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THEME

The *in vitro* evaluation of antioxidant properties of *Cachrys libanotis* L. roots extracts

Soutenu le ……………………..devant le jury :

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Dedication

To my dear parents

To my uncle and my aunt, my "second parents"

To my sisters and my brother

To my family

To my promotion and all my friends

To everybody makes learning as a way to paradise

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Summary

The aim of this study consisted to evaluate the antioxidant activity of *Cachrys libanotis* roots extracts, used in folk medicine. The roots were submitted to extraction and fractionation to give crude extract (CrE), chloroformic extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). RP-HPLC analysis revealed the presence of catechin and epicatechin in ChE, and myrectin and gossypin in EAE. The highest total polyphenols and flavonoids amounts were recorded in EAE (83.23 µg gallic acid equivalent/mg of extract and 0.63 µg quercetin equivalent/mg of extract, respectively). Xanthine oxidoreductase (XOR) was purified from bovine milk with yield of 17.97 mg/l, PFR of 7.2, and XOR specific activity of 2261 nmole/min/mg. Both enzymatic and non-enzymatic methods were used to evaluate the antioxidant activity of extracts. Results demonstrated that EAE had the highest XOR inhibitory effect (IC_{50}= 0.11 mg/ml) followed by ChE, CrE and AqE. ChE presented the highest effect both in cytochrome c test and in NBT assay with an IC_{50} of 0.22 mg/ml and 0.21 mg/ml, respectively. All extracts inhibited linoleic acid oxidation, with remarkable percentage. In fact, the highest effect was showed by EAE (76.21%) followed by CrE and AqE with an approximately similar effect (65.17% and 64.57%, respectively), and ChE (56.95%). DPPH scavenging assay showed that CrE and EAE exhibited the highest effect with an IC_{50} of 0.41 and 0.59 mg/ml, respectively. Both CrE and AqE had a considerable chelating activity on ferrous iron (Fe^{+2}), 9-folds lower than that of EDTA (IC_{50}= 6.1 µg/ml). Reducing power of extracts was evaluated using FRAP. Extracts exhibited a moderate reducing power, and the highest power was recorded for EAE (TAP= 6.12 mM Fe^{+2}/g of extract). These results can be useful as a starting point of view for further applications of *Cachrys libanotis* roots in healthcare. In addition, the ability of roots extracts to inhibit XOR can be of great use in some diseases where the inhibition of XOR is warranted.

Key words: oxidative stress, medicinal plants, XOR, antioxidant activity, polyphenols.

ملخص

تهدف هذه الدراسة إلى تقييم النشاط المضاد للأكسدة لجذور *Cachrys libanotis* المستعملة في الطب الشعبي. تم إستخلاص الفلافونويدات من جذور *Cachrys libanotis*، والذي أعطى أربعة مستخلصات: المستخلص الخام (CrE) ومستخلص الكلوروفورم (ChE) ومستخلص إثيل الأسيتات (EAE) و المستخلص المائي (AqE). و قد كشف تحليل RP-HPLC عن وجود catechin و/أو epicatechin في ChE، وعن وجود myrecetin و gossypin في EAE. كما أظهر تقدير كمية الفينولات و الفلافونويدات أنّ EAE احتوى أكبر كمية (83.23 ميكروغ مكافئ حمض الغاليك/مغ مستخلص و0.63 ميكروغ مكافئ quercetin/مغ مستخلص، على الترتيب). تمت تنقية xanthine oxidoreductase (XOR) من حليب البقر .بمردود قُدر بــ 17.97مغ/ل، وPFR = 7.2، ونشاطية نوعية قدرت بـــ 2261 نانومول/دقيقة/مغ. استُعملت طرق إنزيمية و غير إنزيمية لتقييم النشاط المضاد للأكسدة للمستخلصات. وقد كشفت النتائج أنّ EAE أظهر أعلى تأثير تثبيطي على XOR (IC_{50} = 0.11 مغ/مل) متبوعا بـــ ChE وCrE و AqE . كما بينت أيضا أن ChE يملك أعلى قدرة إزاحية لجذر $O_2^{\cdot-}$ سواءا بـــإختبار cytochrome c أو بـــإختبار NBT (IC_{50}= 0.21 أو 0.22 مغ/مل، على الترتيب). و أما نتائج إختبار تبييض البيتاكاروتين فقد أثبتت أن المستخلصات تملك نشاط مضاد للأكسدة معتبر، حيث أظهر EAE النشاط الأكبر بنسبة 76.21 % يليه كل من CrE و AqE بنسب جد متقاربة: 65.17% و64.57%، على الترتيب؛ في حين كان نشاط ChE الأدنى بنسبة 56.95%. وكذلك بين إختبار إزاحة جذر DPPH أن CrE و EAE يملكان أعلى قدرة إزاحية (IC_{50}= 0.41 و 0.59 مغ/مل، على الترتيب). أظهر كل من CrE و AqE قدرة جد معتبرة في استخلاب أيونات الحديد الثنائي (Fe^{+2})، وهي أقل من قدرة EDTA بـــ9 أضعاف فقط .كما تم تقدير القدرة الإرجاعية لمختلف المستخلصات بتقنية FRAP، والذي بين أنّ المستخلصات لها قدرة إرجاعية متوسطة، وأنّ القدرة الإرجاعية الأفضل تعود لـــــEAE (TAP= 6.12 ميلي مولر/غ من المستخلص). يمكن استغلال هذه النتائج كنقطة بداية لإستعمال جذور *Cachrys libanotis* في مجال التداوي، إضافة إلى أنّ قدرتها التثبيطية لإنزيم XOR قد يُمكّن من استعمالها في علاج بعض الأمراض التي تتطلب تثبيط إنزيم XOR.

الكلمات المفاتيح: الإجهاد التأكسدي، النباتات الطبية، الإنزيم المؤكسد المرجع للكزانثين (XOR)، مضادات الأكسدة، متعددة الفينولات.

Résumé

Le but de cette étude consiste à évaluer l'activité biologique des extraits des racines de *Cachrys libanotis*, utilisées dans la médecine traditionnelle. Les racines ont été soumises à une extraction et fractionnement pour avoir l'extrait brut (CrE), chloroformique (ChE), d'acétate d'éthyle (EAE) et aqueux (AqE). L'analyse par RP-HPLC a révélé la présence du catéchine et épicatéchine dans ChE, et du myréctine et gossypine dans EAE. La quantité en polyphénols et en flavonoids la plus élevée est présenté par EAE (83.23 µg équivalent d'acide gallique/mg d'extrait et 0.63 µg équivalent de quercétine/mg d'extrait, respectivement). La xanthine oxydoréductase (XOR) a été purifiée du lait bovin avec un rendement de 17.97 mg/l, PFR de 7.2, et une activité spécifique de 2261 nmole/min/mg. Des méthodes enzymatiques et non-enzymatiques ont été utilisées pour évaluer l'activité antioxydante des extraits. Les résultats montrent que EAE présent l'effet inhibiteur le plus puissant sur XOR (IC_{50}= 0.11 mg/ml) suivi par ChE, CrE et AqE. ChE montre l'effet le plus élevé dans le test du cytochrome c et le test du NBT avec un IC_{50} de 0.22 mg/ml et 0.21 mg/ml, respectivement. Tous les extraits inhibent remarquablement l'oxydation de l'acide linoleique. En effet, l'effet le plus élevé est observé pour EAE (76.21%) suivi par CrE et AqE ayant un effet approximativement similaire (65.17% et 64.57%, respectivement), et ChE (56.95%). Le test du scavenger du DPPH a montré que CrE et EAE présentent l'effet le plus puissant avec un IC_{50} de 0.41 et 0.59 mg/ml, respectivement. CrE et AqE montrent une activité chélatrice remarquable, 9-fois moins celle d'EDTA (IC_{50}= 6.1 µg/ml). Le pouvoir réducteur des extraits est évalué utilisant la technique de FRAP. Les extraits exercent un pouvoir réducteur modéré, et le pouvoir le plus élevée est celui d'EAE (TAP= 6.12 mM Fe^{+2}/g d'extrait). Ces résultats peuvent être investiguer comme un point de départ pour des applications de ces racines dans la santé. En plus, le pouvoir de ces racines d'inhiber la XOR peut avoir une utilisation importante pour certaines maladies dont l'inhibition de la XOR est nécessaire.

Mots-clés: stress oxydant, plantes médicinales, XOR, activité antioxidante, polyphénols.

Abbreviations

AqE: aqueous extract

BHT: butylated hydroxyl-toluene

BMXOR: bovine milk xanthine oxidoreductase

ChE: chloroform extract

CrE: crude extract

DPPH: 1,2-diphenyl-2-picrylhydrazyl hydrate

DTT: dithiothreitol

DW: dry weight

EAE: ethyle acetate extract

EDTA: etylenediamine-tetraacetic acid

FCR: Folin-Ciocalteu reagent

FlOH: flavonoid antioxidant

FRAP: ferric acid reducing antioxidant power

GAE: gallic acid equivalent

GPx: glutathione peroxidase

GSH: glutathione

IR: ischemia-reperfusion

mt-DNA: mitochondrial DNA

NBT: nitro-blue tetrazolium

NOS: nitric oxide synthase

PFR: protein/flavin ratio

PMS: 5-methylphenazinium methyl sulfate

QE: quercetin equivalent

RE: rutin equivalent

RNS: reactive nitrogen species

RONS: reactive oxygen and nitrogen species

ROS: reactive oxygen species

RP-HPLC: reverse polarity-high performance
liquid chromatography

SOD: superoxide dismutase

TPTZ: 2,4,6- tripyridyl-s-triazine

XOR: xanthine oxidoreductase

XDH: xanthine deshydrogenase

XO: xanthine oxidase

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Introduction

Reactive oxygen and nitrogen species are well recognized for playing a dual role as both deleterious and beneficial species. Beneficial effects of reactive oxygen and nitrogen species occur at low or moderate concentrations and involve physiological roles in cellular signaling pathways. In contrast, the generation of reactive oxygen and nitrogen species, where xanthine oxidoreductase is considered as one of their major sources, beyond the antioxidant capacity of a biological system gives rise to oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA. Oxidative stress facilitates the development of a variety of human diseases such as inflammations, cardiovascular diseases, neurodegenerative diseases, diabetes mellitus and cancer. One solution to this problem is compensating this disequilibrium with antioxidant compounds that are contained in natural plant sources.

Medicinal plants have been used as sources of medicine in virtually all cultures. During the last decade, the use of traditional medicine has expanded globally and is gaining popularity. The interest in many traditional herbs and plants is due to our increasing knowledge of the role of free radicals and antioxidants in human health and disease. However, still there is not enough knowledge and data about most of them.

Plant materials contain secondary metabolites that polyphenols constitute one of the principal classes. These compounds arouse a big interest by their numerous beneficial effects toward human health. Polyphenols are subdivided to simple phenols, flavonoids and tannins. In particular, flavonoids are recognized for their antioxidant properties, antimicrobial, anti-inflammatory, etc. These activities have the most often a link with their antioxidant activity and particularly their capacity to inhibit enzymes responsible for radical generation, scavenge free radicals and chelating metal ions.

The research on plants and herbs with alleged folkloric used as pain relievers, anti-inflammatory agents, should therefore be viewed as beneficial and logical strategy in the search of antioxidants drug. From that came our choice of *Cachrys libanotis* L., having traditional claims for the treatment of rheumatism, as subject of our study.

Up to date, there are no scientific reports neither on the extraction of flavonoids from the *Cachrys libanotis* L. roots nor on its antioxidant properties. In this context, the aim of our study is to investigate its antioxidant properties using the *in vitro* methods.

1. Oxidative stress

Oxidative stress denotes an imbalance between oxidants (reactive species) and antioxidants in favor of the oxidants at the cellular or individual level, leading to a disruption of redox signaling and control and /or molecular damage (Lykkesfeldt and Svendsen, 2007). Oxidative stress might occur when the antioxidant defense system is overwhelmed by an increased oxidant burden or a decrease in antioxidant supply (Kirschvink *et al.*, 2008).

1.1. Oxidants

Oxidants are compounds capable of oxidizing target molecules. This can take place by one of three actions: abstraction of a hydrogen atom, abstraction of an electron or the addition of oxygen (Lykkesfeldt and Svendsen, 2007). Reactive oxygen and nitrogen species (RONS) are the major types of reactive species exist. Some of them are free radicals and some are not (**Table I**). A "free radical" is any atom or group of atoms that contains one or more unpaired electrons in an outer orbital, which is capable of independent existence (Gilbert, 2002). The presence of unpaired electrons can result in a species that is highly reactive. However, the reactivity of RONS, whether free radicals or not, can give an idea about their activity and specificity of reaction with other molecules. The reactivity of RONS will determine their half-life in biological systems and how far they can travel by diffusion from the site of their generation (Aust, 2004).

1.1.1. Important reactive oxygen and nitrogen species (RONS)

Under normal conditions, molecular oxygen (O_2) is present in a triplet, diradical form, having two unpaired electrons of parallel spin. This diradical form is not very reactive to organic molecules because most organic molecules are in a singlet state and due to the quantum mechanical restriction; a spin restriction creates a barrier to the insertion of a pair of electrons simultaneously, preventing its reaction with biomolecules (Laranjinha, 2009). However, O_2

can be reduced to H_2O by a series of reduction reactions, either enzymatically or non-enzymatically, requiring four electrons in total (Auts, 2004; Powers *et al.*, 2008). The incomplete reduction of oxygen leads to formation of reactive oxygen species (ROS) that the most important are superoxide radical (O^-_2, one electron), hydrogen peroxide (H_2O_2, 2 electrons) and hydroxyl anion, OH^- (Berg, 2007; Laranjinha, 2009).

Table I. Nomenclature of RONS (Halliwell and Whiteman, 2004).

Reactive oxygen and nitrogen species	
Free radicals	**Nonradicals**
Superoxide (O^-_2)	Hydrogen peroxide (H_2O_2)
Hydroxyl (OH^-)	Hypobromous acid (HOBr)
Hydroperoxyl (HO^-_2)	Hypochlorous acid (HOCl)
Peroxyl (RO^-_2)	Ozone O_3
Alkoxyl (RO^-)	Singlet oxygen (O^1_2)
Carbonate (CO^-_3)	Organic peroxides (ROOH)
Carbon dioxide (CO^-_2)	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
Nitric oxide (NO^-)	Nitrous acid (HNO2)
Nitrogen dioxide (NO^-_2)	Nitrosyl cation (NO^+)
	Nitroxyl anion (NO^-)
	Dinitrogen tetroxide (N_2O_4)
	Dinitrogen trioxide (N_2O_3)
	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
	Nitronium (nitryl) cation (NO_2^+)
	Alkyl peroxynitrites (ROONO)
	Nitryl chloride (NO_2Cl)

O^-_2 is considered as primary ROS, and can further interact with other molecules to generate secondary ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (Bandyopadhyay *et al.*, 1999): $$O_2 + e^- \rightarrow O_2^-$$
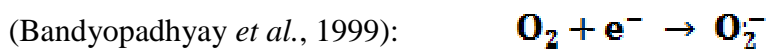
H_2O_2 is formed by dismutation of O_2^- in the presence of superoxide dismutase (SOD) or spontaneously (Valko *et al.*, 2005):

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

H_2O_2 does not contain unpaired electrons in the valence orbitals and, therefore, is not a free radical molecule but, upon lysis of the O-O bond by ferrous iron, known as Fenton reaction, yields the most powerful oxidant known in a biological setting, $HO^{.}$ (Valko *et al.*, 2005):

$$H_2O_2 + Fe^{+2} \rightarrow OH^- + OH^{.} + Fe^{+3}$$

Endogenous iron is usually present in chelated or bound forms as part of hemoglobin, myoglobin, several enzymes and the transport protein, tranferrin, and therefore not readily available for reaction (de Beer *et al.*, 2002).

O_2^- and H_2O_2 can react with transition metals such as iron or copper to form the strong oxidant, $OH^{.}$, following Haber-Weiss reaction (Sorg, 2004):

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^{.} + O_2$$
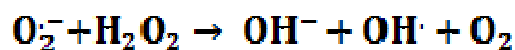
Conversely to O_2^- and H_2O_2, which are less reactive and therefore, more selective in its targets, the $OH^{.}$, upon formation, oxidizes indiscriminately and site-specifically any biomolecule (Sorg, 2004).

As reported by Nivière and Fontecave (1995), $NO^{.}$, an intercellular messenger, is produced from oxygen by various nitric oxide synthase (NOS):

$$O_2 + \text{arginine} + \text{NADPH} \xrightarrow{\text{NOS}} NO^{.} + \text{citrulline} + H_2O + \text{NADP}$$

The simultaneous generation of $NO^{.}$ and O_2^- leads to the formation of $ONOO^-$. ONOOH, formed by its protonation, is a powerful oxidant itself, but can also decompose to yield further

oxidants with the chemical reactivity of $NO_2^.$, $OH^.$ and NO^{+2}. $NO^.$ is poorly reactive with most molecules in the human body (non-radicals), but as a free radical it can react extremely rapidly with other free radicals such as O^-_2 , amino acid radicals, and certain transition metal ions (Gutteridge and Halliwell, 2002).

