الجمهورية الجزائرية الديمقراطية الشعبية

وزارة التعليم العالي و البحث العلمي

Université Ferhat Abbas Sétif 1
Faculté des sciences de la nature et de la vie

جامعة فرحات عباس
سطيف 1
كلية علوم الطبيعة و الحياة

DEPARTEMENT OF BIOCHEMISTRY

N°...../SNV/**2018**

THESIS

Presented by

AOUACHRIA Sana

For the fulfillment of the requirements for the degree of

DOCTORATE OF SCIENCES

BIOLOGY

Special filed: BIOCHEMISTRY

TOPIC

Contribution to the phytochemical study and evaluation of the *in vitro* and *in vivo* antioxidant activity of *Reichardia picroides* L.

Presented publically in: **/ / 2018**

JURY

President:	**DAHAMNA Saliha**	**Pr. UFA Setif 1**
Supervisor:	**BOUMERFEG Sabah**	**Pr. Univ. Bordj bouarreridj**
Co-Supervisor:	**BAGHIANI Abderrahmane**	**Pr. UFA Setif 1**
	LAROUI Saleh	**Pr. Univ. Batna 2**
Examiners:	**KHATEL Bachra**	**Pr. Univ. Bejaia**
	ZERARGUI Fatima	**MCA. UFA Setif 1**

Laboratory of applied biochemistry

ACKNOWLEDGEMENTS

First of all, I am grateful to *Al-mighty ALLAH* who give me strength and perseverance, whatever I am today is just because Him (*ALLAH subhana-Wa-Taala*).

I would like to express my appreciation and grateful thanks to my supervisor **Pr. BOUMERFEG Sabah** for her advice, guidance, encouragements and support throughout my doctoral work.

I would deeply like to thank my co-supervisor, **Pr. BAGHIANI Abderrahmane** for his enriching advices, keen interest, guidance, support and availability throughout my doctoral work.

I would also like to show my gratitude for my committee and their willingness to read my thesis and give critiques and comments in order to make it a better thesis. Thank you to **Pr. DAHAMNA Saliha**, **Pr. LAROUI Saleh**, **Pr. KHATEL Bachra** and **Dr. ZERARGUI Fatima**.

I want to offer my special thanks to **Pr. ARRAR Lekhmissi** for his valuable advices, continuous support, assistance, and to all other technical supports. I am also thankful to **Pr. CHAREF Noureddine, Pr. KHENNOUF Seddik**, **Pr. AMIRA Smain, Pr. DAHAMNA Saliha** and **Dr. AMIRA Fatima** for help and provision of research facilities in their laboratories.

My sincere gratitude to **Dr. BENBACHA Faycal**, Head of anapathology laboratory of Hospitalo-Public Establishment (HPE) of Bordj Bou-Arreridj, for providing all the facilities to realise histological slides and interpreting them. I am also thankful to **Dr. TOUABTI**, Head of Central laboratory of University Hospital of Setif, for providing the facilities to evaluate biochemical parameters.

My thanks are also extended to **Pr. ABU ZARGA Mussa**, Faculty of Science, the University of Jordan, Amman, Jordan for the identification and structural elucidation of the isolated compound.

Many thanks send to all my colleagues in laboratory of applied biochemistry, who provided the most amicable and lively working environment.

ملخص

تستخدم نبتة (*Reichardia picroides* (*R. picroides* أساسا لأغراض غذائية، و تستعمل تقليديا كمخفضة لنسبة السكر في الدم و مدرة للبول و للحليب و منشطة. وعلى حد علمنا، لم تتم الإشارة إلى أي دراسات تتمحور حول سمية النبتة و نشاطها المضاد للأكسدة. تهدف دراستنا إلى معرفة التركيب الكيميائي للنبتة و تقييم سميتها وكذا نشاطها المضاد للأكسدة مخبريا و في الحي. تم استخلاص متعدد الفينول متبوعا بفصل أولي اعتمادا على قطبية المذيب المستخدم لإعطاء المستخلص الخام (CrE) و مستخلص الكلوروفورم (ChE) ومستخلص الإيثيل أسيتات (EAE) و المستخلص المائي (AqE). سمح الفصل على كروماتوغرفيا العمود على هلام السيليكا بالحصول على 12 جزء (F1-F12)، كما خضعت كل تجزئة إلى التحليل على كروماتوغرفيا الطبقة الرقيقة. تم عزل المركب النقي lutéoline 7-O-β-glucoside، الذي ينتمي إلى الفلافون، من F10 وتم تحديد بنيته الكيميائية بواسطة جهاز القياس الطيفي للرنين المغناطيسي النووي. أظهرت نتائج التحليل الكمي أن أعلى كمية من الفينول الكلي والفلافونويد سجلت في EAE (331.64 ± 3.11 ميكروغ مكافئ غرامي حمض الغاليك/ مغ من المستخلص و 48.14 ± 0.70 ميكروغ مكافئ غرامي كارستين/ مغ من المستخلص، على الترتيب). وقد تم إجراء عدة تجارب مخبريا بغية تحديد النشاط المضاد للأكسدة لـ CrE و أجزائه. كشف CrE و أجزاؤه عن نشاط جد معتبر مضاد للانحلال الدموي وذلك بتسجيل زيادة في قيم HT 50 التي تراوحت من 76.92 ± 3.59 دقيقة بالنسبة للشاهد و 188.15 ± 1.91 دقيقة بالنسبة لـ EAE. وُجد أن F12 يملك أقوى نشاط قابط للأيون مساو لحد كبير لنشاط EDTA. أبدى EAE أقوى قدرة إرجاعية مساوية لقدرة Vit C. سجل F5 أعلى قدرة إزاحية لـ H_2O_2 والتي تفوق لحد كبير قدرة BHT. سُجل تثبيط لابيضاض البيتاكاروتين خلال 24 ساعة في وجود كلا من ChE و EAE و F1 و F10 و F11.كشف اختبار إزاحة جذر DPPH أن EAE و ChE و F12 يملكون قدرة إزاحية جد عالية. تم تقييم السمية الحادةللنبتة على الفئران، وذلك لتسجيل الأعراض المصاحبة للسمية و عدد الوفيات المحتملة للحيوانات لمدة أسبوعين لحساب متوسط الجرعة المميتة (LD50) لـ CrE. أظهرت النتائج أن الجرعات المعطاة لم تتسبب في حدوث أي وفيات أو تغيرات في السلوك العام للفئران الذكور و الإناث، كما وجد أن LD50 يفوق 5000 مغ / كغ. وفيما يخص دراسة السمية فوق الحادة، فقد تم إعطاء CrE بجرعات 250 و 500 و1000 مغ / كغ / يوم لمدة 21 يوما متتالية. و تبيّن أن الجرعات بين 500 و 1000 مع / كغ تسبب تغيرات في أنسجة الكبد والكلى. أجريت دراسة على الفئران الذكور من أجل تقييم القدرة المضادة للأكسدة، حيث تم معاملتها بـ CrE بجرعات 250 و 500 و1000 مع / كغ / يوم لمدة 21 يوما متتالية. أثبتت النتائج زيادة ملحوظة في مستوى GSH و انخفاضا في مستوى MDA في كل من الكبد والكلى، فيما لوحظ تحسن طفيف للقدرة المضادة للأكسدة للبلازما و للتأثير المضاد للانحلال الدموي. و عليه نستنتج أن *R. picroides* تملك قدرة مضادة للأكسدة جد عالية سواءا مخبريا أو في الحي، بالإضافة إلى أنها غير سامة و آمنة عند استعمال جرعات ≤ 250 مغ/كغ.

الكلمات المفاتيح : *R. picroides*، البوليفينول، الفلافونويد، الإجهاد التأكسدي، السمية الحادة، السمية فوق الحادة.

ABSTRACT

Reichardia picroides (*R. picroides*) is a species mainly used for daily source; it is used in the traditional medicine as hypoglycemiant, diuretic, depurative, galactagogue and tonic agent. To our knowledge, there are no studies on the antioxidant effect and the toxicity of this plant. The objective of the present study was, therefore, to evaluate the antioxidant activity and the toxicity of crude extract (CrE) and its fractions *in vitro* and *in vivo*. Polyphenols extraction and fractionation from plant material was performed using solvent of different polarity giving the following phases: CrE, chloroform extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). The CrE was then subjected to silica gel column chromatography which results in 12 fractions (F1-F12). Each fraction was subjected to TLC analysis. The pure compound was isolated from F10 and its structure was established by NMR spectroscopy as luteolin 7-O-β-glucoside. Results of polyphenols estimation showed that the highest amount of both total phenols and flavonoids was found in EAE (331.64 ± 3.11 µg gallic acid equivalent (GAE) / mg of extract and 48.14 ± 0.70 µg quercetin equivalent (QE) / mg of extract, respectively). To determine the antioxidant potential of CrE and its fractions, several *in vitro* assays were carried out. The CrE and its fractions had a very important ($p < 0.0001$) antihemolytic activity and revealed an extension of HT 50 from 76.92 ± 3.59 min of control to 188.15 ± 1.91 min of EAE. The F12 was found to have the strongest ion chelating activity comparable to that of EDTA. The EAE appeared to have the strongest reducing power closer to that of Vit C. The F5 found to have the strongest H_2O_2 scavenging effect higher than that of BHT. The bleaching of β-carotene is highly inhibited in the presence of ChE, EAE, F1, F10 and F11.The DPPH scavenging assay revealed that EAE, ChE and F12 possessed a very strong radical scavenging effect. The acute toxicity of CrE was carried out using mice. Signs accompanying toxicity and possible death of animals were monitored for two weeks to ascertain the median lethal dose (LD_{50}) of the CrE. The administrated doses did not resulted mortality or changes in general behaviors. The LD_{50} was found to be superior to 5000 mg/kg. In subacute toxicity study, the CrE was administered by gavage at the doses of 250, 500 and 1000 mg/kg/day for 21 consecutive days. Daily administration of CrE at doses ranged from 500 to 1000 mg/kg resulted in alteration of liver and kidney tisssues. *In vivo* approach was performed by administration three doses 250, 500 and 1000 mg/kg to male mice. The analysis of antioxidant potential parameters revealed that the CrE administration increased significantly the level of GSH and decreased the level of MDA in both liver and kidney. The CrE supplementation improved slightly the plasma antioxidant status and the anti-hemolytic protective effect which remained non significant compared to the control group. It can therefore be suggested that *R. picroides* had potent antioxidant activities both *in vitro* and *in vivo*. The use of this extract is safe at doses ≤ 250 mg/kg.

Key words : *R. picroides*, polyphenols, flavonoids, oxidative stress, acute toxicity, subacute toxicity.

RESUME

Reichardia picroides (*R. picroides*) est une espèce principalement utilisée à des fins alimentaires, et traditionnellement comme hypoglycémiante, diurétique, dépurative, galactagogue et tonique. A notre connaissance, aucune étude n'a signalé à la fois sa sécurité et ses activités antioxydantes. L'objectif de la présente étude était donc de cribler les composés phytochimiques de l'extrait brut de la plante (CrE), d'évaluer son profil de sécurité ainsi que d'étudier l'activité antioxydante de CrE et de ses fractions *in vitro* et *in vivo*. L'extraction des polyphénols a été réalisée en se basant sur la polarité du solvant utilisé pour donner le CrE, l'extrait de chloroforme (ChE), l'extrait d'éthyle d'acétate (EAE) et l'extrait aqueux (AqE). Le CrE a été soumis à une chromatographie sur colonne de gel de silice qui a permis d'obtenir 12 fractions (F1-F12) en suivant leur profil de CCM. Le composé pur a été isolé à partir de F10 et sa structure a été établie par l'utilisation de la spectroscopie RMN. Il s'agit d'un flavone: lutéoline 7-O-glucoside. La teneur en phénols totaux et en flavonoïdes a été quantifiée à l'aide de méthodes colorimétriques. Les résultats ont montré que la plus grande quantité de phénols totaux et de flavonoïdes a été trouvée dans l'EAE (331.64 ± 3.11 µg équivalent d'acide gallique (GAE) / mg d'extrait et 48.14 ± 0.70 µg équivalent de quercétine (QE) / mg d'extrait, respectivement). Pour déterminer le potentiel antioxydant de CrE et de ses fractions, plusieurs essais *in vitro* ont été réalisés. Le CrE et ses fractions ont eu une activité antihémolytique très importante ($p < 0.0001$) et ont révélé une extension de HT 50 varie de 76.92 ± 3.59 min de contrôle à 188.15 ± 1.91 min d'EAE. La F12 s'est avérée avoir l'activité de chélation des ions la plus forte significativement proche à celle de l'EDTA. L'EAE semble avoir le pouvoir réducteur le plus fort, similaire à celui de la Vit C. La F5 s'est avérée avoir l'effet piégeur de H_2O_2 le plus fort qui significativement plus élevé que celui du BHT. Le blanchissement du β-carotène est fortement réduit pendant les 24 heures en présence de ChE, EAE, F1, F10 et F11. Le test de piégeage de DPPH a révélé que l'EAE, ChE et F12 possédaient un très fort effet antiradicalaire. La toxicité aiguë de CrE a été réalisée sur les souris. Les signes d'une toxicité accompagnant la mort possible des animaux ont été suivis pendant deux semaines afin de déterminer la dose létale médiane (DL50) de CrE. Les doses administrées n'ont pas provoqué de mortalité ou de changements dans les comportements généraux des souris testés. La DL50 s'est révélée supérieure à 5000 mg / kg. Dans l'étude de toxicité subaiguë, le CrE a été administré par gavage à des doses de 250, 500 et 1000 mg / kg / jour pendant 21 jours consécutifs. L'administration quotidienne de CrE à des doses allant de 500 à 1000 mg / kg pourrait entraîner une altération de l'histologie du foie et des reins. Une approche *in vivo* a été réalisée par l'administration de trois doses (250, 500 et 1000 mg / kg) à des souris mâles. L'analyse des paramètres du potentiel antioxydant a révélé que l'administration de CrE a augmenté significativement le niveau de GSH et a diminué le taux de MDA dans le foie et les reins. Le statut antioxydant plasmatique et le pouvoir antihémolytique s'est amélioré légèrement par suite à une supplémentation de CrE. En conclusion, on peut suggérer que *R. picroides* a eu des activités antioxydantes puissantes à la fois *in vitro* et *in vivo*. De plus, elle était non toxique en administration aiguë ainsi que l'utilisation de son extrait brut est sûre à des doses ≤ 250 mg / kg.

Mots clés : *R. picroides*, polyphénols, flavonoids, stress oxidatif, toxicité aiguë, toxicité subaiguë

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LIST OF ABBREVIATIONS

AAPH	2,2'-Azobis (2-amidinopropane) dihydrochloride
$AlCl_3$	Aluminium chloride
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AqE	Aqueous extract
AST	Aspartate aminotransferase
BHT	Butylated hydroxytoluene
CAT	Catalase
ChE	Chloroform extract
CrE	Crude extract
DPPH	1,1-diphenyl-2 picrylhydrazyl
DTNB	5,5 0- dithiobis-(2-nitrobenzoic acid)
EAE	Ethyle acetate extract
EC 50	Effective concentration of 50%
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HT50	Half-time of 50% hemolysis
iNOS	Inducible nitric oxide synthase
LD50	Lethal dose 50
MDA	Malondialdehyde
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
$\bullet OH$	Hydroxyl radical
$O_2\bullet^-$	Superoxide
OECD	Organisation for economic co-operation and development
$ONOO^-$	Peroxynitrite
QE	Quercetin equivalent
R. picroides	*Reichardia picroides*
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Vit C	Vitamin C
XO	Xanthine oxidase

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Materials and methods

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INTRODUCTION

Introduction

It is currently hypothesised that many diseases are due to oxidative stress that results from an imbalance between the formation and detoxification of pro-oxidants. Excessive or uncontrolled production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause damage to nucleic acids, proteins and lipids and this is closely associated with human disease pathogenesis such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular, and neurodegenerative diseases.

Under physiologic conditions, cellular ROS and RNS accumulation is controlled by a battery of redundant endogenous antioxidant defence systems, both enzymatic and non-enzymatic. To reinforce the spontaneous antioxidant system that may fail under certain conditions, consumption of natural antioxidants from various food supplements and traditional medicines is necessary. Indeed, there is increased interest among phytotherapy researchers to use medicinal plants with antioxidant activity for protection against oxidative stress since they tend to be accessible to everyone, safer and inexpensive.

Reichardia picroides (*R. picroides*), belongs to Asteraceae family, is one of eight *Reichardia* species growing in Algeria. The plant is known for its alimentary purposes. However, *R. picroides* leaves are also traditionally known to be used as hypoglycemiant, diuretic, galactagogue, emollient, depurative of intestine, and tonic. Furthermore, *R. picroides* roots are used for cough, abdominal pains and kidney problems.

To the best of our knowledge, any work has been carried out on the chemistry, toxicity profile and biological activities of the *R. picroides* (aerial and root parts). Therefore, it was on this basis that this study aimed to achieve the following objectives:

- ✓ Phytochemical screening of plant crude extract (CrE) including: extraction, fractionation, purification, characterization of obtained molecule and quantification of phenolic compounds.

- ✓ Study of antioxidant activity of CrE and its fractions *in vitro* and *in vivo*:
    - Evaluation of the protective effect against oxidative erythrocytes hemolysis.
    - Evaluation of transition metals chelating activity.
    - Evaluation of reducing power.
    - Evaluation of scavenging activity versus hydrogen peroxide (H_2O_2).
    - Evaluation of anti-lipoperoxidation activity using β-carotene bleaching assay.
    - Evaluation of scavenging activity versus a relatively stable free radical (1,1-diphenyl-2 picrylhydrazyl, DPPH).
    - Effect of CrE on improving overall defences antioxidants in mice by determining the total antioxidant capacity of the blood, plasma and tissues.
- ✓ Assessment of acute and subacute toxicity of plant CrE.

REVIEW OF LITERATURE

1. Oxidative stress

1.1. Definition

Oxidative/nitrosative stress results from an imbalance between the formation of ROS/RNS and the impaired ability of an organism to detoxify these reactive intermediates or to repair the damage that they cause (Poprac *et al.*, 2017).

1.2. Forms of ROS and RNS

In the aerobic process, cells metabolize approximately 95% of the oxygen (O_2) to water, without formation of any toxic intermediates. However, a minimal 5% of O_2 is stepwise reduced to a series of intermediate species producing ROS (Ye *et al.*, 2015). Through these steps, three highly toxic species are formed, two of them being free radicals: superoxide ($O_2^{\bullet -}$) and hydroxyl radical (•OH). Hydrogen peroxide (H_2O_2) is still a highly reactive compound, but not a radical in strict sense (Pisoschi and Pop, 2015).

The ROS and RNS are represented by both radical and non-radical forms as showed in table 1.

Table 1. Nomenclature of ROS and RNS (Halliwell and Whiteman, 2004).

Reactive oxygen and nitrogen species	
Free radicals	**Non-radicals**
Superoxide ($O^{\bullet -}_2$)	Hydrogen peroxide (H_2O_2)
Hydroxyl (•OH)	Hypobromous acid (HOBr)
Hydroperoxyl ($HO^{\bullet}_2$)	Hypochlorous acid (HOCl)
Peroxyl ($RO^{\bullet}_2$)	Ozone O_3
Alkoxyl (RO•)	Singlet oxygen (O^1_2)
Carbonate ($CO^{\bullet -}_3$)	Org4anic peroxides (ROOH)
Carbon dioxide ($CO^{\bullet -}_2$)	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
Nitric oxide (NO•)	Nitrous acid (HNO2)
Nitrogen dioxide ($NO^{\bullet}_2$)	Nitrosyl cation (NO^+)
	Nitroxyl anion (NO^-)
	Dinitrogen tetroxide (N_2O_4)
	Dinitrogen trioxide (N_2O_3)
	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
	Nitronium (nitryl) cation (NO_2^+)
	Alkyl peroxynitrites (ROONO)
	Nitryl chloride (NO_2Cl)

The $O^{\bullet -}_2$ is considered as primary ROS. It results from one electron reduction of O_2 by various oxidases such as NADPH oxidase, xanthine oxidase (XO), aminoacid oxidase and cyclooxygenase. It may also be formed in the mitochondrial electron transport chain, in the course of oxidative phosphorylation (Lanciano *et al.*, 2013).

The H_2O_2 is resulted from the conversion of $O^{\bullet -}_2$ either spontaneously or by the superoxide dismutase (SOD). It can be generated by any system yielding $O^{\bullet -}_2$, as the radical anion readily disproportionate (Briben *et al.*, 2012).

