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Impact of Spinach (*Spinacia oleraceae*), Omega3 and Zinc supplementation on fluoride induced hypothyroidism in Wistar rats

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Anfal

Abstract

The main goal of this study was to evaluate the phytotherapeutic impact based on the Spinach (*Spinacia oleraceae*) and also that of biotheraeutic based on the Omega3 and Zinc to reverse the fluoride induced experimental hypothyroid disease and its complications in rats. For this purpose, twenty four female albino Wistar rats were randomly divided into 6 groups (n=4); the first group used as a control group. Group II given fluoride as fluoride sodium (NaF) (400 ppm) to induce hypothyroidism. Group III hypothyroid rats given Spinach powder (17 g/kg) of diet. Group IV hypothyroid rats given Omega 3 as (1.825g Eicosapentaenoic acid (EPA)+1.21g docosahexaenoic acid (DHA)/kg) of diet. Group V hypothyroid rats given Zinc as $ZnSO_4,7H_2O$ (0.230g/kg) of diet. Group VI hypothyroid rats given Spinach, Omega 3 and Zinc association. The exposure to fluoride for the hypothyroidism induction in rats is done during 70 days. As for the Spinach, Omega 3 and Zinc were added to the diet of the animals groups for last 15 days of hypothyroidism induction time. According to the results which have been obtained; there was an increase in serum TSH levels (70.53%) and a decrease in serum FT3 and FT4 levels (26.15% and 17.6%) respectively in hypothyroid group compared to the control. Results show also a significant ($P<0.05$) decrease in serum blood glucose, liver zinc and bone calcium levels and a significant ($P<0.05$) increase in lipid profile, electrolytes levels, markers of liver and renal functions in NaF induction of hypothyroidism group when compared to control rats. Moreover, the findings showed that hypothyroidism affected antioxidant defense system by decreasing Glutathione (GSH) level and Glutathione S transferase (GST) activity and increasing malondialdehyde (MDA) level in brain and kidney tissues. The histological examination of the cellular structure of the thyroid gland and kidney tissues of hypothyroid rats recorded changes in the follicular cells of the thyroid and in glomerulus and tubules of kidney tissues. However, the administration of Spinach, Omega 3 or Zinc alone ameliorated the thyroid function, biochemical parameters and restored the histological alterations while the combined treatment is the best improver for the thyroid dysfunction which had the totally restoring the biochemical parameters studied. In conclusion, present study demonstrated the beneficial effects of phytotherapy and biotherapy associated treatment on hypothyroidism and its metabolic and physiological complications.

Keywords: *Spinacia oleraceae*, Omega3, Zinc, Hypothyroidism, Fluoride.

الملخص

الهدف الرئيسي من هذه الدراسة هو تقدير تأثير العلاج النباتي بالاعتماد أساسا على نبات السبانخ (*Spinacia oleraceae*) وكذا العلاج الحيوي بالاعتماد على التوازن الغذائي اعتمادا على الأحماض الدهنية أوميغا 3 و عنصر الزنك لتحسين قصور الغدة الدرقية التجريبي ومضاعفاته والذي تم استحداثه بإدخال تراكيز عالية من الفلور لدى الفئران. لهذا الغرض، أجرينا دراستنا هذه على 24 فأرة تم تقسيمهن إلى ست مجموعات في كل مجموعة أربع فأرات، الأولى مجموعة شاهدة نظامها الغذائي عادي و مياه شربها خالية من أي إضافات تجريبية، المجموعة الثانية استحدث لديها قصور الغدة الدرقية تجريبيا بإعطائها الفلور على شكل ملح فلور الصوديوم (NaF) في ماء الشرب بتركيز 400 ملغ/ل مع نظام غذائي عادي لمدة 70يوم، المجموعة الثالثة تم علاجها بإضافة مسحوق أوراق السبانخ الجافة 17 غ/كلغ من الغذاء لمدة 15 يوما بعد استحداث قصور الغدة الدرقية لديهن لمدة 55 يوما مع استمراره خلال فترة العلاج، نفس العملية بالنسبة لباقي المجموعات المعالجة لكن في كل مرة نستبدل العلاج حيث عالجنا المجموعة الرابعة بالأحماض الدهنية أوميغا 3 أعطيت على شكل زيت الحوت والذي يحتوي على1.825 غ من الحمض الدهني Eicosapentaenoic acid (EPA) و1.21 غ Docosahexaenoic acid (DHA) /كلغ من الغذاء، المجموعة الخامسة عولجت بالزنك على شكل مسحوق كبريتات الزنك ($ZnSO_4, 7H_2O$) بتركيز 0.230 غ/كيلوغرام من الغذاء في حين أعطيت المجموعة السادسة مزيجا من كل هذه العلاجات بنفس التراكيز و هي منفردة. بالاعتماد على النتائج المتحصل عليها، تم تسجيل ارتفاع في الهرمون الحاث للغدة الدرقية (TSH) بنسبة 70.53% مع انخفاض في مستويات هرمونات الغدة الدرقية FT3 وFT4 بنسبتي 26.15 % و17.6 % على الترتيب، كما أظهرت النتائج أيضا انخفاضا معتبرا ($P<0.05$) في نسب السكر في الدم، الزنك في الكبد والكالسيوم في العظام مع ارتفاع معتبر ($P<0.05$) في مستويات الدهون، الشوارد، مؤشرات وظيفة كل من الكبد و الكلى في أمصال المجموعة المستحدث لديها قصور الغدة الدرقية مقارنة بالمجموعة الشاهدة. بالإضافة إلى هذا، أظهرت النتائج أيضا أن قصور الغدة الدرقية يؤثر على الأنظمة المضادة للإجهاد التأكسدي بتخفيض مستويات الغلوتاثيون (GSH) ونشاط الإنزيم (GST) مع رفع في مستويات النواتج الثانوية لأكسدة الدهون (MDA) في نسيج كل من الكلية والدماغ. المعاينة النسيجية للبُنَى الخلوية لنسيج كل من الغدة الدرقية والكلية لدى المجموعة المستحدث لديها قصور الغدة الدرقية أظهرت تشوهات واضحة على مستوى الخلايا الجريبية للغدة الدرقية وكذا الخلايا الوظيفية للكلية. من جهة أخرى سجلنا أن العلاج بواسطة: السبانخ، الأحماض الدهنية أوميغا3 أو الزنك منفردة حسن في معايير عمل الغدة الدرقية، المعايير البيوكيميائية وكذا في البُنَى النسيجية للأعضاء المدروسة، بينما أعطى مزيج كل هذه العناصر العلاجية أفضل النتائج من حيث تحسن وظيفة الغدة الدرقية والذي أدى إلى استعادة كاملة للمعايير البيوكيميائية و للبُنَى النسيجية قيد الدراسة. أخيرا، بينت هذه الدراسة فوائد دمج كل من العلاج النباتي والحيوي في علاج قصور الغدة الدرقية والتخفيف من مضاعفاتها الاستقلابية و الفزيولوجية.

الكلمات المفتاحية: السبانخ (*Spinacia oleraceae*)، أوميغا3، زنك، قصور الغدة الدرقية، فلور.

Figures List

Tables List

Abbreviation list

ADH: Antidiuretic hormone

AIT : Apical iodide transporter

ALA: α-linolenic acid

$AlCl_3$: Aluminium chloride

ALP: Alkaline phosphatase

ALT : Alanine Amino Transférase

APx: Ascorbate peroxidase

AST: Aspartate Amino Transférase

AVP: Vasopressin

BUN: Blood urea nitrogen

Ca^{++} : Calcium

CAT: Catalase

CDNB: 1-chloro-2,4-dinitrobenzene

DGDG: Digalactosyl diacylglycerol

DHA: Docosahexaenoic acid

DIT: Di-iodotyrosine

DPPH: 2,2-diphenyl-1-picrylhydrazyl

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

EDTA: Ethylenediaminetetraacetic acid

EPA: Eicosapentaenoic acid

F^-: Fluor

FT3 : Free Tri-iodothyronine

FT4 : Free Thyroxine

GDM: Gestational diabetes

GFPs: Goiter female patients

GFR: Glomerular filtration rate

GMPs: Goiter male patients

GOT: Glutamic oxaloacetic transaminase

GPT: Transaminase Pyruvic Glutamic

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced Glutathion

H_2O_2 : Hydrogen Peroxyde

H_2SO_4: Sulfuric acid

HCl: Hydrochloric acid

I^- : Iodide

K^+: Potassium

LDH: Lactate Dehydrogenase

LPO : Lipid peroxydation

MDA: Malondialdehyde

MDH: Malate Dehydrogenase

MGDG: Monogalactosyl diacylglycerol

MIT: Mono-iodotyrosine

Na^+: Sodium

NaCl : Sodium Chloride

NaF: Sodium fluoride

NaOH: Sodium hydroxide

NIS: Sodium/ Iodide transporter

OM3: Omega 3

PHT: Parathyroid hormone

PTU: Propylthiouracil

PUFA: Poly unsutured fatty acids

RAS: Renine angiotensine System

ROS: reactive oxygene species

RPF: Renal plasma flow

SOD: Superoxide dismutase

SUN: Serum urea nitrogen

TBA: Thiobarbituric acid

TBG: Thyroid binding globulin

TBS: Tris buffer Saline

TCA: Trichloroacétic acid

TFCs: Thyroid follicular cells

Tg: Thyroglobuline

TH: Thyroid hormones

TPO: Thyroperoxydase

TRH: Thyrotropin-releasing hormone

TSH: Thyroid stimulating hormone

Tyr: Tyrosine

INDEX

Introduction

Introduction

The human thyroid is a butterfly- shaped gland normally located in the lower part of the neck on either side of proximal aspect of trachea and lower part of thyroid cartilage (Brunová & Bruna, 2014; Harris, 2013). Thyroxine (T4), tri-iodothyronine (T3) and calcitonin are secreted by the thyroid gland. Both T4 and T3 are products of the follicular cells and influence the rate of all metabolic processes. Calcitonin is produced by the specialized C cells and influences calcium metabolism (Crook, 2012).Thyroid hormones play critical roles in differentiation, growth, and metabolism and are necessary for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (Kyriacou *et al.*, 2015).

Alterations in normal thyroid hormonal levels lead to biochemical/clinical abnormalities, such as hypothyroidism and hyperthyroidism (Messarah *et al.*, 2011), while the hypothyroidism is by far the most common thyroid disorder in the adult population and its prevalence increases with age (Ahme *et al*, 2012).

Oxidative stress is defined as the condition occurring when the physiological balance between oxidants and antioxidants is disrupted in favor of the former with potential damage for the organism. Thyroid dysfunction is postulated to be closely related to reactive oxygen species (ROS) formation. These dysfunctions associated with many pathological signs in the body. One of these is lipid peroxidation that develops due to over- or under-secretion of thyroid hormones (Messarah *et al*., 2010).

A good nutrition and a healthy balanced diet in our life can be a main cause of body balance and therefore diminution of disease prevalence. This is why we have based on herbal medicine on one side and the creation of a balanced diet on the other hand.

Health benefits of polyunsaturated fatty acids of the omega-3 family have been largely reported in the last decades. The most common ones are α-linolenic acid (C18:3, ALA), eicosapentaenoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA). They are reported as “essential fatty acids”, seem to be involved in the reduction of inflammation and may help lower risk of chronic diseases such as heart disease, cancer, and arthritis. As they are highly concentrated in the brain, they are also implicated in cognitive and behavioral functions (Crauste *et al.*, 2016)

Zinc is known to be an essential trace mineral that is necessary for health and growth and is also essential for the function and activity of more than 200 metalloenzymes, these enzymes are involved with the metabolism of protein, carbohydrate and lipids (Derouiche & Kechrid, 2016).

Plants have been used medicinally for thousands of years and are an important source of drugs. Some of the most common practices involve the use of crude plant extracts, which may contain a broad diversity of molecules with often unknown biological effects. For these reasons, medicinal plants have become the focus of numerous studies in order to evaluate their safety, efficacy and validate traditional uses. Vegetables play a significant role in the human diet and provide vitamins, fibers and minerals essential for human health and growth. For this reason, in the last decade, their consumption increased in all the socioeconomic groups of people living in rural or urban areas (Botondi *et al*., 2015). One of the most important green leafy vegetables and excellent source of nutritious element is so-called spinach.

