019)**. Renal microscopic examination revealed abnormal nephrotic changes varied from degenerative to necrotic changes in some tubular epithelium besides to atrophy of glomerular tuft which was common after Tol treatment. These results were compatible with that documented by

Shaffie and Shabana (2019) who reported that exposure to 900 mg/kg of toluene causes vacuolar degeneration of renal tubular epithelial lining with dilatation of some tubule.

Furthermore, consistent with the sperm biological parameters, histopathological examination of Tol rats testis presents evidence of poor architecture with several degenerated seminiferous tubules along with the decreases of sperm in their lumen and the increases in interstitial spaces. **Kanter (2010)** also recorded these alterations and mentioned that after toluene inhalation, there was a marked decrease in the seminiferous tubular diameter, with the degeneration of germinal epithelial cells. Also, the epididymis showed mild vacuolar degeneration, necrosis, edema, and lack of sperm in epididymal tubules.

Cellular damage caused by toluene has been previously reported to be a caspase-dependent process. In support to these results, **Yang et al. (2001)** demonstrated in experimental models that Bax/Bcl2 ratio was directly associated with renal and liver apoptosis and progressive diseases in other organs.

As observed in the present study, *P. granatum* has a considerable free radical scavenging ability; thus, it seems that the protective potential of pomegranate may depend on the alleviation of oxidative stress damage. This effect has been affirmed by the modulation of structural organization, decreased MDA levels, and enhanced GSH and GPx activity, especially in rats that received PAE, which agrees with the previous results of **Bouasla et al. (2016)** and **El Bohi et al. (2021)**. The high potential of phenolic components to scavenge radicals might be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups **(Mathew *et al.*, 2015)**. It has been demonstrated that the active constituents of pomegranate, such as flavonoids and ellagic acid, restored the oxidant/antioxidant balance by increasing C-glutamyl cysteine synthetase, which is the critical enzyme in GSH synthesis **(Moskaug *et al.*, 2005)**. The authors also found evidence that punicalagin extracted from PJ and peel increases serum paraoxonase activity, which may protect against lipid peroxidation **(Aviram *et al.*, 2008)**. Moreover, antioxidant activity might not be the only factor responsible for the ability of pomegranates to reduce the severity of Tol toxicity; another potential therapeutic tool is linked to its anti-inflammatory and anti-apoptotic activity. It was revealed that pomegranate peel extract can reduce tissue inflammation and oxidative stress by suppressing the production of pro-inflammatory cytokines and enhancing Nrf2 activation **(El Bohi *et al.*, 2021)**. Thus, Nrf2

up-regulation leads to an elevation in the expression of antioxidant enzymes, to combat ROS generation **(Karthivashan *et al.*, 2015)**. This action could be attributed to bioactive elements, such as punicalagin and punicalin, which contribute to nuclear factor kappa B (NF-κB) down-regulation by blocking NF-κB-driven transcription, in turn, diminishing inducible iNOS and subsequent NO production **(Dell'Agli *et al.*, 2010)**. Furthermore, pomegranates are also able to down-regulate caspase-3and up-regulate the anti-apoptotic protein Bcl-2; therefore protecting cells from death **(El Bohi *et al.*, 2021)**.

Conclusion and Perspectives

Humans are exposed to a greater variety of hazardous chemicals, some of which have gradually been elucidated to be important risk factors for several physiological functions, including those affecting the respiratory, neurological, reproductive, hepatic, renal, and cardiovascular systems. Over the past decade, there has been a growing awareness of the importance of diet for health. Therefore, the majority of current drugs are concentrated copies of herbal remedies. This work focused in the first place on the identification of antioxidant capacity and phytochemical groups, which characterize the *P. granatum* juice and peel aqueous extract *in vitro*. Secondly, the confirmation of this antioxidant therapeutic potential *in vivo* against the deleterious effects of toluene administration on reproductive, hepatic, renal, and hematological systems in male Wistar rats.