The H_2O_2 is able to produce highly reactive radicals as a result of its interaction with metal ions through Fenton reaction. The H_2O_2 is broken down into a hydroxide ion and a •OH. Similarly, the one-electron reduction of H_2O_2 by $O^{\bullet -}_2$ has also been invoked as a potential source of •OH in the presence of metal catalysis as iron or copper through the Haber-Weiss reaction (Toro and Rodrigo, 2009). The •OH has been reported as the most powerful oxidizing radical that can interact at the site of its generation with most organic and inorganic molecules: DNA, proteins, lipids, amino acids, sugars, and metals (Toro and Rodrigo, 2009).

Endogenous NO• is biosynthesized from L-arginine, oxygen and NADPH, by enzymes belonging to nitric oxide synthase (NOS) class or by reduction of inorganic nitrate. It reacts with $O^{\bullet -}_2$, giving a highly reactive specie, namely peroxynitrite ($ONOO^-$), a powerful oxidant versus many biological molecules. The $ONOO^-$ can be decomposed to yield •OH, independently on the presence of transition metals (Pisoschi and Pop, 2015).

1.3. Sources of ROS and RNS

Both endogenous and exogenous sources contribute to intracellular ROS/RNS levels.

1.3.1. Endogenous sources

- ✓ *Mitochondria*

Mitochondria are thought to be the largest contributors to intracellular oxidant production (Holmström and Finkel, 2014). It contains numerous redox enzymes capable of transferring

single electron to the O_2 generating the $O^{\bullet -}_2$ through the tricarboxylic cycle enzymes, electron-transport chain complexes I, II and III, among others enzymes (Murphy, 2009).

- ✓ *Endoplasmic reticulum*

The endoplasmic reticulum contains enzymes that catalyze a series of reactions for detoxifying liposoluble molecules and other toxic metabolic products. The enzymes such as cytochrome p-450 and b5 enzymes and diamine oxidase contribute to the formation of ROS (Toro and Rodrigo, 2009). The ROS may be generated as byproducts of the protein folding machinery in the endoplasmic reticulum (Malhotra and Kaufman, 2007).

- ✓ *Peroxysomes*

The presence of enzymes that produce ROS in peroxysomes indicates that peroxisomes are involved with the metabolism of ROS. Peroxisomes are one of the major sites of intracellular H_2O_2 production since they contain numerous enzymes producing H_2O_2, glycolate oxidase, urate oxidase, aspartate oxidase, XO, NOS and acyl CoA oxidases (del Río and López-Huertas, 2016). A part from H_2O_2, it has been demonstrated that peroxisomes also produce $O^{\bullet}_2^-$ and NO• as a consequence of their normal metabolism. Currently, there is no evidence that mammalian peroxisomes contain enzymes that produce •OH or $ONOO^-$ (Lismont *et al.*, 2015). However, H_2O_2 inside peroxisomes may give rise to •OH through the Fenton reaction. In addition, as these organelles contain enzymatic sources of $O^{\bullet}_2^-$ and NO•, and the reaction of NO• with $O^{\bullet}_2^-$to form $ONOO^-$ is kinetically and thermodynamically favored, it is very likely that peroxisomes also generate $ONOO^-$ (Lismont *et al.*, 2015).

- ✓ *NADPH oxidase*

The NADPH oxidases comprise a family of proteins able to transfer electrons from NADPH across cellular membranes; electrons react with O_2 generating $O^{\bullet -}_2$ and attendant other downstream ROS (Hansen *et al.*, 2018). Several physiological functions have been related with ROS generation by the NADPH oxidases family, including cell signalling, host defence,

control of ventilation, smooth muscle relaxation, control of erythropoietin production and other hypoxia-inducible functions (Holmström and Finkel, 2014)

- ✓ *Xanthine oxidase (XO)*

The XO catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid and is a well-known producer of $O\bullet_2^-$ (Halliwell and Gutteridge 2015). Xanthine oxidoreductases are present in two forms, depending on their electron acceptor. Xanthine dehydrogenase uses NAD^+, and XO uses O_2 to produce $O\bullet_2^-$. Under pathological conditions, such as tissue ischemia, xanthine dehydrogenase can be converted to XO (Toro and Rodrigo, 2009).

- ✓ *Nitric oxide synthase (NOS)*

The NOS are a family of enzymes that convert the amino acid L-arginine to L-citrulline and NO. The NOS family contains three isoforms that are regulated by distinct genes: a constitutive neuronal NOS (nNOS or NOS I), an endotoxin- and cytokine-inducible NOS (iNOS or NOS II) and a constitutive endothelial NOS (eNOS or NOS III). The nNOS performs an important role in intracellular communication. The iNOS uses NO to induce oxidative stress on pathogens. The eNOS plays a major role in the regulation of vascular function (Toro and Rodrigo, 2009; Lee *et al.*, 2016).

- ✓ *Arachidonate cascade enzymes*

The enzyme 5-lipoxygenase has been identified as an inducible source of ROS production in lymphocytes. The 5-lipoxygenase was shown to be involved in the production of H_2O_2 by T lymphocytes after ligation of the CD28 costimulatory receptor and in response to interleukin-1β (Vašková *et al.*, 2012).

Cyclooxygenase-1 has been implicated in ROS production through formation of endoperoxides, which are susceptible to scavenging by some antioxidants in cells stimulated with TNF-α, interleukin-1 or bacterial lipopolysaccharide (Vašková *et al*., 2012).

- ✓ *Auto-oxidation*

The auto-oxidation of molecules such as dopamine, adrenaline, flavins and hydroquinones can also be an important source of intracellular ROS production. The direct product of these auto-oxidations is often $O^{\bullet}{}_2^-$ (Vašková *et al.*, 2012).

1.3.2. Exogenous sources

The ROS are also produced in the biological systems by various exogenous sources.

Cigarette smoke contains many oxidants, free radicals and organic compounds, such as $O^{\bullet}{}_2^-$ and NO•. In addition, inhalation of cigarette smoke into the lung also activates some endogenous mechanisms, such as accumulation of neutrophils and macrophages, which further increase the oxidant injury (Briben *et al.*, 2012).

Ozone exposure can cause lipid peroxidation in the epithelial lining fluid where reactive aldehydes and hydroperoxides are produced and these products may activate epithelial nerve endings or stimulate the release of chemokines from respiratory epithelium that recruit inflammatory cells to the airways. The ROS may be released by polymorphonuclear leukocytes, alveolar macrophages, and other inflammatory cells following ozone exposure, contributing to the epithelial injury (Hiraiwa and van Eeden, 2013).

Hyperoxia refers to conditions of higher oxygen levels than normal partial pressure of O_2 in the lungs or other body tissues. It leads to greater production of ROS and RNS (Berkelhamer *et al.*, 2013).

Ionizing radiation, in the presence of O_2, converts •OH, $O^{\bullet}{}_2^-$, and organic radicals to H_2O_2 and organic hydroperoxides. These hydroperoxide species react with redox active metal ions, such as iron and copper, via Fenton reactions and thus induce oxidative stress (Azzam *et al.*, 2012).

Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, can induce generation of reactive radicals via a Fenton-type reaction (Shahid *et al.*, 2014).

1.4. Biological roles of ROS and RNS

Depending on the concentration, location, and molecular context, ROS can be beneficial or harmful to cells. Increasing evidence indicates that low to moderate concentrations of ROS are indispensable in regulating cellular processes.

They are involved in ion channel/transporter function (Kiselyov and Muallem, 2016), Ca^{2+} spark production (Görlach *et al.*, 2016), protein kinase/phosphatase activation such as the mitogen activated protein kinase and extracellular-signal-regulated kinase pathways that alter gene expression, as well as in coordination with SOD initiates cell death (Son *et al.*, 2011).

The ROS are also involved in gene transcription, signal transduction and regulation of other activities in cell (Zhang *et al.*, 2016). Moreover, ROS are important components of the anti-microbial defence mechanism incorporated by macrophages and neutrophils (Slauch, 2011).

On the other hand, RNS produced by neurons act as neurotransmitters and those generated by phacocytes (monocytes, macrophages and neutrophils) act as mediators of immunity. These are also responsible for leukocyte adhesion, thrombosis, angiogenesis, vascular tone and enzyme activity modulation (Madamanchi and Runge, 2013).

1.5. Molecular targets of ROS and RNS

Since these species are highly reactive, they can damage all the three important classes of biological molecules including nucleic acids, proteins, and lipids.

1.5.1. Nucleic acids

Most of the long-term effects of oxidative stress are inflicted by modifications of DNA which involves degradation of bases, single- or double-stranded DNA breaks, bases or sugar-bound modifications, mutations, deletions or translocations, and cross-linking with proteins. Most of these DNA modifications are highly relevant to carcinogenesis, aging, neurodegenerative, cardiovascular, and autoimmune diseases (Al-Dalaen and Al-Qtaitat, 2014).

1.5.2. Proteins

Oxidation of proteins may occur directly as protein side chains are oxidized leading to a loss of function of proteins and a deactivation of enzymes. Often, thiols of proteins involved in the regulation of enzyme activity are directly oxidized. Conformational changes leading to an increase in hydrophobicity may result in aggregation or precipitation of proteins, which can no longer be subjected to the normal protein degradation pathway. Additionally, oxidative damage of proteins may occur by the adduction of secondary products like glycoxidation or lipoxidation (Stadtman and Levine, 2006).

1.5.3. Lipids

Lipid peroxidation of membranes occurs as a consequence of direct reaction of polar lipids fatty acids with oxygen or a reaction catalyzed either by metals or by NADPH cytochrome P-450 reductase. It leads to the formation of free radical intermediates and semistable peroxide. Increased levels of secondary products like conjugated dienes, hydrocarbon gases and carbonyl compounds, that initiate inflammatory reaction, and decreased levels of polyunsaturated fatty acid have been demonstrated. Oxidation of circulating lipoproteins is an important step in pathogenesis of atherosclerosis (Valko *et al.*, 2006).

1.6. Pathologic implications of oxidative stress

There is a growing body of literature supporting crucial roles for ROS and RNS in the pathogenesis of many diseases. Table 2 lists some of these diseases.

Table 2. Some diseases implicate oxidative stress.

Pathology	**References**
Rheumatoid arthritis	(Mateen *et al.*, 2016)
Cardiovascular diseases	(Kanaan and Harper, 2017)
Diabete mellitus	(Asmat *et al.*, 2015)
Cancer	(Moloney and Cotter, 2017)
Inflammation	(Blaser *et al.*, 2016)
Alzheimer' disease	(Tramutola *et al.*, 2017)
Parkinson disease	(Crotty *et al.*, 2017)

1.7. Defence system against oxidative stress

The concept of biological antioxidant refers to any compound that, when present at a lower concentration compared to that of an oxidizable substrate, is able to either delay or prevent the oxidation of the substrate (Godic *et al.*, 2014).

After ROS and RNS exposure from different sources, living organisms have a series of defence mechanisms against the oxidative stress including: preventative mechanisms, repair mechanisms, physical defences and antioxidant defences. The antioxidant defence mechanisms can be divided into enzymatic and non-enzymatic defences.

1.7.1. Enzymatic antioxidants

- ✓ *Superoxide dismutase (SOD)*

The SODs are classified by their metal cofactors into known types: Cu/ZnSOD and MnSOD, which are localized in different cellular compartments. The Cu/Zn SOD is mainly extracellular and cytosolic, while MnSOD is a mitochondrial enzyme (Vašková *et al.*, 2012). It is considered as a first line of defence against ROS. The SOD catalyses the conversion of $O^{\bullet -}_2$ into H_2O_2 and O_2. It provides protection against ROS induced cellular and histological damages. The SOD reacts very rapidly with NO thereby reducing the bioactivity of NO and producing the $ONOO^-$ (Fukai and Ushio-Fukai, 2011).

- ✓ *Catalase (CAT)*

The CAT maintains the physiological concentration of H_2O_2. It converts H_2O_2 catalytically into H_2O and O_2 and thus neutralizes it (Asmat *et al.*, 2016). The CAT exerts its peroxidase activity *in vivo*. It can also catalyze the reaction of oxidation, by H_2O_2, of numerous metabolites and toxins. Its basic function is to remove H_2O_2 and peroxide ROOH in molecular oxygen in order to prevent irreversible damage to the membranes (Kıvrak *et al.*, 2017). The CAT also binds NADPH as a reducing equivalent to prevent oxidative inactivation of the enzyme by H_2O_2 as it is reduced to water (Briben *et al.*, 2012).

- ✓ *Glutathione peroxidase (GPx)*

The GPx is a selenoenzyme whose catalytic function depends on the presence of the mineral in the enzyme. It has been known to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or the corresponding alcohols, respectively, typically using glutathione (GSH) as reductant. The presence of selenium as the catalytic moiety was suggested to guarantee a fast reaction with the hydroperoxide and a fast reducibility by GSH (Brigelius-Flohé and Maiorino, 2013).

1.7.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants include different chemical compounds such as tocopherol (vitamin E), ascorbic acid (vitamin C, Vit C), caretinoids, GSH, phenolic compounds, ubiquinol (coenzyme Q10), phospholipids (proteoglycans and hyaluronic acid), lipoic acid, proteins binding free iron and copper (ceruloplasmin, transferrin, taurine, albumin), protein hydrolysates, bilirubin, melatonin, uric acid, mucin, surfactant, amino acids, peptides, and phytates (Mirończuk-Chodakowska *et al.*, 2017).

- ✓ *Glutathione (GSH)*

The GSH, a tripeptide (γ-L-glutamyl-L-cysteinylglycine), is an endogenous antioxidant and an important cellular defence agent against oxidative damage. Under normal physiological conditions, GSH is mainly reduced. However, under pathological conditions, the GSH/GSSG ratio can decrease significantly. The pentose phosphate pathway regulates the GSH/GSSG ratio by providing NADPH which is required for the reduction of GSSG to GSH by GSH reductase (Aquilano *et al.*, 2014). The GSH can directly scavenge ROS such as H_2O_2 and •OH or indirectly through the reaction catalyzed by GPx. Moreover, GSH prevents the oxidization of sulfhydryl groups in the protein structure. It is especially important for the activity of GPx, GSH reductase and GSH-S-transferase (Kıvrak *et al.*, 2017). The GSH can regenerate other antioxidants such as Vit C and vitamin E to their active forms (Lü *et al.*, 2010).

- ✓ *Vitamin C (Vit C)*

The Vit C is a water-soluble dietary supplement, thus it acts in the aqueous environments of the body, along with the antioxidant enzymes. The role of Vit C as an antioxidant is indicated by its known free radical-scavenging action. As a reducing and antioxidant agent, it directly reacts with $O_2^{\bullet -}$, •OH and various lipid hydroperoxides. The Vit C cooperates with vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins. By raising intracellular GSH levels, it also plays an important role in protein thiol group protection against oxidation (Im *et al.*, 2014).

- ✓ *Vitamin E*

Vitamin E is the major lipid-soluble component in the cell antioxidant defence system and is exclusively obtained from the diet. It has numerous important roles within the body because of its antioxidant activity. Vitamin E is a potent chain-breaking antioxidant that inhibits the production of ROS when fat undergoes oxidation and during the propagation of free radical reactions (Rivzi *et al.*, 2014). It acts as the first line of defence against lipid peroxidation, protecting the cell membranes from free radical attack (Rivzi *et al.*, 2014). During the antioxidant reaction, α-tocopherol is converted into α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical. Thus, the α-tocopherol radical can be reduced to the original α-tocopherol form by ascorbic acid (Im *et al.*, 2014).

- ✓ *Carotenoids*

Other lipid-soluble antioxidants are β-carotene and related substances called carotenoids. β-carotene is considered as the most efficient scavenger of O_2^1. Carotenoids protect lipid against lipid peroxidation by quenching free radicals and other reactive species. β-carotene traps free radical through its inhibition of lipid peroxidation induced by XO system (Fiedor and Burda, 2014).

- ✓ *Polyphenols*

Polyphenols are a large group of compounds found in plants and uniquely characterize by the presence of more than one phenol group in the molecule. They are considered the most abundant antioxidant in the diet although the diversity of their structures makes them different from other antioxidants. Moreover, the amount of different polyphenols found to date in plants and plant foods, several thousands, make them a complex family of compounds with very interesting therapeutic properties against cancer, cardiovascular diseases, inflammation and other diseases (Rodrigo and Gil-Becerra, 2014).

2. Polyphenols

Polyphenols constitute one of the most important groups of secondary metabolites of plants. They are widely distributed in the plant kingdom. Approximately 10 000 naturally occurring compounds belong to the category of "phenolics", all of which possess a common structural feature: an aromatic ring bearing at least one hydroxyl substituent. From this basic structure, several thousand of naturally occurring compounds have been described, from simple phenolic acids to highly polymerized compounds such as tannins (Goleniowski *et al.*, 2013).

Polyphenols are defined based on the nature of their carbon skeletons, patterns of hydroxylations, existence of stereoisomers, and states of oxidation, glycosylation (of flavonoids), and acylation (of phenolic acids) of heterocyclic rings. The polyphenol content in plants varies between 1 and 3 mg/kg and is influenced by cultivar, maturity, part of the plant, growing conditions, processing, and storage (Martinez *et al.*, 2017).

Polyphenols are synthesized either as soluble or cell wall–bound compounds during plant growth and especially in response to conditions such as infection, wounding, UV radiation, etc. (Goleniowski *et al.*, 2013). They are biosynthesized by several different routes which two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway (Mandal *et al.*, 2010).

Polyphenols provide multiple intracellular functions. They protect plant from UV-B radiation, acting as a screen inside the epidermal cell layer, and by making adjustments to the antioxidant systems at both cell and whole organism level (Agati and Tattini, 2010). They influence also the pools and fluxes of inorganic and organic soil nutrients (Goleniowski *et al.*, 2013). Polyphenols play an important role serving as visual signals by acting as pigments in fruits and flowers, firstly to attract animals as pollinators in flowers, and later to attract animals to eat the fruits and thereby help in seed dispersal (Latanzio *et al.*, 2008).

Phenolics often participate in plant growth. This may be due their roles in lignifications, in functional pollen development, in seed germination and dormancy (Taylor and Grotewold, 2005). One of the undisputed functions of polyphenols is their role in protecting plants against microbial invasion (Mandal *et al.*, 2010). In addition, they play a role in protecting plants from both insect and mammalian herbivory (Pietta, 2000).

2.1. Classes of polyphenols

There are three main classes of polyphenols: phenolic acids, flavonoids, and other phenolics (figure 1).

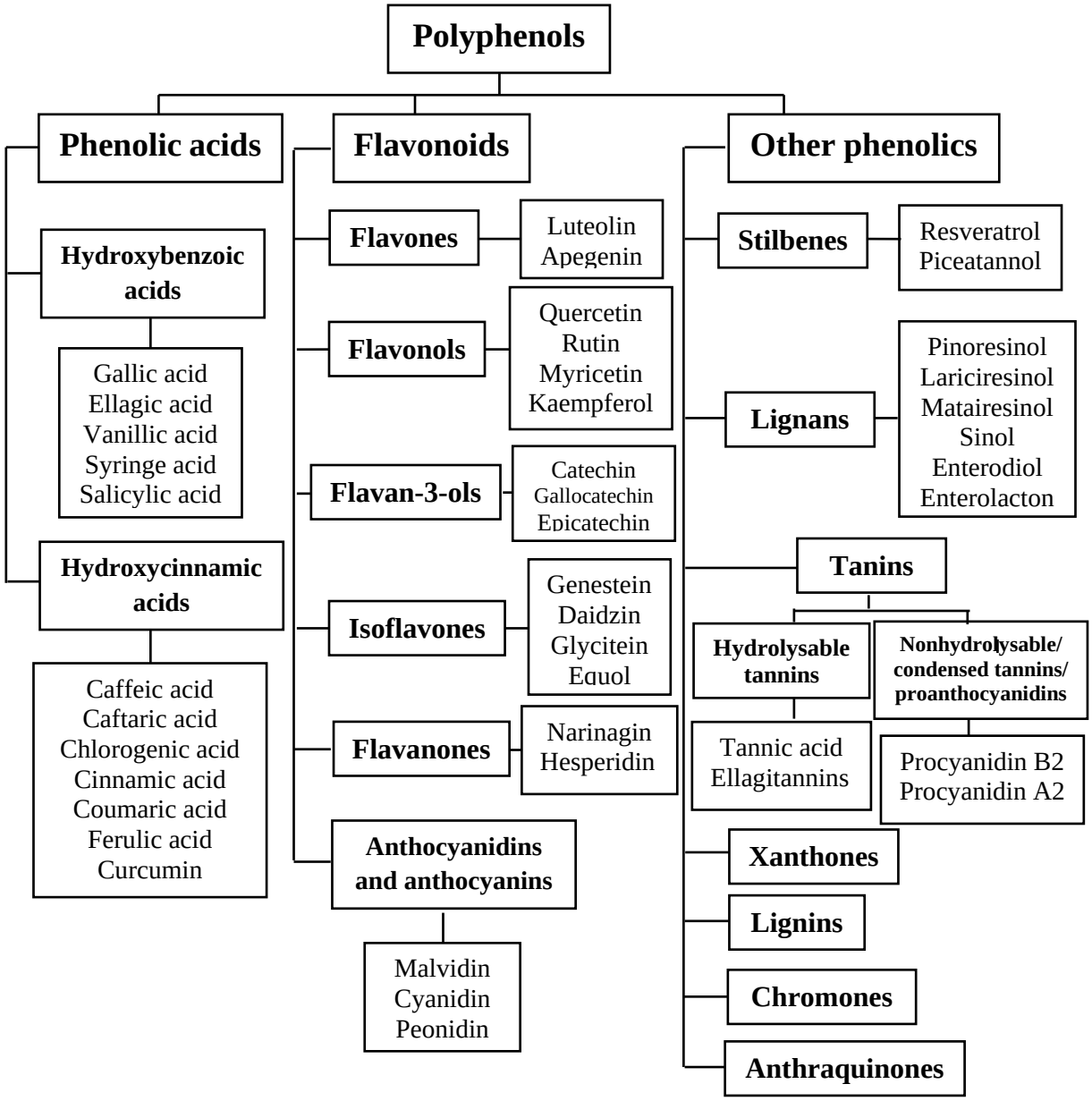

Figure 1. Types of polyphenols (Martinez *et al.*, 2017).