Spinach (*Spinacia oleraceae* L.) is the most important leafy vegetable and also an important source of minerals and is a leafy cool season vegetable that produces a rosette during the vegetative stage (Citak & Sonmez, 2010).

The thyroid disturbance is a big problem that affects human health and trying to find a good treatment has become a necessity as the current drug remain for life. Thus, the present study was carried out to investigate the therapeutic impact of Spinach, Zinc and Omega 3 on hypothyroidism state also to evaluate the therapeutic impact of their combination on this disorder and its complications in the rats.

First part

Bibliographic synthesis

Chapter I

Thyroid gland

1. Thyroid anatomy and appearance

The thyroid is a butterfly-shaped gland located in the front of the neck, just above the trachea; the fully developed thyroid gland in a human is composed of two lobes connected by a thin band of tissue, the isthmus, which gives the gland the appearance of a butterfly (Burtis *et al.*, 2012).The thyroid gland is firmly attached to the trachea from the second to fourth tracheal ring (Fig.1.a). In normal adults, the average weight of the thyroid gland is 15-30 g (Youn *et al.*, 2014). The weight varies with iodine intake, sex, age, and functional status of the gland (Lloyd, 2010), more heavier in women than in men and increases in size during menstruation and pregnancy. It is brownish red in color and is highly vascular (Misra, 2011).

2. Histology of the thyroid gland

The thyroid gland displays peculiar and highly organized architecture characterized by the presence of spheroidal structures known as follicles (Jameson & De Groot, 2015). Which are the functional units of the thyroid for synthesis of T3 and T4. A follicle comprises one single layer of epithelial cells around a cavity, the follicle lumen, in which the colloid with thyroglobulin is stored (Fig.1.b) (Nyström *et al.*, 2011). The principal (or epithelial) cells vary in their appearance according to whether the cell is at the resting (or synthetic) or active (or secretory) phase. In the former phase, the epithelial cells have either a squamous or low cuboidal appearance. When they are in the active phase, these cells tend to be columnar (Kaufman *et al.*, 2010). In addition to the follicular cells, the thyroid gland is composed of other epithelial cell populations of different origin: the parafollicular C cells, devoted to calcitonin production; the epithelial cells, vestiges of the ultimobranchial body; and the thyroid stem cells (Jameson & De Groot, 2015).

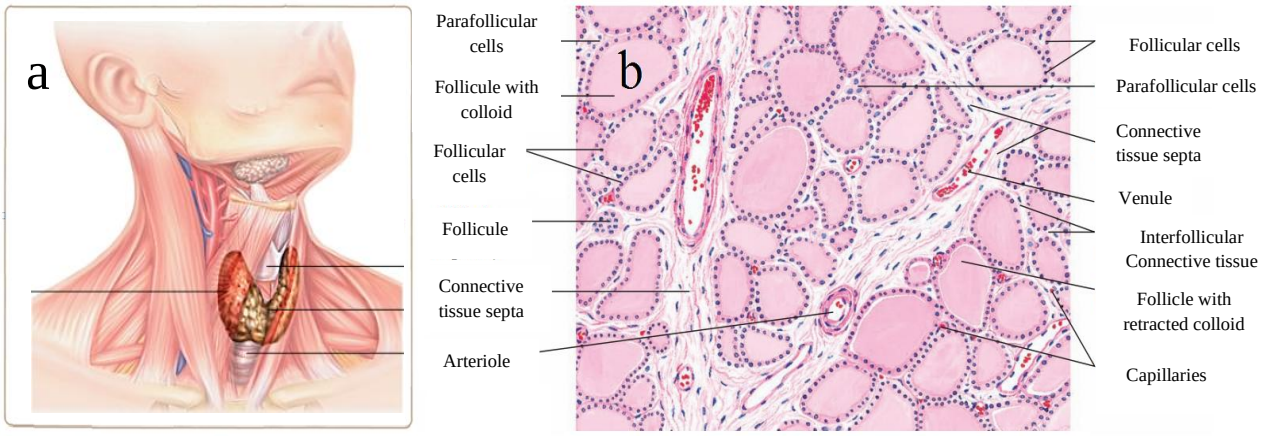

Figure 01: a) Thyroid anatomy (Wainsten, 2012).

b) Thyroid tissue (Victor, 2008).

3. Thyroidal metabolism

3.1. Thyroid hormones Biosynthesis (thyrocyte physiology)

Synthesis of thyroid hormones takes place via the following steps (Nyström *et al.*, 2011; Burman, 2012; Misra, 2011; Mike, 2010):

❖ Tg and TPO are synthesized in the thyroid follicle cells. TPO is then bound to the apical cell membrane.

❖ Iodide organification: in the thyroid gland, the iodide is converted back to iodine and is then organified by binding to tyrosyl residues, which are a part of glycoprotein, Tg that serves as the main storage form of TH.

❖ Iodide uptake into the TFCs, through the sodium iodide transporter NIS which concentrates iodide from the circulation approximately 30 times that of plasma.

❖ Iodide diffusion inside the cell to the apical membrane where it is transported into the follicular lumen through the pendrine and AIT (apical iodide transporter).

❖ Tyrosine iodination: At the apical surface of the follicular cell, the binding of iodine to the tyrosyl residues present in Tg. leads to the formulation of iodothyronines. Initial iodination of the tyrosine molecule occurs in the 3 position as the formation of mono-iodotyrosine MIT and the addition of a second iodide in the 5 position produces 3,5-di-iodotyrosine DIT. The thyroperoxidase TPO enzyme is a key player in this process, together with hydrogen peroxide H_2O_2.

❖ Iodothyronines coupling: MIT+DIT=T3, DIT+DIT=T4. Both of these hormones remain coupled to Tg within the matrix of the colloid.

❖ Hormone release requires the phagocytosis of iodinated Tg from the colloid into the TFCs in the form of vesicles, which are then transported to the lysosomes. Within the lysosomes, Tg undergoes proteolytic degradation releasing T4 and T3, which are then transported out of the cell into the circulation.

❖ Thyroid hormones are tightly bound to various plasma proteins (99%): the thyroid binding globulin (TBG), thyroxine binding prealbumin (transthyretin) and albumin, and the free fraction (1%) is the important active component. T4 is the main hormone released from the thyroid gland and it is transformed into biologically active 3',3,5-triiodothyronine (T3) via 5'-deiodinases of thyroid hormone target cells (Mike, 2010)

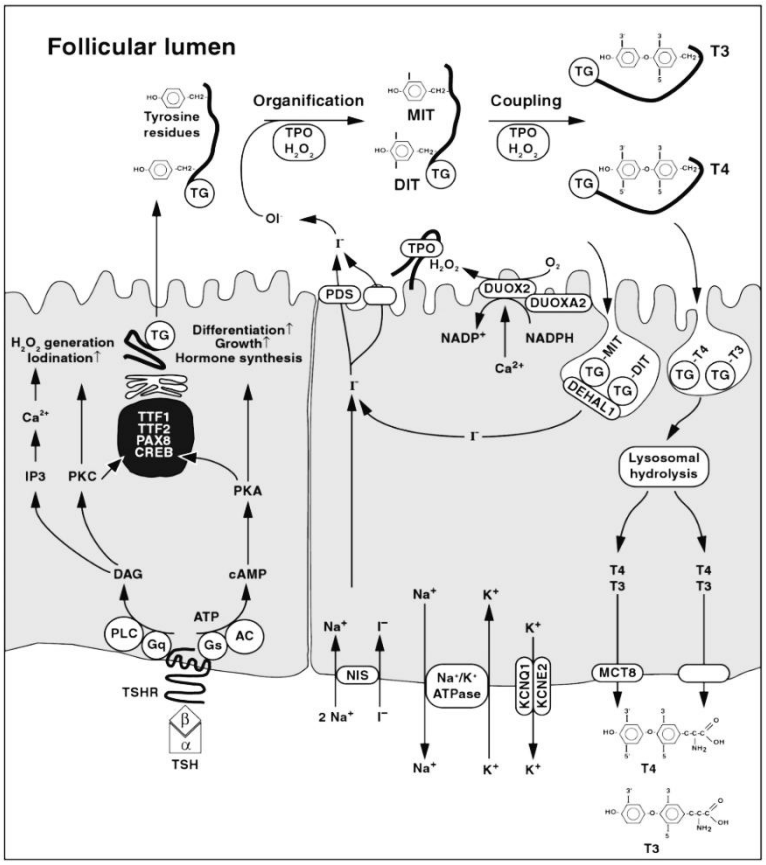

Figure 02: Thyroid hormones synthesis secretion and major signaling pathway in thyrocytes. AC, adenylyl cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; DEHAL1, iodotyrosine dehalogenase 1; DIT, diiodotyrosine; DUOX, dual oxidase 2; DUOXA2, dual oxidasematuration factor 2, KCNQ1 and KCNE2, potassium channel subunits; MCT8, monocarboxylate transporter8; MIT, monoiodotyrosine; NIS, sodium/iodide transporter; PDS, pendrin ; PKA, protein kinase A; PLC, phospholipase C; TG, thyroglobulin; TPO, thyroid peroxidase, TSH, thyrotropin; TSHR, TSH receptor (Braverman & Cooper, 2013).

3.2. Regulation of thyroid hormone production

Biosynthesis and secretion of thyroid hormones are regulated by hypothalamus-pituitary-thyroid axis, with the negative feedback loop at the level of both hypothalamus and pituitary gland. Namely, thyroid gland activity is positively regulated by thyroid stimulating hormone (TSH) synthesized and secreted from pituitary thyrotrophs, which activity is in turn controlled by hypothalamic TSH-releasing hormone (TRH).Under physiological conditions, circulating thyroid hormones suppress the release of TSH and TRH thus providing the so-called long negative feedback loop regulating thyroid function (Rajab *et al.*, 2015). Other mechanisms reported more recently include the inhibitory actions of the released TSH on TRH secretion and on TSH receptors in the pituitary itself. In sum, TSH secretion is influenced by thyroid-to-pituitary, thyroid-to-hypothalamus, pituitary-to-hypothalamus and pituitary-to-pituitary feedback control mechanisms, which combine to reduce fluctuations in circulating T3 and T4. In addition to the hypothalamus-pituitary-thyroid axis there is an intra-thyroid auto-regulatory mechanism; when intra-thyroid iodine concentration are significantly increased, the rate of TH synthesis is decreased, with a reduction in iodothyronine synthesis and decrease in the DIT/MIT ratio. This response is referred to as the Wolff-Chaikoff effect (Elgazzar, 2015).

4. Physiologic role (action)

Anatomically and embryologically, the thyroid gland is two glands in one: the thyroid follicular cells produce thyroid hormones and the parafollicular (or C) cells secrete calcitonin (Burtis *et al.*, 2012). Calcitonin lowers the calcium and phosphate ion concentration of the blood by inhibiting the release of calcium and phosphate ions from the bones and by increasing the excretion of these ions by the kidneys (Rizzo, 2015). Thyroid hormones play crucial roles in regulating development (growth), differentiation of many tissues and organs, as well as energy homeostasis and numerous key metabolic pathways of almost every tissue in the body of mammals, including teeth, bone and brain (Ge *et al.*, 2013; Liu *et al.*, 2015). Thyroid hormones (TH) increases oxygen consumption (Engelking, 2012).

The most important target tissues of thyroid hormones are the central nervous system, the cardiovascular system and the skeleton. Moreover, due to the increasing incidence of obesity worldwide, thyroid hormone action in adipose tissue has regained increasing interest for understanding energy homeostasis (Brix *et al.*, 2011).