1.1.2. Sources of RONS

The principal sources of RONS are devised in two types according to their origin:

a. Endogenous sources of RONS

- **Mitochondria**

ROS are commonly generated as byproducts of the mitochondrial electron transfer reaction for the production of ATP. Approximately 2-4% of the total oxygen consumed during electron transport is reduced not to water by cytochrome c oxidase but rather to $O_2^{.-}$, due to the "leakage" of unpaired electrons to O_2 during the proton-motive quinone cycle. Under the physiological condition, the $O_2^{.-}$ generated by the respiratory chain in mitochondria is scavenged by SOD in the mitochondrial matrix to form H_2O_2 (Chen and Castranova, 2004).

- **NADPH oxidase**

The key component of the respiratory burst system is the membrane-bound multisubunit enzyme complex termed the NADPH oxidase in both phagocytic cells and nonphagocytes, which act as a pivotal defense system against a range of infectious agents (Babior, 1999). NADPH oxidase is inactive in resting cells and upon stimulation by a variety of soluble mediators and by particulate stimuli that interact with cell surface receptors, this oxidase is rapidly activated to produce $O_2^{.-}$ and other ROS, such as H_2O_2, $OH^.$, and, through the one-electron reduction mechanism (Babior, 1999; Robinson, 2009):

$$NADPH + H^+ + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + H^+ + 2O_2^-$$

- **Xanthine oxidoreductase (XOR)**

Xanthine oxidoreductase (XOR) catalyzes the reduction of O_2 to yield O^-_2 and H_2O_2 during uric acid formation from hypoxanthine. Kelley and their collaborators (2010) reported that H_2O_2 is the major oxidant product of XOR. Moreover, XOR has recently been shown to catalyze the anaerobic reduction of inorganic nitrite to $NO^.$. In the presence of oxygen this also is reduced, to O^-_2, which reacts rapidly with $NO^.$ to give $ONOO^-$ (Harrison, 2006).

- **Nitric oxide synthase (NOS)**

Three NOS isoforms have been identified in mammalian tissues: neuronal NOS (nNOS or type I), inducible NOS (iNOS or type II), and endothelial NOS (eNOS or type III). NOS (EC 1.14.13.39) catalyzes the oxygen- and NADPH-dependent oxidation of L-arginine to $NO^.$ and citrulline (del Rio *et al.*, 2002).

In addition, ROS generation can make by enzymes such as membrane oxidases. In fact, cytochrome P-450, P-450 reductase and cytochrome b-5 reductase in the endoplasmic reticulum under certain conditions generate O^-_2 and H_2O_2 during their catalytic cycle (Bandyopadhyay *et al.*, 1999). Peroxisomal oxidases and flavoproteins, as well as D-amino acid oxidase, L-hydroxy acid oxidase, and fatty acyl oxidase participate in ROS generation (Bandyopadhyay *et al.*, 1999; Valko *et al.*, 2007). Furthermore, Reactions catalyzed by lipoxygenases and cyclooxygenase also are important cellular sources of free radicals. Cyclooxygenase react with H_2O_2 producing ferryl heme and tyroxyl radical. Such ''activated enzyme'' can induce one-electron oxidation of arachidonic acid and also other substrates producing free radicals (Bartosz, 2003).

b. Exogenous sources of RONS

A number of environmental factors, such as ultraviolet light, ionizing radiation and ultrasound can stimulate the RONS generation endogenously after interaction with tissues or cells (Mark *et al.*, 2000; Bartosz, 2003; Chen and Castranova, 2004).

Various other exogenous sources also contribute directly or indirectly to the total oxidant load. These include effects of air pollution and natural toxic gases, such as ozone, as well as chemicals and toxins, including oxidizing disinfectants. Moreover, foreign microorganisms induce secondary oxidant formation and release in the host via the immune system, in addition to their sometimes directly oxidizing capabilities. Diets containing inadequate amounts of nutrients may also indirectly result in oxidative stress by impairing cellular defense mechanisms (Lykkesfeldt and Svendsen, 2007).

1.1.3. Physiological roles of RONS

Sorg (2004) reported that an adequate level of certain RONS can have a physiological role, as for instance the catalysis of many biochemical reactions, the defense against invading pathogens or the capacitation of spermatozoa. Moreover, in the nervous system, it acts as a neuromodulator and plays a role in synaptic plasticity and long-term memory, whereas in the vascular system, it controls blood pressure, inhibits platelet aggregation (**Table II**).

Table II. Physiological roles that involve free radicals or their derivatives (Dröge, 2002).

Species	Source	Physiological effects
Nitric oxid ($NO^{\cdot}$)	Different NOS	• Smooth muscle relaxation and others various cGMP-dependent functions
Superoxide ($O_2^{\cdot -}$) and related ROS	NAD(P)H oxidase	• Control of ventilation • Control of erythropoietin production and other hypoxia - inducible functions • Smooth muscle relaxation • Signal transduction from various membrane receptors / enhancement of immunological

Superoxide (O_2^-) and related ROS	Any sources	• Oxidative stress responses and the maintenance of redox homeostasis

1.1.4. Biological consequences of oxidative stress

RONS may cause oxidative damage to proteins, lipids, and DNA, which inhibits the normal functions of proteins and lipids, and facilitates DNA mutagenesis; thus, these species play a pivotal role in various clinical conditions (Rice-Evans, 1994).

DNA and RNA damage are major consequences of oxidative stress. Exposure of nucleic acids to reactive species may result in base modification, cross-linking of DNA-DNA and DNA-proteins, sister chromatid exchange and single- or double-strand breaking leading to disruption of transcription, translation and DNA replication (Goetz and Luch, 2008). Mitochondrial DNA (mtDNA), which is transiently attached to the inner mitochondrial membrane where a large amount of ROS is produced, is particularly vulnerable to oxidative damage. Moreover, DNA repair mechanisms in the mitochondria are less efficient than in the nucleus. Therefore, ROS-mediated mtDNA damages may contribute to mitochondrial dysfunction generated by endogenous reactive intermediates which act directly on mitochondrial proteins (Valko *et al.*, 2006).

Oxidation of proteins may occur directly as protein side chains are oxidized leading to a loss of function of proteins and a deactivation of enzymes. Often, thiols of proteins involved in the regulation of enzyme activity are directly oxidized. Increase of malondialdehyde has been suggested to lead to intra- and inter-molecular cross-links of proteins. Conformational changes leading to an increase in hydrophobicity may result in aggregation or precipitation of proteins, which can no longer be subjected to the normal protein degradation pathway (Stadtman and Levine, 2006; Valko *et al.*, 2006). Additionally, oxidative damage of proteins

may occur by the adduction of secondary products like oxidation of sugar (glycoxidation), or of polyunsaturated lipids (lipoxidation) (Stadtman and Levine, 2006).

Lipid peroxidation of membranes occurs as a consequence of direct reaction of fatty acids of polar lipids with oxygen or a reaction catalyzed either by metals like iron or by NADPH cytochrome P-450 reductase (Devasagayam *et al.*, 2003). Lipid peroxidation is initiated by the formation of a Fenton-derived oxidant that results in the production of a lipid radical. In the presence of oxygen, this radical is converted to a hydroperoxide radical, which then abstracts hydrogen from other lipids. These steps result in chain propagation and, consequently, enhanced lipid peroxidation (Wink *et al.*, 2002).

1.1.5. Pathological implications of oxidative stress

An oxidative stress is often associated to all kinds of diseases, although it is not always easy to determine whether it is a cause or a consequence of the observed condition. A selection of pathologies, for which the mechanism of oxidative stress is well documented, is described in **Table III**.

Table III. Some pathologies result from oxidative stress.

Pathology	References
Cancer	(Goetz and Luch, 2008)
Cardiovascular diseases	(Vijaya Lakshmi *et al.*, 2009)
Metabolic syndrome	(Calhau and santos, 2009)
Diabetes mellitus	(Maritim *et al.*, 2003)
Inflammation	(Libetta *et al.*, 2011)
Rheumatoid arthritis	(Hitchon and El-Gabalawy, 2004)
Ischemic reperfusion injury	(Brennan and Eaton, 2006)
Neurodegenerative diseases	(Anderson, 2004)
Alzheimer' disease	(Cai and Yan, 2007)
Parkinson disease	(Cohen, 2002)

1.2. Antioxidant defense

Antioxidants was defined as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates (Dröge, 2002). According to Shi and their collaborators (2001), there are several lines of defense as follows:

(i) inhibiting the formation of active oxygen species and free radicals,

(ii) preventing chain initiation by scavenging initiating radicals,

(iii) repairing, *de novo* and clearance of oxidatively damaged lipids, proteins and DNA.

1.2.1. Enzymatic antioxidants

They include the glutathione redox system [consisting of glutathione reductase, glutathione-S-transferase, glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase], SOD and catalase.

- **Superoxide dismutase (SOD)**

SOD (EC1.15.1.1.) are a family of metalloenzymes that catalyze the disproportionation of O_2^{-} radicals to H_2O_2 and O_2, and play an important role in protecting cells against the toxic effect of superoxide radicals produced in different cellular compartments (del Rio *et al.*, 2002).

- **Glutathione peroxidase (GPx)**

GPx is a selenoenzyme whose catalytic function depends on the presence of the mineral in the enzyme. It catalyzes the reduction of both H_2O_2 and lipid hydroperoxides. In general terms the enzyme functions in a cycle with glutathione reductase which uses reducing equivalents derived from glucose via the pentose phosphate pathway and NADPH (Diplock, 1994).

- **Catalase**

In human tissues, there is a very high concentration of the enzyme in liver peroxisome and erythrocytes, and there is relatively much less in brain, heart and skeletal muscle (Diplock, 1994). The general reaction catalyzed is the reduction of H_2O_2 to water and O_2. However, H_2O_2 can cross membranes and it may be that the protective effect of GPx is overwhelmed, when intracellular concentrations of H_2O_2 are high. Under these circumstances the protective function of catalase may be of great importance (Diplock, 1994; Forman, 2008).

1.2.2. Non-enzymatic antioxidants

They includes different chemical compounds such as tocopherol (vitamin E), ascorbic acid (vitamin C), Caretinoids**,** glutathione (GSH), phenolic compounds, ubiquinol (Coenzyme Q10), phospholipids (proteoglycans and hyaluronic acid), lipoic acid, proteins binding free iron and copper such as ceruloplasmin, , transferrin, taurine, albumin), protein hydrolysates, bilirubin, melatonin, uric acid, mucin, surfactant, amino acids, peptides, and phytates (Vallyathan, 2004; Kirschvink *et al.*, 2008).

- **Vitamine E**

The vitamine E is a free radical scavenger which yields a long-lived radical upon hydrogen abstraction, thereby interrupting the chain reaction. This property is optimized in α-tocopherol which is a remarkable scavenger of peroxyl radicals in phospholipid membrane bilayers. Vitamin E may reduce the possibility of forming reactive $OH^{.}$ and $ONOO^{.}$, and thus protect against oxidative damage. Also, it may modulate the activation and/or expression of redox-sensitive biological response modifiers, and thereby attenuates the cellular events leading to the onset of cardiovascular, cancer, aging and other degenerative diseases (Chow, 2008).

- **Vitamine C**

The role of vitamin C as an antioxidant is indicated by its known free radical-scavenging action. As a reducing and antioxidant agent, it directly reacts with $O_2^{.-}$, $OH^.$ and various lipid hydroperoxides. In addition, it can restore the antioxidant properties of oxidized vitamin E, suggesting that a major function of vitamin C is to recycle the vitamin E radical. However, vitamin C shown a prooxidant action which resides in its ability to reduce Fe^{+3} to Fe^{+2} state; Fe^{+2} is known as a potent free radical inducer (Yu, 1994).

- **Glutathione (GSH)**

GSH, the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine), has a very important role in protection against free radical damage by providing reducing equivalents for the several key enzymes referred to above. GSH is also a scavenger of $OH^.$ and O^1_2; in the case of $OH^.$, the thiyl radical is produced, which is also a product of the oxidation of GSH by peroxidases or by O_2 in the presence of transition-metal ions. Thiyl radicals may initiate radical reactions although they are much less reactive than $OH^.$. A slow reaction of GSH with $O_2^{.-}$, may also lead to the formation of O^1_2. Thus, although the overwhelming function of GSH in living cells is protective, the possibility of undesirable secondary reactions must be borne in mind (Diplock, 1994). Vitamin E, vitamin C, and GSH may act in combination through NADPH-glutathione reductase system (Bandyopadhyay *et al.*, 1999)

- **Carotinoids**

Carotinoids have long been considered antioxidants because of their capacity to scavenge free radicals. Carotinoids protect lipids against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen. β-carotene displays an efficient biological radical-trapping antioxidant activity through its inhibition of lipid peroxidation induced by the XO system (Yu, 1994).

- **Polyphenols**

Polyphenols are a big group of compounds found in plants and uniquely characterize by the presence of more than one phenol group in the molecule. They are considered the most abundant antioxidant in the diet although the diversity of their structures makes them different from other antioxidants. Moreover, the amount of different polyphenols found to date in plants and plant foods, several thousands, make them a complex family of compounds with very interesting therapeutic properties against cancer, cardiovascular diseases, inflammation and other diseases (Chow, 2008).

2. Xanthine oxidoreductase (XOR)

XOR known initially as Schardinger's enzyme, it was discovered in 1902 by Austrian biochemist Franz Schardinger. It was later isolated, purified, and studied by Malcolm Dixon and Sylva Thurlow in the 1920's, and in 1938 it was suggested by Booth that Schardinger enzyme be there after referred to as xanthine oxidase (XO).

2.1. Structure

XOR belongs to the family of molybdenum-containing hydroxylases comprising a group of closely related metalloflavoproteins (Parks *et al.*, 2002). The enzymes from various sources are proteins of 290 kDa, and are composed of two identical and catalytically independent subunits (Enroth *et al.*, 2000). The 3D structure (**Fig. 1**) of each subunit is organized into three major domains: Mo-domain, Fe-S domain, and flavin adenine dinucleotide (FAD) - domain. The Mo-domain is the largest and contains the Mo active site. The Fe-S domain contains two [2Fe–2S] centers whereas the FAD-domain contains the flavin cofactor (Brondino *et al.*, 2006).

The enzyme occurs in two forms, xanthine dehydrogenase (XDH, EC 1.1.1.204), which predominates *in vivo*, and xanthine oxidase (XO, EC 1.1.3.22). Conversion of XDH to XO

can occur reversibly by oxidation of essential sulfhydryl groups or through limited proteolytic cleavage of the amino terminus (Harisson, 2006; Nishino *et al.*, 2008). XDH shows a preference for nicotinamide adenine dinuclotide (NAD^+) reduction at FAD-domain, whereas XO fails to react with NAD^+ and exclusively uses O_2 as its substrate, leading to the formation of $O_2^{-\cdot}$ and H_2O_2 (Enroth *et al.*, 2000).
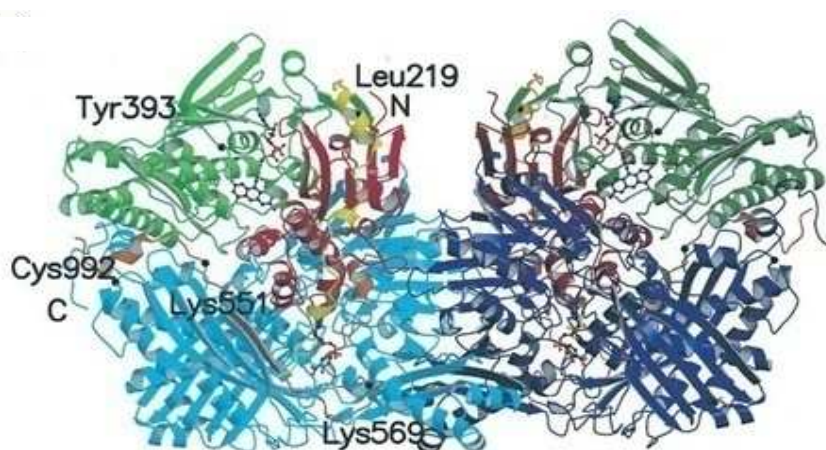
Fig. 1. Molecular structure of the XDH. XDH dimer divided into the three major domains and two connecting loops. The two monomers have symmetry related domains in the same colors, in lighter shades for the monomer on the left and in darker shades for the monomer on the right. From N to C terminus, the domains are: ironysulfur-center domain (residues 3–165; red), FAD domain (residues 226–531; green), and Mo-pt domain (residues 590–1,331; blue). The loop connecting the ironysulfur domain with the FAD domain (residues 192–225) is shown in yellow, the one connecting the FAD domain with the Mo-pt domain (residues 537–589) is in brown, and the N and C termini are labeled. The FAD cofactor, the two ironysulfur centers, the molybdopterin cofactor, and the salicylate also are included (Enroth *et al.*, 2000).

XDH and XO are products of the same gene; however, XOR is transcribed as a single gene product, xanthine dehydrogenase (XDH). The gene encoding human XOR is approximately 60 kb, comprises 36 exons and 35 introns (Xu *et al.*, 1996), and its location was confirmed on chromosome band 2p23 (International Human Genome Sequencing Consortium, 2001). The nucleotide sequence (1,332 residues) is highly homologous with that of the bovine milk enzyme (1332 residues) showing 91% homology (Xu *et al.*, 1994).

2.3. Distribution

XOR was found in liver parenchymal cells and in vesicles in Kupffer cells and sinusoidal endothelial cells, using histochemical techniques which rely on detection of enzyme activity

in tissue homogenates (Frederiks and Vreeling-Sindelarova, 2002). Further, immuno-localization techniques with XOR antibodies recognize active and inactive forms of the enzyme in intact tissues such as human liver, brain and heart (Meneshian and Bulkley, 2002; Martin *et al.*, 2004). Wright and their collaborators (1993) found XOR in human liver, lung, skeleton muscle and pancreas, using tissue hybridation techniques.