2.1.1. Phenolic acids

Phenolic acids are a subclass of the larger phenolics category, occurring in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids, sterols, and glucosides. Structurally, phenolic acids are phenols that possess one carboxylic acid moiety that can be directly attached either to the aromatic ring (benzoic acid derivatives) or attached to an alkyl residue (hydroxycinnamic acid derivatives). Although the basic skeleton remains the same, phenolic acids differ in the number and position of the hydroxyl groups on the aromatic ring (Losada-Barreiro and Bravo-Díaz, 2017). The majority of phenolic acids are linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin) or to larger polyphenols (flavonoids), or smaller organic molecules (glucose, quinic, maleic, or tartaric acids) or other natural products (terpenes) (Goleniowski *et al.*, 2013).

2.1.2. Flavonoids

Flavonoids comprise the most abundant class of plant polyphenols with more than 9000 of identified chemical structures (Wang *et al.*, 2018). They share a carbon skeleton of diphenyl propanes, two benzene rings (A and B) joined by a linear three carbon chain. This central chain usually forms a closed pyran ring (C) with one of the benzene rings (figure 2). According to the degree of oxidation of the C ring, the hydroxylation pattern of the nucleus, and the substituent at carbon 3, the flavonoids can thus be classified into different subclasses: flavones, isoflavones, flavanols (catechins), flavonols, flavanones, anthocyanins, and proanthocyanidins (Losada-Barreiro and Bravo-Díaz, 2017).

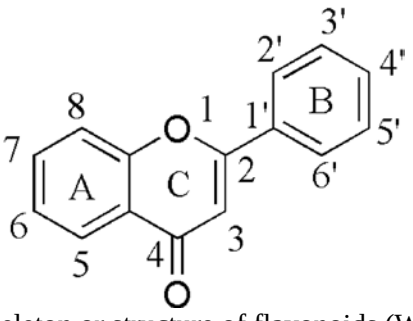

Figure 2. Basic skeleton or structure of flavonoids (Wang *et al.*, 2018).

Flavonoids are the most studied class of polyphenols. Although they are low molecular weight secondary metabolites, their chemical diversity, size, three-dimensional shape, and physical and biochemical properties allow them to interact with multiple targets to influence biological activity in plants, animals, humans and microbes. Consequently, many therapeutic properties have been assigned to flavonoids (Francisco *et al.*, 2014).

2.1.3. Other phenolics

There are at least two major classes of tannins: i: hydrolyzable and nonhydrolyzable (also known as condensed) tannins and ii: proanthocyanidins and procyanidins. Structurally, hydrolyzable and nonhydrolyzable tannins are richly hydroxlyated oligomers or polymers of hydroxybenzoic acids such as gallic acid or flavan-3-ols such as catechin, respectively. High-molecular-weight condensed tannins may contain 50 or more flavan-3-ols subunits attached by carbon–carbon bonds (Selma *et al.*, 2009). They are highly astringent and noticeable in unripe fruits.

Stilbenes are well-known class of naturally occurring phytochemicals. They bear classical C6-C2-C6 structures with two hydroxyl groups on the A ring and one on the B ring. Stilbenes are characterized by a double bond connecting the phenolic rings. These compounds are stress metabolites produced in response to fungal infection. Though known as plant defence compounds, stilbens have an enormous diversity of beneficial human health effects. One of the most relevant and extensively studied stilbene is resveratrol found largely in grapes (Martinez *et al.*, 2014; Losada-Barreiro and Bravo-Díaz, 2017).

Lignans are found in all plants and show enormous structural diversity, with their molecular backbone consisting of two phenylpropane (C3-C6) units. There is a very good recent review of the health effects of lignans. The most lignans that are of special interest owing to their many powerful health benefits are tetrahydrofurofuran and sesamin (Martinez *et al.*, 2014).

2.2. Biological activities of polyphenols

Natural polyphenols have shown numerous biological activities and health benefits for prevention and treatment of age-related diseases, cancers, heart diseases, etc.

2.2.1. Antioxidant activity

Among the notable bioactivities of phenolic compounds, the antioxidant activities have been widely studied especially their acclaimed capability to scavenge ROS and RNS. This activity is frequently cited to be the key property underlying the prevention and/or reduction of oxidative stress-related chronic diseases and age-related disorders (Quideau *et al.*, 2011). Phenolics can also interrupt the propagation stage of the lipid autoxidation chain reactions as effective radical scavengers or act as metal chelators to convert hydro-peroxides or metal pro-oxidants into stable compounds. Plant polyphenols can also act as antioxidants by chelating metal ions such as iron and cupper ions that are involved in the •OH formation through Haber-Weiss/Fenton-type reactions (Procházková *et al.*, 2011). They may reduce the catalytic activity of enzymes involved in ROS and RNS generation. Finally, polyphenols can stimulate antioxidant activities of other enzymes such as CAT (Quideau *et al.*, 2011).

While phenolic compounds are strong antioxidants, it should be pointed out that when a phenolic molecule loses an electron or when it acts as a reducing agent, the molecule itself becomes a radical; its oxidized intermediates can also become pro-oxidants. Interaction between polyphenols and transition metal ions can also result in pro-oxidant formation (Zhang and Tsao, 2016). Polyphenols therefore can be a double-edged sword; on the one hand, when used properly in the form of food or functional food, they are strong antioxidants against excess oxidative stress such as ROS, thus beneficial to health, on the other; they can display pro-oxidant activity when consumed in high doses by taking supplements (Bouayed and Bohn, 2010). The antioxidant potential of phenolic compound depends on the existence of a C_2=C_3 double bond in conjugation with a C_4-carbonyl group, the number and position of

hydroxyl groups in the molecule, the presence of methoxyl groups, and less saccharides connection (Wang *et al.*, 2018).

2.2.2. Anti-inflammation activity

Various investigations have shown that different polyphenols modulate the activity of arachidonic acid metabolizing enzymes such as cyclooxygenase, lipoxygenase, and NOS. Inhibition of these enzymes reduces the production of aminoacids, prostaglandins, leukotrienes, and NO which are among the key mediators of inflammation (Hussain *et al.*, 2016). Moreover, a large number of polyphenols have been reported to inhibit the expression of pro-inflammatory cytokines, which is coupled in some cases to the enhancement of anti-inflammatory cytokines. Polyphenols also exert their beneficial action by modulating monocyte adhesion during the inflammatory process. Flavonoids can modulate the cascade of molecular events leading to the overexpression of inflammatory mediators. These include inhibition of transcription factors, nuclear molecules, protein kinases and mitogen-activated protein kinases (Kim *et al.*, 2014).

2.2.3. Antimicrobial activity

The antimicrobial activity of polyphenols occurring in medicinal plants has been extensively investigated against a wide range of microorganisms. Polyphenols, especially flavan-3-ols, flavonols, and tannins, have wide spectrum and higher antimicrobial activity in comparison with other polyphenols. Cited polyphenols are also able to suppress a number of microbial virulence factors (such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins) and show synergism with antibiotics (Daglia, 2012). For instance, therapeutic activities of polyphenols against influenza virus, canine distemper virus, hepatitis virus, and *Escherichia coli*, have been attributed to chemical structures in particular patterns of methoxylation, glycosylation and hydroxylation (Carvalho *et al.*, 2013).

2.2.4. Anticancer activity

The anti-cancer activities of polyphenols against a wide range of cancers have been documented. Several mechanisms underlying the anticancer activity of polyphenols are proven, including modulation of ROS and RNS, modulation of inflammation- related factors, modulation of estrogen receptor, detoxification of xenobiotics, induction of apoptosis, modulation of cell cycle and their effects on the cellular signaling system. Among these are effects on nuclear factors, such as NF-κB or activator protein 1, which play central roles in cellular signaling cascades, regulating DNA transcription, gene expression in response to different stimuli, cell proliferation and survival (Abdal Dayem *et al.*, 2016; Niedzwiecki *et al.*, 2016).

2.2.5. Cardiovascular protective activity

Numerous studies have demonstrated the health benefits of polyphenols, and special attention has been paid to their beneficial effects against cardiovascular disease. Polyphenols present vasodilator effects and are able to improve lipid profiles and attenuate the oxidation of low density lipoproteins. In addition, they present clear anti-inflammatory and antithrombotic effects and can modulate apoptotic processes in the vascular endothelium. It has been suggested that most of these effects are a consequence of the antioxidant properties of polyphenols (Quinones *et al.*, 2012).

2.2.6. Neuroprotective activity

Recently, a rapidly growing number of polyphenolic compounds with neuroprotective effects have been described. Neuroprotective effects of polyphenols can be divided into the following categories: i: neuroprotective action through antioxidant pathways, ii: interaction with signaling pathways, iii: neuroprotection through modulation of neural mediators and enzymes like acetylcholine and acetylcholinesterase, vi: inhibition of N-methyl-d-aspartate neurotoxicity and v: anti-amyloidogenic effect (Ebrahimi and Schluesener, 2012).

2.2.7. Anti-aging activity

There is great interest in using polyphenol organic compounds to modify or retard the aging process. Several polyphenols augment the lifespan of multiple invertebrate and vertebrate species, in higher organisms in the presence of dietary modification. Polyphenols may influence aging by acting on genes in energy-regulatory intracellular pathways that are believed to play important roles in the aging process. In addition, or together with this process, polyphenols may alter concentrations of ROS, transcription factors, and act via epigenetic mechanisms. Polyphenols may also modulate organ systems through their effect on intercellular signaling molecules including nitrous oxide and pro-inflammatory cytokines (Cherniack, 2016).

2.2.8. Other biological activities

Growing evidence from animal studies supports some other activities of polyphenols such as anti-diabetic activity (Kim *et al.*, 2016), prevention of obesity and obesity-related chronic diseases (Wang *et al.*, 2014), amelioration liver injury (Janel and Noll, 2014), attenuation microarchitecture bone deterioration (Shen *et al.*, 2014).

4. Toxicity of plant

Plants used in traditional medicine are assumed to be safer than pharmaceuticals. This safety is based on their natural origin and also their long usage in the treatment of diseases according to knowledge accumulated over centuries (Philomena, 2011). However, recent scientific reports have demonstrated that several medicinal plants used in phytomedicine are potentially toxic, and some are even mutagenic and/or carcinogenic (Nasri and Shirzad, 2013). It cannot therefore be taken for granted that medicinal plants are void of toxic effects. Some authors have thus recommended that pharmacological studies on medicinal plants should always be accompanied by toxicological screening (Cos *et al.*, 2006).

The toxicity in medicinal natural products may originate from i: dearth of reports on the side effect of medicinal plants, ii: error in botanical identification, iii: accidental ingestion of cardiotonic plants, iv: inappropriate combinations in phytotherapy, v: interference of medicinal plants and vi: conventional pharmacological therapy and contamination with heavy metals (Goldman, 2001; Wojcikowski *et al.,* 2004).

The toxicity for medicinal plant therefore depends on many different factors, such as: their purity, medicinal plants combinations, route of administration, absorption, bioavailability, period of exposure, number of exposures, physical form of the toxicant (solid, liquid, or gas), and reported adverse effects (Ogunlana, 2012 ; Püssa, 2014).

The toxicity of plant is based on their chemical constituents that are classified into: lectins or hemagglutinins, enzyme inhibitors, alkaloids, cyanogenic glycosides, phytoestrogens, glucosinolates, coumarin, toxic amino acids, toxic lipids, oxalates, fluoroacetates, saponins, etc. (Püssa, 2014). They act by altering specific mechanisms involving enzymes, receptors and even genetic material at particular cells and tissues (Chandra *et al.*, 2012).

4.1. Definition of toxicity

Toxicity is defined as "the potential of a substance to exert a harmful effect on humans or animals, and a description of the effect and the conditions or concentration under which the effect takes place" (Yu *et al.*, 2005).

4.2. Assessment of toxicity

Toxicology is an aspect of pharmacology that deals with the adverse effect of bioactive substance on living organisms. In order to establish the safety and efficiency of a new drug, toxicological studies are very essential experiments in animals like mice, rat, etc. No drug substance is used clinically without its laboratory safety assessment at preclinical phase. Toxicological studies help to make a decision whether a new drug should be adopted for clinical use or not (Alam *et al.*, 2006).

The primary aim of toxicological assessment of any medicinal plant is to identify adverse effects that may be associated with its use and to determine limits of exposure level at which such effects occur, therefore avoiding potential harmful effects when used as medicine (Sims *et al.*, 2010; Ifeoma and Oluwakanyinsola, 2013).

Generally, toxicity assessment tests can be divided into two categories: the first category comprises tests that are designed to evaluate the overall effects of compounds on experimental animals. Individual tests in this category differ from each other basically in regard to the duration of the test and the extent to which the animals are evaluated for general toxicity. These tests are classified as acute, sub-acute, chronic and subchronic toxicity tests (Loomis and Hayes, 1996). The last category of tests consists of those that are designed to evaluate specific types of toxicity in detail. The subacute, chronic and subchronic toxicity tests do not detect all forms of toxicity, but they may reveal some of the specific toxicities and indicate the need for more detailed studies. Thus, this last category of tests has been developed for the determination of effects of compounds on the fetus in a pregnant animal (teratogenic tests), on

the reproductive capacity of the animals (reproduction tests), on the genetic system (mutagenic tests), for the determination of the ability of agents to produce tumors (tumorigenicity and carcinogenicity tests), etc. (Loomis and Hayes, 1996; Timbrell, 2002).

4.2.1. Acute toxicity

Acute toxicity is an initial screening step in the toxic assessment and evaluation characteristics of all biological compounds (Akhila *et al*., 2007). Acute toxicity is a term used to describe the adverse effects that are caused by a single exposure to a toxic substance or brief multiple exposures over a very short span of time, usually less than 24 hours. An adverse effect is "a test item-related change in the morphology, physiology, growth, development, reproduction or life span of the animal model that likely results in an impairment of functional capacity to maintain homeostasis and/or an impairment of the capacity to respond to an additional challenge " (Palazzi *et al.*, 2016).

Researchers can carry out the acute toxicity test with any animal species, but they use rats or mice most often because of the low cost, the availability of the animals, and the fact that abundant reference toxicologic data for many compounds in these species are available. In addition, these animals generally metabolize compounds in a similar manner to humans and the compounds may have similar pharmacodynamics in the animals and humans (Mukinda, 2005). Other species include dogs, hamsters, cats, guinea pigs, rabbits, and monkeys. In each case, the choice for the appropriate animal for a particular test is influenced by the test method, which is also linked to the route of product administration (Teke and Kuete, 2014).

The toxicity testing is based on the route of substance administration to the animal: oral, dermal, intraperitoneal or inhalation (Teke and Kuete, 2014). However, two routes of administration for acute toxicity are the most commonly used: intraperitoneal injection or the oral route (Chanda *et al.*, 2015).

Traditionally, acute oral toxicity testing has focused on the immediate determination of the

dose that kills half of the animals (i.e., the median lethal dose or LD50), the timing of lethality following acute chemical exposure, as well as observing the onset, nature, severity and reversibility of toxicity. However, in recent times, after the immediate observation is done, toxicological parameters (biochemical, haematological and histopathological) to assess potential adverse effects are carefully chosen and measured. The fixed single dose, at which signs of toxicity but no deaths are detected, is used to classify the test compounds according to their toxic potential.

LD50 and LC50 (median lethal concentrations) values are used to describe the relative acute toxicity of a chemical substance. The table 3 shows globally harmonized system classification criteria for acute toxicity. Acute toxicity category 1 represents the most severe toxicity.

Table 3. Globally harmonized system classification criteria for acute toxicity (United Nations, 2015).

Exposure route	Category 1	Category 2	Category 3	Category 4	Category 5
Oral (mg/kg)	≤ 5	> 5 ≤ 50	> 50 ≤ 300	> 300 ≤ 2000	
Dermal (mg/kg)	≤ 50	> 50 ≤ 200	> 200 ≤ 1000	> 1000 ≤ 2000	Anticipated oral or dermal LD 50 in the range of 2000 – 5000 mg/kg body weight and equivalent dose for inhalation.
Gases (ppm)	≤ 100	> 100 ≤ 500	> 500 ≤ 2500	> 2500 ≤ 20000	
Vapors (mg/l)	≤ 0.5	> 0.5 ≤ 2.0	> 2.0 ≤ 10	> 10 ≤ 20	
Dusts and mists (mg/l)	≤ 0.05	> 0.05 ≤ 0.5	> 0.5 ≤ 1.0	> 1.0 ≤ 5.0	

Acute toxicity tests can provide preliminary information on the toxic nature of a substance for which no other toxicology information is available. Therefore, they aim to:

- ✓ identify a single dose causing major adverse effects or life-threatening toxicity;
- ✓ obtain information on the biologic activity of a chemical and gain insight into its mechanism of action;
- ✓ determine possible target organs that should be scrutinized;

- ✓ select doses for repeated-dose studies;
- ✓ and for hazard identification and risk management in the context of production, handling, and use of chemicals (Arome and Chinedu, 2013 ; Chanda *et al.*, 2015).

The following alternative animal tests of acute oral toxicity have been developed: fixed dose procedure (FDP)- organisation for economic co-operation and development (OECD) no. 420, acute toxic class (ATC) method-OECD no. 423 and up-and-down procedure (UDP)-OECD no. 425 are methods guarantee significant improvements in animal welfare and permit to reduce dramatically the number of the test animals needed. They have recently undergone a revision to improve their scientific performance but more importantly to increase their regulatory acceptance. These methods can now be used for all types of test substances and for all regulatory and in-house purposes.

4.2.2. Subacute toxicity

Acute toxicity tests are by far not sufficient; they are usually followed by subacute tests. In these tests, repeated doses of substance test are given to animals for period of 14, 21 or 28 days. These studies are to be carried out with three different animal species, tow of which area rodents. Most often, these three species are the mice, rat and dog, since those are easily available and a lot of background information exists concerning these species (Püssa, 2014). Three doses levels are normally used: dose that is high enough to elicit definite signs of toxicity but not to kill many of the animals, low dose that is expected to induce no toxic effect, and intermediate dose. These doses are selected of information obtained in acute toxicity studies using LD50 (Arome and Chinedu, 2013). These tests are intended to:

- ✓ investigate effects on a very broad variety of potential targets of toxicity;
- ✓ provide information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time;
- ✓ determine the principal behavioral changes as well as anatomical, physiological and biochemical manifestations of tissue damage provoked by the substance;

- ✓ and all the knowledge gathered from the studies is used in selecting doses for repeat-dose studies as a source of preliminary identification of target organs of toxicity, and may also reveal delayed toxicity (Kirubi, 2013).

4.3. Target organ toxicity

Results of many subacute toxicity tests of various plant extracts showed that the major organs usually affected are liver and kidneys. Hepatotoxic and nephrotoxic effects are mostly to be expected, as the liver acts as the main detoxifying organ for chemical substances, while the kidney is a principal route of excretion for many chemical substances in their active and/or inactive forms (Abdulrahman *et al*., 2007).