5. Thyroid diseases

Diseases of the thyroid gland are the most common among endocrine disorders (Heymann, 2008). Thyroid dysfunctions comprise a complex network of diseases including malignant, inflammatory and autoimmune diseases of the thyroid gland. Functionally, thyroid diseases can be divided into hypo- and hyperthyroidism, according to thyroid hormone production (Zelzer *et al*., 2015). Thyroid disease can be characterized into four major groups: euthyroid enlargement, thyroid malignancy, thyrotoxicosis, and hypothyroidism. Goiter manifests itself as a nonneoplastic (noncancerous), noninflammatory enlargement of the thyroid gland. Hypothyroidism is a graded phenomenon, ranging from very mild cases in which biochemical abnormalities are present but the individual hardly notices symptoms and signs of thyroid hormone deficiency, to very severe cases in which there is a danger of sliding down into a life-threatening myxedema coma. Worldwide, hypothyroidism is most commonly due to iodine deficiency. However, in developed countries iodine deficiency is rare, and autoimmune thyroiditis is the most common cause followed by destructive therapy (radiation or surgery) for thyrotoxicosis or malignancy. Various drugs may also suppress thyroid function; very rarely, hypothyroidism may have a pituitary cause with failure of TSH production. The most common cause of hyperthyroidism (or thyrotoxicosis) is Graves' disease, but other causes include toxic nodular goiter, thyroiditis, follicular carcinoma, pregnancy(especially molar pregnancy), TSH- secreting pituitary adenoma, and drug-induced hyperthyroidism(e.g. during amiodarone therapy) (Mike, 2010; Colville & Bassert, 2016).

5.1. Signs and Symptoms

Typical symptoms of hypothyroidism include lethargy, fatigue, loss of energy and ambition, slowing of intellectual and motor activity, decreased appetite; increased weight; skin that is dry, cold intolerance, coarse, hair loss including loss of the outer third of eyebrows, bradycardia, hypotension, increased capillarity fragility, osteoporosis, and goiter. Hypothyroid patients show an exaggerated response to central nervous system depressants such as sedatives and narcotic analgesics (Liu *et al.*, 2015; Yagiela *et al.*, 2011). While in hyperthyroidism, reverse symptoms such as: Weight loss, sweating, tremor, maybe a goiter, agitated and nervous, easy fatigability, heat intolerance, tachycardia, Diarrhea, muscle weakness and loss of muscle mass (Tillett & Jewels, 2005)

Chapter II

Fluoride

& oxidative stress

I. Fluoride

1. Definition

Fluorine, a strongly electronegative member of the halogen group is one of the most reactive elements, forming fluoride ions in solution and capable of forming binary compounds with all other elements except helium, neon, and argon. These properties reflect the position of F in the periodic table (Kirk, 1991; Banji *et al.*, 2013). F is element number 9. It is a fairly common element—13th in order of abundance. In Earth's crust, it is found mostly in minerals that contain the fluoride ion (F^-). The fluoride ion, however, is extremely difficult to reduce safely to neutral fluorine, which occurs in the form of gaseous diatomic molecules, F_2. Fluorine gas is extremely hazardous to work with, so only specially trained persons under carefully controlled conditions handle it in that form (Halka, & Nordstrom, 2010).

2. Chemical and physical proprieties

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. Technology and current uses

At one time, liquid fluorine and liquid hydrogen were used as rocket fuel. Other fluorine compounds are used to make industrial acids, refrigeration chemicals, and insecticides. Fluorine is used in the enrichment of uranium (U), a highly radioactive element. Enriched uranium is used as fuel in nuclear reactors. A form of fluorine is also added to toothpaste and water supplies to help prevent cavities (Greg, 2010). Fluoride is an essential trace element to the human body, for decades it has been employed for the prevention of dental caries. Meanwhile, as a pervasive natural pollutant, fluoride can normally enter into the human body through drinking water, food, industrial pollution, drugs, cosmetics, etc. However, drinking water is the major source of daily intake of fluoride (Thangapandiyan & Miltonprabu, 2015).

4. Metabolism

4.1. Absorption and transport

Fluoride compounds can be absorbed via different routes including the gastrointestinal tract which is the major route of fluoride uptake, respiratory tract, and through skin and mucous membranes. In animals, fluoride is absorbed mostly in the stomach (in simple-stomach animals), rumen, abomasum (in ruminants), and upper intestine by a passive process, although its excretion into the mucosa of the intestine is by active transport (Ranjan & Ranjan, 2015).

Fluoride absorption is rapid, diffusing already across the stomach wall into the blood, and it continues in the small intestine, but, when consumed with foods and beverages, fluoride forms complexes with proteins and minerals, which may reduce its absorption to 50–80% (Bhardwaj & Aggarwal, 2013). The rate and extent of F absorption in the gastrointestinal tract is also regulated by several other factors, the most important among them being the chemical nature and solubility of fluoride compounds. In general, inorganic F compounds are more soluble and hence rapidly and extensively absorbed from the gastrointestinal tract. Other factors modulating F absorption include species, sex, and age of the animal and presence of dietary components including minerals (divalent cations), fat, protein, and fiber (Ranjan & Ranjan, 2015). Depending on (Whitford, 2005), Almost all fluoride naturally present in water is absorbed when the gastrointestinal tract is empty. However, the rate and extent of F absorption decreases in the presence of milk or other food items. It was observed that F from sodium fluoride or disodium mono-fluorophosphate was absorbed up to 100% when ingested

along with water in a fasting state. However, a decrease in F absorption by 20–30 % occurs when they are ingested along with milk or baby formula.

4.2. Distribution and elimination

Irrespective of the route of exposure, absorbed fluoride reaches into blood circulation which acts as a transport medium and carries F to different body parts. From plasma, F migrates across cell membranes of nearly all soft tissues which have a steady-state tissue-to-plasma concentration ratio ranging from 0,5 to 0,9. Exceptions to this are brain and fat which have a considerably lower ratio and kidneys which have a higher ratio because fluoride is concentrated in the renal tubular fluid. In general, fluoride concentration in soft tissues is very low and does not vary with age. It is maintained within a narrow limit and shows only marginal variation even during excess F uptake or fluctuations in plasma F levels. Intracellular F in soft tissues is readily exchangeable with F present in extracellular fluid. In a large-scale survey of cattle suffering from industrial fluorosis, it was observed that F accumulated in the liver, lung, kidney, cerebrum, cerebellum, thyroid, and pituitary, but not in the heart, musculature, and spleen; and approximately 99% of the body F burden is associated with the calcified tissues. Bone, as in the case of lead, acts as a natural sink for fluoride; F is not irreversibly bound to bone; it is released either after cessation of excess F uptake or when the diet cannot sustain mineralization of the skeleton (e.g., during lactation). Fluoride is released from bone in two phases. The first phase is a rapid process that takes weeks and involves anion exchange in the hydration shell. The second phase is slower with an average half-life of 8 years and occurs due to osteoclastic resorption of the bone. F is released slowly from compact bones in comparison to trabecular bones (Ranjan & Ranjan, 2015).

Unabsorbed fluoride from the gastrointestinal tract is eliminated through feces, whereas a part of F present in the systemic circulation is excreted through different routes. Once fluoride uptake is reduced, fluoride deposited in bone starts mobilizing slowly and is excreted in urine and feces. In almost all mammals, the kidney plays a key role in fluoride excretion. Other routes of fluoride elimination and excretion include saliva, milk, and perspiration. Some studies on human and laboratory animals suggested that F concentration in hair may serve as an unobtrusive way to determine the body F burden Fluoride concentration in fingernail clippings in humans can also serve as a biomarker of F exposure, although it only reflects F intake about 3–6 months before (Ranjan & Ranjan, 2015).

5. Toxicity

The halogens are toxic materials. Their toxicity together with their reactivity decreases from fluorine to iodine (Leo & Leen, 2014).

4.1. Fluoride and thyroid function

Excess fluoride can accumulate in the thyroid gland, affect iodine metabolism of thyroid follicle cells, disturb synthesis and secretion of thyroid hormone, and then induce incidence of goiter. But time is an important factor for fluoride damage to the thyroid gland (Ge *et al*., 2013)

4.2. Toxicology and Pathological variations

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).

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 (Martínez-Mier, 2011):

- 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.

II. Oxydative stress

1. Definition

Oxidative stress was caused by an imbalance between antioxidant systems and the production of oxidant including reactive oxygen species (ROS) and was involved in alteration of physiologically critical molecules such as proteins, lipids, carbohydrates and nucleic acids and generated damage to the tissues (Assaâd S.*et al.*, 2015). Lipid peroxidation (lipid oxidation) is a phenomenon that takes place in several stages: initiation, propagation and termination (Bauchard & Picard, 2010).

2. 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 ($ONOO^-$) (Lanez & Djouadi, 2015).

3. 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).

4. Fluoride and oxidative stress

Fluoride (F^-) can readily penetrate cellular membranes and cause adverse effects on cellular metabolism and function. Several studies have focused on oxidative stress as one of the accepted mechanisms of F^- toxicity. F^- induces oxidative stress in the brain (Nabavi *et al*., 2012; Sarkar *et al*., 2014), liver (Atmaca *et al*., 2014), kidneys (Qing *et al*., 2014) and blood (Inkielewicz-Stepniak & Czarnowski, 2010). 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).

Chapter III

Spinacia oleraceae

1. Definition

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. Plant 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 03: *Spinacia oleracea*

3. 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) (Youn *et al.*, 2016). Depending on (Gupta & Igamberdiev, 2015) the spinach contain 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 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). Spinach is known to contain significant amounts of oxalates (Wen *et al.*, 2017) and it is contain trace elements such as selenium, cooper, iron and zinc (Güler *et al.*, 2016).

3.1. Biological role of zinc

3.1.1. Role of Zinc 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).

3.1.2. Biological importance of Zn 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).

3.1.3. Zinc and 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).

3.1.4. Zinc and stress oxidative

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).

3.2. Fatty acids (Omega 3)

3.2.1. Definition and physiological role

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).

4. Pharmacological activities of spinach

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 (Peerzada *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.*, 2014); Spinach extracts exhibit significant antigenotoxic activity (Sang-Heui *et al.*, 2014), spinach characterized also by anti-histaminic, CNS depressant, protection against gamma radiation, hepato-protective and antimicrobial effects (Rao *et al.*, 2015).

Second Part

Experimental Part

Chapter I

Materials and methods

1. Materials

1.1. Plant material

In this study, Leaves of spinach (*Spinacia oleraceae*) was obtained from the same farm. 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

In this study, 24 female Wistar rats at the age of 8 weeks old, weighting 180-230g, 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 23± 2°C, relative humidity 65,3%, 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 hypothyroidism induction

Hypothyroidism state was induced by oral administration of sodium fluoride (400 ppm) in drinking water during 70 days. Therefore, treatment of NaF to normal rats is usually considered as a model of hypothyroidism. The effectiveness of NaF in inducing a hypothyroid state is confirmed by noting reduction in serum T with a concomitant increase in TSH level. To proof the efficiency of hypothyroidism fluoride induced model, 8 rats were divided into two groups (n =4 each). Control group received standard rat food and water during the study

and hypothyroidism group received standard rat food and 400 ppm of sodium fluoride NaF in their drinking water during 3 weeks.

1.4. Experimental design

The animals were acclimatized for 2 weeks in the Animal House of molecular and cellular biology department, of El-Oued University, Algeria before induction of hypothyroidism in them. Animals were divided into six groups (n =4), hypothyroidism was induced by oral administration of 400 ppm in drinking water for 70 days:

- Control (C): Received standard diet and water during the study.
- Hypothyroidism (HypoT): Received standard diet and 400 ppm of sodium fluoride (NaF) in their drinking water.
- Hypothyroidism+ Spinach (HypoT+SP): Received standard diet with spinach powder (17g/kg feed) and NaF in their drinking water.
- Hypothyroidism+ Omega3 (HypoT+O3): Received standard diet with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (1,825g EPA+1,21g DHA /kg feed) and NaF in their drinking water.
- Hypothyroidism+ Zinc (HypoT+Zn): Received standard rat food with Zinc (0,230g/kg feed) as $ZnSO_4$, $7H_2O$ and NaF in their drinking water.
- Hypothyroidism+ Spinach/ Omega3,Vitamin E/Zinc (HypoT+Asso): Received standard rat food with the same previously doses and NaF in their drinking water.

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 choloroform (94%) by inhalation; blood sampling was performed during the decapitation and blood samples were transferred into EDTA tubes to carried FNS 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 and bone were rapidly excised, weighed and stored at -20°C for oxidative stress parameters, some minerals levels and histological analysis.

2. Methods

2.1. Crude extract preparation

Aqueous and organic extracts were prepared by putting 10 g of the homogenized dried leaves and 100 mL of distilled water and 100 mL of methanol 60% at 50°C during 30 minutes then the extract is filtered and concentrated using a rotary evaporator.

2.2. Phytochemical analysis

According to the methods of (Harbone, 1986) six assays were used to identify phytochemical compounds of alkaloids, saponins, glycosides, tannins, phenols and flavonoids, in each sample.