Subcellular localization methods have demonstrated the presence of XOR in both the cytoplasm (Frederiks and Vreeling-Sindelarova, 2002) and the cell membranes (Rouquette *et al.*, 1998) with cell surface binding likely mediated by glycosaminoglycans (Radi *et al.*, 1997). In addition, the enzyme is a major protein component of the milk fat globule membrane, which envelops fat droplets in freshly expressed milk (Mather, 2000). XOR is generally present in mammalian blood and, because of endogenous proteases, occurs largely in the XO form (Kooij *et al.*, 1992).

2.4. Catalysis mechanism of XOR

XOR reacts with many substrates including purines, purine ribonucleosides, and 2-deoxyribonucleosides. It plays a pivotal role in the catabolism of purines (**Fig. 2**) when both forms catalyze the terminal two steps of purine degradation in humans, hypoxanthine to xanthine and xanthine to uric acid (Hille and Nishino, 1995).
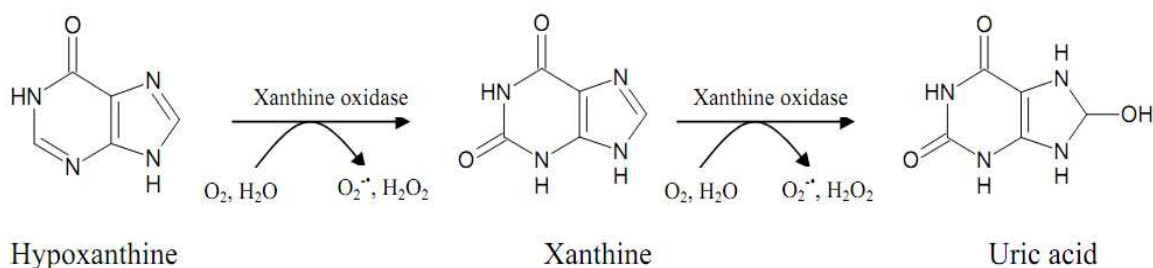
Fig. 2. The enzymatic process catalyzed by XO (Hille and Nishino, 1995).

Reaction mechanism occurs in two phases, the reductive half- reaction and the oxidative half oxidative. The reductive half-reaction involves binding and reaction at molybdenum center of the enzyme of up successive xanthine molecules (Enroth, 2000). In contrast, the oxidative half-reaction takes place at FAD-domain. Electrons are subsequently transferred from FAD to NAD^+ or O_2 (Hille and Nishino, 1995).

2.5. Physiological functions

The primary functions of XOR can be including purine metabolism, oxidant and, antioxidant production, nitric oxide regulation, and drug metabolism (Parks *et al.*, 2002). In addition, XOR implicate in innate immunity (Meneshian and Bulkley, 2002; Harrison, 2006), and it is essential to the process by which the milk fat globule becomes enveloped by the apical cell membrane during secretion (Harrison, 2006).

2.6. Pathological implications

As a result of its ability to generate RONS, implicated in both tissue structural damage and cell signaling interference, XO form has received considerable attention as a patho-physiological cause of some pathological states.

2.6.1. Hyperuricemia

Hyperuricemia is the most cited pathology involving XOR. It is a pathological state that arises from overproduction (by XOR) or under excretion (renal tubule disorders) of uric acid. As a result of hyperuricemia, insoluble uric acid forms microscopic crystals in the capillary vessels of joints. These crystals cause inflammation and sharp pain, which is termed acute gouty arthritis or acute gout (Sachs *et al.*, 2009).

2.6.2. Ischemia-reperfusion injury (IR injury)

Much of the injury sustained by an organ is a consequence of ischemia actually occurred at the time of reperfusion, triggered by ROS generated by XO upon the reintroduction of oxygen (Parks *et al.*, 2002). XO may serve as the initial source of reactive oxygen species generation in post-IR injury (**Fig. 3**). During ischemia, mitochondrial oxidative phosphorylation ceases and ATP levels decrease. The levels of AMP increase, and AMP is metabolized to adenosine, inosine, and hypoxanthine. Normally, hypoxanthine is converted by XDH to xanthine and uric acid. Concurrently, transmembrane ion gradients are dissipated, allowing elevated cytosolic concentrations of calcium that activates various proteolytic and phospholipase enzymes. A rapid Ca^{+2}-dependent proteolytic conversion of XDH to XO takes place, which in the phase of reperfusion uses O_2 as an electron acceptor instead of nucleotide radical, thus catalyzing O^-_2 production. These reactive oxygen species can interact to yield $O_2^{.-}$, and subsequently H_2O_2, OH·, OONO·, HOCl, or the chloramines leading to the cell and tissue injury and organ dysfunction characteristic of post-IR (Meneshian and Bulkley, 2002).
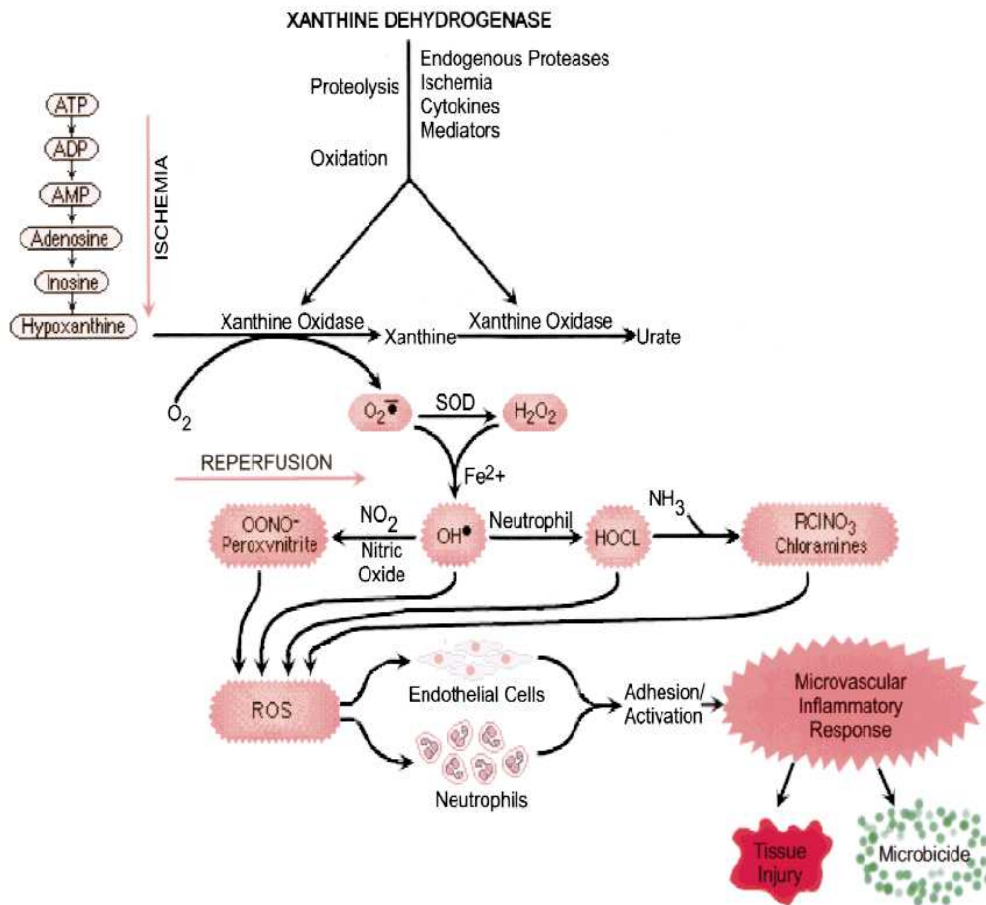
Fig. 3. Role of XOR in ischemia reperfusion- injury (Meneshian and Bulkley, 2002).

In side of these pathologies, XO has been implicated to play a significant role in other pathological states such as cardiovascular diseases (Berry and Hare, 2004), neurological dysfunction (Han *et al.*, 2007), and rheumatoid arthritis and in other joint inflammation (Al-Mouhtaseb *et al.*, 2011).

2.7. XOR inhibition

Allopurinol, a potent XO inhibitor with a purine backbone, has been used clinically for more than 40 years. Unfortunately, this drug has infrequent but severe side effects (Pacher *et al.*, 2006). Febuxostat is a potent non-purine, selective inhibitor of both the oxidized and reduced forms of XOR, and could be useful for the treatment of hyperuricemia and gout, as report Yasuhiro and their collaborators (2005). Currently, no clinically effective xanthine oxidase inhibitor for the treatment of hyperuricemia has been developed since allopurinol. Therefore,

new inhibitor devoid of undesired side effects has been investigated. Many studies of natural polyphenols, especially flavonoids, in the form of plants or purified extracts show that they could be used as XOR inhibitors (Niu *et al.*, 2010).

3. Polyphenols

With nearly 10,000 individual compounds, phenolics compounds are the most abundant secondary metabolites in plants. They are defined, chemically, as compounds that have one or more hydroxyl groups attached directly to a benzene ring and that have more than one phenolic hydroxyl group attached to one or more benzene rings. Plant phenolics are biosynthesized by several different routes which two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway (Taiz and Zeiger, 2003). They can be classified into phenolic acids, tannins and flavonoids.

3.1. Phenolic acids

Phenolic acids are by their structure simple phenols and include two major subgroups: hydroxybenzoic acids and hydroxycinnamic acids. Phenolic acids are commonly present under two principal forms in all plant-derived foods: a free and a bound form. The latter is found more frequently and occurs in the form of esters, glycosides and bound complexes (Andjelkovic *et al.*, 2006).

3.2. Tannins

The term tannin was first used to describe compounds that could convert raw animal hides into leather in the process known as tanning. There are two categories of tannins: condensed and hydrolysable. Condensed tannins are compounds formed by the polymerization of flavonoid units. Hydrolysable tannins are heterogeneous polymers containing phenolic acids, especially gallic acid, and simple sugars. They are smaller than condensed tannins and may be hydrolyzed more easily; only dilute acid is needed (Taiz and Zeiger, 2003).

3.3. Flavonoids

To date, more than 8000 structures have been classified as members this class of natural products (Quideau *et al.*, 2011).

Flavonoids are a group of polyphenolic compounds diverse in chemical structure and characteristics. They occur naturally in fruit, vegetables, nuts, seeds, flowers, and bark and are an integral part of the human diet (Cook and Samman, 1996), although they are considered as non-nutrients (Negrào and Faria, 2009). All flavonoids share a basic C_6-C_3-C_6 structural skeleton, consisting of two aromatic C_6 rings (A and B) and a heterocyclic ring (C) that contains one oxygen atom (**Fig. 4**).
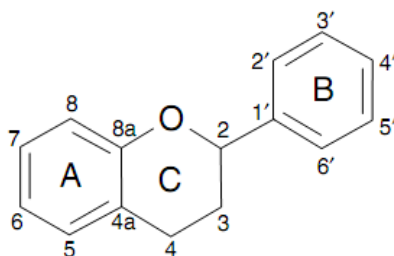
Fig. 4. Basic structure of flavan nucleus (Negrào and Faria, 2009).

On the basis of the position of and the modifications to the A, B and C rings, flavonoids are grouped into the following structural classes: flavanones, dihydroflavonols, leuco-anthocyanidins (flavan-3,4-diols), flavan-3-ols, anthocyanidins, anthocyanins, flavones, flavonols and isoflavones (Negrào and Faria, 2009). Most flavonoid compounds are glycosides, which are often accumulated in the vacuoles of plant cells. Glycosides can be either O- or C-linked (Rice-Evans, 2001).

Many of these flavonoids have potential beneficial effects on human health. They have been found to have effects on: cardiovascular diseases prevention by virtue of their capacity to inhibit lipoproteins oxidation (Mink *et al.*, 2007), neuro-degenerative diseases prevention (Singh *et al.*, 2008) and reduction in cancer incidence (Singh and Khar, 2006). In addition, flavonoids exhibit a wide range of biological activities, including antioxidant, anti-

inflammatory (Negrào and Faria, 2009), antimicrobial, anti-ulcer, hepatoprotective actions and as enzymes inhibitors (Harborne and Williams, 2000). Most of the beneficial health effects of flavonoids are attributed to their antioxidant abilities (Negrào and Faria, 2009).

3.3.1. Antioxidant activity of flavonoids

a. Scavenging activity

Flavonoids express their radical-scavenging action through two mechanisms. The first is based on the capacity of the flavonoids functional group to donate a hydrogen atom to a free radical R· (R represents superoxide anion, peroxyl, alkoxyl, and hydroxyl radicals).

$$Fl - OH + R· \longrightarrow Fl - O· + RH$$
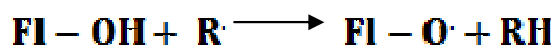
Through this so-called hydrogen-atom transfer mechanism, the flavonoid antioxidant (FlOH) itself becomes a free radical (Fl-O·) which may react with a second radical, acquiring a stable quinone structure (**Fig. 5**). The second mechanism is the single-electron transfer from FlOH to a free radical R· with formation of a stable radical cation $FlOH^{+}$ (Wright *et al.*, 2001). Due to their lower redox potentials ($0.54 \square E_7 \square 0.7V$), flavonoids are thermo-dynamically able to reduce highly oxidizing free radicals with redox potentials in the range (1.0-2.13V), such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals (Jovanovic *et al.*, 1994).
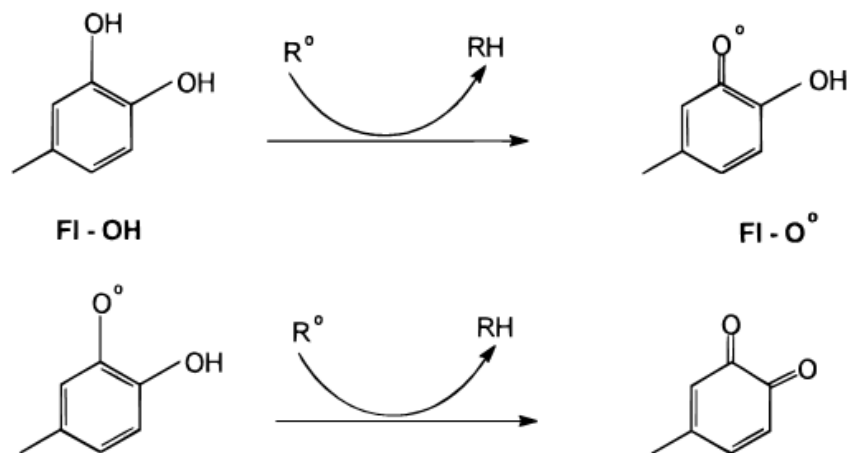
All these reactions provoke inhibition or reduction in the formation of free radicals, they interrupt the propagation of free radical chain reactions, or they delay the start or reduce the reaction rate (Wright *et al.*, 2001). Besides scavenging, flavonoids may stabilize free radicals involved in oxidative processes by complexing with them (Pietta, 2000).

There are three functional groups that have been attributed to an increase in the ROS-scavenging potential among the flavonoids: the o-dihydroxy structure of the B ring; the C2 - C3 double bond in concert with a 4-oxo functionality of the C ring; and the additional presence of both a 3- and a 5-hydroxyl moiety of the C and A rings, respectively (Heim *et al.*, 2002).

b. Chelating activity

Flavonoids can also act as antioxidants by chelating metal ions (**Fig. 6**) such as Fe^{+2}/ Cu^{+} and Fe^{+3}/Cu^{+2} ions that are involved in the conversion of O_2 and H_2O_2 into highly aggressive OH$^{.}$ through Haber-Weiss/Fenton-type reactions (Engelmann *et al.*, 2005). Thus, chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Andjelkovic *et al.*, 2006).
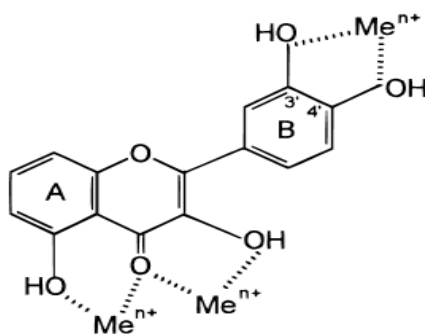
Fig. 6. Binding sites for trace metals (Pietta, 2000).

The flavonoids with better Fe^{+3} reducing activity are those with a 2, 3-double bond and possessing both the catechol group in the B-ring and the 3-hydroxyl group. The copper reducing activity seems to depend largely on the number of hydroxyl groups. However, it must also be recalled that plant polyphenols bearing catechol and/or pyrogallol moieties can, under certain circumstances, exert prooxidant properties, notably by reducing Fe^{+3} or Cu^{+2} ions that they chelate (Mira *et al.*, 2002).

c. Implication in lipidic peroxidation process

The previous two action modes may explain the effect of flavonoids on lipidic peroxidation, since lipidic peroxidation implies the presence of free radicals and metallic ions. They are able to directly scavenge free radicals and then interrupt the propagation step. Moreover, as potent chelators, they chelate the free iron (Engelmann *et al.*, 2005). Finaly, flavonoids present on membrane surface are able to regenerate the vitamin E, one of antioxidant essential to cell membrane protection (Negre-Salvayre *et al.*, 1995).

d. Enzymes inhibition

Flavonoids are also known to be potent inhibitors for several enzymes, such as XO, NOS cyclooxygenase, lipooxigenase, and phosphoinositide 3-kinase. The presence of numerous aromatic hydroxyl groups on flavonoids enables them to easily attach to enzyme surfaces averting potent inhibition (Middleton *et al.*, 2000).

