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Preparation and evaluation of *Spinacia oleracea* extract and zinc Nanosizes effect on experimental biochemical disorders and oxidative stress in rat

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Dédicace

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D. Anfal

Abstract

The main goal of this study was to evaluate nanotherapy based on ZnO nanoparticles and combination between phytotherapy and nanotherapy based on encapsulation of Spinach (*Spinacia oleraceae*) aqueous extract in vasicles to reverse fluoride overdose-induced-experimental biochemical disorders and oxidative stress in rats. For this purpose, thirty six male albino Wistar rats were randomly divided into 6 groups (n=6); the first group (G01) used as a control group. (G02) had given fluoride as fluoride sodium (NaF) in their drinking water to induce systemic toxicity (400 ppm) during 11 weeks. NaF rats injected intraperitoneally 03 times in the last 03 weeks by: Spinach Extract encapsulated in vesicles:7mg/kg body weight (G03), Spinach mediated ZnO nanoparticles green synthesis (SpE-ZnO-NPs): 7mg/kg body weight/week (G04) and Co-treatments (G03+G04) for NaF rats (G05) and control rats (G06). (G01) and (G02) were injected with physiological saline solution. Our biosynthesis ZnO using spinach extract (SpE-ZnO-NPs) characterized as irregular in the morphological shape, rod-like morphology with mean size 30 nm and hexagonal crystallites structure. *In-vitro* SpE-ZnO-NPs present anti-oxidant and anti-inflammatory and narrow antibacterial capacities without any *In vivo* toxicity at the concentrations studied indicating its safety and beneficial therapeutic effect. Spinach extract encapsulated vesicles characterized as regular (uniform in the morphological shape) and spherical with mean diameter 510 ± 27.5 nm. According to the results which have been obtained; fluoride exposure cause hyperthyroidism, hypoglycemia, hyperparathyroidism and anemia. Significant perturbation in markers functions of kidney ($P<0.01$) and liver ($P<0.001$). Results showed also a significant disturbance in electrolytes levels ($P<0.01$) in NaF group when compared to control rats. Moreover, the findings showed that overdose of fluoride affected antioxidant defense system by decreasing Glutathione (GSH) level and altering Glutathione S transferase (GST) and Glutathione peroxidase (GPx) activities simultaneously with malondialdehyde (MDA) level increase in brain, thyroid, liver, kidney, heart, spleen and testis tissues. The histological examination of the cellular structure of the testis, liver and kidney tissues of NaF exposed rats recorded remarkable changes in the tissues' appearance. However, the administration of various treatments ameliorated thyroid function, hematological and biochemical parameters, and restored the histological alterations. Among these treatments, the combined treatment showed the greatest improvement in thyroid, renal, hepatic, hematological, and cardiovascular dysfunctions. It completely restored the studied biochemical parameters as well as that of oxidative stress. In conclusion, the present study demonstrated the beneficial effects of nano-phytotherapy, whether administered separately or in combination, on oxidative stress and its metabolic and physiological complications.

Keywords: *Spinacia oleraceae*, nano-vesicles, ZnO nanoparticles, Fluoride, Oxidative stress.

الملخص

الهدف الرئيسي من هذه الدراسة هو تقييم العلاج بالنانو بالاعتماد على الجسيمات النانوية ZnO والجمع بين العلاج النباتي والعلاج بالنانو اعتمادا على تغليف مستخلص السبانخ (*Spinacia oleraceae*) في حويصلات نانوية لتقليل الاضطرابات البيوكيميائية التجريبية والإجهاد التأكسدي المستحثين بمعاملة الفئران بتراكيز عالية من الفلور. لهذا الغرض، أجرينا دراستنا هذه على 36 فأر تم تقسيمهم إلى ست مجموعات في كل مجموعة ستة (06) فئران، المجموعة الأولى(G01) مجموعة شاهدة نظامها الغذائي عادي و مياه شربها خالية من أي إضافات تجريبية، المجموعة الثانية (G02) استحدث لديها تسمم عام وإجهاد تأكسدي تجريبيا بإعطائها الفلور على شكل ملح فلور الصوديوم (NaF) في ماء الشرب بتركيز 400 ملغ/ل مع نظام غذائي عادي لمدة 11 أسبوع. الفئران بعد تسميمها تم حقنها داخل الصفاق 3 مرات في آخر ثلاث أسابيع بـ: حويصلات تحتوي مستخلص السبانخ بتركيز 7ملغ/كيلوغرام من وزن الجسم (G03) وجسيمات ZnO نانوية مصنعة بيولوجيا بواسطة مستخلص السبانخ بتركيز 7ملغ/كيلوغرام من وزن الجسم (G04)، في حين أعطيت المجموعتين الخامسة (G05) والسادسة (G06) مزيجًا من كل هذه العلاجات بنفس التراكيز وهي منفصلة حيث أن المجموعة (G06) فئران سليمة. تم حقن المجموعتين الأولى (G01) والثانية (G02) بالماء الفزيولوجي. تتميز جسيمات ZnO النانوية المحضرة طبيعيا باستخدام مستخلص السبانخ (SpE-ZnO-NPs) بأنها غير منتظمة في الشكل المورفولوجي، حيث أنها تشبه الأعواد بمتوسط حجم 30 نانومتر وهيكل بلوري سداسي. تُظهِر (SpE-ZnO-NPs) في المختبر فعالية مضادة للأكسدة ومضادة للالتهاب في حين أن الفعالية المضادة للبكتيريا ضعيفة، كما أنها لم تسبب تسمم في الجسم الحي عند التراكيز المدروسة مما يشير إلى أنها تراكيز آمنة ولديها تأثير علاجي فعال. تتميز الحويصلات نانوية الحاوية على مستخلص السبانخ بأنها منتظمة (انتظام الشكل المورفولوجي) وكروية بمتوسط قطر 27.5 ± 510 نانومتر. بناءً على النتائج التي تم الحصول عليها ؛ التعرض للفلورايد يؤدي إلى فرط نشاط الغدة الدرقية، نقص السكر في الدم، فرط جارات الدرقية وفقر الدم. اضطراب كبير في مؤشرات وظيفة كل من الكبد($P<0.001$) والكلى ($P<0.01$)، كما أظهرت النتائج وجود اضطراب معنوي في مستويات الشوارد ($P<0.01$) في أمصال المجموعة المستحدث لديها التسمم بالفلورايد (NaF) مقارنة بالمجموعة الشاهدة. بالإضافة إلى هذا، أظهرت النتائج أيضا أن التسمم بالفلورايد يؤثر على الأنظمة المضادة للإجهاد التأكسدي بتخفيض مستويات الغلوتاثيون (GSH) وتثبيط نشاط الإنزيمين (GST) و(GPx) بالتزامن مع رفع مستويات النواتج الثانوية لأكسدة الدهون (MDA) في نسيج كل من الدماغ، الغدة الدرقية، الكبد، الكلية، القلب، الطحال والخصية. المعاينة النسيجية للبُنَى الخلوية لنسيج كل من الخصية، الكبد والكلية لدى المجموعة المستحدث لديها التسمم بالفلورايد (NaF) أظهرت تشوهات واضحة على مستوى مظهر الأنسجة. من جهة أخرى سجلنا أن حقن مختلف العلاجات منفصلة أو ممتزجة حسَّن في معايير عمل الغدة الدرقية، معايير الدم، المعايير البيوكيميائية وكذا في البُنَى النسيجية للأعضاء المدروسة، بينما أعطى مزيج كل هذه العناصر العلاجية أفضل النتائج من حيث تحسن وظائف الغدة الدرقية، الكلى، الكبد، القلب وفقر الدم و والذي أدى إلى استعادة كاملة للمعايير البيوكيميائية، للبُنَى النسيجية قيد الدراسة وللإجهاد التأكسدي. أخيرا، بينت هذه الدراسة فوائد دمج كل من العلاج النباتي والنانوي بشكل منفصل أو مجتمعة على الإجهاد التأكسدي ومضاعفاته الأيضية والفسيولوجية.

الكلمات المفتاحية: السبانخ (*Spinacia oleraceae*)، حويصلات نانوية، جسيمات ZnO نانوية، ، فلورايد، إجهاد تأكسدي.

Figures List

Tables List

Abbreviation list

ALA: Alpha linolenic acid

ALAT: Alanine Amino Transferase

$AlCl_3$: Aluminum chloride

APx: Ascorbate peroxidase

ASAT: Aspartate Amino Transferase

AVP: Vasopressin

BBB: Blood-Brain-Barrier

BHA: β-hydroxy acid

BHT: Butylated hydroxytoluene

BTB: Blood testes barrier

BWC: Body Weight changes

Ca^{++}: Calcium

CAT: Catalase

CAT: Catalase

CBC: Complete blood count

CDNB: 1-chloro-2,4-dinitrobenzene

CECs: Contaminants of emerging concern

CKD: Chronic Kidney disease

CVDs: Cardiovascular diseases

DHA: Docosahexaenoic

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTNB: 5,5′-Dithiobis(2-nitrobenzoic acid)

EDCs: Endocrine-Disrupting-Chemicals

EDs: Endocrine disruptors

EDTA: Ethylenediaminetetraacetic acid

EDX: Energy disperse X-ray

EPA: Eicosapentaenoic acid

EPO: Erytropoitine

EPs: Emerging pollutants

F^-: Fluoride

FFAs: Free fatty acids

FRAP: Ferric reducing antioxidant power

FT3: Free Tri-iodothyronine

FT4: Free Thyroxine

FTIR: Fourier transform infrared

GFR: Glomerular filtration rate

GFR: Glomerular filtration rate

GOT: Glutamic oxaloacetic transaminase

GPx: Glutathione peroxidase

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced Glutathion

H_2O_2: Hydrogen Peroxyde

H_2SO_4: Sulfuric acid

HCl: Hydrochloric acid

HGB: Hemoglobin

HK: Hyperkalemia

HPT: Hypothalamus- pituitary- testicular

I^-: Iodide

K^+: Potassium

LDH: Lactate Dehydrogenase

LPO: Lipid peroxidation

MDA: Malondialdehyde

MDH: Malate Dehydrogenase

MGDG: Monogalactosyl diacylglycerol

MS: Metabolic syndrome

Na^+: Sodium

NaCl : Sodium Chloride

NaF: Sodium fluoride

NaOH: Sodium hydroxide

NC: Nanocarriers

NDDSs: Nanoparticles-based drug delivery systems

NPs: Nanoparticles

NVs: Nanovesicles

OS: Oxydative stress

PEC: Pollutant of emerging concern

PHT: Parathyroid hormone

PTH: Parathyroid hormone

PUFA: Poly unsutured fatty acids

RAAS: Renine Angiotensine Aldosterone System

RBCs: Red Blood Cells

RBW: Relative Brain Weight

RF: Renal failure

RHW: Relative Heart Weight

RKW: Relative Kidney Weight

RLW: Relative Liver Weight

ROS: reactive oxygene species

RSW: Relative Spleen Weight

RThW: Relative Thyroid Weight

RTW: Relative Testis Weight

SEM: Scanning electron microscope

SOD: Superoxide dismutase

SOD: Superoxide dismutase

SpE: Spinach Extract

T_3: Triiodothyronine

T_4: Thyroxine

TBA: Thiobarbituric acid

TBS: Tris buffer Saline

TCA: Trichloroacetic acid

TH: Thyroid hormones

TSH: Thyroid stimulating hormone

WHO: World health organization

XRD: X-Ray Diffraction

ZnO: Zinc Oxide

Table of Contents

Introduction

Introduction

Among the most frequent and challenging diseases in modern medicine are metabolic disorders. The most common illnesses defined by metabolic failure are obesity, dyslipidemia, diabetes mellitus, osteoporosis, and metabolic syndrome. The development of metabolic disorders occurs when physiological metabolic pathways are disrupted, due to inadequate diet, sedentary work, and lack of physical activity (Yasmina Bourebaba *et al.*, 2022b).

Endocrine system of our body is responsible for secreting hormones that bind with specific receptors. Such interactions control various operations like metabolism, growth and development, mood, fertility sexual function, body weight regulation, etc. (Ghosh *et al.*, 2021).

The term endocrine-disrupting chemicals (EDCs) encompasses a group of "compounds or mixtures of compounds that interfere with hormone action" which are able to interfere with the normal functioning of the endocrine system by mimicking the action of the body's natural hormones or interfering with the endocrine system and metabolism, causing side effects on health (Ho *et al.*, 2022) (Martín-Pozo *et al.*, 2021) (Predieri *et al.*, 2022). In Canada, an EDC is defined as a "substance having the ability to disrupt the synthesis, secretion, transport, binding, action, or elimination of natural hormones in an organism, or its progeny, that are responsible for the maintenance of homeostasis, reproduction, development or behavior of the organism"(Marlatt *et al.*, 2021).

Fluoride is a trace element required to maintain a healthy and normal physiology in humans(Shouyan Wu *et al.*, 2022). Although the benefits of fluoride have been reported for oral health, fluoride pollution in the environment can be a health hazard(Fujiwara *et al.*, 2021). Because it is one of typical environmental pollutant (Liu *et al.*, 2021); which is still unavoidable environmental pollutant negatively influencing human health(Faruk *et al.*, 2021). Fluoridated water is a natural source of fluoride pollution (Adelakun *et al.*, 2022). Moreover, oral care and food products all contribute to the total daily exposure to fluoride but the primary source of fluoride exposure, due to high consumption, is through drinking water, naturally occurring, or added (Dec *et al.*, 2018),(Radovanović *et al.*, 2020). Human activities have resulted in increased contents of fluoride in water (Wang *et al.*, 2019).

Prolonged exposure to high fluoride results in damage to multiple soft tissues of the body inducing morphological changes in many organs, leading to an impairment of their

function (C. X. Wu *et al.*, 2022),(Stawiarska-Pieta *et al.*, 2012). Fluoride exposure produce systemic toxicity (Tian *et al.*, 2019); which targets to teeth (dental fluorosis) and bone (skeletal fluorosis) (Liwen Huang *et al.*, 2022), also soft tissues (Jing-jing Wang *et al.*, 2020) especially the kidney (Saylor *et al.*, 2022). Kidneys, followed by the liver, accumulate more fluoride than any other organ system in the body (Malin *et al.*, 2019). Cardiovascular problems, inculcating arteriosclerosis and arterial calcification, myocardial damage, high blood pressure, heart failure, and cardiac insufficiency have also been attributed to exposure to fluoride(Solanki *et al.*, 2022). Moreover, hematological alteration can induced by fluoride (Anjum *et al.*, 2022) (Djouadi Anfal, Derouiche Samir, 2017). Accumulation of fluoride ion observed in spleen cells (Shashi & Thakur, 2022), and experimental evidence showed that relentless exposure to fluoride affects the formation of blood forming cells i.e., hematopoietic cells in cavities of bone marrow and inhibits the transport of K^+/Cl^- ions altering the hematopoietic process (Ameeramja *et al.*, 2018),(Abbas *et al.*, 2017a). Fluoride can cross the blood-brain barrier (BBB) and accumulate in various brain sections (Niu *et al.*, 2015), and induce harmful effect on endocrine system such as testis (Orta Yilmaz *et al.*, 2018), (Han *et al.*, 2015) and thyroid gland (Adedara *et al.*, 2017),(Abdelaleem *et al.*, 2018).

Thyroid gland is an important endocrine organ located in the anterior neck between the supraclavicular regions and responsible for regulating the body's metabolism via thyroxine (T_4) and triiodothyronine (T_3) hormones(Huang *et al.*, 2021).

Spinach (*Spinacia oleracea*) is one of the most popular and nutrient-rich green leafy vegetables that is commonly consumed fresh in salads or after blanching. Many researchers have reported that spinach contains bioactive secondary metabolites, including flavonoids, phenolic acid derivatives, and primary metabolites such as amino acids and vitamins (Mu *et al.*, 2020),(Yuk *et al.*, 2019). It well known with its anti-inflammatory activity (Vutharadhi *et al.*, 2017) and by virtue of its bioactive phytochemicals, it is able to act as effective radicals and reactive oxygen species scavengers and possess its antimicrobial, anticancer, antiulcer, antidiabetic, antihyperlipidemic and antithrombotic activities (Panda & Shinde, 2017) (Fiorito *et al.*, 2019). Spinach contains high levels of zinc (Thomas *et al.*, 2019).

Phytotherapy has very old origins and it is based on the use of medicinal plants to treat and prevent diseases (Leite *et al.*, 2021). In last decade, phytotherapy, whose therapeutic efficacy is based on the combined action of several active compounds, have been put effort into reducing health risks and improving health conditions(Li *et al.*, 2020).

In recent decades, the application of nanomaterials in biomedicine has been among the most fascinating areas of research, and considerable progress has been made. Nanomaterials exhibit biocompatibility, high targeting, and low toxicity, and hold immense potential for development in biomedicine (Chen *et al.*, 2021).

In nature, Zinc does not exist freely; it occurs in a (+2) oxidation state and almost 55 zinc minerals exist. For human body, Zinc is known to be an essential trace mineral that is necessary for health and growth and is organically known as a helper molecule that assists about 300 enzymes (Derouiche & Kechrid, 2016),(Ali *et al.*, 2018). Zinc is a structural part of key anti-oxidant enzymes such as superoxide dismutase, and Zinc deficiency impairs their synthesis, leading to increased oxidative stress(Derouiche *et al.*, 2017).

Oxidative stress, which is defined as the imbalance between the cell antioxidant defense system and the production of reactive oxygen species (ROS) (Papas *et al.*, 2019). Fluoride induced the generation of ROS and oxidative stress in multiple cell types (Hu *et al.*, 2019).

Nanoparticles (NPs) with at least one dimension sized from 1 to 100 nm exhibit unique features owing to their extremely small size and high surface area to volume ratio (Al-Kordy *et al.*, 2021). Metallic nanoparticles are of particular importance because they often exhibit volume-dependent properties that differ from bulk materials(Samir *et al.*, 2020).

Zinc oxide (ZnO) nanoparticles are one of the most widely used metal oxide nanoparticles in a wide range of products including biomedical applications(Chupani *et al.*, 2017). which considered as one of the most promising and novel magic materials(Sara *et al.*, 2020). ZnO is a bio-safe material that possesses photo-oxidizing and photocatalysis impacts on chemical and biological species (Sirelkhatim *et al.*, 2015). Beside of ZnO NPs there are many zinc nanoparticle types; among them Zinc sulfide NPs (ZnS-NPs) (Iranmanesh *et al.*, 2015), Zinc ferrite NPs ($ZnFe_2O_4$-NPs) (Naseri *et al.*, 2011), Zinc phosphide NPs (Zn_3P_2-NPs) (Mobarok *et al.*, 2014), Zinc selenide NPs (ZnSe-NPs) (Shakir *et al.*, 2009) and Zinc telluride NPs (ZnTe-NPs) (Dwivedi & Dubey, 2009) but the Zinc oxide nanoparticles (ZnO-NPs) are well known due to its multifunctional, non-toxic nature with vast applications in various fields(Govarthanan *et al.*, 2020).

Nowadays, there is a growing urgency to advance ecofriendly nanoparticles synthesis routes(Haglan *et al.*, 2020). The type of plant and plant extract determines the size and shape of the nanoparticles. The increased biological activity of the green synthesized nanoparticles is due to the synergistic effect of the bioactive compounds present in plants

and nanomaterial precursors used for synthesis (Rajendran *et al.*, 2021). The synthesis of nanomaterials mediated by plant extracts has been gaining attention attributed to its simple process, economic feasibility, environmental friendliness, and ease of scale-up(Ahmed *et al.*, 2022).