The *in vivo* experiments confirmed that exposure to a moderate dose of Tol (550 mg/kg BW) elicits reproductive, liver, renal, and hematological injuries, as established based on histological alterations (testes, epididymis, liver, kidneys), pro-oxidants and antioxidant imbalance (GSH, GPx, MDA), sperm (concentration, vitality, motility, velocity), biochemical (AST, ALT, ALP, TBIL, ALB, TP, Ur, Cr) and blood (RBC, HCT, HGB, WBC, LYM, EOS) parameters disruptions. However, supplementation with *Punica granatum* juice and peel extract almost normalized most of the markers. Phytochemically, the results show that PJ and PAE possess a rich and varied composition of secondary metabolites such as polyphenols, flavonoids, and tannins with antioxidant potential. Thus, it can be concluded that exposure to Tol may cause synergistic detrimental health effects in experimental animals; whereas the consumption of pomegranate seems to partially attenuate the toxic effects of this solvent and these beneficial effects are possibly due to its content in phenolic compounds. Nonetheless, further research is needed to ascertain the precise toxic mechanisms of toluene, as well as to give an appropriate dose recommendation of *P. granatum* supplementation for humans.

Finally, the natural antioxidants of local plant species and particularly those from *P. granatum* can be very useful to strengthen the organism in the case of oxidative stress and prevent the different pathologies that occur as a result of a radical attack.

In Perspective, it is interesting to profound these conclusions by:

- Expand the panel of antioxidant activities of plants *in vitro* and *in vivo* using other biological tests: antitumor, anticancer, and anti-inflammatory.
- Characterize and isolate the active ingredients responsible for these pharmacological properties.
- Use other extraction techniques.
- Deepen the study on the radical system and the total antioxidant status by measuring other oxidative stress parameters (Heat Shock Protein, vitamins E and C, catalase, SOD, GST, thioredoxin, free radicals, etc.).
- Evaluate the toxic effect of xenobiotics and the protective effect of phenolic compounds on cells cultured "*in vitro*".
- Study the cytotoxic effect by measuring apoptosis biomarkers (caspase-3 activity and DNA fragmentation).
- In addition, further studies on the effect of chronic exposure and clinical trials on the population exposed to different xenobiotics are recommended.

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Annex

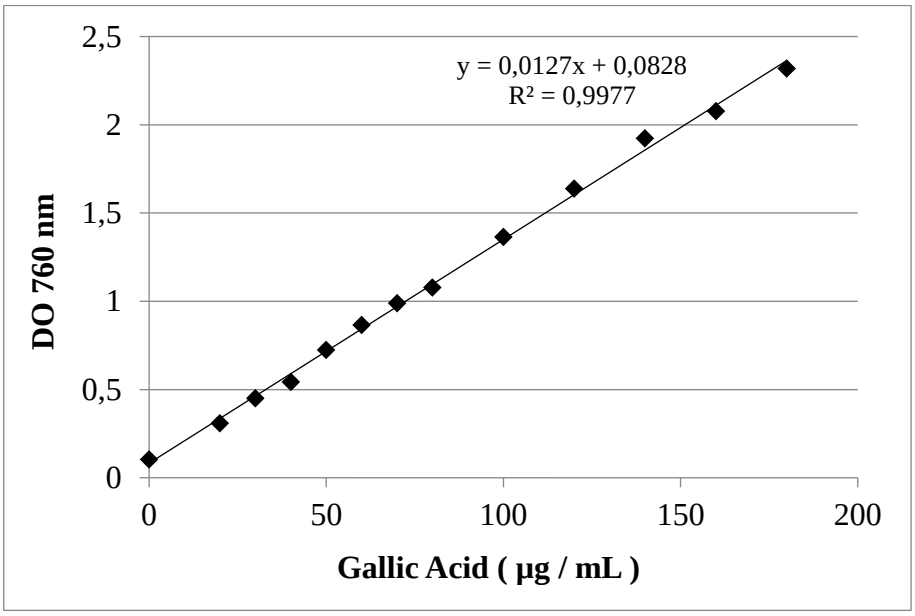
1. Antioxidant activity

- **DPPH antioxidant activity test:**

- Methanolic solution: 0.04 g of DPPH/ liter of methanol.

