

الجمهورية الجزائرية الديمقراطية الشعبية
DEMOCRATIC AND POPULAR REPUBLIC OF ALGERIA
وزارة التعليم العالي والبحث العلمي
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
جامعة فرحات عباس سطيف
UNIVERSITY FERHAT ABBAS SETIF
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THESIS

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SUBMITTED FOR THE DEGREE OF:

DOCTORAT OF SCIENCES

In Biology
Option: Biochemistry

**ANTIOXIDATIVE PROPERTIES OF *Tamus communis*
L., *Carthamus caeruleus* L. AND *Ajuga iva* L. EXTRACTS**

Discussed on: June 29th2010

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To my parents

To my sister and brothers

To all my family

To my husband and his family

To all my

Friends and colleagues

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ACKNOWLEDGEMENTS

First of all great praise and thanks to God for His help in completing this research.

My sincere deepest gratitude is due to my supervisor Professor Abderrahmane Baghiani for the guidance, motivation, and advices during these years not only in the scientific matter but also in the everyday life.

Special thanks to:

Prof. Lekhmici ARRAR, Daoud Harzallah and Dr Seddik Khennouf for their continuous support, assistance, advices and guidance.

I want to acknowledge

The reviewers of my thesis, Professor Daoud Harzallah University Ferhat Abbas of Setif, Professor Hambaba, Professor Salah Laroui University Hadj Lakhdar of Batna and Doctor Fatima Khelifi Touhami University Mentouri of constantine, for critical reading of the thesis, and for their valuable comments, discussions and suggestions.

I'm very grateful to:

The Algerian Ministry of Higher Education and Scientific Research (MESRS) and the Algerian Agency for the development of Research in health (ANDRS) for financial support during the course of the project.

This work was carried out at the Department of Biology, Laboratory of Applied Biochemistry, University Ferhat Abbas, Setif I feel very fortunate for having the opportunity to work in this Laboratory with excellent research facilities and surrounded by supporting people. I wish to thank all of them.

Finally, my warm thanks for the love and support provided by my family, my friends and any one, who has meant more to me than I can ever put into words Provided by my family, my friends and any one has meant more to me than I can ever put into words.

SUMMARY

This work was carried out to study the antioxidant effects of *Tamus communis* L. (Tc), *Carthamus caeruleus* L.(Cc) root extracts and *Ajuga iva* L(Ai) shoot extracts, traditionally used in folk medicine in Algeria. Therefore, subfractions ; Crud (CE), chloroform (CHE) and ethyl acetate (EAE) were obtained by using solvents with different polarity. Total phenolic and flavonoid content of Tc, Cc and Ai extracts were determined by a colorimetric method. The results proved that the extracts represent a significant source of phenolic compounds; value varied from 12.66 ± 0.27 to 208.166 ± 3.12 mg equivalent gallic acid/g lyophilisate. All the extracts exhibited inhibitory properties on xanthine oxidoreductase (XOR), which was purified from fresh bovine milk with yields of 23.21 mg / L, protein / flavine ratio of 5.15, specific activity of 2055.05 nmol / min / mg protein and showed a single major band on SDS-PAGE of Molecular weight around 150 KDa, indicating the high purity of the purified enzymes. Inhibitory concentration of different extracts for 50 % of xanthine oxidase activity range from $0,307 \pm 0,0106$ to 5.835 ± 0.460 μ M/quercetin equivalent, this effect was stronger than that of allopurinol ($IC_{50} = 57.114 \pm 1.093 \mu$ M), with an additional superoxide scavenging capacity which was determined by enzymatic and non enzymatic methods in the same order. In the cellular system, all the extracts showed a protect effect against t-BHP induced hemolysis in human erythrocytes, The most effective were CE of the TC and Cc which significantly decreased hemolysis by 85,75 %, 74,18% respectively, greater than those of Quercetin, rutin and gallic acid. These results were clearly confirmed by a modified thiobarbituric acid-reactive species (TBARS) assay used to measure MDA formed, and β -carotene/linoleic acid assay which demonstrated that CHE of Tc possesses approximately a similar inhibition ratio (94 ,92 %) of the linoleic acid oxidation to that of BHT (96, 77%). In order to more clarify the antioxidants activities of the extracts which can appeared, through their reactivity as hydrogen- or electron-donating agents, and/or metal ion chelating

properties; 1,1- diphenyl-2 picrylhydrazyl (DPPH) radical-scavenging, ferric reducing /antioxidant power (FRAP) and iron chelating ability were realized. The results revealed that the extracts exhibited a potent DPPH radical scavenging activity and powerful Ferric Reducing Ability, which were better than those of quercetin gallic acid and ascorbic acid. In general the iron chelating ability of three plants extracts was very effective, CE of Tc showed excellent effect with IC_{50} at $0,302 \pm 0,053 \mu M$ quercetin equivalent, higher than that of EDTA $IC_{50} = 0,563 \pm 0,053 \mu M$, meanwhile CE of Cc gave approximately the same effect as EDTA. These results showed that *Tamus communis* L, *Carthamus caeruleus* L and *Ajuga iva* L extracts have a notable *anti-oxidant* effects and may have some clinical benefits. Identification of the antioxidative constituents of the plants and evaluation the *in vivo* anti oxidative properties of plants constituents should be the objective of future research.

الملخص

الهدف من هذا البحث هو دراسة التأثير المضادة للأكسدة لجذور نبات *Tamus communis* L و *Carthamus caeruleus* L والجزء الهوائي ل *Ajuga iva* L، هذه النباتات معروفة باستعمالها الواسع في الطب الشعبي. تم استخدام مذيبيات بقطبية مختلفة، و التي سمحت بالحصول على المستخلص الخام (CE) ومستخلص الكلو فورم (CHE) ومستخلص إيثيل الأسيتات (EAE). بين التقدير الكمي لعديدات الفنول الكلية أن هذه المستخلصات غنية بالمرکبات الفنولية إذ تراوحت القيم بين 0.27 ± 12.66 و 208.166 ± 3.12 مغ مكافئ لحمض gallic\غ من الوزن الجفد. أبدت كل المستخلصات تأثير تثبيطي على إنزيم Xanthine oxidoreductase (XOR) الذي تمت تنقيته من حليب البقر، حيث أعطى مردود يقدر ب 23مغ/ل ونسبة الفافين\البروتين مساوية ل 5.15 ونشاطية خاصة قدرت ب 2055.05 نانومول\د\مغ بروتين. كما أظهرت الهجرة الكهربية على هلام متعدد الأكريلاميد شريط واحد في حدود 150 KD ، هذا وقد تراوحت قيم التراكيز المثبطة ل 50 % من نشاطية الإنزيم بين 0.460 ± 5.835 و 0.0106 ± 0.307 μ مول مكافئ Quercetin حيث لوحظ أن هذا التأثير أقوى من تأثير Allopurinol ($IC_{50} = 57.114 \pm 1.093 \mu M$)، إضافة لذلك لوحظ أن لهذه المستخلصات تأثير إزاحي، و الذي تم تأكيده بطرق إنزيمية وغير إنزيمية. من جهة أخرى أبدت كل المستخلصات القدرة على حماية الكريات الحمراء البشرية من التحلل الذي يسببه t-BHP، حيث كان CE لكل من Tc و Cc أكثر المستخلصات فعالية، حيث خفض التحلل الدموي بنسبة 85.75 % و 74.18 على التوالي، والتي كانت أعلى من تلك الخاصة ب Quercetin و rutin و حمض gallic ، هذه النتائج تم تأكيدها بطريقة TBARS assay التي تستعمل في تقدير كمية MDA المتشكل، كما بين اختبار B-carotene/linoleic acid أن CHE الخاص ب Tc قادر على تثبيط أكسدة حمض linoleic بنسبة 94.92 % والتي تقارب تلك الخاصة BHT (96.77%). لاختبار القوة المضادة للأكسدة للمستخلصات التي تظهر من خلال نشاطيتها كعوامل مانحة للهيدروجين و/ أو الإلكترون أو كعوامل ملتقطة للمعادن، تم تطبيق اختبار DPPH و FRAP والقدرة على التقاط الحديد، حيث لوحظ أن لهذه المستخلصات قدرة إرجاعية كبيرة و التي كانت أحسن من تلك الخاصة ب Quercetin و حمض الغاليك و حمض الأسكوربيك. عموما كانت قدرة المستخلصات على التقاط الحديد فعالة إذ أبدى CE الخاص ب TC قوة كبيرة في التقاط الحديد ($IC_{50} 0.302 \pm 0.053 \mu M$ / quercetin equivalent) فاقت

قدرة EDTA ($IC_{50} = 0,563 \pm 0,053\mu M$) في حين CE الخاص ب Cc أعطى قدرة مساوية تقريبا لتلك الخاصة ب EDTA. بينت نتائج هذه الدراسة أن لمستخلصات *Tamus communis* L. و *Carthamus caeruleus* L. و *Ajuga iva* L. تأثير مضادة للأكسدة مهم و الذي يمكن أن يكون له فوائد علاجية، ويبقى التعرف على المركبات المضادة للأكسدة لهذه النباتات ودراسة تأثيرها عند الكائن الحي هدف للدراسة في المستقبل.

RESUME

L'objectif de ce travail est l'évaluation de l'effet antioxydant des extraits des racines des *Tamus communis* L (Tc), *Carthamus caeruleus* L (Cc) et de la partie aérienne de *Ajuga iva* (Ai), utilisées en médecine traditionnelle en Algérie. Des solvants à différente polarité ont été utilisés pour obtenir l'extrait Brut (CE), l'extrait chloroformique (CHE) et l'extrait d'acétate éthyle (EAE). Les polyphénols totaux et les flavonoïdes sont dosés par une méthode colorimétrique. Les résultats ont montré que les extraits de Tc, Cc et Ai représentent une source importante de composés phénoliques avec des valeurs respectives variant de 12.66 ± 0.27 à 208.166 ± 3.12 mg équivalent acide gallique /g lyophilisat. Tous les extraits ont montré un effet inhibiteur sur la xanthine oxydoréductase (XOR) purifiée à partir du lait bovin frais avec un rendement de 23.21 mg /l, un ratio protéine / flavine de 5.15 une activité spécifique de 2055.05 nmol de xanthine / min / mg protéine et une bande majeure d'environ 150 KDa, en SDS-PAGE, indiquant une bonne pureté de l'enzyme. Les valeurs des IC_{50} des différents extraits *vis-à-vis* de cette enzyme varient de $0,307 \pm 0,0106$ au 5.835 ± 0.460 μ M/ équivalent quercétine. Cet effet est plus puissant que celui de l'allopurinol ($IC_{50} = 57.114 \pm 1.093$ μ M), avec un effet scavenger supplémentaire sur le radical superoxyde, qui a été déterminé par des méthodes enzymatiques et non enzymatiques. Dans le système cellulaire, tous les extraits dévoilent un effet protecteur contre le t-BHP qui provoque l'hémolyse. Les extraits les plus effectifs étaient le CE de Tc et Cc qui ont diminué significativement l'hémolyse avec un pourcentage de 85,75 %, 74,18% respectivement, cet effet est plus fort que ceux de la quercétine, la rutine et l'acide gallique. Ces résultats ont été confirmés par la méthode au 'thiobarbituric acid-reactive substance' (TBARS) utilisé pour l'estimation du MDA formé et par le test β -carotène/linoléic acid qui a démontré que le CHE de Tc possède une valeur d'inhibition de l'oxydation d'acide linoléique (94 ,92 %) semblable à celles du BHT (96,77%). Dans le but de mieux éclaircir l'effet antioxydant des extraits *via* leurs réactivités comme des agents réducteur (donneur d'hydrogène ou d'électrons) et / ou comme

des chélateurs des métaux trois techniques; DPPH, FRAP, et Ferrozine sont appliquées. Les résultats ont montré que les extraits ont un pouvoir réducteur plus puissant que ceux de la quercétine, l'acide ascorbique et l'acide gallique. En général, la capacité de chélation du fer des extraits des trois plantes étaient efficace. Le CE de Tc a montré une excellente capacité de chélation (IC_{50} de $0,302 \pm 0,053 \mu M$ / équivalent quercétine) plus élevée que celle de l'EDTA, cependant le CE de Cc a montré approximativement le même effet. Ces résultats démontrent que les extraits de *Tamus communis* L, *Carthamus caeruleus* L et *Ajuga iva* L ont des effets antioxydants notables et peuvent, par conséquent avoir des avantages cliniques. L'identification de constituants antioxydants de ces extraits et l'évaluation de leurs propriétés antioxydantes *in vivo* reste objective pour les recherches futures.

ABBREVIATIONS

AE	Aqueous extract
<i>Ai</i>	<i>Ajuga iva</i> L.
Asc A	Ascorbic acid
BHT	Butylated hydroxytoluene
BXOR	Bovine xanthine oxidoreductase
CAT	Catalase
Cc	<i>Carthamus caeruleus</i> L.
CE	Crud extract
CHE	Chloroform extract
CHF	chronic heart failure
COMT	catechol- <i>O</i> -methyl transferase
Cyt C	cytochrome c
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EAE	Ethyl acetate extract
EDTA	Ethylene diamine tetra acetic acid
FRAP	Ferric Reducing Ability of Plasma
Cat	Catalase
Gall A	Gallic acid
Gal A Eq	Gallic acid equivalents
GPx	Glutathion peroxydase
GR	Glutathion reductase
GSH	Gluthathion reduced
GSSG	Glutathion disulfure oxidized
H₂O₂	hydrogen peroxide
HNE	4-hydroxynonenal
HOCl	hypochlorous acid
IC₅₀ %	Inhibitory concentration for 50% of activity
IR	Ischemia-Reperfusion
LOO°	Lipid peroxide radical

MDA	Malondialdehyde
MeOH	Methanol
Mo	Molybdene
NAC	N-acetylcysteine
NBT	Nitroblue tetrazolium phenazine
NO[°]	Radical nitroxid
NO₂⁻	Nitrite
NOS	nitric oxidesynthase
¹O₂	Singlet oxygen
O₂^{°-}	superoxid Radical
OH[°]	hydroxyl Radical
ONOO⁻	Peroxynitrite
PCs	Penolic compounds
PFR	Protein to flavine ratio
PHGPx	phospholipid hydroperoxide glutathione peroxidase
Quer	Quercetin
Quer Eq	quercetin equivalents
RBC	Human red blood cells
RNS	Reactive nitrogene species
ROS	Reactive oxygen species
RONs	Reactive Oxygen and Nitrogen Species
RO[·]	alkoxyl radicals
ROO[·]	lipo-peroxyl radicals
RO-OH	Lipid hydroperoxides
Rut	Rutin
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamid gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxyde dismutase
TBA	Thiobarbituric acid
<i>t</i>-BHP	<i>tert</i> -butyl hydroperoxide
Tc	<i>Tamus communis</i> L.

TCA	Trichloroacetic acid
TEMED	N,N,N'N' Tetramethylenediamine
TPTZ	tripyridyl-s-triazine
UV-Vis	Ultraviolet-Visible
XDH	Xanthine deshydrogenase
XO	Xanthine oxydase

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INTRODUCTION

The possibility that reactive oxygen species (ROS) and free radicals may be involved in human reproduction was suggested nearly 60 years ago (MacLeod, 1943). Overproduction of such free radicals resulting in oxidative stress, which is defined in general as excess formation and/or incomplete removal of highly reactive molecules such as ROS, thereby can cause oxidative damage to biomolecules (lipids, proteins, DNA) (Farber, 1994). These lead many chronic diseases such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Halliwell, 1994; Poulson et al., 1998). Numerous physiological processes may produce these free radicals. The superoxide radical, the origin of other radicals, is generated from O_2 by multiple pathways: 1) NADPH oxidation by NADPH oxidase; 2) oxidation of reducing equivalents like nicotinamide, NADPH, and $FADH_2$; 3) via the mitochondrial electron transport system; 4) auto-oxidation of many products. One of the very important superoxide-producing enzymes that have been reported to increase during oxidative stress is xanthine oxidase (XO) (Harrison, 2004). There are five different mechanisms known to increase superoxide generation by XO during ischemia-reperfusion (IR). First Granger et al. (1981) suggested that during IR, ROS are enhanced due to increased conversion of xanthine dehydrogenase (XDH) to XO. Secondly, mRNA levels of XDH and XO are upregulated (Saksela et al., 1998). Thirdly, when the liver becomes ischemic and hepatocellular damage occurs, the liver releases XO in the bloodstream (Sanhueza et al., 1992), thus being transported throughout the body. Further, XO can specifically bind to endothelial cells which have been reported to produce inaccessible radicals to CuZn-superoxide dismutase (Houston et al., 1999). Finally, during ischemia, ATP is degenerated to xanthine and hypoxanthine, thereby increasing XO substrate levels, which leads to increase superoxide production. In the context of the inflammatory response, XOR is believed as an agent of innate by generating ROS immunity (Martin et al, 2004). In addition, hyperuricemia

leads to the accumulation of uric acid, produced by XO, in joints and kidneys causing acute arthritis and uric acid nephrolithiasis. One therapeutic approach is the use of XO inhibitors such as allopurinol (Emmerson, 1996). However, allopurinol use can result in a number of adverse side effects (Wallach, 1998), ranging from mild skin allergy to a concerted allopurinol hypersensitivity syndrome (Bouloc et al, 1996; Yale et al, 1996). Thus, there is a need to develop compounds with XO inhibitory activities and scavenging properties, but devoid of the undesirable effects of allopurinol.

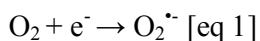
A considerable attention has been paid to antioxidant properties of plants (fruits, vegetables, medicinal herbs, etc.) (Okuda et al, 1994), which contain a wide variety of free radical scavenging molecules, such as phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Cai et al., 2003). Epidemiological studies have shown that many of these compounds possess anti-inflammatory, antiatherosclerotic, antimutagenic, anticarcinogenic, antibacterial and antiviral activities (Halliwell, 1994; Sala et al., 2002), and have been associated with reduced, cardiovascular disease, diabetes, and other diseases associated with ageing (McLarty, 1997; Yang et al., 2001). The goal of this study was to investigate the *in vitro* inhibitory effects of XO and free radical scavenging properties of *Tamus communis* L, *Carthamus caeruleus* L(Cc) roots and *Ajuga iva* L shoots and its active fractions. These plants are used in traditionally folk medicine in Algeria, in the treatment rheumatic diseases. Total antioxidant activities of the plant extracts can not be evaluated by any single method, due to the complex nature of phytochemicals, two or more methods should always be employed in order to evaluate the total antioxidative effects of plants, and therefore many complementary essays have been applied in our study.

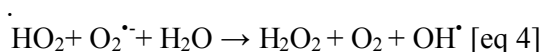
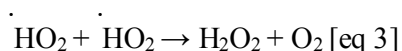
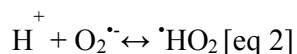
1. Oxidative Stress

Oxidative stress takes place when the balance between free radicals and antioxidants is disrupted by excessive production of ROS, and/or inadequate antioxidant defenses (DeLeo, 1998). Normally there is a balance between the free radicals amount generated in the body and the antioxidant defense systems, which scavenge/quench these free radicals (Nose, 2000) when the free radicals amount is within the normal physiological level (Finkel and Holbrook, 2000), preventing them from causing deleterious effects in the body (Nose, 2000), but when this balance is shifted towards more of free radicals, it leads to oxidative stress.

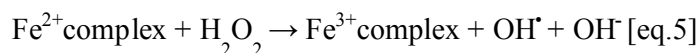
1.1. Biochemistry of Reactive Oxygen and nitrogen Species

Reactive oxygen species (ROS) is a collective term, which includes not only the oxygen radicals but also some non-radical derivatives of oxygen. These include hydrogen peroxide (H_2O_2), hypochlorous radical (HOCl) and ozone (O_3) (Bandhopadhyay et al., 1999). ROS are oxygen-containing molecules that are capable of either accepting or donating a free electron, thus they are unstable and react with other molecules, which may lead to the generation of other, sometimes even more reactive molecules. All aerobic organisms use molecular oxygen O_2 for respiration and /or oxidation of nutrients. The O_2 is therefore indispensable for their life, as electron acceptor in oxidation processes (Vichnevetskaia and Roy, 1999; Scandalios, 2005). In the cellular electron transport chain, oxygen is reduced by four electrons to water. Activation of oxygen is energy dependent and requires an electron donation [eq. 1]. The superoxide anion ($\text{O}_2^{\cdot-}$), produced in the first step, can be protonated at a low pH to yield the hydroperoxyl radical $\cdot\text{HO}_2$ [eq. 2]. Both $\text{O}_2^{\cdot-}$ and $\cdot\text{HO}_2$ undergo spontaneous dismutation to produce hydrogen peroxide (H_2O_2) and hydroxy radical [eq. 3 - 4].

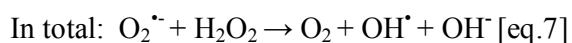
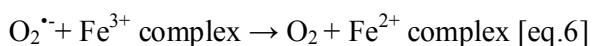




Although H_2O_2 is less reactive than $\text{O}_2^{\cdot-}$, but in the presence of reduced transition metals such as Fe^{2+} (which is the case in biological systems), the formation of hydroxyl radicals (OH^{\cdot}) can occur in the Fenton reaction [eq 5] (Vichnevetskaia and Roy, 1999; Scandalios, 2005):



Fe^{3+} complex can be efficiently reduced by $\text{O}_2^{\cdot-}$ the product closing Fenton reaction [eq. 6] (Fe (II)/Cu (II) metal ion-catalysed Haber-Weiß reaction)



All above reactions (eq. 1 - 7) occur during normal metabolic processes in aerobic cells, yielding the formation of intermediate products: hydrogen peroxide, superoxide anion and hydroxyl radical. These ROS are highly reactive and can directly oxidize macromolecules including DNA, protein, and lipids. Hydroxyl radical and hypochlorite are the most powerful substances involved cytotoxic reactions. Singlet oxygen ($^1\text{O}_2$) is an oxygen form whose electrons are excited at a higher energy level, when returning to the ground state they emit light (chemiluminescence) which may have antimicrobial and cytotoxic effects (Stvrtnova and Hulin, 1995). Although ROS have been shown to cause damage to protein, lipids and nucleic acids, there is also evidence that these molecules have normal regulatory actions. ROS have a major role in the modulation of cellular signal transduction pathways and gene

expression in a variety of biological processes (Sen and Packer, 1996; Esposito et al., 2004). ROS can activate a number of transcription factors (Flohe et al., 1997), increase the activity of a number of protein kinases, and inactivate other enzymes such as protein phosphatases (Klann and Thiels, 1999).

Enzymatic generation of NO^{\bullet} occurs by action of one of three isoforms of nitric oxide synthase (NOS). This enzyme mediates the oxidation of guanidine nitrogen producing NO (Moncada, 1997). Further, it has been reported that under hypoxic conditions and in the presence of NADH, XOR can catalyse the reduction of glyceryl trinitrate (GTN), inorganic nitrate and nitrite to NO (Godber et al, 2000). NO is a ubiquitous signal transduction molecule, can act as intervention in important biochemistry reactions such as: a mediator in a wide range of both antimicrobial and antitumor activities, an endothelial cell derived vasodilator and anticoagulant, a neurotransmitter with a crucial role in neural communications in the brain and peripheral (Zhao, 2005). The interaction of NO and reactive oxygen species (ROS) have assumed greater importance due to the simultaneous generation of both in various pathophysiological conditions (Mancardi et al, 2004). It can be a toxin in the destruction of pathogens (Hummel et al, 2006). During inflammatory conditions, where the production level of $\text{O}_2^{\bullet-}$ and NO is increased, ONOO^- is formed and oxidises a number of target molecules. ONOO^- leads to the nitration of tyrosines and induce the formation of an intermediary compound, with hydroxy-radical properties, able to oxidise DNA and proteins (Salgo et al, 1995).

1.2. ROS sources

One of the main ROS sources under physiologic conditions is mitochondria. In the inner membrane they contain an electron chain transfer system for ATP generation, permanently generating $\text{O}_2^{\bullet-}$. It is estimated that some 1-2% or even 4% of O_2 consumption undergoes

transformation to $O_2^{\cdot-}$ (Cadenas and Davie, 2000). The outer mitochondrial membrane carries monoamine oxidase, producing large amounts of H_2O_2 in the mitochondrial matrix as well as in the cytosol directly.

Another quantitatively important system generating $O_2^{\cdot-}$ is NADPH oxidase, originally found in neutrophils. It consists of 5 subunits, upon stimulation, the cytoplasmic subunits link together and migrate towards the membrane, thus activating superoxide production (Babior, 1999) (Figure. 1). Heerebeek et al (2002) have been shown that many other cellular types (such as endothelia or smooth muscle cells) possess a similar superoxide-generating system, called NADPH-like oxidase, activated by various hormones and cytokines, which producing low amounts of $O_2^{\cdot-}$.

Some cells contain XOR. Under physiological conditions, the XOR is present in XDH form which can be modified, either reversibly or irreversibly to XO generating large amounts of H_2O_2 as well as $O_2^{\cdot-}$. It is speculated that this process gains importance especially after ischemia-reperfusion (Harrison, 2002) (Figure. 1). It has been discovered that XOR can transform nitrates and nitrites to nitrites and NO^{\cdot} , respectively (even in anoxia, in contrast to NOS), the enzyme itself is able to catalyse the reaction of NO^{\cdot} with $O_2^{\cdot-}$ which generating highly reactive peroxynitrite ($OONO^{\cdot}$) (Godber et al 2000; Babior, 2002). It was reported that the increased circulating XO activity leads to the development of endothelial dysfunction via the local formation of peroxynitrite in the vicinity of endothelial cells (Aslan et al., 2001).

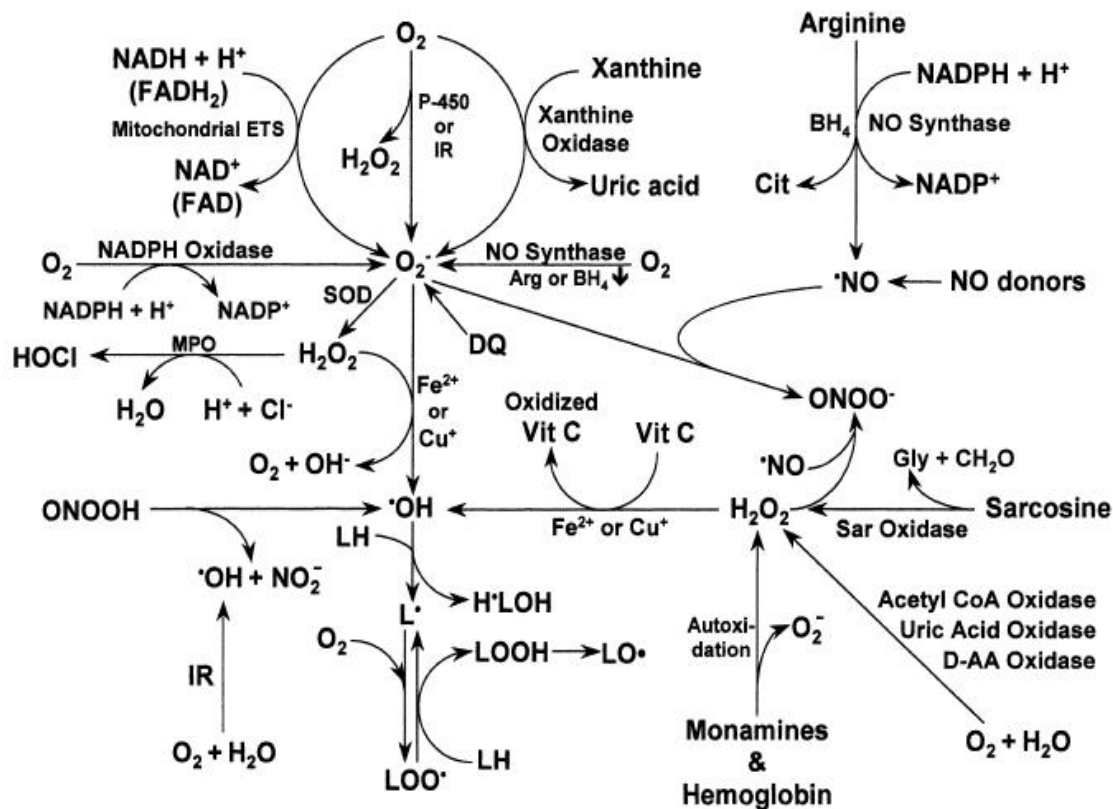


Figure 1. Reactive oxygen species sources (Fang et al, 2002).

ROS can be generated as by-products during metabolism of arachidonic acid. Enzymes participating in the process are cyclooxygenase, lipoxygenase and cytochrome P-450 (Ivanov et al, 2005). Practically all cells possess cytochrome P450, it became clear that P450 isoenzymes participate in the metabolism of steroid hormones, cholesterol, arachidonic acid and eicosanoids (Omura, 1999). They play also an important role in the metabolism of many xenobiotics. The underlying concept of its activity is a multi step transfer of 2 electrons to a substrate while binding one oxygen atom to it, the second being reduced to water and part of the oxygen involved is inevitably reduced to superoxide (Figure1). Lysosomal membrane contains an electron transport system, which helps ensure optimal intralysosomal pH by pumping protons, this system promotes a three-electron reduction of oxygen, thus leading to generation of highly reactive OH^\bullet .

Myeloperoxidase present in neutrophils and eosinophils, where it catalyses the reaction of H_2O_2 with various substrates leading to generation of potent oxidants such as hypochlorous acid (Klebanoff, 2005).

In addition to being generated in the physiological processes, ROS can be produced as primary products under a variety of stress conditions (Scandalios, 2005). For example, numerous toxic environmental chemicals such as xenobiotics, pesticides, herbicides, fungicides, and radiation cause their harmful environmental effects via generation of ROS. (Jhun, 1991, Pohl, 1993) (Figure. 1).

1.3. Targets of Free radicals-Mediated Oxidative Damage

Oxidative stress has the following implications: failure in redox regulation leading to DNA damage, lipid peroxidation and oxidative protein damage (Figure. 2).