The hydroxyl groups at C-5, C-7, C3' and C4' and the carbonyl group at C4, which contribute favorable hydrogen bonds and electrostatic interactions between inhibitors and the active site, and the double bond between C-2 and C-3 in C-ring, which maintain planar structure, were essential for a high inhibitory activity on xanthine oxidase (Cos *et al.*, 1998; Van Hoorn *et al.*, 2002; Lin *et al.*, 2002; Heim *et al.*, 2002).

4. Plant (*Cachrys libanotis* L.)

The Apiaceae (Umbellifereae) is a cosmopolitan family. It is a large family with about 300 genera and more than 3000 species (Heywood *et al.*, 1996). In Algeria, it has about 55 genera and 130 species. Cachrys is a genus belonging to the family of Apiaceae. This genus consists of more than 100 species and is widely distributed in Mediterranean basin (Quezel and Santa, 1963).

4.1. Taxonomy

Kingdom: Plantae, subkingdom: Tracheobionta, super division: Spermatophyta, division: Magnoliophyta, class: Magnoliopsida, subclass: Rosidae, order: Apiales, family: Apiaceae, genus: *Cachrys*, specie: *Cachrys libanotis* L. (*C. libanotis*).

Cachrys libanotis has many synonyme such as *Cachrys echinophora* var. leiocarpa Guss., *Hippomarathrum libanotis* var. typicum Fiori, *Hippomarathrum libanotis* Koch, and *Hippomarathrum bocconei* Boiss.

Cachrys libanotis is called: ighni (local name), amarint(h)e or armarint(h)e à fruit lisse (French), and with the common Persian name of horse fennel. However, only the name of genus (*Cachrys*) was found in English, basil. Cachrys was derived from the romans word *kaio*, to burn, on account of the carminative qualities of the plants (Don, 1834).

4.2. Botanical description

Cachrys libanotis is a perennial plant distributed in Italy, Sardinia, North Africa and South West of Europe. It's of 40-80 cm height; its leaves are divided into linear strips. Flowers are yellow and organized in umbel. Its fruits are smooth and ovoid (10-15 mm) and its seeds are extremely acrid. Its roots are gross, white, branching, and aromatic (**Fig. 7**). The plant grows in arid places and in hedges (Quezel and Santa, 1963).

4.3. Traditional uses

Cachrys libanotis is heating, astringent and anti-jaundice (de Lamarck and Poiret, 1783). Local healers have traditional knowledge about their uses. In fact, they use this plant for treatment of rheumatism. Noting that, in our knowledge, there is no scientific studies on its biologic proprieties, excepting its dermatitis effect (Ena *et al.*, 1989; Ena *et al.*, 1991).

Arial part

Leaves

Flowers

Roots

Fig. 7. *Cachrys libanotis* L. plant. Arial part, leaves and flowers available from: http://www.actaplantarum.org/acta/albums1.php?id=3824

Materials and methods

1. Materials

1.1. Biological materials

The medicinal plant *C. libanotis* L. was gathered in November 2010 from Ain-Touta, Batna. The sample was authenticated   by Pr. Oudjhih Bachir, university Elhadj Lakhdar, Batna. *Cachrys libanotis* roots were washed, cut in thin rings, dried for two weeks in free air and in

dark, and powdered using traditional mill. The powder was conserved in glass bottle at ambient temperature until its use. Bovine milk was obtained in February 2011 from local farm (Guedjel) and stored at 4°C prior to its use.

1.2. Chemicals and materials

Many of chemical materials were used : butanol, methanol, hexane, chloroform, acetate ethyle, acetic acid, Folin-Ciocalteu, aluminium chloride ($AlCl_3$), Caffeic acid, catehin, ellagic acid, epicatechin, feslin, flavon, propyl gallat, gallic acid, gossypin, kaemferol, morin, myricetin, naringin, naringenin, quercetin, rutin, etylenediamine-tetraacetic acid (EDTA), dithiothreitol (DTT), ammonium sulfate, sodium phosphate (Na_2HPO_4 and NaH_2PO_4), bicine, acrylamide, bis-acrylamide, brillant bleu R250, glycine, sodium dodecyl sulphate (SDS), coumassie blue, xanthine, NAD^+, allopurinol, cytochrome c, 1,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), β-carotene, linoleic acid, tween 40, butylated hydroxytoluene (BHT), nitro bleu tetrazolium (NBT), 5-methylphenazinium methyl sulfate (PMS), ferrozine, iron II chloride ($FeCl_2$), 2,4,6- tripyridyl-s-triazine (TPTZ), iron III chloride ($FeCl_3$). All these reagents are from Sigma, Fluka and Prolab. Among used apparatus: rotavapor (Rotavapor Germany, bÜchi461), centrifuge (Sigma) 3K30, spectrophotometer (TechomP, UV/VIS-8500), electro-migration apparatus (pharmacia-LKB), HPLC apparatus (Shimadzu 10 vp).

2. Methods

2.1. Flavonoids extraction

Cachrys libanotis powder was soaked, according to Markham method (1982), in 85% aqueous-methanol with a ratio of plant material and extracting solvent of 1:10 w/v, under agitation overnight at 4°C. The extract was filtered on filter paper then on sintered glass to obtain the first filtrate. This procedure was repeated on the residue using 50% aqueous – methanol under agitation for 4h to obtain the last filtrate. The first and the last filtrates were

combined then the methanol was removed under reduced pressure on a rotavapor below 45°C. The milk white crude extract was coded as CrE. A defined portion of total CrE volume was lyophilized and stored at -20°C until it use.

CrE was subjected to fractionation using liquid-liquid extraction. CrE was successively extracted with different solvents of increasing polarity: hexane for defatting, chloroform for aglycone flavonoids extraction and ethyl acetate for glycoside flavonoids extraction (**Fig. 8**). The obtained organic layer of each partition was evaporated under reduced pressure on a rotavapor below 45°C to dryness and to afford hexane, chloroform, ethyl acetate and aqueous fractions coded as HxE, ChE, EAE and AqE, respectively. All of these fractions were stored at -20°C prior to use.

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                         ┌─────────────────┐
                         │  Roots powder   │
                         └─────────────────┘
                                 │ - Methanol 85% extraction
                                 │ - Agitation for 24h
                                 │ - Filtration on sintered glass
                  ┌──────────────┴──────────────┐
                  ▼                              ▼
          ┌──────────────┐              ┌──────────────┐
          │   Filtrate   │              │   Residue    │
          └──────────────┘              └──────────────┘
                  │                              │ - Methanol 50% extraction
                  │                              │ - Agitation for 4h
                  │                              │ - Filtration on sintered glass
                  │                       ┌──────┴───────┐
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          ┌──────────────────┐    ┌──────────────┐  ┌──────────────┐
          │ Combined filtrates│◄──│   Filtrate   │  │   Residue    │
          └──────────────────┘    └──────────────┘  └──────────────┘
                  │ - Filtration (filtre paper)
                  ▼
          ┌──────────────┐
          │ Crude  extract
          └──────────────┘
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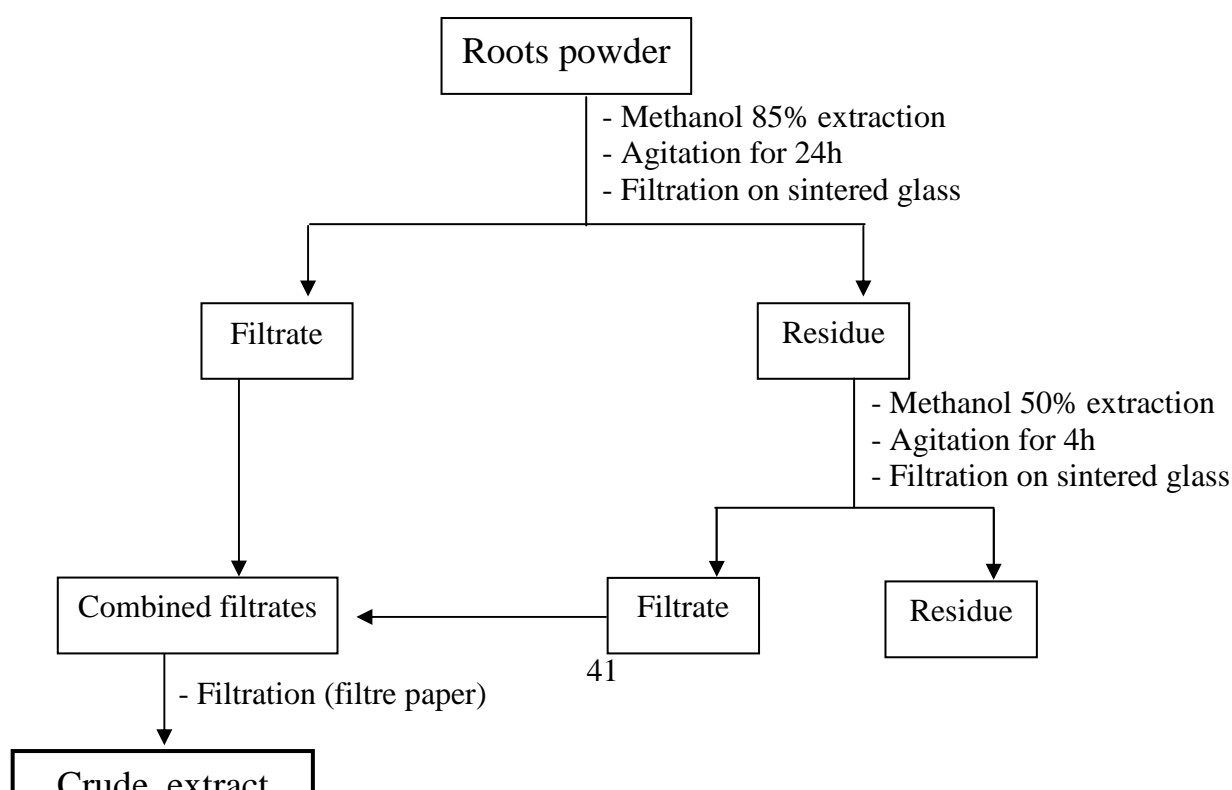
Fig. 8. Extraction procedure of flavonoids from *C. libanotis roots* (Markham, 1982).

2.2. Analyse of *C. libanotis* extracts

2.2.1. Determination of total polyphenols content

Total polyphenols content was estimated by the Folin–Ciocalteu method (Li *et al.*, 2007). This method consists of the phosphotungstic (WO_4^{-2})-phosphomolybdic (MoO_4^{-2}) acid (Folin-Ciocalteu's reagent, FCR) reduction by the phenolic hydroxyl groups, resulting in the formation of a blue product in alkaline solution. Briefly, 200 µl of appropriate dilution of each

extract were added to 1 ml of 1:10 diluted FCR. After 4 min, the reaction mixture was neutralized with 800 µl of saturated sodium carbonate (75 g/l). Subsequently, the shaken mixture was allowed to stand for 2h at room temperature, and then measured at 765 nm using VIS spectrophotometer. Gallic acid (20-140 mg/l) was used for the standard calibration curve (**Fig. 9**). The results were expressed as µg gallic acid equivalent (GAE)/mg of each extract.
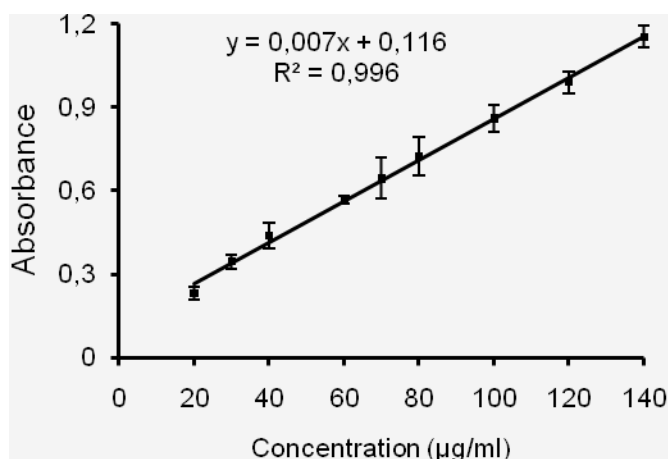
Fig. 9. Calibration curve of gallic acid.

2.2.2. Determination of flavonoids content

The $AlCl_3$ method (Bahorun *et al.*, 1996) was used for determination of the flavonoids content of the *C. libanotis* extracts, employing the reaction of complex formation between flavonoids and aluminum chloride. Aliquots of 1 ml of each extract were added to equal volumes of a solution of 2% $AlCl_3.6H_2O$ (2 g in 100 ml methanol). The mixture was vigorously shaken, and absorbance was read at 430 nm after incubation in dark at room temperature of 10 min. Quercetin and rutin (1-40 mg/l) were used as standards for calibration curve (**Fig. 10**). Flavonoids contents were expressed as µg quercetin and rutin equivalent (QE and RE, respectively)/mg of each extract.
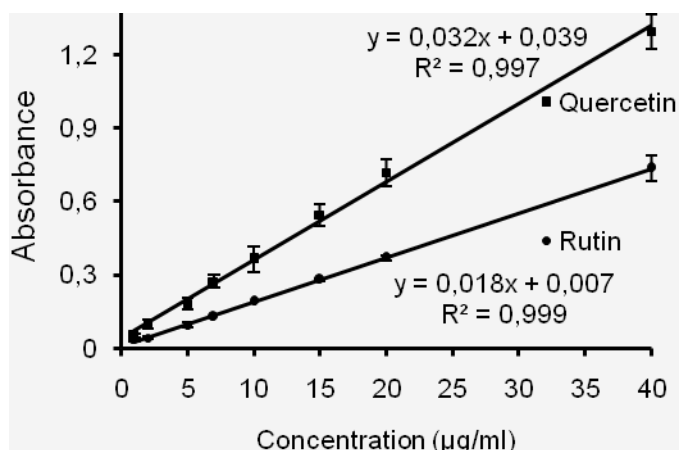
Fig. 10. Calibration curve of quercetin and rutin.

2.2.3. Reverse polarity-high performance liquid chromatography (RP-HPLC)

Reverse polarity-high performance liquid chromatography (RP-HPLC) was performed with a Shimadzu 10 vp class system comprising a pump, a vacuum degasser, a UV-VIS detector, and a 25 µl sample injector. Compounds were separated on a 125 mm × 4.6 mm C18 column. The mobile phase comprised acetonitrile and bidistilled water at a volume ratio 8:2. The flow rate was 1 ml/min. The chromatograms were recorded at 280 nm selected on the specific UV absorption of the assayed compounds. Separated extract peaks were initially identified by direct comparison of their retention times with those of standards : Caffeic acid, catehin, ellagic acid, epicatechin, feslin, flavon, propyl gallat, gallic acid, gossypin, kaemferol, morin, myricetin, naringin, naringenin, quercetin, rutin.

2.3. Purification of bovine milk xanthine oxidoreductase (BMXOR)

Purification of BMXOR was carried out according to Sanders and their collaborators method (1997), slightly modified by Baghiani and their collaborators (2003). The BMXOR was purified in the presence of DTT, by ammonium sulfate fractionation, followed by affinity chromatography on heparin-agarose. XOR concentration was estimated at 450 nm using the FAD extinction coefficient of $36\,000$ $M^{-1}cm^{-1}$ (Venton *et al.*, 1988). The purity of enzyme

was assessed on protein/flavin ratio (PFR= A_{280}/A_{450}) (Bray, 1975), and on a 10% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Total and specific activity of BMXOR was assayed spectrophoto-metrically at 295 nm following the production of uric acid with an extinction coefficient of $9600 M^{-1} cm^{-1}$ (Avis *et al.*, 1956).

2.4. Evaluation of antioxidant activity of *C. libanotis* by enzymatic methods

2.4.1. XOR inhibition

XOR inhibitory activity was carried out using spectrophotometric method, following uric acid production at 295 nm (Boumerfeg *et al.*, 2009). The assay mixture consisted of 840 µl of 50 mM air-saturated phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH 7.4, and containing 0.1 mM EDTA), 100 µl of 0.1 mM xanthine (pH 7.4), 50 µl of corresponding concentration of each extract diluted in buffer or methanol, and 10 µl of enzyme solution. The reaction was initiated by the addition of enzyme and the inhibition was evaluated after 1 min. Allopurinol was assayed as a positive control. Inhibition of XOR was calculated as:

$$Inhibition\% = \frac{A_c - A_e}{A_c} X100$$

Where A_c: XOR activity without extracts or allopurinol, and A_e: XOR activity in the presence of extracts or allopurinol. Results were expressed as extract concentration that inhibits 50% of enzyme activity (IC_{50}).

2.4.2. Cytochrome c test

Superoxide produced by the xanthine-XOR system are able to reduce cytochrome c. Effects of *C. libanotis* extracts on the generation of O_2^- were determined according to (Robak and Gryglewski, 1988). The reaction mixture contained xanthine (100 µM), cytochrome c (25 µM) in air-saturated phosphate buffer (Na_2HPO_4 and NaH_2PO_4, 50 mM, pH 7.4) supplemented

with 0.1 mM EDTA, and corresponding concentration of each extract diluted in buffer or methanol. The reaction was started by the addition of the enzyme. After 1 min, reduced cytochrome c was spectrophotometrically determined at 550 nm against enzyme-free mixture. Reduction cytochrome c inhibition was calculated as:

$$Inhibition\% = \frac{A_c - A_e}{A_c} X100$$

Where A_c: reduced cytochrome c absorbance without extracts, and A_e: reduced cytochrome c absorbance in the presence of extracts. Results expressed as extract concentration that inhibit the reduction of the moiety amount of cytochrome c (IC_{50}).