4.3.1. Liver toxicity

The liver is the largest organ in the body (about 2% of average body weight) and the only organ capable of regeneration. It lies mainly in the right hypochondriac and epigastric regions of the abdominal cavity, below the diaphragm. A thick capsule of connective tissue called Glisson's capsule covers the entire surface of the liver. The liver is divided by the falciform ligament into a large right lobe and a smaller left lobe. Each lobe is further divided into lobules that are the functioning units of the liver (Qin and Crawford, 2018). Each lobule (figure 3) consists of rows of hepatic cells (hepatocytes or parenchymal cells) perforated by specialized blood capillaries called sinusoids. The sinusoid walls contain phagocytic cells, called Kupffer cells, whose role is to engulf and destroy materials such as solid particles, bacteria, dead blood cells and others. The liver receives 75% of its blood supply from the portal vein, which carries blood returning to the heart from the small intestine, stomach, pancreas, and spleen. The blood perfuses the liver and exits through the hepatic vein, merging into the inferior vena cava and return blood to the heart (Abdelmisih and Bloomston, 2010).

The liver is the major organ actively involved in metabolism, biosynthesis and storage. It is the storage organ for glycogen, fat, fat soluble vitamins and other nutrients. It is the site of

metabolism of lipoproteins, functional proteins, such as enzymes and blood-coagulating factors. In addition, the liver, which contains numerous xenobiotic metabolizing enzymes, is the main site of xenobiotics metabolism (Hodgson and Levi, 2004). This function makes it one of the most exposed organs to xenobiotics. Therefore, it is often the targeted organ for chemically induced organ toxicities.

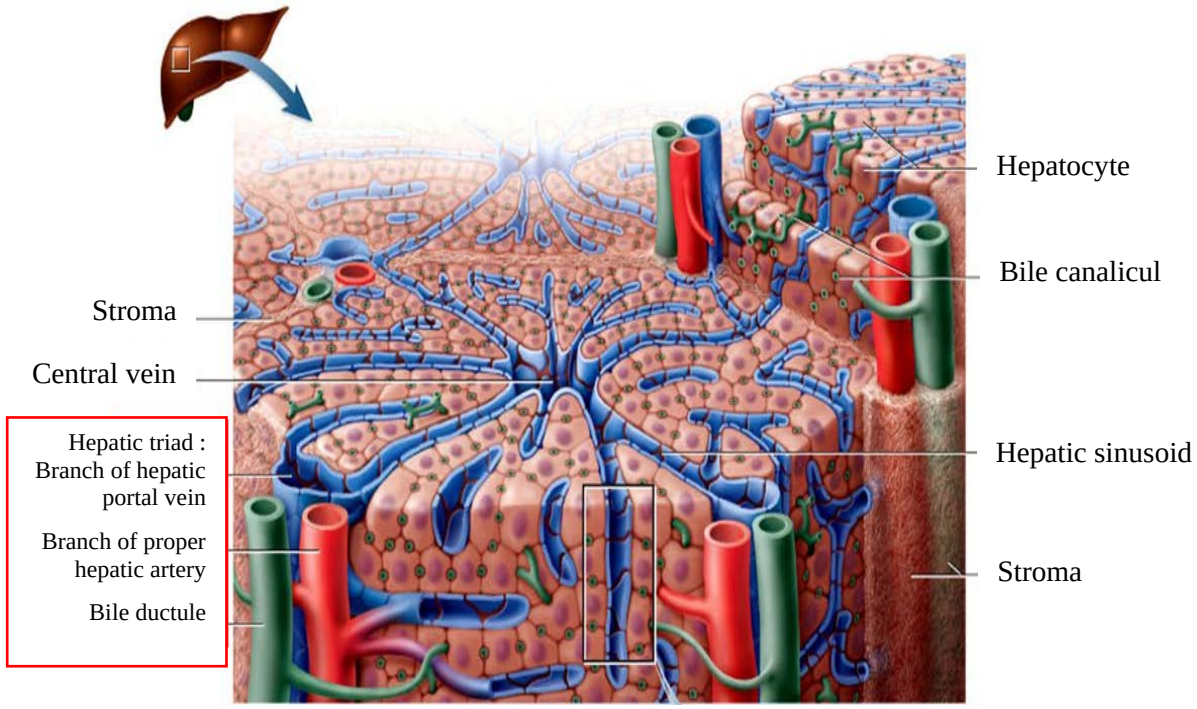

Figure 3. Cross section of a hepatic lobule, illustrating microscopic structure (Saladin, 2010).

There are many plants and herbal extracts which have shown to possess hepatoprotective activities. About 170 phytoconstituents isolated from 110 plants belonging to 55 families have been reported to possess liver protective activity (Girish and Pradhan, 2017). However, over the last fifty year, approximately 21 herbs have been reported to be associated with liver toxicity in case report publications, where the spectrum of herb-induced hepatotoxicity researched included elevated liver enzymes, hepatitis, steatosis, cholestasis, hepatic necrosis, hepatic fibrosis, hepatic cirrhosis, veno-occlusive disease, acute liver failure requiring a liver transplant, and death (Brown, 2017).

4.3.2. Kidney toxicity

The Kidneys are paired retroperitoneal bean-shaped organs weighting together about 300 g (about 0.4% of body weight) and they are found along the posterior muscular wall of the abdominal cavity with the left kidney located slightly more superior than the right because of the larger size of the liver on the right side of the body (Ekor, 2014; Feher, 2017). They are distinguished into cortex with granular appearance surrounded by fibrous capsule and medulla with striated appearance. Kidneys are surrounded by adipose tissue. Medulla is composed of renal pyramids whose bases are attached to the cortical tissue and define the bordeline between cortex and medulla. The pyramidal vertices are heading to hilum and create renal papillae surrounded by smooth muscle cells allowing evacuation of urine to the renal pelvis. Cortex is about 5 to 7 cm thick. Between the renal pyramids, cortical tissue penetrates to the renal sinus and created renal columns. The cortex sends strips penetrating the medulla, called striae medullares (figure 4a).

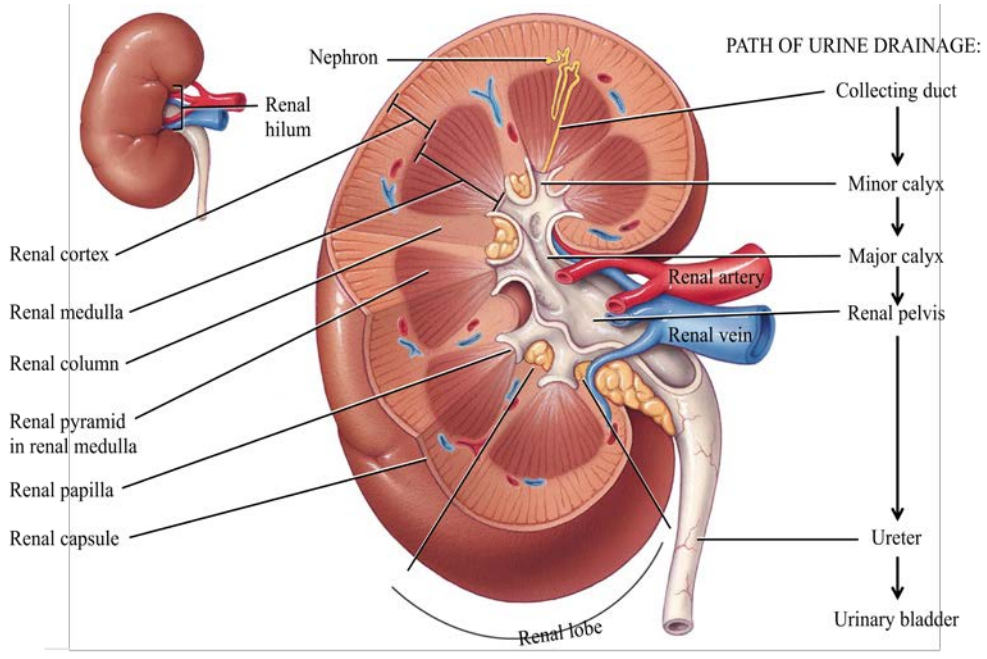

Figure 4a. Anterior view of dissection of right kidney (Feher, 2017).

The nephron is principal structural and functional unite of kidney. Each kidney contains about 1 to 1.3 million nephrones. Each nephrone (figure 4b) is composed of glomerulus, Bowman's capsule, proximal tubule, loop of Henle, distal tubule and collecting tubules (Feher, 2017).

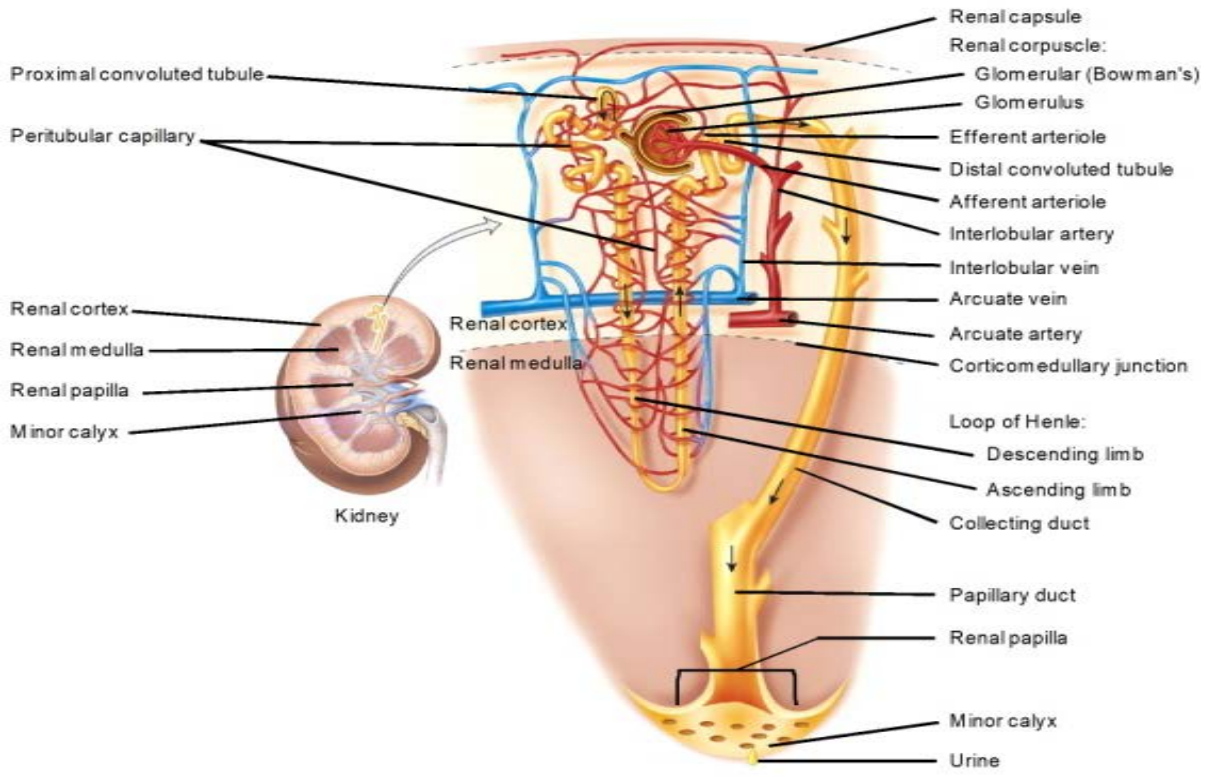

Figure 4b. Cortical nephron and vascular supply (Feher, 2017).

The kidneys serve several essential regulatory roles. They remove excess organic molecules from the blood, and it is by this action that their best-known function is performed: the removal of waste products of metabolism. Kidneys are essential to the urinary system and also serve homeostatic functions such as the control of volume status, regulation of electrolytes, maintenance of acid-base balance, and regulation of blood pressure. They are also responsible for the reabsorption of water, glucose, and amino acids. The kidneys also produce hormones including calcitriol and erythropoietin. An important enzyme rennin, a major stimulus of the rennin-angiotensin-aldosterone system and an important regulator of extracellular fluid volume and blood pressure, is also produced in the kidneys (Feher, 2017).

The role of the kidney as the primary eliminator of exogenous drugs and toxins, in addition to the fact that it is characterized by a large volume of blood supply (20 to 25% of the cardiac output) which ensures a high level of toxicant delivery over a period of time, predisposes this important organ to nephrotoxicity and enhances its vulnerability to developing various forms of injury (Ekor, 2014).

Plants medicines and related natural products are an important and unregulated source of potentially nephrotoxic substances and a large number of them have long been recognized as capable of causing renal failure with transient or chronic requirement for dialysis (Ekor, 2014). Generally, they have been classified into i: agents which are directly nephrotoxic, ii: herbal drugs which can cause electrolyte abnormalities by acting on the kidney, iii: agents which can predispose to formation of stones (oxalate stones), iv: agents which act as diuretics, v: herbal drugs which contain heavy metals or other drugs; and vi: herbal agents which can interact with other drugs, especially in the renal transplant subject (Singh and Prakash, 2011).

3. *Reichardia picroides*

Asteraceae (Compositae, the daisy family) is one of the largest plant families. The family currently has 32 913 accepted species names, in 1 911 genera. Most members of Asteraceae are herbaceous, but a significant number are also shrubs, vines, or trees. The family has a worldwide distribution, from the Polar Regions to the tropics, colonizing a wide variety of habitats (Dinde *et al.*, 2017). *Reichardia* genus comprises eight species which are distributed throughout the Mediterranean area, although some of them can also be found in certain parts of India and Africa (Recio *et al.*, 1992).

3.1. Taxonomy

- **Kingdom** : Plantae
  - **Subkingdom** : Viridaeplantae
    - **Division** : Magnoliophyta
      - **Class** : Equisetopsida
        - **Sub-Classe** : Magnoliidae
          - **Super-Order** : Asteranae
            - **Order** : Asterales
              - **Family** : Asteraceae
                - **Genus :** *Reichardia*
                  - **Specie** : *Reichardia picroides* (L.) Roth, 1787.

The *R. picroides* has many synonymes : *Picridium istriacum* Gand., *Picridium lesbiacum* Candargy, *Picridium mixtum* Gand., *Picridium picroides* (L.) H.Karst., *Picridium rupestre* Pomel, *Picridium sadleri* Gand., *Picridium vulgare* Desf., *R. hypochoeriformis* Ginzb., *R. integrifolia* Moench, *Scorzonera picroides* L., *Scorzonera variifolia* Salisb., *Sonchus chondrilloides* Sm., *Sonchus picroides* (L.) Lam.

The *R. picroides* is called: Halhal (local name), Cousteline or Picridie (French), Coustelina (Italien). Reichardia: dedicated to German doctor and botaniste Johann Jakob Reichard (1743-1782). Picroide, Picris: was derived from the Greek word 'pikris', bitter plant.

3.2. Botanical description

The *R. picroides* (figure 5) is glabrous perennial herb. Stems 10-45 cm. Leaves smooth or more or less papillose; basal 2-13 x 0-5-2-5 cm, oblanceolate or linear-lanceolate; lower cauline similar to basal but sessile, amplexicaul, the upper smaller and often more or less entire. Capitula 1-5; peduncles long, with numerous small, ovate, acuminate bracts which have a scarious margin. Involucre 10-20 x 8-14 mm, glabrous; outermost bracts 3-5 x 1-5-2-5 mm, ovate, with a scarious margin up to 0-5 mm wide, the inner lanceolate, obtuse, usually with a narrow scarious margin. Ligules yellow, the outer usually with a dark stripe on outer face, l-2 times as long as involucre. Achenes 2-3 mm, the outer 4- to 5-angled, transversely rugose, the inner smooth and appearing sterile (Sell, 1976; Thompson, 2007).

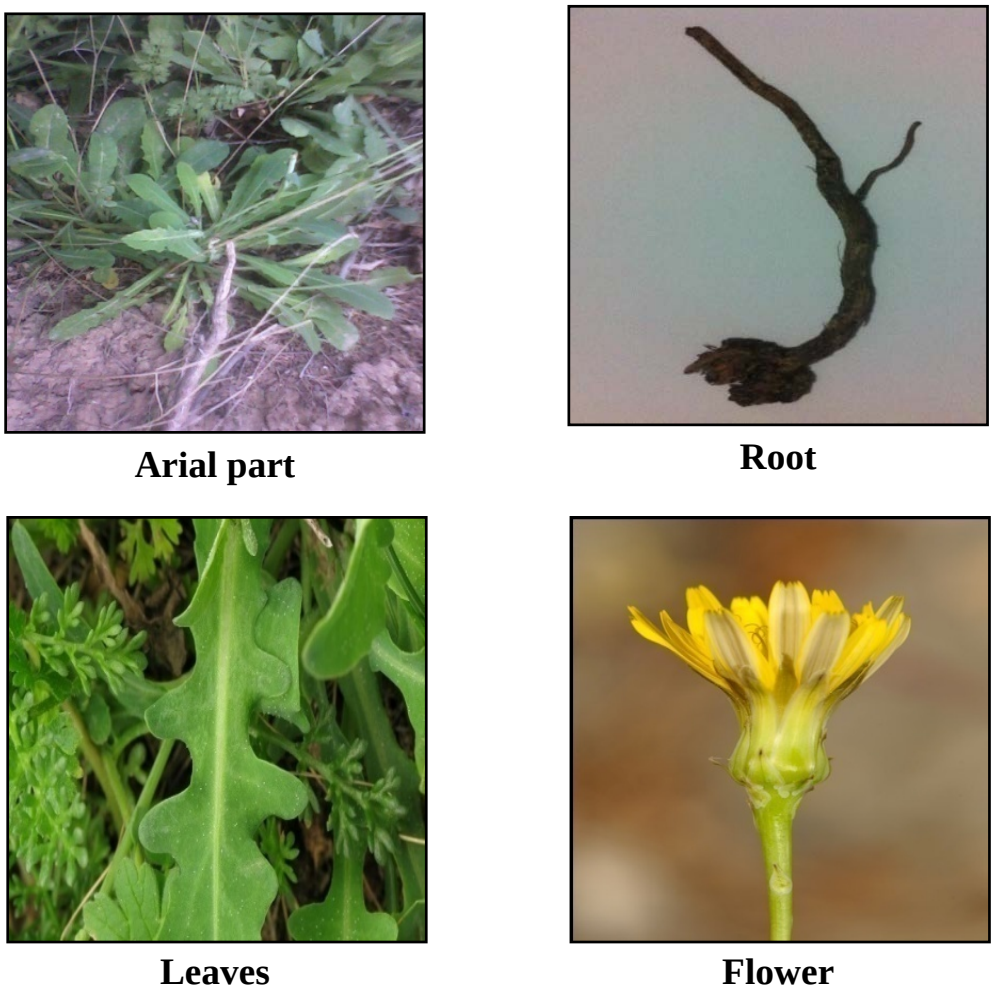

Figure 5. The *R. picroides* L. Roth plant. Leaves and flowers are available from https://www.urbanturnip.org/perennial-lettuce-salad-leaves/ and http://www.florealpes.com/comparaison.php?zoomph2=2#visiga, respectively.

3.3. Traditional use

The *R. picroides* leaves are mainly used for alimentary purposes (Cornara *et al.*, 2009; Di Novella, 2013; Guarrera and Savo, 2013), but it is also traditionally known to be used as hypoglycemiant (Bonet *et al.*, 1999), diuretic, galactagogue and emollient (Guarrera and Savo, 2013), depurative of intestine (Cornara *et al.*, 2009), and tonic (Loi *et al.*, 2004). Furthermore, herb roots are used for cough, abdominal pains and kidney problems (Savo *et al.*, 2011).

3.4. Chemical composition

In our knowledge, there are no scientific studies on its chemical composition, excepting the study of Recio and their collaborators (1992). In this study, they had isolated 13 substances from the Methanolic extracts of the aerial parts of *R. picroides* : nine flavonoid glycosides: luteolin-7-O-glucoside, luteolin-7-O-rhamnoside, luteolin-7-O-rutinoside, luteolin -7-O-galactosylglucoside, luteolin-3',7-O-diglucoside, luteolin-4'-O-glucoside, apigenin-7-O-glucoside, apigenin-7-O-rutinoside and apigenin-7-O-neohesperidoside; and four cinnamic derivatives: caffeic acid, chlorogenic acid, isochlorogenic acid and 3,4-dicaffeoylq cuinic acid.

MATERIALS AND METHODS

1. Materials

1.1. Collection of plant

The plant *R. picroide* was collected from Bouguaâ (36°19′57″Nord 5° 05′ 19″Est), Setif (Algeria), in March-April 2013. It was authenticated by Pr. Oudjhih Bachir, Institut of nutrition and agronomy, Batna (Algeria), and the voucher specimen was deposited to the University Herbarium with the reference number 333/isvsa/uhl/13. The whole plant was shade dried at free air for two weeks, and powdered using electrical grinder (Retsch SK 100). The powder was conserved in clean dried glass bottle at room temperature until experimentation.