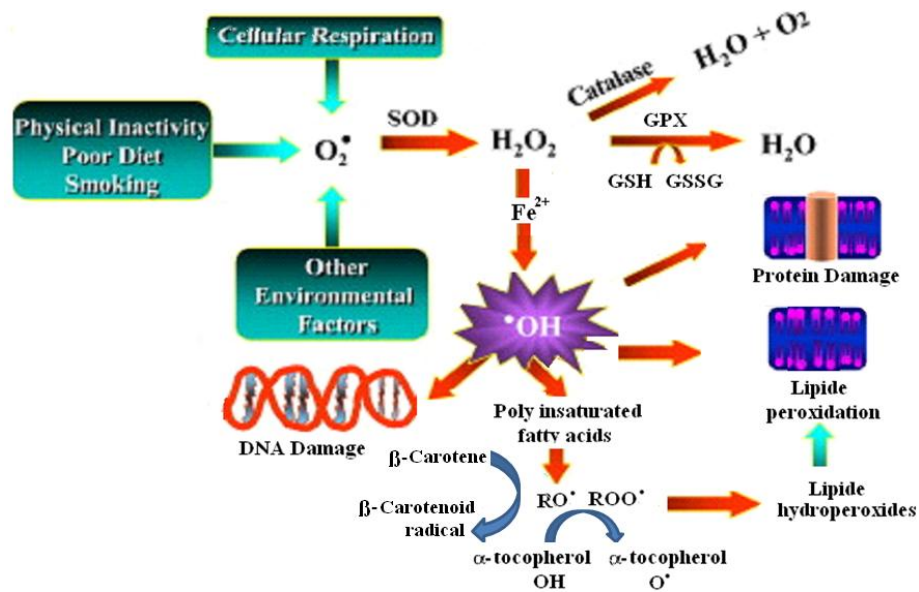


Figure 2. Oxidative damage to cellular macromolecules and relevant relationships between ROS and antioxidants (adapted from Aruoma, 1994).

1.3.1. Lipids

The lipoperoxidation could have a major, or even causal, influence on the pathogenesis of the disease (Busciglio and Yankner, 1995). Free radicals interact with unsaturated sites in lipids, resulting in the production of hydroperoxides (Verma and Sonwalker, 1991). The hydroperoxide residues change the hydrophobic interactions between adjacent chains of phospholipids, allowing easier penetration of water molecules, thereby altering the electric constant across the lipid bilayer, which in turn leads to changes in membrane permeability as well as lipid peroxidation (Parasassi et al., 1994). Pioneering studies of lipid peroxidation by an iron complex started with the observations of Bucher et al (1983). Since then, the study of lipid peroxidation has been a topic of many studies (Eaton and Qian, 2002). The mechanism, proposed, involves the formation of a Fe (II): Fe (III). The maximal rates of lipid peroxidation are observed when the ratio of Fe (II): Fe (III) is 1:1 (Bucher et al., 1983).

Lipid peroxidation consists of three stages: initiation, propagation and termination (Valko et al., 2005) (Figure. 3). Initiation, the first stage, involves the attack of a reactive oxygen species capable of abstracting a hydrogen atom from a methylene group in the lipid. The presence of a double bond adjacent the methylene group weakens the bond between carbon and hydrogen so the hydrogen can be more easily removed from the fatty acid molecule. The process of hydrogen abstraction leaves behind a fatty acid having one unpaired electron (Figure. 3). When oxygen is present in the surrounding tissues, the fatty acid radical can react with it leading to the formation of lipo-peroxyl radicals ($\text{ROO}\cdot$) during the propagation stage (Figure. 3).

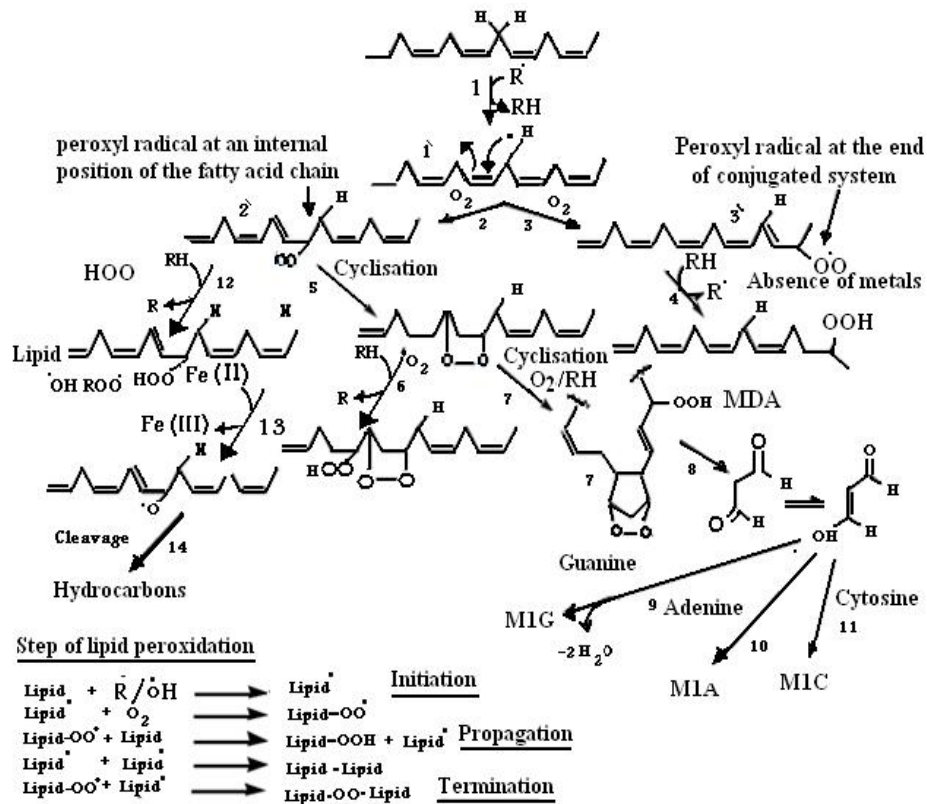


Figure 3. The main Steps of lipid peroxidation (Valko et al., 2005).

ROO· radicals are themselves very reactive and are capable of abstracting another hydrogen from the neighboring fatty acid molecule thus creating lipid hydroperoxides (ROO· + H· → RO-OH). Lipid hydroperoxides (RO-OH) are a relatively short-lived species, they can either be reduced by glutathione peroxidases to unreactive fatty acid alcohols or they react with redox metals to produce a variety of products which are themselves reactive (e.g. epoxides, aldehydes, etc.). The reaction of RO-OH with for example Fe(II) and Fe(III) ions lead to the formation of very reactive alkoxy radicals (RO·) and lipo-peroxy radicals (ROO·), respectively. Once formed, (ROO· radicals can be rearranged via a cyclization reaction to endoperoxides (precursors of malondialdehyde) with the final product of peroxidation process being malondialdehyde (MDA) (Figure. 3). The major aldehyde product of lipid peroxidation besides MDA is 4-hydroxynonenal (HNE) (Marnett, 1999).

MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. In addition, HNE has powerful effects on signal transduction pathways which in turn have a major effect on the phenotypic characteristics of cells. Peroxidation of lipids is an autocatalytic process and is ended by the termination process, for example by the recombination of radicals (Valko et al., 2005).

1.3.2. DNA

An oxidative stress is often associated to all kinds of diseases. Many experiments suggest that the most aggressive species responsible for oxidising or modifying DNA is the OH^\bullet and $\text{O}_2^{\bullet-}$. (Eaton and Qian, 2002). Although it is not always easy to determine among the bases in the DNA, target for oxidative reactions mediated by free radicals, guanine is the most susceptible, the resulting product, 8-hydroxy-2-deoxyguanosine (8-OHdG), is an abundant and measurable lesion and is reported to be a key biomarker of carcinogenesis (Floyd, 1990). Free radicals also attack the sugar-phosphate backbone of DNA, resulting in the production of single- and double-strand breaks, as well as DNA-DNA and DNA-protein cross-links (Breen and Murphy, 1995; Cadet et al., 1999). The single-strand breaks can be repaired quickly using the undamaged DNA strand as a template, whereas double-strand breaks are not easily repairable and are considered the primary lesion leading to carcinogenesis, and cell death (Halliwell and Aruoma, 1990).

MDA can react with DNA to form adducts to dG, dA and dC (Figure. 3). (Marnett, 1999). M1G and presumably M1A and M1C also can be made by the reaction of the corresponding bases, providing an alternate route for their generation by direct oxidation of DNA. M1G residues were detected in tissue at levels ranging from below the limit of detection to as high as 1.2 adducts per 10⁶ nucleosides (which corresponds approximately 6000 adducts per cell).

M1G has also been detected in human breast tissue as well as in rodent tissues. Site-specific experiments confirmed that M1G is mutagenic in *E.coli* (Valko et al., 2005).

1.3.3. Proteins

Proteins are resistant to damage by H_2O_2 and simple oxidants unless transition metals are present. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine cross links, the introduction of carbonyl groups, and the formation of protein-centered alkyl (R^\cdot), alkoxyl (RO^\cdot), and alkylperoxyl (ROO^\cdot), radicals (Eaton and Qian, 2002). Studies on the metal-induced protein denaturation led to the discovery that degradation occurs when the protein has been oxidized. Iron-mediated oxidation of a protein may be a site-specific process as proline, histidine, arginine, lysine, and cysteine residues in proteins which are highly sensitive to oxidation by iron (Stadtman, 1990). The Fe (II)-protein complex reacts with H_2O_2 via the Fenton reaction to yield an active oxygen species, e.g., OH^\cdot , at the site, which represents the major species responsible for the oxidation of protein.

Experimental studies revealed oxidised side chains of amino acids involving carbonyl derivatives, loss of catalytic activity, and increased susceptibility of the protein to proteolytic degradation (Stadtman, 1990). Welch et al. (2001) demonstrated the site-specific of ferritin modification by iron which involved the oxidation of cysteine, tyrosine, and also some other residues. The oxidation of myoglobin by H_2O_2 yields ferrylmyoglobin, which contains two oxidising equivalents: the ferryl complex and an amino acid radical. The aromatic amino acid radical was observed to be relatively long lived and in close proximity to the heme iron. Hence, it has been proposed that this is the first site of the protein radical. Reduction of the ferryl complex by Tyr leads to Tyr- O^\cdot formation (Baud et Karin, 2001) and alternatively by other amino acids leads to the subsequent formation of other amino acid radicals within an electron-transfer process throughout the protein.

1.3.4. Glycooxidation

Glycooxidation is considered to cause gradual deterioration in the structure and function of tissue proteins and to contribute to the pathophysiology of diabetes and its complications (Takagi et al., 1995; Brownlee, 2001; Stirban et al., 2006). It has been reported that autooxidation of glucose under the influence of transition metal ions can lead to ketoaldehyde and H₂O₂ formation. The ketoaldehydes can in turn react with amino groups of proteins forming ketoimines. These ketoimines may finally lead to advanced glycation. The highly reactive hydroxyl radicals can induce further oxidative protein degradation (Wolff and Dean, 1987).

1.4. Free radicals and their role in diseases

Over about 100 disorders like rheumatoid arthritis, hemorrhagic shock, cardiovascular disorders, metabolic disorders, neurodegenerative diseases, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediated (Table.1).

The brain is particularly vulnerable to oxidative stress because it consumes large amounts of oxygen, has abundant lipid content and a relative paucity of antioxidant levels compared to other organs (Halliwell, 1992). Numerous studies have reported correlations between age and the accumulation of oxidative damage to cellular macromolecules (Floyd and Hensley, 2002). Cell damages provoked by free radicals are common during aging; once in this life step cells produce less concentrations of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), etc. (Ferrari, 2001).

Table1. Some diseases caused by free radicals.

<i>Diseases</i>	<i>References</i>
Atherosclerosis	(Mitchinson and Ball, 1987)
Autoimmune diseases	(Halliwell and Gutteridge, 1999).
Cancer	(Cerutti, 1994).
Diabetes	(Wolff and Dean, 1987).
Inflammation	(Valentao et al., 2002)
Parkinson's disease	(Halliwell and Gutteridge, 1999).
Rheumatoid arthritis	(Krane et al., 1990)
Alzheimer's disease	(Rao and Balachandran, 2002)
Ageing	(Finkel and Holbrook, 2000)

1.5. Main antioxidant defense systems

Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (De Groot, 1994; Grace, 1994). The antioxidants may be defined as “any substance, when present at low concentrations compared with that of an oxidisable substrate that significantly delays or prevents oxidations of that substrate” (Nose, 2000). Gutteridge and Halliwell (2000) classified antioxidants as primary; preventing oxidant formation, secondary; scavenging ROS and tertiary; removing or repairing oxidatively modified molecules.

Antioxidant defense can be parted in two groups: the Enzymatic and Non-enzymatic the first one include superoxide dismutase (SOD), peroxidases such as catalase and glutathione peroxidase, glutaredoxins, thioredoxins and glutathione S-transferases (GST) (Halliwell and Gutteridge, 1999), the second one typically consist of small molecules which are soluble in an

aqueous environment or in a lipid environment, which include glutathione, ascorbate, tocopherols, phytochelatins, polyamines and metallothioneins and other products (Meister, 1995).

1.5.1. Enzymatic antioxidant defense system

1.5.1.1. Superoxide dismutases

First in the line of enzymatic ROS degradation is superoxide dismutase (SOD) (Figure. 4), which was discovered in 1969 by McCord and Fridovich in bovine blood.

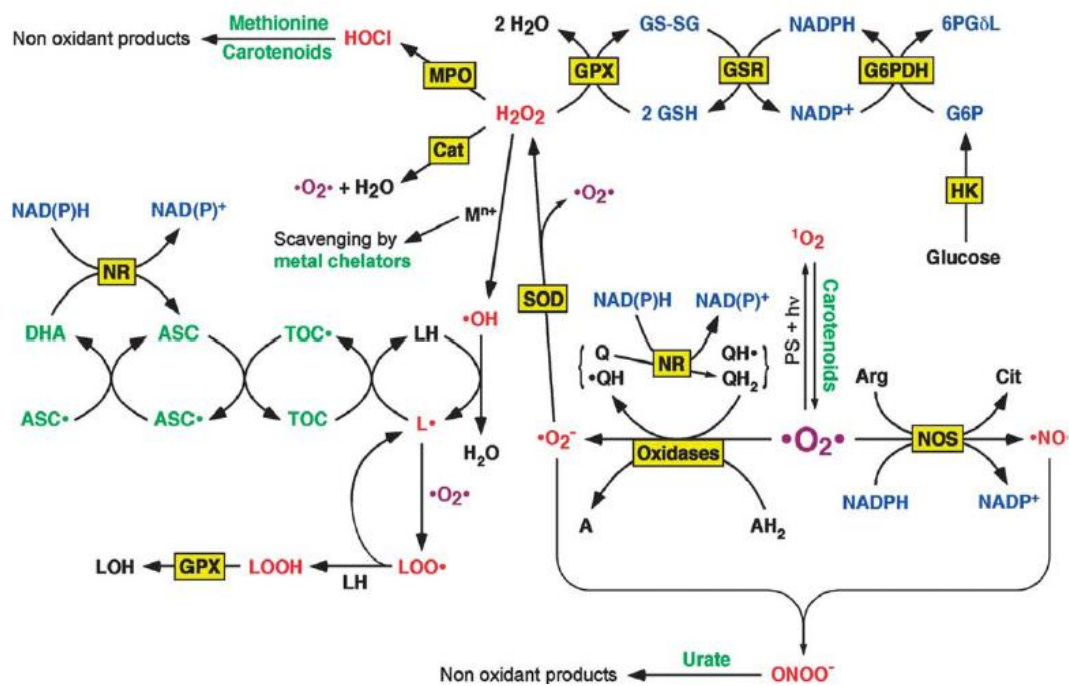
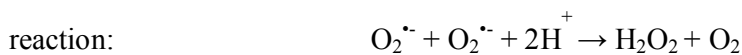


Figure 4. Free radical and ROS elimination (Sorg, 2004)

SOD activity is presented in all aerobic and in some anaerobic organisms as well as in all subcellular compartments susceptible to oxidative stress. This enzyme catalyzes the following



SOD exists in 3 forms a) Cu/Zn SOD present mainly in cytosolic matrix, b) MnSOD localized preferentially in mitochondria and c) extracellular SOD. All SODs efficiently catalyse transformation of $O_2^{\cdot-}$ into H_2O_2 . Adding SOD was reported to enhance as well as to limit lipid peroxidation and membrane damage (Mccord, 1985), which may result from different local concentrations of Fe^+ (Figure. 4). Apart from their localisation, these isoenzymes differ in their sensitivity to inhibitors: Cu/Zn SOD is sensitive to KCN, whereas H_2O_2 inhibits both the Cu/Zn SOD and Mn SOD (Fridovich, 1986).

1.5.1.2. Glutathione peroxidases

Glutathione peroxidases (GPxs) represent the major enzymatic defense against oxidative stress caused by hydroperoxides. They reduce H_2O_2 and organic hydroperoxides, such as fatty acid hydroperoxides, to the corresponding alcohols, using reducing power provided by GSH (Herbette et al., 2007). Two principal forms of GPxs have been characterized: classical GPx (cGPx or GPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Arthur, 2000). PHGPx is monomeric and partly membrane-associated, often evident in both soluble and membrane fractions, whereas GPx is tetrameric. Moreover, PHGPx is functionally distinct from GPx, as they reduce lipid hydroperoxides esterified in membranes in addition to the peroxides that are substrates of both Gpx and PHGPx. Phospholipid hydroperoxides are central intermediates in the lipid peroxidation chain reaction. Thus, PHGPx is generally considered to represent the main line of enzymatic defense against oxidative membrane damage (Avery et Avery, 2001). The ability of the glutathione system to reduce H_2O_2 depends on the ratio between GSH and the oxidized form (GSSG), which in turn depends on two processes: transport of GSSG out of the cell and the capacity of GSH reductase (Cantin, 1987) (Figure. 4).

1.5.1.3. Catalases

Catalase is localized intracellularly, especially in peroxisomes, and works efficiently under high concentrations of H₂O₂. In low concentrations it is mainly the reduced form of glutathione (GSH) that transforms hydrogen peroxide (Kelly, 1999). The final step in the detoxification of ROS (the elimination of excess H₂O₂) is carried out by catalases (Figure. 4),

It was reported that NiCl₂-induced increase the generation of hydroxyl radical, and was prevented significantly by catalase; in addition this enzyme inhibited the 8-OH-dG formation, (Valko et al, 2005). The catalase activity is significantly reduced in tumors of the liver (Litwin et al., 1999) and other organs (Lauer et al., 1999), as well as in a variety of pathological processes such as liver allograft rejection (Steinmetz et al., 1998), and after ischemia-reperfusion injury (Singh, 1996).

1.5.1.4. Peroxiredoxin I

Peroxiredoxin I is an antioxidant protein which has thioredoxin-dependent peroxidase activity with strong affinity for the pro-oxidant heme (Iwahara et al., 1995). The presence of peroxiredoxin 1 was reported in rat liver peroxisomes, in addition to cytoplasm, mitochondria and nucleus (Immenschuh et al., 2003). The peroxiredoxins play an important role in cellular protection against oxidative stress and in cell signaling (Hofmann et al., 2002).

1.5.2. Non-enzymatic defense system

Among non-enzymatic defenses, the tripeptide glutathione (γ -glutamyl-cysteinyl-glycine; GSH) (Pocsi et al., 2004). Glutathione is one of the most important intracellular mechanisms protecting normal cells from oxidative injury (Bounous, 2003). Under normal conditions, glutathione is primarily present in its reduced form (GSH), with only a small proportion being present in the fully oxidised state (GSSG) (Pocsi et al., 2004). Glutathione has multifunctional

roles in both regular cellular metabolism and in defense against ROS (Figure. 4). It reacts nonenzymatically with a series of ROS including OH^\bullet , HOCl , RO^\bullet , ROO^\bullet , $^1\text{O}_2$, as well as with many nitrogen and carbon containing radicals through the formation of GS^\bullet radicals (Halliwell and Gutteridge, 1999). In addition to its role as a free-radical scavenger in cells, glutathione serves as a co-factor for several enzymes, include glutathione peroxidases, glutathione reductase and glutathione S-transferases.

N-acetylcysteine (NAC) is a sulphhydryl donor, which is a potent drug acts directly by reacting with ROS (forming NAC disulfides in the end) and indirectly, serving as a GSH precursor by donating cysteine; however, the GSH synthesis enzymes are necessary (Aruoma et al, 1989; Victor et al 1999; Dekhuijzen, 2004). In addition NAC shows *in vitro* and *in vivo* stimulatory effect on the phagocytic process of macrophages and controls the functions of lymphocytes (Victor et al, 1999).

Vitamin E has been described as the major chain-breaking antioxidant in mammalian cellular membranes, as a result of its extremely efficient antioxidant capacity interrupting the chain of membrane lipid peroxidations (Traber and Packer, 1995). Because of its ability to localize in the bi-lipid cell membrane, vitamin E has several additional biologically important effects, including the inhibition of protein kinase C activity (Azzi et al., 1993). Vitamin E is found to be the free radical sweeper in the prevention of chronic diseases (Herrera and Barbas, 2001) Biological function of ascorbic acid can be defined as an enzyme cofactor, a radical scavenger, and as a donor/acceptor in electron transport at the plasma membrane. Ascorbic acid is able to scavenge the superoxide and hydroxyl radicals, as well as regenerate α -tocopherol (Davey et al., 2000). Vitamin C plays an important role in the defense against oxidative damage especially in leukocytes (Figure. 4) (Jariwalla and Harakech, 1996). Carotenoids (carotens and xanthophylls) are yellow, orange, and red pigments present in

many fruits and vegetables. Several of them are precursors of vitamin A (i.e. β -carotene, σ -carotene, and β -cryptoxanthin), and due to conjugated double bonds they are both radical scavengers and quenchers of singlet oxygen. Lower serum β -carotene levels have been linked to higher rates of cancer and cardiovascular diseases, as well as to increased risk of myocardial infarction among smokers (Rice-Evans et al, 1997). Research suggests β -carotene may work synergistically with vitamin E (Jacob, 1995; Sies and Stahl, 1995).

Vitamin A (retinol) is lipid-soluble vitamins essential for human health. Vitamin A has free-radical-scavenging properties that allow it to function as physiologic antioxidants in protecting a number of chronic diseases such as cancer and cardiovascular disease (Wakai et al., 2005). The parent compound of vitamin A is all-trans-retinol. It is the most abundant dietary form of vitamin A, and occurs naturally in the form of fatty acid esters such as retinyl palmitate, there are other naturally occurring forms of vitamin A of minor dietary components such as retinal, retinoic acid, etc. (Bates, 1995).

In the past, some studies estimated that uric acid contributes to 35- 65% of the total plasma antioxidant capacity (Geisinger et al, 1979). Uric acid is a physiological antioxidant effective inhibitor of the formation of $O_2^{\cdot-}$, H_2O_2 , that are ROS formed by the action of XO during the catalysis of xanthine and hypoxanthine (Kand'ar et al., 2006).

Lipoic acid, yet another important endogenous antioxidant, categorised as a “thiol” or “biothiol,” is a sulfur-containing molecule that is known for its involvement in the reaction that catalyses the oxidative decarboxylation of σ -keto acids, such as pyruvate and α -ketoglutarate, in the Krebs cycle (Chen et al., 1997). Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), are capable of quenching free radicals in both lipid and aqueous domains and as such has been called an universal antioxidant (Chen et al., 1997). Packer and Witt (1995) have demonstrated that supplemental lipoic acid protect against the symptoms of

vitamin E or vitamin C deficiency. Further, Aposhian et al (2003) tested the hypothesis as to whether GSH, vitamin C, or lipoic acid, alone or in combination with DMPS (2,3-dimercapto-1-propanesulfonate), would decrease brain mercury in young rats.

The antioxidant enzymes - glutathione peroxidase, catalase, and superoxide dismutase (SOD) metabolise oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity, it has been suggested that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms (Duthie and Brown, 1994). Research indicates that consumption and absorption of these important trace minerals may decrease with aging.

Selenium is a nutritionally essential element to the life of human and animals (Thomson, 2004). A component of selenoproteins, some of which have important enzymatic functions. This element plays key roles in redox regulation and antioxidant function, energy metabolism and protection against DNA damage (Rayman, 2000; Daniels, 2004).

More recently, a number of epidemiological studies have investigated the potential protective role of selenium in the prevention of many degenerative conditions including cancer, inflammatory diseases, thyroid function, cardiovascular disease (Van den Brandt et al., 2003; Faure et al., 2004).

Metal sequestration is another important mechanism of antioxidant defence. Proteins such as caeruloplasmin, and transferrin and its related protein lactoferrin, are found in extracellular fluids such as blood plasma, and have been shown to exert efficient protection against lipid peroxidation, simply by chelation of iron ions (transferrin and lactoferrin), or by their ferroxidase activity (caeruloplasmin) (Vacas, 1988; Martinez-Cayuela, 1995).

A large number of substances present within the cell, including also antioxidants, can chelate iron *in vivo* thus limiting its participation in free radical reactions. For example desferrioxamine mesylate (DFO) is a powerful ironchelating substance capable of almost completely suppressing iron-mediated oxidations in biological systems. DFO is often used as a therapeutic tool in the treatment of iron overload (Kasprzak et al., 1990)

A number of other dietary antioxidant substances (Phytonutrients) exist beyond the traditional vitamins discussed above. Many plant-derived substances, collectively termed “phytonutrients,” or “phytochemicals,” are becoming increasingly known for their antioxidant activity. Phenolic compounds are ubiquitous within the plant kingdom (Briviba and Sies, 1994). Flavonoids, tannins, anthocyanins and other phenolic constituents present in food of plant origin are potential antioxidants (Saskia et al., 1996).

2. Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) commonly called xanthine oxidase, is a dimeric enzyme with a molecular weight of 300 KDa. XOR is generally recognized as the terminal enzyme of purine catabolism in man, catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid. In addition to hypoxanthine and xanthine, XOR catalyzes the hydroxylation of a wide range of N-heterocyclic and aldehyde substrates. It can also act as an NADH oxidase (Bray, 1975).

2.1. Distribution and expression of XOR in organs and cells

XOR is found in a wide range of species, even, sometimes in very low amounts. It has been detected in a variety of bacterial species, both Gram-negative and Gram-positive (Woolfolk and Downard, 1977). XOR activity has also been detected in invertebrates, amphibians, reptiles and birds (Krenitsky et al, 1974). The enzyme has been shown, immuno-

histochemically, to be widely distributed in mammalian. Immunolocalisation techniques and sensitive radioimmunoassays on bovine tissue have revealed XOR in lactating mammary tissue, in liver, lung endothelial cells and in tissue macrophage. The lowest levels were found in the brain, testis and cornea (Bruder et al., 1983). XOR is localised particularly in the microvascular endothelial and certain epithelial cells in mammals including human (Bruder et al, 1983; Moriwaki et al., 1996). In mammals, the liver and intestine have the highest XOR activity of any tissue (Al-Khalidi and Chaglassian, 1965). The discrepancies in XOR expression between species may be explained by significant differences in the promoter regions (Xu et al., 1996). In one previous study bovine hepatocytes and intestinal epithelial cells lacked demonstrable XOR protein, whereas mammary gland epithelial cells and capillary endothelial cells of a variety of tissues including the liver, lung, skeletal muscle, heart, and intestine showed XOR immunoreactivity (Jarasch et al., 1981). In contrast, several other studies have localized XOR in the hepatocytes of rat (Ichikawa et al., 1992) and chicken liver (Hattori, 1989).

2.2. Basic reactions catalyzed by XOR

Xanthine oxidase (EC 1.1.3.22) and xanthine dehydrogenase (XDH) (EC 1.1.1.1) are interconvertible forms of the same enzyme (XOR). Both enzymes can reduce molecular oxygen to superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), while the ideal electron acceptors are NAD^+ and molecular oxygen for XDH and XO, respectively (Hille and Nishino, 1995). Physiologically XO and XDH participate in a variety of biochemical reactions including the hydroxylation of various purines, pterins, and aromatic heterocycles, as well as aliphatic and aromatic aldehydes, thereby contributing to the detoxification or activation of endogenous compounds and xenobiotics (Massey et al., 1970).

The major purine compounds in the cell are adenine and guanine ribo- and deoxyribonucleotides, they play an essential role in energy requiring reactions, nucleic acid synthesis, and as signaling molecules. Hypoxanthine is quantitatively the most important purine catabolic product. XOR catalyzes the two last steps in the catabolic pathway in humans, with uric acid as the end product (Figure. 5), whereas in all other animal species uric acid is further metabolized into allantoin by uricase (Raivio et al., 2001).

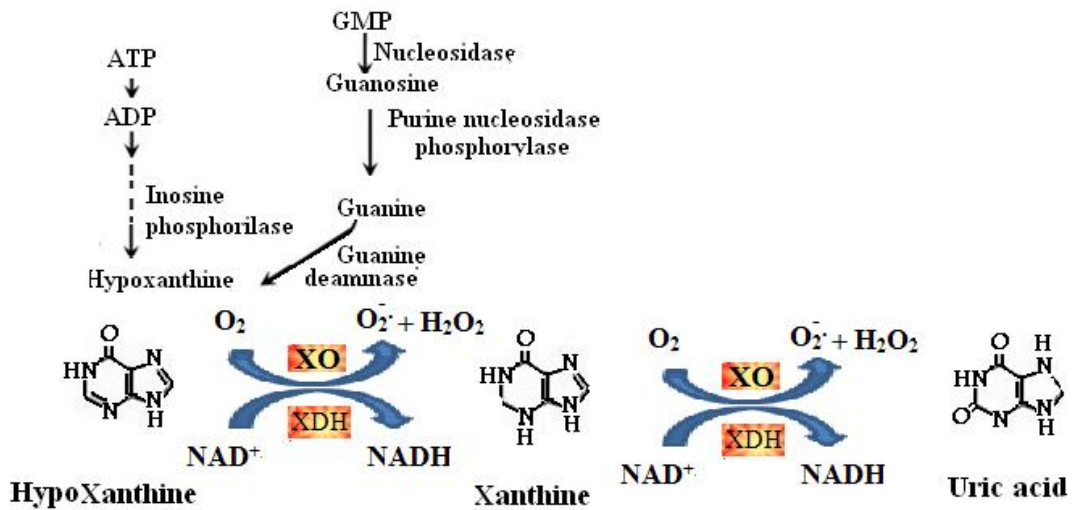


Figure 5. Pathways of human purine metabolism (Adapted from Raivio et al., 2001)

The reactive oxygen species, which include O₂⁻ and H₂O₂ produced by XO alter the structure and function of macromolecules including proteins, lipids, carbohydrates, and nucleic acids (Castro and Freeman, 2001). However, the primary product of XOR, uric acid is an antioxidant and has been ascribed a protective role against free radical effects (Ames et al., 1981).