With the rapid development of nanomedicine, nanoparticle-based drug delivery systems (NDDSs) have been widely studied. Liposomes, polymers, albumins, and micelles have shown considerable potential for clinical application (Zhenggang Wang *et al.*, 2022). Nano-carrier (NC) drug delivery helps in improving bioavailability, enhances permeability, reduces drug dosage, prevents degradation, active and passive targeting, reduces the risk of side effects due to aggregation and speed up the curing of disease. Apart from various advantages of NC, its application is limited due to toxicity, difficulty in scale-up process and low drug loading efficacy (Afreen Sultana *et al.*, 2022).

Based on this observation, the original idea of our thesis work was to assess phyto-nano-therapy, *Spinacia oleracea* aqueous extract encapsulated in nano-vesicles, and nanotherapy based on ZnO nanoparticles against experimental biochemical alterations and oxidative stress induced by overdose of fluoride in rats.

First part

Bibliographic synthesis

Chapter I

Environmental pollutants

1. Environmental pollutant

Environmental pollution is a global issue and it is badly affecting the human development. The water quality has been deteriorated because of the direct release of wastes from agriculture, household and industries in water sources such as rivers, ponds, etc. Heavy metals, antibiotics, and pesticides present in aqueous system pose serious health issues (Shakeel *et al.*, 2022). Environmental pollution, a hidden culprit, deserves more concern in unraveling the etiology of obstinate human diseases (Hanqing Xu, Jia, Sun, *et al.*, 2022).

In recent years, the increase in industrial manufacturing processes, agricultural practices, and new technological development, have exposed the environment and humans to many new chemical compounds, defined as emerging pollutants (EPs) or pollutants of emerging concern (PEC). Some organic EPs are also classified as endocrine-disrupting compounds (EDCs) (Souza *et al.*, 2022).

2. Emerging pollutants (EPs)

Pharmaceutically active compounds, personal care products**,** endocrine-disrupting chemicals and pesticides are some of Contaminants of Emerging Concerns (CECs) that have been increasingly detected in water. They are ubiquitous, very persistent and not easy to remove by classic wastewater treatment plants (Rigoletto *et al.*, 2022).

2.1. Organic pollutants

Pharmaceutical compounds and pesticides play a relevant role in health protection and agricultural activities. Pharmaceuticals are relevant for medicine and disease prevention and are consumed worldwide, while pesticides are applied to control crop-damaging pests and disease vectors. In terms of water treatment, these organic compounds are considered emerging pollutants. The manufacturing of pharmaceuticals and their human consumption cause their subsequent entry into aquatic ecosystems. In the case of pesticides, their presence in the environment has a common origin in agricultural activities and reaches water bodies after being dissolved and transported mainly by stormwater runoff. Some studies have indicated that these emerging organic pollutants can be still identified in the effluents obtained from traditional wastewater treatment because they are not completely removed and, consequently, their persistence generates environmental pollution that could imply a negative effect on the ecosystems (Sellaoui *et al.*, 2023).

In the environment, the commonly occurring pharmaceuticals include antibiotics, painkillers, lipid regulators, β-blockers and neuroactive compounds. Till date, there are different types of pharmaceutical compounds have been listed that exhibit potential risk to the aquatic life and human beings as they are gradually discharged into the environment depending on their mode of use in variety of applications (e.g. discharge of wastes from industry, medical and household products) (Prasenjit Kar *et al.*, 2021).

Environmental risk assessment studies of these pharmaceutical chemical evaluate their acute (lethal) effects and, in some cases, study their long-term effects (reproduction). Most of the chemicals are generally found in aquatic system at very low concertation (ng/L) range. However, they exhibit several effects in human health like metabolic change, endocrine disruption as well as ecotoxicological effect in aquatic environment. Till date, wide variation of emerging pharmaceuticals chemicals were analyzed in aquatic environment at varying levels ranging from ng/L to mg/L (Prasenjit Kar *et al.*, 2021).

2.2. Inorganic pollutants

Inorganic pollutants (acid, salts, heavy metals, etc.); its presence in drinking water is one of the most serious environmental problems. Based on the concentration of chemicals in the water bodies, pollutants are categorized as macro-pollutants and micro**-**pollutants**.** Commonly found inorganic contaminants (micro-pollutants) in water include fluoride, chromate, perchlorate, nitrate**,** phosphate and heavy metals like arsenic, copper, lead, etc. (Gogoi & Pulikkal, 2022).

3. Environmental pollution-related public health

Environmental epidemiology is concerned with the relation between environmental exposures and human health. Environmental health risk assessment and management consist of different steps**)** hazard identification, dose-response assessment, exposure assessment**,** risk characterization, risk evaluation, risk perception and communication, control of exposure and risk monitoring) and provides indispensable information for setting preventive action priorities to resolve environmental health problems and can help to monitor and evaluate the effectiveness of such action. However, in order to manage environmental health in an effective way and to develop successful prevention and mitigation strategies, it is key to understand public health risk perceptions. Especially when

it comes to effective communication about exposures and the effects of this exposures towards affected communities (Noël *et al.*, 2021).

3.1. Notorious public health events

3.1.1. Air pollution

Air pollution is a serious public health issue globally. Particulate air pollution has been associated with increased morbidity including chronic and acute respiratory diseases, cardiovascular diseases, and lung cancer (He *et al.*, 2022).

The World Health Organization (WHO) reports that almost 99% of humanity breathe polluted air that exceeds the World Health Organization guideline limits, and that ambient air pollution exposure results in 4.2 million deaths annually. Beyond the lungs, increasing evidence highlights the link between ambient air quality and stroke deaths and a prevalence of a variety of mental diseases. Epidemiological studies have linked air pollution exposure to increment in dementia incidences and increased risk of neurodegenerative diseases, including Alzheimer's disease (AD) (Puris *et al.*, 2022). Moreover, air pollution is a major risk factor for diabetes (Burkart *et al.*, 2022).

Gaseous pollutants, such as nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and ozone (O_3) have been also associated with hazardous health effects and increased mortality (Baranyi *et al.*, 2022).

Air pollution could increase the incidence, prevalence or mortality of metabolic disorder (Yan *et al.*, 2022).

3.1.2.Heavy metal pollution

Heavy metals pose the most significant threat among various leachate compositions due to their non-biodegradable, toxicity, environmental persistence, bioaccumulative, endocrine-disrupting, and carcinogenic nature. Due to its persistent nature, heavy metals can be amassed in the environment over a period leading to possible contamination of the food chain. Accumulation of potentially toxic heavy metals in biota causes a potential health threat to their consumers, including humans (Afolabi *et al.*, 2022).

3.1.3. Chemical poisoning

Chemical substances are widely used in the medical, farming**,** and manufacturing fields. Chemical poisoning is one of the major causes of admission to emergency rooms and hospitalization as in cases of mortality in developed and developing countries. Many studies

have exposed different factors that can affect the incidence of chemical poisoning and mortality, including nature, age and amount of poison, administration route, and other factors. Drug and chemical poisoning are either by group or individually or both of the orally or intravenously use of a drug or chemicals in higher amounts lead to poisoning or may lead to death (Alnasser *et al.*, 2020).

3.1.3.1. Organic pollutants

The organic chemicals, classified as legacy or emerging pollutants, have included additives, benzophenones, bisphenol, polychlorinated dibenzo-p- dioxins and dibenzofurans (PCDD/Fs), estrogens (17α-ethinylestradiol and 17β-estradiol), flame retardants, illicit drugs, microplastics, parabens**,** per- and polyfluorinated alkyl substances (PFASs), pesticides, pharmaceutical, personal care products, antibiotics, phenolic compounds, phthalates**,**polycyclic aromatic hydrocarbons (PAHs), surfactants, and volatile organic compounds (VOCs) (Souza *et al.*, 2022).

3.1.3.2. Inorganic pollutants

Inorganic pollutants (acid, salts, heavy metals, etc.); its presence in drinking water is one of the most serious environmental problems. Based on the concentration of chemicals in the water bodies, pollutants are categorized as macro-pollutants and micro**-**pollutants. Commonly found inorganic contaminants (micro-pollutants) in water include fluoride, chromate, perchlorate, nitrate**,** phosphate and heavy metals like arsenic, copper, lead, etc. (Gogoi & Pulikkal, 2022).

3.2. Endemic diseases

Brucellosis is most commonly spread to humans by the inhalational route or through close human contact with infected animals. It can also be transferred among laboratory workers through direct mucosal contact with infected fetal products**.**Brucellosis is endemic to many developing countries, particularly Asia**,** Africa, the Mediterranean rim, the Middle East, central and south America. The most common clinical presentations of brucellosis include constitutional symptoms such as recurrent fever, profuse sweating, weakness, enlargement of the reticuloendothelial organs as lymphadenopathy, hepatosplenomegaly and osteoarticular involvement including arthralgia, back pain and occasionally spondyloartheritis and peripheral artheritis (Sharif *et al.*, 2022).

Kashin-Beck disease (KBD) is an endemic, deforming, degenerative cartilage injury disorder with unclear aetiology and pathogenesis. The main pathological feature of KBD is multiple focal necroses of the endochondral ossifying hyaline cartilage during the growth and development of children, which leads to endochondral ossification disorders and secondary osteoarthropathy. Some patients with advanced disease manifest joint deformity, short stature, and even permanent disability, which severely affect their quality of life (Ning *et al.*, 2022).

3.3. Occupational exposure-related diseases

Occupational use of chemical substance is the origin of a wide variety of occupational injuries (Schenk *et al.*, 2018).

4. Toxic effect of environmental pollutant in living organisms

Toxic pollution, any form of environmental pollution that causes harm to living beings, is a major global health threat which does not affect all equally. Exposure to toxic pollution is complex and can take many forms (i.e**,.** skin contact, inhalation, ingestion, and even prenatal exposure). Toxic pollution can be imperceptible, its effects surpassing temporal and spatial scales. For instance, some synthetic pesticides used in agriculture move beyond their target location into the soil, water and air, affecting the health of communities living miles away; Likewise, toxins can be inherited (i.e., in utero exposure or through breastfeeding), increasing intergenerational effects. These complexities mean that environmental conflicts that report health impacts follow a different pattern than those that do not (Navas *et al.*, 2022).

Human exposure to environmental organic pollutants is of great concern, as ample evidence shows that these compounds have adverse effects on animals and human beings. Monitoring the levels of organic pollutants and endogenous metabolites in biota are crucial for exploring their bioaccumulation and toxicity in both environmental toxicology and clinical analysis (Gong *et al.*, 2022).

Food and beverages are composed of a diverse and complex set of substances which are essential for humans (such as proteins, carbohydrates, lipids, vitamins and minerals), along with an extensive number of microconstituents which can either be beneficial (e.g., micronutrients, probiotics) or toxic (e.g. pathogens, toxins) for the human organism. However, the environmental and industrial contamination to which these foods and beverages are exposed during their production and processing causes concerns in the

international community, since it affects their composition and quality. In this context, assessing food safety through precise, selective and reliable determinations of toxic substances is demanded by regulatory organisms, thus requiring the constant development of analytical methods which are in turn respectful with the environment (Fiorentini *et al.*, 2022).

Recently, scientific advances have shed more light on this issue: it has emerged that, along with genetic predisposition, incorrect lifestyles, and psychological stressors, exposure to a mix of pollutants, significantly increases the likelihood of developing tumours. Global patterns of cancer incidence and the high number of deaths in the worst-polluted places on Earth serve as unequivocal proof (Cazzolla Gatti *et al.*, 2023).

4.1. Cardiovascular disease related to environmental pollution

Globally, nearly 20% of cardiovascular disease deaths were attributable to air pollution. Further, air pollution was the 4th highest ranking risk factor for mortality, with more attributable deaths than high LDL cholesterol, high body-mass index, physical inactivity, or alcohol use (Brauer *et al.*, 2021).

Hypertension is an increasingly important global health challenge. Besides lifestyle risk factors, environmental factors, such as air pollution, may influence hypertension risk. More exposure to traffic may increase people's risk of hypertension (Jing Xu, Niehoff, White, *et al.*, 2022).

More importantly, the current obesity epidemic is mainly driven by environmental factors. Recently, air pollution has attracted more attention regarding health outcomes, especially obesity (Shi *et al.*, 2022).

4.2. Liver disease related to environmental pollution

Metabolic dysfunction-associated fatty liver disease (MAFLD) related to air pollution (AP) could be mediated by a variety of AP components leading to both systemic inflammatory effects and liver-targeted toxicity elicited by specific pollutants (Colombo & Barouki, 2022).

Numerous scientific studies have identified that environmental contaminants can induce steatosis and steatohepatitis in organisms. Therefore, environmental pollutants need to be considered as a class of risk factors for nonalcoholic fatty liver disease (NAFLD) (Zheng *et al.*, 2021). NAFLD is a clinicopathological disease caused by some specific factors (non-alcoholic), which is characterized by obvious lipid deposition of liver parenchymal

hepatocytes and persistent abnormal liver enzymes. If symptoms persist, it would develop into cirrhosis, or even hepatocellular carcinoma (Yao Zhang *et al.*, 2021).

4.3. Kidney disease related to environmental pollution

Air pollutants exposure is emerging as a novel risk factor for chronic kidney disease (CKD) Mounting experiment studies have suggested that inhaled pollutants might result in kidney injury through vascular inflammation, oxidative stress, and atherosclerosis (Jianing Wang *et al.*, 2022).

Chronic kidney disease (CKD) is a common term for heterogeneous conditions that affect the function and structure of the kidney. The CKD definition is centered on the presence of Kidney damage or decreased kidney function (i.e. glomerular filtration rate [GFR]<60 mL/min per 1·73 m^2) for > 3 months (Valdivia-Rivera *et al.*, 2018).

4.4. Hematological disease related to environmental pollution

Changes in structure and irregularities in erythrocyte are thought to be key indicators of oxidative stress (Kole *et al.*, 2022). Occupational and acute exposures to selected volatile organic compounds (VOCs), benzene, toluene, ethyl-benzene, xylenes, and styrene, have been associated with changes in hematological profiles (Cakmak *et al.*, 2020).

4.5. Osteoporosis disease related to environmental pollution

Osteoporosis and its consequence of fragility are increasingly recognized as a major public health burden in contemporary populations. Important aspect of osteoporosis is that patients with a pre-existing fracture are at increased risk of refractures and mortality (Ho-Pham & Nguyen, 2017). Vitamin D deficiency (VDD) is common and implicated in many diseases among them osteoporosis. Some population-based studies indicated that air pollution contributed to VDD in both adults and children (Haofan Zhang *et al.*, 2022).

4.6. Neurological disease related to environmental pollution

New research shows exposure to high levels of air pollutants at critical points in our lives, particularly in early life, is detrimental to brain health across the life course and increases the risk of dementia syndrome and related disorders. Examples include global cognition and neurodegenerative disorders, learning in childhood and stroke-related brain damage, and reduction in white matter and neuroinflammation (Castellani *et al.*, 2022). Air pollution is

considered a potent risk factor for Alzheimer's disease (AD)-relevant neuropathology (Dutta *et al.*, 2022).

4.7. Endocrine disruption related to environmental pollution

The development of Endocrine-related noncommunicable disease is thought to primarily depends on the complex interaction of human genome and exposome which is a relatively new paradigm in environmental science research that includes all lifelong environmental factors contributing to disease development, among these factors, exposure to Endocrine disrupting compounds (EDCs), including toxic metals, have a significant impact on human endocrine health (Javorac *et al.*, 2023).

EDCs are emerging environmental micropollutants that cause serious environmental pollution and consequently affect wildlife and public health concerns due to their hormone like behavior (Werkneh *et al.*, 2022). EDCs interfere with hormone synthesis, metabolism, excretion and functions in exposed organisms, these interactions eventually produce deleterious consequences on reproduction**,** development, metabolism, physiology, neurobehavioral disorders, and other health and disease effects (Ibor *et al.*, 2023).

Endocrine disruptors (EDs) are known to provoke disarray of hormonally controlled physiological parameters (such as mineral and osmotic balances) or functions (such as growth**,** development and reproduction). There are different mechanisms through which EDs can act, either by binding to hormone receptor or altering the levels of endogenous hormones, or by modulating gene networks. This leads to stimulation/inhibition of downstream cellular and molecular pathways, thereby, affecting the normal parameters and functions (Sonika Kar *et al.*, 2021).

5. Fluoride as environmental pollutant

Fluorine is the most reactive element in the halogen group, and occurs naturally as fluoride. Fluoride is released from agriculture and industrial processes, and travels in the atmosphere and water. Fluoride has become one of the most serious environmental pollutants, and poses a risk to human health worldwide (Junlin Yang *et al.*, 2022).

Fluoride (F) exists in nature in combination with the other elements and these fluorinated compounds are the constituents of minerals in soils and rocks, which are the natural contributors of fluoride (Pal *et al.*, 2022). It can enter the human body through food,

water, tea and other ways causing varying degrees of damage to many tissue systems, such as the nervous system, immune system, reproductive system, etc. Drinking water containing fluoride is the main way in which the fluoride recognized worldwide as the most serious inorganic contaminants (Hui Zhao *et al.*, 2022).

Chapter II

Fluoride & oxidative stress

I. Fluoride

1. Definition

Fluorine is a natural component of the biosphere. It is the thirteenth (13^{th}) most abundant element in the earth's crust. Due to the small radius of the fluorine atom, its effective surface charge is the highest among all elements. As a consequence, fluorine is the most electronegative and reactive of all elements and hardly occurs in nature in its elemental form. Instead, it is found most frequently as inorganic fluoride that is widely distributed (Afonso *et al.*, 2011)**.**

2. Chemical and physical proprieties

Halogen elements: fluorine (F), chlorine (Cl), bromine (Br), iodine (I), and astatine (At) constitute Group VIIA of the periodic table (Han *et al.*, 2023). The high electronegativity of fluorine makes it the most reactive element and enables it to form compounds with almost all elements except inert gases. However, the electronegativity of halogen decreases in the order of F (3.9) > Cl (3.0) > Br (2.8) > I (2.5) (El Zokm *et al.*, 2022).

The chemical and physical properties of elements are determined by their electronic configurations. Certain electronic configurations are especially stable with respect to their energies (Halka, & Nordstrom, 2010).

Fluorine is the most reactive element in the family because the very small size of fluorine atoms causes a fluorine nucleus to exert an extremely strong force of attraction on an electron of a nearby atom (This strong force of attraction is the reason; fluorine is the most electronegative element in the periodic table). The result is that a fluorine atom in contact with atoms of almost any other element will react so as to pull an electron either completely away, or at least partially away, from the other atom, resulting in either a fluorine ion or in a polar bond between the atoms. Doing so gives fluorine an effective electron configuration of $1s^2 2s^2 2p^6$, which is the same stable configuration that a neon atom has.

Table 01: Some physical Fluorine proprieties (Halka, & Nordstrom, 2010; Pauling, 1970).