1.2. Experimental animals

Experiments were performed using young adult male and female (nulliparous and non-pregnant) *Swiss albinos* mice and weighing 20-30 g. They were obtained from Pasteur institut (Algeria) and housed in plastic cages under normal laboratory conditions (12 h light / dark cycle, 23 ± 2 °C) for an acclimatization period of 7 days prior to the experiments. All the animals were given food and water *ad libitum*. The experiments were conducted in accordance with the internationally acceptable guidelines for evaluating the safety and efficacy of plant medicines [Organisation of Economic Co-operation and Development (OECD), 2008].

Experiments were performed on adult male *Swiss albinos* weighing 25-30 g, from Pasteur Institute of Algeria. Animals were maintained on a 12 hour light / dark cycle in a temperature regulated room (20 - 25°C) during the experimental procedures. They fed a normal laboratory diet. They were cared according to the guiding principles in the care and use of animals.

1.3. Reagents and apparatus

All used reagents were purchased from Sigma (Germany), Fluka, prolab and Biochem.

Silica gel for column chromatography was Silica gel 60 (0.063-0.200 mm, Fluka, Steinheim, Germany).

Routine thin layer chromatography (TLC) was done on silica gel G-UV 254 aluminium plates (Fluka, Steinheim, Germany).

Among used apparatus: rotavapor (Rotavapor Germany, bÜchi461), centrifuge (Sigma, 3K30), spectrophotometer (TechomP, UV/VIS-8500), electrical grinder (Retsch SK 100), and Bruker 500 MHz Avance III spectrometer.

2. Methods

2.1. Preparation of crude extract and fractions

Powedred materials were extracted three times at ratio of 1:10 (w/v) by maceration in methanol for seven days each time at room temperature and with intermittent shaking, according to Sharma and their collaborators (2008) method with slight modifications. The methanol mixture was then filtered and concentrated at 45 °C under reduced pressure using a rotary evaporator. The crude extract, coded as CrE, was further concentrated by allowing it to stand in an oven at 40 ◦C.

A quantity of CrE was subjected to fractionation using liquid- liquid extraction (Markham, 1982). The CrE was successively fractionated with different solvents of increasing polarity: hexane for defatting, chloroform for aglycone flavonoids extraction and ethyl acetate for glycone flavonoids extraction, as shown in figure 6. The combined organic layer of each partition was evaporated under reduced pressure on a rotavapor below 45°C to dryness and to afford hexane, chloroform, ethyle acetate and aqueous fractions coded as HexE, ChE, EAE and AqE, respectively.

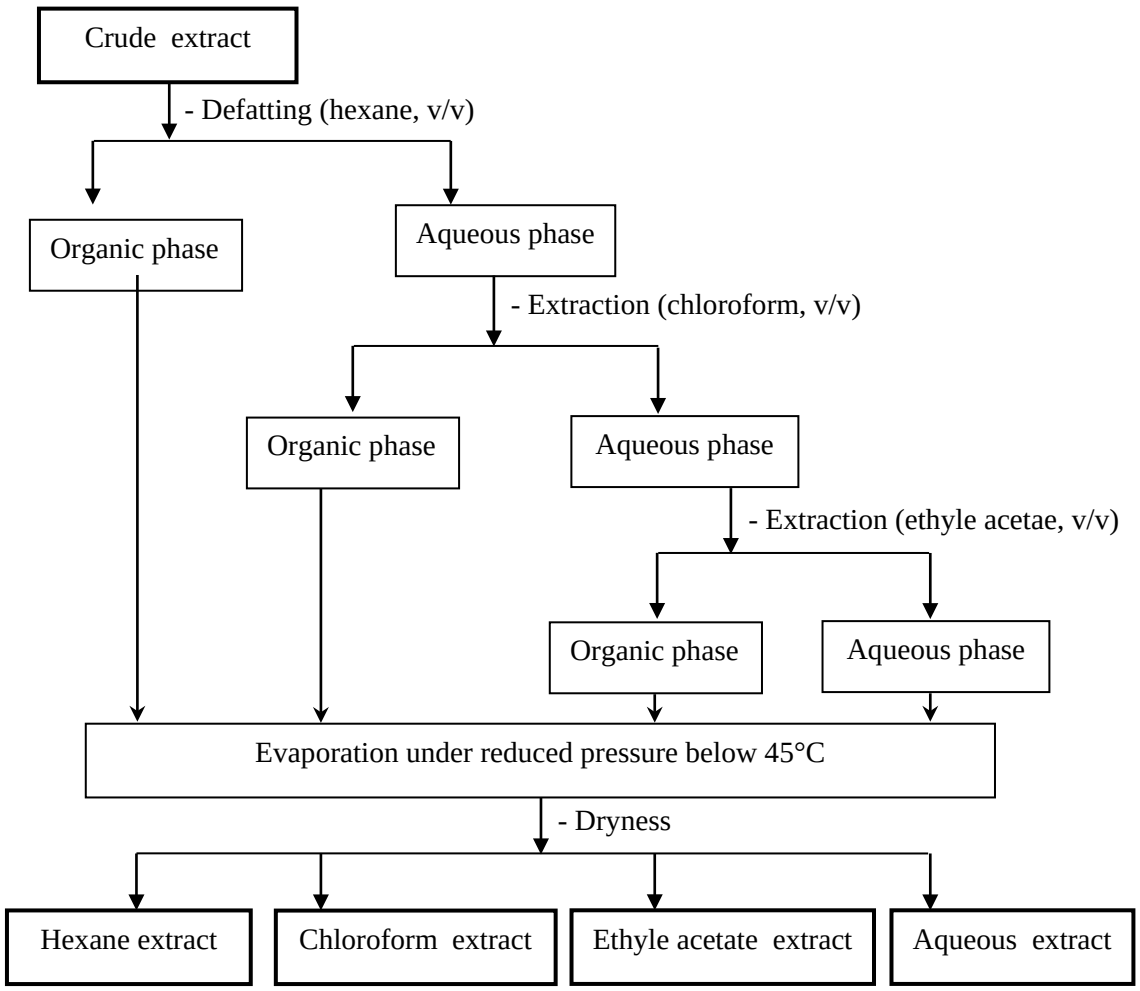

Figure 6. The *R. picroides* CrE fractionation.

2.2. Phytochemical characterization

2.2.1. Analytic chromatography method

Activated silica gel (60-120 mesh) was packed onto glass column (800 mm × 40 mm) using dichloromethane as a packing solvent. A 150 g of the CrE was loaded on top of the silica gel. The CrE was fractionated by elution with dichloromethane and then with dichloromethane/ methanol mixtures of increasing polarity to obtain 660 fractions (400 ml for each) that were collected into properly conical flasks. The fractions were concentrated using a rotary evaporator at 40°C to reduce the volume and then poured into a bottle and evaporated to dryness under a fan. Fractions then combined according to their TLC profile into 12 fractions which were later dried. These 12 fractions were the object of our study.

- ✓ *Isolation*

By washing with methanol, only the F10 allowed to obtain a pure compound in the form of white crystals (C1), identified as luteolin 7-O-β-glucoside.

- ✓ *Techniques for determining the structure of isolated compounds*

The NMR spectra are recorded on a Bruker 500 MHz Avance III spectrometer (Department of Chemistry, university of Jordan), resonating respectively at 500 MHz (^{1}H) and 125 MHz (^{13}C). The chemical shifts are recorded in δ (ppm) by considering the chemical shift of tetramethylsilane (TMS, as reference) δTMS = 0. The coupling constants (J) are expressed in Hertz (Hz). The samples were solubilized in DMSO solvent.

2.2.2. Determintion of total phenolics contents

Total phenolics contents of *R. picroides* CrE and fractions were estimated by the Folin-Ciocalteu method (Li *et al.*, 2007). Such method consists of the phosphotungstic (WO_4^{-2})-phosphomolybdic (MoO_4^{-2}) acid (Folin-ciocalteu's reagent) reduction by the phenolic hydroxyl groups, resulting in the formation of a blue product in alkaline solution. Briefly, 200µl of appropriate dilution of CrE or each fraction were added to 1 ml of 1:10 diluted Folin-ciocalteu's reagent. After 4 min, the reaction mixture was neutralized with 800 µl of saturated sodium carbonate (75 g/l). Subsequently, the shaken mixture was allowed to stand for 2 h at room temperature, and then measured at 765 nm. Gallic acid (20-140 mg/l) was used for the standard calibration curve. The results were expressed as µg gallic acid equivalent (GAE)/mg of CrE or each fraction.

2.2.3. Determination of flavonoids contents

The aluminium chloride ($AlCl_3$) method (Bahorun *et al.*, 1996) was used to determine the flavonoids contents of the *R. picroides* CrE and fractions, employing the reaction of complex formation between flavonoids and $AlCl_3$. Briefly, 1 ml of CrE or each fraction was added to equal volume of a solution of $AlCl_3$ (2%). The mixture was vigorously shaken, and

absorbance was read at 430 nm after incubation in dark at room temperature for 10 min. Quercetin (1-40 mg/l) was used as standard for calibration curve. Flavonoids contents were expressed as µg quercetin equivalent (QE)/mg of CrE or each fraction.

2.3. The *in vitro* evaluation of antioxidant activity

2.3.1. Anti-hemolytic assay

The inhibition of mice erythrocytes hemolysis by CrE and fractions was assessed according to the method described by Girard and their collaborators (2006) with slight modifications. Mice erythrocytes were isolated by centrifugation at 3000 rpm for 10 min and washed three times with phosphate buffer (10mM, pH 7.4) until the supernatant became colourless. The erythrocytes were then diluted with phosphate buffer to give 2% (v/v) suspension. Briefly, 80 µl of 2% erythrocytes suspension was added to 20 µl of CrE or each fraction (0.1 mg/ml), the mixed were treated then by 136 µl of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH, 300 mM). We have controlled the whole blood hemolysis with a 96-well microplate reader device. The kinetics of erythrocytes resistance to hemolysis was determined at 37°C by continuous monitoring by measuring the rate of decrease at 630 nm. Results were expressed as the time corresponding to 50% of maximal hemolysis (half-hemolysis time, HT50 in min). Vit C was used as standard.

2.3.2. Ion chelating assay

Ferrous iron-chelating activity of CrE and fractions was measured by inhibition of the formation of Fe^{2+}-ferrozine complex after treatment of test extract with $Fe^{2+,}$ following the method of Decker and Welch (1990) modified by Le and their collaborators (2007). The reaction mixture contained 250 µl CrE or each fraction, 50 µl $FeCl_2$ (0.6 mM in water) and 450 µl methanol. The control contained all the reaction reagents except the extract. The mixture was shaken and allowed to react at room temperature for 5 min. An aliquot of 50 µl of ferrozine (5 mM in methanol) were then added; the mixture shaken again, followed by

further reaction at room temperature for 10 min to complex the residual Fe^{2+} ion. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm against a blank contained all the reaction reagents except ferrozine. Lower absorbance indicates a higher chelating power. Ethylene diamine tetraacetic acid (EDTA) was used as reference chelator. The chelating activity was calculated as percentage:

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

Where A_c: control absorbance and A_e: absorbance in the presence of CrE or each fraction. EC50, effective concentration which produces 50% Fe^{2+} ions chelating, was calculated.

2.3.3. Reducing power assay

The reducing power of CrE as well as their fractions was determined according to the method of Oyaizu (1986). An aliquot of CrE or each fraction (125 µl) was mixed with 125 µl of sodium phosphate buffer (0.2 M, pH 6.6) and 125 µl of 1% $K_3Fe(CN)_6$ followed by incubation at 50°C for 20 min. After adding 125 µl of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 3750 g for 10 min. The supernatant solution (100 µl) was mixed with 100 µl of distilled H_2O and 20 µl of 1% ferric chloride to react for 10 min. Subsequently, the absorbance was measured at 700 nm. The results were expressed as EC 50 which means effective concentration at which the absorbance is 0.5. Vit C was used as standard.

2.3.4. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity of CrE and fractions was determined according to the method of Ruch and their collaborators (1989) with slight modifications. A solution of H_2O_2 (43 mM) was prepared in sodium phosphate buffer (0.1 M, pH 7.4). The CrE or each fraction at different concentration in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution. The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the sodium phosphate buffer without H_2O_2. The H_2O_2 scavenging activity was calculated as follows:

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

Where A_c: control absorbance and A_e: absorbance in the presence of CrE or each fraction. EC50 values of the CrE or fractions, concentration of sample necessary to decrease the initial concentration of H_2O_2 by 50%, were calculated.

2.3.5. β- carotene bleaching assay

Antioxidant capacity is determined by measuring the inhibition of CrE and fractions compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Aslan *et al.*, 2006). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1ml of chloroform, and then 25 µl linoleic acid and 200 mg Tween 40 were added in flask. Chloroform was evaporated using a rotavapor. A volume of 100 ml distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking to form emulsion. Aliquot 350 µl of CrE or each fraction, prepared in methanol and/or distilled water at concentration of 2 mg/ml, were added to 2500 µl aliquot of reaction mixture, and the emulsion system was incubated up to 24h in dark at room temperature. Control samples (BHT, H_2O and methanol) received only the emulsion, while blank consisted only of corresponding sample or control. After this incubation period, absorbance of the mixtures was measured at 490 nm after 0h, 1h, 2h, 4h and 24h of incubation. The rate of bleaching of β-carotene was calculated as antioxidant activity and calculated using the equation:

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

Where A_c: absorbance at t = 0, and A_e: absorbance at t = x (1h, 2h, 4h or 24h).

2.3.6. DPPH scavenging assay

The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free-radical scavenging antioxidant activity. The DPPH is a purple-colored stable free radical; it becomes reduced to the yellow-colored, diphenyl picryl-

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

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

Where A_c: control absorbance and A_e: absorbance in the presence of CrE or each fraction. Effective concentration (EC50) values of the extract, concentration of extract necessary to decrease the initial concentration of DPPH by 50%, were calculated.

2.4. Acute oral toxicity of crude extract

The acute toxicity study was conducted to determine the adverse effects of CrE on the target organs (liver and kidney) and the biochemical parameters (urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phaosphatase (ALP)). The CrE was administered in a single dose by gavage, at a dose of 2000 and 5000 mg/kg, to one male and one female under fasting for 4 h. Sequentially, at intervals of 48 h, the same dose was administered to four males and four females, five treated animals in total for each group. In parallel, five males and five females were treated with vehicule (distilled water) in order to establish a comparative negative control group (OECD, 2008).

2.4.1. Observation

The animals were observed periodically during the first 24 hours after administering the CrE and then once a day for 14 days. Observation was carried out focusing attention on animal response and general physical condition. The observation was also carried out the palpation of the abdomen and the thorax searching for palpable masses. We checked the integrity of the

mucous membranes and the skin, as well as hair characteristics and possible alterations in feces and urine coloration (Malone and Robichaud, 1962). Body weights were recorded at the beginning, then once after every seven days during the study. At the end of the observation period, all animals were sacrificed.

2.4.2. Plasma preparation and biochemical analysis

Biochemical analysis were investigated at the end of the experiments period. Animals were fasted 4 h and then sacrificied. The blood sample was collected in heparine-tubes and then centrifuged at 3000 rpm for 10 min. Plasma biochemical parametres : glycemia, urea, creatinine, cholesterol, triglycerides, AST and ALT were analysed using Beckman coulter Synchron (CX 9 PRO) and commercial kits (Spinreact, Spain).

2.4.3. Organs weight

Organs (liver, spleen, kidneys, heart, lungs, brain and stomach) were quikly removed and weighed. The relative organ weight of each animal was then calculated relating the absolute organ weight and body weight of the animal on the day of sacrifice.

2.4.4. Histological analysis

Liver and kidneys were fixed in 10% formol and embedded in parrafin. Sections at 5µm were stained with hematoxylin and eosin and examined under light microscopy (Martey *et al.*, 2010). The analyses of the different organs (liver and kidneys) were performed using an Optika B-500T i-5 microscope (10 and 40×) in order to verify alterations in the tissues. Digital images were obtained using the Optika camera associated to the microscope, and all the images were analyzed using the image processing software, Optika Vision Pro.

2.5. Subacute orale toxicity of crude extract and its *in vivo* antioxidant activity

2.5.1. Animals treatment

Siwss albinos male mice of 25-30 g were used. The animals were distributed randomly into four groups of nine to ten animals each with similar average body weight.

Group C: served as a neutral control, received distilled water with 4% DMSO.

Group Vit C: received 100 mg/kg of Vit C with 4% DMSO.

Group D1: received 250 mg/kg of CrE with 4% DMSO.

Group D2: received 500 mg/kg of CrE with 4% DMSO.

Group D3: received 1000 mg/kg of CrE with 4% DMSO.

Distilled water, Vit C, CrE (250, 500 and 1000 mg/kg) were given orally once daily for 21 days. Weight of all mice was noted before treatment (day 0), on day 7, day 14 and day 21 of treatment (Baghiani *et al.*, 2013).

2.5.2. Collection of blood and plasma

On day 21, blood samples were collected in an EDTA centrifuge tube for oxidative hemolysis inhibition assay. Plasma was separated by centrifugation at 3000 rpm for 10 min and then divided in two aliquot. One plasma aliquot was reserved to analyze the antioxidant activity using DPPH scavenging assay, and the last one was reserved to analyze the biochemical parameters for repeated-dose toxicity.

2.5.3. Oxidative hemolysis inhibition assay

The assay is based on the inhibition of free radical-induced membrane damage in erythrocytes by antioxidants. The advantage of this method is that it uses peroxyl radicals as pro-oxidants and erythrocytes as oxidizable targets so that the results obtained reflect biologically relevant radical-scavenging activity and micro-localization of antioxidants.

The anti-hemolysis activity of treated mice whole blood was evaluating using the same *in vitro* protocol with slight modification. Briefly, 136 µl of AAPH (300 mM) was added to 80µl of mice blood in 96-well microplate. We have controlled the whole blood hemolysis with a 96-well microplate reader device. The kinetics of erythrocytes resistance to hemolysis was determined at 37°C by continuous monitoring of changes in absorbance at 630 nm. Results

were expressed, for the time corresponding to 50% of maximal hemolysis (half-hemolysis time, HT50 in min).

2.5.4. DPPH radical-scavenging activity of plasma

Plasma ability to scavenge DPPH radical was measured with the same *in vitro* method. An aliquot of 25 µl of plasma was added to 625 µl of DPPH solution (0.004%), and the reaction mixture was shaken vigorously. After incubation at room temperature for 30 min, the absorbance of this solution was determined at 517 nm.

2.5.5. Plasma biochemical analysis

Plasma biochemical analysis: urea, creatinine, AST, ALT and ALP were analysed using Beckman coulter Synchron (CX 9 PRO) and commercial kits (Spinreact, Spain).

2.5.6. Organs weight, histological analysis and preparation of tissue homogenates

After sacrifice, organs (liver, spleen, kidneys, heart, lungs, brain and stomach) were quikly removed, washed by NaCl 0.9 %, and weighed. The relative organ weight of each animal was then calculated relating the absolute organ weight and body weight of the animal on the day of sacrifice.

Each animal liver and kidneys were divided in two pieces: one piece was fixed in 10% formol for histological analysis and the last one was immediately stored at -20°C until further antioxidant analyses.

Pieces of liver and kidneys fixed in 10% formol were embedded in parrafin. Sections at 5µm were stained with hematoxylin and eosin and examined under light microscopy (Martey *et al.*, 2010). The analyses of the different organs (liver and kidneys) were performed using an Optika B-500T i-5 microscope (10 and 40×) in order to verify alterations in the tissues. Digital images were obtained using the Optika camera associated to the microscope, and all the images were analyzed using the image processing software, Optika Vision Pro.

After a swift thawing, pieces of livers and kidneys were weighed and homogenized in ice cold KCl (1.15%) to prepare 10% (w/v) homogenate, using electric homogenizer. Then, the homogenate was centrifuged twice at 4000 rpm/min for 10 min at 4°C to purge cellular debris and supernatant was collected and stored at -20°C until quantification of antioxidant enzyme (CAT), GSH and malondialdehyde (MDA). The protein concentration in the supernatant was measured using commercial Kit.

2.5.7. Estimation of CAT activity

The CAT catalyzes H_2O_2 into H_2O and O_2. At 240 nm, absorption of H_2O_2can be measured. Absorbance value of H_2O_2 decreases when it is decomposed by CAT. Thus, CAT activity can be calculated by measuring decrease in absorption of H_2O_2. Estimation of CAT activity was carried out according to Claiborne (1986). The activity of CAT was calculated by taking the extinction coefficient of H_2O_2 to be 43.6 $M^{-1}cm^{-1}$ at 240 nm and expressed as µmole/min/mg protein.