2.3. Structure of XOR protein

Xanthine oxidoreductase (XOR) is molybdopterin-containing flavoproteins, use a molybdenum cofactor and a flavin to oxidize their substrates (Garattini et al., 2003). The

active form of the enzyme is a homodimer, with each of the monomers acting independently in catalysis. Each subunit molecule is composed of an N terminal 20-kDa domain containing two iron sulfur centers, a central 40-kDa FAD domain, and a C-terminal 85-kDa molybdopterin-binding domain (Hille and Nishino, 1995) and the subunits are connected to each other with a linker peptide (Enroth et al., 2000) (Figure. 6).

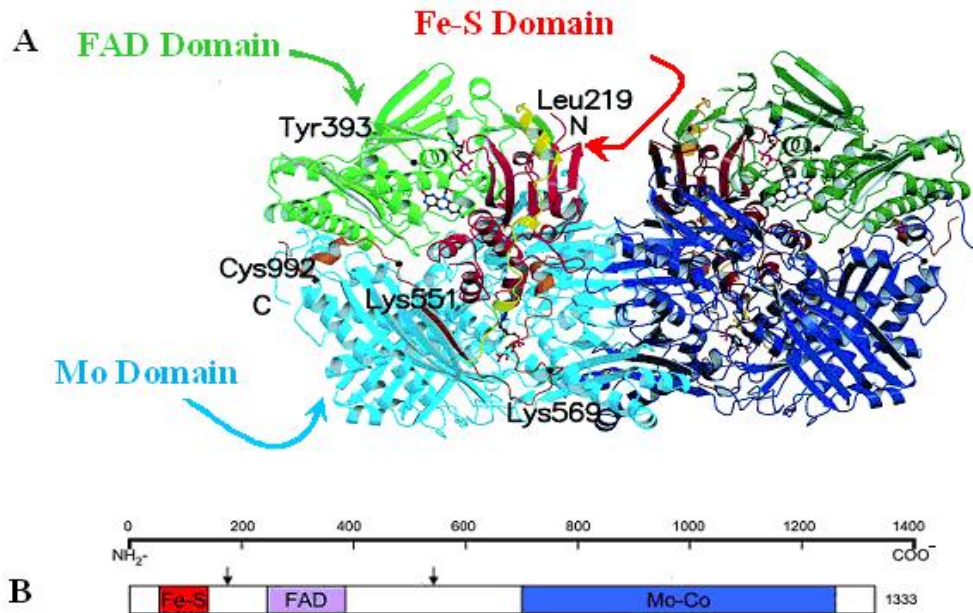


Figure 6. Molecular structure of XDH. The Fe₂S₂, FAD and Mo domains are shown in red, green and blue respectively (A). Linear arrangement of cofactors and salicylic acid into one subunit of XDH (B) (Enroth et al., 2000).

The oxidation of hypoxanthine and xanthine takes place at the molybdopterin site, where the purine substrate is bound to the molybdenum and donates two electrons, reducing Mo (VI) to Mo (IV) (Hille and Nishino, 1995). The arrangements of the cofactors indicate that the Fe/S clusters provide an electron transfer pathway from the molybdopterin cofactor to FAD (Enroth et al., 2000; Hille and Nishino, 1995). Finally, the reduction of the co-substrates NAD⁺ or molecular oxygen occurs at the FAD site (Hille and Nishino, 1995).

2.4. Dehydrogenase to Oxidase interconversions

XOR is synthesized as XDH form, which appears to be the predominant enzyme type in intact and freshly prepared mammalian tissue (Stirpe et al., 1969). However, the XDH form can be converted to the oxidase XO form, reversibly by sulfhydryl (SH) oxidation or irreversibly by proteolysis. Reversible XDH to XO conversion is due to SH-group oxidation of specific cysteine residues and generation of disulfide bridges (S-S), which can be reversed by SH-reducing agents, such as dithiothreitol (Amaya et al., 1990).

2.5. Inactive forms of XOR

There are two naturally occurring inactive forms of XOR, demolybdo and desulpho. Their presence was noted in most preparation of XOR from bovine milk (Hart et al., 1970) and rat liver (Ikegami and Nishino, 1986). Both the desulpho and demolybdo forms are inactive with respect to reducing substrate interacting directly with the molybdenum centre, such as hypoxanthine or xanthine, but can, nevertheless, catalyse the oxidation of NADH, which binds to the FAD centre (Hille and Nishino, 1995). The desulpho form of the enzyme has an essential Mo=S group at the molybdenum active site replaced by Mo=O (Gutteridge et al., 1978). Desulpho-sulpho conversion was found to be reversible *in vitro*. The total inactivation of XOR by treatment with cyanide was accompanied by the extraction of sulphur from the enzyme (Massey and Edmondson, 1970). It is possible that, *in vivo*, this form may serve to regulate the enzyme; being reactivated by sulphur-incorporating enzymes such as rhodanases (Ikegami and Nishino, 1986). The demolybdo form of the enzyme lacks the molybdenum atom. Hart et al (1970) mentioned that the molybdenum content of XOR varied depending on the regional variation in the nutritional status of the dairy cattle. The third inactive form of XOR (deflavo form), a non-naturally occurring form, lacks the FAD cofactor. Deflavo-XOR can be made by incubation in 2M CaCl₂ (Komai et al., 1969) or 3M KI (Kanda et al., 1972).

This de flavo form is inactive towards NADH and can only oxidase other reducing substrates in the presence of artificial electron acceptors such as ferricyanide (Komai et al., 1969).

2.6. XOR catalysed Reactive Oxygen and Nitrogen Species generation

The ability of XOR to generate a diverse range of Reactive Oxygen and Nitrogen Species (RONS) has been the driving force for the intense study of the XOR enzyme. The XOR-generation of ROS and the reactive nitrogen species (RNS) have been demonstrated (Godber et al, 2000). The hypothesised scheme for $O_2^{\cdot-}$ and H_2O_2 generation during the re-oxidation of reduced XO with O_2 was confirmed in 1981, it was concluded that a fully reduced XO monomer could generate two molecules of H_2O_2 and $O_2^{\cdot-}$, respectively (Porras et al, 1981). The discovery of XOR nitric oxide synthase and peroxynitrite synthase activity has added further interest to the XOR. The enzyme XOR is capable of reducing organic and inorganic nitrate to nitrite, and nitrite to NO^{\cdot} , under hypoxic conditions. (Millar et al, 1998), all redox centres need to be inserted and be active. However, in the absence of active cofactors, for example the Mo, it is feasible to suggest that the FAD redox centre alone could generate RONS by virtue of XOR–NADH oxidase activity (Abadeh et al, 1992).

2.7. Xanthine Oxidase and Ischemia-Reperfusion (IR) Injury

The mechanism of ischemia and tissue injury during reperfusion has been extensively studied, especially in the heart (Ceconi et al, 2003). Thus, it is a good example of the involvement of oxidative stress in a frequent pathology. During ischemia, blood glucose falls; glycolysis and oxidative phosphorylation rates decrease, and subsequently ATP stores are consumed. The two consequences of ATP depletion are (i) accumulation of AMP (Van Bilsen et al, 1998), (ii) a decrease in the activity of Na^+/K^+ -ATPase, the ATP-dependent pump that maintains the physiological gradients of sodium and potassium ions between both sides of cellular

membranes. AMP is metabolised to hypoxanthine; thus ATP depletion leads to hypoxanthine accumulation (Vander Heide et al., 1996) (Figure. 7).

On the other hand, the decrease of the activity of ATP-dependent ionic pumps induces a depolarisation of the plasmic membrane, promoting a massive influx of calcium ions within cells (Katsura et al., 1993). High cytoplasmic Ca^{2+} lead to the activation of calcium dependent proteases such as calpains (Budd, 1998). Calpains can convert the enzyme XDH to XO, (Nishino, 1997). This alteration does not change anything for its substrates. The difference concerns the electron acceptor: the dehydrogenase form has a binding site for NAD^+ , and substrate oxidation regenerates NADH, whereas the oxidase form cannot bind NAD^+ , and the enzyme transfers the electrons coming from the substrates to oxygen, leading to the formation of superoxide, and then hydrogen peroxide. Furthermore, due to hypoxanthine accumulation in response to ischaemia, XO is activated and gives rise to a dramatic production of superoxide ions as soon as oxygen is reperfused (Rees et al., 1994; Nishino, 1994). Finally, the activity of antioxidant enzymes such as catalase and SOD, are decreased following ischaemia (Homi et al., 2002) (Figure. 7).

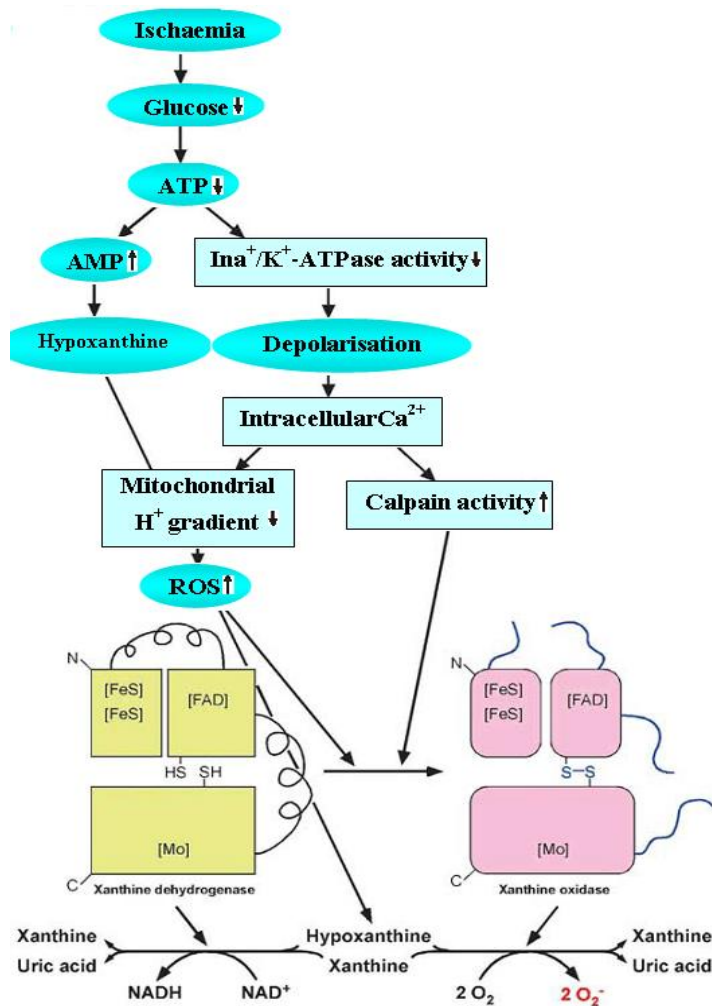


Figure 7. Mechanism of ROS production during reperfusion after ischemia (Sorg, 2004).

2.8. The role of Xanthine Oxidase in disease

Inherited XOR deficiency leads to xanthinuria and a multiple organ failure syndrome characterized by the deposition of xanthine in various tissues. In the mid-1970s, it was observed that various hepatotoxic agents, such as halothane and alcohol, induce the systemic release of XO from the liver (Giler et al., 1977; Zima et al., 1993), initially, it was considered that circulating XO could be used as a sensitive marker to quantitate liver injury. Subsequent many work demonstrated that circulating XO is not only a marker of hepatic and intestinal damage, but it can also act as a circulating mediator that is responsible for remote organ injury

in a variety of pathophysiological conditions including hepatic ischemia and reperfusion, hemorrhagic shock and atherosclerosis (Aslan et al., 2001).

2.8.1. Xanthine Oxidase in cardiovascular disease

Recent work has indicated a role for XO and XO related oxidant species in the pathogenesis of chronic heart failure (CHF) (Berry and Hare, 2004). *In vitro* studies in isolated hearts have demonstrated that the progressive development of heart failure is associated with increased myocardial XO levels, which contribute to an enhancement of oxidative stress in the heart (Ferdinandy et al., 2000). In patients with CHF, elevated circulating uric acid levels have been noted as well as an increase in myocardial XO activity (Cappola et al., 2001). In patients with coronary artery disease, heart failure, or diabetes, elevated serum uric acid levels are highly predictive of mortality. Although the mechanisms by which uric acid may play a pathogenetic role in cardiovascular disease are poorly understood, hyperuricemia is associated with deleterious effects on vascular function. It has recently been demonstrated that patients with hyperuricemia had impaired flow mediated dilation, which was normalised by 3 months of therapy with the XO inhibitor allopurinol (Mercurio et al., 2004).

2.8.2. Xanthine Oxidase and Inflammatory Diseases

There is a significant evidence for the pathogenetic role of XO in some experimental models of colitis and inflammatory bowel disease and duodenal ulceration (Ben-Hamida et al., 1998). It is possible that basal levels of XO (when converted from XDH) contribute to the development of the disease. Alternatively, it is also possible that the beneficial effects of allopurinol in colitis are unrelated to XO inhibition and are actually related to oxyradical scavenging.

Neutrophils contain the complex enzyme, NADPH oxidase, which is a rich source of both superoxide and the HOCl (Kurose and Granger, 1994). Interaction of neutrophils with the vascular endothelium involves a complex sequence of rolling, adhesion, and transendothelial migration of neutrophils, mediated by a range of cell adhesion molecules (CAMs) (Grisham et al, 1998). In view of the considerable destructive potential of neutrophils in IR, XOR-derived ROS have been increasingly seen as mediating neutrophil adhesion rather than as primary agents of tissue damage (Kurose and Granger, 1994). ROS, especially $O_2^{\cdot-}$, can react with cell membrane components, such as arachidonic acid, to produce chemotactic lipids which attract more neutrophils to the site of infection, amplifying the inflammatory response (Perez et al, 1980).

The localisation of XOR in several epithelial cell types (Rouquette et al., 1998) provides a possible connection between the mechanisms involved in epithelial and endothelial cell injury. Page (1999) has shown that the inflammatory molecules, IL-1, IL-6, TNF- α , and IFN- γ increase the activity of XOR, these cytokines upregulate the gene expression of this enzyme. Furthermore, XO-derived ROS have been proposed to act as mediators of inflammatory signal transduction pathways and proinflammatory gene expression (Matsui et al., 2000).

2.9. Inhibition of XOR

Inhibitors of XOR can be divided into two categories; molecules which are structural analogs to purine substrates and molecules which are unrelated to physiologic substrates. Allopurinol and its major metabolite oxypurinol are purine analogs that form a tight-binding complex with molybdenum at the active site, leading to the inhibition of XOR (Massey et al., 1970). Allopurinol was approved by Drug Administration for treatment of gout and remains a cornerstone in the therapy of primary and secondary hyperuricemia (Wortmann, 2005). Allopurinol is rapidly oxidised by XO in vivo to its active oxypurinol, which also inhibits

XO. The formation of this compound, together with its long persistence in tissues, is responsible for much of the pharmacological activity of allopurinol. In terms of pharmacokinetics, allopurinol is rapidly absorbed, reaching peak plasma concentrations within 30 to 60 min, following oral administration. Allopurinol has relatively short half-life in plasma (2-3 h), whereas the half-life of oxypurinol is much longer (14-30 h) due to renal reabsorption (Pea, 2005).

Cyanide is also capable of modifying the molybdopterin by removing the essential sulfur atom, thus inactivating the enzyme (Coughlan et al., 1980). This may also be the mechanism by which the enzyme is inactivated in hyperoxia by oxygen metabolites in vivo (Terada et al., 1988). Desulfo-XOR can be reactivated by reinsertion of the sulfur atom at the molybdenum site, which is catalyzed by a sulfurase present in human tissues (Ichida et al., 2001).

2.10. Role and adverse effects of allopurinol

By lowering the uric acid concentration in plasma, allopurinol prevents the development of chronic gouty arthritis (Rott and Agudelo, 2003; Schlesinger, 2004; Pea, 2005). The formation of uric acid stones gradually disappears with therapy, and this prevents the development of nephropathy. In addition to the gout and hyperuricemia, there are numerous potential therapeutic applications for allopurinol and oxypurinol in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases, and chronic heart failure.

The most common adverse effects of allopurinol are gastrointestinal distress, hypersensitivity reactions, and skin rash. The hypersensitivity reaction may occur even after months or years of medication. Symptoms of allopurinol toxicity include fever, rash, vasculitis, and worsening of renal function, which can lead to a fatal outcome especially in elderly patients with renal insufficiency (Rott and Agudelo, 2003; Bieber and Terkeltaub, 2004).

2.11. Novel Xanthine Oxidase Inhibitors

The early search for novel XO inhibitors, focused on synthetic purine and pyrimidine derivatives. However, the drug structural framework based on the purine and pyrimidine motifs is responsible for some side effect caused by allopurinol, i.e., rashes, which are sometimes severe (occurring in 2-8% patients). The rashes result from the metabolic conversion of the drugs to corresponding nucleotide through the action of phosphoribosyl transferase (Borges et al., 2002). This prompted a search for new XO inhibitors that are structurally distinct from purines (Borges et al., 2002). During the past decade, definite progress has also been achieved in the understanding of the XO enzyme structure, and rational drug development approaches led to the discovery of new powerful XO inhibitors of various classes, including imidazole and triazole derivatives, and flavonoids among many others. Two of these very potent new compounds, febuxostat [2-[3-cyano-4-(2-methylpropoxy) phenyl]-4-methylthiazole-5-carboxylic acid; TMX-67, TEI-6720] and Y-700, are reported to have a favorable toxicology profile, high bioavailability, and more potent and longer-lasting hypouricemic action than allopurinol. These new compounds are currently in human clinical trials for the treatment of hyperuricemia and gout (Okamoto et al., 2003; Hashimoto et al., 2005; Takano et al., 2005).

Thus, it appears that the hegemony of allopurinol, as a drug in its own league, which essentially had no competitors in clinical use for half a century, is about to change. There are a few structural classes of compounds that are many hundreds times more potent than allopurinol *in vitro* (both of a purine and nonpurine types). Several drug candidates are either in the development phase or are moving toward clinical testing (Borges et al., 2002; Fukunari et al., 2004; Hashimoto et al., 2005; Takano et al., 2005).

3. Phenolic compounds

Polyphenols are a wide and complex group of secondary plant metabolites, which can be defined as compounds possessing an aromatic ring bearing one or more hydroxy substituents (Harborne, 1967). Phenolic compounds are also commonly referred to as polyphenolics, a term that originally meant “many phenolics” and probably referred to a compound having many phenolic groups (Wrolstad, 2005). Structures of the compounds range from simple molecules such as phenolic acids, to highly polymerized compounds like proanthocyanidins (Wrolstad, 2005).

In plants, phenolic compounds occur primarily in their mono-glycosylated form (Shahidi and Naczk, 1995). Glucose is the predominant glycosyl moiety; however arabinose, galactose, rhamnose and xylose are also common. Phenolic compounds may also be conjugated with aliphatic organic acids, amines, lipids, oligosaccharides or other substituents (Bravo, 1998).

3.1. Occurrence of phenolic compounds

Polyphenols are ubiquitous in plant kingdom and practically all plant foods and beverages contain at least some amounts of these compounds. However, the types and amounts of compounds may vary greatly between different plants (Manach et al., 2004). Several environmental factors, such as light, climate, and seasonal variation also affect the types and the amounts (Erdman et al., 2007). The polyphenol content in plants is mainly determined by genetic factors such as plant phyla, order, family and population variations within species (Harborne, 1986; Aherne et O'Brien, 2002). Flavonols occur widely in fruits and vegetables as well as in beverages (Scalbert and Williamson, 2000; Mattila, 2005).

Flavan subclass, which consists of both monomeric (catechins) and polymeric (proanthocyanidins) forms, are one of the most ubiquitous flavonoids in plant foods (Manach

et al, 2004). Citrus fruits and citrus juices are the main sources of flavanones. Flavones are less common, but significant amounts can be found in parsley and celery.

3.2. Types of Phenolic compounds

Variations in complexity of structure, conjugation, hydroxylation and methoxylation contribute to the wide range of naturally occurring phenolic molecules, and more than 8000 phenolic compounds have been identified in plants (Harborne and Williams, 2000). Out of the large variety of compounds, the most common groups of phenolic compounds are phenolic acids and flavonoids (Figure. 8) (Bravo, 1998; Harborne, 1986).

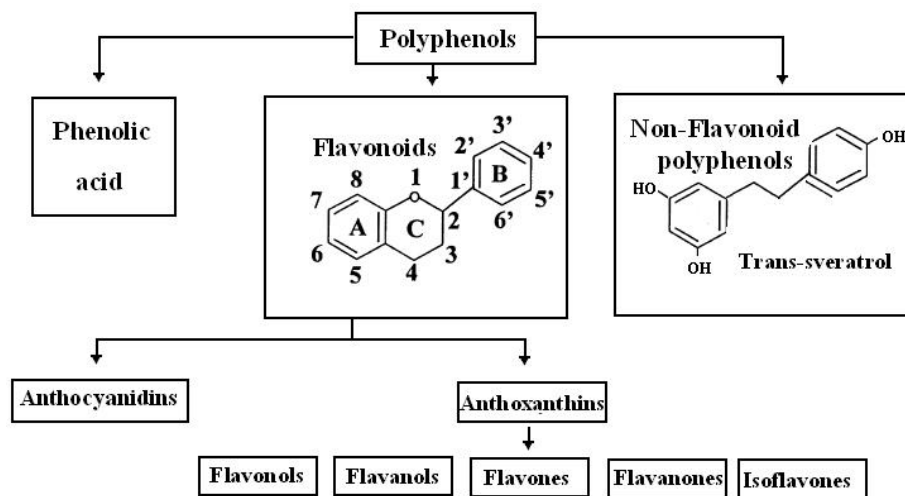


Figure 8. Classification and structure of natural polyphenols. (Modified from Weinreb et al., 2004).

3.2.1. Flavonoids

The flavonoids are a diverse group of phenolic compounds widely distributed in the plant kingdom. There are more than 6400 known flavonoid compounds (Harborne and Williams, 2000). The term flavonoids are a collective noun for plant pigments, mostly derived from benzo- γ -pyrone, which is synonymous with chromone (Hassig et al., 1999). In general, all

flavonoids are derivatives of the 2- phenylchromone parent compound composed of three phenolic rings referred as A, B, and C rings, which exhibit various levels of hydroxylation and methoxylation (Figure. 8) (Harborne and Turner 1984; Beecher, 2003).

The basis of the great variability of the flavonoids is: 1) differences in the ring structure of the aglycone and in its state of oxidation/reduction; 2) differences in the extent of hydroxylation of the aglycone and in the positions of the hydroxyl groups; 3) differences in the derivatisation of the hydroxyl groups, e.g., with methyl groups, carbohydrates, or isoprenoids (DuBois and Sneden, 1995). The main substituents are hydroxyl, methoxyl, or glycosyl, which can be further substituted forming very complex structures. Classification of flavonoids into subclasses is based on the functional groups in the C ring. Subclasses include anthocyanidins, flavanols, flavones, flavonols, flavanones, and isoflavonoids (Figure. 8, 9) (Cheynier, 2005).

The flavanols largely occur in the aglycone form, contrary to the fact that most flavonoids exist in plants as glycosides (Harborne, 1967). Flavanones are characterized by the presence of a carbonyl group at C-4 of ring C. the most commonly occurring aglycones are hesperetin, eriodictyol and naringenin (Figure. 9) (Grayer and Veitch, 2005).

Flavones are less common in plants as compared to other flavonoids but are prominent in citrus fruits (Kim and Lee, 2005), however Flavonols are the most prevalent flavonoid in the plant kingdom. More than 1400 glycosylated flavonols have been identified in plants (Figure. 9) (Harborne and Williams, 2000)

Isoflavones are structural isomers of flavanones that are often treated separately from the former sub-classes of flavonoids because they typically occur only in plants of the family Leguminaceae (Mann, 1987). Isoflavones commonly occur as aglycones, glycosides, acetyl-glycosides and malonyl-glycosides (Mann, 1987).

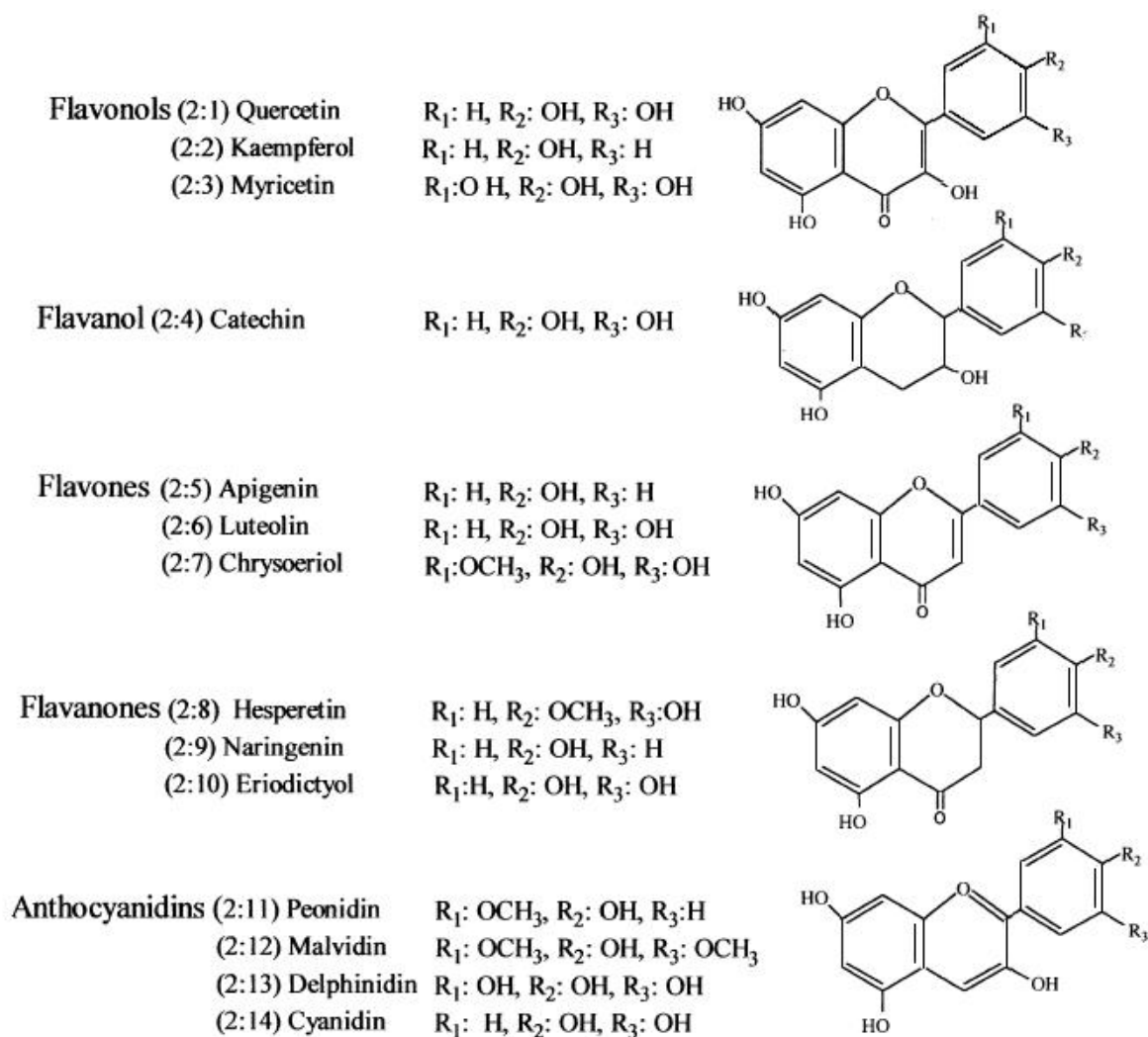


Figure 9. Chemical structures of some flavanoids (O'Connell and Foxa, 2001).

In addition to the aforementioned main subclasses of flavonoids there are three minor flavonoid subclasses that include chalcones, dihydrochalcones and aurones. The chalcones are structurally one of the most diverse groups of flavonoids and are found as monomers, dimers, oligomers and conjugates of various types. The unique feature that distinguishes chalcones and dihydrochalcones from other flavonoids is the open chain three-carbon structure linking the A- and B-rings in place of a heterocyclic C rings (Harborne, 1967). Aurones are not common in dietary plants although they provide strong yellow colours in a variety of flowers (Grayer and Veitch, 2005).

The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Harborne, 1986). Flavonoids can be sugar-free (aglycones) or glycosylated (usually at either the C-3 or C-7 position) except flavanols which occur as aglycones (Beecher, 2003). The sugar moiety is usually glucose but flavanoid glycosides containing also rhamnose, xylose, arabinose, galactose, galacturonic acid or glucuronic acid also exist. Polysaccharides, amines and lipids have also been reported to be esterified to PCs (Bravo, 1998).

3.2.2. Phenolic acids

Phenolic acids are plant secondary metabolites widely spread in plant kingdom (Bruneton, 1993). Phenolic acids are derivatives of benzoic or cinnamic acid (Mehansho et al. 1987). The main subclasses of phenolic acids include Hydroxybenzoic acids and Hydroxycinnamic acids (Cheynier, 2005).

3.2.2.1. Hydroxybenzoic acids

Hydroxybenzoic acids have a general structure derived directly from benzoic acid (Figure. 10A). Variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring (Macheix et al. 1990). Four acids occur commonly: hydroxybenzoic, vanillic, syringic, and protocatechuic acid. They may be present in soluble form conjugated with sugars or organic acids as well as bound to cell wall fractions, e.g. lignin. A common hydroxybenzoic acid is also salicylic acid (2- hydroxybenzoate). Gallic acid (Figure. 10A) is a trihydroxyl derivative which participates in the formation of hydrolysable gallotannins (Strack 1997). Its dimeric condensation products, (hexahydroxydiphenic acid, ellagic acid) (Figure. 10B) are common plant metabolites which is usually present as esters of diphenic acid analogue with glucose (Maas et al. 1992).

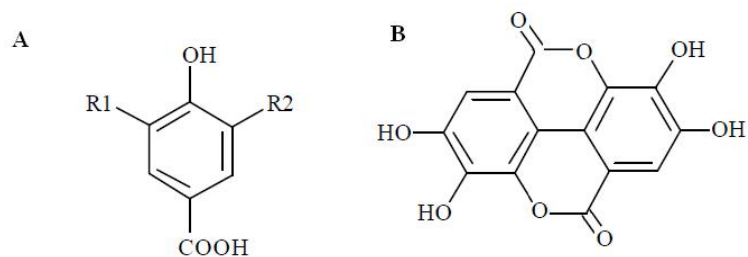


Figure 10. Chemical structures of hydroxybenzoic acids: p-hydroxybenzoic acid, R1=H, R2=H; gallic acid, R1=OH, R2=OH (A) and ellagic acid (B).