Property	Atomic number Z	Symbol	Atomic Weight A (g/mol)	Color and form	Melting point (˚C)	Boiling point (˚C)	Electronic configuration
Fluorine	9	F	19	Pale yellow gas	-223	-187	$1n^2 2s^2 2p^5$

3. Application of fluoride

Fluorine is a ubiquitous element worldwide and has been detected in human urine and blood samples. Because fluoride has been widely applied in industrial processes, including fossil combustion, fertilizer production, electroplating, glass and ceramic production, high levels of fluoride are detected in soil, water and the atmosphere. Fluoride pollution inevitably results in its transfer into plants, animals, and the human body (Hui Wang *et al.*, 2022).

Several different fluoride products are currently available on the market. There is strong evidence that daily use of fluoride-containing toothpaste prevents caries disease in permanent dentition (Enerbäck *et al.*, 2022).

Oral care and food products all contribute to the total daily exposure to fluoride but the primary source of fluoride exposure, due to high consumption, is through drinking water, naturally occurring, or added (Dec *et al.*, 2018),(Radovanović *et al.*, 2020)

Several foods, such as rice, bread, beans, and food prepared using fluoride-containing water are also potential sources of fluoride (Sawangjang & Takizawa, 2023).

4. Metabolism of fluoride

According to (Leite, 2015) Fluoride metabolism includes absorption, distribution and excretion, where each step depends on the pH.

By definition, at pH 3.4, 50% of fluoride is in the undissociated form (HF), while the remaining 50% is in the ionic form (F-) As pH decreases from 3.4, the concentration of HF increases, and as pH increases, the concentration of F– increases. This means that fluoride permeates cell membranes as HF, in response to a pH gradient between adjacent body-fluid compartments, going from the more acidic to the more alkaline compartment.

After ingestion, plasma fluoride reaches a peak within 20–60 min, due to its pH-dependent gastric absorption, followed by a rapid decline as a result of both uptake in calcified tissues and urine excretion. The small intestine also contributes to fluoride absorption in a pH-independent mechanism. Non absorbed fluoride is excreted in feces.

From plasma, fluoride is distributed to both hard and soft tissues followed by its renal excretion.

A minor portion of absorbed fluoride is found in soft tissues through a steady-state distribution between extracellular and intracellular fluids. However, about 50% of the absorbed fluoride is incorporated in calcified tissues, mainly in bone, where 99% of the fluoride content in the body is found.

Its incorporation in calcified tissue is not irreversible, from where it can be released back to plasma compartment. The fluoride excreted in urine is mostly a result of the total absorbed amount subtracted by the incorporated quantity in hard tissues. Each step related to the fluoride metabolism will be described in sequence.

5. Fluoride toxicity and oxidative stress

Fluoride has a systemic effect on the body, involving a variety of organs and tissues, but there is still a lack of systematic and comprehensive studies on the observation of metabolite changes from the perspective of metabolomics. Metabolomics are not only sensitive to organ-specific toxicity but also provide information about the mechanisms involved in organs and tissues (Shiyuan Zhao *et al.*, 2022). Fluoride in blood exists in two forms, organic and inorganic fluorides. The inorganic F is only important from a toxicological point of view, as it is the only active form (Ranjan & Ranjan, 2015).

5.1. Acute toxicity

Common signs and symptoms of acute fluoride toxicity include nausea, vomiting, and a drop in blood calcium, causing local or general signs of muscle tetany. Signs also include abdominal cramping and pain and increasing hypocalcaemia and hyperkalemia, leading coma, convulsions, and cardiac arrhythmias. Generally, death from excessive fluoride ingestion will occur within 4 hours; if the individual survives for 24 hours, the prognosis is guarded to good. The toxic effects of fluoride are mainly due to 4 different actions (Martı´nez-Mier, 2012):

- Burning the tissues, it forms hydrofluoric acid when it comes in contact with moisture, which has a corrosive action.
- Impeding nerve function, through its affinity for calcium, which is needed for nerve function.
- Cellular poisoning, through the inhibition of enzyme systems.
- Impeding cardiac function, by causing an electrolyte imbalance leading to hyperkalemia.

5.2. Chronic and sub-chronic toxicity

Fluoride, a necessary mineral element for our health, could prevent dental cavities and be beneficial for the development of bone in appropriate dosage. However, the excessive consumption of fluoride could lead to the detrimental effect on human body, such as endemic dental, skeletal fluorosis, oxidative stress, and neurotoxicity (Guijie Chen *et al.*, 2022).

Prolonged exposure to high fluoride results in damage to multiple soft tissues of the body inducing morphological changes in many organs, leading to an impairment of their function (C. X. Wu *et al.*, 2022),(Stawiarska-Pieta *et al.*, 2012). Kidneys, followed by the liver, accumulate more fluoride than any other organ system in the body (Malin *et al.*, 2019).

The fluoride exposure pathways are through (Sawangjang & Takizawa, 2023):

- Inhalation
- dermal contact
- through ingestion of food
- beverages
- dental products
- drinking water

Reviewed the literature on cellular toxicity of fluoride and reported many toxicity phenotypes including oxidative stress, organelle damage, and apoptosis in single cells, and skeletal and soft tissue damages in multicellular organisms. The mechanisms of fluoride toxicity can be attributed to inhibition of proteins, organelle disruption, altered pH and electrolyte imbalance (Sawangjang & Takizawa, 2023).

Oxidative stress is the main proposed mechanism of fluoride toxicity; which confirmed by many studies (Djouadi & Derouiche, 2022a),(Djouadi & Derouiche, 2022b).

F^- is known to induce free radical generation and consequently results in oxidative stress due to its high electronegativity. F^- interacts with proton donors such as biomolecules, enzymes, antioxidants etc. by forming H-bonds and also decrease the levels of antioxidant markers as they continuously neutralize the reactive oxygen species generated on F^- exposure (Shivarajashanker & Shivashankara, 2012; Chinoy, 2003).

The literature suggests that fluoride toxicity induces oxidative stress which has immense effect on the levels of neurotransmitter's and also on pro and anti-oxidative markers such as

glutathione, Superoxide dismutase (SOD), Lipid peroxidation (LPO), vitamins (C, D and E) (Banala & Karnati, 2015).

II. Oxydative stress

1. Reactive Oxygen Species

Reactive Oxygen Species (ROS) are charged, uncharged or free radical oxidants mainly produced by oxidative metabolism. ROS are involved in physiological and pathological processes. At low concentrations they act as signaling molecules, through oxidative post-translational modifications they regulate cell proliferation, hypoxic environment adaptation and cell shape; they play a role in vascular tone and angiogenesis regulation. At high levels, ROS are harmful molecules which oxidize macromolecules and provoke structural cell damage which can lead to cell apoptosis. This condition is called oxidative stress (OS) (Soragni *et al.*, 2022).

2. Oxidative stress

In the organism, the delicate balance between the production of ROS and the antioxidant mechanism of cells makes the body function normally. Once this balance is broken, the harmful accumulation of ROS will put the body in a state of oxidative stress (OS), which in turn, leads to a variety of adverse effects, including cell and tissue damage, DNA, lipid and protein peroxidation and modification as well as neutrophil extracellular bactericidal network formation. These changes may account for cancer, neurodegenerative diseases, diabetes, and many other serious diseases (Hu *et al.*, 2022)**.**

3. Lipid peroxidation

Oxidative stress reflects an imbalance between systemic manifestation of oxidants and antioxidants in favor of the former, leading to a disturbance of redox homeostasis and molecular damage. One of the major targets of oxidant injury is lipids, which undergo peroxidation. Of concern, over the past few decades, numerous evidence has suggested that lipid peroxidation may play critical roles in several pathologies such as cancer, neurodegenerative diseases, cardiometabolic diseases, and muscular disorders (Yingya Zhao *et al.*, 2022)**.**

Lipid peroxidation (lipid oxidation) is a phenomenon that takes place in several stages: initiation, propagation and termination (Bauchard & Picard, 2010).

4. Free radicals

A free radical is a chemical species (atom or molecule) which has a single (or unpaired) electron on its external electronic orbitals which gives them a very great instability. Free radicals capable of reacting with different molecules, in particular during chain reactions, the best-known example of which is the peroxidation of lipids (Christophe & Christophe, 2011), most free radicals are produced by the mitochondria (Sylvia, 2010).

Oxygen is a low-reactive free radical, usually present as a dioxygen. Under physiological conditions 2% to 5% of the oxygen used by the mitochondria is partially reduced by electrons that escape from the respiratory chain carriers, thereby forming more reactive derivatives called reactive oxygen species (ROS) (Ichai *et al*., 2011).

These molecules are a family of chemical entities regrouping oxygenated free radicals (chemical species possessing an unpaired electron) such as the anion superoxide ($O•_2^-$), The hydroxyl radical (OH•), Nitrogen monoxide (NO•) … and the oxygen derivatives known as active oxygen species (not possessing a single electron) they are not free radicals but they are also reactive and can be precursors of radicals (peroxide anion (O_2^{-2}), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) (Djouadi, 2015).

5. Anti-oxidant systems

We pay attention to two types of antioxidant system : the enzymatic antioxidant system and the nonenzymatic antioxidant system. Antioxidant enzymes include glutathione peroxidase (GR), catalase (CAT) and superoxide dismutase (SOD); while the nonenzymatic antioxidant system are the dietary and endogenous antioxidant chemicals, which include vitamin A, carotenoids, vitamin C, vitamin E, glutathione (GSH), alpha-lipoic acid, coenzyme Q_{10}**,** L-carnitine and polyphenolic compounds derived from plants, fruits and vegetables (Kedar, 2015; Xuejun *et al*., 2015).

The most abundant non-protein thiol is glutathione in its reduced (GSH) and oxidized (GSSG) form. GSH is a tripeptide present in millimolar concentrations within cells, which acts directly as an antioxidant or as a substrate in enzymatic antioxidant defense mechanisms, involving mainly GSH-peroxidases. Another important non-protein thiol is cysteine (CSH), a precursor of GSH and the amino acid that supplies the protein thiol groups (PS) (Grintzalis *et al.*, 2022).

Chapter III

Oxidative stress

&

biochemical disorders

1. Oxidative stress and biochemical disorders related Metabolic disease

Blood biochemical parameters are decisive tool for predicting the metabolic diseases (Nasr *et al.*, 2022). ROS represent an important topic and their role in the pathogenesis of certain disorders was comprehensively studied (Rotariu *et al.*, 2022). Therefore, both oxidative stress and biochemical disorders observed in metabolic diseases

2. Metabolic disorders

Metabolic disorders are among the most common and challenging diseases in modern medicine. Obesity, dyslipidemia, diabetes mellitus, nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steato-hepatitis (NASH), osteoporosis and metabolic syndrome among others, have emerged as a major global health problem and are the most widespread pathologies characterized by metabolic failure (Tabatabaei-Malazy *et al.*, 2015), (Xu Wang *et al.*, 2022).

Glucolipid metabolic disorders (GLMD) are a series of diseases characterized with glucose and lipid metabolic disorders, which are influenced by multiple elements including environmental, genetic and mental factors. The manifestations of GLMD are diverse including diabetes, hyperlipidemia, hypertension, obesity, non-alcoholic fatty liver disease (NAFLD) and atherosclerosis (Xiao *et al.*, 2022).

2.1. Obesity

Obesity is a well-documented risk factor for several chronic diseases, including type 2diabetes, hypertension, cardiovascular diseases (CVDs), and certain cancers (LIU *et al.*, 2022). The prevalence of obesity continues to rise, suggesting a need for new treatment options. Difficulties in treating obesity are directly related to the complex causes, which are biomedical, environmental, and socio-cultural in nature (Kleiboeker & Lodhi, 2022).

2.2. Diabetes

Type 2 diabetes mellitus (T2DM) accounts for 90% of diabetes cases worldwide and is considered a major public health challenge. T2DM and prediabetes can cause multiple damages to body systems, with undiagnosed and untreated patients having a greater risk of complications than those who receive treatment leading to morbidity and mortality (Kontochristopoulou *et al.*, 2022).

2.3. Dyslipidemia

Dyslipidemia is a common metabolic disorder that associated with changes in the plasma lipid profile and manifests as an elevation of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), or triglycerides (TGs) or a reduction in high-density lipoprotein cholesterol (HDL-C) in blood, leading to major clinical conditions, such as cardiovascular disease (CVD), and the global burden has significantly increased over the past 30 year (Zhou *et al.*, 2022), (Pengpid & Peltzer, 2022). Dyslipidemia refers to either lipoprotein overproduction or deficiency, which is a consequence of abnormal lipoprotein metabolism. Dyslipidemia could be Primary (genetic defect in the lipid metabolism that causes abnormal lipid levels) and Secondary (caused due to modifiable lifestyle and environmental factors, diseases, and medications) (Misra *et al.*, 2022).

Dyslipidemia is one of the main risk factors of cardiovascular disease (Jinqiu Yao *et al.*, 2022). Therefore it is important for patients to manage dyslipidemia, which is also a major risk factor for arteriosclerotic disease (Katsuyama *et al.*, 2022).

2.4. Atherosclerosis

Atherosclerosis is a complex pathological process. Endothelial dysfunction, inflammation, and thrombogenesis all play a role in this process. Endothelial dysfunction is one of the first steps within the pathogenesis of atherosclerosis and can be caused by known risk factors for acute coronary syndrome and cardiovascular disorder (Izoe *et al.*, 2022).

2.5. Osteoporosis

Osteoporosis is a common metabolic disease of bone tissue. According to statistics, approximately 200 million people worldwide suffer from osteoporosis, mainly including postmenopausal women, diabetic patients and obese people (Keda Yang *et al.*, 2022). In addition to some recognized risk factors for osteoporosis, such as ageing, body mass index (BMI), current smoking, family history of fragility fractures, excessive alcohol use, and chronic corticosteroid use, environmental factors, especially air pollution, are possibly linked to the incidence of osteoporosis (Cheng Xu *et al.*, 2022).

2.6. Metabolic syndrome

Metabolic syndrome (MS) is a multifaceted disease condition, which includes cardiac abnormalities as cardiometabolic disease conditions, abnormalities in insulin metabolism

and obesity. The MS is also known as insulin syndrome, as it has been known to prone to insulin resistance. Also, it includes an increase in abdominal fats, hyperglycemia, problems in lipid metabolism and many more (Patil *et al.*, 2020). MS can be defined as a clusters of interconnected biochemical, physiological, metabolic, and clinical factors including central obesity, visceral adiposity, impaired glucose tolerance, hyperglycemia, insulin resistance (IR), high triglyceride, atherogenic dyslipidemia, low high-density lipoprotein (HDL) cholesterol levels, and/or hypertension, which collectively increase the risk of type 2 diabetes mellitus (T2DM), atherosclerotic cardiovascular disease (ASCVD), as well as vascular and neurological consequences like cerebrovascular accident. Insulin resistance and obesity appear to be the most relevant factors in the MetS pathogenesis, in addition to the proinflammatory state that likely participate to the condition's development (Yasmina Bourebaba *et al.*, 2022a).

The two major components of metabolic syndrome are weight gain and fat accumulation at intra-abdominal sites with abnormal fat in the liver, pancreas, and heart. Psychosocial stress and our lifestyle also contribute to metabolic syndrome (Patil *et al.*, 2020).

3. Metabolic disease pathophysiology

A block in a metabolic pathway is the biochemical hallmark of inherited metabolic disorders (IMD), and it is generally caused by a defect in either an enzyme or transport protein. The pathophysiology of metabolic diseases is related to excessive accumulation of toxic metabolites and/or deficiency of essential product (Altassan *et al.*, 2022). Metaflammation, a chronic low-grade inflammatory state in metabolic tissues, is a hallmark of metabolic disease (Hotamisligil, 2017). Therefore, metabolic disorders are caused by abnormal metabolic processes

4. Metabolic disorders causes

The development of metabolic disorders occurs when physiological metabolic pathways are disrupted, due to inadequate diet, sedentary work, and lack of physical activity (Yasmina Bourebaba *et al.*, 2022a).

Inherited metabolic disorders (IMDs), also known as inborn errors of metabolism, are genetic conditions that affect the metabolism of biological molecules such as protein, carbohydrates, and fat. These pathogenic mutations reduce enzyme activity, leading to

accumulation of toxic compounds, reduced reaction product, or synthesis or catabolism of complex molecules (Hansen *et al.*, 2022).

Infection is considered a major triggering factor for metabolic decompensation in inherited metabolic disorders (IMD), especially in patients with intoxication disorders and energy metabolism disorders. This is attributed to the increased metabolic demand during infection leading to catabolic state in these patients overwhelming the block in the metabolic pathway (Altassan *et al.*, 2022).

Being overweight during childhood is a risk factor related to several comorbidities, developed during the early stages of life and/or adult life; among which metabolic disorders (MetDis) stand out, mainly hyperglycemia, dyslipidemia, and high blood pressure. Also, an unhealthy diet promotes the modification of the gut microbiota, conditioning the development of metabolic disorders (Bahena-Román *et al.*, 2022).

Centuries of research into the pathobiology of CVDs and metabolic diseases have gracefully led to the discoveries of diverse altered physiological processes, transports, and metabolic systems essential to host health. Recurrent sterile inflammation (chronic production of pro-inflammatory cytokines) triggered by metabolic noxiae is a classic example of pathophysiological conditions discovered to occur in patients with heart, vascular, and metabolic diseases (Oduro *et al.*, 2022).

5. Metabolic disorders complications in biochemical levels

According to (Kleiboeker & Lodhi, 2022), improving our biochemical understanding of obesity pathogenesis could help improve obesity treatment and aid in prevention of associated diseases. We suggest that this is for all metabolic disorders.

When an Inherited Metabolic Disorders (IMDs) is suspected without any obvious clinical diagnosis, a first-line biochemical screening is generally proposed (lactate and pyruvate levels, plasma amino acids, urine organic acids, acyl-carnitines, ketone bodies and very long chain fatty acids). Usually, these initial results drive specific secondary investigations, mainly based on enzymatic studies and/or targeted genetic analyses. This strategy offers an overall diagnostic yield around 50%, when clinical features are highly suggestive of IMDs (i.e. encephalopathy, coma, hypotonia or organomegaly and are associated with biological marker elevation (Delanne *et al.*, 2021).

5.1. Metabolic associated liver disease

Metabolic associated fatty liver disease (MAFLD), formerly named nonalcoholic fatty liver disease (NAFLD), is the most common chronic liver disease. MAFLD is associated with increased risk of cirrhosis, liver cancer, type 2 diabetes (T2DM), and atherosclerosis (Ye *et al.*, 2022).

The term metabolic (dysfunction)-associated fatty liver disease (MAFLD) has been advocated as an overarching definition for fatty liver diseases associated with metabolic dysregulation to more accurately reflect the underlying pathogenesis of the disease (VoPham *et al.*, 2022).

5.2. Metabolic associated kidney disease

Lysinuric protein intolerance (LPI) is a rare metabolic disease, impaired absorption in the intestine and decreased reabsorption in the tubules of the kidney of cationic amino acids causes low plasma levels of ornithine, lysine and arginine. Symptoms most often present in childhood and mimic primary urea cycle defects consisting of protein intolerance, delayed growth, osteoporosis, hepatosplenomegaly, muscle hypotonia, macrophage activation, pulmonary fibrosis and chronic hemophagocytic lymphohistiocytosis (IJzermans *et al.*, 2022).