2.5.8. Estimation of GSH

The GSH levels were determined in the homogenates of liver and kidneys using Ellman's reagent (5, 5 0- dithiobis-(2-nitrobenzoic acid), DTNB) (Ellman, 1959). A volume of 5 ml of sodium phosphate buffer (0.1 M, pH = 8) was added to 25 µl of supernatant homogenate, then 1.5 ml of mixture was added to 10 µl of DTNB (0.01 M). Determination of GSH is based on the reaction of DTNB with GSH and yield a yellow colored chromophore (TNB) with a maximum absorbance at 412 nm. The amount of GSH present in the tissue was calculated was calculated using TNB extinction coefficient to be 1.36 $x10^4$ $M^{-1}cm^{-1}$ at 412 nm and expressed as µmoles /g tissue.

2.5.9. Estimation of MDA

The hepatic and renal MDA content was an indicator to determine hepatic and renal lipid peroxidation levels. According to Okhawa (1979), 125 µl of TCA (20%) and 250 µl of

thiobarbituric acid (TBA, 0.67%) were added to 125µl of tissue homogenate. The mixture was incubated at 100 C° during 20 min. After incubation, the sample was cooled by raining the cold water, and MDA-TBA complex was extracted with 1 ml of butanol. The organic phase was separated by centrifugation at 3000 rpm for 15 min and then measured at 530 nm. The concentration of MDA was calculated using MDA-TBA adduct extinction coefficient to be $1.56 \times 10^5\ M^{-1}cm^{-1}$ at 530 nm and expressed as nanomoles MDA/g tissue.

2.6. Statistical analysis

The *in vitro* experimental results are expressed as mean ± SD of triplicate. The data were analysed by Student's *t*-test to determine statistical significance. The *p* values less than 0.05 ($p < 0.05$) were considered as indicative of significance. The EC_{50} values were calculated from linear regression analysis. On the other hand, *in vivo* experimental results are expressed as mean ± SEM. Statistical analysis of data was accomplished using one-way analysis of variance (ANOVA; $p < 0.05$) and "Dunnett's multiple comparison test" was used to compare with control group followed by "Tukey's multiple comparisons test" were used to determine significant differences between groups. The $p < 0.05$ were considered as indicative of significance. The Pearson correlation analysis was performed between antioxidant activity and total phenolic and flavonoids contents, and also between total phenolic content and flavonoids content. All statistical analysis and graphing of data were performed using Graph pad prism 5 Demo.

RESULTS AND DISCUSSION

1. Preparation of crude extract and fractions

Extraction is a very important step in the isolation, identification and use of phenolic compounds and there is no single and standard extraction method. Solvent extraction is the most common used techniques for the extraction of phenolic compounds due to their ease of use, efficiency, and wide applicability. It, as a function of the biomass status, may be solid-liquid extraction or liquid-liquid extraction (Ignat *et al.*, 2011).

Extraction efficiency is known to be a function of process conditions. Several factors affect the concentration of the desired components in the extract: solvent types, solvent strength, extraction time, agitation speed, sample-solvent ratio, temperature and particle size. However, it is reported that the most influential factor is solvent types (Azwanida, 2015).

Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest. In order to extract different polyphenols (a class of rather hydrosoluble molecules) with a high degree of accuracy, scientists have discovered that using highly polar solvents, such as methanol, is the most efficient extraction (Altemimi *et al.*, 2017). Furthermore, it is reported that methanol was more selective than ethanol for extracting polyhenols (Barla *et al.*, 2007). For the purpose of antioxidant extraction, studies have demonstrated that methanol has a high effectiveness as antioxidants (Dhawan and Gupta, 2017). For these raisons, the solvent that is retained for our study is the methanol.

A preliminary fractionation of CrE was carried out as sequential extraction with a number of solvents of increasing polarity: hexane, chlorophorm and ethyle acetate. This can lead to the separation of glycosides from aglycones polyphenols and to the separation of polar from non-polar aglycones. Although a certain degree of overlap could occur.

The appearance, color, and yield of CrE and fractions relative to the weight of the powder and relative to the CrE are shown in the table 4.

Table 4. Appearance, color and yields of *R. picroides* CrE and its fractions.

Extracts	Appearence	Color	Yield (relative to 100g of CrE)	Yield (relative to to 100 g of dry weight)
CrE	Pasty	dark green	100 %	19.87 %
Hex	Oily	dark green	8.50 %	1.67 %
ChE	Powder	brownish	7.70 %	1.53 %
EAE	Powder	Brownish	7.67 %	1.52 %
EAq	Pasty	Brownish	56.67 %	11.26 %

CrE: crude extract, Hex: hexane extract, ChE: chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract.

The yield of the CrE was calculated from the initial weight of the used plant powder. Ressults showed that CrE registered a yield of 19.87 ± 6.75 % which was significantly higher than the one (8.50 %) found by Recio and their collaborators (1992). Such difference may be due to the extraction method used. While AqE gave the highest yield relative to dry weight of used powder with 11.26%, Hex, ChE and EAE gave approximately the same yield (1.67, 1.53 and 1.52%, respectively). The yields sum of the four fractions relative to the CrE is 80.53 %, which may explain by losses during the high number of wash performed for the entire fractionation.

2. Phytochemical characterization

2.1. Analytic chromatography method

More conventional methods of separation are the use of column and thin layer chromatography. Column chromatography remains the single most useful technique for the isolation of large quantities of flavonoids from crude plant extracts.

In our study, we applied a conventional chromatographic technique using a column on silica gel for separating CrE based on its polarity. We chose dichloromethane and dichloromethane/ methanol of increasing polarity (1 %, 2 %, 3 %, 5 %, 7 % 10 %, 15 %, 20 %, 25 %, 30 %, 35 %, 40 %, 45 %, 50 %, 60 %, 80 % and 100 % v / v methanol / dichloromethane) as elution system. In total, 660 fractions (400 ml each) are collected and combined on 12 different sub-fractions (F1 - F12) according to their TLC profile. Indeed, finding a good solvent system is

usually the most difficult part of TLC. For this reason, multiple trials were carried out to find the appropriate elution system for each fraction, resulting in three solvent systems (table 5).

Table 5. Elution system for TLC and yield of each fraction from *R. picroides* CrE column chromatography.

Fractions	Elution system (TLC)	Yield (relative to 150 g of CrE)
F1	Hexane/Ethyl acetate/Acetic acid (80/20/2)	0.66 %
F2	"	0.98 %
F3	"	1.49 %
	"	
F4	"	0.77 %
F5	"	1.77 %
F6	"	1.35 %
F7	"	2.30 %
F8	"	2.05 %
F9	"	3.97 %
F10	Chloroform/Methanol/Acetic acid (70/30/2)	12.50 %
F11	"	27.56 %
F12	Chloroform/Methanol/Acetic acid (70/30/10)	25.69 %

2.1.1. Isolation and identification of compound C1

Treatment of F10 with methanol afforded pure white crystals, which was identified as luteolin 7-O-β- glucoside (figure 7). Upon further comparison with published literature (Lin *et al.*, 2015), we were able to confirm the chemical structure of C1. In fact, NMR spectrum of C1 showed a great similarity with that obtained by Lin and their collaborators (2015).

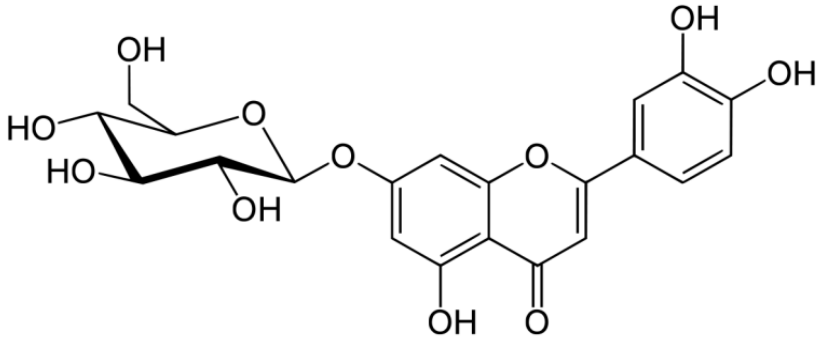

Figure 7. Luteolin 7-O-β-glucoside.

Data obtained from NMR spectroscopic analysis of luteolin-7-O-β-glucoside was represented in table 6.

Table 6. The NMR spectroscopic data of luteolin-7-O-glucoside.

Position	δ_H	Multiplicity, J(Hz)	δ_C	Position	δ_H	Multiplicity, J (Hz)	δ_C
2	/	/	164.96	3′	/	/	146.28
3	6.71	s	103.60	4′	/	/	150.49
4	/	/	182.35	5′	6.86	d, J = 8.35	116.66
5	/	/	161.61	6′	7.41	m	119.64
6	6.41	s	99.99	1″	5.04	d, J = 7.45	100.36
7	/	/	163.42	2″	Nd	/	73.59
8	6.75	s	95.18	3″	Nd	/	76.86
9	/	/	157.41	4″	Nd	/	70.02
10	/	/	114.01	5″	Nd	/	77.63
1′	/	/	126.86	6″	Nd	/	61.08
2′	7.39	d, J = 7.7	116.45	OH-5	12.95	/	/

The ^{1}H NMR (DMSO, 500 MHz), ^{13}C NMR (DMSO, 125 MHz) and DEPT 135 (DMSO, 500 MHz) spectra were presented, respectively, in figure 8, 9 and 10.

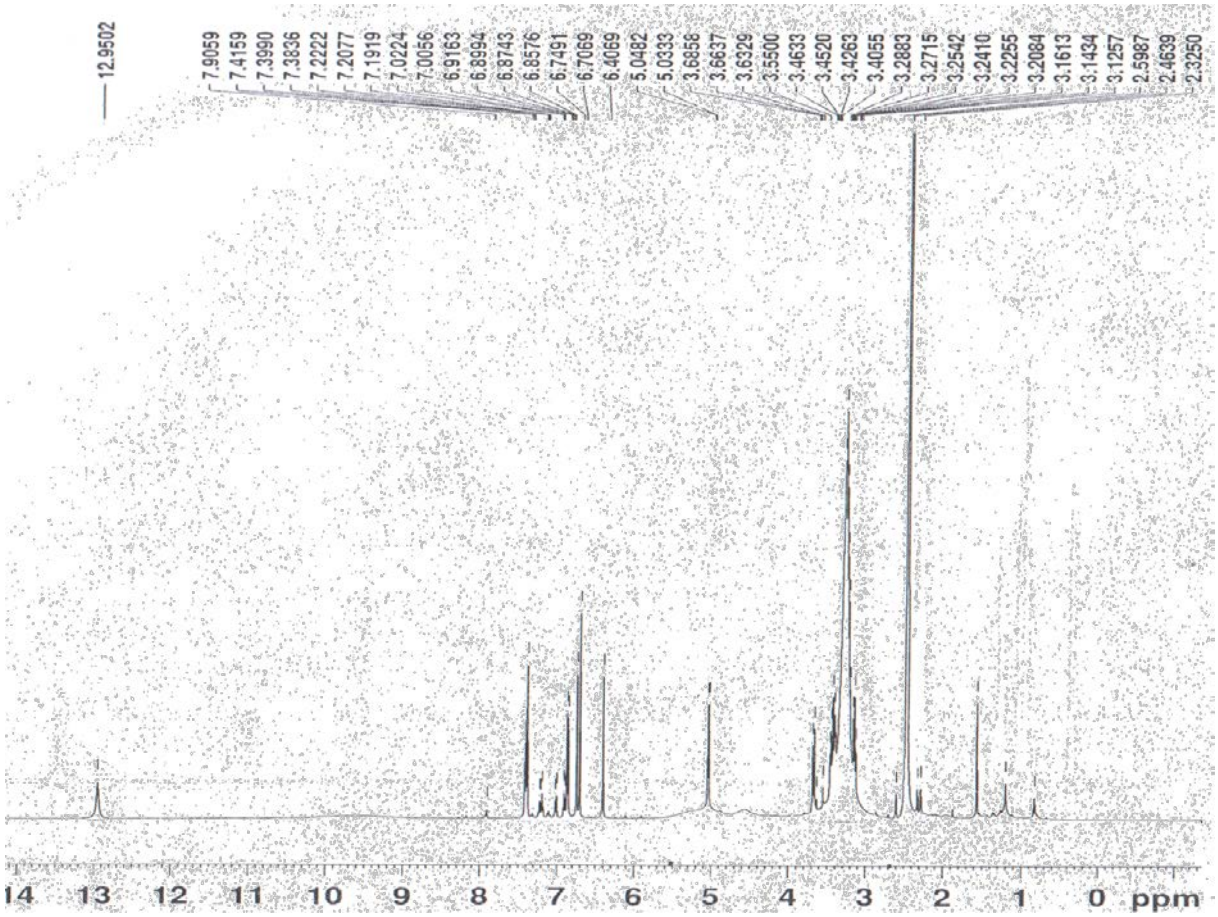

Figure 8. The ^{1}H NMR (DMSO, 500 MHz) of luteolin 7-O-β-glucoside.

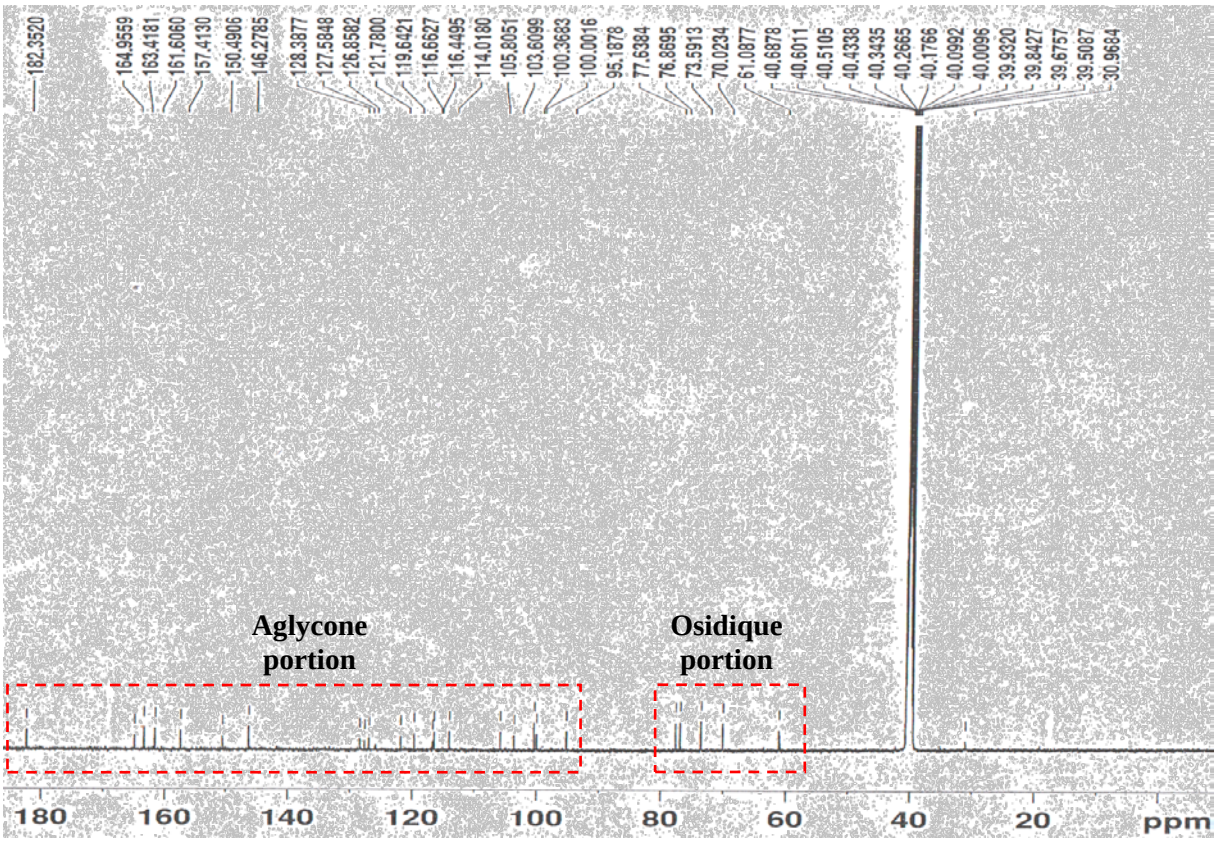

Figure 9. The ^{13}C NMR (DMSO, 125 MHz) of luteolin 7-O-β-glucoside.

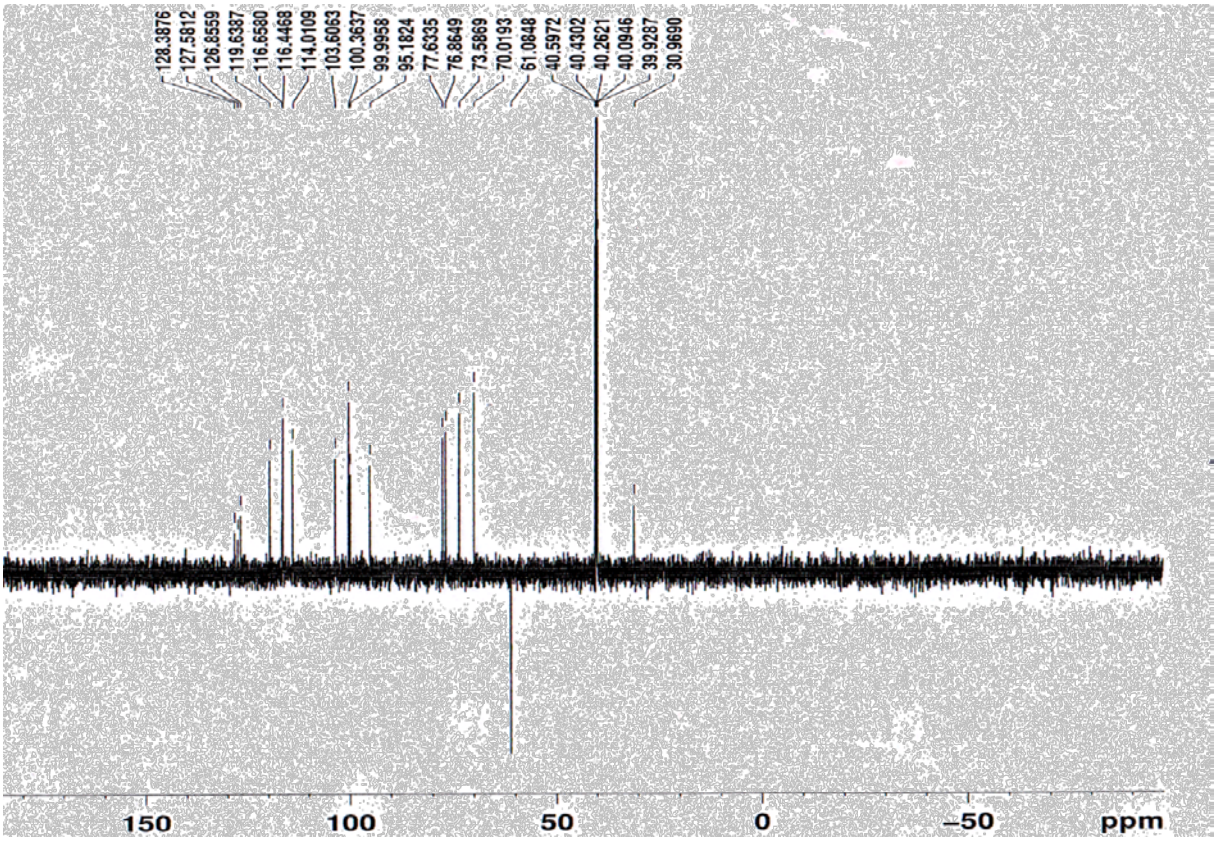

Figure 10. The DEPT (DMSO, 500 MHz) of luteolin 7-O-β-glucoside.

2.2. Determintion of total phenolics and flavonoids contents

As phenolic compounds constitute one of the major contributors to the antioxidant capacity of plant, it was reasonable to determine their total amount in the selected plant extracts. The total phenolics content of CrE and fractions obtained from preliminary fractionation and column chromatography was estimated by the Folin–Ciocalteu method (Li *et al.,* 2007), expressed as GAE. Folin- Ciocalteu method is very popular, convenient, simple, reproducible and economic for the measurement of phenolics (Agbor *et al*., 2014).

The recovery of phenolic contents in different samples is influenced by the polarity of extracting solvents and the solubility of this compound in the solvent used for the extraction process (Sulaiman *et al.,* 2011).