2.5. Evaluation of antioxidant activity of *C. libanotis* by non-enzymatic methods

2.5.1. NBT assay

Antiradical activity was determined according to Ani and their collaborators (2006) method. The assay involves the production of $O_2^{.-}$ from O_2, using NADH as a reductant, and PMS as a catalyst, in the presence of an indicator (NBT), which turns blue when reduced by $O_2^{.-}$. The color change can be monitored spectrophotometrically at 560 nm. Briefly, the reaction mixture consisted of 300 µl of buffer (NaH_2PO_4 and Na_2HPO_4, pH 7.8), 50 µl β-NADH (3 mM), 50 µl of varying concentrations of each extract, 50 µl NBT (1 mM) and 50 µl PMS (0.3 mM). The reaction was conducted at room temperature for 2 min and initiated by the addition of PMS for 3 min. Superoxide scavenging by extracts was determined by comparing the extent of NBT reduction with controls. Evaluating the superoxide-scavenging was based on IC_{50}. The IC_{50} values were expressed as mg/ml. The inhibition percentage of superoxide anion generation was calculated using the following formula, where A_c is the control absorbance, and A_e is the absorbance in the presence of extracts:

$$Inhibition\% = \frac{A_c - A_e}{A_c} X100$$

2.5.2. β-carotene-linoleic acid bleaching method

Antioxidant capacity is determined by measuring the inhibition of *C.libanotis* extracts compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Aslan *et al.*, 2006). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1ml of chloroform, and then 25 µl linoleic acid and 200 mg Tween 40 were added in flask. Chloroform was evaporated using a rotavapor. 100 ml distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking to form emulsion. 350 µl of each extracts, prepared in methanol and/or distilled water at concentration of 2 mg/ml, were added to 2500 µl aliquot of reaction mixture, and the emulsion system was incubated up to 48h in dark at room temperature. Control samples (2 mg/ml) received only the emulsion without any sample, while blank consisted only of corresponding extract or control. After this incubation period, absorbance of the mixtures was measured at 490 nm after 0h, 1h, 2h, 4h, 6h, 12h, 24h and 48h of incubation. The rate of bleaching of β-carotene was calculated as antioxidant activity and calculated using the equation:

$$Antioxidant\ activity\% = \frac{A_e}{A_c} X100$$

Where A_c: control absorbance (BHT), and A_e: absorbance in the presence of extracts.

2.5.3. DPPH scavenging assay

The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free-radical scavenging antioxidant activity. DPPH is a purple-

colored stable free radical; it becomes reduced to the yellow-colored, diphenyl picryl-hydrazine (Prior *et al.,* 2005). According to Cuendet and their collaborators (1997) method with slight modification, 50 µl of various dilutions of each extract or standards were mixed with 1250 µl of a 0.004% methanol solution of DPPH. After an incubation period of 30 min in dark at room temperature, the absorbance of the samples was read at 517 nm. BHT, gallic acid, quercetin, rutin were used as standards. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The ability to scavenge the DPPH radical was calculated by using the following equation:

$$Scavenging\ effect\% = \frac{A_c - A_e}{A_c} X100$$

Where A_c: control absorbance and A_e: absorbance in the presence of extracts. IC_{50} values of the extract, concentration of extract necessary to decrease the initial concentration of DPPH by 50%, was calculated.

2.5.4. Ion chelating assay

Ferrous iron-chelating activity was measured by inhibition of the formation of Fe^{+2}-ferrozine complex after treatment of test extract with $Fe^{+2,}$ following the method of Decker and Welch (1990) modified by Le and their collaborators (2007). The reaction mixture contained 250 µl extract, 50 µl $FeCl_2$ (0.6 mM in water) and 450 µl methanol. The control contained all the reaction reagents except the extract. The mixture was shaken and allowed to react at room temperature for 5 min. 100 µl of ferrozine (5 mM in methanol) were then added, the mixture shaken again, followed by further reaction at room temperature for 10 min to complex the residual Fe^{+2} ion. The absorbance of the Fe^{+2}-ferrozine complex was measured at 562 nm against a blank contained all the reaction reagents except ferrozine. Lower absorbance indicates a higher chelating power. EDTA and quercetin were used as reference chelators. The chelating activity was calculated as percentage:

$$Chelating\ activity\% = \frac{A_c - A_e}{A_c} X100$$

Where A_c: control absorbance and A_e: absorbance in the presence of extracts. EC_{50}, effective concentration which produces 50% Fe^{+2} ions chelating, was calculated from curve of chelating percentage in function of *C. libanotis* extracts concentrations.

2.5.5. FRAP assay

The total antioxidant potential of each extract was determined using an Fe^{+3} reducing ability of plasma (FRAP) (Benzie and Strain, 1996) modified by Pulido and their collaborators (2000). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue-colored Fe^{+2}-TPTZ compound by the reaction of colorless Fe^{+3} and electron-donating antioxidants. FRAP reagent was prepared by mixing 10 volume of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ in 40 mM HCl and 1 volume of 20 mM $FeCl_3$. Freshly prepared FRAP reagent was warmed to 37°C, and a reagent blank reading was taken at 593 nm. Subsequently, 90 µl of deionized water and 30 µl of sample, water or methanol as appropriate for the reagent blank, were added to the FRAP reagent. The initial blank reading with the FRAP reagent alone was subtracted from the final reading selected of the FRAP reagent with the sample to determine the FRAP value of the sample. A standard curve (**Fig. 11**) was prepared using different concentrations (0-2 mM) of $FeSO_4 .7H_2O$. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$, EC1 (Pulido *et al.*, 2000). EC1 was calculated as the concentration of antioxidant giving absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1mM concentration of Fe^{+2} solution determined using the corresponding regression equation. In this study the reaction was monitored for 30 min, but the 4 and 30 min readings were selected for calculation of FRAP values. Total antioxidant power (TAP) was calculated from EC1. All solutions were used on the day of preparation.
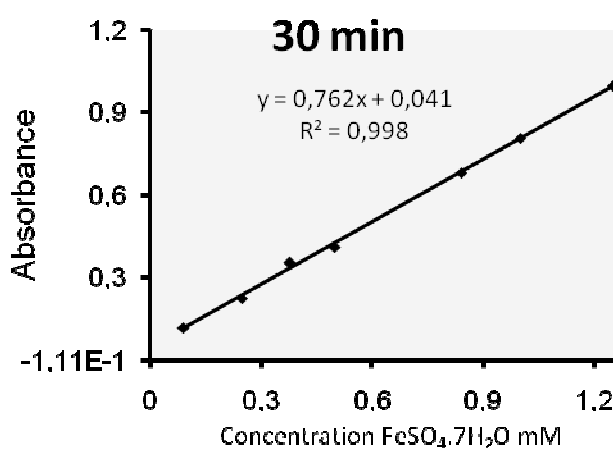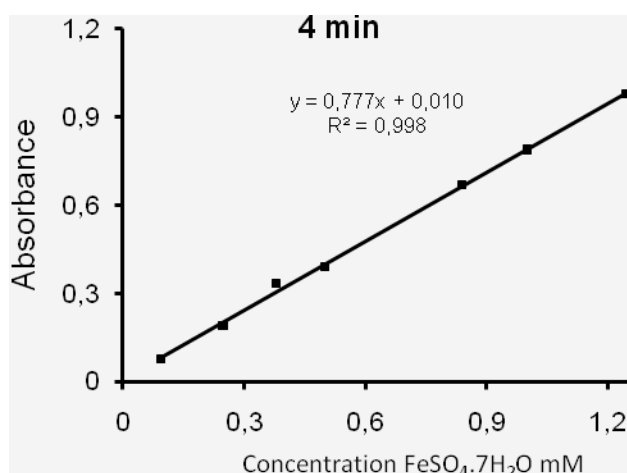
Fig. 11. Calibration curve of FeSO$_4$.7H$_2$O.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) of two or three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA), where the differences between extracts to each other were determined by Dunnett's multiple comparison test, and Tukey's multiple comparison test for comparison between extracts and standards, using GraphPad program. *p* value ≤ 0.05 was regarded as significant.

Results and discussion

1. Flavonoids extraction

Extracts preparation from *C. libanotis* roots was carried out according to Markham method (1982) using solvents with different polarities. In this extraction, methanol was used to obtain CrE due to its polarity and because it was anticipated that alcohol solvent would more effectively extract the polyphenols which possessed antioxidant activity from plant samples (Mohammedi and Atik, 2011). CrE is fractionated successively with solvents of increasing polarity: hexane, chlorophorm and ethyle acetate leading to separate CrE extract compounds according to their solubility in solvents and thus their glycosylation degree (aglycone flavonoids, mono, di and tri glycosulated). In total, this extraction allows to obtain five extracts: CrE, HxE, ChE, EAE, and AqE. Yields of extraction (**Table IV**) showed that CrE gave the highest yeild with 12.14% followed by AqE (7.38%), ChE (0.26%), EAE (0.21%) and HxE (0.17%).

Table IV. Yields of extraction of *C. libanotis* roots.

Extract	Yield (%)
CrE	12.141 ± 0.338
HxE	0.173 ± 0.006
ChE	0.260 ± 0.008
EAE	0.208 ± 0.008
AqE	7.380 ± 0.101

Solvent extractions (solid-liquid and liquid-liquid extraction) are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability (Stalikas, 2007).Preparation of CrE was carried out in the dark and under cold conditions (4°C) to minimize transformation reaction of phenol induced by UV and to avoid phenol oxidation and hydrolysis (Cseke *et al.*, 2006), respectively.

2. Analyse of *C. libanotis* extracts

2.1. Determination of total polyphenols and flavonoids content

As phenolic compounds constitute one of the major contributors to the antioxidant capacity of plant, it was reasonable to determine their total amount in the selected plant extracts. The total polyphenols content of extracts was estimated by the Folin–Ciocalteu method (Li *et al.*, 2007), and the amount of polyphenols in the extracts was expressed as µg GAE/mg of extract.

According to the results shown in **Table V**, the total polyphenols contents in decreasing order were: 83.23, 49.64, 15.64 and 14.98 GAE µg/mg, corresponding to EAE, ChE, CrE and AqE, respectively. In other words, approximately 1.7-folds, 5.3-folds and 5.6-folds higher amounts of phenolic compounds existed in EAE extract relative to that of ChE, CrE and AqE, respectively.

Table V. Total polyphenols and flavonoids content of *C. libanotis* extracts.

Extract	Total phenols content	Flavonoids content	
	µg GAE/mg of extract	µg QE/mg extract	µg RE/mg extract
CrE	15.635 ± 3.585	0.099 ± 0.007	0.248 ± 0.030
ChE	49.637 ± 6.014	0.109 ± 0.007	0.259 ± 0.022
EAE	83.228 ± 2.034	0.634 ± 0.281	0.977 ± 0.297
AqE	14.977 ± 2.018	0.054 ± 0.011	0.145± 0.003

The choice of Folin-Ciocalteau method as total polyphenols determination method is due to their advantages: convenient, simple, and reproducible. Although FCR is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds, the measurement of color changes after two hours storage and under basic conditions could be used to determine the existence of phenols in extracts (Huang *et al.*, 2005). The determination of polyphenols level is not based on absolute measurements of the amounts of phenolic compounds, but is in fact based on their chemical reducing capacity relative to gallic acid (Amzad Hossain and Shah, 2011).

Since flavonoids are probably the most important natural polyphenols (Djeridane *et al.*, 2010), its amount in each *C. libanotis* extract was measured. The AlCl$_3$ method is simple, inexpensive, offer sigh sensitivity, which makes it preferred in quality control and analytical laboratories. In addition, this method allows the total flavonoids content to be determined even in the presence of other polyphenolic compounds not forming complexes with AlCl$_3$ (Matyushchenko and Stepanova, 2003). The flavonoids content was estimated using AlCl$_3$ method (Bahorun *et al.*, 1996), and calculated as µg QE and RE/mg of extract (**Table V**). The content of flavonoids decrease in the order: EAE, ChE, CrE and AqE, where the highest values found in EAE, 0.63 µg QE/mg extract and 0.98 µg RE/mg extract. In other term, flavonoids existing in EAE as QE/RE are, approximately, 5.8-folds/ 4-folds, 6.4-folds/3.8-folds and 11.7-folds/ 6.8-folds higher than those of ChE, CrE and AqE, respectively. It is noted that this order is similar to that of total polyphenols contents.

Cachrys libanotis roots (CrE) showed a similar polyphenols content (1.897 mg GAE/g dry weight (DW)) to that of *Thapsia garganica* (1.84 mg GAE/g DW), plant belongs to the same family of *C. libanotis* (Apiaceae). In contrast, its flavonoids content (0.032 mg RE/g DW) is lower than that of *Thapsia garganica* (1.25 mg RE/g DW), according to the Djeridane and their collaborators (2010), worked on 18 Algerian medicinal plants. Moreover, by 2-folds lower, polyphenols content of CrE was equal to that of *Plumbago rosea* (4.41 mg GAE/g DW), roots that showed the highest level of polyphenols, in the work of Surveswaran and their collaborators (2007) on 133 Indian medicinal plants. Also, *C. libanotis* roots might be considered as poor source of phenolic compounds, when it compared to the highest polyphenols content (*Acacia catechu*, 41.47 mg GAE/g DW), in the previous work.

2.2. RP-HPLC

In the present study, we conducted preliminary identification of representative natural phenolic compounds from selected *C. libanotis* extracts by chromatography with phenolic

standards. RP-HPLC currently represents the most popular and reliable technique for analysis of phenolic compounds (Dai and Mumper, 2010).

By comparison with T_R of standards (**Table VI**), ChE profile (**Fig. 12**) revealed the presence of catechin or epicathechin (2.81%; T_R= 1.583 mn) and probably gossypin (0.42%, T_R= 0.917 mn) and myricetin (8.57%; T_R= 1.967 mn). Whereas, EAE profile showed the presence of gossypin (3.68%) and myricetin (5.03%; T_R= 1.950 mn). In contrast of ChE and EAE profiles, both CrE and AqE profiles did not present any phenolic compounds corresponding to used standard.

Table VI. Retention time of standards.

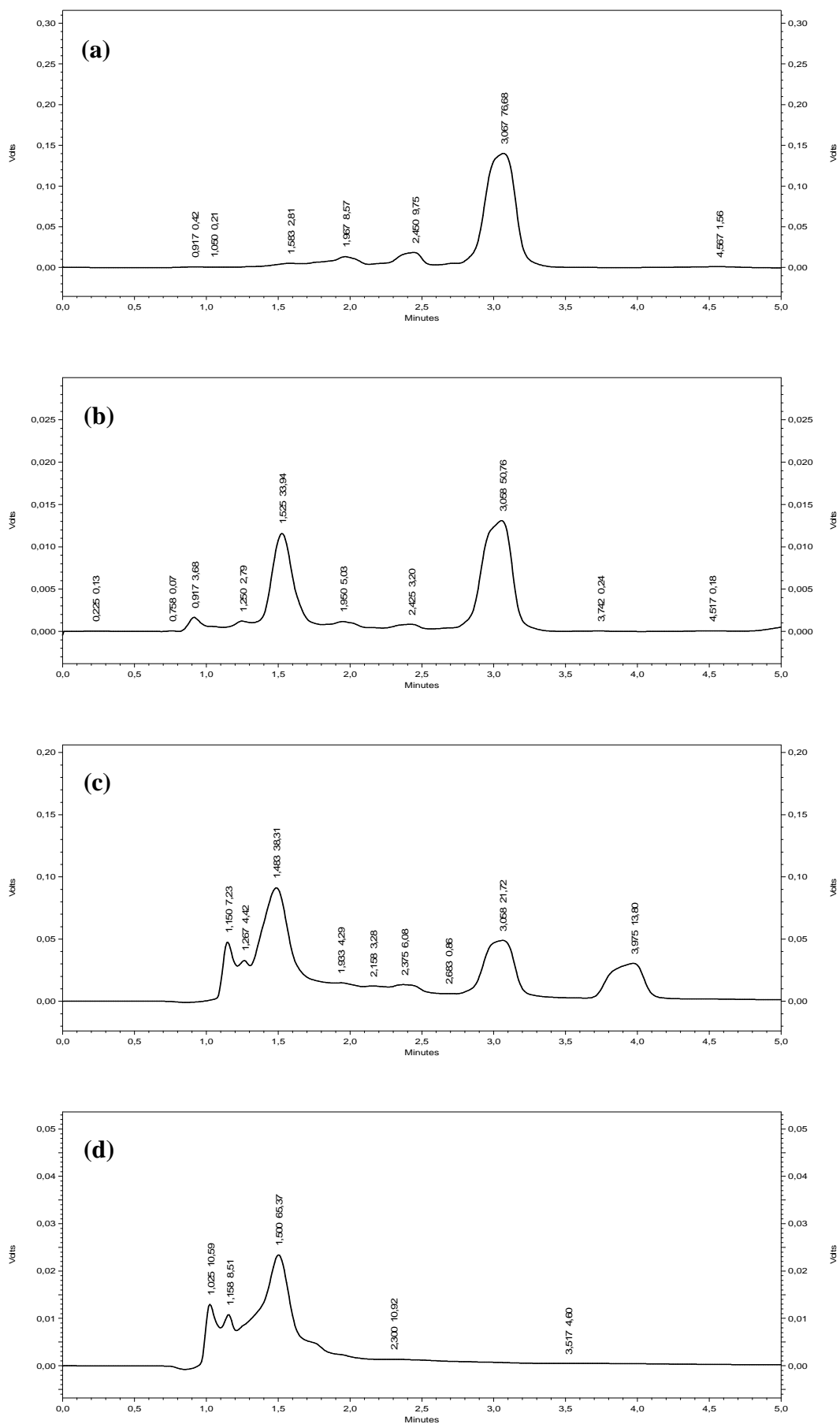| Standards	Retention time (mn)
Gossypin	0,908
Naringin	1,533
Gallic acid	1,567
Rutin	1,567
Epicatechin	1,583
Catechin	1,583
Morin	1,592
Ellagic acid	1,600
Caffeic acid	1,633
Gallat propyle	1,755
Naringenin	1,800
Quercetin	1,917
Myricetin	1,958
Kaempferol	1,992
Fisetin	2,467
Flavon	3,250

Fig. 12. Separated extracts peaks in RP-HPLC (a): ChE, (b): EAE, (c): CrE and (d): AqE.