Metabolic acidosis is common in later-stage CKD because of the metabolism of dietary protein, generation of nonvolatile acids, and impaired net acid excretion. The prevalence of metabolic acidosis, defined by a reduced serum bicarbonate level, increases as glomerular filtration rate (GFR) decreases. Metabolic acidosis is postulated to be a CKD-specific cardiovascular risk factor because of its negative effects on the cardiovascular system, including inflammation and activation of the renin-angiotensin-aldosterone system (RAAS) (Collister *et al.*, 2021).

5.3. Metabolic associated cardiovascular disease

Cardiovascular disease (CVD), including coronary artery disease (CAD) and ischemic stroke, is a major public health issue globally. CVD is a consequence of multiple etiologies and a series of modifiable factors (metabolic risk factors and lifestyle behaviors) are implicated in the development of CVD, such as hypertension, diabetes, hyperlipidemia, obesity, smoking, physical inactivity and unhealthy die (Jia *et al.*, 2022).

The link between obesity and metabolic abnormalities and chronic diseases is well understood. Obesity-related metabolic disorders, including hyperglycaemia, dyslipidemia, or hypertension, contribute to an elevated risk of cardiovascular diseases (CVDs) (Abiri *et al.*, 2022). The risk of developing CVD increases two-fold when dyslipidemia sets in (Thongtang *et al.*, 2022).

5.4. Metabolic associated brain disease

Metabolic disease is one of the leading causes of the burden of Bipolar disorder (BD); BD is a complex group of severe and chronic mental disorder which is mainly manifested by alternating depressive episodes and manic episode (Sujuan Li *et al.*, 2022).

A variety of metabolic disorders have been associated with cognitive dysfunction, including diabetes, diabetic ketoacidosis (DKA), and hyperlipidemia. Considering that a series of metabolic changes occurs after traumatic brain injury (TBI), including glucose, lipid, and acid–base balance, by reviewing how metabolic diseases cause cognitive impairment it may be possible to better understand and extend how central metabolic changes affect cognitive function after TBI (Lai *et al.*, 2022).

5.5. Metabolic associated bone disease

Bipolar disorder and major depressive disorder are related to bone metabolic disease. Abnormal bone metabolism, including osteoporosis and low bone mass, is one of the complications of BD, second only to obesity and cardiovascular disease (Sujuan Li *et al.*, 2022).

5.6. Metabolic associated hematological disease

It has been observed that the hematological parameters play a crucial role in the development of macro and micro-vascular complications and are the reasons behind the increased morbidity and mortality in type 2 DM patients (Sampathkumar *et al.*, 2021).

6. Dysthyroidism status involvement in metabolic diseases

Thyroid gland is an essential component of endocrine system which is one of the pivotal players of regulating the homeostasis by providing chemical signals between a diversity of cells (Sadoughi *et al.*, 2022). Thyroid hormone plays an important role in regulating human body metabolism (Wen Zhang *et al.*, 2022). Untreated thyroid disease can produce severe consequences like elevated cholesterol levels, hypertension, cardiovascular complications,

decreased fertility, and depression. The thyroid hormones regulate critical biochemical functions in the developing brain (Das *et al.*, 2022).

Subclinical thyroid dysfunction is associated with various adverse cardiovascular outcomes, including atherosclerotic disease, altered total and low-density-lipoprotein cholesterol (LDL-C), and atrial fibrillation (Lai *et al.*, 2011).

Thyroid hormones (THs) play an important role in liver lipid metabolism by regulating de novo lipogenesis (DNL), fatty acid oxidation, and cholesterol and carbohydrate metabolism (Ruifang Li *et al.*, 2022).

6.1. Hypothyroidism role in metabolic diseases

Hypothyroidism is the disease state in which there is a deficiency of thyroid hormone or a failure of its action. This leads to a variety of non-specific symptoms and signs such as tiredness, dyspnoea, constipation, weight gain, hair loss, dry skin, intolerance to cold and hoarseness of voice. As a result, a high index of suspicion is required to make the diagnosis, which is confirmed on biochemical tests.

Thyroid hormones play a role in the activity of important enzymes in the cholesterol synthesis and metabolism pathway. One of the first enzymes in the cholesterol synthesis pathway, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, is induced by thyroid hormones. Thus, in the setting of hypothyroidism, the synthesis of cholesterol is impaired (Mansfield *et al.*, 2022).

Hypothyroidism-induced non-alcoholic fatty liver disease (NAFLD) has been attributed to decreased lipid utilization, including reduced β-oxidation of free fatty acids (FFAs) and the clearance of TGs, with a consequent increase in the accumulation of triglycerides (TGs) and low-density lipoprotein within hepatocytes (Ruifang Li *et al.*, 2022).

6.2. Hyperthyroidism role in metabolic diseases

Various studies, including those performed on HepG2 models, established the potential factors influencing the SHBG levels. Furthermore, circulating levels of SHBG are affected by different hormonal factors - for example, thyroid hormones influence plasma levels of SHBG by altering its production. However, SHBG plasma levels are high in patients with hyperthyroidism and this can be explained by the poor functioning of thyroid hormones in the liver. Indeed, HepG2 cells express iodothyronine deiodinase type I, which converts the

prohormone thyroxine (T4) into active receptor triiodothyronine (T3) in the healthy liver; however, the key enzyme responsible for deactivating thyroid hormones in the liver and other tissues, iodothyronine deiodinase type III, is lacking. On the other hand, HepG2 cells express glucuronidases responsible for the metabolic clearance of thyroid hormones in the liver. The human SHBG gene is also expressed in HepG2 cells, so its production is enhanced by thyroid hormone treatments which increase the levels of SHBG mRNA. Other factors have also been demonstrated to influence the bioavailability of SHBG, including dietary lipids; in fact, it was noted that increased cholesterol levels correspond with decreasing SHBG levels (Nabila Bourebaba *et al.*, 2022).

Sex hormone binding globulin (SHBG) is a hepatokine that binds to circulating steroid hormones (testosterone, oestradiol) to regulate their concentration in the bloodstream. Recently SHBG was recognized as an essential biomarker for metabolic syndrome (MetS) and hepatic steatosis development. At the hepatic level, the production of SHBG is mainly regulated by sex steroids and thyroxine (Nabila Bourebaba *et al.*, 2022).

7. Phytotherapy & nanotherapy

Therapeutic agents managing endocrine disorders are overlooked; they are vital elements within the protective measures for patients suffering from diabetes mellitus, thyroid disorders, infertility, and adrenal gland diseases. In addition to the conventional approaches in this field, medicinal plants acquire exceptional consideration from professionals to develop potent phytomedicine for optimizing curative outcomes. Therefore, the rising interests of researchers in nutrition and natural products are fast rising to the frontier of research priorities.

The most effective medicinal plants used for the management of common endocrine diseases taking into account the green-formulated nanoparticles, which are more effective than the crude extracts. Their higher efficacy can be attributed to their higher surface area and better solubility, which in turn enhance the therapeutic activity and thus overcome the limitations of insolubility**,** low bioavailability, and incapability to reach their sites of action (Al Zarzour *et al.*, 2022).

Chapter IV

Phytotherapy

& *Spinacia oleraceae*

1. Spinach

Spinach (*Spinacia oleracea*) is one of the most widely grown vegetables in temperate climates around the world (Navazio, 2012). It is an edible flowering plant (Amaranthaceae family) native to central and south-western of Asia, now cultivated all over the world (Vázquez *et al.*, 2013).

Different varieties of *Spinacia oleracea* have been selected for their resistance to bolting and for dark leaves that are rich in vitamins. Depending on climate and season, either cold tolerant or long day tolerant varieties can be grown (Ashworth & Whealy, 2002). Among the best varieties, 'Melody' and 'Tyee' are both cold-tolerant, with lightly savoyed leaves. 'Bloomsdale Savoy' is a bolt-resistant variety whose deeply savoyed leaves have good flavor. 'Indian Summer' and 'Steadfast' are bolt-resistant with flat leaves. 'Giant Spinach of Viroflay' (aka 'Monstrieux de Viroflay') and 'Giant Nobel' both have flat, tender leaves that are very large (Lively, 2011).

2. Spinach profile

2.1. Scientific Classification (Rao *et al*., 2015)

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Caryophyllidae

Order: Caryophyllales

Family: Amaranthaceae

Genus: Spinacia

Species: *Spinacia oleracea*

Figure 01: *Spinacia oleracea*

2.2. Chemical compounds

Spinach is an excellent source of dietary vitamins and minerals (Xu *et al.*, 2016). Besides carotenoids (mainly lutein and carotene), other bioactive substances identified in spinach are phenolic compounds, such as flavonoids and phenolic acids (p-cumaric, gallic and ferulic acids) and fatty acid derivative compounds, such as glycoglycerol lipids and lipoic acid (Vázquez *et al.*, 2013).

Spinach is low in calories and a good source of vitamin C, vitamin A and minerals especially iron. Vitamin C is one of the most important nutritional quality factors in many horticultural crops and has many biological activities in the human body. Vitamin C is the most important vitamin in fruits and vegetables for human nutrition and more than 90% of the vitamin C in human diets is supplied by fruits and vegetables. Today, ascorbic acid is a well-known antioxidant and enzyme cofactor with many roles in human health (Citak & Sonmez, 2010).

Five soluble sugars, namely sucrose, fructose, glucose, maltose and raffinose were identified and their concentrations were assessed in this study. The most abundant sugar in spinach was found to be sucrose. Also free amino acids (34 compounds, essential and non-essential) (Yoon *et al.*, 2017).

Depending on (Gupta & Igamberdiev, 2015) the spinach contains the follow anti-oxidative enzymatic system: superoxide dismutase SOD, ascorbate peroxidase (APx), glutathione reductase (GR), Catalase (CAT), glutathione peroxidase (GPx), Ascorbate, glutathione (GSH).

Green leafy vegetables such as spinach, are mainly composed of monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) rich in Alpha linolenic acid (ALA). ALA is an essential fatty acid that must be consumed through the diet. There have been many epidemiological and clinical studies on the cardiovascular-protective effects of ALA. ALA is a precursor of eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids. Both n-3 EPA and DHA have sometimes been regarded as active forms of ALA in biological systems.

EPA and DHA have been shown to cause significant biochemical and physiological changes in the body that often result in a positive influence on human nutrition and health. EPA and DHA consumption have benefits of reducing the risk of cardiovascular disease,

probably due to regulation of membrane structure, lipid metabolism, blood clotting, blood pressure, and inflammation. Thus, the bioconversion of ALA to EPA and DHA is important for understanding the biological importance of ALA (Kuroe *et al.*, 2016).

Omega-3 fatty acids are one of the main components of the functional food ingredients, because they play a positive role against many degenerative and inflammatory complications such as heart disease, stroke, rheumatoid arthritis, asthma, and some types of cancer, and contribute towards development and functioning in infants' brain and retina (Belayneh *et al.*, 2015).

Structurally poly unsutured fatty acids (PUFA) are made up of long hydrocarbon chains which terminate with a hydroxyl group. Depending on the position of their first double bond, they are classified as omega-3 or omega-6 fatty acids. PUFA play a variety of important functional roles in organisms, including as structural components of the phospholipid bilayer in cell membranes. The fatty acid composition of this bilayer is important for the regulation of the membrane fluidity and the dietary intake can strongly influence the fatty acid composition of cell membranes. The deficiency of omega-3 fatty acids of the western diet has been identified as one of the main causes of numerous chronic dis-eases (Schmid *et al.*, 2016).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) show the most powerful anti-inflammatory effects, suggesting it could be used for treatment of many chronic diseases, including coronary heart disease, diabetes, arthritis, cancer, osteoporosis, mental health, dry eye disease and age-related macular degeneration (Sánchez-Camargo *et al.*, 2012).

Spinach is known to contain significant amounts of oxalates (Wen *et al.*, 2017) and it is contained trace elements such as selenium, cooper, iron and zinc (Somer *et al.*, 2016).

3. Zinc's biological role

3.1. Zn role in Plants

The Zn plays very important role in plant metabolism, stabilization of ribosomal fractions and synthesis of cytochrome. Plant enzymes activated by Zn are involved in metabolism, preservation of the integrity of cellular membranes, protein synthesis and pollen formation. The regulation and maintenance of the gene expression essential for the tolerance of environmental stresses in plants are Zn dependent (Cakmak, 2000).

3.2. Zinc biological importance in animal and human

Zinc has been revealed to be essential for microorganisms, plants and animals. More than 30% world's population suffers from Zinc deficiency. Zinc plays a part in the basic roles of cellular functions in all living organisms. The optimum dietary intake for human adults is 15 mg Zn per day. Zinc acts as a catalytic or structural component in various body enzymes (Hafeez *et al*., 2013). Inadequate intake and inappropriate absorption of Zn in the body may cause deficiency of Zn. in Zn deficiency; the human body will suffer from hair and memory damage, skin problems and feebleness in body muscles (Morley, 2004).

3.3. Zn & hormonal action

The biology of zinc is related extensively to hormone metabolism. For example, the zinc finger motifs of regulatory proteins essential for hormonal signals to regulate gene transcription. It has been reported that Zinc is essential in the synthesis, transport, and action of previous hormones. Low dietary zinc status has been related with low circulating concentrations of several hormones including testosterone, free T4, and IGF-1. (Wada & King, 1986).

3.4. Zn and oxidative stress

Zinc is a cofactor for important enzymes that contribute to the antioxidant defense system such as superoxide dismutase present in the cytoplasm of cells. In addition, this mineral protects cells against oxidative damage because it acts in the stabilization of membranes, inhibits the enzyme nicotinamide adenine dinucleotide phosphate oxidase (NADPH-Oxidase), a pro-oxidant enzyme, and induces metallothionein synthesis (Dilina *et al*., 2017).

4. Spinach's pharmacological activities

There are many various pharmacological activities of *Spinacia oleracea*; this last is recognized as one of the functional foods due to their whole-some nutritional, antioxidant and anti-cancer composition (Hussain *et al.*, 2016), also depending on (Maeda *et al.*, 2008) spinach glycolipid fraction can effectively suppress mouse colon tumor growth, influenced by the anti-angiogenesis and anti-proliferation of tumor cells without side-effects. It has anti-inflammatory activity (Jaime *et al.*, 2015); Spinach extracts exhibit significant antigenotoxic activity (Ko *et al.*, 2014), spinach characterized also by anti-histaminic, CNS depressant, protection against gamma radiation, hepato-protective and antimicrobial effects (Rao *et al*., 2015).

Chapter V

Nanotherapy & ZnO nanoparticles

1. Nanoscience

Nanoscience is a fusion of physics, materials science, and biology involving the manipulation of materials at the atomic and molecular levels. Nanotechnology is one among the most promising technologies of the 21st century. It involves translating nanoscience theory into useful applications by observing, measuring, manipulating, assembling, controlling, and manufacturing matter at the nanoscale; the basis for various applications of nanomaterials is 1–100 nm (Yongshuai Yao *et al.*, 2022).

2. Nanomaterials

European Union agency defines nanomaterial as "A natural or manufactured material that contains particles in an unbound state or as an aggregate or as an agglomerate where 50% or more of the particles has dimension is in the range of 1-100 nm".

Nanomaterial has great prominence and more significance than the bulk material due to their greater surface area and quantum effects (Ahire *et al.*, 2022). Compared to their bulk counterparts, NPs have higher surface charge, absorption, reactivity, surface area, sensitivity, stability, and strength (Khan *et al.*, 2022).

Table 02: Characteristics of the nanomaterial vs bulk material (Ahire *et al.*, 2022)

Nanomaterial	Bulk material
Large surface area	Small surface area
Chemically more reactive	Chemically less reactive
Faster in performance	It is Slower in performance
Lighter material	Heavier material
Cheaper than bulk	Expensive
More energy efficient	Not energy efficient
Shows different properties at a small scale	Properties remain same

3. Applications of nanotechnology

Nanotechnology has piqued people's interest all across the world. Nanotechnology has a huge influence and is a game-changing and useful technology in a variety of industries which has been exploited in many different fields of applications**,** such as medicine, energy, and electronics (Cardoza *et al.*, 2022) (Qiang Zhu *et al.*, 2022).

In the early 1980s, articles relating to the research of the promising applications of nanomaterials in beauty goods began to appear. Nanoscience in Cosmetics strives to use nano-enabled solutions in the research and creation of novel cosmetics (Cardoza *et al.*, 2022).

Nanotechnology has penetrated practically every industry and astounded the world by giving various possible applications in several fields. Nanostructure materials are favored over macrostructure equivalents because of their unique chemical and physical features and better performance. Apart from this, the food sector is one of the fields where nanotechnology has changed the way foods are preserved, processed, tested, and packaged. However, advanced packaging based on nanotechnology has made it feasible to carry food products securely without compromising flavor, nutrition, or quality (Gupta *et al.*, 2022).

The application of nanotechnology in agriculture can change agricultural research and introduce cutting-edge tools for the rapid identification of plant diseases and plant pathogens at an early stage (Shivashakarappa *et al.*, 2022).

Nanomaterials are extensively used for several food applications such as sensors for food analysis, biodegradable packaging, edible food packaging, intelligent packaging, and active packaging. Additionally**,** nanomaterials are used in food sectors as nano additives, nanocapsules**,** gelating agents, nanocarriers, anticaking agents, etc. The main function of nanotechnology in food security includes food safety, preservation**,** and functionalization (Ghosh *et al.*, 2022).

Convergence of artificial intelligence with nanotechnology has the potential to pave the way for several technical advances in the area of material sciences that will depend on novel computer structures and data demonstrations. Computer science, generally, and artificial intelligence specifically, may profit from the enhanced power that nanomaterials, Nano-devices, and Nano-computing will give as suitable frameworks for implementing machine learning algorithms in the near future (Taha Basheer Taha *et al.*, 2022).

Functional coating materials range from organic polymers to hybrid composites and inorganic nanoparticles, depending on the properties and functionality of these materials. In particular, the rapid development of nanotechnology in recent years has translated into a profound growth in the innovation of coatings that incorporate the use of such technology (Qiang Zhu *et al.*, 2022).

4. Role of nanomaterials in medical applications

Nanoparticles have good potential for preventing, diagnosis and treatment of cancer (Tao Huang *et al.*, 2022). Its application in the medical field is mainly aimed at modifying nanoparticles'surface by using nanoparticles' unique properties to form targeted**,** controllable release, easy-to-detect drug transport carriers, and a new method for treating local lesions (Danyang Li *et al.*, 2022).

4.1. Nanotherapy

Study performed by (Meng *et al.*, 2022) revealed that Nanoscale ZnO NPs (≤50 nm) at low concentration (5 µg/mL) did not induce cytotoxicity and exerted anti-inflammatory effects *In-vitro* and *In-vivo*. Zinc acts predominantly in the early (inflammatory) phase of wound healing which associated with accelerated epithelialization of wounds (Tarnow *et al.*, 2009). Several works have highlighted that ZnO nanostructures may successfully promote the growth, proliferation and differentiation of several cell lines (Laurenti & Cauda, 2017).

Tissue regenerative properties related to the ability of ZnO to form reactive oxygen species (ROS) which is a crucial for angiogenesis (Formation of new blood vessels). In engineered tissue constructs, the formation of new blood vessels enables the supply of oxygen and nutrients to the cells that are colonizing the scaffold, and this is vital for tissue regeneration. It was shown that endogenous ROS increased VEGF or VEGFR2 expression and stimulated EC proliferation and migration (Chong *et al.*, 2022). Beside of the formation of ROS, ZnO nanoparticles induce endogenous electric fields (EF) which play a major functional role during wound healing (Funk, 2015). Electrical stimulation can activate many intracellular signaling pathways, and influence intracellular microenvironment, as a result, affect cell migration, cell proliferation, and cell differentiation of different cell groups (Chen *et al.*, 2019).