Measured (figure 11) illustrated that significant difference ($p < 0.0001$) in total phenolic contents was observed compared to CrE (81.21 ± 0.51 µg GAE / mg of extract). Also, results showed that the total phenolic compounds varied greatly among different fractions obtained from preliminary fractionation. The EAE contained the highest amount 331.64 ± 3.11 µg GAE / mg of extract followed by ChE (146.82 ± 2.96 µg GAE / mg of extract) and then AqE (37.27 ± 0.77 µg GAE / mg of extract). This indicated the possible influence of fractionating solvent on total phenolic contents. Furthermore, among all fractions obtained from column chromatography, F10 (133.55 ± 2.96 µg GAE / mg of extract), F11 (91.70 ± 2.22 µg GAE / mg of extract) and F12 (106.18 ± 2.06 µg GAE / mg of extract) were found to contain the highest amount of phenolic compounds which were significantly higher than that of CrE. From the same figure, it is clear that the rest of fractions presented a total phenolic content lower then CrE.

Since flavonoids are probably the most important natural polyphenols (Djeridane *et al.,* 2010), its amount in CrE and each fractions was measured. The $AlCl_3$ method is simple, inexpensive, offer sigh sensitivity, which makes it preferred in quality control and analytical

laboratories. In addition, this method allows the total flavonoids content to be determined even in the presence of other polyphenolic compounds not forming complexes with $AlCl_3$ (Matyushchenko and Stepanova, 2003).

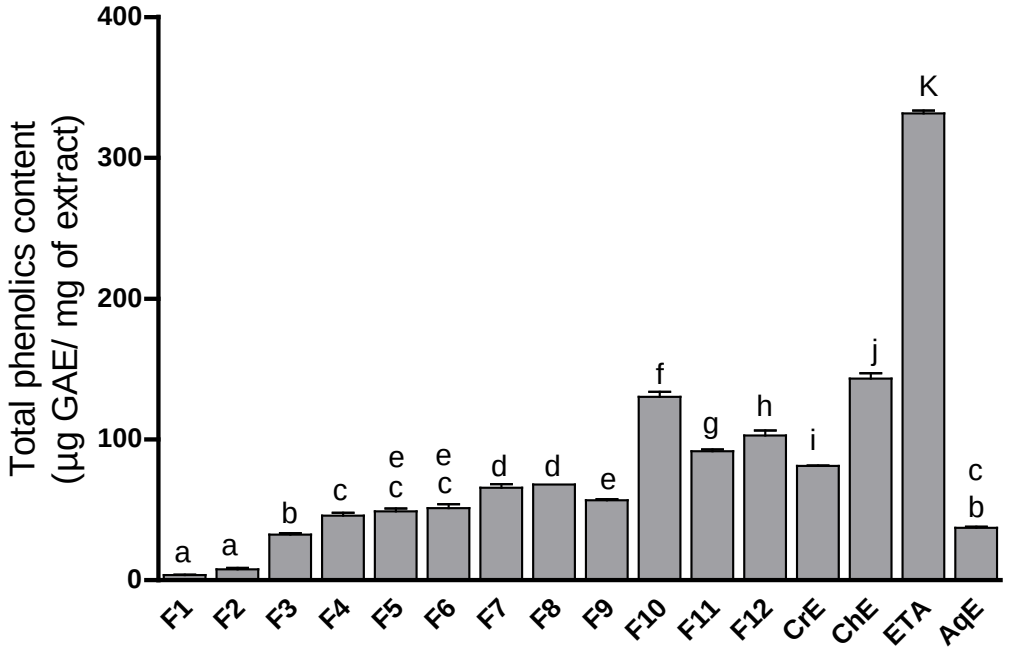

Figure 11. Total phenolic content in *R. picroides* CrE and its fractions. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction.

Results (figure 12) showed that significant difference ($p < 0.0001$) in flavonoids contents was observed compared to CrE (12.77 ± 0.10 µg QE / mg of extract). Also, results showed that the flavonoids content varied greatly among different fractions obtained from preliminary fractionation. The EAE contained the highest amount 48.14 ± 0.70 µg QE / mg of extract followed by ChE (22.55 ± 0.46 µg QE / mg of extract) and then AqE (5.16 ± 0.07 µg QE / mg of extract). This indicated the possible influence of fractionating solvent on flavonoids contents. Furthermore, among all fractions obtained from column chromatography, F9 (16.12 ± 0.77 µg QE / mg of extract) and F10 (33.47 ± 0.24 µg QE / mg of extract) were found to contain the highest amount of flavonoids which were significantly higher than that of CrE. From the same figure, it is clear that the rest of fractions presented flavonoids content lower than CrE.

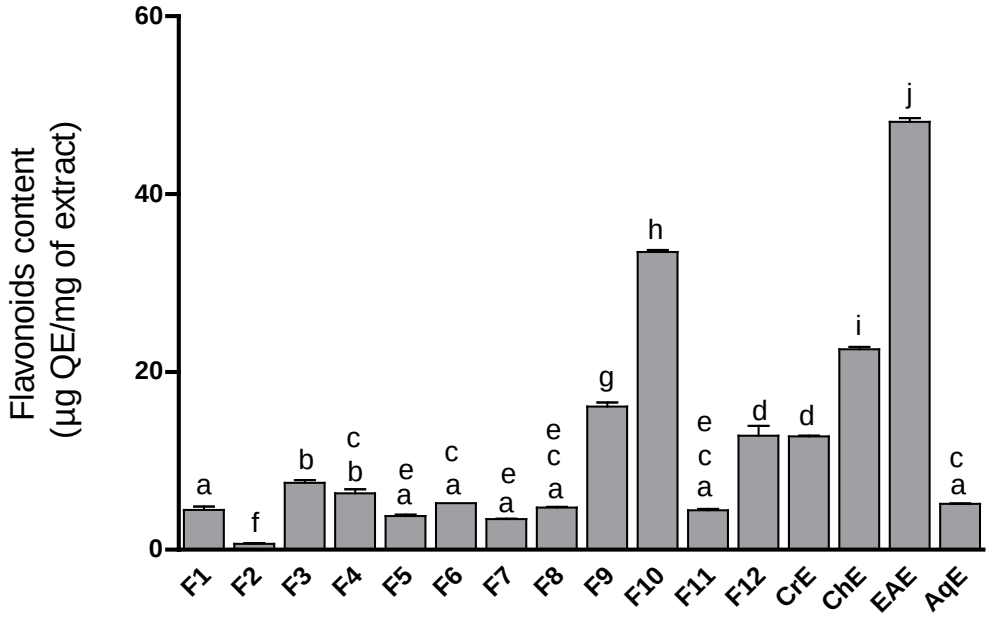

Figure 12. Flavonoids content in *R. picroides* CrE and its fractions. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction.

A correlation analysis was performed on total phenolics and flavonoids contents using Pearson's correlation coefficient. Pearson's correlation coefficient was positively high if $0.610 < r < 0.974$ and negatively high if $-0.610 < r < -0.974$ (Fitriansyah *et al.*, 2017). As expected, total phenolics content of CrE and fractions had strong significant and positive correlation with flavonoids content ($r = 0.9034$, $p < 0.0001$). This indicates that flavonoids are the dominating phenolic group in CrE and its fractions.

3. The *in vitro* evaluation of antioxidant activity

3.1. Anti-hemolytic assay

The most plentiful cells in the human body are found to be the erythrocytes, which own copious biological and morphological characteristics, hence they have been widely exploited in drug transport. The polyunsaturated fatty acids and hemoglobin molecules which are redox active oxygen transport molecules and potent promoters of activated oxygen species mainly target the erythrocytes. Oxidative mutilation to the erythrocyte membrane lipids and proteins may be responsible for hemolysis accompanying with several factors (Afsar *et al.*, 2016).

Thus, oxidation of erythrocytes serves as a good model for the oxidative damage of biological membrane of their ease of isolation, their simplicity, the richness of their polyunsaturated fatty acid membranes and the high oxygen and hemoglobin cell concentration (Hammoudi *et al.*, 2017). This experiment was aimed to assess whether CrE and fractions prevented oxidative damages to erythrocyte membrane or not.

Results showed that CrE, fractions and Vit C significantly ($p < 0.05$) protected the erythrocyte membrane from hemolysis in a time-dependent manner. It appeared from the hemolysis sigmoid curves that CrE, fractions and Vit C lead to hemolysis delay which is evident as deviation of the curves to the right (figure 13). In our study, the incubation of erythrocyte together with AAPH led to remarkable hemolysis that was agree with previous findings (Phrueksanan *et al.*, 2014). In addition, the results from the AAPH-induced hemolysis of mice erythrocyte are similar to hemolysis of human erythrocytes (Wang *et al.*, 2009).

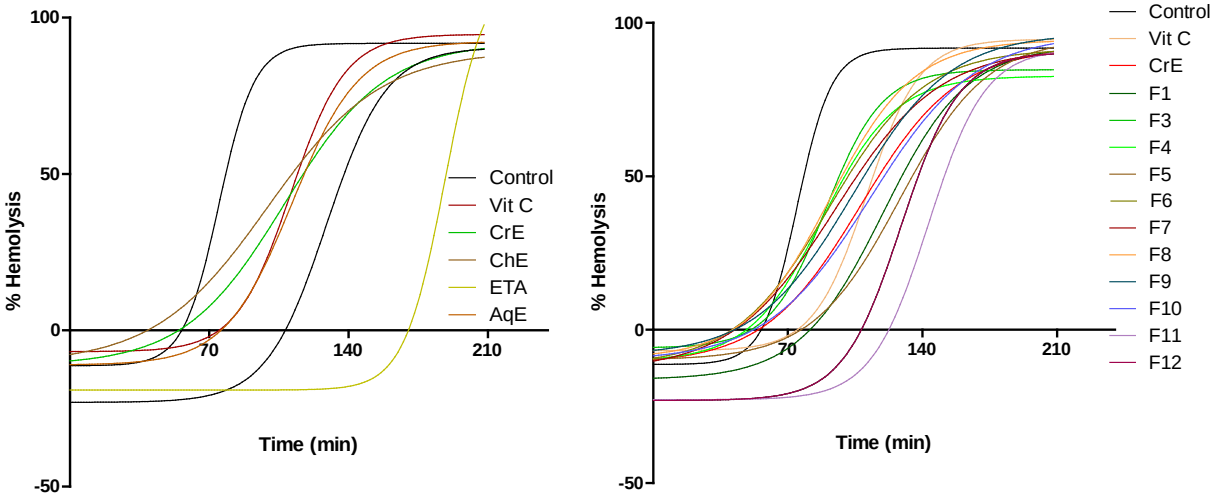

Figure 13. Kinetics of erythrocytes hemolysis in the presence of CrE, fractions, control and Vit C during 208 min. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, Vit V : vitamin C.

Results presented in figure 14 showed that CrE, fractions and Vit C had a very important ($p < 0.0001$) antihemolytic activity and revealed an extension of HT 50 from 76.92 ± 3.59 min of control to 188.15 ± 1.91 min of EAE which presented the greatest protective effect. Statistically, significant difference was not observed between CrE (HT 50 = 108.03 ± 2.65

min), AqE (HT 50 = 111. 83 ± 6.83 min) and Vit C (111.10 ± 1.51 min), while the ChE showed the lowest activity (HT 50 = 99.36 ± 2.49 min). Among column chromatography fractions, F5 (HT 50 = 134.65 ± 2.90 min) and F11 (HT 50 = 143.40 ± 3.12 min) exhibited the highest antihemolytic activity which significantly ($p < 0.0001$) higher than that of Vit C.

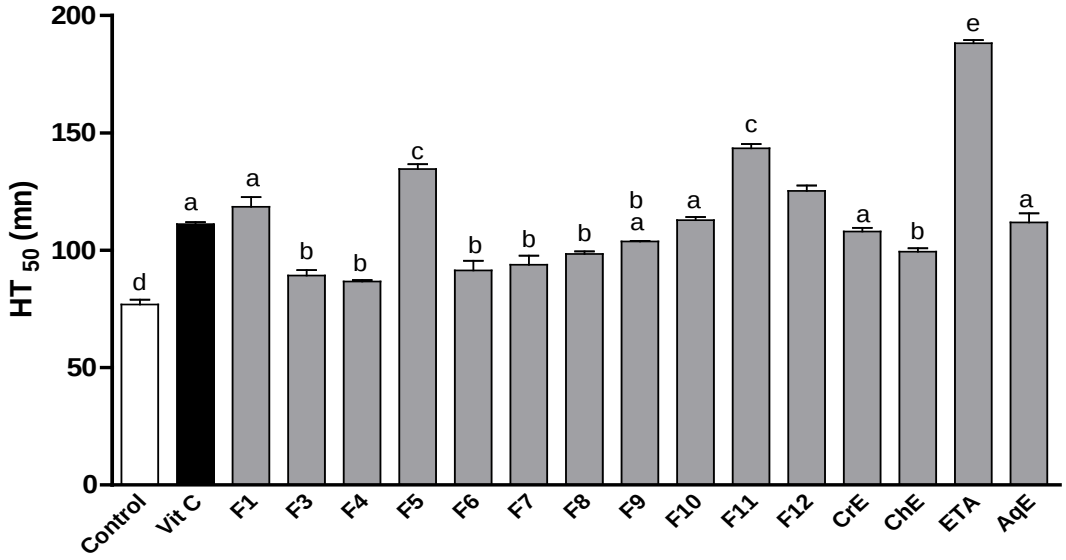

Figure 14. The HT 50 values of antihemolytic activity of CrE, fractions, control and Vit C. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, Vit V : vitamin C.

Biological membranes can be affected by many natural products present in medicinal plants. Various authors mentioned that phenolic compounds and flavonoids had beneficial effect on the erythrocyte membrane stability (Ramchoun *et al.*, 2015). Since phenolic compounds appear to function as good electron and hydrogen atom donors, and therefore, be able to terminate radical chain reaction by converting free radicals and ROS to more stable products (Loganayaki, 2013). The correlation analysis indicated that there is a significant positive correlation between total phenolics content and HT 50 of antihemolytic activity (r = 0.7217, $p = 0.001$) but it was weak with flavonoids content (r = 0.5753, $p = 0.0248$). A good part of the resistance of the erythrocytes to hemolysis inducted by CrE and fractions can be linked up to the content of polyphenols. Our findings are in agreement with studies showing that polyphenols protect erythrocytes from oxidative stress or increase their resistance to oxidative damage (Khalili *et al.*, 2014; Ramchoun *et al.*, 2015; Joujeh *et al.*, 2017).

3.2. Ion chelating assay

The metal chelating capacity is important since it reduces transition metal acting as catalysts to generate the first few radicals and initiate the radical mediated oxidative chain reactions in biological or food systems. Ion chelating agents also may inhibit the Fenton reaction and lipid peroxidation (Apak *et al.*, 2016). Thus, an antioxidant's ability to chelate metals is an important antioxidant property to measure. Ferrous ions are generally present in food systems and are considered as effective pro-oxidants. Ferrozine and Fe^{2+} form coloured complex (violet colour). In the presence of chelating agents, ferrozine-Fe^{2+} ion complex is disturbed, resulting in a decrease in color of the complex. Measurement of the color reduction allowed for estimate the metal chelating activity for the coexisting chelator (Kumar and Jain, 2015).

The chelating abilities increased as concentration increased. The strongest chelating activity was showed by F2 (EC50 = 0.14 ± 0.00 mg/ml), F3 (EC50 = 0.17± 0.03 mg/ml) and F12 (EC50 = 0.05 ± 0.01 mg/ml) which were similar to that of EDTA (figure 15). Interestingly, F2 and F12 showed the same chelating activity although the difference in polyphenols and flavonoids composition was clear ($p < 0.001$), noting that F2 was the less rich. We suppose that the compounds containing nitrogen were the responsible for this activity since they are generally more powerful chelating than phenolic compounds (Fadavi *et al.*, 2011).

Another important finding was that AqE (EC50 = 0.56 ± 0.01 mg/ml) was significantly similar to EDTA (EC50 = 0.006 ± 0.00 mg/ml). Then, the sequence for the chelating activity (figure 15) was CrE (EC50 = 0.90 ± 0.01 mg/ml) = EAE (EC50 = 1.29 ± 0.05 mg/ml) > ChE (EC50 = 7.18 ± 0.60 mg/ml). Noting that AqE contained the lowest amount of both total phenolics and flavonoids compared to CrE, ChE and EAE; however, it exhibited the highest chelating activity. This result can be explained by the work of Sahreen and their collaborators (2010), showing that plant fractions induce chelating activity directly proportional to the polarity of their solvents.

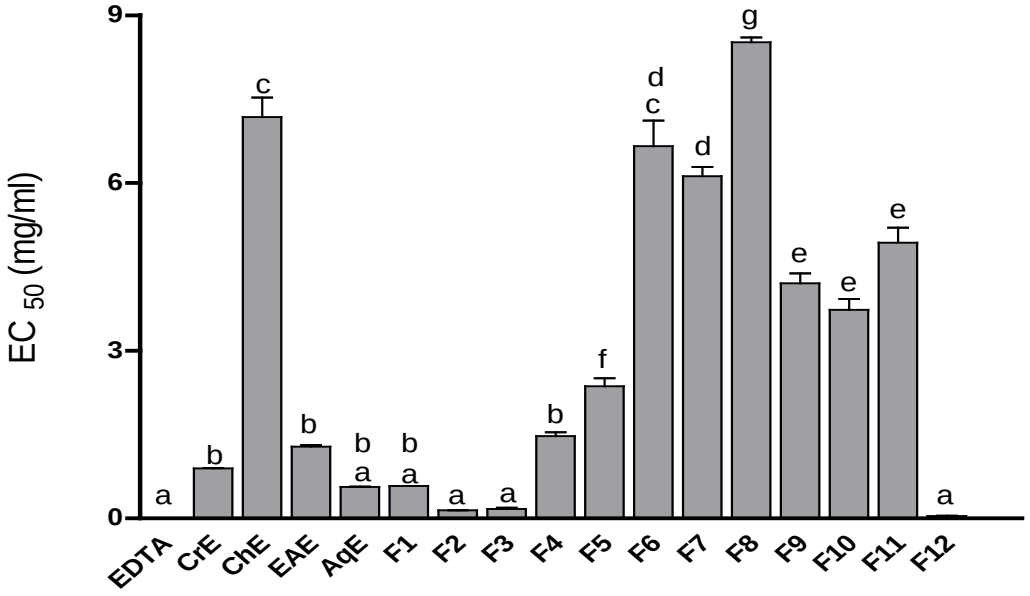

Figure 15. The EC50 values of ferrous iron chelating activity of CrE, fractions and EDTA. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, EDTA : ethylenediaminetetraacetic acid.

According to the literature, studies undertaken in recent years have demonstrated that the polyphenols and flavonoids can be excellent chelators for iron and copper (Papuc *et al.*, 2017). However, through the correlation analysis, the chelating activity of CrE and fractions did not point to any correlation with phenolic compounds content (r = 0.0908, $p = 0.7379$) and flavonoids content (r = -0.3905, $p = 0.1348$). The lack of correlation indicated that phenolic compounds and flavonoids played a weak role on the ferrous chelating activity. Our results are consistent with those found by Esmaeili and their collaborators (2015) who reported that there is no correlation between phenolic compounds and flavonoids contents of the extract from the *in vitro* grown *Trifolium pretense* and ion chelating activity.

Several studies show that only phenolic compounds with a certain structure and functional groups can act as transition metals chelators and show a chelating activity (Morel *et al.*, 1998; Mira *et al.*, 2002; Mladěnka *et al.*, 2011; Papuc *et al.*, 2017). From our findings, we can suggest that the chelating activity of CrE and fractions did not return only to the amount of polyphenols but also to their structure and to the possible synergism with other compounds.

3.3. Reducing power assay

Reducing power assay is often used to evaluate the ability of antioxidant to donate electron. Many reports have revealed that the electron-donating ability of polyphenols reflects the reducing power of these biomolecules and is also associated with their antioxidant activity. Polyphenols can break the free radical chain by donating electrons and, for this reason; the transition metal reducing power of polyphenols is correlated with their antioxidant activity (Papuc *et al.*, 2017). The reducing power assay was ascertained using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the colour of the test solution, which was yellow, transforms to various hues of green and blue, based on the extent of the reducing power of the samples. The presence of the antioxidants in the samples leads to Fe^{3+}/ferricyanide complex reduced to the Fe^{2+} form, and Fe^{2+} can be monitored through the measurement of the formation of Perl's Prussian blue at 700 nm (Esmaeili *et al.*, 2015; Kumar and Jain, 2015).