3. Purification of bovine milk xanthine oxidoreductase (BMXOR)

XOR was prepared from bovine milk (Sanders *et al.*, 1997), method slightly modified by Baghiani and their collaborators (2003). The yield of purification is up to 17.97 mg per liter of milk, comparable to that found by Baghiani and their collaborators (2003). The purified BMXOR showed an UV/VIS spectrum with classical maxima at 280, 325 and 450 nm. SDS-PAGE of BMXOR showed a major band of around 150 kDa (Result not showen). As generally accepted, the most sensitive indicator of XOR purity is the PFR (A_{280}/A_{450}), which, for the purest enzyme preparations, is close to 5.0 (Bray, 1975). The purified BMXOR gave a PFR of 7.2, indicating an acceptable purity (**Table VII**).

The XOR activity was determined following the amount of uric acid formed without NAD^+ (oxidase activity) and in the presence of NAD^+ (oxidase plus dehydrogenase activity, total activity). Xanthine oxidase specific activity was 2011 nmole/min/mg of enzyme; whereas total specific activity was 2261 nmole/min/ mg of enzyme (**Table VII**). These results are similar to those obtained by Baghiani and their collaborators (2003).

Table VII. Characteristics of purified BMXOR.

PFR	XO ratio	Concentration (mg/ml)	Specific activity (nmole/min/mg of enzyme)	
			XO	Total activity
7.2	89%	1.198	2011	2261

Around 10% of the purified enzyme was in the dehydrogenase form. Since the solubilization of BMXOR, as described herein, was done in the presence of 5mM of DTT (reducing agent), it is reasonable to assume that the solubilized BMXOR will be primarily in XDH form and this form can be retained during the purification procedure (Hunt and Massey, 1992). However, in the process of purification of BMXOR, by means of ammonium sulfate fractionation, dialysis, and chromatography, the reducing agent will be progressively eliminated from the protein solution. Under this non-reduced condition the XDH form will be

converted to the XO. The isolated BMXOR will therefore be mostly, if not completely, in the XO form.

4. Evaluation of antioxidant activity of *C. libanotis* by enzymatic methods

4.1. XOR inhibition

Inhibition of XOR by *C. libanotis* extracts results in a decreased production of uric acid, which was measured spectrophotometrically at 295nm. Results were expressed as IC_{50}Values.

Extracts of *C. libanotis* inhibited XOR in a concentration-dependent manner (**Fig. 13**). The most potent XOR inhibitors observed were EAE and ChE with an IC_{50} of 0.110 ± 0.006 and 0.139 ± 0.002 mg/ml, respectively, followed by CrE ($IC_{50} = 1.049 \pm 0.001$ mg/ml) then AqE ($IC_{50} = 2.349 \pm 0.076$ mg/ml). The *in vitro* inhibition of XOR by EAE and ChE showed a remarkable effect by 13-folds and 16-folds, respectively, comparing to allopurinol as a specific inhibitor for XO (**Fig. 14**).
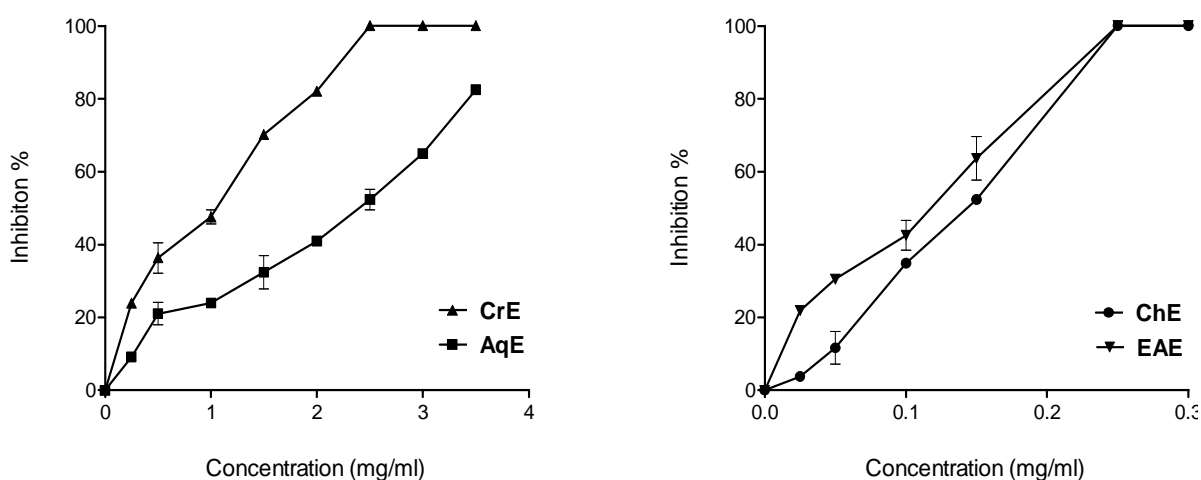

Fig. 13. BMXOR inhibition by *C. libanotis* roots extracts. Values were expressed as mean ± SD of duplicate.

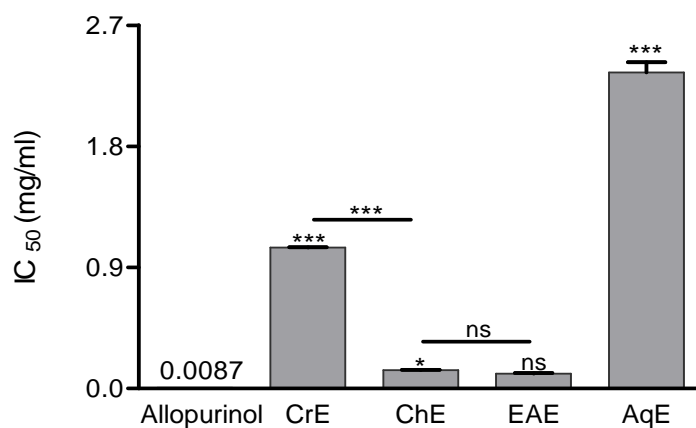**Fig. 14.** IC_{50}, Inhibitory concentration of *C. libanotis* extracts for 50% of BMXOR activity. Comparison was realized against allopurinol. *: $p \leq 0.05$, ***: $p \leq 0.001$, and ns: non significance.

Comparing our results to that of Boumerfeg and their collaborators (2009), EAE presented an inhibitory effect higher than that observed with EAE of *Tamus communis* L. roots (0.316 mg/ml), while ChE showed a similar effect compared to that of ChE (0.138 mg/ml). Thus, *C. libanotis* roots are more effective in inhibiting uric acid formation than *Tamus communis* L.

Since flavonoids are a group of polyphenolic compounds, which have been reported to possess XO inhibitory activity (Costantino *et al.*, 1992), the presence of phenolics and flavonoids content in the extracts would have contributed towards XOR inhibition. The IC_{50} values were plotted versus the polyphenol and flavonoid contents. The rough trend: the higher the polyphenol and flavonoid contents, the lower IC_{50} value. In the view of this correlation between the polyphenol and flavonoid contents and XOR inhibition activity of *C. libanotis* extracts, our results were confirmed by those obtained with *Tamus communis* L. roots (Boumerfeg *et al.*, 2009), *Geranium sibiricum* L. (Wu *et al.*, 2010) and *Carthamus caurulis* (Baghiani *et al.*, 2010), extracts contained high phenolic compounds level were the most active. In addition, our results showed that the XOR inhibition could be linked not only on the polyphenols and flavonoids contents of the extract, but also to the nature (structure) of these compounds (Cos *et al.*, 1998), where, glycosidic (EAE) and non-glucosidic polyphenols (ChE) showed the highest inhibitory effect.

Previous studies (Selloum *et al.*, 2001; Van Hoorn *et al.*, 2002; Lin *et al.*, 2002) indicate that flavones (e.g. Myrectin) show a high potential for inhibition of XO with competitive mode. However, catechin and epi-catechin does not show any inhibitor activity on XO (Cos *et al.*, 1998; Hsieh and Chang, 2010). Or, RP-HPLC results showed the presence of myrectin in both EAE and ChE, catechin and epicatechin were recorded in ChE. Thus, we can assume that a part of *C. libanotis* inhibition of XOR is due to presence of myrectin binding to XO allosteric center, thereby replacing xanthine. In addition, *C. libanotis* may content other components exercising XOR inhibition action. In fact, some authors reports the possible synergism between polyphenolic compounds and other components present in an extract, may contribute to its overall antioxidant activity (Ordonez *et al.*, 2006).

4.2. Cytochrome c test

The O_2^- generated from the xanthine/XOR system can be measured by their ability to reduce cytochrome c^{+3}. Thus, the initial rate of cytochrome c reduction will directly reflect the level of O_2^- (McCord and Fridovich, 1968). The effects of CrE and all fractions of *C. libanotis* roots were studied for their ability to scavenge O_2^-.

The O_2^- scavenging activity of the *C. libanotis* extracts are increased markedly with increasing concentrations (**Fig. 15**). Results showed that ChE present the most potent scavenger of O_2^- with an IC_{50} of 0.223 ± 0.001 mg/ml followed by EAE with IC_{50} of 0.471 ± 0.003 mg/ml, which is approximately 2-folds higher than that of ChE. While CrE present a high IC_{50} of 2.685 ± 0.084 mg/ml, IC_{50} value of AqE could not be determined, since the highest concentration tested did not lead to inhibition as high as 50%. The reduction of cytochrome c^{+3} was almost totally inhibited by SOD (330 U/mL). As comparison, *C. libanotis* extracts showed a less superoxide scavenger activity than that of *Tamus communis L.* roots extracts (Boumerfeg *et al.*, 2009).

Fig. 15. Superoxide- scavenging effect of *C. libanotis* extracts using cytochrome c assay, where $O_2^{.-}$ was generated by xanthine-XOR system. Values were expressed as means $\pm$ SD in duplicate.

Since an inhibitory effect on the enzyme itself would also lead to a decrease in reducing cytochrome c^{+3}, we cannot confirm that this decrease in presence of extracts is only due to their $O_2^{.-}$ scavenger abilities. In this regard, the previous results were compared with that obtained from extracts inhibitory effect on XOR, demonstrating that CrE, ChE and EAE exhibited a dual effect (XOR inhibition and $O_2^{.-}$ scavenger) on cytochrome c^{+3} reduction. These extracts present a predominance of an enzyme inhibition activities rather than a scavenging of $O_2^{.-}$. In contrast, AqE shows an inhibitory effect on the enzyme only (**Fig. 16**).

Fig. 16. Comparison between IC_{50} of *C. libanotis* extracts resulting from XOR inhibition and $O_2^{.-}$ scavenging assays, where IC_{50} is the inhibitory concentration for 50% of BMXOR activity and for 50% of cytochrome c activity, respectively. Values were expressed as means $\pm$ SD of duplicate.

Robak and Gryglewski (1988) reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. Myrecetin inhibits stronger the cytochrome c^{+3} reduction than the production of uric acid as found by Selloum and their collaborators (2001). Thus, it was selected into category C (XO inhibitors with an additional superoxide scavenging activity), according to classification of XO inhibitor of Cos and their collaborators (1998); however, catechin and epicatechin are selected into category A (superoxide scavengers without inhibitory activity on XO).

5. Evaluation of antioxidant activity of *C. libanotis* by non-enzymatic methods

5.1. NBT assay

To confirm the scavenger effect of *C. libanotis* extracts on O^{-}_2, the NBT assay was carried out (Ani *et al.*, 2006). The different concentrations of extracts showed antioxidant activities in a dose-dependent manner (**Fig. 17**). At concentration of 120 to 300 µg/ml, ChE inhibited the reduction of NBT by 29.15 to 75.15%, showing considerable superoxide radical scavenging activity higher than those of EAE and CrE by, approximately, 20 and 33-folds, respectively (**Fig. 18**). The results were statistically significant ($p \leq 0.05$).
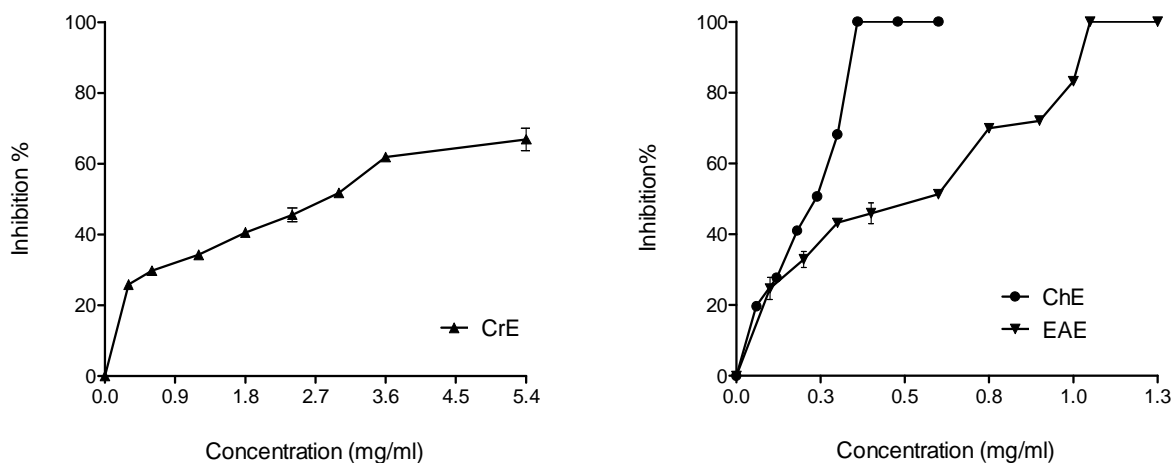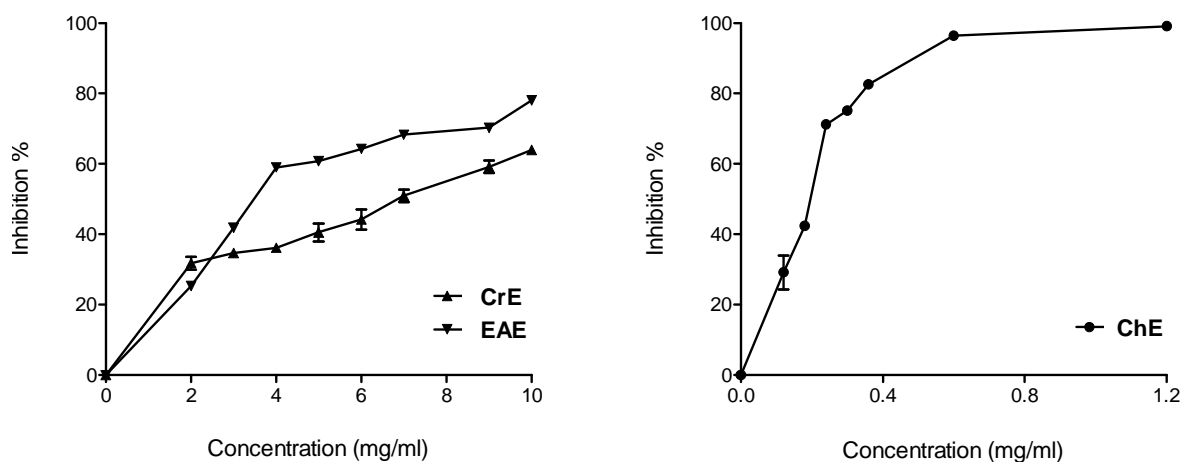

Fig. 17. Superoxide- scavenging effect of *C. libanotis* extracts using NBT assay, where O_2^{-} was generated by NADH/PMS system. Values were expressed as mean ± SD (n=2).
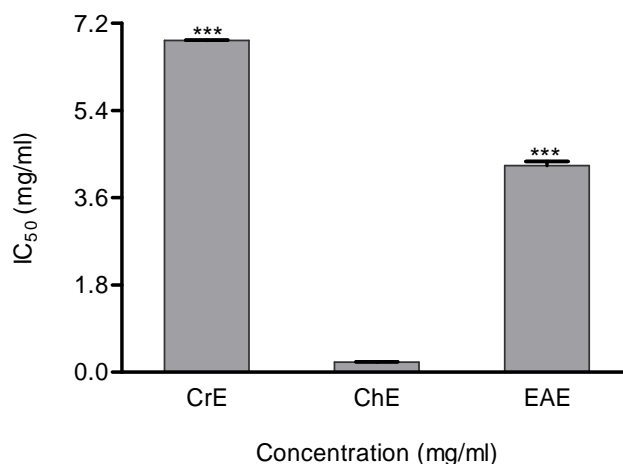
61

Fig. 18. IC_{50}, inhibitory concentration of *C. libanotis* extracts for 50% of superoxide generation using NBT assay. Comparison was realized against ChE, ***: $p \leq 0.001$.