4.2. Delivery vehicles

The production materials of nanoparticle delivery vehicles are mainly derived from various biocompatible and biodegradable materials including lipids, proteins, polymers and inorganic compounds (Chen & Cong, 2023).

5. Classification of nanomaterials

5.1. Based on structural dimensions (Figure 02)

The structural dimensions of nanoparticles are categorized as 0D, 1D, 2D and 3D materials. The examples of each dimension are shown in Fig. 2. Each of dimensionality has both benefits and limitations for fuel cell applications (Fatina & Shaari, 2022).

5.1.1. 0D nanomaterials

The class of zero-dimensional materials (0D) primarily consists of fullerenes, organic molecules, quantum dots, and atomic clusters (Sinha *et al.*, 2021). Zero-dimensional (0D) nanoparticles refer to materials that reach nanoscale (below 100 nm) in all dimensions (Hongrui Yao *et al.*, 2022).

5.1.2. 1D nanomaterials

One-dimensional (1D) nanowires are materials with nanoscale in two dimensions, which have a large length-diameter ratio, including nanoscale fibers, rods and tubes (Hongrui Yao *et al.*, 2022).

5.1.3. 2D nanomaterials

Two-dimensional (2D) nanomaterials mean the layered nanosheets with a dimension at the nanoscale. In 2D nanosheets, the atoms within the layer are closely bonded by covalent bonds, while the atoms between the layers are connected by vander Waals forces, so the interatomic interactions within the layer are much larger than those between the layers (Hongrui Yao *et al.*, 2022). Nano-plates, nano-sheets, nano-ribbons, nano-walls**,** multilayer and network nanostructures are categorized in 2D nanomaterials structures. Another 2D nanomaterials is graphene (Fatina & Shaari, 2022).

5.1.4. 3D nanomaterials

Pyramids, stars, flowers, multi-pod, nanourchins, tadpole**,** nanocages, nanorice, nanocorn, nanoboxes, nanocubes are the examples of 3D structures (Fatina & Shaari, 2022).

5.2. Based on shape and existence of nanopores

Nanomaterials are available in a variety of shapes, including spherical particles, cubes, rods, wires, plates, prisms, core-shell structures, and more three-dimensional structures. In response to different external stimuli, nanomaterials can change their shape that alters physical and chemical properties (Shivashakarappa *et al.*, 2022), Dendrimers, nanotubes, nanocapsules, nanoshells**,** nanorings, nanobelts, nanospheres, fullerenes, nanowires, nanorods, liposomes, and quantum dots are examples of diverse nanomaterials that can be classified based on their shape and the existence of nanopores (Umapathy *et al.*, 2022).

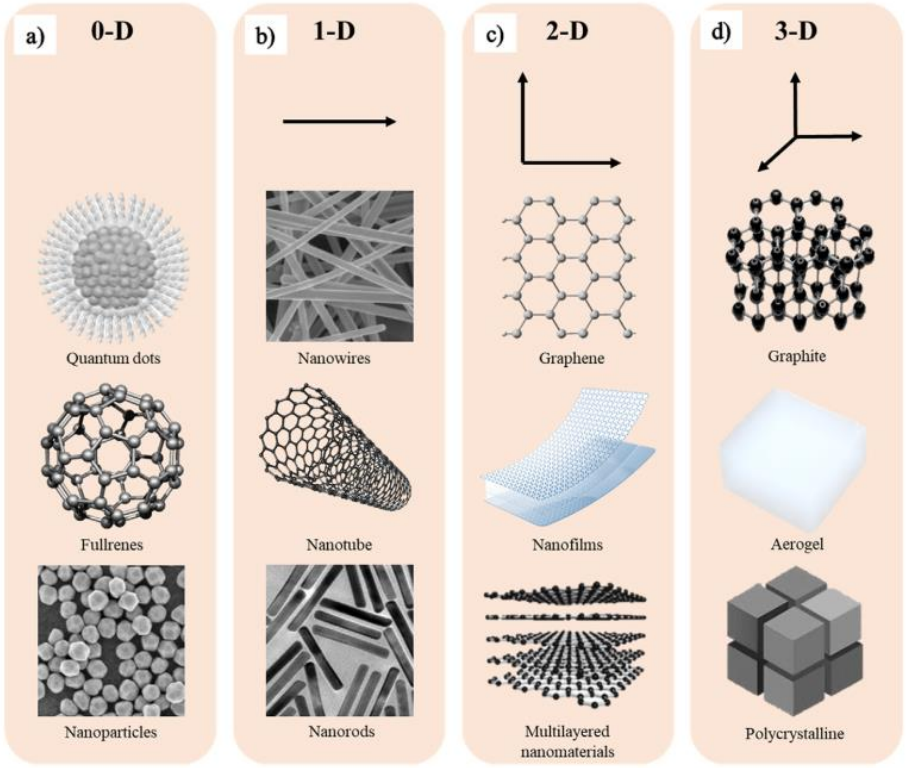

Figure 02: Overview of a) 0D b)1D, c)2D and d)3D nanomaterials (Fatina & Shaari, 2022).

5.3. Based on Components

Nanoparticles (NPs) are the essential building components of nanotechnology, they are made of carbon, metal, metal oxides, or organic matter (Khan *et al.*, 2022).

5.3.1. Inorganic nanomaterials

5.3.1.1. Metal- and Metal oxide-based nanomateials

Among various nanomaterials, metal and metal oxide have attracted a lot of attention due to their special structure and considerable mechanical, thermal, optical, and electrical properties (Jouyandeh *et al.*, 2022). NPs of transition metals and their oxides are widely

used in industries and exhibit diverse biological activities (Maria S.Kuyukina, Marina V.Makarova, Olga N.Pistsova, Grigorii G.Glebov, Mikhail A.Osipenko, 2022). There are many metal nanoparticles among them: Titanium NPs (Tabassum *et al.*, 2020), Silver NPs (Derouiche, 2022); Gold NPs (Al-Radadi, 2021), Copper NPs (Ouidad *et al.*, 2020), Selenium NPs (Samir *et al.*, 2021), Magnesium NPs (Samir *et al.*, 2020), Iron NPs (Attia *et al.*, 2022), Nickel NPs (Iftikhar *et al.*, 2023), Aluminum NPs (Rzayev *et al.*, 2022) and Zinc NPs (Sun *et al.*, 2022).

ZnONPs is one of the commonly used/studied nanoparticles; compared to other metal oxides, they have superior chemical and thermal stability, robustness, and long shelf life. They can be used as an antimicrobial agent in food production, UV repellents in sunscreens, and drug carriers in medicine (Dinga *et al.*, 2022).

In nature, Zinc does not exist freely; it occurs in a (+2) oxidation state and almost 55 zinc minerals exist. For human body, Zinc is known to be an essential trace mineral that is necessary for health and growth and is organically known as a helper molecule that assists about 300 enzymes (Derouiche & Kechrid, 2016),(Ali *et al.*, 2018).

There are many zinc nanoparticle types; among them Zinc sulfide NPs (ZnS-NPs) (Iranmanesh *et al.*, 2015), Zinc ferrite NPs ($ZnFe_2O_4$-NPs) (Naseri *et al.*, 2011), Zinc phosphide NPs (Zn_3P_2-NPs) (Mobarok *et al.*, 2014), Zinc selenide NPs (ZnSe-NPs) (Shakir *et al.*, 2009) and Zinc telluride NPs (ZnTe-NPs) (Dwivedi & Dubey, 2009) but the Zinc oxide nanoparticles (ZnO-NPs) are well known due to its multifunctional, non-toxic nature with vast applications in various fields (Govarthanan *et al.*, 2020). ZnO is a bio-safe material that possesses photo-oxidizing and photocatalysis impacts on chemical and biological species (Sirelkhatim *et al.*, 2015).

5.3.2. Organic nanomaterials

The organic nanomaterials such as polymeric nanomaterials in the form of nanostructured films, nanostructured hydrogel, dendrimer, hyperbranched polymeric nanoparticles, covalent organic frameworks, molecularly imprinted polymeric nanoparticles, polymer nanocomposites, etc.

5.3.2.1. Carbon-allotrope

Carbon exists in several forms (allotropes) with distinctive physical and chemical properties and has been used in many solar selective applications (Fatma Taha *et al.*, 2022).

Because carbon can form *sp*, sp^2 and sp^3 hybridized bonds and stable covalent bonds can be formed between carbon atoms with the same or different hybrid modes. Therefore, there are a large number of carbon allotropes in nature, including graphite, diamond, lonsdaleite. A variety of novel carbon allotropes have been discovered and synthesized, for instance fullerenes, carbon nanotubes and graphene etc. (Wang *et al.*, 2021).

Graphene has seen a tremendous increase in interest since it was first isolated in 2004. This 2D single layer allotrope of carbon boasts exceptional thermal, electrical, and mechanical properties**,** which has sparked interest in electronics, catalysis, and composite fillers.2 Using graphene as a polymer filler offers the promise of inexpensive materials with increased strength and enhanced thermal and electrical properties (Ward & Adamson, 2022).

Due to their superior features, carbon-based nanomaterials have garnered considerable interest in comparison to other materials**,** which include extraordinary mechanical strength, large specific surface**,** superior bioactivity, high biocompatibility, as well as plentiful. Carbon-based nanomaterials are natural or artificial materials consisting of carbon elements with at least one three-dimensional measurement of 1–100 nm, mainly including fullerene, carbon dots, carbon nanotubes, and graphene in the order of dimensional quantum properties. Due to the Sp2 or Sp3 hybrid orbitals, each carbon nanomaterial has its own unique structure and function (Chen & Li, 2022).

5.3.2.2. Nanovesicules (NVs)

Over the past few years, several types of nanocapsules have been synthesized from polymers for the delivery of various bioactive compounds. These nanomaterials present different characteristics (e.g., malleability, size, surface charge) that are important for the incorporation of bioactive molecules and their interaction with plants (Preisler *et al.*, 2022).

Recent decades, encapsulation based food nanotechnology are the emerging field for entrapment of foods or their bioactive constituents into biopolymers to achieve controlled, targeted release and improvement in nutritional qualities. Besides, the nanoencapsulation has additional benefits like greater penetrance capacity, unique surface to volume ratio, and better reaction kinetics. Currently, several nanocarriers like polysaccharides, proteins, and lipids are commonly used to improve the bioactivities of food components either through controlled delivery or by absorbance through gastrointestinal tracts (Soni *et al.*, 2022).

5.4. Niosome

Niosomes are widely used as promising vehicles for drug delivery. They are bilayer vesicles obtained by mixing a non-ionic surfactant and cholesterol with subsequent hydration in an aqueous medium. They have a number of advantages in many aspects such as high stability and purity, content uniformity, reduction of drug degradation, post-administration stability, increased drug bioavailability, low-cost, and a large number of surfactants available for their preparation. Niosomes have the capability of entrapment of lipophilic or hydrophilic drugs in their lipid membrane or in their aqueous cores, respectively (Nazari-Vanani *et al.*, 2018).

5.5. Liposome

Liposomes, one of the most widely used nanoparticle forms, have high biocompatibility, easy surface modification and good pharmacokinetic properties after surface or size modification (Hengyan Zhang *et al.*, 2022).

6. Synthesis of nanomaterials

The two approaches "Bottom-Up" and "Top-down" are employed for the generation of NPs. In the ''Bottom-Up'' approach by using oxidation/ reduction, NPs are produced from nanoscopic entities, like atoms and molecules. In ''Top-Down'' synthesis by using various physicochemical methods. NPs are synthesized by a size reduction reaction (Azim, Singh, Khare, Singh, Amist, Niharika & Yadav, 2022).

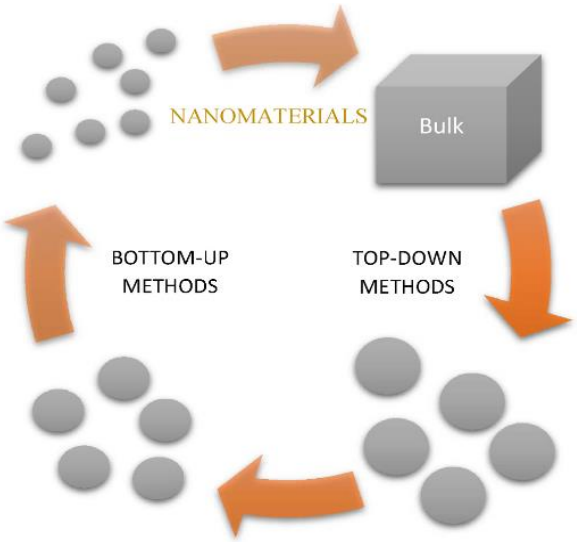

Figure 03: Top-down and bottom-up methods of synthesis nanoparticles (Alharbi *et al.*, 2022).

6.1. Green nanotechnology

Nanoparticles are synthesized by using numerous approaches viz., physical, chemical, biological/green, and hybrid methods. In these approaches, biological methods of NPs synthesis are eco-friendly and less intricate in comparison to traditionally used chemical and physical methods due to the entanglement of unsafe and lethal chemicals which cause serious environmental risks (Azim, Singh, Khare, Singh, Amist, Niharika, Yadav, *et al.*, 2022).

Nowadays, there is a growing urgency to advance ecofriendly nanoparticles synthesis routes (Haglan *et al.*, 2020). The biosynthesis for obtaining nanoparticles using naturally occurring reagents such as vitamins, sugars, plant extracts, biodegradable polymers, and microorganisms as reductants and capping agents could be considered attractive for nanotechnology (Ouidad *et al.*, 2020). The type of plant and plant extract determines the size and shape of the nanoparticles. The increased biological activity of the green synthesized nanoparticles is due to the synergistic effect of the bioactive compounds present in plants and nanomaterial precursors used for synthesis (Rajendran *et al.*, 2021).

Previous studies use *Pelargonium odoratissimum* (Abdelbaky *et al.*, 2022), *Rhododendron Ponticum* (Jayachandran *et al.*, 2021), *Anoectochilus elatus* (Vijayakumar *et al.*, 2021), *Thymbra Spicata* (Gur *et al.*, 2022), *Hibiscus subdariffa* (Bala *et al.*, 2015) and *Coriandrum sativum* (Asmat-Campos *et al.*, 2022) *Punica granatum* (Abdelmigid *et al.*, 2022), *Aquilaria malaccensis , Portulaca oleracea and Coriandrum sativum* (Samir *et al.*, 2022) and *Spinacia oleraceae* (Djouadi & Derouiche, 2021) for ZnO nanoparticles.

Second Part

Experimental Part

Chapter I

Materials and methods

1. Materials

1.1. Plant collection

In this study, Leaves of spinach (*Spinacia oleraceae*) was obtained from the same farm (Guemar region). The plant materials were washed thoroughly using mild warm water to remove contamination from insects and fungal dust and dried at room temperature at shadow condition. The completely dried spinach leaves was powdered by using a mechanical grinder.

Figure 04: *Spinacia oleraceae* leaves (original photo)

1.2. Animals care

In this study, 36 male Wistar rats at the age of 10 weeks old, weighting 153–205 g, obtained From the Institute Pasteur of Algiers, and were housed in an animal room of molecular and cellular biology department, of El-Oued University, Algeria; with a temperature of 22.27±0.15 ˚C, relative humidity 72±1.62 %, and given free access to standard rat food and tap water during the study. The rats were adapted to an inverse 12:12 h light/dark cycle. All experimental procedures employed, as well as rat care and handling, were in accordance with guidelines provided by the local ethics.

1.3. Experimental systemic toxicity

Systemic toxicity induced by oral administration of sodium fluoride (400 ppm) in drinking water during 80 days. Control group received standard rat food and water during the study and NaF exposed group received standard rat food and 400 ppm of sodium fluoride NaF in their drinking water during 11 weeks.

1.4. Experimental design

1.4.1. In-vivo acute toxicity testing of SpE-ZnO-NPs

To assure the safety of ZnO nanoparticles; an acute toxicity testing was carried out before the experiment using (Lorke, 1983) method with modifications. Twelve (12) rats were divided into three groups of four each (n = 4); all rats were maintained fasting for 12h; each group of rats was injected intraperitoneally with a single dose of SpE-ZnNPs (Control, 35 and 70 mg/Kg body weight). The rats were observed for 24h to monitor their behavior as well as mortality. The rats were placed under observation for 20 days after **(Figure 05)**.

1.4.2. In-vivo biological activities assessment

The animals were acclimatized for 2 weeks in the Animal House of molecular and cellular biology department, of El-Oued University, Algeria before systemic toxicity's induction. Animals were divided into six groups (n=6), systemic toxicity was induced by oral administration of 400 ppm in drinking water for 80 days **(Figure 06)**

1.5. Sacrifice, Blood sampling and tissue collection

At the end of each treatment, and after 12 hours of fasting, animals were dissected under slight anesthesia by chloroform (94%) by inhalation; blood sampling was performed during the decapitation and blood samples were transferred into EDTA tubes for hematological parameters and dry tubes. The serum was prepared by centrifugation, for 10 min at 3000 revolutions/min and utilized for hormonal and biochemical analysis assays; fasting blood glucose level obtained using glucometer for each rat. Liver, kidneys, brain, thyroid, spleen, heart and testis were rapidly removed, weighed, washed, homogenized and stored at -20°C for oxidative stress parameters, enzymes activities. Liver, kidneys and testis conserved in formolaldehyde for histological analysis.

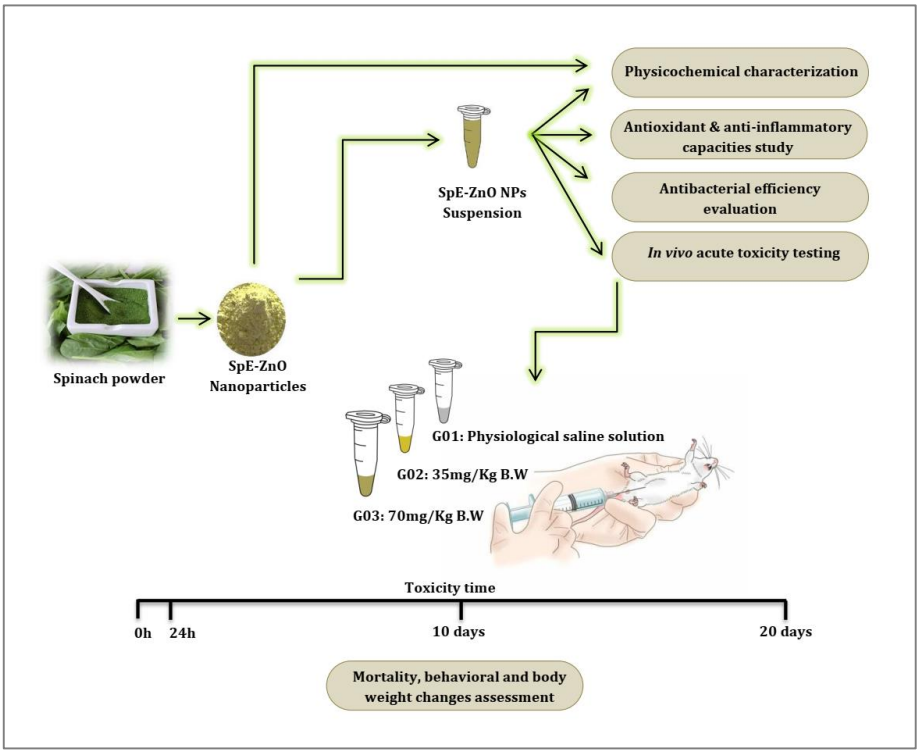

Figure 05: SpE-ZnO-NPs green synthesis, characterization and ZnO nanoparticles' In-vitro biological activity evaluation and toxicity testing

Figure 06: Experimental design of study

1. Methods

1.1. Crude extract preparation

Aqueous extracts were prepared by putting dried powder of *Spinacia oleraceae* (05 g) and 100 mL of distilled water at 50°C during 30 minutes. The filtrate was obtained finally; the extract was collected and stored for further investigation.