3.2.2.2. Hydroxycinnamic acids

The four most widely distributed hydroxycinnamic acids in fruits are p-coumaric, caffeic, ferulic and sinapic acids (Figure. 11) (Macheix et al. 1990). Hydroxycinnamic acids usually occur in various conjugated forms, the free forms being artifacts from chemical or enzymatic hydrolysis during tissue extraction. The conjugated forms are esters of hydroxyacids such as quinic, shikimic and tartaric acid, as well as their sugar derivatives (Macheix et al. 1990, Shahidi and Naczki, 1995).

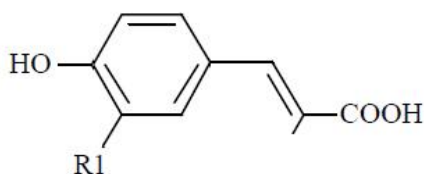


Figure11. Chemical structure of three common hydroxycinnamic acids: p-coumaric acid R1=H; caffeic acid, R1=OH; ferulic acid, R1=OCH₃

3.2.3. Tannins

The term “tannin” comes from the capacity of these compounds to transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen (Bravo, 1998). Tannins are phenolic compounds of intermediate to high molecular weight ranging from 500 to > 20.000 Da (Santos-Buelga and Scalbert, 2000). These compounds can form insoluble

complexes with carbohydrates and protein through hydrogen bonding of the hydroxyl groups (Salunkhe et al., 1989). Tannins in meals and flours from some oilseeds, grains and legumes possess antinutritional properties by binding and precipitating carbohydrate and protein, thereby reducing food digestibility (Shahidi and Naczki, 1995). Tannins may also precipitate a wide range of essential minerals, thus lowering their bioavailability (Faithful, 1984). There are two main types of tannins which include the hydrolysable tannins, based on gallic acid polymers, and condensed tannins or proanthocyanidins which are composed of flavanol polymers (Manach et al., 2004).

3.2.4. Biflavonoids and Triflavonoids

Biflavonoids and triflavonoids are dimers and trimers, respectively of flavonoids. The dimers and trimers are products of oxidative coupling mainly of flavones, flavanones and/or aurones (Ferreira et al., 2006). Biflavone, 2, 3-dihydroapigeninyl-(I-3',II-3')-apigenin has a C-C interflavanyl linkage between the C-3' carbons on each flavone moiety. Several biflavonoids with C-O-C interflavanyl bonds have recently been identified (Bennie et al., 2001; Ferreira et al., 2006)

3.2.5. Lignins and Lignans

Lignin is formed by oxidative polymerization of cinnamic acid derivatives, particularly hydroxycinnamyl alcohol monomers (Mann, 1987). The most prominent of cinnamic acid derivatives found in lignins are 4-hydroxycinnamyl alcohol, coniferyl alcohol and sinapyl alcohol, although these monomers vary with the plant type (Mann, 1987; Dewick, 2002). Lignin is held within a cellulose matrix and is utilised to strengthen the cell wall of the plant against external physical and chemical stresses. On the contrary, lignans are dimeric compounds also formed from cinnamic acid derivatives coupled in a specific manner, thus forming specific compounds such as secoisolariciresinol (Dewick, 2002).

3.3. Properties of Phenolic Compounds

Phenolic compounds antioxidant activity is regarded to be related to 1) scavenging free radicals, 2) chelating transition-metals involved in free-radical production and 3) inhibiting the enzymes participating in free-radical generation (Yang et al., 2001; Aruoma, 2002). Due to their antioxidant activity, PCs and fruit extracts have been reported to have positive effects on cancer, cardiovascular disease, immune disorders, microbial infections, neurodegenerative disease and viral infections (Xu et al., 2000; Le-Marchand et al., 2000). The correlation between ingesting phenolic compounds and improved health has been reported in epidemiological studies (Nichenam et al., 2006). Measurement of plasma and urine antioxidant power after ingestion of green tea has shown that absorption of antioxidants is rapid (Benzie et al., 1999). The interest in phenolic acids stems from their potential as protective factors against cancer (Breinholt, 1999) and heart diseases in part because of their potent antioxidative properties (Nardini et al., 1995; Breinholt., 1999). These phytochemicals are able to scavenge a wide range of reactive species, including hydroxyl radicals, peroxy radicals hypochlorous acid and superoxide radical. PCs can also inhibit biomolecular damage by peroxynitrite *in vitro* (Pannala et al, 1997; Santos and Mira., 2004), They are also known to exhibit antibacterial effects (Weston et al., 1999), enzyme inhibition, antimicrobial activity (Havsteen, 1983), antimutagenic and anti-inflammatory activities in bacteria and mammalian, respectively (Kaur et al., 1997). Proanthocyanidin-rich extracts from grape seeds also display anticataract activity in rats (Yamakoshi et al., 2002). Several flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity (Shoskes, 1998).

Certain phenolic acids were reported to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase (Middleton et al., 2000), Quercetin has been

reported to be a potent inhibitor (HIV)-1 protease (Xu et al., 2000), and Some flavonoids can inhibit degranulation of neutrophils without affecting superoxide production (Ferrandiz et al., 1996). But Caffeic acid is known to selectively stop the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma and allergic reactions (Koshihara et al., 1984), other Flavonoids can be stimulate the production of antibodies (Goodwin and Webb, 1980).

3.4. Structure-Antioxidants activity relationships of Phenolic Compounds

The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim et al., 2002; Mathew and Abraham, 2006). They can participate in protection against the harmful action of ROS, Further they have multiple applications in food, cosmetic and pharmaceutical industries (Kahkonen et al., 1999). The antioxidant capacity of PCs is mainly due to their redox properties, (Balasundram et al. 2006), which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Amarowicz et al., 2004).

The structure of PCs is a key determinant of their antioxidants activities (Robards et al., 1999). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation. However, substitution of the hydroxyl groups at the 3- and 5-position with methoxyl groups reduces the activity (Rice-Evans et al., 1996). Hydroxycinnamic acids exhibit higher antioxidant activity compared to the corresponding hydroxybenzoic acids (Andreasen et al., 2001). The higher activity of the hydroxycinnamic acid could be due to the $\text{CH}=\text{CH}-\text{COOH}$ group, which ensures greater H-donating ability and radical stabilisation than the $-\text{COOH}$ group in the hydroxybenzoic acids (Rice-Evans et al., 1996). Some of the structural features and nature of substitutions on rings B and C which determine the antioxidant activity of flavonoids include the following: (i) The degree of hydroxylation and

the positions of the -OH groups in the B ring, in particular an ortho-dihydroxyl structure of ring B (catechol group) results in higher activity (van Acker et al., 1996), or acts as the preferred binding site for trace metals (Pietta, 2000). (ii) The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions of ring B (a pyrogallol group) has been reported to enhance the antioxidant activity (Van Acker et al., 1996). (iii) A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C enhances the radical scavenging capacity of flavonoids (Pietta, 2000). (iv) A double bond between C-2 and C-3, combined with a 3-OH, in ring C, also enhances the active radical scavenging capacity of flavonoids (Van Acker et al., 1996). (v) Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids (Pietta, 2000; Seeram and Nair, 2002).

From ancient time, plants are rich source of effective and safe medicines. Herbal medicines have been main source of primary health care in many nations. About 80% of world populations are still dependent on traditional medicines (WHO, 1998). Herbal medicines defined as preparations derived from plants and fungi, for example by alcoholic extraction, used to prevent and treat diseases, which are an essential part of traditional medicine in almost any culture (Vickers and Zollman, 1999). Herbal medicines materials include juices, gums, fatty oils, essential oils and any other substances of this nature, contain as active ingredients, aerial or under ground part of plants or other plant materials, whether in the crude state or as plant preparations.

4. *Tamus communis* L.

Tamus communis L. belongs to a plant of twining and climbing plants which generally spring from large tubers, some of which are cultivated for food, as the Yam, which forms an important article of food in many tropical countries. *T. communis* is a very common plant in woods and hedges, with weak stems twining round anything, and thus ascending or creeping among the trees and bushes to a considerable distance. *T. communis*, Family of Dioscoreacea have many common names such as El-Karma Saouda, herbe aux femmes battues, raisin du diable, black bryony, bryony, blackeye, albarri, brionia negra, nueza negra, lady's, seal and siyah akasma.

4. 1. Geographic distribution

T. communis (Tc) displays a broader distribution in South, South-Central and West Europe (Tutin et al., 1972), as well as in Asia (Iran) and South West Africa (Tutin et al., 1980) in Morocco and Algeria (Maire, 1959). *T. communis* grows in the form of ordered shrubs, it is found particularly in the mountain forests of 1600m height.

4. 2. Morphological description

T. communis is a climbing plant with large tubers (Schmidt and Moul, 1983). The leaves are alternate, heart-shaped pointed, smooth and generally shining as if they had been varnished. Late in autumn they turn dark purple or bright yellow, making a very showy appearance. In winter, the stems die down, though the root is perennial. The flowers are small, greenish-white of two kinds, barren and fertile on different plants, the latter being succeeded by berries of a red color when ripe. The large fleshy root is black on the outside, like that of the yam, thick and tuberous nearly cylindrical, and abounding in starch (Figure. 12) (Boullard, 2001).



Figure12. Different part of *Tamus communis* L.

4. 3. Uses in traditional medicine and reported activities

T. communis or El-Karma Saouda as known in Algeria, causes irritation when rubbed on the skin (Schmidt and Moul, 1983). The scraped pulp was applied as a stimulating plaster, and has been found serviceable in gout, rheumatism and paralysis (Duke, 2002). Tincture made from the root and fruits prove a most useful application to unbroken chilblains. Both the rhizomes and the berries have a reputation for the treatment of arthrosis, lumbago and dermatosis; therefore they have been traditionally used in several countries (Duke, 2002). The juice of the root, with honey, has also been used as a remedy for asthmatic complaints (Kovacs et al., 2007). The berries act as an emetic, moreover; different parts of the plant have been applied in traditional medicine for the treatment of polyps (Hartwell, 1969). Black Bryony is a popular remedy for removing black eyes and discoloration caused by bruises (Boullard, 2001).

4.4. Chemical compositions

The distribution of phenanthrene and dihydrophenanthrene in the plant kingdom appears to be limited; their occurrence has been reported to date in only a few plant families: Orchidaceae, Dioscoreaceae, Combretaceae, etc. These compounds are known to be endogenous plant growth regulators. As concerns their pharmacological profile, a number of phenanthrenes have been reported to exert antiviral activity against vesicular stomatitis virus and human rhinovirus serotype 1B (Aquino et al., 1991), and smooth muscle-relaxing activity (Estrada et al., 1999). Some natural phenanthrenes and dihydrophenanthrenes have also been reported to display an antitumour effect (Pettit et al., 1988; Shagufta et al., 2006). Kovacs results (2007) provide further evidence that hydroxy/alkoxy-substituted phenanthrenes from *Tamus* are promising antitumour agents. Previous phytochemical investigations revealed the presence of spirostane and furostane glycosides (Aquino et al., 1991), sterols (Capasso et al., 1983), histamine (Schmidt and Moulton, 1983) and hydroxy/alkoxy- substituted phenanthrenes and dihydrophenanthrenes (Aquino et al., 1985 a, b).

5. *Carthamus caeruleus* L.

The genus *Carthamus* of the family Asteraceae, includes 14 species of annual or perennial grasses, including blue Carduncelle or kendjar, which has many scientific names; *Carthamus caeruleus* L., *Kentrophyllum caeruleum* (L.), *Carduncellus caeruleus* L., *Onoborma caerulea* L., and *Carthamus tingitanus*. However the local noun is Gares Mars.

5.1. Geographic distribution

It is a rare species that prefers sunny site in the Mediterranean basin, it is from the south - west Asia (Mioulane, 2004), but widespread in the Asia, north Africa, Australia and in both Americas, and Europe (Boullard, 2001).

5.2. Morphological description

Carthamus caeruleus L. is a biennial or annual herb with ascending stems simple or little ramous, hairless (high of 0.2 to 0.6 m)), leaves are dentate or pinnatisect, with 13-21 spinose teeth or lobes (Mioulane, 2004). The flowers are blue usually solitary with size of 3 cm wide and 3 to 4 cm long. They have a tubular corolla, in addition to the fruits of *Carthamus caeruleus* L. achenes are significantly shorter than the pappus (about 2 times, 1 cm long) (Figure. 13) (Boullard, 2001).



Figure13 : Different parts of *Carthamus caeruleus* L.

5.3. Uses in traditional medicine and reported activities

C. caeruleus roots have been used in folk medicine in Algeria for its useful action against painful joints of the limbs, and as cicatriser to treat injures result from burn. Roots are applied as powder or cream prepared with milk.

6 . *Ajuga iva* L.

A. iva comprises many terms; Arabic: Chendgoura or Shandgoura; Berber: Touf et-toulba (best that doctors). English: Bugle iva, Herb ivy, Musky bugle. French: Yvette musquée, Bugle, *Ajuga iva pseudoiva* , Bugle faux iva.

6. 1. Geographical Distribution

This aromatic plant, *A. iva*, Chendgoura, develops in deep soil of 2700m height. It grows in period from spring to late summer. The flowering period is between May and June (Batanouny et al. 1999; Halimi, 2004). It is widely distributed in the Mediterranean region: southern Europe and northern Africa, particularly in Algeria, Morocco, Tunisia, and Egypt (Halimi, 2004).

6. 2. Morphological Description

A. iva is a small aromatic perennial bitter taste of 5 to 10cm, with green stems creeping and hairy. Leaves are narrowly oblong to linear, pubescent, 14-35 mm long. *A. iva* grows in rocky slopes up to 2700 m of altitude (Gordon et al. 1997; Batanouny et al., 1999). The flowers are purple, pink, or yellow, 20 mm of long, the upper lip of the corolla is absent or reduced and the lower lip is divided into three lobes hairy. The side lobes are small, while the central lobe is relatively larger, decorated in base with a central yellowish spots with the same color of the flower, usually in purple. Within the flower there are four stamens related to four carpels black. The seeds are brown and have the size of the seeds of *Nigella sativa* (Figure.14) (Halimi, 2004).



Figur14: Different part of *Ajuga iva* L.

6.3. Uses in traditional medicine and reported activities

Ajuga iva L. is one of the plants widely used to treat diabetes and other disorders (Ziyyat *et al*, 1997; Bnouham *et al*, 2002; El-Hilaly *et al*, 2003; Tahraoui *et al*, 2007). It is ingested for its useful action against stomach and intestinal pains, enteritis, fever, sinusitis and headache (Ghedira *et al* 1991). Pharmacological studies have shown that *A. iva* has anti-ulcerous (Habib *et al*, 1990) and anti-inflammatory activities (Hilaly *et al*, 2002). It has been used to treat dysuria and painful joints of the limbs. *Ajuga iva* extract decreases plasma cholesterol and triglycerides (El-Hilaly *et al*, 2006). The Powder of dried plant or its infusion taken after meals against diabetes and hypertension, in addition the infusion of flowering branches is considered as antidiarrheic, depurative, and very effective for feminine sterility (Ghedira *et al* 1991). In addition *Ajuga iva* induce an Inhibition of calcium oxalate monohydrate crystal growth (Beghalia *et al*, 2008) and no apparent toxicity was observed for this plant (El-Hilaly *et al*, 2004).

6.4. Chemical compositions

Ajuga iva L. was found to contain a large number of compounds. such as 8-O-acetyl harpagide, ajugarine, apigenin-7-O neohesperidoside, barpagide, caffeine, clorogenes, cyasterone, diglycerides, 14,15-dihydroajugapitin, ecdysones, ecdysterones, flavonoids, iridoides, makisterone A, neohesperidoside, phenylcarboxylic acids and tanninpolyphenols (Khafagy et al., 1979; Jannet et al., 1997). It was also reported that *A. iva* contain tannins, phytoecdysteroids, polyhydroxylated-sterols, few essential oil (Wessner et, 1992; Ghedira et al 1991). Bondi et al (2000) reported that *A. iva* includes a large amounts of three major ecdysteroids (makisterone A, 20-hydroxyecdysone and cyasterone) with several minor compounds including 24,28-dehydromakisterone A and two new phytoecdysteroids (22-oxocyasterone and 24,25-dehydroprecyasterone). In addition it contains polypodine B and 2-deoxy-20-hydroxyecdysone. The occurrence of the antifeedant 14, 15-dihydroajugapitin in the aerial parts of *A. iva* from Algeria was also shown (Bondi et al., 2000).

MATERIALS AND METHODES

1.MATERIALS

1.1. Biological material

Roots of *T. communis* (Tc) *C. caeruleus* (Cc) and shoots of *A. iva* (Ai) were collected in spring 2007, from Bouandas, Bougaa (W. Setif) and Biskra , respectively. authenticated by Dr Daniel Jeamonod, conservator, Geneva University Switzerland. A voucher specimen was deposited at the Laboratory of Biochemistry, Department of Biology, Faculty of Sciences, University Ferhat Abbas of Setif, Algeria. Leaves and roots were dried under shadow at room temperature, then powdered and stocked in darkness until use. Bovine milk and eggs were obtained from a local farm. Blood samples were obtained from healthy donors at university Hospital of Setif.

1.2. Chemicals

Bovine erythrocytes superoxide dismutase (SOD), dithiothreitol, Allopurinol, Horse heart Cytochrome c, NAD, Phenylmethylsulfonyl fluoride (PMSF), Xanthine, linoleic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), sodium dodecyl sulphate (SDS), *tert*-butyl hydroperoxide (*t*-BHP), Ferrozine, Phenazine methosulfate (PMS), different polyphenols; Rutin (Rut), Quercetin (Quer), Gallic acid (Gall A), Tween 40, Trichloroacetic acid (TCA), Ascorbic acid (Asc A), EDTA, β -Caroten, FeCl₃, 2,20-diphenylpicrylhydrazyl (DPPH), Thiobarbituric acid (TBA), Butylated hydroxytoluene (BHT) Nitroblue tetrazolium phenazine (NBT) wer purchased from Sigma (Germany), and all other reagents salts and solvents were obtained from Prolabo, Aldrich, and Organics. All common chemicals and solvents used were of analytical grade.

2.METHODES

2.1. Phenolic compounds Extraction

The extractions were carried out using various polar and non polar solvents, according to the method of Markham (1982). 100g of dried *Tamus communis* L roots (Tc) , *Carthamus caeruleus* L (Cc) roots and *Ajuga iva* L (Ai) shoots were ground in warring blender. They were mixed with a 10-20 volume of 85 % aqueous methanol (MeOH). The slurry was placed on shaker for 24 hours. The extracts were filtered through a Buchner funnel and the MeOH was removed on rotary evaporator (BÜCHI) to give crude extract (fraction labelled CE). The aqueous solution was extracted with hexane several times to eliminate lipids. The aqueous fraction was partitioned against chloroform to give chloroform extract (fraction labelled CHE). The remaining aqueous phase was exhaustively extracted with ethyl acetate (EtOAc) until the final EtOAc extract was colorless (fraction labelled EAE), the remaining aqueous extract was labelled AE. All the solvents were removed by evaporation under reduced pressure and the extracts were stored at -20 until use (Figure 15).

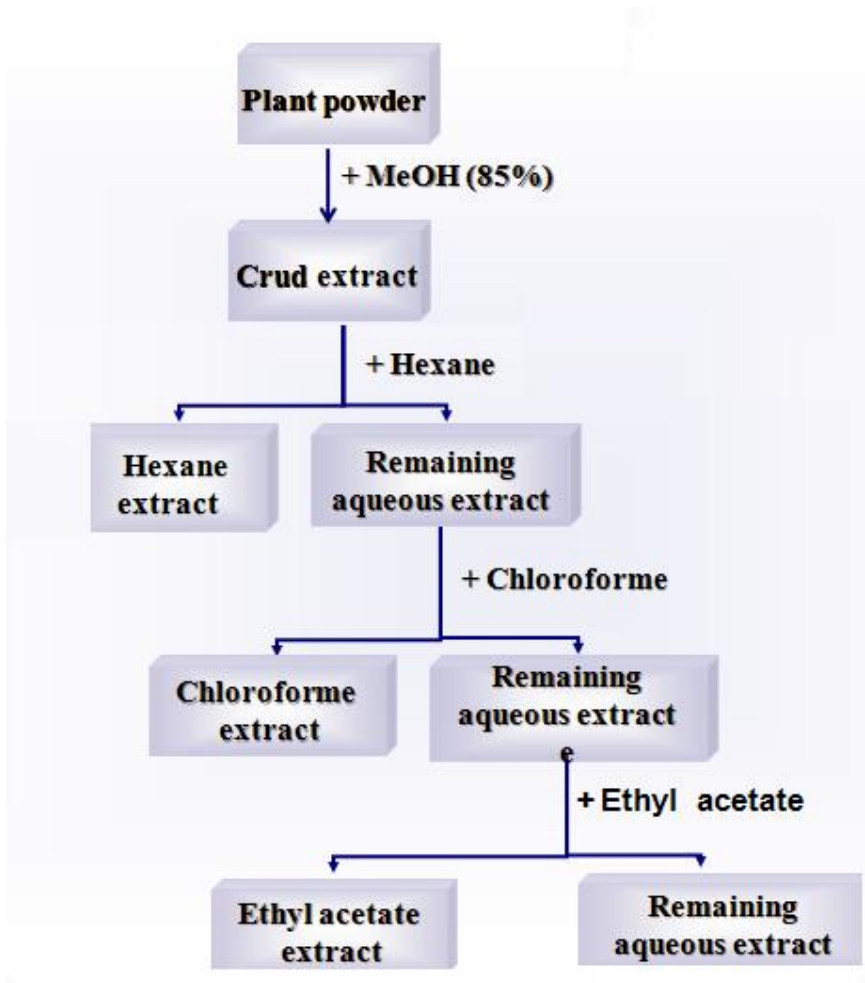


Figure 15. Different steps of phenolic compounds extraction.

2.2. Determination of total polyphenol

Total polyphenols were measured using Prussian blue assay methods described by Price and Butler (1977) modified by Graham (1992). Phenolics were expressed as gallic acid equivalents (Gal A Eq). Briefly 0.1 mL of Tc ,Cc root extracts and Ai shoot extracts are dissolved in methanol, 3 mL distilled water were added and mixed then 1 mL of $K_3Fe(CN)_6$ (0.016 M) was added to each sample, followed by the addition of 1 mL of $FeCl_3$ (0.02 M dissolved in 0.1 M HCl). It was immediately mixed using a vortex, after adding the reagents to the sample, 5 mL stabilizer (30 mL gum Arabic, 1 %; 30 mL H_3PO_4 , 85 % and 90 mL of

distilled water) were added to the sample and mixed. The absorbance was measured at 700 nm using a UV/VIS-8500 Techom spectrophotometer. The amount of total polyphenols in different extracts was determined from a standard curve of gallic Acid ranging from 0.00 to 200 µg / mL (Figure 16).

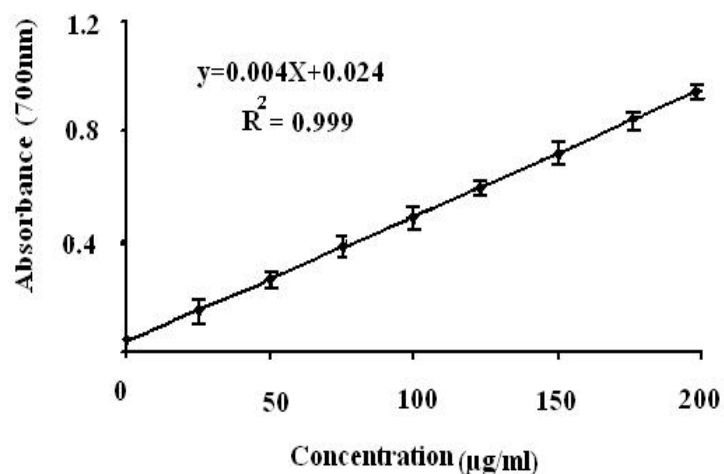


Figure 16. Standard curve of gallic acid for determination of total polyphenols in *T. communis*, *C. caeruleus* and *A. iva*. Each value represent mean \pm SD (n = 5).

2.3. Determination of flavonoid

Flavonoids were quantified using aluminium chloride reagent $AlCl_3$ (Baharun et al., 1996). Flavonoids were measured as quercetin equivalents (Quer-Eq). 1 mL of Tc, Cc and Ai samples are dissolved in methanol, then 1 ml of $AlCl_3$ (2 % in MeOH) was added, after incubation for 10 min, the absorbance was measured using a UV/VIS-8500 Techom spectrophotometer at 430 nm (Figure 17).

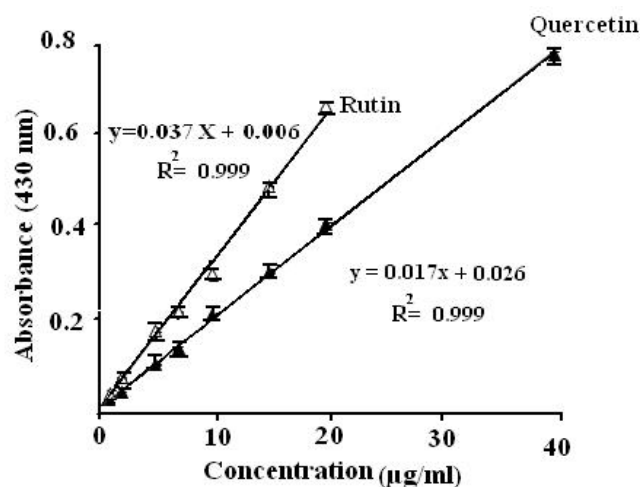


Figure 17. Standards curve of Rutin and Quercetine for determination of total flavonoids in *T.communis*, *C. caeruleus* and *A. iva*. extracts. Each value represent mean \pm SD (n=3).

2.4. Purification of milk xanthine oxidoreductase

XOR was routinely purified, in our laboratory from different mammalian milk, according to the previously described protocol for human milk (Sanders et al., 1997), with slight modification which consists of the omission of any supplementation to the raw milk and the addition of 10 mM DTT to the cream washing buffer (Baghiani et al, 2002; Baghiani et al, 2003). Briefly, the cream of fresh or thawed milk (1500ml) was collected by centrifugation at 5000 g (Sigma 3K30C centrifuge) for 20 min at 4 °C and all the following steps were carried out at 4 °C. The cream was resuspended in a double volume of 0.2 M K_2HPO_4 containing 1mM EDTA, 10mM DTT, 1mM sodium salicylate and 0.1mM PMSF, and stirred for 2 hours and centrifuged at 6000 g for 30min. The aqueous supernatant was collected, filtered through glass wool, and supplemented with 15% (v/v) chilled butanol. Ammonium sulphate (15% w/v) was slowly added to the mixture and left stirring for one hour and then centrifuged at 11.000 g for 30 min. The aqueous supernatant was filtered through glass wool. A further 20 % (w/v) ammonium sulphate was added slowly and allowed to stir for one hour. The brown pellet was collected by centrifugation for 30 min. at 13.000 g, and resuspended in heparin

buffer (25mM NaH₂PO₄/Na₂HPO₄, pH 6.2 containing 1mM EDTA) and dialysed overnight against the same buffer to remove excess ammonium sulfate. The remaining precipitate was discarded by centrifugation at 40000 g for one hour and followed by filtration of the supernatant through a 0.22 µm filter. The obtained filtrate is referred to as crud enzyme preparation.

Crud enzyme was further purified using a heparin chromatography. Heparin column was prepared by packing heparin immobilized on cyanogens bromide-activated 4 % cross-linked agarose in heparin buffer into a 1 cm x 10 cm column under gravity. The gel was allowed to settle before being washed with heparin buffer containing 1 M NaCl and equilibrated with heparin buffer (30 ml) at a flow rate of 10-15 ml / hour. Crud enzyme extract was slowly applied to the column at a flow rate of 10 ml/ hour. Unbounded proteins were washed off with heparin buffer. The gel was washed with heparin buffer containing 0.02 M NaCl to elute weakly bound proteins. The enzyme was then eluted in heparin buffer containing 0.1 M NaCl. Fractions were collected and those containing the enzyme were pooled and dialysed against Na-bicine buffer (50 mM), pH 8.3, for overnight and stored at -20°C.

2.4.1. Enzyme concentration estimation

Purified XOR concentration was estimated using an extinction coefficient of $\epsilon=36000 \text{ M}^{-1}\text{cm}^{-1}$ at 450 nm using $\Delta A = \epsilon \cdot l \cdot C$ (Beer-Lambert law) (Bray, 1975).

2.4.2. Protein / Flavin ratio (PFR) Calculation

The purity of enzyme was assessed by protein / flavin ratio ($\text{PFR} = A_{280} / A_{450}$) (Bray, 1975). PFR is the ratio of $A_{280\text{nm}} / A_{450\text{nm}}$ of the sample. An enzyme sample with a PFR value around 5 is widely accepted to be pure.

2.4.3. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out, using vertical slab gels according to the method of Laemmli (1970). The separating gel was made up of distilled H₂O, 10 % (w/v) acrylamide [9.75 % (w/v) acrylamide, 0.25 (w/v) N,N'-methylene bisacrylamide] in 0.375 M Tris-HCl, pH 8.8, containing 0.1 % (w/v) SDS, polymerised by the addition of 0.4 µl/ml TEMED and 0.1 % ammonium persulphate. The stacking gel consisted of distilled H₂O, 5 % (w/v) acrylamide [4.876 % (w/v) acrylamide, 0.124 % (w/v) N,N'-methylene bisacrylamide] in 0.125 M Tris-HCl, pH 6.8 and 0.1 % (w/v) SDS, polymerised by the addition of 1 µl/ml TEMED and 0.1 % (w/v) ammonium sulphate was layered onto the separating gel. The samples were mixed with reducing sample buffer [62 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 5 % β-mercaptoethanol, 0.1 % (w/v) bromophenol blue] in a ratio of 1:1 to 3:1 depending on sample concentration. The samples were boiled for 5min before loading onto separate wells in the stacking gel. Molecular weight markers (MW-SDS-200 Kit, Sigma) were treated in the same way. The gels were run at 70 Volts until the samples had entered the stacking gel in a tank containing running buffer [25 mM Tris-HCl, pH 8.3, containing 0.192 M glycine and 0.1 % SDS] than at 200 Volts. They were then fixed and stained using 0.1 % (w/v) Coomassie Brilliant Blue R250 in 45 % (v/v) methanol and 10 % (v/v) acetic acid. The gels were destained in 5 % (v/v) methanol and 7.5 % (v/v) acetic acid.