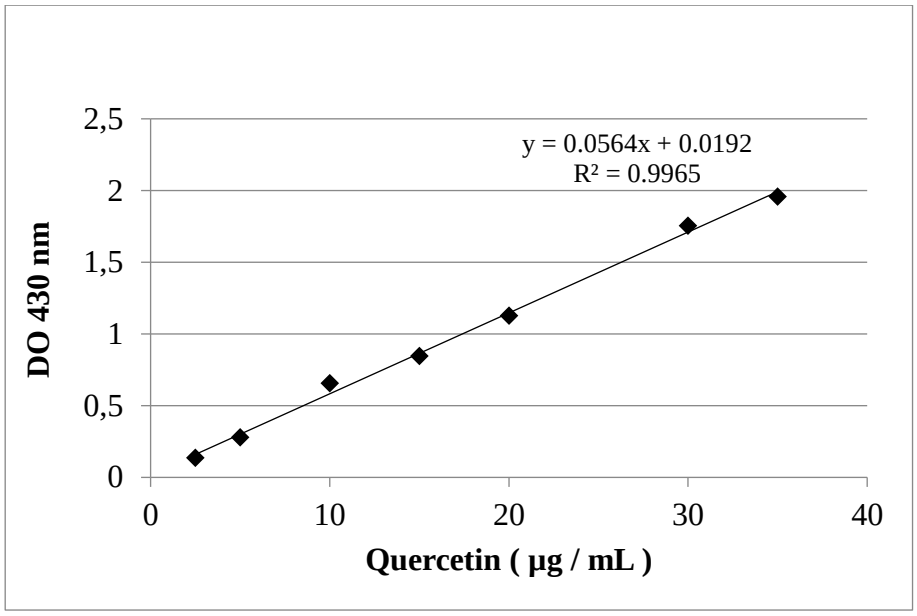
- **Total Polyphenols :**

- Dilution of Folin ciocalteu 1/10: 1 mL of Folin dissolved in 9 mL of distilled water.
- Solution of sodium carbonate Na2Co3 (7.5 %): 7.5 g in 100 mL of distilled water.
- Standard: Gallic acid: 1 mg A. g./1 mL of distilled water.



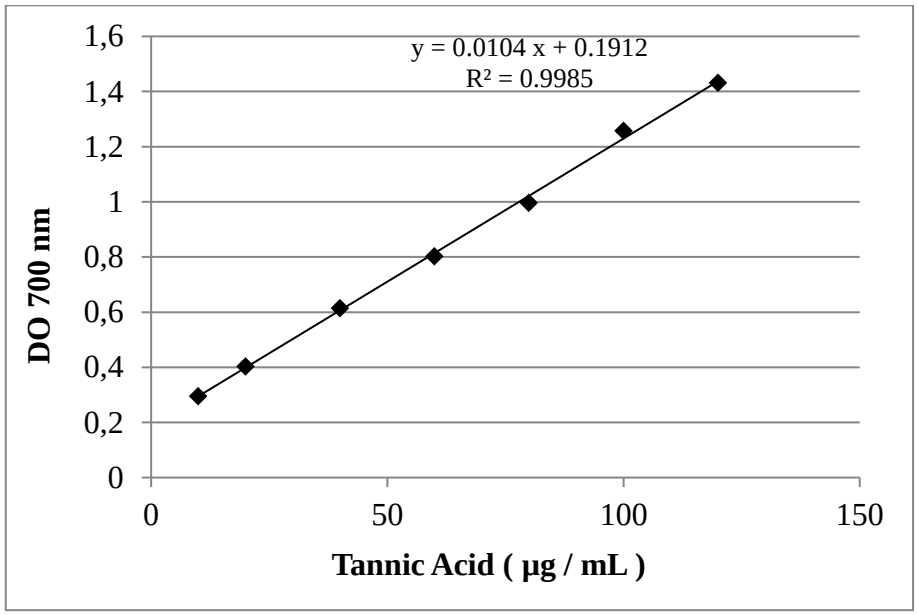
- **Total Flavonoïds:**

- $NaNO_2$: 0.5g in 10 mL of distilled water.
- NaOH (1N) = 4% solution: 4g in 100 mL of distilled water.
- $AlCl_3$ Solution: 10g dissolved in 100 mL of distilled water.
- Etalon: Quercetin / Catechin: 1mg / 1mL of distilled water.



- **Tanin Contents :**

- $Na_2 CO_3$ solution (5%): 0.5 g dissolved in 100 mL of distilled water.
- Tannic acid standard: 1mg / 1mL of distilled water.



2. Oxydantive stress

- **Glutathione (GSH):**

-EDTA solution (0.02 M): Dissolve 5.613 g EDTA in 750 ml of distilled water.

- DTNB solution (0.01 M): Dissolve 200 mg DTNB in 50 ml of absolute methanol.