2.2.1. 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.

2.2.2. 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.

2.2.3. 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.

2.2.4. 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%).

2.2.5. 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.

2.2.6. 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.

2.3. Total phenolic and flavonoid compounds

2.3.1. Total phenolic

Polyphenols are determined by the Folin-Ciocalteu method. This method, initially described by (Slinkard & Singleton, 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.

2.3.2. 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.

2.4. DPPH test

The DPPH• solution is prepared by solubilizing 2.4 mg of DPPH• in 100 ml of methanol. 1mL of each phenolic extract is added to 1mL of the DPPH• solution prepared previously. The reaction mixture is shaken immediately, and then maintained in the dark for 30 min at an ambient temperature for the reaction is accomplished. The absorbance of the reaction medium is measured at 517 nm against the control (Mansouri *et al.*, 2005). Inhibition percentage (IP) = $[(A_{control} - A_{sample}) \times 100]/A_{control}$ where $A_{control}$ is the absorbance of the control (containing all reagent except the sample), and A_{sample} is the absorbance of the sample, both measured at517 nm.

2.5. Determination of IC_{50} for DPPH activity

The IC_{50} (DPPH) value, which represents the concentration of extract that gives 50% reduction in DPPH absorbance (free radicals level), was determined by linear regression analysis of inhibition percentage versus concentration.

2.6. Method of chromatographic analysis by HPLC

The extracts of *Spinacia oleraceae* were filtered before injection. An HPLC system was used with a detector at λ = 280 nm for the polyphenols and 360 nm for the flavonoids, the experimental conditions being as follows:

The column used is of length 150 mm and diameter 4.6 mm, the stationary phase C18; Mobile phase: A: acetonitrile; B: 2% glacial acetic acid solution (pH = 2.6); Gradient: 0-5min: 5 % A ; 25-30min: 35% A ; 35-45min: 70%A ; Debit: 0.5 ml / min; Injection volume: 20µL; Temperature: 30 °C.

2.7. Hormonal parameters

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

2.8.Tissue zinc and calcium analyses

Dried livers and bone were heated in silica crucibles at 600°C for 6 hours, and the ash was dissolved in hot nitric acid for zinc and calcium using a flame atomic absorption spectrophotometer (Shimadzu AA-6200; Somerset, New Jersey, USA) and Flame photometer (Jenway PFP7) respectively. In the zinc and calcium tissues was determined after 20-fold

dilution. The zinc standards were prepared from a 1 mg/mL zinc sulfate and calcium standards were prepared from a 1 mg/mL calcium carbonate. All tubes were soaked in nitric acid (10%) and rinsed with doubly distilled water.

2.9. Hematological parameters

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

2.10. Biochemical parameters

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

2.10.1. 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).

2.10.2. 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

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

2.10.3. 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

2.10.4. 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:

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

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

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

2.10.5. 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

2.10.6. 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)

2.10.7. Uric acid

Uric acid is dosed according to the following reactions, Wavelength: 510 nm (490-550):

$$\text{Uric acid + } 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoine + } CO_2 + H_2O_2$$

$$2H_2O_2\text{+Amino-4-antipyrine+Dichloro2-4 Phenolsulfonate} \xrightarrow{\text{Peroxydas}} \text{Quinone rose+ } 4H_2O$$

2.10.8. Cholesterol

Cholesterol is measured after enzymatic hydrolysis and then oxidation. The quinoneimine indicator is formed from hydrogen peroxide and amino 4 antipyrin in the presence of phenol and peroxidase.

Enzymatic determination according to the following reactions:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesten-4-one-3} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{Amino-4-antipyrine} \xrightarrow{\text{Peroxydas}} \text{Quinoneimine rose}$$

The amount of quinoneimine formed is proportional to the concentration of cholesterol. Wavelength: 505 nm (500 - 550)

2.10.9. Triglyceride

The triglycerides are determined according to the following reactions, Wavelength: 505 nm (490-550):

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Gylcerol} + \text{ATP} \xrightarrow{\text{Glycerokinase, } Mg^{++}} \text{Glycerol-3-P} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{Glycerol-3- Phosphate oxydase}} H_2O_2 + \text{Dihydroxyacétone-P}$$

$$H_2O_2 + \text{Amino-4-Antipyrine} + \text{chloro-4-phenol} \xrightarrow{\text{Peroxydase}} \text{Quinone rose} + H_2O$$

2.10.10. Sodium and potassium

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

2.11. Oxidative stress parameters

2.11.1. 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 5000 revolutions/min for 15 min at 4°C, and the obtained supernatant was used for the determination of antioxidant activity.

2.11.2. 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.

2.11.3. Determination of reduced glutathione (GSH) level

GSH concentration was performed with the method described by (Weckbercker, 1974) 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.

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

Glutathione-S-transferase(GST) activity of tissues was measured spectrophotometrically by the method of (Habig *et al*., 1988) 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.

2.12. Histopathological study of the thyroid and kidney tissues

At the end of the experimental period, the rats were sacrificed after inhalation anesthesia and thyroid and kidney tissues were removed and immersed for 48 h in a fixative solution (solution 4% formaldehyde, in phosphate buffer, pH＝7.6), dehydrated in ascending graded series of ethanol, cleaned with toluene, immersed in paraffin, and colored with hematoxylin and eosin. Histopathological evaluation was performed with a light microscope.

2.13. Statistical analysis

Our statistical study is carried out by the software program (Minitab) 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 24 rats divided into 6 groups. Differences were considered statically significant at $p<0.05$.

Conclusion and perspectives

Conclusion and perspectives

Because of its importance in all body parts, the disturbance of thyroid gland generates many harmful problems and complications may results in the death. Therefore, the main goal of this study was to assess the phytotherapy impact based on the spinach (*spinacia oleracea*) and also that of biotherapy based on the zinc and omega3 to reverse the experimental hypothyroid disease induced by fluoride in rats and its complication in the body. In conclusion, our data showed that:

The phytochemical findings demonstrate that this Spinach variety is rich source of phenolic and flavonoids compounds, beside of his contenant on calcium and zinc.

The observed abnormalities in thyroid gland profile corrected partially by the alone administration of our different treatments spinach, omega 3 and zinc; whereas the combined treatment is the best improver for the thyroid dysfunction which had totally restoring the markers of the thyroid function.

Our treatments alone or combined ameliorate the hepatic function by decreasing ALT and ALP activities; also the metabolic marker: glucose, cholesterol and triglycerides were regulated after different treatments administration when those last present a big anti-inflammatory impact.

In the level of bone alteration, the individual or associated treatments regulate the balance between bone and serum calcium hence improving the bone alteration.

Overall, we demonstrated that every our alone treatment ameliorate a part of hypothyroidism complications whose Omega-3 fatty acids had more beneficial effects on lipid perturbation associated to hypothyroidism state while the Spinach revealed the best improving impact on bone alteration, so the zinc is the best corrector of the thyroid function itself and the histological appearance of thyroid tissue supported this.

All our treatments attenuated the oxidative damages by inhibiting ROS generation and reversing antioxidant enzyme activities in the brain and kidney, whereas the Omega 3 counteract strongly the lipid peroxidation associated with fluoride induced hypothyroidism in the brain but in the kidney the combined treatment present the main anti-oxidant defense system which confirmed by the histological analysis of kidney tissues.

The renal function, as a main biological system in the body, not restored neither in alone nor combined treatments. For this purpose, and to complete this study future studies with longer duration of the treatments intervention, or with a higher or miner doses of our different supplements and bigger sample size are needed to confirm the validity of our original findings.

In our next study, therefore, we will try to examine the influence of these treatments individual or combined in the hyperthyroidism state. Also we will try to detect all compounds responsible to the improver impact of thyroid function and their appropriate doses too the cellular and molecular mechanism of these molecules to obtain a final drug against both hyper- and hypothyroidism disorders and helping the people ill.

References

References

Ahme, O.M., Gabar, M.A. Tarek & Ali, M. (2012). Impacts of the coexistence of diabetes and hypothyroidism on body weight gain, leptin and various metabolic aspects in albino rats. *Journal of Diabetes and Its Complications*, 26 (2012), 491– 500.doi: 10.1016/j.jdiacomp.2012.05.021

Ahn, M.-R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T., *Food Chem.* 101(2007) 1383–1392

Amir Reza, K. B.(2011). Effect of the spinach (Spinacia oleracea) on blood glucose and insulin levels in diabetic rats. Doi: 10.1016/j.clinbiochem.2011.08.817

Ashworth, S. & Whealy, K.(2002). Seed to Seed: Seed Saving and Growing Techniques for the Vegetable Gardener. USA: Chelsea Green Publishing.

Atmaca, N., Atmaca, H.T., Kanici, A. & Anteplioglu, T. (2014). Protective effect of resveratrol on sodium fluoride-induced oxidative stress, hepatotoxicity and neurotoxicity in rats. *Food and Chemical Toxicology*,70 (2014), 191–197. doi: org/10.1016/j.fct.2014.05.011

Azuma, M.M., Gome s-Filho, J.E., Ervolino, E., Pipa, C.P., Morais Cardoso, C., Andrada, A.C., Kawai, T., & Angelo Cintra, L.T. (2017). Omega 3 Fatty Acids Reduce Bone Resorption While Promoting Bone Generation in Rat Apical Periodontitis. *J Endod*, 2017(),1–7. doi: org/10.1016/j.joen.2017.01.006

Banala,R.R., Karnati, P.R.(2015). Vitamin A deficiency: An oxidative stress marker in sodium fluoride (NaF) induced oxidative damage in developing rat brain. *Int. J. Devl Neuroscience*, 47 (2015), 298–303.doi: org/10.1016/j.ijdevneu.2015.08.010

Banji, D., Otilia, J.F., Gouri, P. & Annamalai, A.R. (2013). Investigation on the role of Spirulina platensisin ameliorating behavioural changes, thyroid dysfunction and oxidative stress in offspring of pregnant rats exposed to fluoride. *Food Chemistry*, 140 (2013), 321–331. doi: org/10.1016/j.foodchem.2013.02.076

Barbara, S. P., Beata B., Katarzyna B.& Ewa B. (2012).The influence of vitamin E and methionine on the activity of enzymes and the morphological picture of liver of rats intoxicated with sodium fluoride. *Food and Chemical Toxicology* , 50 (2012), 972–978.doi: 10.1016/j.fct.2012.01.014

Bashandy,S.A., Omara, E.A., Ebaid, H., Amin, M.M. & Soliman, M.S. (2016). Role of zinc as an antioxidant and anti-inflammatory to relieve cadmium oxidative stress induced testicular damage in rats. *Asian Pacific Journal of Tropical Biomedicine,* doi: 10.1016/j.apjtb.2016.08.016

Bauchard, D.& Picard, B. (2010). Muscle et viande de ruminant. Paris: Ed. Quae.

Belayneh, H.D., Wehling, R.L., Cahoon, E. & Ciftci, O.N.(2015). Extraction of omega-3-rich oil from Camelina sativaseed using supercritical carbon dioxide. *J. of Supercritical Fluids* ,104 (2015), 153–159.doi: org/10.1016/j.supflu.2015.06.002

Bhardwaj, M. & Aggarwal, S.(2013). Evaluation of biochemical interaction and correlation between high fluoride ingestion and protein metabolism. *Biomedicine & Preventive Nutrition*, 3 (2013), 129–137.doi: org/10.1016/j.bionut.2012.11.003.

Bhatia A.L.& Jain M.(2004). Spinacia oleracea L. protects against gamma radiations: a study on glutathione and lipid peroxidation in mouse liver. *Phytomedicine*, 11(2004), 607–615.doi: 10.1016/j.phymed.2003.07.004

Botondi, R., Bartoloni, S., Baccelloni, S. & Mencarelli, F.(2015). Biodegradable PLA (polylactic acid) hinged trays keep quality of fresh-cut and cooked spinach. *J Food Sci Technol* , 52(9), 5938–5945.doi: 10.1007/s13197-014-1695-x

Brandao-Neto , J., Conceiçao Ribeiro Dantas Saturnino, A., Dantas Leite, L., Dantas de Medeiros Rocha, E., Passos Marcos, C.M., Bruno da Silva, C.A., Sergio Marchini, J., Augusto de Rezende, A., Graças Almeida, M. & Cunha Medeiros, A. (2006). Lack of acute zinc effect on thyrotropin-releasing hormone–stimulated thyroid-stimulating hormone secretion during oral zinc toleranc test in healthy men. *Nutrition Research*, 26 (2006), 493 – 496.doi: 10.1016/j.nutres.2006.08.010

Braverman, L.E. & Cooper, D.S. (2013). Werner & Ingbar's The Thyroid: A Fundamental and Clinical Text. Chaina: Lippincott Williams & Wilkins.