2.4.4. Xanthine oxidase activity

xanthine oxidase was determined at 295 nm (UV/VIS-8500 Techom spectrophotometer), by measuring the oxidation rate of xanthine to uric acid, using an absorption coefficient of 9600 M⁻¹ cm⁻¹ (Avis et al., 1956). Assays were performed at room temperature, in 1 cm width quartz cuvette, in air-saturated 50 mM Na-bicine, pH 7.4, containing 100µM xanthine as reducing substrate and 500 µM NAD as electron acceptor.

2.4.5. Superoxide (O_2^-) production

Superoxide production was determined in terms of superoxide-dismutase-inhibitable reduction of cytochrome c (from horse heart, Sigma), monitored at 550 nm and calculated by using an extinction coefficient for reduced cytochrome c of $21000\text{ M}^{-1}\text{cm}^{-1}$ (Massey, 1959). The assays were carried out at room temperature in air-saturated 50 mM sodium phosphate, pH 7.4 containing 0.1 mM EDTA, in the presence of 25 μM cytochrome c and 100 μM of reducing substrate (xanthine).

2.5. Effects of Tc, Cc and Ai extracts on the generation of O_2^- radicals

Anti-radical activity was determined spectrophotometrically according to Robak and Gryglewski (1988), by monitoring the effect of Tc, Cc and Ai extracts on superoxide anion radicals produced by xanthine/xanthine oxidase system. These radicals are able to reduce cytochrome c. The reaction mixture contained xanthine (100 μM), horse heart cytochrome c (25 μM), in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1mM EDTA and various concentrations of Tc, Cc and Ai extracts. All concentrations indicated are final ones. The reactions were started by addition of XO. Within 2 min, reduced cytochrome c was spectrometrically determined at 550 nm against enzyme-free mixture using a UV/VIS-8500 Techom spectrophotometer. The cytochrome c activity was calculated using an absorption coefficient of $21.100\text{ M}^{-1}\text{cm}^{-1}$, and the sensibility of the reaction was determined by using bovine erythrocytes superoxide.

2.6. Effects of Tc, Cc and Ai extracts on Xanthin Oxidase activity

The effect of Tc, Cc and Ai on the xanthine oxidation was examined spectrophotometrically at 295 nm (UV/VIS-8500 Techom spectrophotometer) following the production of uric acid using an absorption coefficient of $9600\text{ M}^{-1}\text{cm}^{-1}$ (Avis et al., 1956). Assays were performed

at room temperature, in presence of final concentration of 100 μ M of xanthine, in air saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1 mM EDTA, with various amounts of plants extracts dissolved in MeOH. Control experiments revealed that solvent didn't influence the activity of XOR at this concentration. The reaction was started by the addition of XO (1176 nmol of urate /min/mg protein) for Enzyme activity of the control sample was set to 100 % activity. The percent inhibition was calculated by using the following formula.

$$\text{The inhibition (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

2.7. Measurement of superoxide anion scavenging activity

The superoxide scavenging ability of the Tc, Cc and Ai extracts was assessed by a modified method (Nishikimi et al., 1972). Superoxide anions were generated in samples that contained 100 μ l each of 1 mM NBT, 3mM NADH and 0.3mM PMS and the final volume was adjusted to 1ml with 0.1M phosphate buffer (pH 7.8) at ambient temperature. The reaction mixture (NBT and NADH) was incubated with or without extracts at ambient temperature for 2 min and the reaction was started by adding PMS. The absorbance at 560 nm was measured, using a UV/VIS-8500 Techom spectrophotometer, against blank samples for 3 min. Decrease in absorbance in the presence of various plants extracts indicated superoxide anion scavenging activity. The percent scavenging activity was calculated by using the following formula.

$$\text{Superoxide scavenging activity (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

2.8. Evaluation of RBC Hemolysis

Blood (10-20 ml) was obtained from healthy volunteers. Human erythrocytes from blood were isolated by centrifugation (Sigma 3K30C centrifuge) at 3000g for 10 min and washed four times with phosphat buffer, and then re-suspended using the same buffer to the desired hematocrit level. Cells stored at 4°C were used within 6 h of sample preparation. In order to induce free-radical chain oxidation in erythrocytes, Human red blood cells (RBC) suspensions (2% hematocrit) were treated with *tert*-butyl hydroperoxide (*t*-BHP) (500 μ M final concentration) (Manna et al, 1999). At the end of 2 h of incubation, the extents of hemolysis were evaluated as described below. To assay the antioxidant protective effect on RBC from oxidative injury, the cells were pre-treated for 15 min, in the absence or presence of 2 mg /ml of each extract of Tc, Cc and Ai or antioxidant, before the induction of oxidative stress. After the oxidative treatment, samples were centrifuged at 1500g for 10 min, and the absorption (*A*) of the supernatant (*S1*) at 540 nm, using UV/VIS-8500 Techom spectrophotometer, was measured. The precipitates (packed RBC) were then haemolysed with 40 volumes of ice-cold distilled water and centrifuged at 1500g for 10 min. The supernatant (*S2*) was then added to *S1* and the absorption (*B*) of the combined supernatants (*S1* + *S2*) was measured at 540 nm; the percentage of haemolysis was calculated from the ratio of the readings (*A/B*) x 100.

2.9. Thiobarbituric acid-reactive substances (TBARS) assay

A modified thiobarbituric acid-reactive species (TBARS) assay (Ohkowa et al., 1979) was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid rich media (Ruberto et al., 2000). Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm.

Egg homogenate (0.5 ml of 10% v/v) and 0.1 ml of various concentration Tc, Cc and Ai extracts were added to a test tube and made up to 1 ml with distilled water, 0.05 ml of FeSO₄ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 ml 20% Trichloroacetic acid (TCA) were added and the resulting mixture was vortexed then heated at 95 C for 60 min. To eliminate this non-MDA interference, another set of samples was treated in the same way, incubating without TBA, to subtract the absorbance. After cooling, 5.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm using a UV/VIS-8500 Techom spectrophotometer. Inhibition of lipid peroxidation (%) by the extract was calculated according to;

$$\text{Inhibition of lipid peroxidation (\%)} = \frac{1 - E}{C} \times 100$$

Where; C is the absorbance value of the fully oxidised control and E is:

$$(\text{Abs}_{532+\text{TBA}} - \text{Abs}_{532-\text{TBA}}).$$

2.10. β -Caroten-Linoleic acid

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β -carotene-linoleic acid mixture was prepared as follows: 0.5 mg β -carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 μ l linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen (30 min 100 ml/min) were added with vigorous shaking. 2500 μ L of this reaction mixture

were dispensed into test tubes and 350 µl of the Tc, Cc and Ai extracts, prepared at 2 g/l concentrations, were added and the emulsion system was incubated for 48 h at room temperature. The same procedure was repeated with synthetic antioxidant, BHT, as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm using a UV/VIS-8500 Techom spectrophotometer. Antioxidative capacities of the extracts were compared with those of BHT and blank.

2.11. DPPH assay

The hydrogen atom or electron donation abilities of Tc, Cc and Ai extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,20-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits et Bucar, 2000; Cuendet et al., 1997). 50µl of various concentrations of the extracts in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm using a UV/VIS-8500 Techom spectrophotometer. Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.12. Ferrous ion chelating activity

Ferrous ion chelating activity was measured by inhibition of the formation of iron (II)-ferrozine complex after treatment of various concentration of Tc,Cc and Ai extracts with Fe^{2+} , following the method of Decker and Welch (1990). The reaction mixture (1.50 ml) contained 500 μl test material or EDTA, 100 μl FeCl_2 (0.6 mM in water) and 900 μl MeOH. The control contained all the reaction reagents except the extract and EDTA. The mixture was shaken well and allowed to react at room temperature for 5 min; 100 μL of ferrozine (5 mM in methanol) was then added. The absorbance of the Fe^{2+} -ferrozine complex was measured at 562 nm, using a UV/VIS-8500 Techom spectrophotometer, against a MeOH blank. The chelating effect was calculated as a percentage, using the equation below, an IC_{50} value defined as the effective concentration of test material which produces 50% of maximal scavenging effect.

$$\text{Chelating effect (\%)} = \left(1 - \frac{A_{\lambda_{562 \text{ nm}}}^{\text{Sample}}}{A_{\lambda_{562 \text{ nm}}}^{\text{Control}}} \right) \times 100$$

2.13. Ferric reducing ability of plasma assay (FRAP)

The antioxidant capacity of each sample was estimated according to the procedure described by Benzie and Strain (1996) with some Modifications (Pulido et al., 2000).

Briefly, 900 μL of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with 90 μL of distilled water and 30 μL of test sample (Benzie and Strain, 1996). Readings at the absorption maximum (593 nm), using a UV/VIS-8500 Techom spectrophotometer, were taken every 15s, the reaction monitored for up to 30 min. Methanolic solutions of known $\text{Fe}(\text{II})$ concentrations in the range of 100-2000 $\mu\text{mol/L}$ ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were used for calibration. (Figure 18).

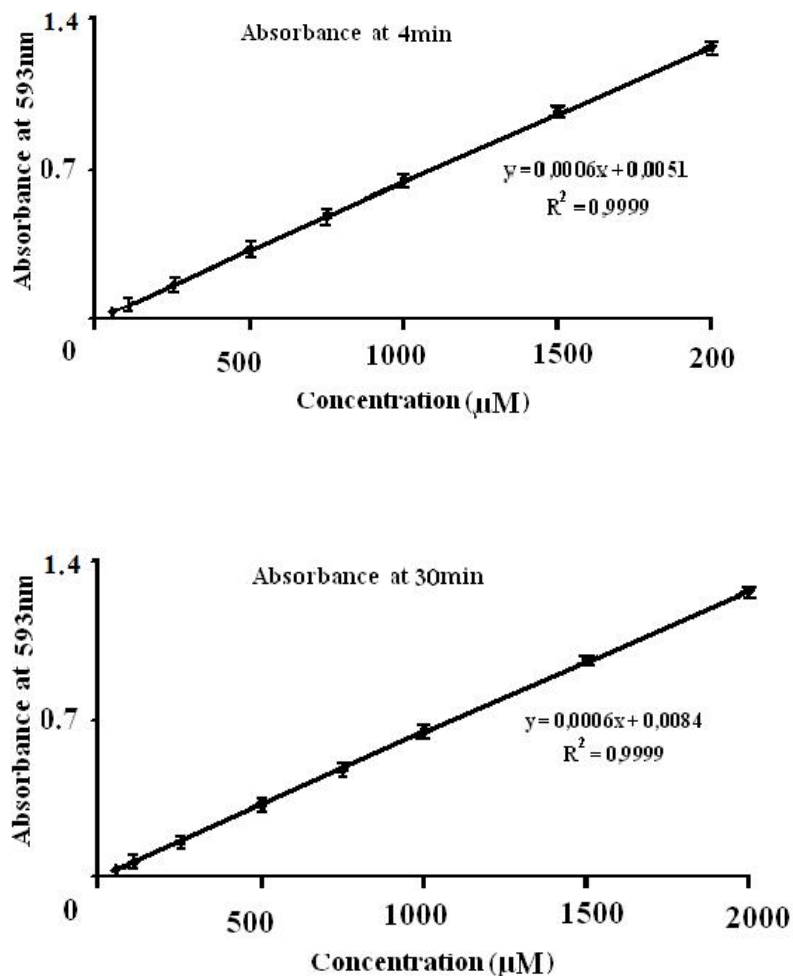


Figure 18. FeSO₄·7H₂O Calibration Curves at 4min and 30min for determination of Equivalent Concentration 1 (EC1) of *T.communis*, *C. caeruleus* and *A. iva*. extracts. Each value represent mean ± SD (n=3)

The parameter *Equivalent Concentration 1* or EC1 was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L FeSO₄·7H₂O. EC1 was calculated as the concentration of antioxidant or extract giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/L concentration of Fe (II) solution determined using the corresponding regression equation (Figure 18).

2.14. Statical analysis

All determinations were conducted in triplicate or more and all results were calculated as mean \pm standard deviation (SD). In this study Statistical analysis was performed using Student's t-test for significance and analysis of variance (ANOVA) followed by Dunnet's test were done for the multiple effects comparison of the different extracts. The P-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

1. Extraction and Determination of total polyphenol contents

Plants extracts preparations of *T. communis* (Tc), *C. caeruleus* (Cc) and *A. iva* (Ai) were carried out using various polar and non polar solvents (Markham, 1982). The most commonly used solvent is methanol (Chanda and Dave, 2009). According to the method, dried plant material was ground in warring blender. The powder was mixed with 10-20 volume of 85 % aqueous methanol (MeOH). The crud extract (CE) has been split by a series of extractions with solvents of increasing polarity; hexane, Chloroform, Ethyl acetate respectively, this procedure allowed their separation in to three subfractions; chloroform, ethyl acetate and aqueous extracts, which labelled CHE, EAE, AE, respectively. The estimate of yields in relation to the total powder weight (100g) of plants, shows that the CE of different plants has the high yield which arrange from 8.172 ± 0.327 to $12.16 \pm 1.471\%$ followed by AE with 6.508 ± 0.802 to $9.24 \pm 0.495\%$ while the other extracts displayed a lower yields with rat varied from 0.210 ± 0.016 to $1.253 \pm 0.370\%$ (Table2).

Table 2. The yields of extraction of *T. communis*, *C. caeruleus* and *A. iva*.

%Yield of extracts	Tc	Cc	Ai
CE	8.172 ± 0.327	10.91 ± 1.338	12.16 ± 1.471
CHE	0.573 ± 0.0639	0.661 ± 0.129	1.253 ± 0.370
EAE	0.210 ± 0.016	0.715 ± 0.069	0.897 ± 0.117
HEX	0.276 ± 0.064	0.390 ± 0.057	0.94 ± 0.062
AE	6.508 ± 0.802	9.24 ± 0.495	7.79 ± 0.520

Each value represents the mean \pm SD (n = 3).

It is well known that polyphenols are widely distributed in plants, they are sometimes present in high concentrations (Harborne, 1993), especially in medicinal plant and many edible plants (Hagerman et al., 1998). Flavonoids and phenolic compounds are the main antioxidative compounds of fruits and vegetables (Huang et al, 1998), which exhibit considerable free radical scavenging activities, through their reactivity as hydrogen or electron-donating agents, and metal ion chelating properties (Rice-Evans et al, 1996). The biological properties, scavenging and antioxidant activities of flavonoids have been extensively reviewed (Frankel et al, 1993).

In this regard the Tc, Cc and Ai extracts phenolic content was investigated using Prussian blue assay (Graham, 1992), and $AlCl_3$ method described by Bahorun and al (1996). Total phenolic contents were expressed as mg gallic acid equivalents per gram dry weight (mg GA-Eq/g) and total flavonoids contents as mg quercetin equivalents per gram dry weight (mg Q-Eq/g) (Table3: A, B).

There was a wide range of phenol concentration in different extracts. The value varied from 39.54 ± 0.53 to 208.166 ± 3.12 , 12.66 ± 0.27 to 38.791 ± 0.587 and 20.125 ± 2.251 to 42.979 ± 1.6 mg GA-Eq/g lyophilisate for Tc, Cc and Ai extracts, respectively (Table. 3A). The highest level of polyphenols were recorded in the Tc extracts especially in CHE and EAE followed by Ai extracts which EAE exhibited a value higher than those of CE and CHE, while total polyphenol levels were particularly low in CE of Cc, however CHE and EAE, were approximately similar (Table 3. A). Flavonoids were quantified using $AlCl_3$ method described by Bahorun and al (1996). Quercetin (quer) and Rutin (Rut) (Figure. 17) are used as standards. The flavonoids content in the three plants were varied from 0.42 ± 0.02 to 12.48 ± 0.69 mg Q-Eq/g lyophilisate. In general flavonoids levels haven't exhibited a great difference among various plant extracts (Table. 3B).

Table 3. Total phenolic^A and flavonoid content^B of *T. communis*, *C. caeruleus* and *A. iva* extracts.

Extracts	Total phenolic content mg gallic acid equivalents/g dried extract		
	Tc	Cc	Ai
CE	39,54 ± 0.53	12.66 ± 0.27	20.125 ± 2.251
CHE	208.166 ± 3.12	36.64 ± 0.732	30.46 ± 3.72
EAE	100.25 ± 1.29	38.791 ± 0.587	42.979 ± 1.6

Extracts	Total flavonoïdes content					
	mg quercetin equivalents/g dried extract			mg Rutin equivalents/g dried extract		
	Tc	Cc	Ai	Tc	Cc	Ai
CE	0.402 ± 0.02	0.7 ± 0.08	5.33 ± 1.02	19.35 ± 0.030	3.44 ± 0.19	10.24 ± 1.07
CHE	5.16 ± 0.17	1.82 ± 0.407	12.48 ± 0,69	15.25 ± 0.678	18.50 ± 0.02	23.42 ± 1.93
EAE	4.17 ± 0.40	2.22 ± 0.22	7.07 ± 0,91	12.79 ± 0.687	13.73 ± 0.02	13.38 ± 0.87

Each value represents the mean ± SD (n = 3).

The higher flavonoid content were recorded in Ai extracts followed by EAE and CHE of Tc then those of Cc, however the lowest value was found in CE of TC and Cc, respectively (Table. 3B).

2. Xanthine oxidase purification

Using Nakamura and Yamazaki (1982) procedure modified by Baghiani et al, (2003). bovine milk was found to yield around 23.21 mg XOR protein per litre milk, comparable to the amounts reported by Baghiani et al (2002; 2003), the obtained enzyme was largely (more than 90%) under the oxidase form as shown in (Table. 4).

Table 4. Purification of xanthine oxidoreductase (XOR) from bovine milk

Specific activity (nmol/min/mg of enzyme)		XO form (%)	Protein / Flavin Ratio (PFR)	yield (mg of enzyme/liter of milk)
XDH + XO	XO form			
2055.05	1988.55	91.89	5.15	23.21

Enzymatic activity was assessed throughout in term of production of uric acid from xanthine (100 μ M). Type XDH + OX and type OX activities were determined in air saturated 50 mM of Na-bicine buffer, pH 8.3, at room temperature, respectively in the presence and absence of 500 μ M NAD⁺.

The freshly purified bovine milk XOR showed an ultraviolet / visible spectrum with three major peaks at 280, 325, 450 nm, with protein to flavin ratio (A_{280}/A_{450}) (PFR) of 5.15 indicating a high degree of purity (Bray, 1975). Run on SDS-PAGE purified enzymes showed one major band of approximately molecular weight of 150 KDa and traces of degradation bands appeared on storage (Figure. 19). This is analogous to the well studied bovine (Bray, 1975), human (Abadeh et al., 1992) and Camel XOR (Baghiani et al., 2003). The activity of XOR was spectrophotometrically determined by measuring the production of uric acid from xanthine (100 μ M, final concentration) in the presence and absence 500 μ M NAD⁺ at 295 nm, using an absorption coefficient of 9600 M⁻¹ cm⁻¹ (Avis et al, 1956) (Table.4).

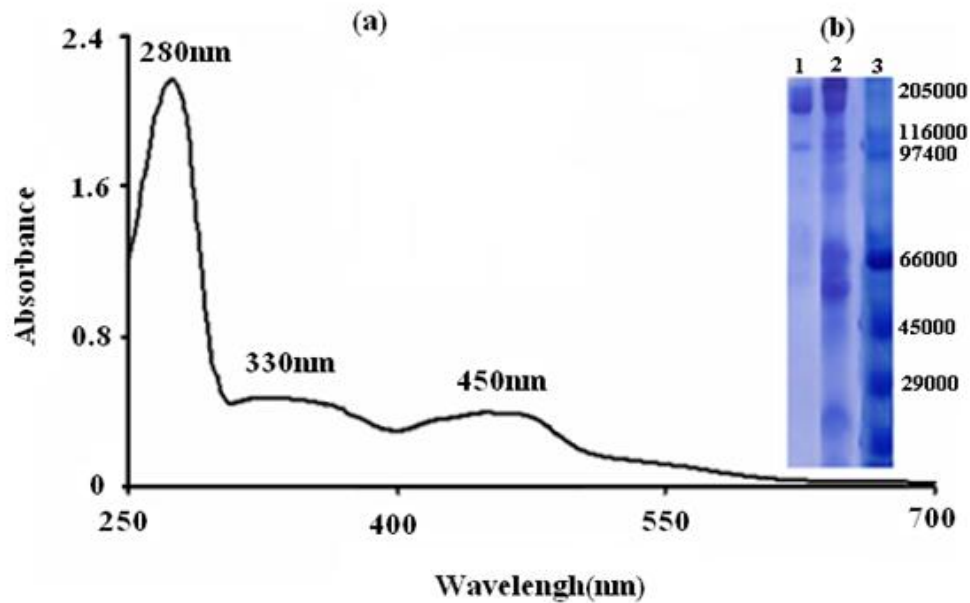


Figure 19. UV/Visible absorbance spectrum of BXOR (a) 10% SDS-PAGE of XOR preparation (b). Conditions were as described in materials and methods. Lan1: purified bovine milk XOR; Lan2: crud bovine milk XOR and Lan3: molecular weight markers; Myosin 205 000; β -galactosidase 116 000; Phosphorylase 97 400; Serum albumin 66 000; Ovalbumin 45 000; Carbonic anhydrase 29 000.

3. Effects of TC, Cc and Ai extracts On the generation of $O_2^{\cdot-}$ radicals by XOR

T. communis, *C. caeruleus* and *A. iva* are traditionally recognised in folk medicine plants, mostly used in the treatment of many pathologies in North Africa. This test, examined $O_2^{\cdot-}$ radicals scavenger effects of Tc, Cc and Ai extracts. Cytochrome c^{3+} (Cyt C^{+3}) has been extensively used for the detection of $O_2^{\cdot-}$ produced in biological systems due to its fast superoxide-mediated reduction to cytochrome C^{2+} (Cyt C^{+2}) (McCord et Fridovich, 1968). The effect of Tc, Cc and Ai extracts at different concentrations were studied for their ability to scavenge $O_2^{\cdot-}$ generated by the xanthine/xanthine oxidase system. The amount of generated $O_2^{\cdot-}$ was determined by measuring the reduction of Cyt C. Under our experimental conditions the activity of Cyt C, in the absence of extracts, reduced by $O_2^{\cdot-}$ generated from XO was 2135.91 nmols/min/mg protein.

The reduction of Cyt C⁺³ was almost totally inhibited by SOD (330 U/mL). The results demonstrated that all the extracts were able to inhibit Cyt C⁺³ activity ($P \leq 0.05$). This scavenging effect was found to increase with increasing concentration of *Tc*, *Cc* and *Ai* extracts (Figure.20).

The most potent scavengers of superoxide anion radical were observed for CE of *Tc* followed by that of *Cc* with IC_{50} (μ M Q-E) of 0.163 ± 0.0013 and 1.297 ± 0.162 respectively ($p \leq 0.01$), then EAE of *Tc* which gave an $IC_{50} = 1.992 \pm 0.15$, however *Ai* extracts showed the lowest effect in compared with previous extracts ($p \leq 0.05$), meanwhile their effect still efficient and significant compared with control ($p \leq 0.01$). In general *Tc* extracts were the most potent followed by *Cc* then *Ai* extracts ($p \leq 0.05$) (Figure. 21).

Xanthine oxidase activity, has been reported to increase during oxidative stress (Adkins and Taylor, 1990; Matsumura et al., 1998), this enzyme is considered to be an important biological source of superoxide radicals which has been linked to post-ischaemic tissue injury and edema (Hearse et al, 1986; Matsumura et al., 1998). These and other reactive oxygen species (ROS) contribute to the oxidative stress on the organism and are involved in many pathological processes such as inflammation, atherosclerosis, cancer, aging, etc.

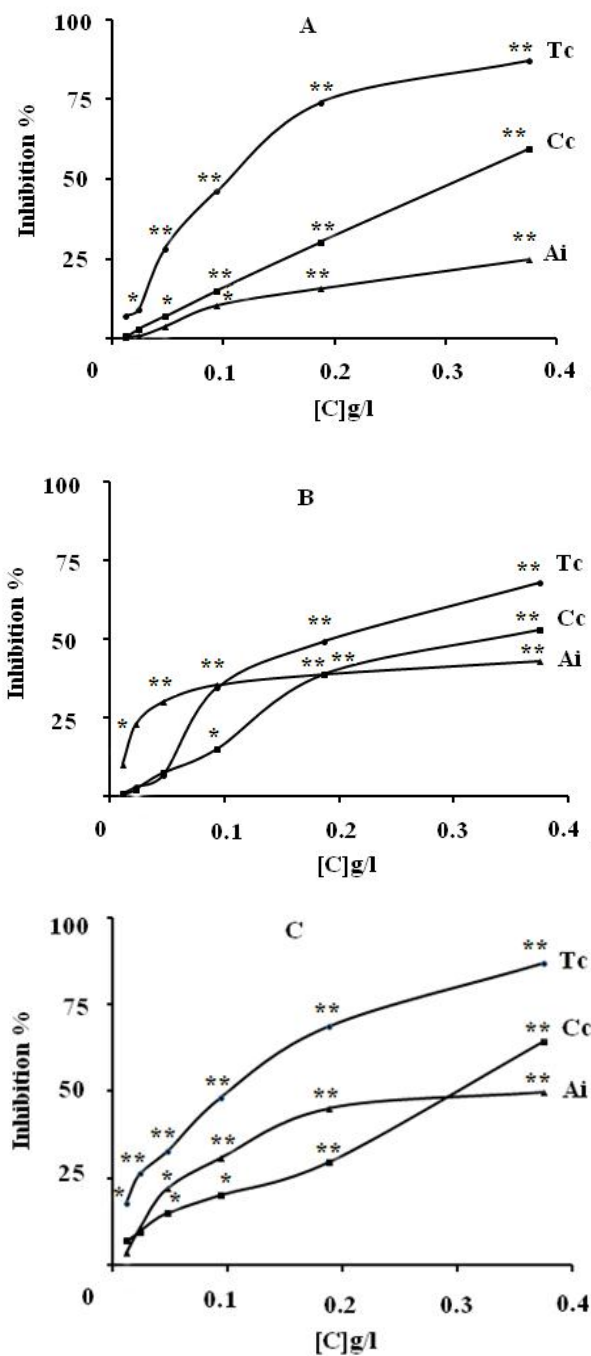


Figure 20: Inhibition of the superoxide anion free radical generation from xanthine/xanthine oxidase by *T. communis*, *C. caeruleus* and *A. iva* extracts as measured by the cytochrome C test. Curve **A** represent crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts (** $p \leq 0.01$; * $p \leq 0.05$). Results are expressed as the percentage of activity inhibition compared with control where no extract was added. Each value is represented as mean \pm S.D (n = 3).

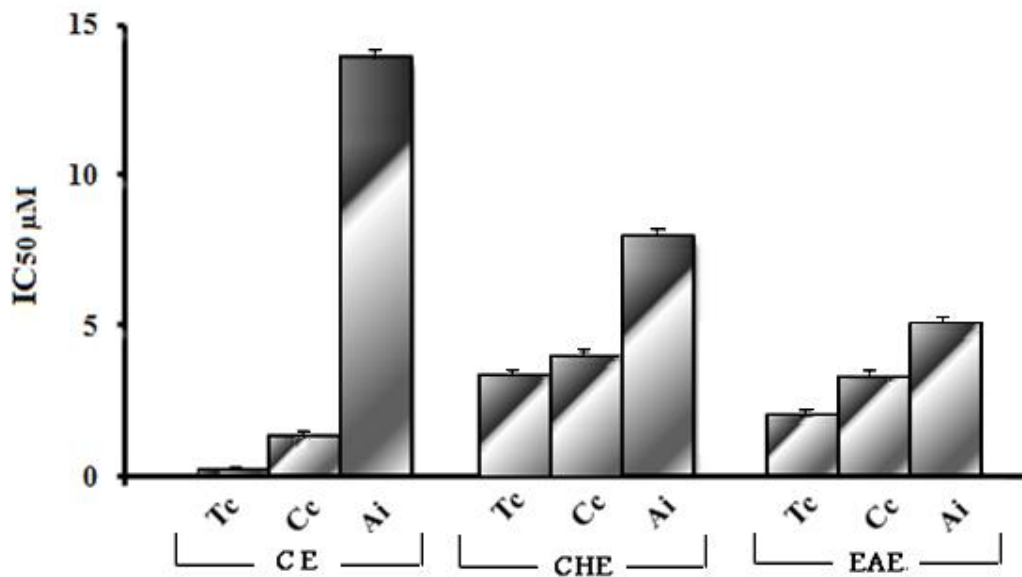


Figure 21. Comparison of inhibitory concentration of *T. communis*, *C. caeruleus* and *A. iva* extracts for 50% of Cyt C activity which reduced by $O_2^{\cdot-}$ generated from XO. Amount of superoxide anion radicals in the control sample without extract was set to 100%. Values are mean \pm SD of three independent experiments. The effects of extracts on Cyt C activity were compared with SOD.

To study the possibility that the TC, Cc and Ai extracts suppressed the rate of conversion of xanthine to uric acid and to account for this $O_2^{\cdot-}$ scavenging ability, the effect of all extracts on the XO activity were checked.

4. Effects of TC, Cc and Ai extracts on Xanthine Oxidase activity

In order to prove the previous scavenger effects of Tc, Cc and Ai extracts on $O_2^{\cdot-}$ generated by XO, the effects of these extracts on XO activity was checked. Using the initial concentrations which gave scavenging effects, we observed significant inhibition of XO activity ($p \leq 0.05$). The results demonstrated that all the extracts were effective in inhibiting the uric acid production in a concentration dependent manner. This effect was compared with allopurinol, clinically used as XO inhibitor (Figure. 22).