-Salicylic acid solution (0.25%): Dissolve 250 mg of salicylic acid in 100 ml of distilled water.

- **Glutathione peroxidase (GPx):**

- TBS solution: Tris (50 mM, NaCl (150 mM): Dissolve 8.775g NaCl in 1l of distilled water, then add 6.057g Tris and complete the volume to 1l with the NaCl solution (150 mM) and adjust the pH to 7.4 by adding HCl or NaOH

- GSH solution (0.1 mM): Dissolve 3.073 mg GSH in 100 ml of distilled water - TCA solution (1 %): Dissolve 1 g TCA in 100 ml of distilled water

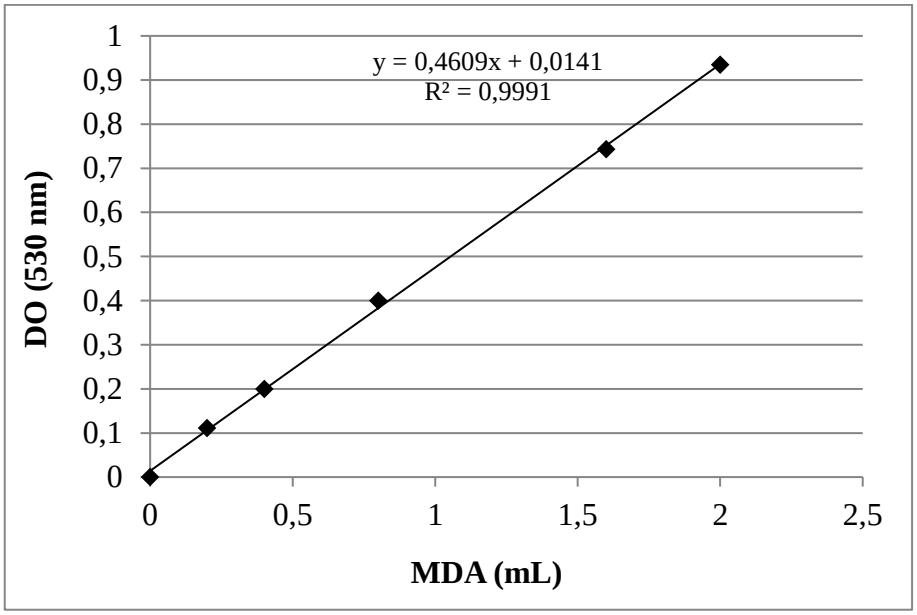
- DTNB solution (1.0 mM): Dissolve 100 mg DTNB in 250 ml of absolute methanol.

- **Malondialdehyde (MDA):**

- TCA solution (20%): Dissolve 20 g of TCA in 100 ml of distilled water.

- Tris solution: Dissolve 0.15 g of tris in 50 ml of distilled water.

- TBA solution (0.67%): Dissolve 0.33 g of TBA in 50 ml of the tris solution.



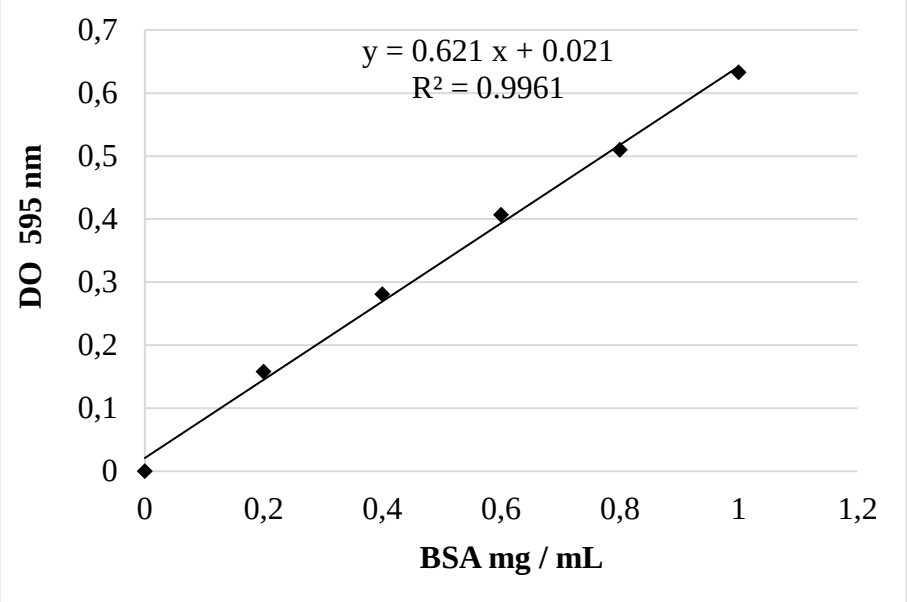
- **Protein assay by the Bradford method:**