Brix, K., Führer, D. & Biebermann, H. (2011). Molecules important for thyroid hormone synthesis and action - known facts and future perspectives. *Thyroid Research*, 4(1),1-6.doi: 10.1186 /1756-6614-4-S1 -S9

Brunová, J. & Bruna, J. (2014). Clinical Endocrinology and Diagnostic Imaging. Karolinum Press.

Burman, K.(2012). Thyroid Disorders and Diseases, An Issue of Medical Clinics (volume 96). **USA:** Elsevier Health Sciences.

Burtis,C.A., Ashwood,E.R.& Bruns; D.E.(2012). Tietz Textbook of Clinical Chemistry and Molecular Diagnostics.USA: Elsevier Health Sciences.

Cakmak I.(2000). Role of zinc in protecting plant cells from reactive oxygen species. *New Phytol*,146:185–205.

Calvo, R.M. & Obregon, M.J. (2011). Presence and regulation of D1 and D2 deiodinases in rat white adipose tissue. Metabolism clinical and experimental, 60 (2011), 1207– 1210.doi: 10.1016/j.metab ol.2011.01.014

Cao,J., Chen, J., Xie, L., Wang, J., Feng, C. & Song, J.(2015). Protective properties of sesamin against fluoride-induced oxidative stress and apoptosis in kidney of carp (Cyprinus carpio) via JNKsignaling pathway. *Aquatic Toxicology*, 167(2015),180–190.doi: org/10.1016/j.aquatox.2015.08.004

Chattopadhyay, S., Choudhury, S., Roy, A., Chainy, G.B.N. & Samanta, L. (2010). T3 fails to restore mitochondrial thiol redox status altered by experimental hypothyroidism in rat testis. *General and Comparative Endocrinology*, 169 (2010), 39–47.doi: 10.1016/j.ygcen.2010.07.014

Chinoy, N.J.(2003). Fluoride stress on antioxidant defence systems. *Fluoride* 36, 138–141.

Christophe, P. & Christophe, S.(2011). Physiologie pathologie et thérapie de la reproduction chez l'humain . paris : Ed. spriner-verlag.

Citak, S.& Sonmez, S.(2010) Effects of conventional and organic fertilization on spinach (Spinacea oleraceaL.) growth, yield, vitamin C and nitrate concentration during two successive seasons. *Scientia Horticulturae*, 126 (2010), 415–420.doi: 10.1016/j.scienta.2010.08.010

Colville, T.P. & Bassert, J.M. (2016). Clinical Anatomy and Physiology for Veterinary Technicians (3rd ed.). Canada: Elsevier Health Sciences.

Crauste, C., Rosell, M., Durand, T. & Vercauteren, J.(2015). Omega-3 polyunsaturated lipophenols, how andwhy?.*Biochimie,*120(2016),62–74.doi: org/10.1016/j.biochi.2015.07.018

Crauste, C., Rosell, M., Durand, T.&Vercauteren, J.(2016). Omega-3 polyunsaturated lipophenols, how and why?. *Biochimie*, 120 (2016), 62–74.doi: org/10.1016/j.biochi.2015.07.018

Crook, M.A.(2012).Clinical Biochemistry and Metabolic Medicine (8th ed.). CRC Press.

De Souza dos Santos, M.C., Lima Gonçalves,C.F., Vaisman, M. Freitas Ferreira, A.C. & de Carvalho, D.P.(2011). Impact of flavonoids on thyroid function. *Food and Chemical Toxicology*, 49 (2011), 2495–2502.doi: 10.1016/j.fct.2011.06.074

DeLong, L. & Burkhart, N.(2013). General and Oral Pathology for the Dental Hygienist (2nd ed.). China: Lippincott Williams & Wilkins.

Derouiche, S. & Djouadi, A.(2017). An evaluation of stress oxidative and serum electrolytes in female hypothyroid patients. *International Journal of Biological & Medical Research*, 8(1),5861-5865. doi:

Derouiche, S. & Kechrid, Z. (2013). Influence Of Calcium Supplements On Zinc Status, Carbohydrate Metabolism And The Liver Activity Of Detoxifying Glutathione Enzymatic System In Alloxan-Induced Diabetic Rats. *Journal of Experimental Biology and Agricultural Sciences*, 1(6),425-429.

Derouiche, S. & Kechrid, Z.(2016). Zinc Supplementation Overcomes Effects of Copper on Zinc Status, Carbohydrate Metabolism and Some Enzyme Activities in Diabetic and Nondiabetic Rats. *Can J Diabetes*, xxx (2016), 1–6.doi: org/10.1016/j.jcjd.2016.02.005

Derouiche, S., Djermoune, M., Abbas, K.(2017). Beneficial Effect of Zinc on diabetes induced kidney damage and liver stress oxidative in rats. Journal of Advances in Biology, 10(1),2050-2055.

Dilina do Nascimento Marreiro, Kyria Jayanne Clímaco Cruz, Jennifer Beatriz Silva Morais, Jéssica Batista Beserra, Juliana Soares Severo and Ana Raquel Soares de Oliveira. (2017).Zinc and Oxidative Stress: Current Mechanisms. *Antioxidants.*, 6(2): 24-33.

Elgazzar, A.H. (2015). The Pathophysiologic Basis of Nuclear Medicine (3rd ed.). London: Springer.

El-Moghazy, M., Zedan, N.Z., El-Atrsh, A.M., El-Gogary, M.& Tousson, E.(2014). The possible effect of diets containing fish oil (omega-3) on hematological,

biochemical and histopathogical alterations of rabbit liver and kidney. *Biomedicine & Preventive Nutrition*, xxx (2014), xxx–xxx.doi: .org/10.1016/j.bionut.2014.03.005

Eman, S.H., Gomaa, A. & Manal, M.S. (2014). The effect of omega 3 on cognition in hypothyroid adult male rats. *Acta Physiologica Hungarica*, 101 (3), 362–376. doi: 10.1556/APhysiol.101.2014.3.11

Engelking L.*(*2012).Metabolic and endocrine physiology.USA: Ed. Tenton New Media.

Fatma, Y. G., Suleyman, A., Hayriye, E. A., Betul,C. T., Faik, C.,Fatih, T., Rabia, Y.M. & Mustaf a, Y.(2016). FT3/FT4 ratio predicts non-alcoholic fatty liver disease independent of metabolic parameters in patients with euthyroidism and hypothyroidism. *CLINICS*, 71(4), 221-225. doi: 10.6061/c linics/2016(04)08

Félicien, M. K., Adrien, T. M., Justine K. M., Archimède, B. M., Damien, S.T T., Koto-Te-Nyiwa, N., Justin, N. K. & Pius, T. M. (2016). *International Journal of Herbal Medicine*, 4(6), 14-19.

Galecka, E., Talarowska, M., Maes, M., Su, K.P., Gorski, P. & Szemraj, J. (2016). Polymorphisms of iodothyronine deiodinases (DIO1, DIO3) genes are not associated with recurrent depressive disorder. *Pharmacological Reports*, 494, 1–5.doi: org/10.1016/j.pharep.2016.04.019

Ge, Y., Ning H., Gu X., Yin M., Yang X., Qi Y. & Wang J. (2013). Effects of High Fluoride and Low Iodine on Thyroid Function in Offspring Rats. *Journal of Integrative Agriculture*, 12(3), 502-508. doi: 10.1016/S2095-3119(13)60251-8

Ghulam, A. K., Tasneem, G. K., Hassan I. A., Naveed K., Jameel, A. B., Mohammad, B.A., Sirajuddin, Abdul Qadir, S.,Raja A.S., Mohammad, K. J. & Nasreen, S. (2009) .Effect of zinc supplementation on the zinc level in serum and urine and their relation to thyroid hormone profile in male and female goitrous patients. *Clinical Nutrition*, 28 (2009), 162–168.doi: 10.1016/j.clnu.2009.01.015

Gill, K.K. & Vinod, K. D.(2013). Biochemical alterations induced by oral subchronic exposure to fipronil, fluoride and their combination in buffalo calves. *Environmental Toxicology And Pharmacology*, 36 (2013), 1113–1119.doi: org/10.1016/j.etap.2013.09.011

Giuliani, C., Bucci, I., Di Santo, S., Cosmo, R., Antonino, G., Mauro, P., Fabrizio, M. & Giorgio N.(2014).The flavonoid quercetin inhibits thyroid-restricted genes expression and thyroid function. *Food and Chemical Toxicology*, 66 (2014), 23–29.doi: Food and Chemical Toxicology 66 (2014) 23–29

Godini, A., Ghasemi, A., Karbalaei, N. & Zahediasl S.(2014). The Effect of Thyroidectomy and Propylthiouracil-Induced Hypothyroidism on Insulin Secretion in Male Rats. *Horm Metab Res*, 46(10), 710-716.doi: 10.1055/s-0034-1370962

Godini,A., Ghasemi, A.& Zahediasl, S.(2015). The Possible Mechanisms of the Impaired Insulin Secretion in Hypothyroid Rats. *PLoS ONE*,10(7).doi: org/10.1371/journal.pone.0131198

Greg, R. (2010). The Halogen Elements: Fluorine, Chlorine, Bromine, Iodine, Astatine.USA: The Rosen Publishing Group.

Gregory E. T. , Michelle E. Ross , Lihai Song , Robert W. Grundmeier , James Massey , Michelle R. Denburg , Lawrence Copelovitch , Steven Warner , Thomas Chi , David W. Killilea , Marshall L. Stoller , Susan L. Furth . (2016). Dietary Zinc and Incident Calcium Kidney Stones in Adolescence. *The Journal of Urology,* , doi: 10.1016/j.juro.2016.11.096

Guillot, M., Charles, A.L., Chamaraux-Tran, T.N., Bouitbir, J., Meyer, A., Zoll, J., Schneider, F. & Geny, B . (2013).Oxidative stress precedes skeletal muscle mitochondria l dysfuncti on during experiment al aortic cross-clamping but is not associated with early lung, heart, brain, liver, or kidney mitochondria l impairment. *Jou Rnal Of Vascular Surg Ery*, 1-9.doi: org/10.10 16/j.jvs.2013.0 7.100

Güler, S., Şükrü, K. & Olcay Ş. (2016). A new and direct method for the determination of trace elements in spinach using differential pulse polarography. *Journal of Electroanalytical Chemistry*, 778 (2016), 49 –52. doi: org/10.1016/j.jelechem.2016.07.041

Gupta, K.J. & Igamberdiev, A.U. (2015). Reactive Oxygen and Nitrogen Species Signaling and Communication in Plants. Springer.

Habig, W.H., Pabst, M.J. and Jakoby, W.B., (1974). Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem 249: 7130-7139.

Hafeez B., Khanif Y. M. and Saleem M.(2013). Role of Zinc in Plant Nutrition. *American Journal of Experimental Agriculture,* 3(2),374-391.

Halka, M. & Nordstrom, B. (2010). Halogens and Noble Gases. USA: Library of Congress Cataloging-in-Publication Data.

Hanhineva, K., Törrönen ,R., Bondia-Pons, I., Pekkinen,J., Kolehmainen ,M., Mykkänen, H. & Poutanen K.(2010). Impact of Dietary Polyphenols on Carbohydrate Metabolism. *International Journal of Molecular Sciences*, 11, 1365-1402.doi: 10.3390/ijms11041365

Harbone, J. (1986). Phytochemical Method (2nd ed.). Chapman Hall, New York, Slinkard, K., Singleton, V., *Am. J. Enol. Viticult.* 28(1977) 49–55.

Harris, R.E.(2013). Epidemiology of Chronic Disease. Jones & Bartlett Publishers.

Hassan, A.H.& Yousef, M.I. (2009). Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats. *Food and Chemical Toxicology* , 47 (2009), 2332–2337.doi: 10.1016/j.fct.2009.06.023

Henningsen,C., Kuntz, K., Youngs, D.J. (2014). Clinical guide to sonography: Exercices for critical thinking (2nded.). USA: Elsevier Health Sciences.