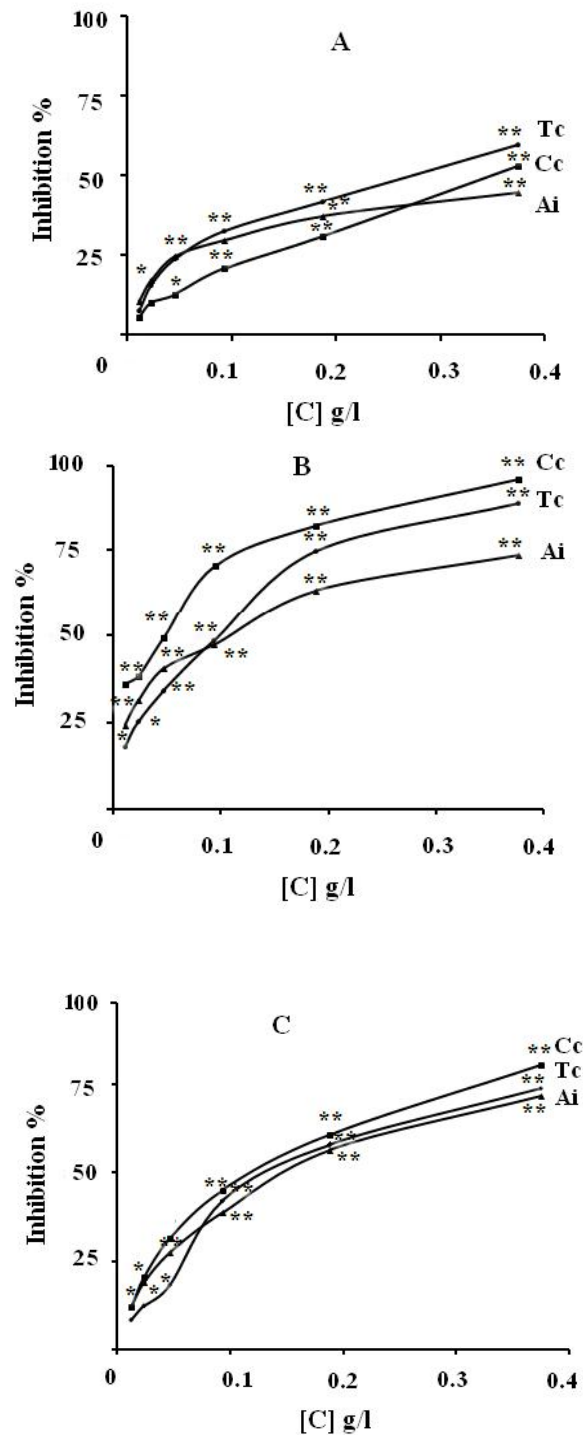


Figure 22. Inhibitory actions of *T. communis*, *C. caeruleus* and *A. iva* extracts on xanthine oxidase activity. Different concentrations of extracts were incorporated in the assays. Results are expressed as percentage of control where no inhibitor was added. Each value is represented as mean \pm S.D (n = 3). Curve **A** represent crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts (** p \leq 0.01; * p \leq 0.05)

All the extracts exhibited a great effect on the XO activity which was more efficient than allopurinol ($IC_{50} = 57.117 \pm 1.093 \mu M$) with approximately 9 to 186 folds (Figure. 23). The concentrations of these extracts required to inhibit 50% of the inhibitable xanthine oxidase activity (IC_{50}) indicated that the highest effect was for CHE of Cc with IC_{50} (μM Q-E of extract) of $0,307 \pm 0, 0106$ followed by those of Tc and Ai with 7 and 17 folds respectively.

The effect of CE of Tc ($IC_{50} = 0.421 \pm 0.0125$) was greater than those of Cc and Ai with approximately 3.5 and 14 folds respectively, however EAE of Cc showed the highest inhibitory effect with IC_{50} of $0,692 \pm 0,192$ followed by those of Ai then TC which were too close to each other (Figure. 23). In general Cc extracts were more effective than those of Tc and Ai ($p \leq 0.05$), nevertheless Tc and Ai extract were very effective in compared with allopurinol which gave IC_{50} of $57.114 \pm 1.093 \mu M$ ($p \leq 0.01$).

Xanthine oxidase-derived superoxide anion has been linked to post-ischaemic tissue injury and edema (McCord and Fridovich, 1969). Further, Studies have shown that XO inhibitors may be useful for the treatment of hepatic disease and gout, which is caused by the generation of uric acid and superoxide anion radical (Lin et al, 2001). Allopurinol is an allosteric xanthine oxidase inhibitor, which can decrease the damaging effect of xanthine oxidase in radical- mediated diseases, but the use of allopurinol is known to have many side effects (Hamanaka et al., 1998). The most common adverse effects of allopurinol are gastrointestinal distress, hypersensitivity reactions, and skin rash (Bieber and Terkeltaub, 2004; Schlesinger, 2004; Pea, 2005).

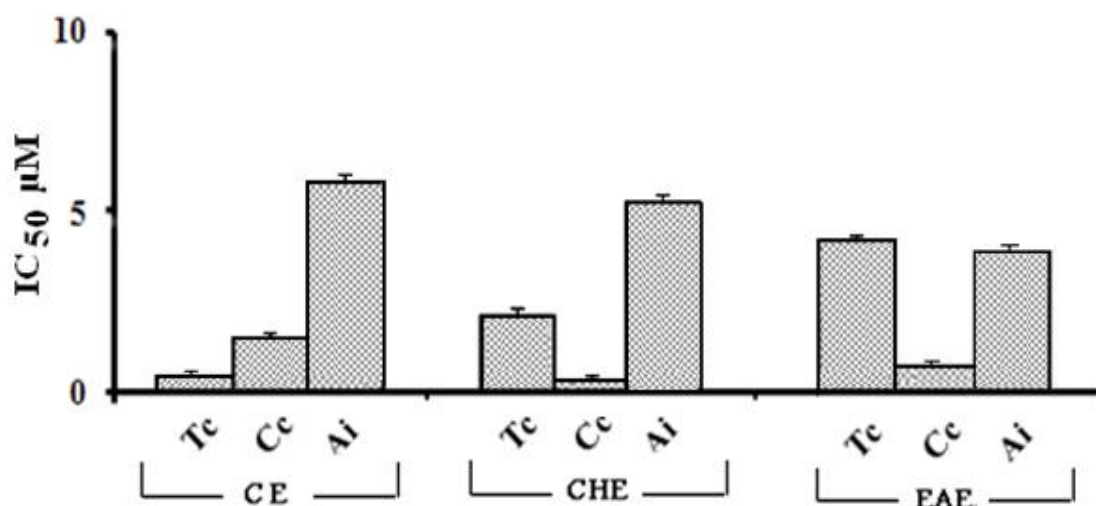


Figure 23. Relationship among inhibitory concentration of *T. communis*, *C. caeruleus* and *A. iva* extracts for 50 % of xanthine oxidase activity as measured spectrophotometrically at 295 nm by the production of uric acid. XO was added to xanthine (100 μ M) preincubated with extract for 2 min. Enzyme activity of the control sample without extract was set to 100%. The concentration of extracts is expressed as μ M quercetin equivalent of extract in the reaction mixture. Each value is represented as mean \pm S.D (n = 3).

The hypersensitivity reaction may occur even after months or years of medication. These effects generally occur in individuals with decreased renal functions. (Bieber and Terkeltaub, 2004; Schlesinger, 2004; Pea, 2005). Allopurinol may inhibit the metabolism of oral coagulants. The symptoms of allopurinol toxicity include fever, rash, vasculitis, eosinophilia, and worsening of renal function, which can lead to a fatal outcome especially in elderly patients with renal insufficiency (Bieber and Terkeltaub, 2004; Schlesinger, 2004; Pea, 2005). Thus, a prompted search for new XO inhibitors that are structurally distinct from pureness is a necessity (Borges et al., 2002). Hence, phytochemicals or extracts, which inhibit XO and the superoxide anion regeneration by the enzymatic pathway, would be beneficial in preventing these side effects.

5. Evaluation of inhibition and scavenging effects of Tc, Cc and Ai extracts

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation (Benavente-Garcia, 1997). Thus Both inhibition of XO and the scavenging effect on the superoxide anion were measured. Inhibition of xanthine oxidase results in a decreased production of uric acid and superoxide. For each extract tested two IC_{50} values; 50% inhibition of XO (50% decrease of uric acid production) and 50% reduction of the superoxide level. The half-maximal inhibitory concentrations of the extracts for xanthine oxidase inhibition and reduction of the superoxide level are illustrated in (Figure. 24).

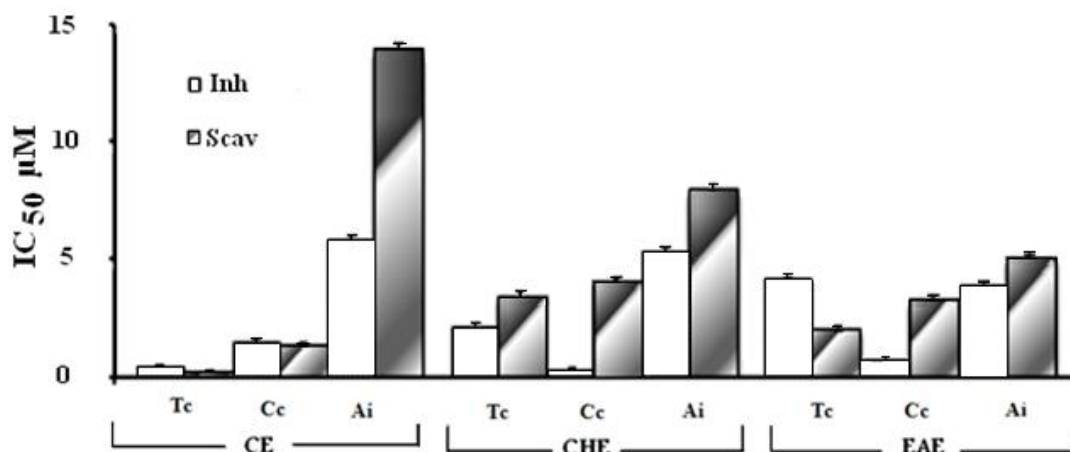


Figure 24. Evaluation of *T. communis*, *C. caeruleus* and *A. iva* extracts as inhibitors of xanthine oxidase and as scavengers of superoxid produced by the action of XO enzyme. Each value is represented as mean \pm S.D (n = 3).

Cos et al (1998) have classified phenolic compound into six groups according to their effect on XO and as superoxide scavengers, which can be useful in the search for better compounds against gout than, the widely used drug, allopurinol (category B). These six categories are

superoxide scavengers without inhibitory activity on xanthine oxidase (category A), xanthine oxidase inhibitors without any additional superoxide scavenging activity (category B), xanthine oxidase inhibitors with an additional superoxide scavenging activity (category C), xanthine oxidase inhibitors with an additional pro-oxidant effect on the production of superoxide (category D), flavonoids with a marginal effect on xanthine oxidase but with a prooxidant effect on the production of superoxide (category E), and finally, flavonoids with no effect on xanthine oxidase or superoxide (category F). The result illustrated in figure 24 showed that the CE of Tc possesses the highest activities in both $O_2^{\cdot-}$ scavenging and XO inhibition. In other hand EAE of the same plant have an IC_{50} values for the scavenging of the superoxide level lower than that for the inhibition of xanthine oxidase. According to Cos et al (1998) the classification indicating that CE and EAE extracts of Tc show inhibition of xanthine oxidase and possesses an additional superoxide-scavenging activity (category C). However CE of Cc has approximately the same IC_{50} for scavenging of the superoxide level and for the inhibition of XO which means that the rate of uric acid reduction equals the rate of superoxide reduction, this indicating that CE can be classified into category B, which considered as pure inhibitors of xanthine oxidase, without any additional superoxide-scavenging activity in agreement with Costantino et al (1992) finding, which reported that polyphenolic crude extracts are known to have a certain inhibitory activity towards xanthine oxidase. Meanwhile all the other extracts exhibited a IC_{50} levels of superoxide scavenging higher than those for uric acid reduction, The relative percentage of $O_2^{\cdot-}$ detected is higher than the relative uric acid level, and the superoxide curve is situated above the uric acid curve, this is the case of a xanthine oxidase inhibitor with a pro-oxidant effect, generation of superoxide radicals during uric acid production (category D).

The potent capacity in inhibition of XO and $O_2^{\cdot-}$ scavenging activity exhibited by Tc, Cc and Ai extracts mainly CE and EAE extracts of Tc can be explained by their high level and/or the

type of phenolic compounds, tannin and other yet to be discovered antioxidant compounds (**Table. 3**). The scavenging action of plants constituents is related to polyphenolic compounds (Kimura et al., 1985; Hatono et al., 1989), and flavonoids (Hatono et al., 1989). Several data has been reported in several experimental models showed that phenolic compounds are considered as antioxidants not only because they act as free radical scavengers, but also because of their ability to inhibit XO (Cos et al., 1998; Boumerfeg et al., 2009, Baghiani et al., 2010). Furthermore, the Flavonoids might provide an interesting alternative for treatment of radical-mediated diseases (Matsumura et al., 1998; Manach et al., 1999). Enroth et al. (2000) confirm existing structure-activity relationship. In phenolic compound, especially flavonoid, the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 were essential for a high inhibitory activity on xanthine oxidase. Furthermore, they suggested that phe 1009 interacts with the six-membered ring of xanthine and phe 914 interacts with the five-membered ring of xanthine, it may be hypothesised that the planar A and C ring of flavones have interactions with phe 1009 and phe 914, which are necessary for inhibition of xanthine oxidase. A planar flavone skeleton alone is insufficient to induce xanthine oxidase inhibition. It may be speculated that at least one hydroxyl group is necessary, favorable at position 7, to achieve xanthine oxidase inhibition by flavones (Enroth et al., 2000).

6. Measurement of superoxide anion scavenging activity (PMS-NADH-NBT)

The inhibition of cyt C reduction is due to dual effect of extracts as demonstrated above. Firstly, these compounds inhibit the XO activity and secondly, some of them scavenge superoxide radical proximally at the same concentrations. Thus, to give a clear cut if the Tc, Cc and Ai extracts have a superoxide anion scavenging activity or not, the PMS-NADH-NBT system, was used as another method to measure $O_2^{\bullet -}$ scavenging activity, superoxide anion derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT.

The decrease in the absorbance at 560 nm with antioxidants indicates the consumption of the generated superoxide anion in the reaction mixture. Figure 25 demonstrated that Tc, Cc and Ai extracts had a concentration-dependent scavenging activity by neutralizing superoxide radicals in the same order as shown in the results obtained by using enzymatic method (Cyt c). TC, Cc and Ai extracts are more efficient scavengers than gallic acid (IC_{50} at $5.10^2 \pm 0.923\mu\text{M}$) ($P \leq 0.01$), which have an antioxidant capacity, determined by both ABTS and DPPH scavenging assays, higher than that of vitamin C and other phenolic constituents such as quercetin, epicatechin, catechin, rutin and chlorogenic acid (Kim et al., 2002).

The most potent scavenger of $O_2^{\cdot-}$ radical observed was CE and EAE of Tc with IC_{50} (μM Q-Eq) of 4.078 ± 1.029 and 6.063 ± 1.148 followed by CE of Cc in the same order as found in enzymatic method, however the other extracts gave a scavenging effects lower than those of previous extracts ($p \leq 0.01$) (Figure. 26), but they have a greater activity than quercetin (IC_{50} at $1.3.10^3 \pm 0,053\mu\text{M}$) ($P \leq 0.01$). CHE of three plants gave a prooxydant effects at high concentration, in agreement with the results showing by Jayaprakasha and Patil (2007). Zubia et al (2007) showed that extract from marine macroalgae species (*Chondria baileyana*) had a scavenger effect on $O_2^{\cdot-}$ at low concentration, however it had a prooxydant activity using concentrations over than 1 mg/ml. In the other hand all the extracts present a good correlation between the scavenger effects on superoxide and phenolic compound content ($r^2 = 0.85$, $p \leq 0.05$) (Figure. 26) comparable to the results mentioned by Maisuthisakul et al (2007).

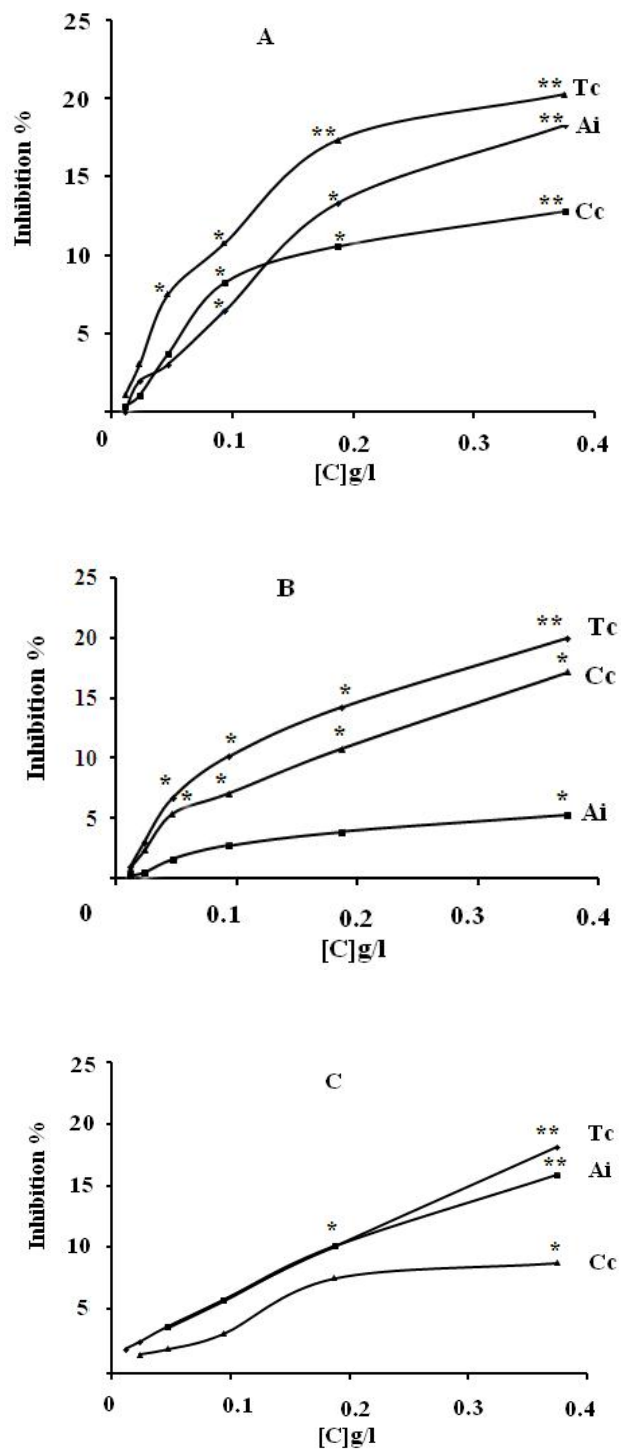


Figure 25. Scavenging effects of *T. communis*, *C. caeruleus* and *A. iva* extracts on superoxide anion radicals derived from the dissolved oxygen by PMS/NADH coupling reaction reduces as determined as IC₅₀. Values mean ± SEM (n = 3). Curve **A** represent crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts (** p ≤ 0.01; * p ≤ 0.05).

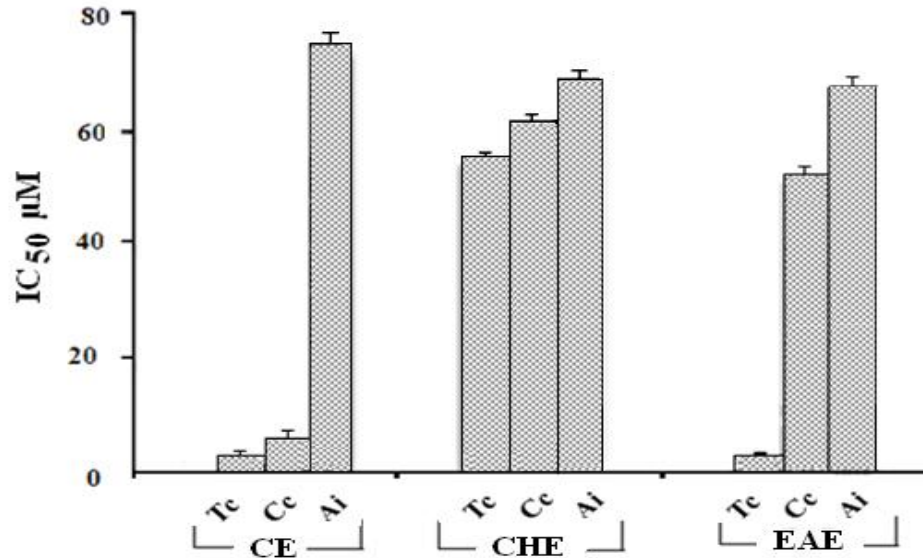


Figure 26. Evaluation of *T. communis*, *C. caeruleus* and *A. iva* extracts Scavenging capacity on the superoxide radical derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT. Results are expressed as percentage of control where no extract or standards were added. Values are: mean \pm SEM (n = 3).

Although $O_2^{\cdot-}$ is a weak oxidant, it gives rise to generate a powerful and dangerous hydroxyl radicals as well as singlet oxygen, hydrogen peroxide and hydroxyl radical, that have the potential of reacting with biological macromolecules and inducing tissue damages (Aruoma, 1998; Stief, 2003). The inhibitory effect of TC, Cc and Ai extracts on $O_2^{\cdot-}$ radical generation clearly indicated that TC, Cc and Ai extracts are a potent scavenger of superoxide radicals, thereby can prevent the formation of ROS.

7. Evaluation of RBC Hemolysis

Erythrocytes are the most abundant cells in the human body and possess desirable physiological and morphological characteristics (Hamidi and Tajerzadeh, 2003). Since the richness of their membranes in polyunsaturated fatty acids, hemoglobin and high cellular concentration in oxygen, the erythrocyte are extremely susceptible to oxidative damage (Arbos et al., 2008; Çimen, 2008).

Oxidative damage of erythrocyte membrane (lipid/protein) may be implicated in hemolysis (Ko et al., 1997). Therefore this cellular system could be very useful to study oxidative stress and the protective effect of TC, Cc and Ai extracts against hemolysis. The antioxidant activity of studied extracts were confirmed in Human erythrocytes (RBC) exposed to 500 μM *t*-BHP, by measuring the erythrocyte membrane resistance to free radical-induced hemolysis. When control RBC were incubated with extracts (2mg/ml), no significant hemolysis was observed within 2 h. In RBC exposed to 500 μM *t*-BHP, the hemolysis started after 60 min incubation and stabilised between 90 and 120 min (27% hemolysis which was considered as 100%) (Figure. 27).

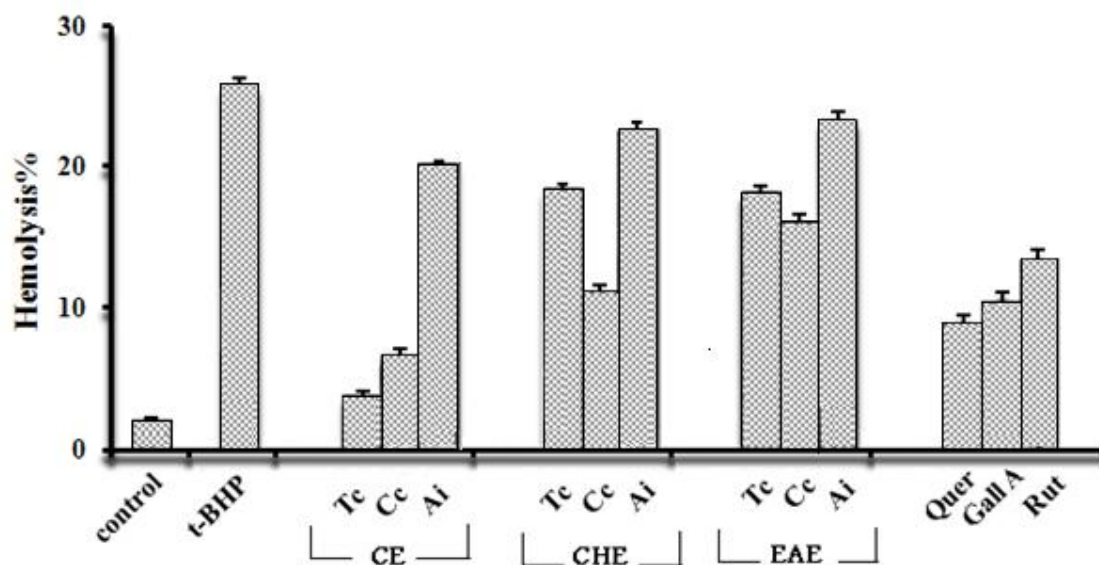


Figure 27. Effect of *T. communis*, *C. caeruleus* and *A. iva* extracts (2mg/ml) on *t*-BHP (500 μM) induced oxidative stress in red blood cells. Hemolysis percentage were measured as described under Materials and Method section and values are means \pm SEM; n = 5. CE: crud extract; CHE: chloroform extract; EAE: ethylacetat extract.

In the present study the strongest effect was observed with CE of the TC, Cc extracts which decreased significantly the hemolysis ($p \leq 0.01$), at 120 min, as shown in Figure 27 and Table 5 with 85,75 %, 74,18 %, respectively. They were more potent than quercetin, rutin and gallic acid ($P \leq 0.01$), previous data reported that quercetin is very efficient in inhibiting

erythrocyte hemolysis (Ng and al., 2003) and more potent than rutin in inhibiting lipid oxidation (Chen and Ahn, 1998), which indicate the potency of Tc, Cc and CE, these results are comparable with those shown by Ferreira et al (2007), studying the effect of *Olea europaea* leaves methanolic extracts on erythrocytes hemolysis. CHE and EAE of Tc and Cc also significantly protect RBC against hemolysis ($P \leq 0.01$), where the most effective were Cc with 56.99 %, 37.84 % followed by Tc extracts with 28.61 % 29.48 %, respectively. Meanwhile Ai extracts exhibited the lowest effect (Figure 27, Table 5).

Table 5. Percentage inhibition of red blood cells Hemolysis by *T. communis*(Tc), *C. caeruleus*(Cc) and *A. iva*(Ai) extracts (2 μ g/ml). Quer: quercetine; Gal A: gallic acid; Rut: rutin; CE : crud extract ; CHE : chloroforme extract ; EAE : ethylacetate extract

Extracts	% Inhibition		
	Tc	Cc	Ai
Quer	65.464 \pm 0.9 59	65.464 \pm 0.959	65.464 \pm 0.959
Gal A	59.620 \pm 0.939	59.620 \pm 0.939	59.620 \pm 0.939
Rut	47.733 \pm 0.799	47.733 \pm 0.799	47.733 \pm 0.799
CE	85.750 \pm 1.029	74.183 \pm 1.24	21.924 \pm 0.533
CHE	28.611 \pm 0.517	56.993 \pm 0.592	12.476 \pm 0.561
EAE	29.486 \pm 0.686	37.848 \pm 0.592	9.697 \pm 1.028
Control	92.450 \pm 0.786	92.450 \pm 0.786	92.450 \pm 0.786

Values are means \pm SEM; n = 5. Hemolysis induced by t-BHP (45 μ g/ml)

The results of the present study shows that TC, Cc and Ai extracts, especially CE of TC and Cc can effectively protect erythrocytes against haemolytic injury induced by t-BHP. the extracts are a powerful scavenger as previously shown; it could have provided a defence against haemolytic injury by suppressing t-BHP related fall in reduce glutathione and other antioxidants. GSH oxidation, which can be the result of direct radical attack, can also occur indirectly through GSH-requiring repair processes such as the reduction of oxidized

membrane-protein thiol groups (Ko et al., 1997). GSH appeared to provide the primary antioxidant defense in stored erythrocytes and their decline was concurrent with an increase in oxidative modifications of membrane lipids and proteins which may destabilise the membrane skeleton (Yamamoto et al, 1985)

The protective effects of plants may be due to: i. their kind of phenolic content, because there is no significant correlation between antihemolytic effect of extracts and their phenolic compound content ($r^2 = 0.47$, $p > 5$), ii. and/or the difference in the degree of the penetrations of the flavonoid molecules in intact erythrocytes (Chaudhuri et al, 2007). It was demonstrated that binding of the flavonoids to the RBC membranes significantly inhibits lipid peroxidation, and at the same time enhances their integrity against lysis (Kitagawa et al, 2004) (Table 3) confirmed this hypothesis; although phenolic contents in CHEs of Cc and Ai are lower than EAE of the same plants, the CHEs (which contain apolar compounds so more soluble in RBC membranes) exhibit an anti-hemolytic action stronger than those of EAE (less apolar than CHE), however the crude extract (more polar) was more efficient, this may be due to the synergistic effect of different compounds present in (Zhishen et al., 1999).

Studies involving plasma have indicated that flavonoids have the ability to delay the consumption of some endogenously present antioxidants in the human body (Lotito and Fraga, 1998). It has been reported that antioxidant enzymes such as superoxide dismutase, catalase, peroxidase and glutathione peroxidase were elevated in RBCs treated with *m*-chloroperbenzoic acid, a novel photosensitizer (El-Missiry and Abou-Seif, 2000).

Our results indicate that t-BHP-induced oxidative hemolysis of erythrocytes was suppressed by the extracts of TC, Cc and Ai extracts *in vitro*. However, the behavior of these extracts against the radical attack is always difficult to explain because the RBCs are a complex matrix in which the substances pharmacologically evaluated could be involved in many reactions in

cell membrane (Djeridane et al., 2007). It is known that erythrocytes have a high content of polyunsaturated lipids which along with their rich oxygen supply and transition metals make them more susceptible to lipid peroxidation (Puppo and Halliwell, 1988). Any process which increases peroxidation of unsaturated bonds in membrane lipids also causes an increase in erythrocyte fragility and their susceptibility towards haemolysis (Brzezinska-Slebodzinska, 2001). Lipid peroxidation is one of the consequences of oxidative damage, and it has been proposed as a general mechanism for cell injury and death (i.e., hemolysis) (Miki et al., 1987). Since Malondialdehyde (MDA), the well-characterized product of the lipid peroxidation of erythrocytes, is a highly reactive which has been shown to cross-link erythrocyte phospholipids and proteins, to impair a variety of the membrane-related functions, which ultimately lead to diminished erythrocyte survival (hemolysis) (Sugihara et al., 1991). Total antioxidant activities of the plant extracts can not be evaluated by any single method, due to the complex nature of phytochemicals (Chu et al, 2000). Two or more methods should always be employed in order to evaluate the total antioxidative effects of vegetables (Nuutila, et al 2003). In this regard and/or to confirm previous results two supplementary essays were performed, lipid Peroxidation by using MDA as a marker of lipid peroxidation and β -carotene/linoleic acid essays with linoleic acid as a model of lipid peroxidation and as a source of radical.

8. Thiobarbituric acid-reactive substances (TBARS) assay

To confirm the effects of TC, Cc and Ai extracts against lipid peroxidation as a direct antioxidant, Thiobarbituric acid-reactive substance (TBARS) assay was used. The extent of lipid peroxidation was estimated by the levels of MDA measured using the thiobarbituric acid. FeSO₄ was added to egg yolk homogenates, as lipid rich media, to induce lipid peroxidation. MDA a secondary end product of polyunsaturated fatty acids oxidation, reacts

with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm. The result illustrated in figure 28 showed that phospholipid peroxidation induced by FeSO₄ was significantly counteracted by TC, Cc and Ai extract in dose dependent manner.