-BSA solution (1 mg/ml): Dissolve 5 mg BSA in 5 ml of distilled water. *Bradford reagent:

Dissolve 100 mg of Coomassie blue (G 250) in 50 ml of ethanol (95%). Shake with the

shaker for 2 hours, then add 100 ml of orthophosphoric acid (85%) and 850 ml of distilled water (to obtain 1 l of solution).

This reagent must be filtered and then stored for a maximum of 1 month at a temperature of 4°C and protected from light



3. Calibration ranges

- Realization of the calibration range of Gallic Acid:

Gallic Acid (µg/mL)	5	10	25	100	150	200	250	300
DO at 760 nm	0.08	0.14	0.266	0.901	1.262	1.701	1.979	2.339

- Realization of the quercetin calibration range:

Quercetin (µg/mL)	25	50	75	100	150	200	250	300
DO at 510 nm	0.018	0.061	0.138	0.206	0.293	0.422	0.566	0.665

- Realization of the calibration range of the Tannic Acid:

Tannic Acid (µg/mL)	10	20	40	60	80	100	120
DO at 700	0.295	0.403	0.615	0.803	0.997	1.258	1.432

- Realization of the protein calibration range:

BSA (mg/mL)	0	0.2	0.4	0.6	0.8	1.0
DO at 595 nm	0	0.158	0.281	0.407	0.510	0.633

- Realization of the MDA calibration range:

1,1,3,3-tetraetoxypropane (mL)	0	0.20	0.40	0.80	1.60	2.00
DO at 530 nm	0	0.111	0.2	0.4	0.743	0.935

4. Results

Table 8: Total phenolic, flavonoid and tannin contents of *Punica granatum* juice and peel aqueous extract (mean±SEM). (GAE: Gallic Acid Equivalent, EQ: Equivalent Quercetin, TAE: Tannic Acid Equivalent).

	Total phenolic (mg GAE/g)	Flavonoids (mg EQ/g)	Tannins (mg TAE/100g)
PJ	0.52±0.005	0.22 ± 0.029	0.44 ± 0.042
PAE	0.78±0.01	0.49±0.002	0.74± 0.033

Table 9: Variation of organs absolute weight in different experimental groups (Mean±SEM). C: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene+Pomegranate Juice, **PAE-Tol**: Toluene+Peel Aqueous Extract).

Groups	Testis weight (g)	Epididymis weight (g)	Liver weight (g)	Kidney weight (g)
C	1.69±0.02	0.64± 0.01	8.43±0.2	0.77±0.01
CO	1.69±0.01	0.64± 0.01	8.48±0.1	0.80±0.01
PJ	1.72±0.05	0.67± 0.01	8.31±0.2	0.77±0.01
PAE	1.77±0.02	0.70±0.008	8.31±0.2	0.76±0.024
Tol	1.54±0.03^{acd}	0.55±0.03^{cd}	9.7±0.2^{abcd}	0.92±0.006^{abcd}
PJ-Tol	1.60±0.03	0.61± 0.01	9.08±0.3	0.85±0.01
PAE-Tol	1.66±0.02	0.63±0.003	8.7±0.1	0.82±0.004

Table 10: Variation of sperm concentration, vitality, and testosterone levels in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	concentration (10^6/ml)	vitality (Alive sperm) (%)	vitality (Dead sperm) (%)	Testosterone (ng/ml)
C	88.66±4.71	90.04±0.41	9.94±0.41	3.49±0.12
CO	84.78±4.34	90.45±0.30	9.46±1.27	3.48±0.26
PJ	92.45±5.74	91.72±1.01	8.25±1.01	3.6±0.06
PAE	96.18±8.16	91.77±1.34	8.23±1.34	3.73±0.04
Tol	51.33±1.84^{abcd}	29.22±1.13^{abcd}	70.71±1.11^{abcd}	1.88±0.24^{abcd}
PJ-Tol	69.23±4.3	57.86±4.31^{ce}	42.13±4.31^{ce}	2.92±0.03
PAE-Tol	79.9±2.15^{e}	86.66±0.71^{e}	11.83±0.29^{e}	3.30±0.24^{e}