Heymann, W.R.(2008). Thyroid Disorders with Cutaneous Manifestations. London: Springer Science & Business Media.

Huether S., Mcance K.(*2012*). Understanding Pathophysiology. USA: Ed. Mosbey Inc,.

Ibrahim, N.M., Eweis, E.A., El-Beltagi, H.S. et Abdel-Mobdy, Y.E. (2011). The Effect of Lead Acetate Toxicity on Experimental Male Albino Rat. Biological Trace Element Research, 144: 1120-1132.

Ichai, C. et al. (2011). Désordres métaboliques et réanimation : de la physiopathologie au traitement. France : Ed. spriner-verlag.

Inkielewicz-Stepniak, I. & Czarnowski, W. (2010). Oxidative stress parameters in rats exposed to fluoride and caffeine. *Food and Chemical Toxicology*,48 (2010), 1607–1611. doi: 10.1016/j.fct.2010.03.033

Jaime, L., Vázquez, E., Fornari,T., López-Hazas,M.C., García-Risco,M.R., Santoyo, S.& Reglero, G.(2014). Extraction of functional ingredients from spinach (Spinacia oleracea

L.) using liquid solvent and supercritical CO_2extraction. *Journal of the Science of Food and Agriculture*, 95, 722–729.doi: 10.1002/jsfa.6788

Jalal, D.I., Chonchol, M., Chen, W. & Targher, G.(2012). Uric Acid as a Target of Therapy in CKD. *Am J Kidney Dis*,61(1),134-146.doi: org/10.1053/j.ajkd.2012.07.021

Jameson, J.L.& De Groot, L.J. (2015). Endocrinology: Adult and Pediatric (7th ed.). Elsevier Health Sciences.

Jamilian, M., Dizaji, S.H., Bahmani, B., Taghizadeh, M., Memarzadeh, M.R., Karamali, M., Akbari, M. & Asemi, Z. (2016). A Randomized Controlled Clinical Trial Investigating the Effects of Omega-3 Fatty Acids and Vitamin E Co-Supplementation on Biomarkers of Oxidative Stress, Inflammation and Pregnancy Outcomes in Gestational Diabetes. *Can J Diabetes* ,xxx (2016), 1–7.doi: org/10.1016/j.jcjd.2016.09.004

Jan, C. A. B., Vishwa, D., Wei-Hang, C., Marlena, C. K., Annie M. J. & Magdalena C.(2014).Effects of ω3- and ω6-Polyunsaturated Fatty Acids on RANKL-Induced Osteoclast Differentiation of RAW264.7 Cells **:**A Comparative in Vitro Study. *Nutrients*, 2014(6), 2584-260.doi:10.3390/nu6072584

Jena,S., Nityananda Chainy,G.B., Dandapat, D.(2012). Hypothyroidism modulates renal antioxidant gene expression during postnatal development and maturation in rat. *General and Comparative Endocrinology*, 178 (2012), 8–18.doi: org/10.1016/j.ygcen.2012.03.012

Karbalaei, N., Noorafshan, A. & Hoshmandi, E.(2016).Impaired glucose-stimulated insulin secretion and reduced β-cell mass in pancreatic islets of hyperthyroid rats. *Exp Physiol*, 00.00 (2016), 1–14.doi: 10.1113/EP085627

Kastelein, J.J.P., Maki, K.C., Susekov, A., Ezhov, M., Nordestgaard, B.G., Machielse, B.N., Kling, D. & Davidson, M.H.(2014).Omega-3 free fatty acids for the treatment of severe hypertriglyceridem ia: The EpanoV a fOr Lowering Very high triglyceridEs (EVOLVE) trial. *Journal of Clinical Lipidology* ,8(1) , 94–106.doi: org/10.1016/j.jacl.2013.10.003

Kaufman,M., Nikitin, A.Y.& Sundberg,J.B.(2010). Histologic Basis of Mouse Endocrine System Development: A Comparative Analysis. CRC Press.

Kedar, N. P.(2015). Neurodegenerative Disease and Micronutrients: Prevention and Treatment. CRC Press.

Keerthi, M., Prasanna, J., Aruna, M. & Rao, N. (2014). Review on polyphenols as nature's gift world. *Journal Of Pharmacy And Pharmaceutical Sciences, 3 (4),*445-455.doi:

Kirk, K.L. (1991). Biochemistry of the Elemental Halogens and Inorganic Halides.New york: Springer Science & Business Media.

Kuroe, M., Kamogawa, H., Hosokawa, M. & Miyashita, K.(2016). Dietary ALA from Spinach Enhances Liver n-3 Fatty Acid Content to Greater Extent than Linseed Oil in Mice Fed Equivalent Amounts of ALA. *Lipids*, 51 (2016),39–48.doi: 10.1007/s11745-015-4086-9

Kyriacou, A., McLaughlin, J.& Syed, A.A(2015). Thyroid disorders and gastrointestinal and liver dysfunction: A state of the art review. *European Journal of Internal Medicine*, 26 (2015), 563 –571.doi: org/10.1016/j.ejim.2015.07.017

Lanez, T. & Djouadi A. (2015).Evaluation de l'activité antioxydante des polyphénols extraits de deux variétés de Solanummelongena L. de la région d'El-Oued par voltampérométrie cyclique et ondes carrées. Allemagne: Éditions universitaires européennes.

Leo, M.L. N. & Leen S. P. D.(2014). Handbook of Water Analysis, Third Edition. CRC Press.

Leqing, Z., Qiang, W., Liming, Z., Benxiang, N.,Yifang, Z. & Xuehua,C. (2016). Determination of component s in traditional Chines e medicines associated with promoting or inhibiting urinary stone formation. *Journal of Traditional Chinese Medical Sciences,* 2(2015), 99 -104.doi: org/10.1016/j.jtcms.2016.01.011

Liu, C., Zhao, L., Wei, L. & Li, L. (2015). DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. *Environ Sci Pollut Res,*22(2015),12711–12719. doi: 10.1007/s11356-015-4567-7

Liu, X.,Yang, X., Wang, L., Duan, Q.& Huang, D. (2016) Comparative analysis of metabolites profile in spinach (Spinacia oleracea L.) affected by different concentrations of gly and nitrate. *Scientia Horticulturae* , 204 (2016), 8–15.doi: org/10.1016/j.scienta.2016.02.037

Lively, R. (2011). Taunton's Complete Guide to Growing Vegetables & Herbs. Taunton Press.

Lloyd, R.V.(2010). Endocrine Pathology: Differential Diagnosis and Molecular Advances (2nd ed.). London: Springer Science & Business Media.

Maeda, N., Kokai, Y., Ohtani, S., Sahara, H., Kumamoto-Yonezawa,Y., Kuriyama, I., Hada, T., Sato,N., Yoshida, H. & Mizushina, Y.(2008). Anti-Tumor Effect of Orally Administered Spinach GlycolipidFraction on Implanted Cancer Cells, Colon-26, in Mice. *Lipids*, (2008) 43,741–748.doi: 10.1007/s11745-008-3202-5

Mansouri, A., Embarek, G., Kokkalou, E., Kefalas, P. (2005) Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Phoenix dactylifera).Food Chemistry.89: 411-420.

MartínezMier, E.A. (2011). Fluoride: Its Metabolism, Toxicity, and Role in Dental Health. *Journal of Evidence-Based Complementary & Alternative Medicine*, 000(00), 1-5.doi: 10.1177/2156587211428076

Messarah, M., Boumendjel, A., Chouabia, A., Klibet, F., Abdennour, C., Boulakoud, M.S.& El Feki, A.(2010).Influence of thyroid dysfunction on liver lipid peroxidation and antioxidant status in experimental rat. *Experimental and Toxicologic Pathology*, 6 2 (2010), 301–310. doi: 10.1016/j.etp.2009.04.009

Messarah, M., Saoudi, M., Boumendjel, A., Boulakoud, M.S. & El Feki, A.(2011). Oxidative stress induced by thyroid dysfunction in rat erythrocytes and heart. *Environmental toxicology and pharmacology*, 31 (2011), 33–41.doi: 10.1016/j.etap.2010.09.003

Miguel, M.G. (2010). Antioxidant and Anti-Inflammatory Activities of Essential Oils: A Short Review. *Molecules*, *15*, 9252-9287.doi :

Mike, J.(2010). Anaesthesia for Patients with Endocrine Disease.India: OUP Oxford

Misra, S. (2011). Thyroid Dysfunction and Pregnancy. India: Elsevier Health Sciences.

Miyawaki, I., Matsumoto, I., Horie, H., Inada, H., Kimura, J., Funabashi,H. & Seki, T. (2011).Toxicological approach for elucidation of clobazam-induced hepatomegaly in male rats. *Regulatory Toxicology and Pharmacology* ,60 (2011), 323–331.doi: 10.1016/j.yrtph.2011.04.009

Mogulkoc R., Baltaci A.(2010). Effect of melatonin supplementation on plasma vasopressin response to different conditions in rats with hyperthyroidism induced by L-thyroxine. Regulatory Peptides.vol. 161 (1-3): 38–42.

Mogulkoc R. et al. (1999).Influence of hypovolemic and hypertonic treatments on plasma vasopressin levels and fluid balance in the propylthiouracil-induced hypothyroid rat. Neuro endocrinol Lett.vol. 20 (5):311– 314.

Mogulkoc R. et al., (2000).Influence of hypovolemic and hypertonic treatments on plasma vasopressin levels and fluid balance in thethyroidectomy-induced hypothyroid rats. Neuro endocrinol Lett .vol. 21(1): 35 –38 .

Mogulkoc R., Baltaci A. K.(2006). Influences of isotonic, hypertonic and hypovolemic treatments on vasopressin response and fluid–electrolyte balance in L-thyroxine-induced hyperthyroid rat. Life Sci. vol.79 (9):817 – 821.

Mohamed, H.M., Abd El-Twab, S.M. (2016). Gallic acid attenuates chromium-induced thyroid dysfunction by modulating antioxidant status and inflammatory cytokines. *Environmental Toxicology and Pharmacology*, 48(2016), 225–236.doi: org/10.1016/j.etap.2016.08.019

Monica, D., Alessandro, M., Ann, M. Z., Reed Larsen, P. & Domenico, S. (2012). The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. *Biochimic a et Biophysica Acta* ,1830 (2013), 3937– 3945.doi: 10.1016/j.bbagen.2012.05.007

Morley JE.(2004). The top 10 hot topics in aging. *J Gerontol*, 59:24–33.

Nabavi, S.M., Nabavi, S.F., Eslami, S. & Moghaddam, A.H. (2012). In vivo protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue. *Food Chemistry*,132 (2012), 931–935. doi: 10.1016/j.foodchem.2011.11.070

Navazio, J.(2012). The Organic Seed Grower: A Farmer's Guide to Vegetable Seed Production. USA: Chelsea Green Publishing.

Neela, M., Sreenivas, N., Shubha, J., Sangamesh, K.K., Manjula, R., Mamatha, S. & Arti Ganiger, M.B.(2015) Study of serum electrolytes in female thyroid patients: a case control study. *Journal of Evidence based Medicine and Healthcare*, 2(8), 1023-1028.

Nyström, E., Berg, G.E.B., Jansson,S.K.G., Torring,O. & Valdemarsson, S.V.(2011). Thyroid Disease in Adults. Berlin : Springer Science & Business Media.

Olvera, A., Mendoza, A. ,Villalobos,P., Martínez, L.M.,Orozco,A.& Valverde-R, C.(2015). The variable region of iodothyronine deiodinases directs their catalytic properties and subcellular localization, *Molecular and Cellular Endocrinology* , 402 (2015), 107–112.doi: org/10.1016/j.mce.2015.01.011

Özkan, G. & Ersus Bilek, Z.(2014) Enzyme-assisted extraction of stabilized chlorophyll from spinach. *Food Chemistry*, 176 (2015), 152–157.doi: .org/10.1016/j.foodchem.2014.12.059

Pal, S. & Sarkar, C. (2014). Protective effect of resveratrol on fluoride induced alteration in protein and nucleic acid metabolism**,** DNA damage and biogenic amines in rat brain. *Environmental Toxicology And Pharmacology*, 38 (2014), 684–699.doi: org/10.1016/j.etap.2014.07.009

Pauling, S. (1970). General Chemistry. : Courier Corporation.