The CHE extracts of Cc exhibited the highest inhibitory potency with IC₅₀ of 0.157 ± 0.012 μ M Q-Eq higher than those of Quer, Rut and Gall, Asc. respectively, ($p \leq 0.01$) followed by the CHE of Tc which was very close to previous extract, meanwhile, the CHE of Ai didn't elicit a marked inhibition of peroxidation, compared with Tc and Cc extracts ($p \leq 0.01$).EAE of Ai showed a remarkable effect in suppressing lipo-peroxidation, which was not far to those of Tc and Cc. CE of Tc Strongly inhibited peroxidation followed by that of Cc and certainly more than CE of Ai ($p \leq 0.01$), although it still significantly more active than reference substance quercetin ($p \leq 0.01$) (Figure 29).

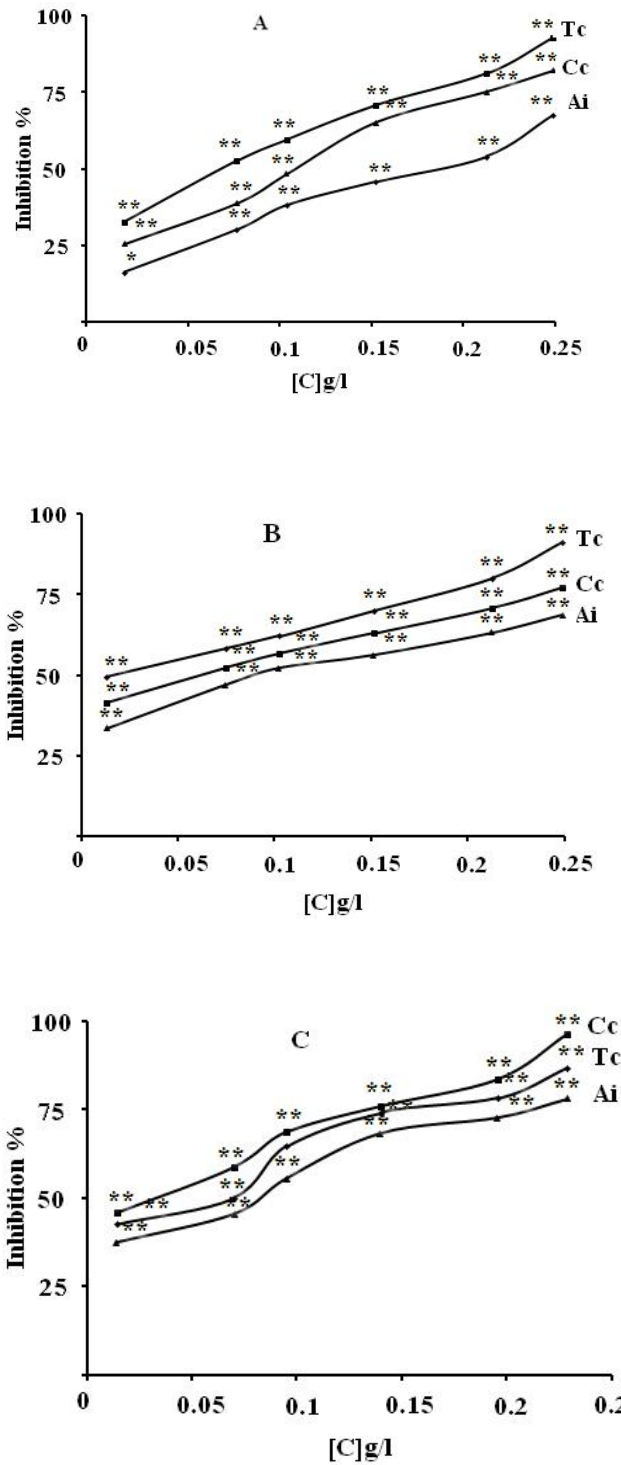


Figure 28. Dose-response effect of the *T. communis*, *C. caeruleus* and *A. iva* extracts against lipid peroxidation in egg yolk homogenates after incubating with FeSO₄ (0.07 M) and extracts. Curve A represent crud extracts; B: chloroform extracts; C: ethyl acetate extracts (** p ≤ 0.01; * p ≤ 0.05).

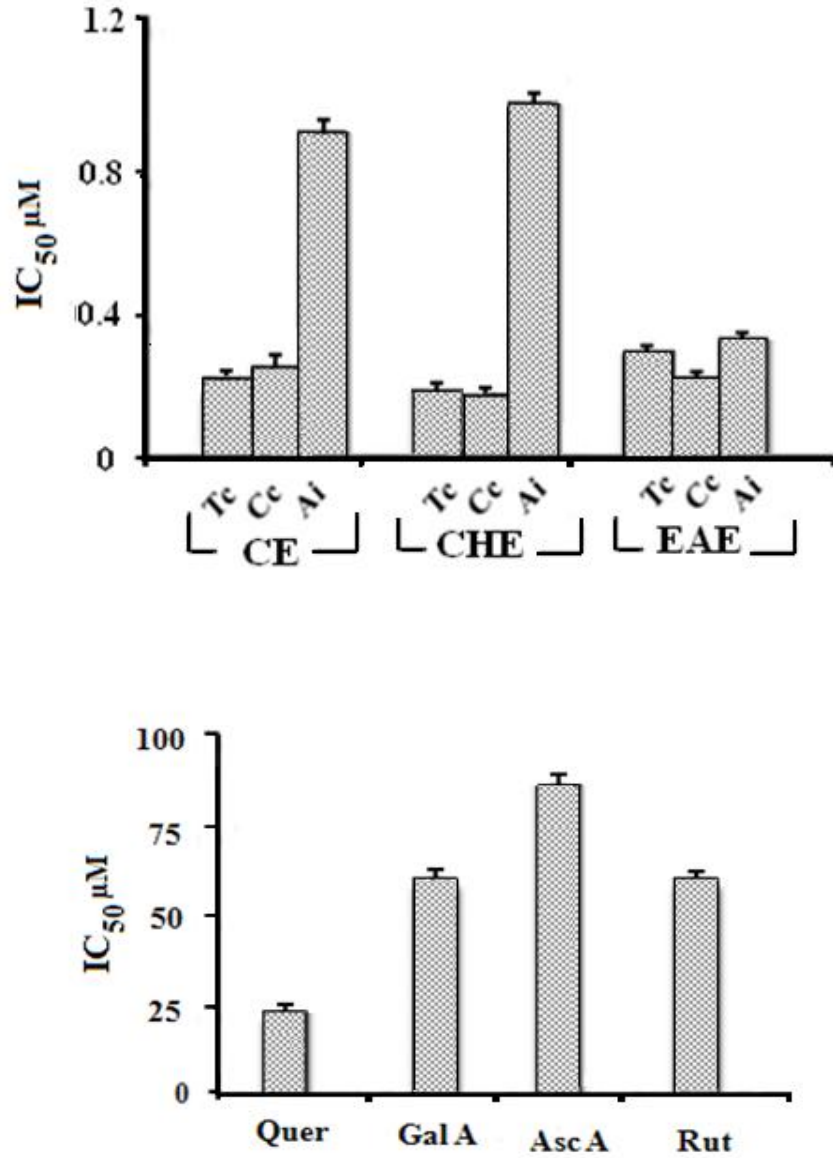


Figure 29. Antilipoperoxidative effects of the *T. communis*, *C. caeruleus* and *A. iva* extracts at IC₅₀ concentration against FeSO₄ (0.07 M) induced lipid peroxidation in egg yolk homogenates. After incubating the yolk homogenates with FeSO₄ (0.07 M) and/or extracts/standards the lipid peroxidation measured by TBARS assay. Values are mean ± SD., *n* = 3.

The results showed a feeble correlation between antioxidant potent and CE, EAE phenolic content ($r^2 = 0.39$, $p > 0.5$) (Table. 3), similar to the results of Agbor et al (2005) working on some herbs/spices from Cameroon and the results of Demiray et al (2009) working on Turkish medicinal plants: *Tilia argentea*, *Crataegi folium* leaves and *Polygonum bistorta* roots, therefore the potent effect of Ai especially EAE may due to other products and /or the type of phenolic compound. Hall and Cuppett (1997) showed that plant tissues contain a wide variety of compounds with antioxidant activity, phenolic compounds, nitrogen compounds, carotenoids, lignans and terpenes were reported to possess antioxidative activity in suppressing the initiation or propagation of the chain reactions. However the studied CHEs exhibited a good correlation between antioydant activity and phenolic content. In agreement with Maksimović et al (2005) results, which showed that there is a positive and significant correlation existed between antioxidant activity and total phenolic content, revealing that phenolic compounds were the dominant antioxidant.

Our results support the view that some medicinal plants are promising sources of natural antioxidants. Further it is believed that plants which are having more phenolic content show good antioxidant activity, leading to the conclusion that there is a direct correlation between total phenol content and antioxidant activity (Biglari et al., 2008; Saravana et al., 2008). Previous reports noted that polyphenolics are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993). The chemical natures and structures of the antioxidant principles present in Tc, Cc and Ai extracts may include sternly phenolic compounds, so the difference in potency between the extracts in inhibiting lipid peroxidation was caused by the different compounds present in these plants. Since the differences in properties constituents of plant extracts, the activity could be different in kinds of lipid occurrence (Porter et al. 1989). Thus it is possibly the hydrophobic nature of CHE,

which resulted in its greater in the cell membrane which make it a potent anti lipid peroxidation more than EAE and CE which are more polar than CHE.

9. β -carotene/linoleic acid

According to several authors, the test of linoleic acid oxidation inhibition coupled with β -carotene, appears very useful as a mimetic model of lipid peroxidation in biological membranes (Ferreria et al., 2006). β -carotene/linoleic acid assay determines the inhibition ratios of the oxidation of linoleic acid as further methods to confirm the antilipoperoxidation of Tc, Cc and Ai extracts. Lower absorbance indicates a higher level of antioxidant activity. Figure 30 shows the changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of Tc, Cc and Ai extracts (2mg/ml) compared to that of synthetic antioxidant BHT as a positive control during 24 h under the same condition.

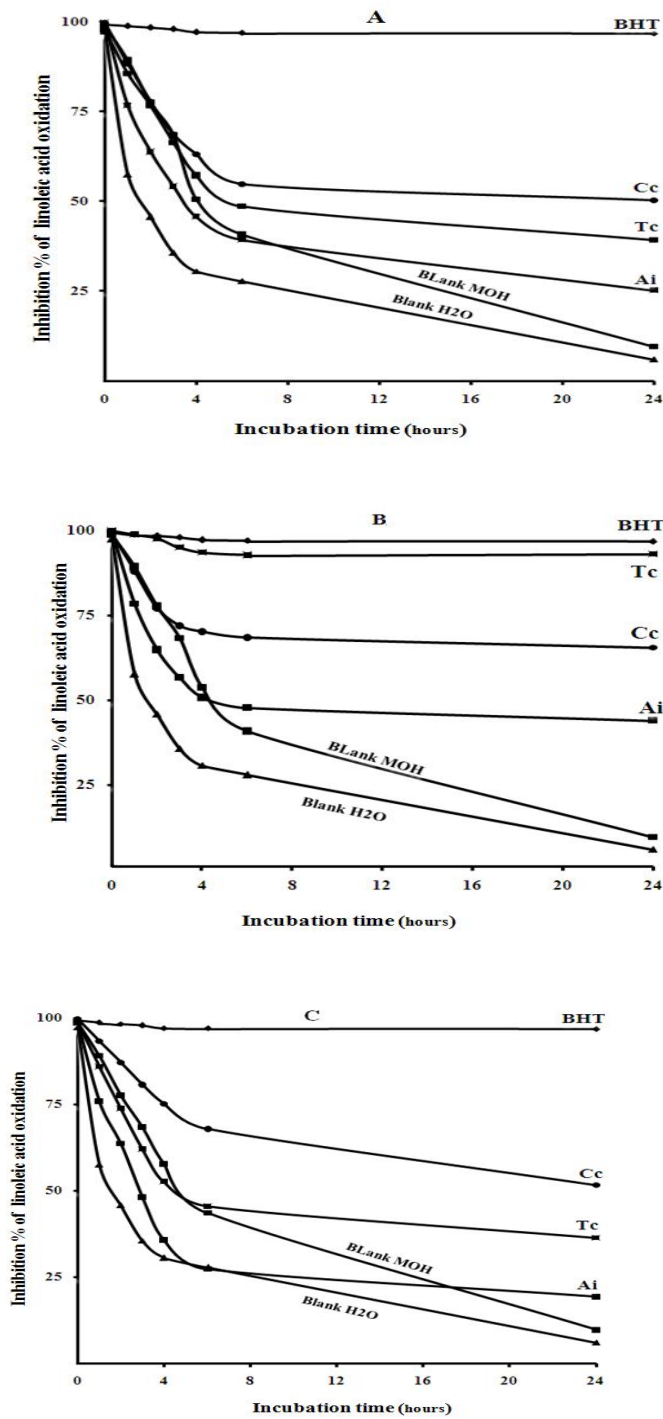


Figure 30. The changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of *T. communis*, *C. caeruleus* and *A. iva* extracts (2mg/ml), compared to BHT as a positive control during 24 h. For all values statistically different ($p \leq 0.01$) the curve **A** represent: crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts. (Extracts of the three plants)

The inhibition extent of lipid oxidation by Tc, Cc and Ai extracts when compared to BHT, which had 96,773 % at the same concentration (2mg/ml) showed a marked activity effects ($p \leq 0.01$) (Figure 31). The high inhibition ratios of linoleic acid oxidation by the CHE, were showed for Tc (92. 94 %), similar to that of BHT, followed by that of Cc (65.578%) ($p \leq 0.01$), however CHE of Ai exhibited the lowest ratio with 43. 94 % ($p \leq 0.01$). For EAE and CE the most efficient were those of Cc 79. 21 and 50.29 % respectively, followed by Tc then Ai extracts (Figure 31).

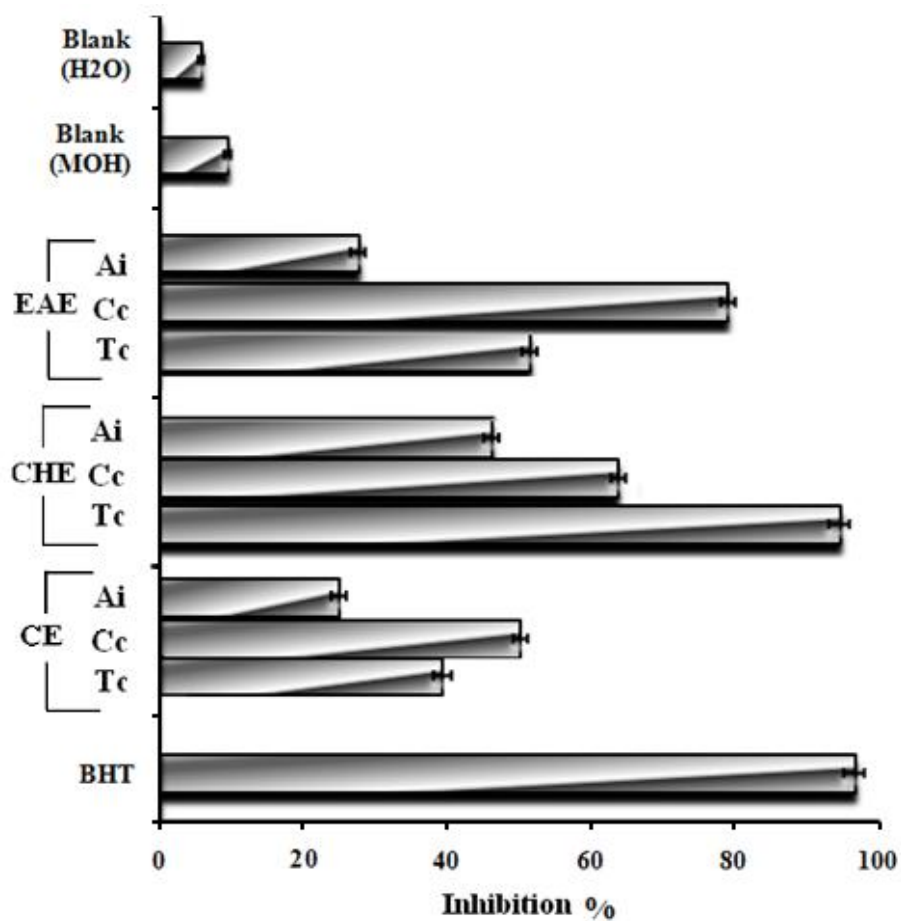


Figure 31. Percentage inhibition of the linoleic acid oxidation by the *T. communis*, *C. caeruleus* and *A. iva* extracts after 24h. ($p \leq 0.01$). CE: crud extract; CHE: chloroform extract; EAE: ethylacetat extract.

We can summarize the descending order of extracts tested in terms of polarity activity the apolar extracts or moderately apolar (CHE, EAE) are more active than the polar extract (CE). On the other hand, the richest extracts in phenolic compounds are more assets (CHE and EAE) so there is a good correlation between antioxidants activity and polyphenols contents in the same plant ($r^2 = 0.82$, $p \leq 0.05$). In multiphase systems antioxidants localization depends on their solubility and polarity (Huang et al. 1994). According to Frankel et al. (1994) the test β carotene bleaching is similar to a lipid emulsion in water; Frankel and Meyer (2000) have suggested that antioxidants which exhibit apolar properties are most important because they are concentrated in the lipid-water interface, thereby preventing the formation of lipid radicals and β -carotene oxidation. While polar antioxidants are diluted in the aqueous phase and are thus less effective in protecting lipids. It was found that ethanol extracts constituting of higher amount of lipophilic fraction showed higher antioxidative activity than aqueous extracts, constituting of higher amount of hydrophilic fraction (Frankel and Meyer 2000). Thus the antioxidant activity of extract is based on the radical adducts of extracts with free radicals from linoleic acid. The linoleate-free radical attacks the highly unsaturated β -carotene models. The presence of extracts shows a decrease of the free radical concentration. The presence of different antioxidants can hinder the extent of β -carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al, 2001).

Lipid oxidation is one of the major factors causing deterioration of foods during the storage. Although there are some synthetic antioxidant compounds such as BHT and butylated hydroxyanisole (BHA) which are commonly used in foods processing, it has been reported that these synthetic antioxidants are not devoid of biological side effects and their consumption may lead to carcinogenicity and causes liver damages (Branien, 1975; Linderschmidt et al, 1986).

Therefore, the development of alternative antioxidants mainly from natural sources has attracted considerable attention because of their potential antioxidant activity (Lopez-Velez et al., 2003). There are numerous investigations showing that plant extracts, rich in polyphenols, might exhibit strong antioxidative properties in lipid systems (Gramza et al., 2005). It was reported that sample that inhibits or retards the bleaching β -carotene can be described as a scavenger of free radicals and as a primary antioxidant (Liyana-Pathirana et al., 2006). Literature reported that reducing power is associated with antioxidant activity (Oktay et al., 2003).

The antioxidative properties of flavonoids are due to several antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to catch metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1997; Benavente-Garcia, 1997). Further, Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

From our results we may deduce that the anti-haemolytic effect of TC, Cc and Ai extracts in t-BHP induced haemolysis and anti lipoperoxidation were due to its ability to scavenge free radicals and/or chelating metal. In order to correlate and understand the mechanism of the observed antioxidant activity of TC, Cc and Ai extracts, we have applied DPPH, Ferrous ion chelating, and FRAP assays for testing scavenging (hydrogen donating ability), chelating and reducing proprieties respectively.

10. DPPH assay

The DPPH radical is a stable organic free radical with an absorption maximum band around 515-528 nm, so it is a useful reagent for evaluation of antioxidant activity of compounds (Sanchez-Moreno, 2002). In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants (Bondent et al., 1997).

The effect of Tc, Cc and Ai extracts at different concentrations were studied for their ability of hydrogen donating measured using DPPH test. Results showed that these extracts exhibited a dose-dependent activity in scavenging DPPH radicals (Figure 32). The IC₅₀ of all extracts indicating that these extract are more potent than quercetin, rutin and gallic acid (Figure 33). The CE of Cc which showed the lower effect than other extracts was more effective than quercetin and gallic acid ($p \leq 0.01$). The results revealed a moderate linear correlation between antiradical potent and phenolic content.

In general the most effective extracts were CHEs, in agreement with the results described Rice-Evans et al (1996) reporting that the phenolic acids and flavonoids especially aglycone flavonoids, are effective hydrogen donors, which make them good antioxidant. Plant phenolics present in fruit and vegetables have received considerable attention because of their potential antioxidant activity (Lopez-Velezet al, 2003). Although there is not a great difference between Cc and Ai extracts in phenolic content, but the Ai extracts exhibited an antiradical effect higher than that of Cc extract, this may be due to the kind of phenolic compound present in Ai extracts or other products. Phytochemical screening demonstrated the presence of isoflavonoids, and different types of compounds like tannins, flavonoids and others. (Akroum., 2009).

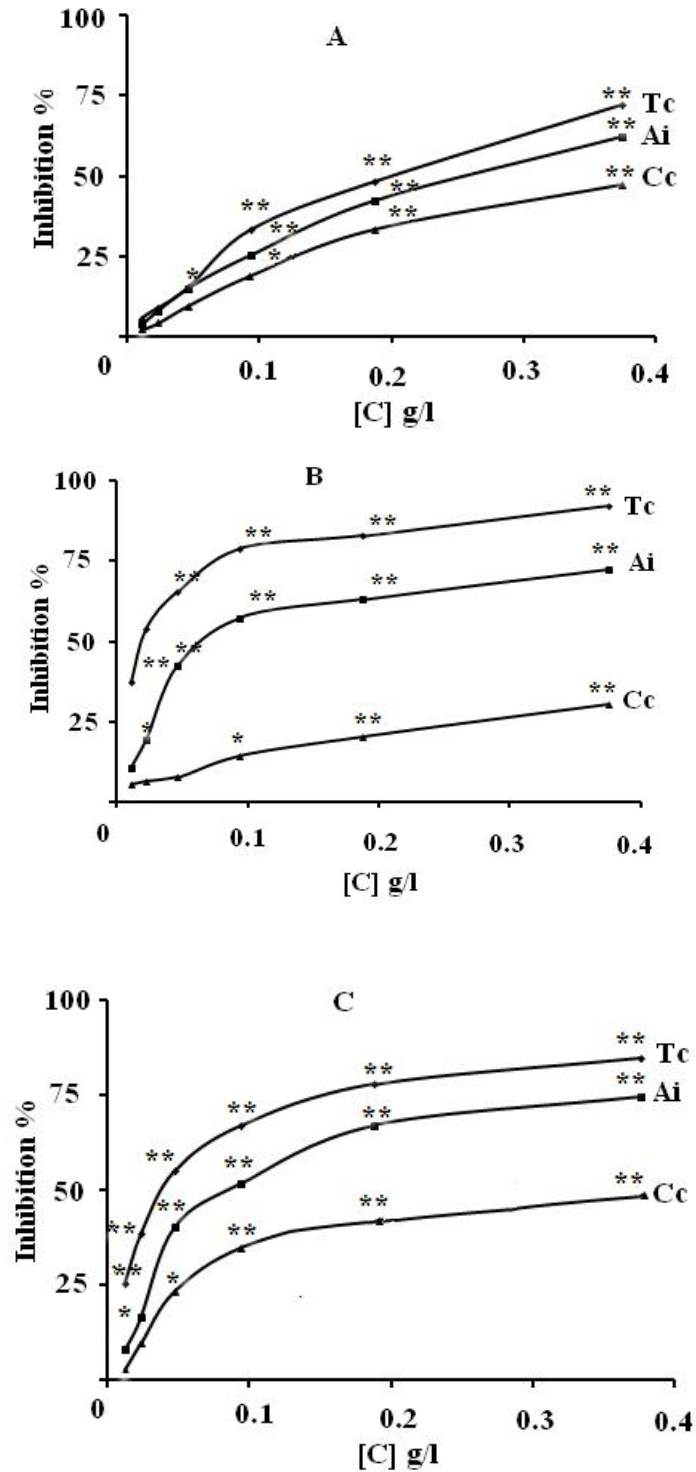


Figure 32. The percentage inhibition of free DPPH radical in the presence of different concentration of *T. communis*, *C. caeruleus* and *A. iva* extracts. Curve **A** represent crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts (** $p \leq 0.01$; * $p \leq 0.05$). Each value represents the mean \pm S (n=3).

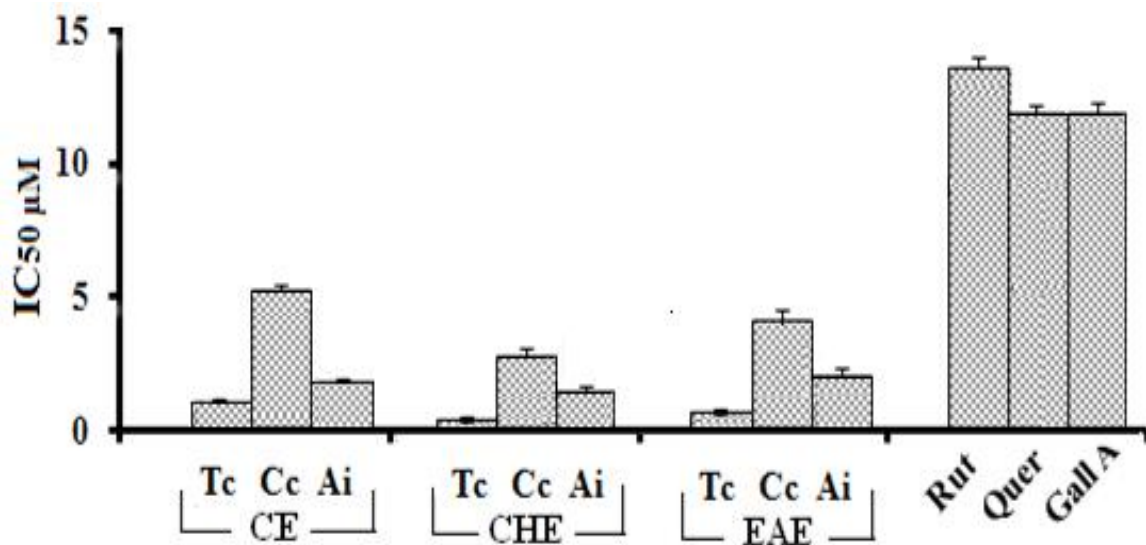


Figure 33. DPPH radical scavenging activity of different *T. communis*, *C. caeruleus* and *A. iva* extracts. CE: crud extract; CHE: chloroform extract; EAE: ethylacetat extract; Data are presented as IC₅₀ values. Each value represents the mean ± SD (n = 3).

The results obtained from these test suggested that Tc, Cc and Ai extracts are a good free radical scavenger, and contributes significantly to the antioxidant capacity of these plants. The relationship between the molecular structure of a series of structurally related flavonoids (flavones, flavonols and flavanones) and their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radicals was studied, it was demonstrated that the free radical scavenger potential of polyphenolic compounds closely depends on the particular substitution pattern of free hydroxyl groups on the flavonoid skeleton, the highly active flavonoids possess a 3', 4'-dihydroxy occupied B ring and/or 3-OH group. (Amič et al., 2003).

11. Ferrous ion chelating activity

The xanthine/xanthine oxidase system and other system are known to generate $O_2^{\cdot-}$ and H_2O_2 . There is some evidence that $O_2^{\cdot-}$ alone formed extracellularly does not cause cell death. However, its contribution to a metal-ion catalysed Haber-Weiss reaction, by reducing transition metal ions and thereby accelerating the cycle of Fenton-type reaction in which the

reduced ions are oxidized by H_2O_2 generating HO^\cdot radicals the most powerful radical involved cytotoxic reactions which can directly oxidize macromolecules including DNA, protein, and lipids. (Halliwell and Gutteridge, 1985), so the chelating of metal-ions lead to stop the Fenton-type reaction thereby the reduction of HO^\cdot production. Further, extracts or compounds with chelating activity are believed to inhibit lipid peroxidation by stabilizing transition metals (Wu and Ng, 2008). Ferrozine can quantitatively form complexes with metal-ion Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted and as a result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows the estimation of the chelating activity of the coexisting chelator (Decker et Welch, 1990). In this study, TC, Cc and Ai extracts and EDTA showed chelating activity as demonstrated by their effectiveness in inhibiting the formation of Fe^{2+} -ferrozine complex, the absorbance of this complex was dose dependently decreased (Figure. 34).

In general the iron chelating ability of TC, Cc and Ai extracts were not far from EDTA (Figure 35). CE of Tc showed excellent chelating ability with IC_{50} ($0.302 \pm 0,053$) μ M Q-Eq, higher than that of EDTA ($IC_{50} = 0,563 \pm 0,053\mu$ M) ($p \leq 0.01$), meanwhile CE of Cc gave approximately the same effect as EDTA, however CE of Ai exhibited a chelating effect lower than that of EDTA with approximately 5.7 folds ($p \leq 0.01$).

In other hand CHE of Cc was the most effective than those of Tc and Ai by 3 and 5 folds respectively, ($p \leq 0.01$) and less effective than EDTA by 15 folds $p \leq 0.01$ (Figure. 35). while the EAE showed the lowest chelating abilities, the IC_{50} of EAE of Tc and Cc were too close to each other, and more potent than that of Ai with approximately 1.5 folds and lower than EDTA by 62 times ($p \leq 0.01$) (Figure 35).