Table 11: Variation of sperm motility in the different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	Total motility (%)	Progressive (%)	Non-progressive (%)	Immotile (%)
C	62.81±5.59	6.13±1.47	56.67±3.78	37.19±5.11
CO	67.36±5.46	3.89±0.49	63.47±4.67	32.64±4.88
PJ	66.75±6.23	8.34±0.41^{b}	58.4±5.87	33.25±5.83
PAE	67.17±0.64	9.48±0.8^{b}	57.68±1.23	32.83±0.36
Tol	26.25±2.99^{abcd}	0.79±0.29^{acd}	22.65±1.68^{abcd}	76.55±1.48^{abcd}
PJ-Tol	50.2±3.31	2.86±0.37^{c}	47.34±0.45	49.8±3.31
PAE-Tol	57.25±7.24^{e}	3.21±0.04^{d}	54.03±5.2^{e}	42.76±7.24^{e}

Table 12: Variation of sperm kinematic parameters in the different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqu eous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	BCF (Hz)
C	77.19±2.37	16.03±0.4	33.82±0.75	20.25±0.93	3.66±0.12
CO	78.85±1.14	16.54±1.25	34.07±0.83	20.58±1.68	3.78±0.01
PJ	78.71±1.75	16.73±0.22	35.03±0.24	21.28±1.6	3.89±0.05
PAE	79.12±0.39	16.96±0.74	35.9±0.31	21.55±0.97	3.96±0.02
Tol	74.07±3.5	9.17±0.62^{abcd}	30.78±0.38^{cd}	12.9±0.34^{abcd}	2.65±0.16^{abcd}
PJ-Tol	75.62±0.47	13.19±0.33^{e}	33.07±0.08	17.37±0.05	3.25±0.03
PAE-Tol	75.32±1.63	14.49±0.68^{e}	33.8±0.47	19.25±0.18	3.5±0.03

Table 13: Hepatic biochemical parameters of rats in the different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	AST (µl/L)	ALT (µl/L)	ALP (µl/L)	Albumin (g/L)	Total Protein (g/L)	Total Bilirubin (mg/L)
C	138.6±3.91	31.9±0.91	86±6.17	33.82±1.18	92.63±2.49	5.76±0.09
CO	139.8± 2.25	31.4±0.97	86.89±2.66	33.79±1.55	91.07±5.5	5.65±0.13
PJ	138.6±3.48	31.6±0.7	86.83±2.17	34.17±0.55	93.11±1.47	5.59±0.46
PAE	138.3±0.54	31.5±1.25	86.24±46.89	34.38±1.01	93.38±2.88	5.75±0.39
Tol	175.7±4.09^{abcd}	47.63±0.64^{abcd}	129.2±7.95^{abcd}	26.48±1.05^{abcd}	71.65±2.4^{abcd}	8.3±0.97^{abcd}
PJ-Tol	161± 6.65^{ce}	40.33±0.88^{ce}	106±4.54	29.46±1.06^{e}	81.01±4.95	7.3±0.52
PAE-Tol	157.5±1.18^{de}	37.710.3^{de}	96.25±2.56^{e}	31.94±1.54^{e}	87.54±0.52^{e}	6.53±0.32

Table 14: Renal biochemical parameters of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	Urea (g/L)	Creatinine (mg/L)	Uric Acid (mg/L)
C	0.38±0.005	7.8±0.43	14.2±0.8
CO	0.39±0.01	8.44±1.18	14.44±1.34
PJ	0.36±0.01	7.78±0.78	13.91±0.57
PAE	0.36±0.02	7.54±0.8	13.09±0.58
Tol	0.52±0.01^{abcd}	9.95±0.05^{abcd}	17±0.98
PJ-Tol	0.46±0.003^{c}	9.95±0.05	15.69±0.78
PAE-Tol	0.42±0.02^{e}	7.66±0.88^{e}	14.51±0.44