Peerzada, R. H., Prashant, S., Sadiya, J., Heena, A.& Sadaf P. (2016). Influence of postharvest gamma irradiation treatment on the content of bioactive compounds and antioxidant activity of fenugreek (Trigonella foenum–graceum L.) and spinach (Spinacia oleracea L.) leaves. *Innovative Food Science and Emerging Technologies,* 33 (2016), 268 – 28. doi: org/10.1016/j.ifset.2015.11.017

Peter H.et al . (2006). Biologie . Ed. De boeck Supérieur, Bruxelles. 1256p.

Polin, R.A., Fox, W.W.& Abman, S.H.(2011).Fetal and Neonatal Physiology (4thed.). USA: Elsevier Health Sciences.

Qing, S.L., Deng, J., Lou, D.D., Yu, W.F., Pei, J. & Guan, Z.Z. (2014). The decreased expression of mitofusin-1 and increased fission-1 together with alterations in mitochondrial morphology in the kidney of rats with chronic fluorosis may involve elevated oxidative stress. *Journal of Trace Elements in Medicine and Biology*.doi.org/10.1016/j.jtemb.2014.06.001

Rajab, N.M.A., Ukropina, M. & Cakic-Milosevic, M.(2015). Histological and ultrastructural alterations of rat thyroid gland after short-term treatment with high doses of

thyroid hormones. *Saudi Journal of Biological Sciences,*xxx (2015), xxx–xxx.doi: org/10.1016/j.sjbs.2015.05.006

Ranjan, R. & Ranjan, A. (2015). Fluoride Kinetics and Metabolism. *Springer Briefs in Animal Sciences,*21-34.doi: 10.1007/978-3-319-17512-6_3

Rao, K.N.V. , Tabassum,B., Babu, S.R., Raja,A.& Banji, D.(2015). PRELIMINARY PHYTOCHEMICAL SCREENING OF SPINACIA OLERACEA L. *World Journal Of Pharmacy And Pharmaceutical Sciences,* 4(6), 532-551.

Rizzo, D.C., (2015). Fundamentals of Anatomy and Physiology(4th ed.).USA: Cengage Learning.

Rohit, A.S., Brijesh, K. S. & Paul, M. Y. (2014). Thyroid hormone regulation of hepatic lipid and carbohydrate metabolism. *Trends in Endocrinology and Metabolism*, xx (2014), 1–8.doi: org/10.1016/j.tem.2014.07.001

Sahoo, D.K., Roy, A., Bhanja, S. & Chainy G. B.N. (2008). Hypothyroidism impairs antioxidant defence system and testicular physiology during development and maturation. *General and Comparative Endocrinology,* 156 (2008), 63–70.doi: 10.1016/j.ygcen.2007.11.007

Saleh, A.A.S. (2015).Lipid profile and levels of homocysteine and totalantioxidant capacity in plasma of rats with experimental thyroid disorders. *The Journal of Basic & Applied Zoology,*1-6.doi: org/10.1016/j.jobaz.2015.01.001

Sánchez-Camargo,A.B., M. Ângela A., Meireles, Ana L.K. Ferreira, Saito ,E., Fernando A. Cabral.(2012). Extraction of omega 3 fatty acids and astaxanthin from Brazilian redspotted shrimp waste using supercritical CO2+ ethanol mixtures. *J.of Supercritical Fluids* 61 (2012) 71– 77.doi: 10.1016/j.supflu.2011.09.017

Sang-Heui, K., Jae-Hee, P., So-Yun, K., Seon, W. L., Soon-Sil, C.& Eunju, P.(2014). Antioxidant Effects of Spinach (Spinacia oleracea L.) Supplementation in Hyperlipidemic Rats. *Prev. Nutr. Food Sci,*19(1),19-26.org/10.3746/pnf.2014.19.1.019

Santi, A., Ivana, B.M., Vânia, L.L., Marta, M. M. F.D., Fernanda, B., Thiago, D. & Anahy, G.P. (2015). Overt hypothyroidism is associated with blood inflammatory biomarkers dependent of lipid profile. *Journal Of Applied Biomedicine* , (77), 1–6. doi: org/10.1016/j.jab .2015.10.002

Sarkar,C., Pal, S., Das, N.& Dinda, B. (2014). Ameliorative effects of oleanolic acid on fluoride induced metabolic and oxidative dysfunctions in rat brain: Experimental and biochemical studies. *Food and Chemical Toxicology*, 66 (2014), 224–236.doi: org/10.1016/j.fct.2014.01.020

Sastre, J., Pallardo, F.V., Asuncion, J., Vina, J.(2000). Mitochondria, oxidative stress and aging. *Free. Radic. Res*. 32(3):189-198.

Schmid, M., Guihéneuf, F.& Stengel, D.B.(2016). Evaluation of food grade solvents for lipid extraction and impact of storage temperature on fatty acid composition of edible seaweeds Laminaria digitata (Phaeophyceae) and Palmaria palmata (Rhodophyta). *Food Chemistry* ,208 (2016), 161–168.doi: org/10.1016/j.foodchem.2016.03.123

Shivarajashankara, Y.M., Shivashankara, A.R.(2012). Neurotoxic effects of fluoride in endemic skeletal fluorosis and in experimental chronic Fluoride.Toxic. *J.Clin. Diagn. Res.* 6(4), 740–744.

Simone, M. W., Helena, C. R., Tulio, S. & Ana, L. M.(2015). Sodium selenite supplementation does not fully restore oxidative stress-induced deiodinase dysfunction: Implications for the nonthyroidal illness syndrome. *Redox Biology*, 6 (2015), 436 – 445.doi: org/10.1016/j.redox.2015.09.0 02

Singh, G., Kawatra, A. & Sehgal,S. (2001). Nutritional composition of selected green leafy vegetables, herbs and carrots. *Plant Foods Hum Nutr*., 56(4),359-64.

Singh, N., Verma, K.J., Verma ,P., Sidhu, G.K. & Sachdeva, S.(2014). A comparative study of fluoride ingestion levels, serum thyroid hormone & TSH level derangements, dental fluorosis status among school children from endemic and non-endemic fluorosis areas. *Springer plus*,3(7), - .doi:

Srinivasan, S., Pragasam, V. , Jenita, X. , Kalaiselvi, P., Muthu, V. & Varalakshmi, P.(2004). Oxidative stress in urogenital tuberculosis patients: a predisposing factor for renal stone formation—amelioration by vitamin E supplementation. *Clinica Chimica Acta*, 350 (2004), 57 – 63.doi: 10.1016/j.cccn.2004.07.001

Stanley, M. & Beare, B.G.(2005). Soins infirmiers en gériatrie: vieillissement normal et pathologique. Belgique: De Boeck Supérieur.

Sylvia, *S.M. (*2010).Biologie humaine. Bruxelles: Ed. De Boeck & Larcier s.a.

Taal, M.W., Chertow, G.M., Marsden, P.A., Skorecki, K., Yu, A.S.L. & Brenner B.M. (2011). Brenner and Rector's The Kidney E-Book (9th ed.).USA: Elsevier Health Sciences.

Taghizadeh, M., Jamilian,M., Mazloomi, M., Sanami,M.& Asemi, Z. (2016). A randomized-controlled clinical trial investigating the effect of omega-3 fatty acids and vitamin E co-supplementation on markers of insulin metabolism and lipid profiles in gestational diabetes. *Journal of Clinical Lipidology*, doi: org/10.1016/j.jacl.2015.12.017

Thangapandiyan, S. & Miltonprabu, S. (2015). Epigallocatechin gallate exacerbates fluor ide-induced oxidative stress mediated testicular toxicity in rats through the activation of Nrf2 signaling pathway. *Asian Pacific Journal of Reproduction*, 4(4), 272–287.doi: org/10.1016/j.apjr.2015.07.005

Thongprayoon,C., Cheungpasitporn, W., Kittanamongkolchai, W., Harrison, A.M. & Kashani, K. (2016). Prognostic Importance of Low Admission Serum Creatinine Concentration for Mortality in Hospitalized Patients. *The American Journal of Medicine*, 9343(16),31234-7.doi: 10.1016/j.amjmed.2016.11.020

Tillett, A. & Jewels, C.(2005). Endocrine and Reproductive Systems.British: CRC Press

Tonello, L., Bueno Godinho Ribeiro, D., Yukio Asano, R., Marques da Silveira, J. & Fernandes de Miranda, E. (2013).Aerobic training and lipid profile of hypothyroid rats. *Rev Andal Med Deporte*,6(2),47-51.doi:

Toprak Aktas,E., Yildiz, H. (2011). Effects of electroplasmolysis treatment on chlorophyll and carotenoid extraction yield from spinach and tomato. *Journal of Food Engineering*, 106 (2011), 339–346.doi: 10.1016/j.jfoodeng.2011.05.033

Urden, L.D., Stacy, K.M., Lough, M.E.(2015). Priorities in Critical Care Nursing (7thed.). USA: Elsevier Health Sciences.

Vázquez, E., García-Risco, M.R., Jaime, L., Reglero, G. & Fornari, T. (2013). Simultaneous extraction of rosemary and spinach leaves and its effect on the antioxidant activity of products. *Journal of Supercritical Fluids*, 82 (2013), 138– 145.doi: org/10.1016/j.supflu.2013.07.004

Victor P.E.(2008).Di Fiore's atlas of histology with functional correlations. Philadelphia:Ed. Lippincott Williams & Wilkins.

Wada L, King JC. (1986). Effect of low zinc intakes on basal metabolic rate, thyroidhormones and protein utilization in adult men. *J Nutr* 116:1045–1053.

Wainsten J.(2012). Le larousse médical. Paris: Ed. Larousse.

Wang, H., Cheng, L., Lin, D., Ma, Z., Deng, X.(2017) .Lemon fruits lower the blood uric acid levels in humans and mice. *Scientia Horticulturae,* 220 (2017),4–10. doi: org/10.1016/j.scienta.2017.03.023

Wang,Z., Hou,L., Huang,L., Guo,J. & Zhou, X. (2017). Exenatide Improves Liver Mitochondrial Dysfunction and Insulin Resistance by Reducing Oxidative Stress in High Fat Diet-induced Obese Mice. *Biochemical and Biophysical Research Communications,* 291 (17),30449-7.doi: 10.1016/j.bbrc.2017.03.010

Weckbercker, G., Cory, J.G ., (1988). Ribonucleotide reductase activity and growth of glutathione-depleted mouse leukemia L1210 cells in vitro. Cancer Letters 40(3): 257-264.

Wen, C.B., Leo P. V. & Geoffrey P. S.(2017). Addition of calcium compounds to reduce soluble oxalate in a high oxalate food system. *Food Chemistry,* 221 (2017), 54–57.doi: org/10.1016/j.foodchem.2016.10.031

Whitford, G.M.(2005). Fluoride metabolism when added to salt. *Schweiz Monatsschr Zahnmed,* 115 (8), 675–678.

Wu, J.H., Cahill, L.E. & Mozaffarian D.(2013). Effect of fish oil on circulating adiponectin: a systematic review and meta-analysis of randomized controlled trials. *J Clin Endocrinol Metab*,98(6),2451-9. doi: 10.1210/jc.2012-3899

Xu, H., Zhao, X., Guo, C., Chen, L.& Li, K. (2016). Spinach 14-3-3 protein interacts with the plasma membraneHþ -ATPase and nitrate reductas e in response to excess nit rate stress. *Plant Physiology and Biochemistry,* 106 (2016), 187-197.doi: org/10.1016/j.plaphy.2016.0 4.0 43

Xuejun, S.,Shigeo, O. & Atsunori, N.(2015) Hydrogen Molecular Biology and Medicine. New york: Springer.

Yagiela, J.A., Dowd, F.J., Johnson, B.S., Mariotti, A.J. & Neidle, E.A. (2011). Pharmacology and Therapeutics for Dentistry(6th ed.). USA: Elsevier Health Sciences.

Yarbro, C.H., Wujcik, D. & Gobel, B.H.(2011). Cancer Nursing: Principles and Practice (7thed.). USA: Jones & Bartlett Publishers.