The antioxidant properties of flavonoids are effective mainly via the scavenging of superoxide anion (Robak and Gryglewski, 1988). In addition, Hanasaki and their collaborators (1994) showed that flavonoids such as epicatechin, myricetin and catechin are able to scavenge superoxide radical. Accordingly, our result showed that the superoxide-scavenging effect return to the non-glucosidic phenolic compounds (ChE).

Since the IC_{50} of ChE (0.208 mg/ml, in the NADH-PMS system) similar to that found in the xanthine-XOR system (ChE, 0.223 mg/ml), it might be suggested that ChE has a direct scavenging effect on superoxide radicals. Thus, this result suggests that the possible mechanism of inhibition effect was through direct superoxide radicals- scavenging action instead of XOR inhibition.

5.2. β-carotene-linoleic acid bleaching method

It has long been known that β-carotene reacts with the peroxyl radical to produce β-carotene epoxides (Kennedy and Liebler, 1991). Therefore, β-carotene has received attention as a radical scavenger or antioxidant (Tsuchihashi *et al.*, 1995). The inhibition of β-carotene bleaching in a coupled oxidation with linoleic acid is a well-known methodology used for

evaluating the antioxidant activity. The mechanism of bleaching of β-carotene is a free-radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-carotene undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation; the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically (Naidu *et al.*, 2011).

The kinetic activity of different extracts of *C. libanotis*, BHT, methanol and water during a range of time between 1h to 48h was showed in **figure 19**. EAE exhibited the highest antioxidant activity (76.21 ± 6.12%), which was near to that of BHT (96.17 ± 2.05%) used as positive control, following by CrE and AqE with, approximately, an equal antioxidant activity (65.17 ± 1.88 % and 64.57 ± 1.68%, respectively). Whereas, ChE exhibited the lowest antioxidant activity (56.95 ± 3.40%) (**Fig. 20**).
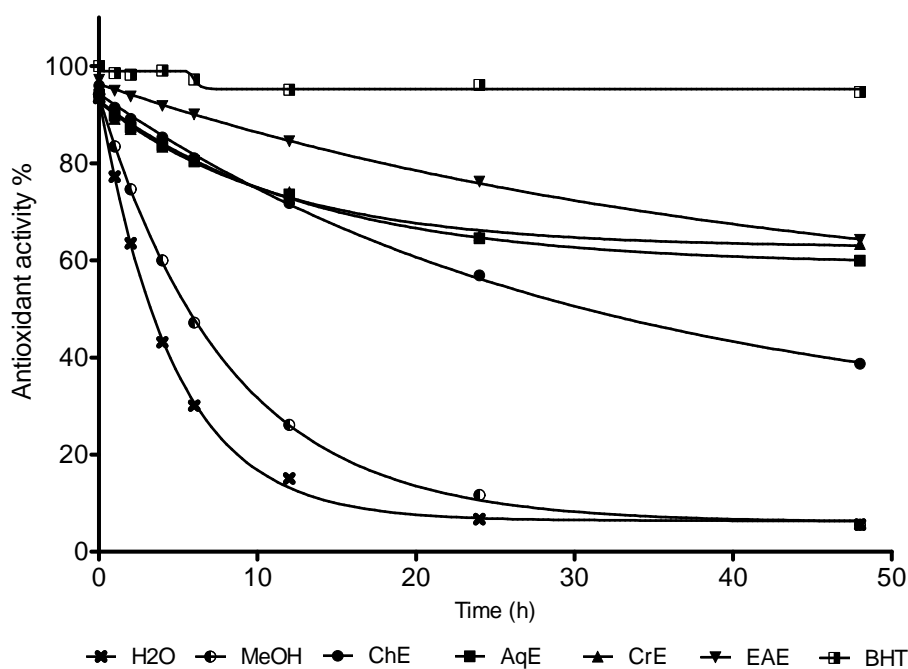

Fig. 19. Kinetics of β-carotene bleaching in the presence of *C. libanotis* extracts, water, methanol and BHT during 48h, using a β-carotene-linoleic acid bleaching assay. Values were expressed as means of triplicate.
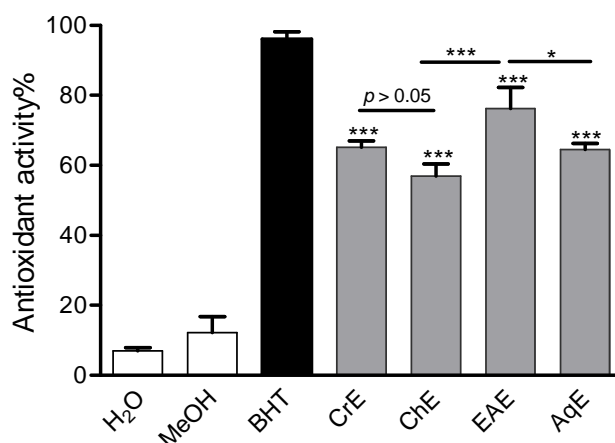
Fig. 20. Antioxidant activity of *C. libanotis* extracts compared with BHT, methanol and water. Comparison was realized against positive control (BHT); $*$: $p \le 0.05$, $***$: $p \le 0.001$.

All extracts of *C. libanotis* roots exhibited an antioxidant activity which was much higher than that of methanolic extract of *Hippomarathrum microcarpum leaves* ($22.9 \pm 2\%$), according to Özer (2007). It was probable that the antioxidative components in these extracts were found to hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

Flavonoids and other polyphenols have the ability to scavenge free radicals and, therefore, delay lipid auto-oxidation (Gao *et al.*, 2000). Thus, the highest activity of EAE could be attributed to its highest contents, and nature (glycosidic) of polyphenol. Moreover, Burda and Oleszek, (2001) reported that the differences in solubility of flavonoids, both aglycones, glycosides, and methoxylated derivatives, in a micellar water–lipid system may influence the results obtained from this test, and the partition of the compounds between the two phases can influence the oxidation results. In this regard, we remarked that mild-polar and polar extracts (EAE, CrE, AqE) were more potent that apolar extract (ChE), which was not adequate with phenomenon of polar paradox (Porter, 1993).

Although ChE contains a high polyphenols amount (apolar antioxidant) than CrE and AqE, it showed the lowest antioxidant activity. According to previous remarks, these results indicate that the polyphenols and flavonoids content and antioxidants polarity are not the only factor

related to this antioxidant activity. The possible synergism of polyphenols with other components present in the extracts may be responsible for this observation.

The antioxidant activity measured as the inhibition of oxidation of linoleic acid can simulate the oxidation of the membrane lipid components (Tepe *et al.*, 2005). Thus, *C. libanotis* roots might exhibit a high effect against peroxidation of lipids. Or, the inhibition of lipid peroxidation implies radicals-scavenging and/or ion chelating, DPPH-scavenging assay and ferrous iron chelating assay were carried out.

5.3. DPPH-scavenging assay

DPPH-scavenging assay is based on a mixed mechanism of free radical stabilization: hydrogen-atom transfer and single-electron transfer. The method presents several analytical critical points (Prior *et al.*, 2005), but it has the great advantage of being easy to implement and not requiring special equipment (just a simple spectrophotometer).

DPPH radical scavenging activity of extracts increased with concentration (**Fig. 21**). The CrE was found to exhibit the greatest scavenger activity with IC_{50} of (0.414 mg/ml), which is lower by 5-folds than that of BHT, followed by that of EAE (0.593 mg/ml). The radical scavenging activity in the plant extracts decreased in the following order: CrE □ EAE □ AqE □ ChE (**Table VIII**). The DPPH scavenging activities of all plant extracts were significantly lower than that of BHT ($p \leq 0.001$).

Table VIII. DPPH scavenging activity of *C. libanotis* extracts and standards.

Standards	IC_{50}(mg/ml)	Extracts	IC_{50}(mg/ml)
BHT	0.087 ± 0.001	CrE	$0.414 \pm 0.010^{***}$
Gallic acid	$1.267 \pm 0.001^{\#}$	ChE	$1.143 \pm 0.024^{***}$
Quercetin	$2.565 \pm 0.000^{\#}$	EAE	$0.593 \pm 0.013^{***}$
Rutin	$5.586 \pm 0.000^{\#}$	AqE	$0.685 \pm 0.006^{***}$

$^{\#}$: µg/ml. Comparison was realized against BHT, ∗∗∗: $p \leq 0.001$.
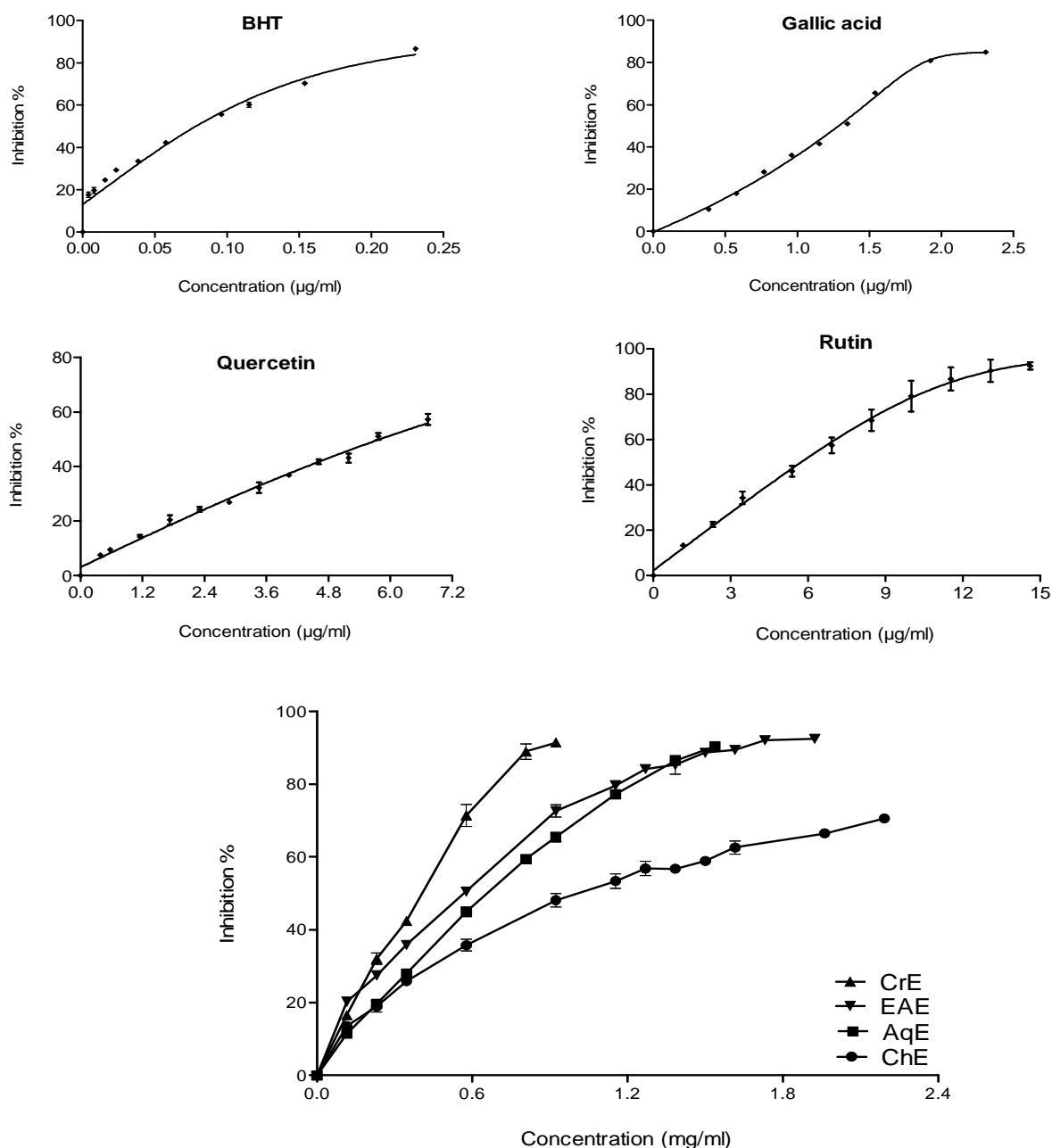
Fig. 21. DPPH scavenging activity of *C. libanotis* extracts and standards. Values were expressed as the mean ± SD of triplicate.

The CrE extract showed a strong scavenging activity compared to methanolic extract of *Hippomarathrum microcarpum* (Bieb) leaves which possess no scavenger activity towards DPPH (Özer *et al.*, 2007).

Surveswaran and their collaborators (2007) reported that the radical scavenging activity of plant extracts depends on the amount of polyphenolic compounds in the extracts. A high

radical scavenging activity of polyphenols can be attributed to their high degree of hydroxylation of aromatic rings, the arrangement of the hydroxyl group, as well as the number of galloyl group and ortho-hydroxyl groups, on benzene nucleus structure (Cai *et al.*, 2006). However, in our study, the order of decreasing scavenging activity among the *C. libanotis* extracts was found to be CrE > EAE > AqE > ChE, which is not in accordance with the amount of phenolic compounds present in these extracts. These results are not in agreement with that found by Amzad Hossain and Shah (2011) and Abdille and their collaborators (2005). Despite the fact that AqE contain the lowest amount of polyphenols and flavonoids, it showed a scavenger activity 1.7-folds higher than that of ChE (IC_{50}= 1.143 mg/ml). This could be explained by the presence of other compounds in AqE beside the polyphenols and also, their specificity against the antioxidant activity.

The measurement of consumption of DPPH radical allows determining exclusively the intrinsic ability of a substance or a complex mixture to donate hydrogen atoms or electrons to this reactive species in a homogeneous system. This technique does not consider the partition in organized mediums or antioxidant mechanisms other than radical scavenging (Chaillu and Nazareno, 2006).

5.4. Ion chelating assay

In this study, ferrous iron chelating activity was measured by inhibition of the formation of Fe^{+2}-ferrozine complex after treatment of test material with Fe^{+2} following the method of Decker and Welch (1990) modified by Le and their collaborators (2007). Ferrozine produces a violet complex with free Fe^{+2}. In the presence of chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased.

The chelating effects of the extracts on ferrous increased as a function of concentration (**Fig. 22**).The extracts exhibited an excellent chelating ability, which was even much better than

that of quercetin ($IC_{50}= 950.4 \pm 11.0$ µg/ml). CrE and AqE showed the highest chelating activity with an IC_{50} values of 52.6 ± 0.3 and 52.7 ± 0.2 µg/ml, respectively, followed by EAE (503.5 ± 3.8 µg/ml) and ChE (635.5 ± 18.4 µg/ml). Moreover, both CrE and AqE had a chelating effect lower by, approximately, 9-folds than EDTA, which demonstrate the excellent chelating ability of these extracts (**Fig. 23**).
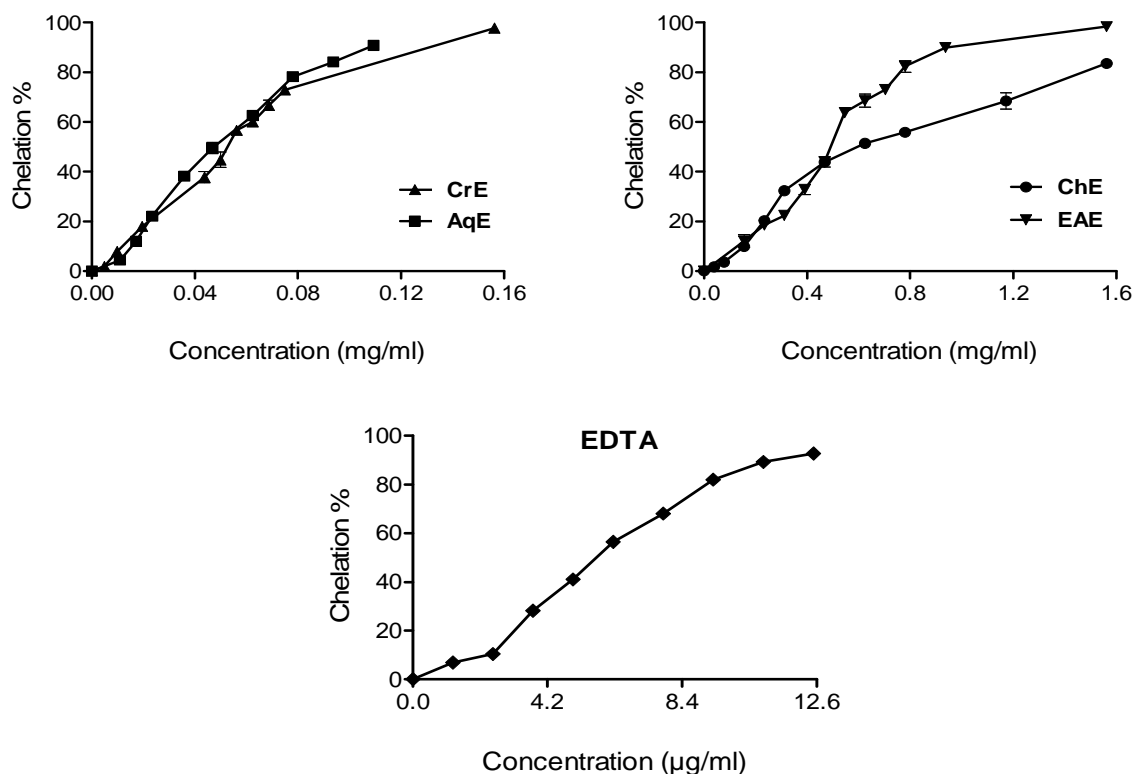

Fig. 22. Ferrous iron-chelating ability of extracts from the roots of *C. libanotis*. Results were expressed as mean $\pm$ SD (n= 3).
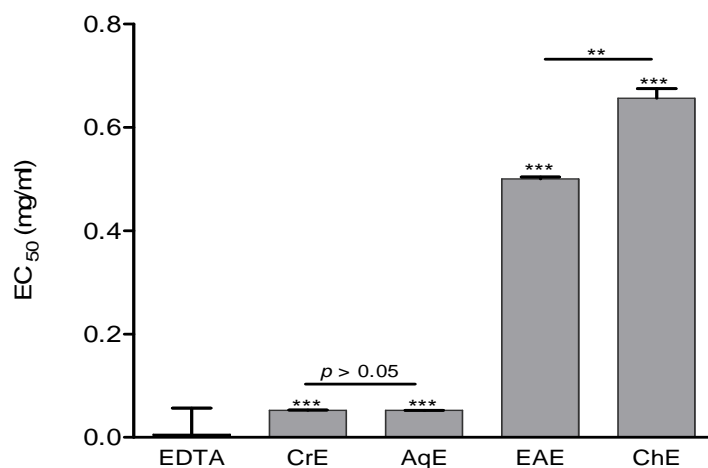

Fig. 23. EC_{50}, effective concentration of *C. libanotis* extracts and EDTA for 50% of chelating activity. Comparison was realized against EDTA; **: $p \leq 0.01$, ***: $p \leq 0.001$.