Table 15: GSH content of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	GSH Testis (nmoL/mg Prts)	GSH Liver (nmoL/mg Prts)	GSH Kidney (nmoL/mg Prts)
C	27.23±0.62	61.71±1.71	53.18±0.16
CO	26.31±0.46	61.24±0.89	52.85±1.35
PJ	27.90±0.51	63.57±1.48	55.1±1.71
PAE	28.73±0.39	64.03±1.28	56.25±1.29
Tol	22.01±0.29^{abcd}	47.06±1.86^{abcd}	41.77±1.73^{abcd}
PJ-Tol	24.47± 0.68^{c}	52.85±1.4^{c}	47.6±2.53^{c}
PAE-Tol	25.64±0.68^{de}	57.42±0.47^{de}	49.9±0.47^{de}

Table 16: GPx activity of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	GPx Testis (nmoL GSH/mg Prts)	GPx Liver (nmoL GSH/mg Prts)	GPx Kidney (nmoL GSH/mg Prts)
C	0.34±0.005	0.46±0.02	0.44±0.007
CO	0.34±0.0007	0.45±0.01	0.43±0.02
PJ	0.37±0.009	0.46±0.007	0.45±0.01
PAE	0.40±0.010	0.46±0.01	0.46±0.01
Tol	0.22±0.008^{abcd}	0.28±0.02^{abcd}	0.31± 0.02^{abcd}
PJ-Tol	0.26±0.009^{c}	0.37±0.02	0.38±0.005
PAE-Tol	0.28±0.005^{de}	0.40±0.009^{e}	0.40±0.009^{e}

Table 17: MDA levels of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	MDA Testis (nmoL/mg Prts)	MDA Liver (nmoL/mg Prts)	MDA Kidney (nmoL/mg Prts)
C	0.27±0.01	0.30±0.01	0.32±0.01
CO	0.27±0.01	0.31±0.01	0.34±0.01
PJ	0.26±0.01	0.28±0.01	0.30±0.01
PAE	0.24±0.01	0.28±0.007	0.31±0.009
Tol	0.49±0.009^{abcd}	0.53±0.02^{abcd}	0.53±0.01^{abcd}
PJ-Tol	0.41±0.002^{c}	0.46±0.01^{c}	0.46±0.03^{c}
PAE-Tol	0.36±0.01^{de}	0.40±0.01^{de}	0.44±0.01^{de}

Table 18: RBC count, hemoglobin and hematocrit levels, and MCV of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	RBC (10^{12}/L)	HGB (g/L)	HCT (%)	MCV (fL)
C	9.40±0.04	149.5± 0.86	44.96± 0.87	45.67± 0.88
CO	9.273±0.04	149.2 ± 1.06	44.88 ±0.71	45.33 ±0.88
PJ	9.57±0.04	150.2± 1.59	45.7±0.21	46±0.40
PAE	9.56±0.30	149.7±2.02	45.23±0.45	46±0.70
Tol	8.48±0.08^{abcd}	0.79±0.29^{abcd}	38.13±0.36^{abcd}	45.8±0.79
PJ-Tol	9.38±0.15^{e}	146.7±0.33^{e}	42.99±0.56^{e}	45.75±0.478
PAE-Tol	9.12±0.15^{e}	144.8± 2.35	41.97±0.6^{e}	45.5±0.763

Table 19: WBCs count, lymphocytes, eosinophils, neutrophils, platelets levels, and MCV of rats in different experimental groups (Mean±SEM). (**C**: Control, **CO**: Corn Oil, **PJ**: Pomegranate Juice, **PAE**: Peel Aqueous Extract, **Tol**: Toluene; **PJ-Tol**: Toluene + Pomegranate Juice, **PAE-Tol**: Toluene + Peel Aqueous Extract).

Groups	WBC (10^{9}/L)	LYM (%)	PLT (10^{9}/L)
C	8.05± 0.44	55.62±0.82	729±26.88
CO	8.08 ±0.31	56.4±0.82	729.1±46.61
PJ	8.033±0.34	54.78±1.89	728±17.66
PAE	8.24±0.63	55.88±1.92	728.3±42.36
Tol	13.96±0.7^{abcd}	0.79±0.29^{abcd}	621.8±8.37
PJ-Tol	10.33±0.66^{e}	58±2.04^{e}	714.4±0.72
PAE-Tol	10.86±0.82^{e}	59.01±1.68^{e}	711.1±2.25