Yoon, Y.E., Kuppusamy, S., Cho, K., Kim, P., Kwack,Y.B.& Lee, Y.B. (2016). Influence of cold stress on contents of soluble sugars, vitamin C and free amino acids including gamma-amino butyric acid (GABA) in spinach (Spinacia oleracea). *Food Chemistry*. doi:org/10.1016/j.foodchem.2016.07.167

Youn,Y.K., Lee,Y.E. & Choi, J.Y. (2014). Color Atlas of Thyroid Surgery: Open, Endoscopic and Robotic Procedures.London: Springer Science & Business Media

Zelzer, S., Mangge, H., Pailer, S., Ainoedhofer, H., Kieslinger, P., Stojakovic, T., Scharnagl, H., Prüller, F., Weghuber, D., Datz, C., Haybaeck, J., Obermayer-Pietsch, B., Trummer, C., Gostner, J.& Gruber, H.J. (2015). Oxidized LDL Is Strictly Limited to Hyperthyroidism Irrespective of Fat Feeding in Female Sprague Dawley Rats. *International Journal of Molecular Sciences,* 16(2015), 11689-11698. doi:10.3390/ijms160511689

Zheng,X., Sun, Y., Ke, L., Ouyang, W. & Zhang, Z.(2016).Molecular mechanism of brain impairment caused by drinking-acquired fluorosis and selenium intervention. *Environmental Toxicology and Pharmacology* , 43 (2016), 134–139.doi: org/10.1016/j.etap.2016.02.017

Zhou, B.H., Zhao, J., Liu, J., Zhang, J.L., Li, J. &Wang, H.W.(2015). Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice. *Chemosphere,139* (2015), 504–511.doi:org/10.1016/j.chemosphere.2015.08.030

Annex

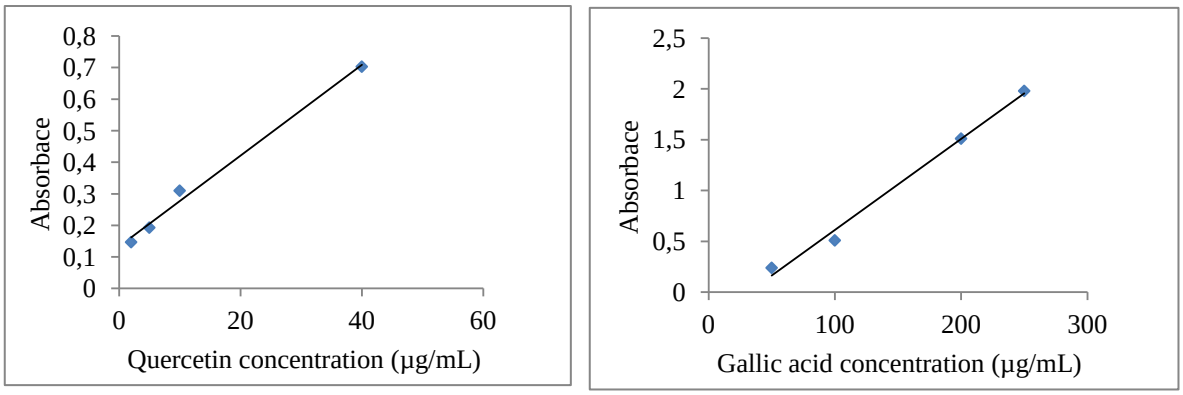

Figure: Calibration curves of Quercetin and Gallic acid for the determination of total flavonoids and polyphenols respectively.

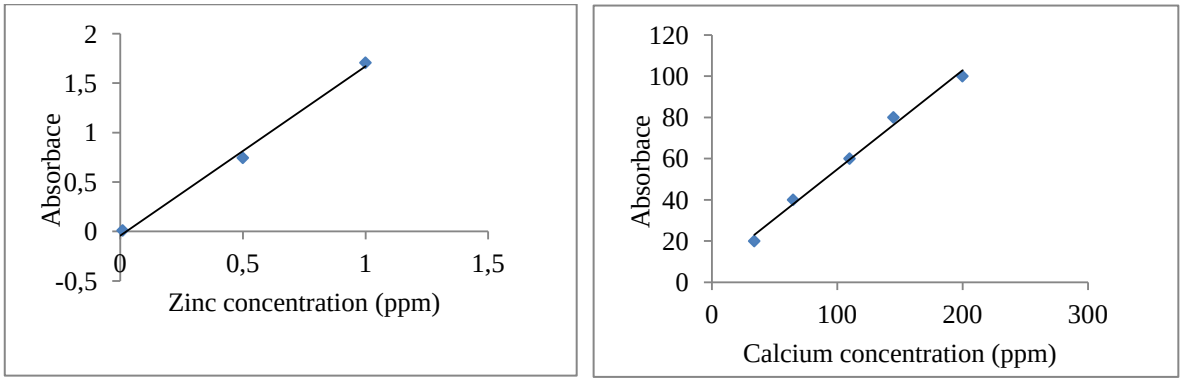

Figure: Calibration curves for the Zinc and Calcium determination.

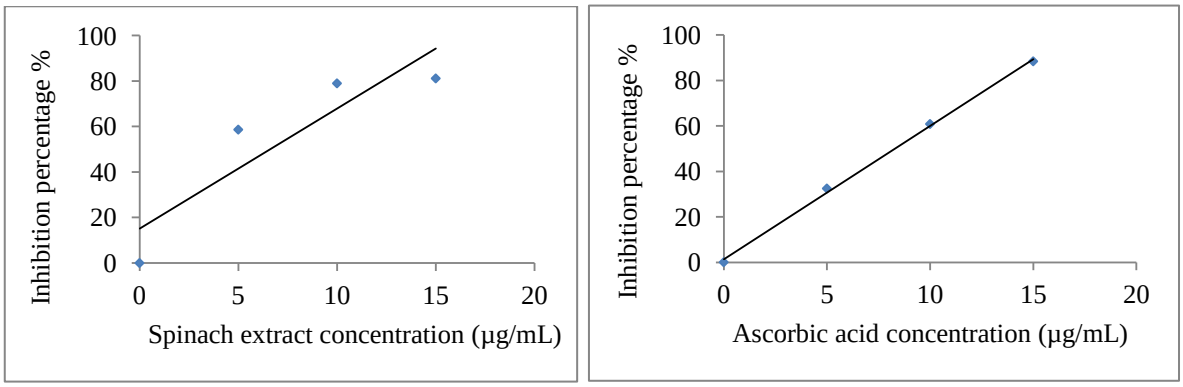

Figure: Inhibition percentage versus Spinach extract and ascorbic acid concentrations for the determination of IC_{50} for DPPH activity.

The identification of the peaks of polyphenols and flavonoids was made for the standard realized by pure components by comparing the retention times

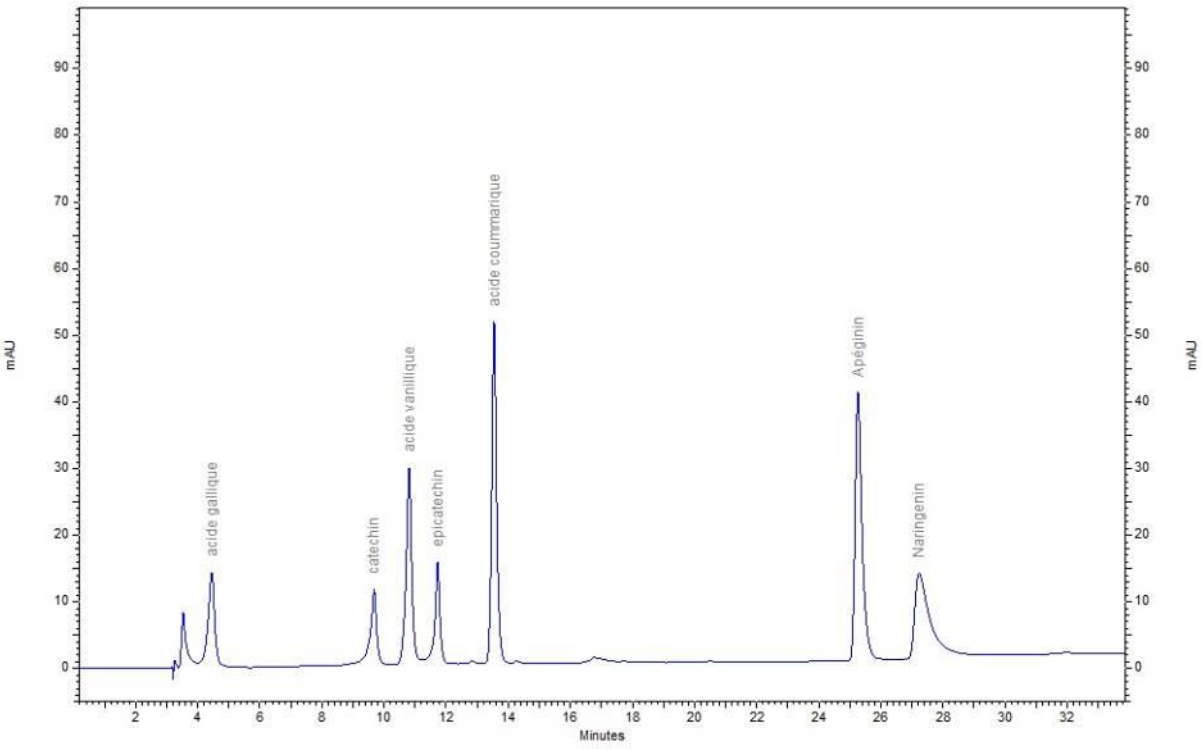

Figure: Chromatogram for polyphenols standards

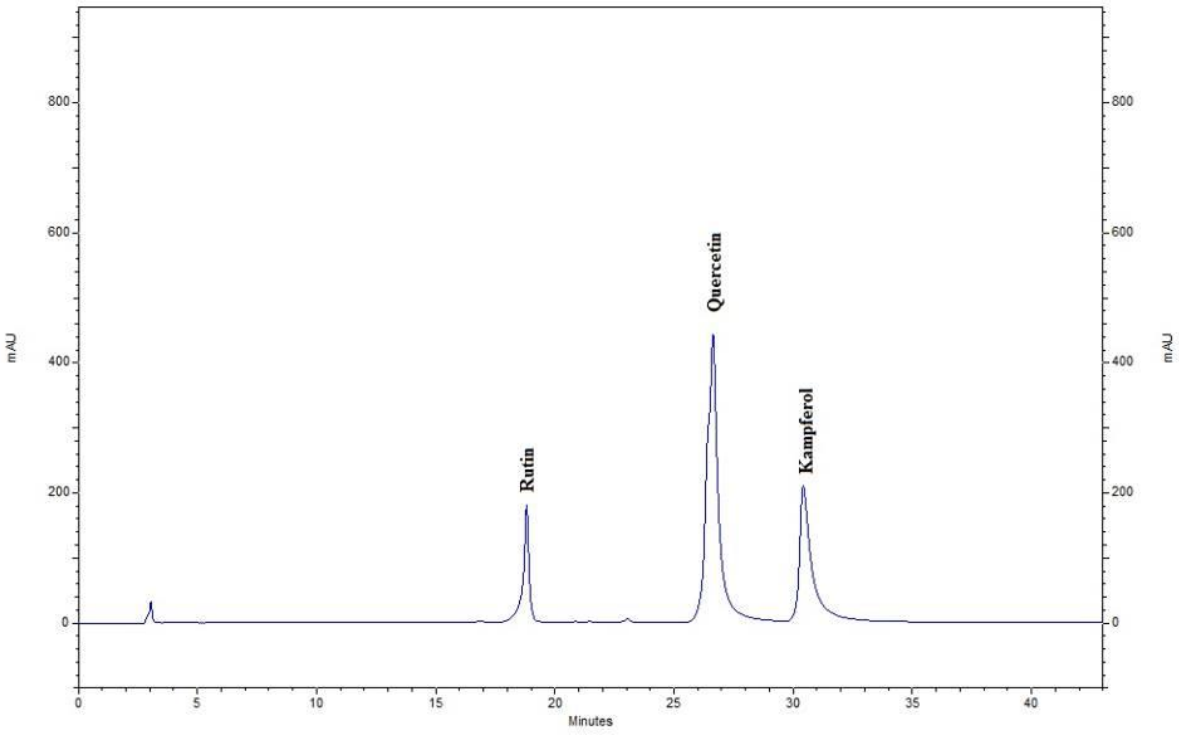

Figure: Chromatogram for flavonoïds standards