1.2. Green synthesis of SpE-ZnO nanoparticles

SpE-ZnO NPs were synthesized according to (Patrón-Romero et al., 2020). The synthesis of ZnONPs process was prepared using 2 g of Zinc Nitrate ($Zn(NO_3)_2*6H_2O$) as a Zn precursor, added to 42 mL of Spinach extract and was placed in a round-bottomed flask (100 mL) and stirred until it was completely dissolved. Following this, the sample was placed in a water bath at 60 ◦C until the sample presented a pasty consistency and later was calcined at 400 ◦C for 1 h.

1.3. Characterization of SpE-ZnO-NPs

ZnO sample absorbance was measured using a UV–Visible spectrophotometer (JENWAY 6705) at the wavelength of (200–500 nm) for confirmation of ZnO nanoparticles green synthesis. To identify the functional groups presented in Sp-ZnO-NPs, infrared scan by (Agilent technologies cary 630 FTIR) was performed within the range of (4000–400 cm^{-1}, resolution 4 cm^{-1}). Scanning Electron Microscope (SEM) micrographs of ZnO NPs were taken by (Phenom-World) device operated at an accelerating voltage of 5 kV, these micrographs detected the morphology, size and shape of the green synthesized ZnO nanoparticles. Energy disperse X-ray (EDX) analysis of ZnO-NPs was carried using the same instrument to examined the element composition of sample. The nanoparticles diameter distribution function was drawing by statistical analysis of SEM images obtained. XRD was applied in the scaling angle (2θ) range of 10–80° with CuKα radiation (kλ=1.5406Å) to determine crystallite structure, purity, crystallinity and crystallite size of synthesized SpE-ZnO NPs using X-ray diffractometer, *PROTO AXRD Benchtop*.

1.4. Phytochemical analyses

Identification of phytochemical compounds in *Spinacia oleraceae* aqueous crude extract (alkaloids, saponins, glycosides, tannins, phenols and flavonoids) carried up using standard method as described in the study of (Altemimi et al., 2015) .

1.4.1. Alkaloids

The 50 mg of crude extracts were treated with 1–2 mL of hydrochloric acid (2N) and then 1–3 drops of newly prepared Mayer's reagent were added. The appearance of red residue in the test liquid indicated alkaloids in the sample.

1.4.2. Saponins

Exactly 25 mL of distilled water were added to 2 mL of the spinach samples with manual shaking for 15–20 min. The appearance of a steady foam indicated the presence of saponins.

1.4.3. Glycosides

Hydrochloric acid, 5 mL of 70% (v/v) was added to 1 g spinach for hydrolysis in water bath at 100 ˚C. Afterward spinach extracts were treated with chloroform, and then 5 mL of dilute ammonia were added to the supernatant layer. A pink color indicated the existence of glycosides in the samples.

1.4.4. Tannins

Drops of distilled water were added to the crude spinach extracts with approximately 0.25 g NaCl. The appearance of tannins was indicated when a blue green color developed after treating samples with 1 mL of ferric chloride (2%).

1.4.5. Phenols

The presence of phenolic compounds with intense green color was observed when 5 mL of 6% (w/v) of ferric chloride was mixed with 1 mL of samples.

1.4.6. Flavonoids

In the first assay, approximately 3–4 drops of absolute H_2SO_4 and a few drops of 10% (w/v) NaOH were added to the spinach samples. Brown and orange colors were indicative of flavonols and flavones respectively. In a second assay, about 0.5 mL of the spinach extract was added to test tube, then 7–10 drops of 80% (v/v) HCl with a small amount of magnesium ribbon to reach the boiling point after 5–10 min. Either reddish pink or foggy brown color in samples indicated the presence of flavonoids.

1.5. Phytochemicals estimation

1.5.1. Total phenolic

Polyphenols are determined by the Folin-Ciocalteu method. This method, initially described by (K. Slinkard, 1977), makes it possible to know the total polyphenolic content of a given sample. The sample of the leaves methanolic extract (0.5 ml) and 2 ml of sodium

carbonate (75g/L) were added to 2.5 ml of 10% (v/v) Folin-Ciocalteau with gallic acid as standard. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm. The tests were carried out three times in order to ensure the reproducibility of the results. The total phenolic content was expressed as mg Equivalent of Gallic Acid per gram of sample.

1.5.2. Total flavonoid

The determination of the total flavonoid content of the methanolic extract of the leaves is carried out by the method described by (Ahn et al., 2007). 0.5 ml of a 2% AlCl 3 -ethanol solution was added to 0.5 ml of sample or standard. After 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for plotting the calibration curve. The tests were carried out three times in order to ensure the reproducibility of the results. The results were expressed in milligrams equivalent to Quercetin per gram of sample.

1.5.3. Total Sugar

Total sugar was estimated according to the method described by (Dubois et al., n.d.). 01 ml of 5% phenol was added with 01 ml of aqueous extract and mixed thoroughly. 05 ml of concentrated H_2SO_4 was added rapidly with constant stirring in the next step. This was allowed to stand at room temperature for 30 min. The color of the solution was changed to yellow orange and the OD was measured at 490 nm against a blank. Known concentration of glucose was used to prepare the standard curve of the blank.

1.6. Antioxidant activity: Ferric Reducing Antioxidant Power « FRAP » Assay

The reducing power of the both *Spinacia oleraceae* aqueous extract and SpE-ZnO-NPs were determined as described by (Rahman et al., 2015) with some modifications. 250 µL of samples or standard solution (5–500µg/mL) was mixed individually with 625µL mL of potassium buffer (0.2 M, pH 6.6) and 625 µL of 1 % potassium ferricyanide [$K_3Fe(CN)_6$] solution into test tubes. The reaction mixtures were incubated for 20 min at 50 ˚C to complete the reaction. Then 625 µL of 10 % trichloroacetic acid (TCA) solution added and the mixtures were centrifuged at 3000 rpm for 10 min, Thereafter, 625 µL of the upper layer solution (supernatant) was withdrawn and mixed with 625 µL of distilled water and 125 µL of ferrous chloride $FeCl_3$ solution. The absorbance was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution

mixture without plant extracts/standard and was incubated under the identical conditions. Increased absorbance of the reaction mixture indicates increased reducing capacity.
Ferric Reducing Antioxidant Power (FRAP) values calculating as follows:

$$100- (OD_{control} \times 100/ OD_{sample})$$

1.7. Anti-inflammatory activity: Inhibition of albumin denaturation assay

Both Spinach crude extract and SpE-ZnO-NPs were screened for their anti-inflammatory activities as a measure of protein denaturation inhibition. At room temperature for 30 min, equal volumes of Bovine serum albumin solution (1%) were incubated with different samples concentrations (10–70 µg/ml). The pH of the solution was adjusted to 2 using drop-wise addition of concentrated HCl. After incubation, the mixture was heated at 72 °C for 30 min, cooled for 10 min and the turbidity was measured at a wavelength of 660nm. Diclofenac was used as standard (Vennila et al., 2018). The results expired by IC_{50}, The percentage inhibition of protein denaturation was calculated as follows:

$$ADIP (\%) = (A_{sample} - A_{control}) \times 100/A_{sample}$$

1.8. Antibacterial testing

Green synthesized ZnO-NPs and Spinach aqueous extract (SpE) antimicrobial activities were evaluated against different pathogenic bacteria using disk diffusion method (Hudzicki, 2012). A positive (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATTC 6633) and negative (*Esherichia coli* ATCC 8737, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* ATCC 14028) Gramm bacteria were used. The Petri Dishes were incubated at 37 °C for 24 h. Zone diameter of inhibition was measured in millimeter (mm). The antibacterial testing performed 3 times.

1.9. Hormonal parameters

Hormonal parameters (Parathormone, calcitonine, FT3, FT4 and TSH) were determined by methods using automated immunoassay by chimiluminescence- Vitros ECIQ 2.

1.10. Hematological parameters

The determination of hematological parameters performed using fully Auto Blood Cell Counter (ERMA).

1.11. Biochemical parameters

Biochemical parameters were determined by methods using commercial reagent kits from Biomaghreb (Tunisia) using auto-analyzer.

1.11.1. Calcium

Calcium forms with the cresolphthalein complexing agent in an alkaline medium a compound colored in violet whose intensity is proportional to the concentration of calcium. Wavelength: 570 nm (550-590).

1.11.2. Alkaline phosphatase (ALP)

Kinetic determination of alkaline phosphatase activity according to the method recommended by the German Society of Clinical Chemistry (DGKG) Wavelength: 405 nm

$$\text{Nitro-4 phenylphosphate } H_2O \xrightarrow{\text{ALP}} \text{nitro 4 phenol+ phosphate}$$

1.11.3. Aspartate aminotransférase (GTO-ASAT)

Kinetic determination of aspartate aminotransferase activity. The reaction is initiated by adding the patient sample to the reagent. The reaction is as follows:

$$\text{2 oxoglutarate + L-Aspartate} \xrightarrow{\text{GTO}} \text{Glutamate + oxaloacetate}$$

$$\text{Oxalocetate + NADH + H}^+ \xrightarrow{\text{MDH}} \text{Malate + NAD}^+$$

The rate of NADH concentration decrease is directly proportional to the aspartate aminotransferase activity in the sample. Wavelength: 340 nm

GOT: Glutamic oxaloacetic transaminase

MDH: Malate Dehydrogenase

1.11.4. Alanine aminotransférase GTP-ALAT

Kinetic determination of Alanine aminotransferase activity. The reaction is initiated by adding the patient sample to the reagent. The reaction is as follows:

$$\text{2-oxoglutarate + L-Alanine} \xrightarrow[\text{LDH}]{\text{GPT}} \text{Glutamate +Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \longrightarrow \text{Lactate} + \text{NAD}^+$$

The rate of NADH concentration decrease is directly proportional to the alanine transferase activity in the sample. Wavelength: 340 nm

GPT: Transaminase Pyruvic Glutamic

LDH: Lactate Dehydrogenase

1.11.5. Urea

The urea is measured in kinetics according to the following reaction:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

The ammonium ions in the presence of salicylate and sodium hypochlorite react to form a green compound (Dicarboxylindophenol) whose the intensity is proportional to the urea concentration. Wave length: 590 n

1.11.6. Creatinine

Creatinine forms in an alkaline medium a complex colored with picric acid. The rate of formation of this complex is proportional to the concentration of creatinine. Wavelength: 492 nm (490-510)

1.11.7. Sodium, potassium and chloride

Serum sodium, potassium and chloride levels were determined using electrolyte auto analyzer (Easylyte PLUS Na/K/CL de Medica)

1.12. Oxidative stress parameters

1.12.1. Homogenates' preparation

About 1g of each organs was homogenized in 9ml of buffer solution of Tris buffer saline (TBS, pH=7.4). Homogenates were centrifuged at 9000 revolutions/min for 15 min at 4°C, and the obtained supernatant was used for the determination of antioxidant activity.

1.12.2. Determination of malondialdehyde (MDA) level

Tissue homogenates were prepared at 10% (w/v) in 0.1 mol/L Tris-NaCl buffer, pH 7.4, and MDA steady-state level was determined. MDA was measured according to the method described by (Sastre et al., 2000). Thiobarbituric acid 0.67% (w/v) was added to a liquots

of the homogenate previously precipitated with 10% trichloroacetic acid (w/v). Then the mixture was centrifuged, and the supernatant was heated (100°C) for 15 min in a boiling water bath. After cooling, n-butanol was added to neutralize the mixture, and the absorbance was measured at 532 nm. The results were expressed as µmol of MDA/g tissue.

1.12.3.Determination of reduced glutathione (GSH) level

GSH concentration was performed with the method described by (Weckbecker & Cory, 1988) based on the development of a yellow color when DTNB is added to compounds containing sulfhydryl groups. In brief, 0.8 mL of tissue homogenate was added to 0.2 mL of 0.25% sulphosalylic acid and tubes were centrifuged at 2500g for 15min. Supernatant (0.5 mL) was mixed with 0.025 mL of 0.01 M DTNB and 1 mL TBS (pH 7.4). Finally, absorbance at 412 nm was recorded. Total GSH content was expressed as mmol GSH/g tissue.

1.12.4.Determination of Glutathione-S-transferase (GST) activity

Glutathione-S-transferase(GST) activity of tissues was measured spectrophotometrically by the method of (Habig et al., 1974) using CDNB as electrophilic substrate that binds to GSH with the participation of the enzyme and forms a colored GSH-substrate complex, detected at 340 nm. The activity of GST was expressed in terms of µmol CDNB/GSH conjugate formed/min/g tissue.

1.12.5. Determination of Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activities in tissues was measured spectrophotometrically by the method of (Flohé & Günzler, 1984).

1.13. Histopathological study of the liver kidney and testis tissues

After fixation, liver and kidney samples were dehydrated with a gradient series of ethanol, cleared by xylen and then embedded in paraffin. After that, the paraffin was cut into the sections of 4 µm on a rotary microtome and collected on slides. Then the slides were stained with hematoxylin and Eosin. Finally, the histological changes were observed using the optical microscope.

1.14. Statistical analysis

Our statistical study is carried out by the software program (Minitab 19) using Student t test to compare means among our different experimental groups; the results are in the form

of mean and standard error (ES) for 36 rats divided into 6 groups. Differences were considered statically significant at $p<0.05$.

Chapter II

Results & Discussion

1. Results

1.1. Phytochemical study

1.1.1. Qualitative phytochemical analysis of spinach

Standard phytochemical analysis of *Spinacia oleraceae* aqueous extract showed in indicate that this water extract contain Phenos, Flavonoids, Tanins and Saponins **(Table03)**

Table 03: Phytochemical essays of *Spinacia oleraceae* extract

Phenols	+++
Flavonoids	+++
Glycosides	+++
Alkaloids	+++
Tannins	+++
Saponins	+++

1.1.2. Total phenols, flavonoids and sugar estimation

(Table04) revealed quantitative phytochemical analysis demonstrating phenols, flavonoids and sugar levels in *Spinacia oleraceae* aqueous extract.

Table 04: Phenols, flavonoids and sugar concentrations in *Spinacia oleraceae* aqueous extract

Phenols (mg of GAE/g of crude extract)
Flavonoids (mg of QE/g of crude extract)
Total sugar (mg of GluE/g of crude extract)

1.1.3. Zinc and calcium in spinach

Table 05: Zinc and calcium concentrations in Spinacia oleraceae plant.

Elements	**Zinc** (µg /g of Spinach powder)	**Calcium** (mg /g of Spinach powder)
Spinacia oleraceae		

1.2. Nano-materials' formulation and Characterization

1.2.1. UV- visible & Fourier transform infrared (FTIR) spectra analyses

Spectrophotometric measurements show the UV–Vis absorption of SpE-ZnO NPs presenting the maximum absorption peak around 300nm **(Figure 07. A.)**. SpE-ZnO NPs FTIR spectrum illustrate clearly that transmittance band of SpE-ZnO-NPs corresponds to 400 cm^{-1} identify the ZnO **(Figure 07. B.)**

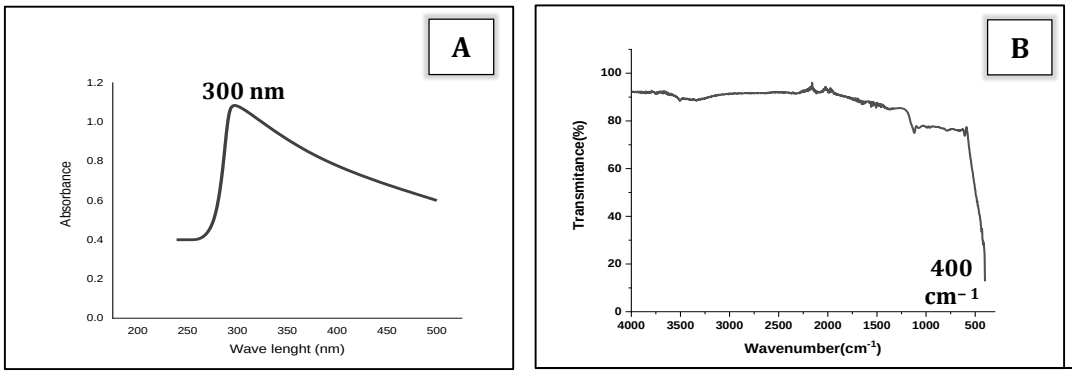

Figure 07: SpE-ZnO-NPs Characterization: UV–vis spectrum (A); FTIR spectrum (B)

1.2.2. Scanning Electron Microscope (SEM)-coupled Energy Despersive X-ray (EDX) analysis

Average particle size and morphology of SpE-ZnO NPs obtained and calculated by SEM images analysis **(Figure 08)**, our biosynthesis ZnO characterized as irregular in the morphological shape and rod-like morphology with mean diameter 30 nm. Compositional analysis by EDX revealed the presence of the elements: Zinc, Oxygen. Along with Zn and O atoms, small signal was exhibited from Calcium, Sulfur, Silicon, Phosphorus, Nitrogen and Carbon. **(Figure 09)**, so this data confirm ZnO nanoparticles formation.

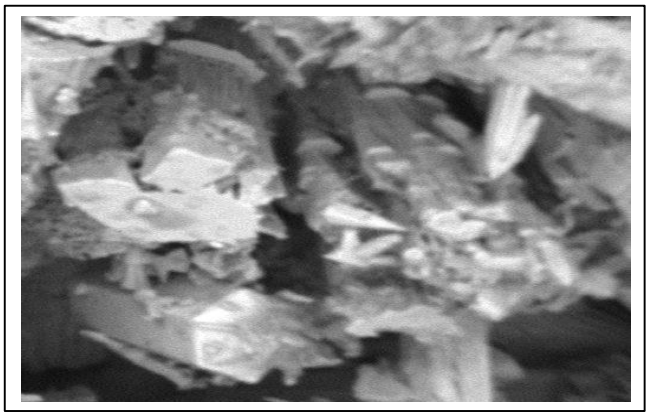

Figure 08: SpE-ZnO-NPs Characterization: SEM micrographs

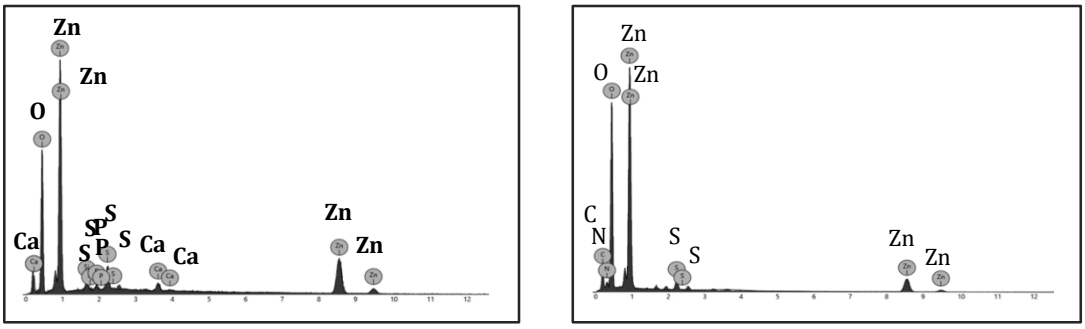

Figure 09: SpE-ZnO-NPs Characterization: EDX detection in different sample regions

1.2.3. X-ray Diffraction

Pattern peaks of XRD given in **(Figure 11. A)** revealed (in agreement with JCPDS card N° 01-075-1526

1.3. Biological activities

1.3.1. Antioxidant and anti-inflammatory activities

1.13.1.1. IC_{50} for FRAP values and BSA denaturation Inhibition

Results presented in **(Figure 12.A.B.)** showed the antioxidant and anti-inflammatory activities in comparison with (Ascorbic acid, Butylated hydroxytoluene "BHT" and β-hydroxy acid "BHA") and (Physiolone and Diclifenac) repectively.