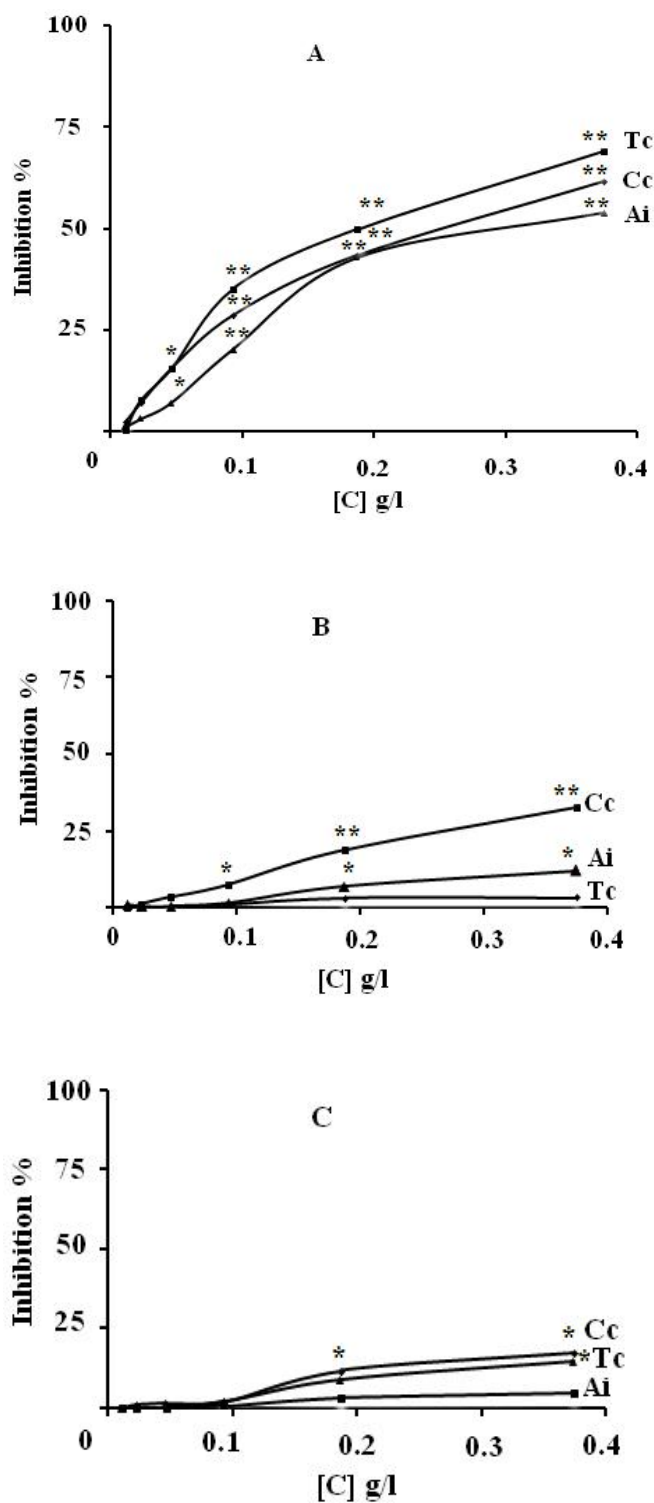


Figure 34. Metal chelating activity of different *T. communis*, *C. caeruleus* and *A. iva* extracts. Data were presented as mean \pm SD (n= 3) Curve **A** represent crud extracts; **B**: chloroform extracts; **C**: ethyl acetate extracts (** p \leq 0.01; * p \leq 0.05).

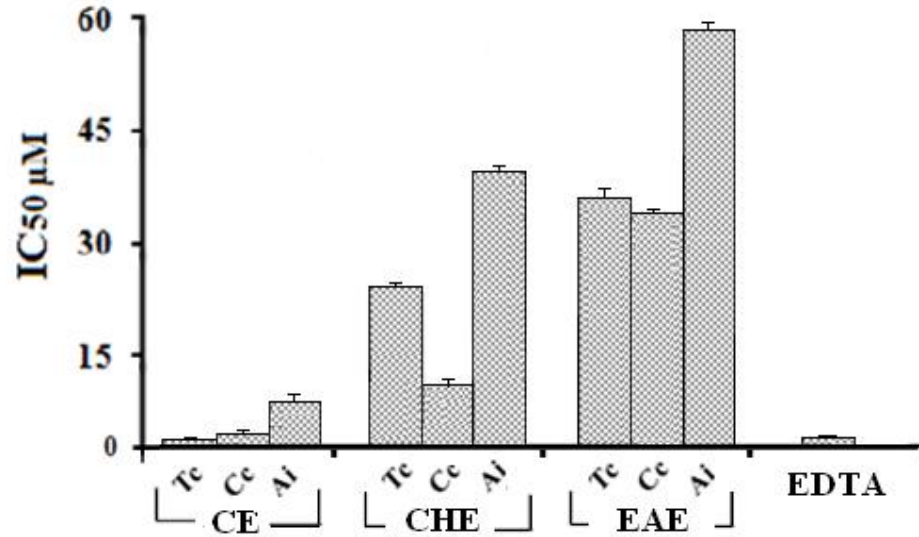


Figure 35. Comparison in metal chelating activity of different *T. communis*, *C. caeruleus* and *A. iwa* extracts. CE: crud extract; CHE: chloroform extract; EAE: ethylacetat extract; Data were presented as IC₅₀ Values mean \pm SEM (n= 3).

Metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Suresh-Kumar et al., 2008). Results revealed a poor correlation between Metal chelating capacity and phenolic content in different extracts ($P > 0.05$), indicating that polyphenols might not be the main ion chelators suggested that partly responsible for the antioxidant activities. These results were comparable to those of Zhao et al (2008) working on typical malting barley varieties.

The data obtained from figure 35 reveal that CE proved to be better as metal-chelating ability than CHE and EAE. This may be explained by the interaction of the different flavonoids (Zhishen et al., 1999), and/or other compound present in the extracts which are in transition metals (Wong et al. 2006). It is reported that the terpenoids and flavonoids having glycosidic linkage are likely to be extracted into aqueous extracts (Satyanarayana et al., 2004). The compounds containing nitrogen are generally more powerful chelating than

phenolic compounds (Chan et al., 2007). Moreover, a sample rich in compounds phenolic could not chelate transition metals if the polyphenols don't have the necessary functional groups for chelating activity (Wong et al., 2006).). It is reported that the terpenoids and flavonoids having glycosidic linkage are likely to be extracted into aqueous extracts (Satyanarayana et al., 2004). The compounds containing nitrogen are generally more powerful chelating than phenolic compounds (Chan et al., 2007). Moreover, a sample rich in compounds phenolic could not chelate transition metals if the polyphenols don't have the necessary functional groups for chelating activity (Wong et al., 2006). These results clearly demonstrated the capacity of TC, Cc and Ai extracts to chelate the iron metal ions, which has a critical role in the in generating hydroxyl radicals, which are an extremely reactive oxygen species, capable of modifying almost every molecule in the living cells, and has the capacity to cause damages, leading to carcinogenesis, mutagenesis, and cytotoxicity (Hwang and Kim, 2007). Moreover, hydroxyl radicals are capable of the quick initiation of lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids (Aruoma, 1998; Kappus, 1991).

12. Ferric reducing ability of plasma assay (FRAP)

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003; Hsu et al., 2006). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). For the measurement of the reductive ability, we used the FRAP assay which was developed to determine the ferric reducing ability of biological fluids and aqueous solutions of pure compounds and can be applied to study the antioxidant activity of plant extracts and beverages. The FRAP values calculated using the respective Fe (II)

calibration curves. Gallic acid, quercetin, rutin and ascorbic acid were used for comparison of ferric reducing ability of plants extracts used in the present study. Conditions for the determination of the ferric reducing ability of antioxidants in the original method established at 4 min interval as suitable for such measurements, since the absorbance of the reduced ferrous-TPTZ complex was stable at this time (Benzie and Strain, 1996). When these conditions were used in the present study, we observed that the reduction of the ferric-TPTZ complex was not established after 4min reaction time and most of the compound shows a steady increase in the absorbance with time, therefore we prolonged the reaction time for several hours and the continuous increment of absorbance at 593 nm was determined in interval time. However 30 min was maintained as one of the best time showing the antioxidant efficiency of the studied samples, this was in agreement with Pulido et al (2000) studying the anti oxidant effect of many pure polyphenols. The 4 min absorbance recording established in the original method was also kept (Benzie and Strain1996) for comparison (Figure 36).

As showed in figure 36, some antioxidants even doubled their initial absorbance after 30 min of reaction, such as the most standards and extracts. Nevertheless, these increments in the absorbance readings were not caused by alterations of the reaction mixture with time, since blank samples showed no significant modification of their initial absorbance. The equivalent concentration 1 or EC_1 , which is the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of a 1 mmol/L concentration of $FeSO_4 \cdot 7H_2O$ was measured. The lower EC_1 values are expressed as $\mu mol/L$, indicate the higher antioxidant activity.

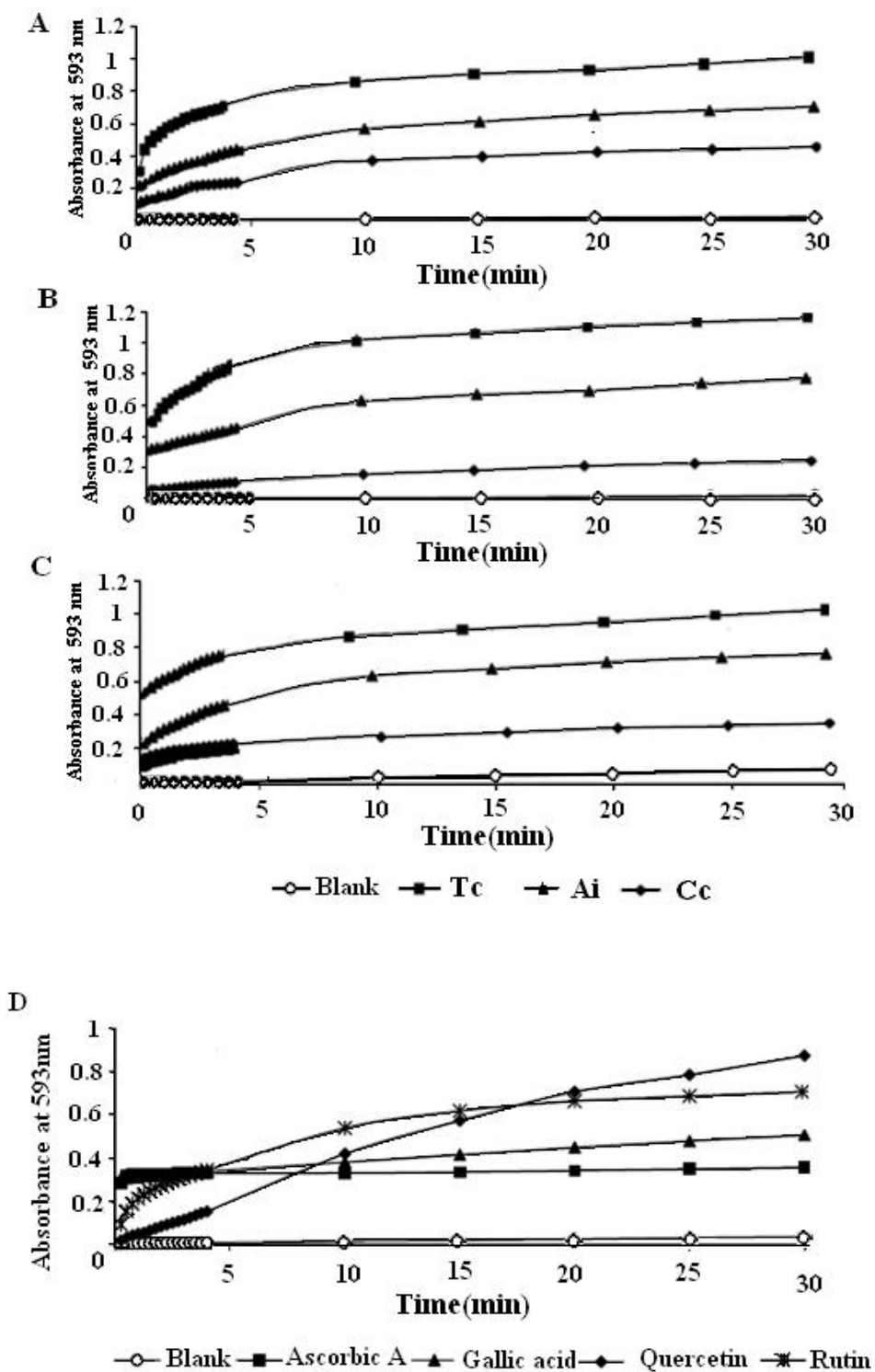


Figure 36. FRAP reaction kinetics of reagent blanks, (0.8g/l) of *T. communis*, *C. caeruleus* and *A. iva* crud extracts A; chloroform extracts B; ethyl acetate extracts C; and (250 μ M) standards D.

The data obtained (Figure 37) shows that the EC_1 values at 4 and 30 min of the studied extracts and standards. EC_1 values, and therefore ferric reducing ability, were lower at longer reaction times as expected from the kinetic behaviour of the compounds at 4min. At 30 min the results indicated that the Ai and Tc extracts, which were approximately too close to each other, were more potent than those of Cc with 2 to 6 folds, ($p \leq 0.01$). Although the lowest ferric reducing ability of Cc extracts was showed for EAE with EC_1 of 60.898 ± 1.076 (μM Q-E of extract) but it was more efficient than quercetin, gallic acid and ascorbic acid by 2. 2.5 and 5.5 folds respectively, ($p \leq 0.01$) (Figure 37). These results demonstrated that Tc, Cc and Ai extracts possess a powerful reducing ability.

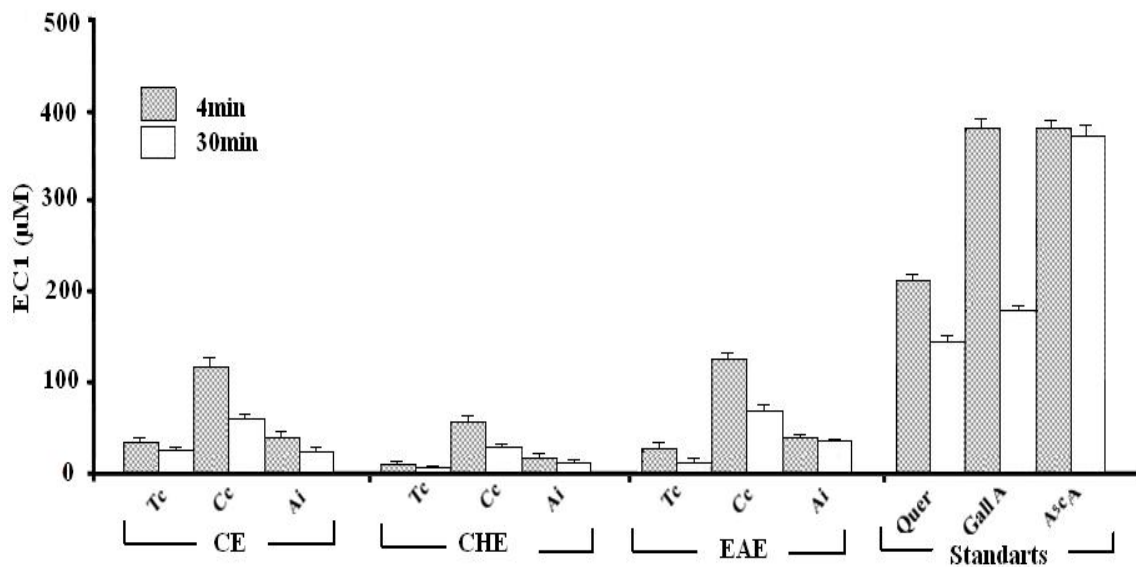


Figure 37. Comparison the EC_1 values of *T. communis*, *C. caeruleus* and *A. iva* extracts with gallic acid rutin ascorbic acid and quercetin. Data are the Mean values \pm SD, $n=3$. EC_1 : concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ CE: crud extract; EAE: ethyl acetate extract; CHE: chloroform extract.

Our results revealed a proportionall increasing the antioxidant activity with the polyphenol content (Figure 36, Table 3) agreeing with results obtained by Maksimovic'et al (2005) working on various polyphenol classes in the silks of fifteen maize hybrids. These results are

in agreement with previous results of Pulido et al (2000). For standards, quercetin showed the lowest EC₁ values, followed by gallic, however Ascorbic acid had a ferric reducing ability lower than those of most polyphenols.

In general, the classification of antioxidants according to their ferric reducing ability reported in this study agreed with results reported by other authors using different methods to estimate antioxidant power. Wang and Goodman (1999) reported a decreased effect of polyphenols inhibiting peroxidation of LDL in the order quercetin > rutin > gallic acid.

GENERAL DISCUSSION

Free radicals such as superoxide radical, peroxide radical and hydroxyl radical play an important role in the genesis of various diseases such as cirrhosis of liver, arthritis, rheumatism, cataract and ischemia (Halliwell and Gutteridge, 1985).

Medicinal plants constitute one of the main sources of new pharmaceuticals and healthcare products. A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional ingredients (Demiray et al., 2009). Natural products play a highly significant role in the drug discovery and development process. They not only serve as drugs or templates for drugs directly, but in many instances lead to the discovery of novel mechanisms of action that provide a better understanding of the targets and pathways involved in the disease process. Herbal drugs containing antiradical constituents are gaining importance in prevention and treatment of such diseases and free radical scavengers like phenolics (Nagarjan et al., 1987). The presence of flavanoids and polyphenolic structures are reported to have antioxidant activity (Kinsella et al., 1993). In the present study, Tc, Cc and Ai extracts produced good antioxidant activity and was comparable with that of used standards, this activity is probably due to its phenolic contents. It is well known that phenolic compounds are found in many plants and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants (Hollman and Katan, 1999; Rice-Evans et al., 1997). Many assays and methods for antioxidant activity *in vitro* and *in vivo* have been developed, but only a few rapid and reliable methods applicable to antioxidant activity assay for a huge number of plant extract samples exist (Miller et al., 1993). Flavonoids and phenolic compounds are the main antioxidative compounds of fruits and vegetables (Huang et al., 1998), which exhibit considerable free radical scavenging

activities, through their reactivity as hydrogen- or electron-donating agents, and metal ion chelating properties (Rice-Evans et al., 1996). Because of the high reactivity of the hydroxyl group of the phenolic compounds, radicals are made inactive (Korkina et Afanas'ev, 1997). It is generally believed that plants which have more phenolic content show good antioxidant activity, to be precise there is a direct correlation between total phenol content and antioxidant activity (Salazar et al., 2008). It can be stated that phenolic content of the plant may be a good indicator of its antioxidant capacity. Thus, it would be valuable to determine the total phenolic and flavonoid content in the plant extracts. Total phenolic and flavonoid contents of TC, Cc and Ai were determined and expressed in terms of gallic acid and Quercetin equivalents (Table 3). The present study confirms that extract from TC, Cc and Ai represent a significant source of phenolic compounds; value varied from 12.66 ± 0.27 to 208.166 ± 3.12 mg equivalent gallic acid/g lyophilisate and from 0.402 ± 0.02 to 12.48 ± 0.69 mg equivalent quercetin/g lyophilisate for polyphenols and flavonoids, respectively. Firstly, in Crud and ethyl acetate extracts the highest level of polyphenols compounds were recorded in Tc followed by Ai then Cc. Secondly for the CHE great values were showed in Tc next Cc then Ai. Meanwhile the highest level of flavonoids was showed in Ai in all the extracts followed by Tc then Cc extracts. The data obtained from this study reveal that CE proved to be better as antioxidant than CHE and EAE; this may be explained by the interaction of the different phenolic compounds and other compounds such as tannins. The difference in antioxidant activities of the extracts could be due to the different polarities of the solvents, and thus different extractability of the antioxidative compounds (Maisuthisakul et al., 2007). CE of Tc was the most potent antioxidant than those of Cc and Ai this may be due to there high content of phenolic compound (Table 3), and other products such as Some natural phenanthrenes and dihydrophenanthrenes, which have been reported to display an antioxidant and antitumour effect (Pettit et al., 1988; Shagufta et al., 2006). These phenanthrenes are a rather uncommon

class of aromatic metabolites which are presumably formed by oxidative coupling of the aromatic rings. Besides these derived compounds, phenanthrenes most likely originated from diterpenoid precursors (Kovacs et al., 2008). Adria results (2007) provide further evidence that hydroxy/alkoxy-substituted phenanthrenes from *Tamus* are also promising antitumour agents. other study revealed that from fresh rhizomes extract of *Tamus communis*, the 7-hydroxy-2,3,4,8-tetramethoxyphenanthrene (1) was isolated, together with the known 2,3,4-trimethoxy-7,8-methylenedioxyphenanthrene(2), 3-hydroxy-2,4,-dimethoxy-7,8 methylene dioxypheanthrene (3), 2-hydroxy-3,5,7-trimethoxyphenanthrene (4) and 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (5). Phenanthrenes were tested in an *ex vivo* leukotriene biosynthesis inhibition assay, the potency of phenanthrenes in this test system suggests that these substances might be the anti-inflammatory and antiasthmatic principles *Tamus* species (Adams et al., 2005; Bordat et al., 2006). Although, the mechanisms of action and the structure- activity relationships of these compounds have been reported only rarely and are worthy of future investigations, the analyses revealed that the dimerization of phenanthrenes and the presence of other products are a very important factors for the antioxidants activities (Zhang et al., 2005)

Although the phenolic content in CHE and EAE of Cc were lower than those of Tc and Ai, the antioxidant activity of these extracts were more efficient in some states. Antioxidant properties of single compounds within a group can vary remarkably, so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses (Parejo et al., 2002). This confirmed that in this case there wasn't significant correlation between phenolics concentration and anti oxidant activity which suggesting that this powerful antioxidant showed in CHE and EAE of Cc are due to the kind of phenolic content and/or other substance not determined. Our primary phytochemical analysis indicated that phenolic compounds are the major constituents of the Cc. Although no previous work was realized on Cc extracts

antioxidant activities and phytochemical structure, the studies which tested other species from the same genus, *Carthamus tinctorius*, reported that five flavonol glycosides (6-hydroxykaempferol 3-O-glucoside, quercetin 3-O-rutinoside, quercetin 3-O-glucoside, kaempferol 3-O-rutinoside, kaempferol 3-O-glucoside) and caffeic acid were isolated from EAE extract (Lim et al., 2007), which showed a strong antioxidant activities against the ABTS radical system. In the other hand the phytochemical screening demonstrated the presence of isoflavonoids, and different types of compounds like tannins, flavonoids and others (Akroum, 2009), which could be responsible for the diverse obtained antioxidant activities.

Even though the Ai extracts gave an anti oxidant activities lower than those of TC and Cc extracts it remain very effective compared with the used markers. This activity observed for Ai extracts may be due to one or more of the compounds found in *Ajuga iva* L. such as 8-O-acetyl harpagide, ajugarine, apigenin-7-O neohesperidoside, barpagide, caffeine, clorogenes, cyasterone, diglycerides, 14,15-dihydroajugapitin, ecdysones, ecdysterones, flavonoids, iridoides, makisterone A, phenylcarboxylic acids, neohesperidoside, tanninpolyphenols and naringin (Khafagy et al., 1979; Jannet et al., 1997). In addition no apparent toxicity was observed for this plant (El-Hilaly et al., 2004). The common responsible factor for antioxidant activity for three plants is the presence of phenolic compounds which were correlated to their chemical structures. Previous studies found that there was structure-activity relationship between antioxidant activity and total phenolic content in selected herbs and vegetables and fruits, phenolic compounds had a major contribution to antioxidant activity (Rice-Evans et al., 1996; Velioglu et al., 1998; Sun et al., 2002; Son and Lewis, 2002). Different reports are found on the literature; whereas some authors found correlation between the total phenolic content and the antioxidant activity, others found no such relationship. For instance, Velioglu et al (1998) reported a strong relationship between total phenolic content and total antioxidant

activity in selected fruits, vegetables and grain products, whereas Kahkönen et al. (1999) found no correlation on the same plant extracts containing phenolic compounds. In addition, in another study with citrus residues, no correlation was found (Bocco et al., 1998). There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants. The different antioxidant activities of phenolic extracts rich in phenolic compounds can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, the purity of active compounds, as well as the test system (Meyer et al, 1998). Determination of an absolute value for the antioxidant capacity of an extract is difficult because it depends on the actual concentration of the radical, its degradation during analysis, or matrix interference (Almela et al, 2006).

In general the antioxidant activity of phenolic compounds (PCs) reportedly varies with the structure and degree of hydroxylation of the aromatic ring (Burda and Oleszek, 2001; Aruoma, 2002). It is associated with the number of hydroxyl groups and the most active possess from 3 to 6 hydroxyl groups. Hydroxylation in the C₃ position seems to be detrimental for their antioxidant potency (Huguet et al., 1990). Fukumoto and Mazza (2000) reported that for benzoic and cinnamic acid derivatives, flavonols and anthocyanidins, an increase in the number of hydroxyl groups on the aromatic ring lead to higher antioxidant activity *in vitro*. Compounds with three hydroxyl groups on the phenyl ring of phenolic acids or the B-ring of flavonoids had high antioxidant activity. The loss of one hydroxyl group decreased activity slightly. The data obtained provide clear evidence that the radical-scavenging activity depends on the structure and the substituents of the heterocyclic and B rings, as suggested by Bors et al (1990). It has been proposed that PCs bearing a substituent in the ortho position form an intramolecular hydrogen bond, which is energetically favorable, resulting in easier donation of hydrogen atoms to free radicals (De Heer et al., 1999).

More specifically, the major determinants for radical-scavenging capability are (i) the presence of a catechol group in ring B, which has the better electron-donating properties and is a radical target, and (ii) a 2,3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. (iii) The presence of a 3-hydroxyl group in the heterocyclic ring also increases the radical-scavenging activity, while additional hydroxyl or methoxyl groups at positions 3, 5 and 7 of rings A and C seem to be less important (Figure 8).

Burda and Oleszek (2001) investigated the relationship between the structure of 42 flavonoids and their antioxidant and antiradical activities. They reported that flavonols with a free hydroxyl group at the C-3 position of the flavonoid skeleton showed the highest inhibitory activity to β -carotene oxidation. Antiradical activity depended on the presence of a flavonol structure or free hydroxyl group at the C-4' position. The effect of the 4'-hydroxyl was strongly modified by other structural features, such as the presence of free hydroxyls at C-3 and/or C-3' and a C2-C3 double bond.

In contrast glycosylation of flavonoids reduces their ability to scavenge radicals (Fukumoto and Mazza, 2000). It was reported that glycosylation of quercetin, cyanidin, pelargonidin and peonidin resulted in lower antioxidant activity and the addition of a second glycosyl moiety decreased activity further. This decrease in antioxidant effect was attributed to steric hindrance by carbohydrate moiety. Yang et al., (2001) found that quercetin exhibited the highest antioxidant activity among eight flavonols studied. When the 3-hydroxyl group of quercetin was glycosylated, as in rutin, the result was a significant decrease in antioxidant activity. It is important to note that under certain conditions phenolic antioxidants may act as promoters of free radicals and thus, act as pro-oxidants. Such conditions have been reported to include high concentrations of the phenoxy radicals resulting from low concentrations of synergistic antioxidants or a lack of reducing enzymes to regenerate the antioxidant from its phenoxy radical state (Patel et al., 2001).

The high chemical reactivity of phenolics compound is expressed in the binding affinity to biological polymers and heavy metal ions, and the ability to catalyze electron transport and to scavenge free radicals (Havsteen, 1983). PCs antioxidant activity is regarded to be related to 1) scavenging free radicals, 2) chelating transition-metals involved in free-radical production and 3) inhibiting the enzymes participating in free-radical generation (Yang et al., 2001; Aruoma, 2002). These compounds may also enhance the antioxidant activity of nonphenolic antioxidants by regenerating the oxidized forms of these compounds. For example, phenolic compounds have been reported to regenerate dehydroascorbic acid to its reduced form (Cossins et al., 1998). Furthermore, Pedrielli and Skibsted (2002) reported synergistic antioxidant interactions between flavonoids and α -tocopherol *in vitro*. Phenolic compounds may also impart antioxidant properties by functioning as chelators of metal ions that are capable of catalyzing oxidation (Gordon, 1990; Aruoma, 2002) which have been shown to complex with iron (Rice-Evans et al., 1996; van Acker et al., 1996) and copper ions (Brown et al., 1998) to provide secondary antioxidant effects. Many studies have reported that polyphenols show marked inhibition of enzymes responsible for the generation of ROS such as nitric oxide synthase (Wang and Mazza, 2002), tyrosine kinase (Traxler et al., 1999) and xanthine oxidase (Plessi et al., 1998; Boumerfeg et al., 2009, Baghiani et al., 2010). Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in reactive oxygen species generation (Korkina and Afanas'ev, 1997).

Based on our results and these reports, there can't be little doubt that extracts of Tc, Cc and Ai can inhibit oxidative processes leading us to conclude that the extract has substantial antioxidant activity *in vitro*. The antioxidative activity of phenolic compounds is mainly due to their redox properties, which can play an important role in neutralizing free radicals,

quenching oxygen species. These antioxidant activities could have contributed, at least partly, to the therapeutic benefits of traditional use of these plants.

Conclusion and perspectives

The aim of this work was to investigate the antioxidants activity of *T. communis*, *C. caeruleus* and *A. iva* extracts. Antioxidants activity screening leads to the conclusion that the extracts contain active compounds. The obtained fractions were tested for their antioxidant activity. The results suggest that the various antioxidant mechanisms of Tc, Cc and Ai extracts may be attributed to xanthine oxidase inhibition, metal chelating ability, hydrogen donating ability, reducing power and their effectiveness as strong scavengers of superoxide and other free radicals. The enzymatic method had not gave a clear cut that the extracts have a real scavenger effects on $O_2^{\cdot-}$, because they exhibited a high scavenging effects on $O_2^{\cdot-}$ generated by XO and a strong inhibitory effects on XO. Therefore, a nonenzymatic method for scavenging effects on $O_2^{\cdot-}$ was realized. The results showed that the extracts of the studied plants, are a good superoxide radical scavengers. The erythrocyte membrane contains abundant polyunsaturated fatty acids which are very susceptible to free radical induced peroxidation, data obtained in this study proved a relationship between the inhibition percentage of hemolysis and chelating power of plants extracts suggesting that their action as anti hemolytic may be related to their iron-binding capacity. Further, to confirm the possibility that extracts can act as direct antioxidant by possessing antilipoperoxidative mechanism, which may prevent RBC hemolysis, tow other assay were applied; TBARS assay and β -carotene/linoleic acid which displayed an antiradical effect approximatel in the same array, which indicate that Tc, Cc and Ai extracts possesses a substantial protective effect and free radical scavenging mechanism against lipid peroxidation. Since the compounds with reducing power indicate that they are electron /or hydrogen donors can reduce the oxidized intermediates of lipid peroxidation, therefore the DPPH and FRAP assay were realised. Results obtained in this study demonstrated that these extracts possess a good reducing power in the same order and that can prevent lipide peoxidation then prevent many oxidative

damage. The various antioxidant mechanisms action of the extracts in their antioxidant activity appeared to be identical, throughout the good correlation observed between different applied techniques however, the magnitude of antioxidative potency varies with the type of extracts, which being related to the content in phenolic compounds and other yet to be discovered antioxidant compounds. These results are preliminary, it would be interesting to test the activity of high purified fractions and isolate the responsible molecules underlie the various detected activities in different extracts by more efficient methods. In any case it is important to highlight that the majority of the test were performed *in vitro*. It is thus mandatory to confirm these findings by *in vivo* studies so as to obtain useful information for eventual therapeutic or dietary interventions

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