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EDTA exhibited a strong chelating ferrous iron ability (IC_{50}= 6.1 µg/ml) which is comparable to that found by Le and their collaborators (2007), 5.6 µg/ml. It is because the unique structure of EDTA possesses two nitrogen atoms and four oxygen atoms bearing to carboxyl moiety, which can chelate ferrous ion in the centre and block the Fe^{+2}-ferrozine complex formation (Luo *et al.*, 2011). However, quercetin showed a low activity of chelating ferrous ion, which may be due to the presence of $FeCl_2$ in the system to induce its degradation, indicated that the antioxidant abilities of quercetin were associated with their strong radical scavenging capacity, but not related to their metal chelating capacity (Tung *et al.*, 2009). This result let us suggest that the inhibition of lipidic peroxidation might be due to the chelating effect rather than scavenging one.

The relationship between polyphenols complexation for a range of metals has been widely reported in the literature. As a class, they tend to be good metal complexing agents. It is the structure of polyphenols that ultimately determines their effectiveness as complexing agents as well as the number of metal cations they can bind. Binding ratios vary depending on the molecules involved (Fernandez *et al.*, 2002), where bidentate ligands are more effective complexes than monodentate ligands (Hider *et al.*, 2001). Therefore, phenolics possessing the structural elements necessary to form bidentate complexes with metals would be expected to be good metal chelators. It was also proposed that optimum metal-binding and antioxidant activity is associated with the structures which contain hydroxy-keto group (a 3-OH or 5-OH plus a 4-C=O), as well as a large number of catechol or gallol groups (Khokhar and Owusu Apenten, 2003; Perron and Brumaghim, 2009).

The possible mechanism of chelating Fe^{2+} by phenolics is as follows: phenolics, which possess a number of hydroxyl groups, firstly lose protonated hydrogen and then form strong coordination oxygen ion complexes with a ferrous ion (Fernandez *et al.*, 2002).

Although polyphenols was considered as potent chelators agents, our study on *C. libanotis* extracts showed a weak relation between polyphenols contents and chelating activity. This result combined with IC_{50} values let us suggest that the noticeable chelating activity of *C. libanotis* roots was the result of synergic effect of all fractions' compounds that include a high amount of nonphenolic hydrosoluble compounds.

The metal chelating capacity is important since it reduces the concentration of transition metals acting as catalysts to generate the first few radicals and initiate the radical mediated oxidative chain reactions in biological or food systems. Ion chelating agents also may inhibit the Fenton reaction and hydroperoxide decomposition (Liu *et al.*, 2010). Thus, an antioxidant's ability to chelate metals is thus an important antioxidant property to measure.

5.5. FRAP assay

On the basis of the reports that showed the antioxidant activity of plants being closely associated with their reducing power (Singh, 2004; Zhu *et al.*, 2009), we further evaluated the reducing powers extracts using FRAP assay. In contrast to Benzie and Strain (1996) method, the absorbance did not stabilize after 4 min; therefore the measurements were continued for 30 min, in agreement with Pulido (2000) (**Fig. 24**).
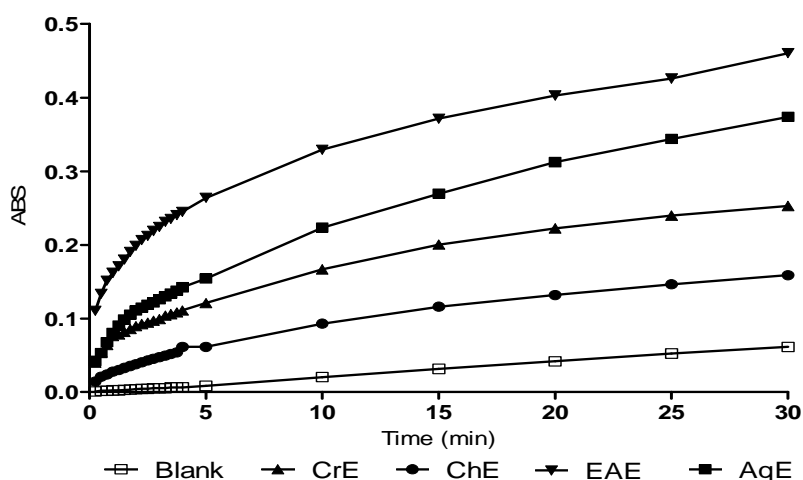
Fig. 24. FRAP reaction kinetics of reagent blank and *C. libanotis* extracts (73.5 µg/ml). Values were expressed as mean (n= 2).

Some extracts even doubled their initial absorbance after 30 min of reaction, as was the case with CrE and EAE. However, while ChE showed only a minor increase beyond 4 min, AqE showed a high increase (**Fig. 25**). *Cachrys libanotis* extracts increased their reducing power with time might imply an ability to maintain their antioxidant activity for longer times, helping to maintain an adequate antioxidant status *in vivo*. Another remark that the reducing powers of *C. libanotis* extracts increased with the increase in concentration.
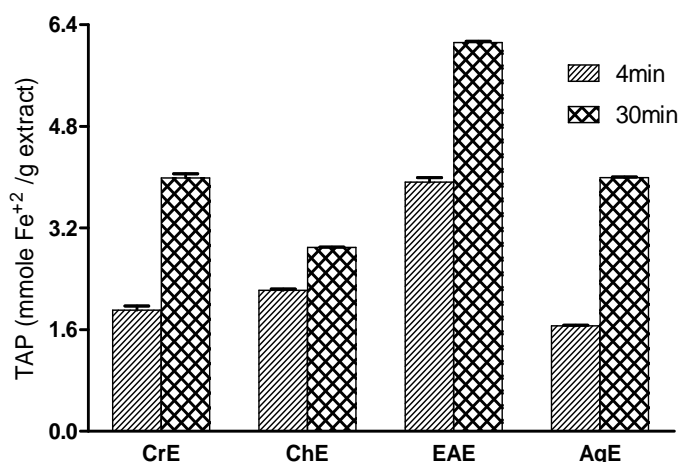
Fig. 25. Comparison between TAP of *C. libanotis* extracts at 4 min and 30 min. TAP values were expressed as mean ± SD of duplicate.

The results of reducing power of extracts and gallic acid is presented in **Table IX**. At 4 min, EAE showed the highest TAP, which was, approximately, 5.5-folds lower than that of gallic acid, referring a good reducing power. EAE was followed by ChE, CrE and AqE with TAP lower than that of gallic acid by 9.6-folds, 11.1-folds and 12.8-folds, respectively. However, this order was changed at 30 min to: EAE (4.6-folds) ☐ CrE=AqE (7-folds) ☐ ChE (9.8-folds) lower than gallic acid. ChE appears having the strongest reducing power compared to CrE and AqE at 4 min; however, at end of reaction (30 min), it presented the less reducing power (**Fig. 26**). The matter might return to the nature of phenolic and non-phenolic compounds of each extract.

Table IX. EC1 and TAP values at 4 min and 30 min of reaction using FRAP assay.

	EC1 (4mn) (mg/ml)	EC1 (30mn) (mg/ml)	TAP (4mn) mM Fe^{+2} /g extract	TAP (30mn) mM Fe^{+2} /g extract
Gallic acid	0.048 ± 0.003	0.035 ± 0.002	21.287 ± 1.179	$28,312 \pm 1.458$
CrE	0.524 ± 0.018	0.250 ± 0.004	1.907 ± 0.066	3.991 ± 0.065
ChE	0.450 ± 0.004	0.3453 ± 0.0004	2.220 ± 0.020	2.896 ± 0.003
EAE	0.255 ± 0.005	0.1634 ± 0.0005	3.924 ± 0.071	6.120 ± 0.020
AqE	0.602 ± 0.003	0.2503 ± 0.0006	1.661 ± 0.008	3.994 ± 0.010
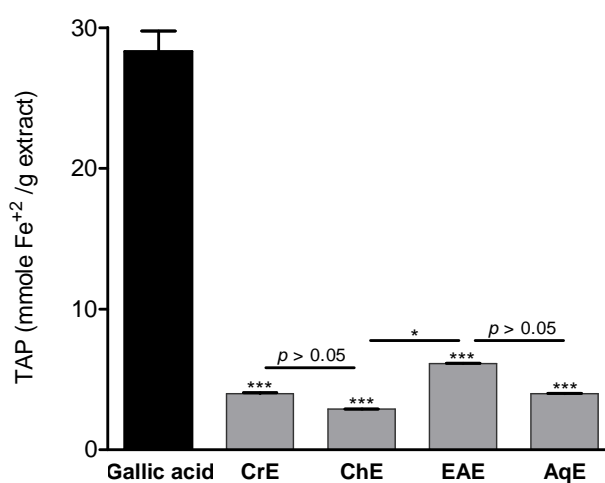
Fig. 26. TAP, total antioxidant power of *C. libanotis* extracts and gallic acid at 30 min. comparison was realized against gallic acid. *: $p \leq 0.05$, ***: $p \leq 0.001$.

CrE showed a low antioxidant capacity (231.5 µmole/g DW, at 4 min) compared to commercially available *Gingo biloba* extract (9254.2 µmole/g DW), according to Borneo and their collaborators (2009), i.e. CrE reducing power was 40-folds lower than *Gingo biloba*. In other hand, antioxidant capacity of CrE was, approximately, 2-folds lower than *Sargentodoxa cuneata* (453.53 µmole/g DW), plant was found to have the highest antioxidant capacity among 44 others (Li *et al.*, 2008). The surprising matter is that *Sargentodoxa cuneata* polyphenol contents, in the same work, equal to 27-folds (52.35 mg GAE/g DW) that of CrE (1.897 mg GAE/g DW). Thus, *C. libanotis* is more potent than *Sargentodoxa cuneat*, when compare reducing capacity versus polyphenols content. Moreover, the last comparison

showed that reducing power of CrE might be return to a synergism effect of both phenol and non phenol compounds.

The reducing power and the total phenolic content of *C. libanotis* extracts showed a high correlation at 4 min ($r^2 = 0.938$; $p \leq 0.05$). Therefore, the presence of phenolic compounds contributed significantly to the antioxidant activity of the *C. libanotis* plants. High phenolic content is thus an important factor in determining the antioxidant activity. This result is in agreement with previous reports that the phenolic compounds contribute significantly to the antioxidant activity in different medicinal plants (Wong *et al.*, 2006; Borneo *et al.*, 2009).

General discussion

Medicinal plants have been in use all over the world to treat various diseases including inflammation, heart diseases, cancer, etc. A great number of medicinal plants contain compounds exhibiting antioxidant properties. To enable medicinal plants to be use in modern medicine, researches and development are important for the advancement of traditional medicines. However, scientific information on antioxidant activities from plants that are less widely used for medicinal purposes, including *C. libanotis* is still rather scarce. This fact partially inspired this study. An increasing demand for naturalness of healthcare was another important motivation. As the antioxidant properties of *C. libanotis* had been not studied before, it was selected for investigations.

In the present work, the antioxidant capacities of *C. libanotis* roots extracts were evaluated using both enzymatic and non-enzymatic methods. According to enzymatic method results, extracts inhibited XOR in a concentration-dependent manner. EAE and ChE exhibited an effective effect in inhibiting XOR (IC_{50}= 0.110 ± 0.006 and 0.139 ± 0.002 mg/ml, respectively), whereas CrE and AqE presented a weak effect by 10-folds and 21-folds lower than EAE. The XOR inhibition could be linked not only on the polyphenols and flavonoids contents of the extract (Wu *et al.*, 2010), but also to the nature (structure) of these compounds (Cos *et al.*, 1998). These results suggest that *C. libanotis* roots possess XOR inhibitory activity that might be helpful in preventing or slowing the progress of several diseases. On the other hand, ChE (IC_{50}= 0.223 ± 0.001 mg/ml) was the most potent extract in scavenging superoxide radical generated by XOR, followed by EAE, which gave a scavenging effect lower than that of ChE by 2 folds, whereas, AqE showed no effect. CrE, ChE and EAE presented a predominance of an enzyme inhibition activities rather than a scavenging of O_2^{-}. In contrast, AqE shows an inhibitory effect on the enzyme only. According to classification of Cos and their collaborators (1998), CrE, ChE and EAE were selected in category C (XO

inhibitors with an additional superoxide scavenging activity), and AqE in category B (XO inhibitors without any additional superoxide scavenging activity).

As non enzymatic methods, $O_2^{\cdot-}$ scavenging activity using NADH-PMS system, β-carotene bleaching, DPPH scavenging activity, ferrous ion chelating, and reducing power have been used to investigate antioxidant activity.

Results of $O_2^{\cdot-}$ scavenging using NADH-PMS system showed a high scavenging activity for ChE followed by EAE and CrE (with 20-folds and 33-folds lower than ChE, respectively), an order similar to that found in $O_2^{\cdot-}$ scavenging using xanthine-XOR system. Compared to result obtained in $O_2^{\cdot-}$ scavenging using xanthine-XOR system, we can suggest that the possible mechanism of inhibition of both NBT and cytochrome c reduction was through direct superoxide radicals- scavenging action ChE, and through dual action of XOR inhibition and superoxide radicals- scavenging with favor of XOR inhibition for the rest of extracts.

Followed by CrE, AqE and ChE, respectively, EAE exhibit the highest activity in β-carotene bleaching assay (76.21%). This activity could be attributed to its highest contents of phenols compounds as well as to the compound's nature (glycosidic polyphenols). In contrast, antioxidant activity of CrE (65.17%), AqE (64.57%) and ChE (56.95%) indicate that the polyphenols and flavonoids content and antioxidants polarity are not the only factor related to this antioxidant activity. The possible synergism of polyphenols with other components present in the extracts may be responsible for this observation. The antioxidant activity measured as the inhibition of oxidation of linoleic acid can simulate the oxidation of the membrane lipid components (Tepe *et al.*, 2005). Thus, *C. libanotis* roots might exhibit a high effect against peroxidation of lipids. Or, the inhibition of lipid peroxidation implies radicals-scavenging and/or ion chelating, DPPH-scavenging, and ferrous ion chelating assays were carried out.

CrE had the highest activity toward DPPH scavenging (IC_{50}= 0.414 mg/ml) followed by EAE, AqE and ChE with scavenging activity lower by 1.4-folds, 1.7-folds and 2.8-folds than CrE. Such order is not in accordance with polyphenols and flavonoids contents in extracts. This may due to supplement activity of other components than polyphenols as well as to inadequate structure of phenolics compounds to scavenge DPPH radicals in these extracts.

Moreover, CrE and AqE exhibited an excellent activity on ferrous ion chelating ($\approx$ 53 µg/ml) which was lower by only 9-folds than EDTA, a strong chelator. This activity could be contributed to the nature of phenolic compounds as well as the synergic effect of all fractions' compounds. As conclusion of these results, *C. libanotis* roots might be used as a potential source of natural chelating agent.

The reducing power of *C. libanotis* extracts was determined by FRAP assay. EAE exhibit the highest reducing power followed by CrE, AqE and ChE. The reducing power and the total phenolic content of *C. libanotis* extracts showed a high correlation at 4 min (r^2= 0.938; $p \leq$ 0.05). Therefore, the presence of phenolic compounds contributed significantly to the antioxidant activity of the *C. libanotis* roots.

In conclusion, *C. libanotis* roots extracts showed a good XOR inhibition, ferrous iron chelating and inhibition of linoleate oxidation, and they present a medium free radical scavenging and reducing power. These results can be useful as a starting point of view for further applications of *C. libanotis* roots or its constituents in area of healthcare after performing clinical *in vivo* researches.

Generally after antioxidant activity evaluation assays, it is still rather difficult to unambiguously identify investigated plant materials as a new source of antioxidants. This work open thus a large field for *C. libanotis* study, plant used traditionally but that its biologic activities still until now uninvestigated.

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