Our findings indicate that both of spinach aqueous extract and SpE-ZnO-NPs showed remarkable anti-inflammatory impact using *In vitro* method. **(Figure12.A.).** IC_{50} value represents sample concentration that gives 50% inhibition of albumin denaturation, was determined by linear regression analysis of inhibition of albumin denaturation percentage versus concentration of sample or standard. Diclofenac and Physiolone, a standard anti-inflammatory drugs revealed a high percentage of albumin denaturation inhibition at the concentration of 12.84 µg/ml and 16.40 µg/ml respectively. Spinach extract and SpE-ZnO-NPs exhibit an anti-inflammatory impact at the concentration 36.8µg/ml and 39.1µg/ml respectively in comparison with standard**.**

The ferric reducing antioxidant power (FRAP) results of both Spinach aqueous extract and SpE-ZnO NPs in comparison with Ascorbic acid, BHT and BHA as standard demonstrated that Spinach extract showed concentration dependent reducing power (Increase in sample concentration led to increased reaction mixture absorbance and hence increased reducing efficiency). Whereas, in comparison with Ascorbic acid, which exhibited the strongest reducing activity; its reducing power was narrow and higher than that of BHT and BHA. While, Bioynthesized SpE-ZnO NPs exhibits concentration independent reducing

power and don't exhibit any antioxidant activity neutralize the Fe^{3+}/ferricyanide complex by this antioxidant pathway although the spinach extract exhibit that. **(Figure 12 B.)**

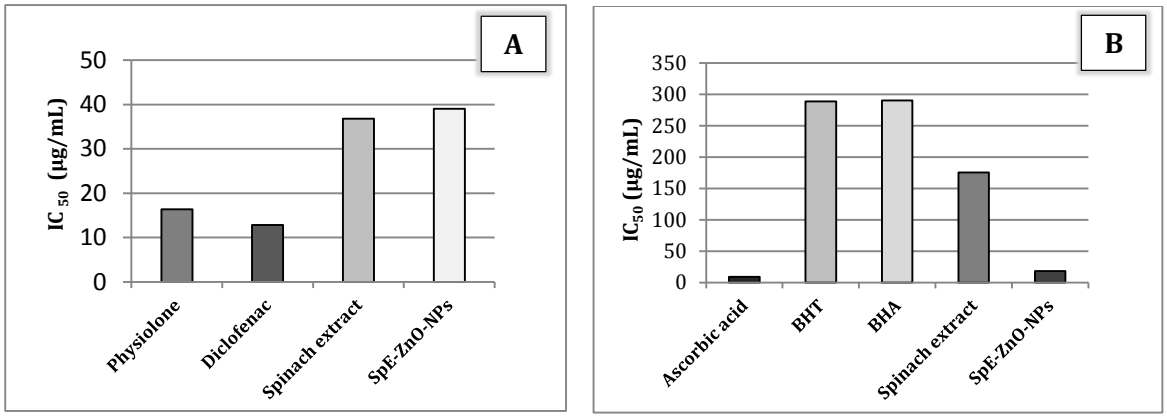

Figure 12: IC_{50} for BSA denaturation inhibition (A) and FRAP values (B) of spinach extract and SpE-ZnO-NPs

1.3.2. Antimicrobial analysis

Results of antibacterial activities were given in **(Table 06)**. There is no effective antibacterial effect against both positive and negative gram bacteria in comparison with antibiotics. Thus, the SpE-ZnO-NPs and spinach extract exhibit very narrow efficiency against bacteria strains

Table 06: Antimicrobial activity of ZnO-NPs synthesized by aqueous crude extract of *Spinacia oleraceae* compared to antibiotics and negative controls against bacterial strain

Treatments	Concentration (µg/mL)	Inhibition zone (mm)				
		ATCC 6538	ATTC 6633	ATCC 8737	ATCC 9027	ATCC 14028
		Gram +	Gram +	Gram-	Gram-	Gram-
		S.a.	B.s.	E.c.	P.a	S.t.
SpE-ZnO-NPs	25	08.00 mm	No zone	07.33 mm	07.33 mm	08.00 mm
	50	09.00 mm	No zone	08.33 mm	08.33 mm	07.67 mm
	100	09.00 mm	No zone	06.83 mm	09.00 mm	07.33 mm
	200	09.33 mm	No zone	07.67 mm	09.50 mm	No zone
SpE	200	07.66 mm	No zone	07.33 mm	08.00 mm	07.00 mm
Antibiotics	Gentamicin	22.33 mm	30.00 mm	21.00 mm	22.00 mm	21.33 mm
	Ciprofloxacin	34.50 mm	31.00 mm	38.50 mm	32.50 mm	36.00 mm
	Amoxicillin	18.33 mm	25.67 mm	14.67 mm	16.33 mm	14.00 mm
Negative	Distilled water	No zone	No zone	No zone	No zone	No zone

controls	DMSO	No zone	No zone	No zone	No zone	No zone

Values are mean of three replicates.
No zone i.e. the pathogen was resistant (R)

1.4. Toxicity testing

No deaths were noted on acute toxicity testing at any dose tested during 24 h. No signs of behavioral toxicity including diarrhea, salivation, lacrimation, defecation, urination, over activity, aggressiveness, piloerection, twitches, tremors and convulsions were recorded during this period. A narrow slowly movement (decreased motor activity) was observed in the rats only in the first 4 h after SpE-ZnO-NPs injection (G03: 70 mg ZnO /Kg of body weight) and an epistaxis after 8 h of SpE-ZnO-NPs injection in all animals of G03 (70 mg ZnO /Kg of body weight) and a half of G02 (35 mg ZnO /Kg of body weight).

All animals administered with any dose tested did not show any signs of toxicity, their behavioral response was normal with no mortality recorded after 36 h just the feed consumption in G03 which injected by 70 mg ZnO/Kg of body weight. Abnormal changes in feed and water consumption and at the end of the experimental period (20 days) the feed and water consumption normal state restored **(Table 07)**.

Table 07: Behavior observations, Mortality and body weight monitor after the acute toxicity using Spinach mediated Zinc Oxide nanoparticles

Behavior observations	Group/ ZnO dose	Toxicity time						
		0h	2h	4h	8h	24h	36h	10 days after	20 days after
Mortality	**G01:** Control: Physiological Saline Solution		None	None	None	None	None	None	None
	G02: 35 mg ZnO /Kg of body weight		None	None	None	None	None	None	None
	G03: 70 mg ZnO /Kg of body weight		None	None	None	None	None	None	None
Feed (F) and water (W) consumption	**G01:** Control: Physiological Saline Solution		Fasting	Fasting	Normal	Normal	Normal	Normal	Normal
	G02: 35 mg ZnO /Kg of body weight		Fasting	Fasting	Abnormal(<<)	Abnormal(<<)	Abnormal(<)	Normal	Normal
	G03: 70 mg ZnO /Kg of body weight		Fasting	Fasting	Abnormal(No)	Abnormal(No)	**F:** Abnormal(<<<) **W:** Abnormal (<<)	**F:** Abnormal(<<) **W:** Normal	Normal
Diarrhea	**G01:** Control: Physiological Saline Solution		None	None	None	None	None	None	None
	G02: 35 mg ZnO /Kg of body weight		None	None	None	None	None	None	None
	G03: 70 mg ZnO /Kg of body weight		None	None	None	None	None	None	None
Epistaxis	**G01:** Control: Physiological Saline Solution		None	None	None	None	None	None	None
	G02: 35 mg ZnO /Kg of body weight		None	None	Half	None	None	None	None
	G03: 70 mg ZnO /Kg of body weight		None	None	All	None	None	None	None
Movement	**G01:** Control: Physiological Saline Solution		Normal	Normal	Normal	Normal	Normal	Normal	Normal
	G02: 35 mg ZnO /Kg of body weight		Normal	Normal	Normal	Normal	Normal	Normal	Normal
	G03: 70 mg ZnO /Kg of body weight		Slow	Slow	Normal	Normal	Normal	Normal	Normal

1.5. Body and relative organs weights

1.5.1. Body weight changes

All experimental groups in adaptation phase revealed no significant alteration in body weight gain. After sodium fluoride administration there was a significant diminution in body weight then we observe that body weigh became to rise again and then decreased. All treatments decreased the body weight while the control group showed a normal elevation **(Figure 13).**

1.5.2. Relative Organs Weights (ROW)

Relative Liver Weight (RLW) altered after NaF exposure which showed high significant reduction (P<0.01) in RLW of NaF exposed rats compared to control, which ameliorated in all treated rats, while both of (ZnO-NPs)

Relative Kidney Weight (RKW) altered after NaF exposure which showed significant elevation (P<0.05) in RKW of NaF exposed rats compared to control, which ameliorated in all treated rats compared to control group (P>0.05). Non-significant (P>0.05) variation in healthy rats combined treatment (SpE-ZnO-NVs) compared to control rats **(Figure 14.D.)**.

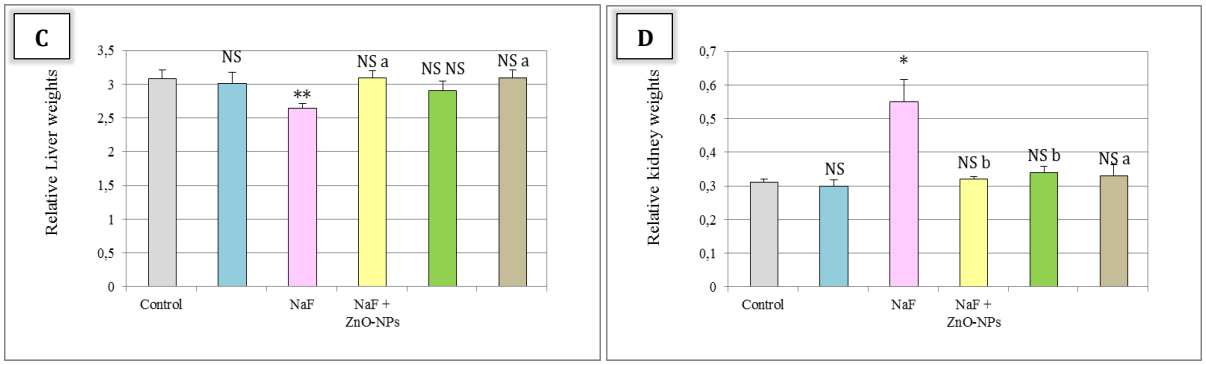

Figure 14: Relative organs weights: in experimental rats groups

Comparison with the control group: * P < 0.05, ** P < 0.01, *** P < 0.001
Comparison with NaF exposed group: a P < 0.05, b P < 0.001, c P < 0.001

1.6. Hematological disorders

1.6.1. hematological profile

In current study **(Figure 15)** revealed hematological profile which present that NaF exposure reduce significantly (p<0.05) red blood cells (RBCs), hematocrit (HCT) (p<0.05) and hemoglobin (HGB) (p<0.01) in comparison with control **(Figure 15.A.B.C.)**. Significant elevation in Mean Corpuscular Volume (MCV) (p<0.05), Mean Corpuscular Hemoglobin

(MCH) ($p<0.05$) and mean corpuscular hemoglobin concentration (MCHC) ($p<0.01$) in (NaF) exposed fluoride rats compared to control **(Figure 15.D.E.F.)**.

1.7. Biochemical disorders

1.7.1. Blood sugar and liver functions

Hypo-glycemia observed in NaF exposed rats and all treated rats compared to control rats. Albumin level increased significantly ($p<0.05$) in NaF rats compared to control rats. All treatment decreased its level **(Figure 23)**.

Liver function alterations caused by sodium fluoride exposure are confirmed by histopathological examination, which showed necrosis, edema and degeneration in the hepatocytes. All of these abnormalities appear to be removed after all treatments **(Figure24)**.

1.7.2. Liver oxidative stress status

Oxidative stress results of liver given in **(Figure 25)** showed significant ($P<0.05$) increase in MDA level in NaF exposed rats compared to control rats; in that group significant decrease observed in GSH levels ($P<0.05$), GPx ($P<0.001$) and GST activity ($P<0.05$).

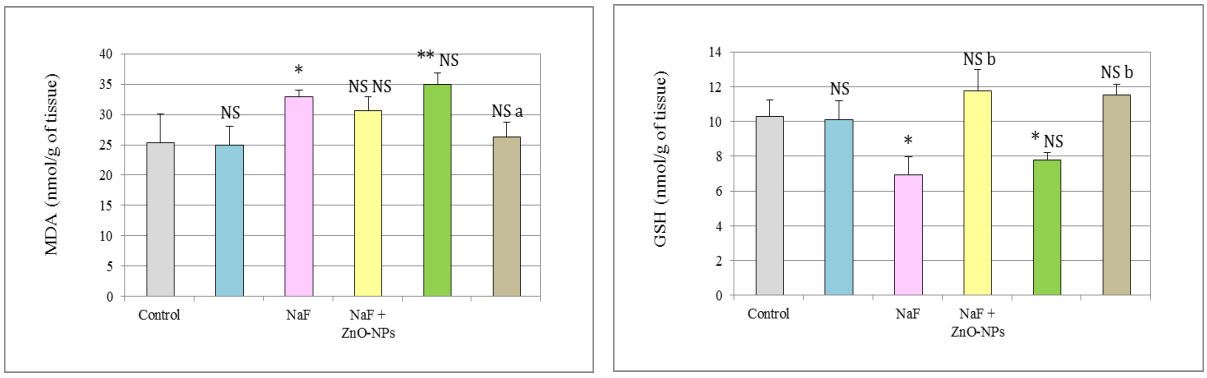

Figure 25: Oxidative stress status of liver in experimental rats groups

Comparison with the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
Comparison with NaF exposed group: a $P < 0.05$, b $P < 0.001$, c $P < 0.001$

1.7.3. Renal function

Compared to control group, high significant ($p<0.01$) increase in renal function biomarkers (Urea and creatinine) indicate the renal dysfunction in NaF exposed groups. **(Figure 28)**.

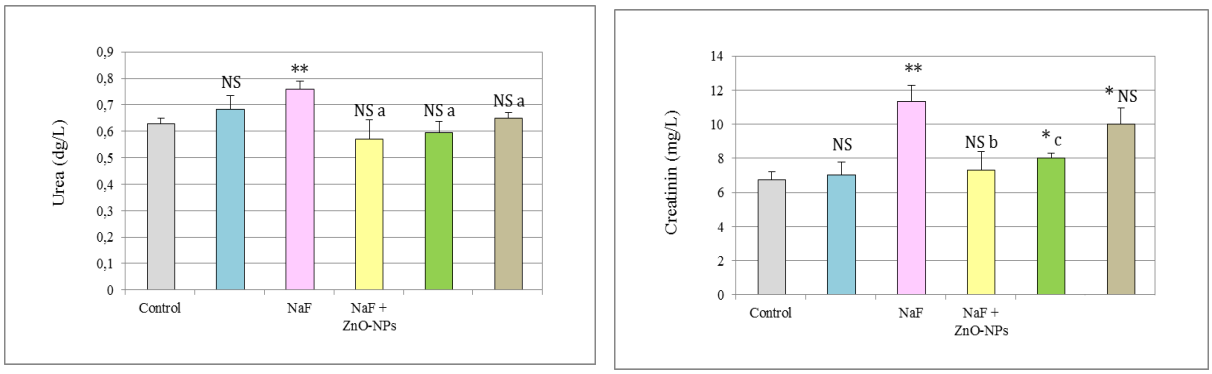

Figure 28: Serum renal function biomarkers in experimental rats groups

Comparison with the control group: * P < 0.05, ** P < 0.01, *** P < 0.001
Comparison with NaF exposed group: a P < 0.05, b P < 0.001, c P < 0.001

Renal function alterations caused by sodium fluoride exposure are confirmed by histopathological examination; histological sections revealed various tissue damages in NaF exposed rats: necrosis, tubule dilatation and degeneration, glomerular atrophy and inflammation; treatment by all treatments eliminate all this injury .The morphological changes of kidney tissue were examined by H&E. Tissue kidney of treated groups present architecture narrow to that of control, where the tissue became more orderly and the degree of injury was gradually alleviated **(Figure 29)**.

Figure 29: Kidney histopathological changes of experimental rats groups

1.7.4. Kidney oxidative stress status

Oxidative stress results of kidney given in **(Figure 30)**. Our results present elevation of MDA level (P<0.05) and reduction of that of GSH (P<0.05) and GPx activity (P>0.05) with increase in GST activity (P<0.05) indicating oxidative stress status in kidney of NaF exposed rats.

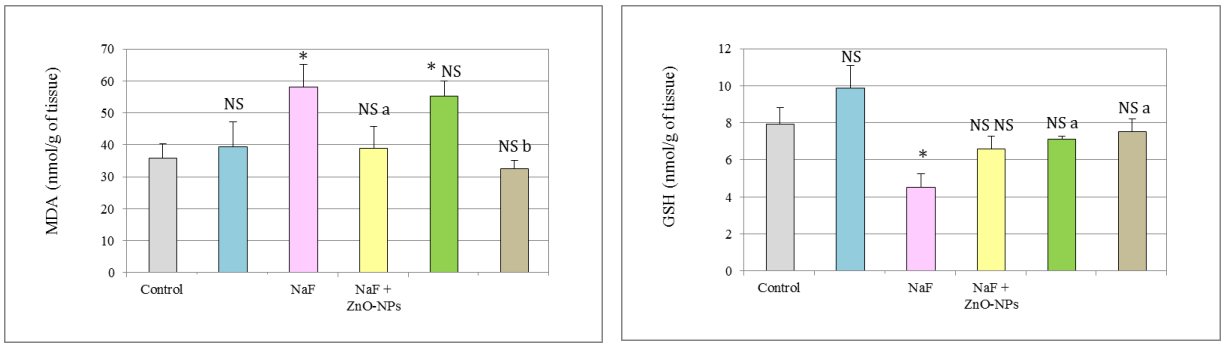

Figure 30: Oxidative stress status of kidney in experimental rats groups

Comparison with the control group: * P < 0.05, ** P < 0.01, *** P < 0.001
Comparison with NaF exposed group: a P < 0.05, b P < 0.001, c P < 0.001

2. Discussion

2.1. SpE-ZnO NPs' characterization

Characterization of SpE-ZnO-NPs biosynthesized carried up using UV- visible, Fourier transform infrared (FTIR) spectroscopies analysis, Scanning Electron Microscope (SEM) coupled Energy Disperse X-ray (EDX) analysis and X-Ray Diffraction.

In the present study, Average particle size and morphology of SpE-ZnO NPs obtained and calculated by SEM images analysis **(Figure 08)**, our biosynthesis ZnO characterized as irregular in the morphological shape and rod-like morphology with mean size 30 nm.

Compositional analysis by EDX demonstrates the presence of ZnO **(Figure 09)**, therefore supporting the UV-Vis and FTIR data. An absorption peak of ZnO NPs at 308 nm, 298 nm and 274 nm are observed in UV–Vis absorption spectra in the studies of Saravanadevi *et al.* (Saravanadevi *et al.*, 2020), Sara *et al.* (Sara *et al.*, 2020) and Helmy *et al.* (Helmy *et al.*, 2020) respectively. In other study realized by Naveenkumar *et al.* (Naveenkumar *et al.*, 2019) the absorption bands of ZnO NPs at 289, 366 nm and 375 nm where the ZnO synthesized from fructose, dextrose and maltose carbohydrates respectively. These results of previous studies are in agreement with the present result (300nm) **(Figure 07. A.)**. The shape of the band was symmetrical, suggesting uniform scattering of spherical shape that also confirms that particle existence and stability(Ding *et al.*, 2020).

FTIR spectrum illustrate clearly that the transmittance band of SpE-ZnO-NPs corresponds to 400 cm^{-1} as obtained in the studies of Prashanth *et al.* (G.K. *et al.*, 2015) . In another study performed by Jeyabharathi *et al.* (Jeyabharathi *et al.*, 2019) FTIR peak at 414.70 cm^{-1} elucidates the presence of ZnO NPs; hence, the band at 400 cm^{-1} corresponds also to SpE-ZnO-NPs **(Figure 07. B.)**.

Similarly to our EDX findings, previous study obtain three peaks between 01 kV and 10 kV and those maxima are directly related to zinc in its tested material indicating that the reaction product was composed of high purity zinc nanoparticles (Dobrucka & Długaszewska, 2016), but In our EDX spectrum, along with Zn and O atoms, small signal was exhibited from Calcium, Sulfur, Silicon, Phosphorus, Nitrogen and Carbon **(Figure 09)**, which may be linked to the presence of carbohydrates and proteins present in the plant extract (Ashokan *et al.*, 2017).

Measurements using SEM micrographs were found to be coherent with XRD calculations. Then, SpE-ZnO NPs mean size calculated using XRD data was 30 nm.

Our results similar to previous literature(Huanliang Liu *et al.*, 2020),(Diallo *et al.*, 2015),(Ying Chun Liu *et al.*, 2020) although the minute differences in 2 Theta (2θ) values.

Moreover, according to (Muzzio *et al.*, 2019); quality control of ZnO nanoparticles assessed by monodispersity determination, which their increase considered as a measure of quality control. To define NPs as monodisperse, their standard deviation in diameter (or in one dimension) should be less than ten percent. Our findings (PDI=0.07) indicate that SpE-ZnO NPs is monodispersed (well quality).

2.2. SpE-ZnO NPs' biological activities

The ferric reducing antioxidant power (FRAP) results of both Spinach aqueous extract and SpE-ZnO NPs in comparison with Ascorbic acid, BHT and BHA as standard demonstrated that Spinach extract showed concentration dependent reducing power (Increase in sample concentration led to increased reaction mixture absorbance and hence increased reducing efficiency). Whereas, in comparison with Ascorbic acid, which exhibited the strongest reducing activity; its reducing power was narrow and higher than that of BHT and BHA. While, SpE-ZnO NPs exhibits concentration independent reducing power. The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe^{3+}/ferricyanide complex to ferrous form (Dhandapani *et al.*, 2020). Bioynthesized SpE-ZnO NPs don't exhibit any antioxidant activity neutralize the Fe^{3+}/ferricyanide complex by this antioxidant pathway although the spinach extract exhibit that **(Figure 12 B.)**.

Inflammation is generally referred to as a complex biological response of vascular tissues to harmful stimuli. As well, inflammation is associated with pain, and it involves in an increase of protein denaturation(Gunathilake *et al.*, 2018). Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation(Karthik *et al.*, 2013). IC_{50} value represents sample concentration that gives 50% inhibition of albumin denaturation, was determined by linear regression analysis of inhibition of albumin denaturation percentage versus concentration of sample or standard. Diclofenac and Physiolone, a standard anti-inflammatory drugs revealed a high percentage of albumin denaturation inhibition at the concentration of 12.84 µg/ml and 16.40 µg/ml respectively. Spinach extract and SpE-ZnO-NPs exhibit an anti-inflammatory impact at the

concentration 36.8µg/ml and 39.1µg/ml respectively in comparison with standard. Our findings indicate that both spinach aqueous extract and SpE-ZnO-NPs showed remarkable anti-inflammatory impact using *In vitro* method **(Figure12.A.)**. This anti-inflammatory impact of SpE-ZnO-NPs perhaps refer to its adsorption with BSA which led to its protection against heat although the change of BSA structure after the adsorption on nanoparticles was confirmed (Bukackova & Marsalek, 2020).

As shown in **(Table 06),** there is no effective antibacterial effect against both positive and negative gram bacteria in comparison with antibiotics. Our findings are in contradiction with several studies which found that ZnO NPs present a potent antibacterial effect (Iqbal *et al.*, 2019) (Zafar *et al.*, 2020) (Rambabu *et al.*, 2021) . It is well known that Spinach (*Spinacia oleraceae*) have a significant antibacterial activity (Salehi *et al.*, 2019). In the study of (Altemimi *et al.*, 2017) the *Spinacia oleraceae* methanolic extract exhibit higher efficacy against Gram-positive and negative bacteria; this also in contrast with our findings. According to our XRD results and SEM results which revealed that ZnO sizes was in the mean of 30 nm, Our antibacterial results perhaps refer to the ZnO-NPs sizes (Lallo da Silva *et al.*, 2019), concentrations (Seshadri, 2021) (Asamoah *et al.*, 2020) or to the method of synthesis (Hamouda *et al.*, 2020).

2.3. *In-vivo* acute toxicity testing of SpE-ZnO NPs

Motor activity has been widely used as a behavioral assay in toxicology, pharmacology, and in aging-neurobiology research (MacPhail *et al.*, 2012). Results of previous studies showed that prenatal exposure to ZnO NPs was involved with increased levels of depression-like behaviors increasing the immobility duration of the offspring (Alimohammadi *et al.*, 2019). Many symptoms of depressive disorder among them sleep disturbances and weight/appetite changes (Becker *et al.*, 2021). Although the underlying pathophysiological mechanisms of depression have not been clarified, animal and human studies have shown that intracerebral neurotransmission dysfunction such as serotonin, norepinephrine and dopamine plays a pivotal role in the development of depression(Du *et al.*, 2020). Acetylcholine is known to participate in the mechanisms of conditioned choice of motor activity(Sergutina & Rakhmanova, 2014). Blood–brain barrier (BBB) is a specialized system that separates blood from cerebrospinal fluid However, NPs made of different materials could cross the BBB and induce their potential neurotoxicity (Hu & Gao, 2010). So, It is possible that ZnO nanoparticles has an important effect on the monoaminergic and cholinergic systems and cause their temporary dysfunctions led to the decreased motor

activity in the first 4h and changes in feed and water consumption which rapidly restored because of the rats were in healthy state and because of the NPs toxicity related to many factors including the mode of entry (dermal, oral, inhalation or injection), time of exposure, size and on the surface to mass ratio (Vysloužil *et al.*, 2020) **(Table 07)**.

Blood is promptly affected by environmental pollutants and toxicants that can cause many metabolic disorders (Abbas *et al.*, 2017b). Fluoride is one of important environmental pollutant (Liu *et al.*, 2021). Oxidative stress, which is defined as the imbalance between the cell antioxidant defense system and the production of reactive oxygen species (ROS) (Papas *et al.*, 2019). Fluoride is related to excessive intracellular reactive oxygen species (ROS)(Ge *et al.*, 2018). Prolonged exposure to high fluoride results in damage to multiple soft tissues of the body inducing morphological changes in many organs, leading to an impairment of their function (C. X. Wu *et al.*, 2022),(Stawiarska-Pieta *et al.*, 2012). Fluoride exposure produce systemic toxicity (Tian *et al.*, 2019); Oxidative stress is the underlying pathophysiological mechanisms by which fluoride induce systemic toxicty

NaF–induced hepatotoxicity

Liver is the first and most easily invasive organ for poisons as vital digestion and detoxification organ(Yangfei Zhao *et al.*, 2022); it is an important metabolic organ in the body, secreting bile and processing various nutrients into proteins. Most importantly, the liver functions in detoxifying tissue by transforming, neutralising, and eliminating toxins through hepatocyte mediated enzymatic detoxification systems (Zhou *et al.*, 2015). The liver is the most important organ for metabolism, contributing to the homeostasis of glucose (Zhang *et al.*, 2023).

Transaminases alterations could be expected to occur associated with pathology involving necrosis of the liver. Abnormal concentrations of some biomarkers in serum such as ALT, AST and ALP, which are caused by the destruction of hepatocyte membrane structure, are widely used to evaluate normal liver functions (Zhou *et al.*, 2015).

Albumin level increased significantly ($p<0.05$) in NaF rats compared to control rats. All treatment decreased its level **(Figure 23)**.

In the present study hepatic function biomarkers (ALT, AST) activities are very high significantly ($p<0.001$) increased in fluoride exposed group; these results are in accordance with the findings of (Akinrinde *et al.*, 2021) and (Raina *et al.*, 2015) who found the same results like us; but the ALP activity present high significant ($p<0.01$) decrease. Plasma

enzymes activities rise when the membranes of only very few cells are damaged. Liver cells contain more AST than ALT, but ALT is confined to the cytoplasm in which its concentration is higher than that of AST. In inflammatory or infective conditions: the cytoplasmic membrane sustains the main damage; leakage of cytoplasmic contents causes a relatively greater increase in plasma ALT than AST. In infiltrative disorders, in which there is damage to both mitochondrial and cytoplasmic membranes, there is a proportionally greater increase in AST activity than ALT (Hassan & Yousef, 2009).

Our hematological findings confirm the inflammation status which revealed that both LYM and WBC were significantly increased in this rats group, These results in the line of that obtained by (Ameeramja *et al.*, 2018), who found that exposure to fluoride can associated to WBC level elevation.

Our results present liver oxidative stress status **(Figure 25)**, where elevated MDA has been considered as a reliable biomarker for oxidative stress in animals exposed to environmental contaminants (Cao *et al.*, 2020); hence the liver dysfunction caused by the fluoride induced inflammatory in addition of that of oxidative stress because AST activity is greater than that of ALT.

Oxidative stress results of liver given in **(Figure 25)** showed significant ($P<0.05$) increase in MDA level in NaF exposed rats compared to control rats; in that group significant decrease observed in GSH levels ($P<0.05$), GPx ($P<0.001$) and GST activity ($P<0.05$).

Relative Liver Weight (RLW) altered after NaF exposure which showed high significant reduction ($P<0.01$) in RLW of NaF exposed rats compared to control, which ameliorated in all treated rats, while both of (ZnO-NPs) administrated rats were the better ($P<0.05$) compared to (NaF) group. **(Figure 14.C.)**.

Reduction in RLW observed in NaF group is conflicting with our previous study(Djouadi Anfal, Derouiche Samir, 2017) when we observed hepatomegaly; but the results of present study is in accordance with the study of S.A. Adelakun *et al*. (Adelakun *et al.*, 2022) which present also reduction of RLW after fluoride exposure. Based on results obtained by Zhuanxu Ouyang *et al*. (Ouyang *et al.*, 2021), which suggested from the findings of their experimental that sodium fluoride can cause liver damage by inducing cell apoptosis, RLW reduction may be refer to apoptosis of hepatocytes.

Liver function alterations caused by sodium fluoride exposure are confirmed by histopathological examination, which showed necrosis, edema and degeneration in the

hepatocytes. These data are in line with the previous studies of (Pereira *et al.*, 2018), (Atmaca *et al.*, 2014). All of these abnormalities appear to be removed after all treatments **(Figure24)**.

(ZnO-NPs) injection reversed the oxidative stress status increasing and decreasing the GSH and MDA levels respectively **(Figure 24)**, and modulating liver function biomarkers. Many previous studies indicate that ZnO nanoparticles exhibit this biological activities (Jayappa *et al.*, 2020) (Ali *et al.*, 2021) (Velsankar K.,Venkatesan A., Muthumari P.,Suganya S., Mohandoss S., 2022).

2.3.1. NaF–induced Nephro-toxicity

Kidney is the main tissue for the both elimination and retaining of fluoride(Caglayan *et al.*, 2021), because renal excretion of fluoride is one of the most important mechanisms for the regulation of fluoride levels in the body(Cárdenas-González *et al.*, 2013). Kidneys, followed by the liver, accumulate more fluoride than any other organ system in the body (Malin *et al.*, 2019).

Fluoride is freely filtered through the glomerulus and undergoes a variable degree of proximal tubular re-absorption(Cárdenas-González *et al.*, 2013). Fluoride induced nephrotoxicity in present study supported by previous studies (Nabavi *et al.*, 2013), (Tian *et al.*, 2020). The long-term accumulation of F in the kidney can lead to tissue structure and functional damage(Haojie Li *et al.*, 2021). Therefore, histological sections revealed various tissue damages in NaF exposed rats: necrosis, tubule dilatation and degeneration, glomerular atrophy and inflammation; treating by all treatments eliminates all this injury. The morphological changes of kidney tissue were examined by H&E. Tissue kidney of treated groups present architecture narrow to that of control, where the tissue became more orderly and the degree of injury was gradually alleviated **(Figure 29)**.

According to results of (Tian *et al.*, 2019) which revealed that F caused mitochondrial damage in the kidney; they speculate that fluoride toxic effects can cause excessive production of ROS and interfere with the kidney antioxidant defense system, resulting in oxidative stress and renal tissue damage.

Oxidative stress results of kidney given in **(Figure 30)**. Our results present elevation of MDA level ($P<0.05$) and reduction of that of GSH ($P<0.05$) and GPx activity ($P>0.05$) with increase in GST activity ($P<0.05$) indicating oxidative stress status in kidney of NaF exposed rats.

Relative Kidney Weight (RKW) altered after NaF exposure which showed significant elevation (P<0.05) in RKW of NaF exposed rats compared to control, which ameliorated in all treated rats compared to control group (P>0.05). **(Figure 14.D.)**.

2.3.2. NaF–induced hemato-toxicity

The complete blood cell count (CBC) is one of the most commonly ordered blood tests. It provides insight into several disease processes as well as normal hematopoietic physiology(Patel, 2019). Anemia is associated with a lower level of hemoglobin than the normal range which decreases the oxygen-carrying capacity of RBCs to tissues; anemia also lowers the number of circulating RBCs (Fatima *et al.*, 2022). There are many reasons of anemia, such as chronic diseases and nutritional deficiencies (Pan *et al.*, 2021).

We suppose that NaF-induced anemia is the result of a wide variety of causes that often coexist together to induce an experimental anemia model by altering many key sites.

In current study **(Figure 15)** revealed hematological profile which present that NaF exposure reduce significantly (p<0.05) red blood cells (RBCs), hematocrit (HCT) (p<0.05) and hemoglobin (HGB) (p<0.01) in comparison with control **(Figure 15.A.B.C.)**. Significant elevation in Mean Corpuscular Volume (MCV) (p<0.05), Mean Corpuscular Hemoglobin (MCH) (p<0.05) and mean corpuscular hemoglobin concentration (MCHC) (p<0.01) in (NaF) exposed fluoride rats compared to control **(Figure 15.D.E.F.)**.

Packed cell volume (PCV) is an alternative to hematocrit (HCT) for quantifying RBC mass. PCV is assessed by determining the percentage of RBCs per volume of blood present in centrifuged microhematocrit tubes(Goodrich & Behling-Kelly, 2019). Thus the reduction of HCT may be referring to reduction to RBCs number in those rats. Significant elevation in Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in NaF exposed fluoride rats compared to control. Those three parameters calculated as **MCV**= (HCT × 10)/RBC, **MCH**=(Hb × 10)/RBC and **MCHC** =(Hb/HCT)× 100 (Goodrich & Behling-Kelly, 2019).

Spleen functions include storing blood cells in response to hemorrhage and hypoxia or other stress, filtering or clearing old or damaged blood cells and pathogens, and maintaining iron metabolism(Wei *et al.*, 2022).

We observed in **(Figure 17)** high significant (p<0.01) increase in spleen MDA levels with very high significant decrease (p<0.001) of that of GSH indicating an oxidative state in NaF rats compared to control. (ZnO-NPs) administration decreased significantly MDA levels

($p<0.01$) simultaneously with increase of GSH levels ($p<0.001$) compared to NaF exposed rats.

Relative Spleen Weight (RSW) altered after NaF exposure which showed significant reduction ($P<0.05$) in RSW of NaF exposed rats compared to control, which ameliorated just in (ZnO-NPs) treated rats which showed high significant elevation ($P<0.01$) compared to (NaF) group, Reduction of RSW Maybe refer to cellular death and spleen damage as a result of accumulation of fluoride ion in spleen cells (Shashi & Thakur, 2022).

Conclusion & perspectives

Conclusion and perspectives

- The phytochemical findings demonstrate that this Spinach variety is rich source of phenolic and flavonoids compounds and other secondary metabolic, besides of his continent on calcium and zinc.
- We succeeded to realize Spinach extract mediated ZnO nanoparticles with a high quality and purity.
- The ZnO nanoparticles developed in this study were able to induce *In vitro* antioxidant and anti-inflammatory properties without any *In vivo* toxicity at the concentrations studied indicating its safety and beneficial therapeutic effect.
- But on the other hand, the study indicated that the prepared ZnO NPs had very low antibacterial activities compared to antibiotics at the concentrations studied.
- Fluoride could promote an imbalance of redox status. A mechanism of how NaF cause disturbance in redox balance and systemic toxicity in the testis, brain, thyroid gland, liver, heart, kidney, spleen and blood.
- Current study will attract people who under exposure to fluoride in drinking water to risk of anemia, dysthyroidism, renal failure, heart failure, bone alteration, reproduction perturbation , changes of electrolyte profile, neurological disorders and metabolic diseases.
- It will also bring awareness among the people to ovoid a long-term and high concentration of fluoride although the beneficial effect of this element on human body because actually it is one of important environmental pollutant.
- Hematological toxicity of sodium fluoride on blood component, spleen, heart and kidney explain the possible experimental NaF-induced anemia mechanism in rats.
- ZnO nanoparticles synthesized eco-friendly from spinach aqueous extract able to prevent sodium fluoride-induced hepato-nephrotoxicities through oxidative stress and apoptosis in rats, which could be due to its antioxidant and anti-inflammatory activities.
- ZnO nanoparticles correct organs weight coefficient and correct histological structure restoring complete normal appearance of different tissues.
- ZnO nanoparticles injection present very powerful antioxidant effect *In-vivo* translated by decreasing MDA level, increasing GSH level and increasing GST and GPx activities more even than control rats.

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