The CrE and studied fractions showed reducing power in a concentration-dependent manner. The EAE exhibited the highest reducing power (EC 50 = 50.16 ± 0.50 µg/ml) which was significantly ($p < 0.0001$) similar to that of Vit C (EC 50 = 48.96 ± 1.50 µg/ml). Thus, it is suggested that EAE has a remarkable potency to donate electron to reactive free radicals, converting them into more stable nonreactive species and terminating the free radical chain reaction. The sequence for this reducing power, as shown in figure 16, was as follows: ChE (EC 50 = 81.75 ± 5.50 µg/ml) > CrE (EC 50 = 233.05 ± 4.23 µg/ml) = AqE (EC 50 = 260.35 ± 5.29 µg/ml). Also in terms of column chromatography fractions, the reducing power of fractions was increased gradually with increasing polarity of elution system. Among fractions (figure 16), F11 (88.97 ± 1.25 µg/ml) showed the highest reducing power, although, in comparison with Vit C, it is significantly lower. A non significant correlation between EC50 and phenolics content (r = -0.5645, $p = 0.0588$) was found. It might be feasible to suggest that phenolics did not act as the major reducer compounds. There might be possibility of

synergism with other active constituents, which are nonphenolic in nature. Moreover, the lack of correlation between EC50 and flavonoids content may be linked to the assumption that most of the flavonoids were in their glycoside forms and thus less effective compared to their aglycone forms (Anwar *et al.*, 2013). Therfore, it can be said that the reducing power cannot always be attributed to phenolics, especially to flavonoids (Nur Arina and Azrina, 2016).

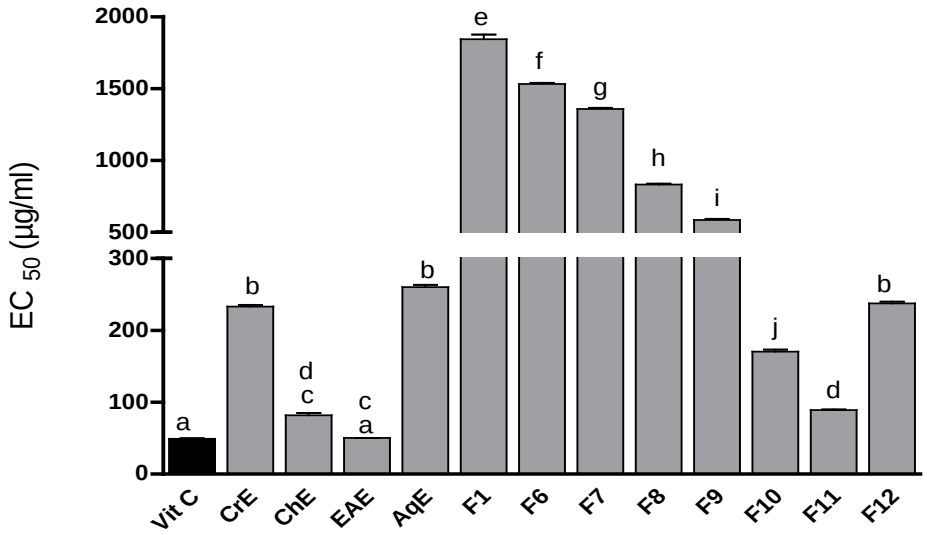

Figure 16. The EC50 values of reducing power of CrE, fractions and Vit C. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly (p < 0.05) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, Vit C : vitamin C.

3.4. Hydrogen peroxide scavenging assay

While H_2O_2 itself is not very reactive, it can sometimes be toxic to cell because it may give rise to •OH radical in the cells that can initiate lipid peroxidation and cause DNA damage (Sumathi and Anuradha, 2016). Therefore, removing of H_2O_2 is very important for antioxidant defence in cell system.

The scavenging effect of CrE and studied fractions on H_2O_2 was concentration-dependent. Results showed an important finding which was the strongest effect of F3 (EC50 = 28.66 ± 0.48 µg/ml) and F5 (EC50 = 19.18 ± 0.51 µg/ml) among the rest of the other fractions, significantly higher than that of BHT (p < 0.0001). As compared the EC50 values, the H_2O_2 scavenging activity of CrE (EC50 = 92.84 ± 1.21 µg/ml), ChE (EC50 = 98.16 ± 0.77 µg/ml)

and EAE (EC50 = 83.38 ± 0.73 µg/ml) was similar and significantly ($p < 0.0001$) higher than that of BHT (EC50 = 138.10 ± 1.44 µg/ml). However, AqE (EC50 = 406.62 ± 15.04 µg/ml) showed a weak effect (figure 17).

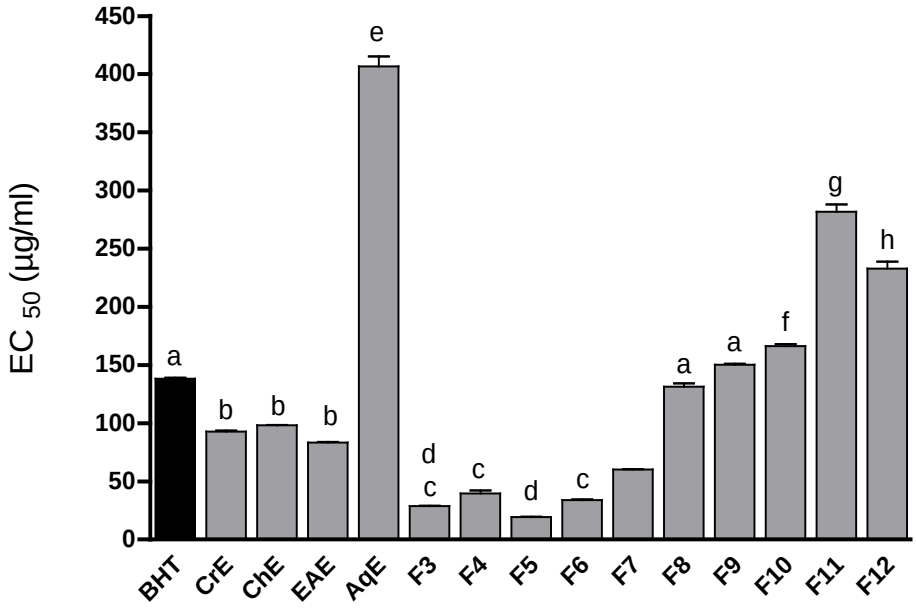

Figure 17. The EC50 values of H_2O_2 scavenging activity of CrE, fractions and BHT. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, BHT : butylated hydroxytoluene.

According to recent reports, a highly positive relationship between total phenols and H_2O_2 scavenging activity appears to be the trend in many plant species (Ruskin *et al.*, 2017), suggesting that phenolics compounds may be a responsible for this antioxidant activity. However, in our study, correlation analysis revealed no significant correlation between both total phenols and flavonoids contents and EC 50 of H_2O_2 scavenging activity (r = -0.0466, p = 0.8742 and r = -0.0544, p = 0.8534, respectively). Similar correlation analysis was done in *Caesalpinia pulcherrima* flowers where the total phenol content showed no significant correlation with H_2O_2 scavenging activity (Dela Torre *et al.*, 2017).

3.5. β- carotene bleaching assay

The antioxidant activity of CrE and fractions was also evaluated by the β-carotene-linoleate bleaching assay because β-carotene shows strong biological activity and constitutes

physiologically important compound (Msaada *et al.*, 2017). In this system, the peroxyl free radicals were generated due to oxidation of linoleic acid by abstraction of hydrogen atom from diallylic methylene groups of linoleic acid located on carbon-11 between two double bonds. The generated peroxyl radicals decolorize the highly unsaturated β-carotene in the absence of antioxidant (Kumar and Jain, 2015). 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. Hydroperoxides formed in this system will be degraded by the antioxidants from the extract/fractions. Accordingly, the absorbance decreased rapidly in samples without antioxidant whereas, in the presence of an antioxidant, they retained their color, and thus absorbance, for a longer period of time (Pandey *et al.*, 2018).

The bleaching kinetics of β-carotene in the presence CrE, fractions, controls and standard are shown in figure 18 and 19. The CrE, fractions, controls and standard inhibited the oxidation of β-carotene. This effect is due to either the inhibition of linoleic acid peroxidation or the radical scavenging of hydroperoxides formed during the peroxidation of linoleic acid.

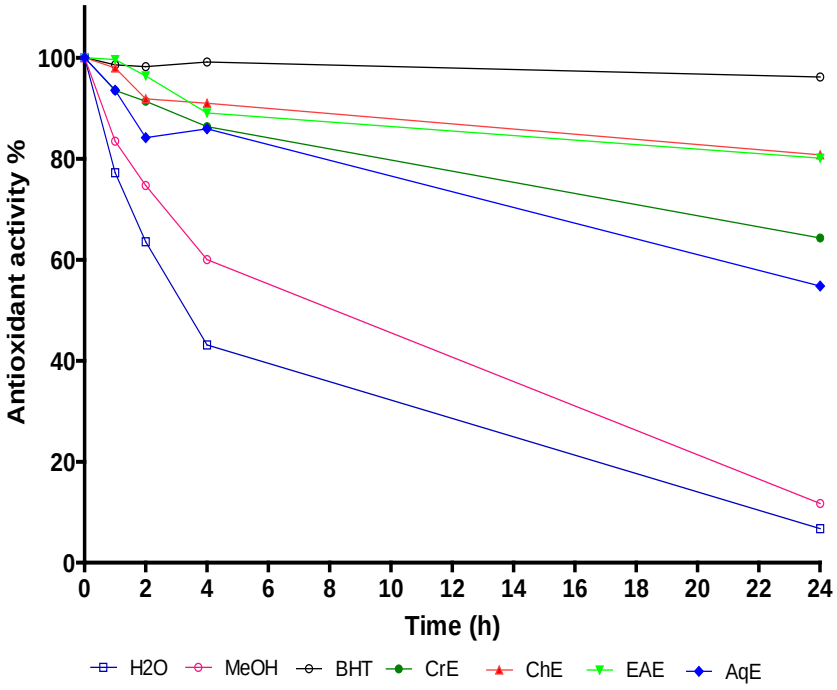

Figure 18. Kinetics of β-carotene bleaching in the presence of CrE, ChE, EAE, AqE, water, methanol and BHT during 24h. CrE : crude extract, ChE : chloroform extract, EAE: ethyle acetate extract, AqE : aqueous extract, BHT : butylated hydroxytoluene, MeOH : methanol.

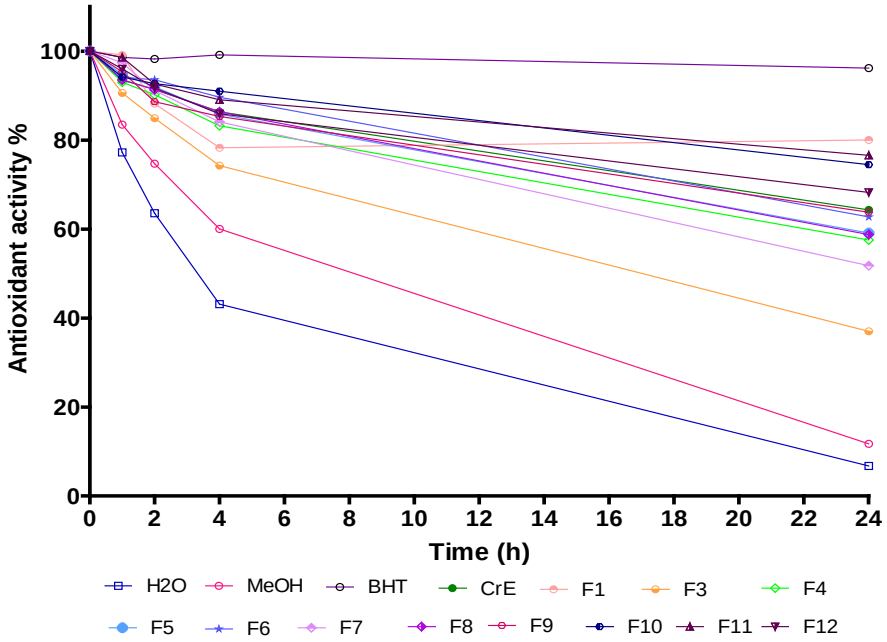

Figure 19. Kinetics of β-carotene bleaching in the presence of CrE and its column chromatography fractions, water, methanol and BHT during 24h. CrE : crude extract, F : fraction, BHT : butylated hydroxytoluene, MeOH : methanol.

Results showed that the ChE (AA % = 80.78 ± 4.57 %) and EAE (AA % = 80.11 ± 4.24 %) were more powerful in inhibiting the percentage of β-carotene bleaching than CrE (64.30 ± 13.81 %) and AqE (AA % = 54.76 ± 7.52%). Furthermore, fractions obtained from column chromatography separation showed different degrees of antioxidant activity. The F1 (AA % = 80.02 ± 7.36 %), F10 (AA % = 74.49 ± 11.14 %), F11 (AA % = 76.58 ± 8.26 %) and F12 (AA % = 68.23 ± 4.50%) exhibited maximum antioxidant activity with significant differences than others. As described in figure 20, there were significant differences ($p < 0.05$) between the CrE, fractions, and BHT effect. From these results, the plant fractions exhibiting the greatest antioxidant potential were those with the highest levels of total polyphenols. However, with reference to correlation analysis, there was a weak correlation between both phenolic compounds and flavonoids contents and antioxidant activity based on β-carotene bleaching assay (r = 0.5217, $p = 0.0461$ and r = 0.5258, $p = 0.0441$, respectively). This showed that the phenolic compounds or flavonoids from the plant in this study weakly inhibited the oxidation of β-carotene by hydroperoxides. Possibly, other bioactive compounds were responsible for the antioxidant activity determined (Nur Arina and Azrina, 2016).

Our findings were consistent with the studies conducted by Othman and their collaborators (2014) and Sahareen and their collaborators (2017) who showed that there was no correlation between antioxidant activity based on β-carotene bleaching assay and total phenolics contents for four Malaysian local herbs (*Melicope Iunu-ankenda*, *Polygonum minus*, *Murraya Koenigii* and *Eugenia polyantha*) and *Carissa opaca*, respectively.

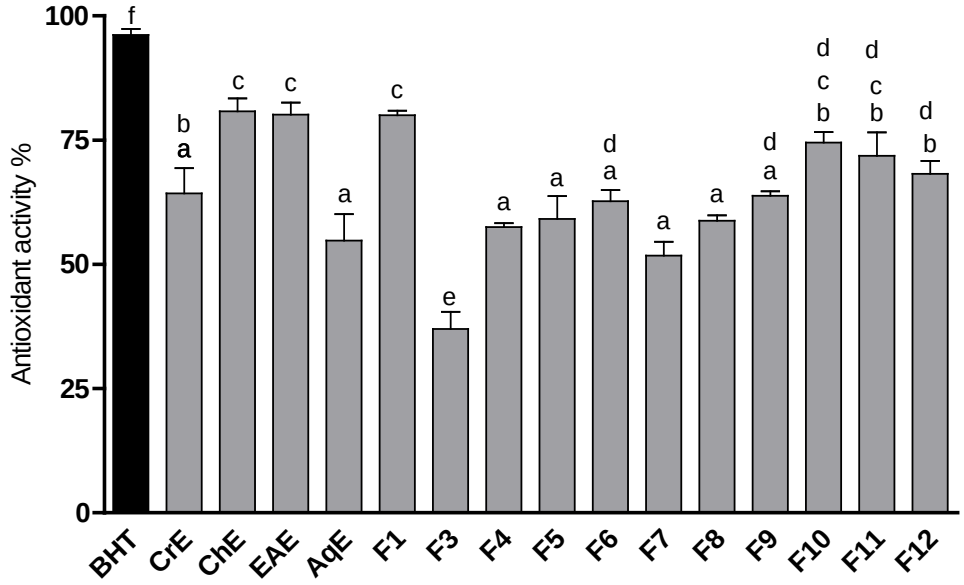

Figure 20. Percentage values of antioxidant activity (β-carotene bleaching assay) of CrE, fractions and BHT. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, BHT : butylated hydroxytoluene.

3.6. DPPH scavenging assay

DPPH radical scavenging assay is one of the most popular and frequently employed methods among antioxidant assays. The method is useful in light of its high reproducibility, easy measurements taken at ambient temperature without risk of thermal degradation, efficient, relatively inexpensive, and quick (Akar *et al.*, 2017). Furthermore, it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations. During this assay, the purple chromogen radical reacts with both electron and hydrogen donors to the corresponding pale yellow hydrazine, and the reaction is monitored at 517 nm (Musa *et al.*, 2016). In other words, the DPPH scavenging assay is based on both electron transfer and hydrogen atom transfer pathways (Liang and Kitts, 2014).

Results showed that CrE and all fractions scavenge DPPH radical in concentration dependent manner. The EC50 of an extract is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis (Oliveira *et al.*, 2016).

The EAE and ChE were found to exhibit significantly ($p < 0.0001$) a strong scavenger activity (EC50 = 6.41 ± 0.51 and 28.94 ± 0.16 µg/ml, respectively) which was surprisingly similar to that of quercetin (EC50 = 2.57 ± 0.00 µg/ml). Then, the DPPH scavenging activity decreased in the following order: CrE (EC50 = 38.56 ± 0.18 µg/ml) >AqE (EC50 = 66.87 ± 1.02 µg/ml). Interestingly, all the previous extracts except AqE showed significantly a higher DPPH scavenging activity than that of BHT (EC50 = 87.27 ± 0.68 µg/ml) as showed in figure 21.

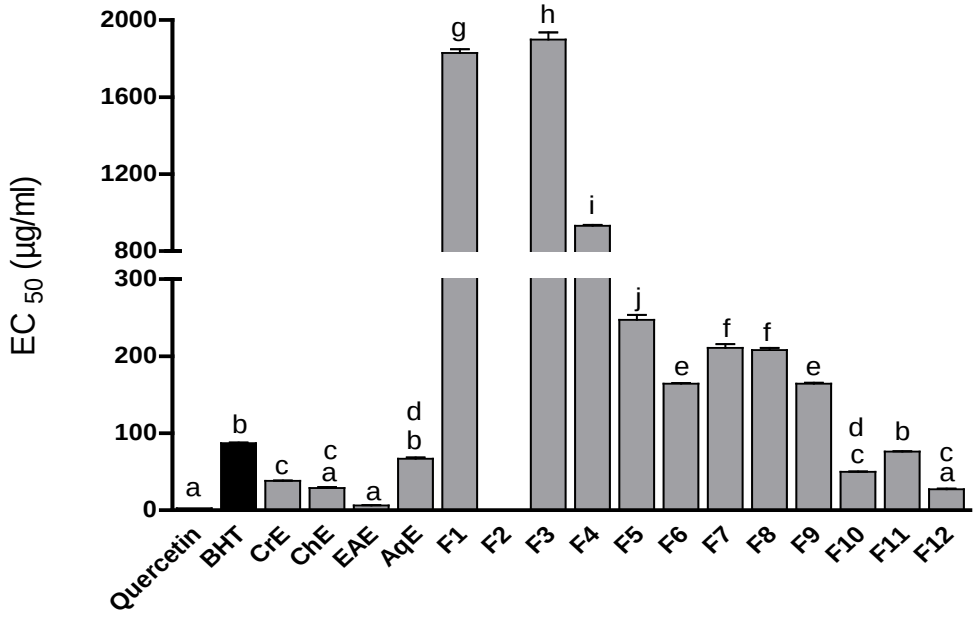

Figure 21. The EC50 values of DPPH scavenging activity of *R. picroides* CrE, fractions, quercetin and BHT. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. CrE: crude extract, ChE: chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract, F : fraction, BHT : bautylated hydroxytoluene.

Another important found is the higher scavenging activity of F12 (EC50 = 27.33 ± 1.67µg/ml) that was significantly similar to that of quercetin. Moreover, F10 showed significantly a similar scavenging activity to that of CrE. However, all the other fractions except F2, that did not show any scavenging activity, had significantly a lower scavenging activity (figure 21).

Total phenolic content in CrE and fractions had a significant and negative correlation with EC50 of DPPH scavenging activity (r = - 0.5011, p = 0.0480). It might be supposed that phenolic compounds in CrE and fractions were the major contributor in its antioxidant activity by DPPH scavenging assay. Several studies reported that the phenolic contents plant extracts are responsible for their radical scavenging activity. The phenolic compounds may act as free radical scavengers because of their hydrogen-donating ability and scavenging ability (Saha and Verma, 2016). Whereas, Pearson's correlation coefficient between flavonoids content of CrE and fractions and DPPH scavenging activity gave no significant correlation (r = 0.3905, p = 0.1348). This may be linked to the assumption that most of the flavonoids were in their glycoside forms and thus less effective compared to their aglycone forms (Anwar *et al.*, 2013). It means that flavonoids had no influence in antioxidant activity by DPPH scavenging method.

Our results are in agreement with Fitriansyah and their collaborators (2017) who reported that total phenolic content in *Sesbania sesban* leaves extracts had significant and negative correlation with their EC50 DPPH scavenging activity; whereas, flavonoids content showed no correlation.

4. Acute oral toxicity of crude extract

4.1. Behavioral observations and mortality patterns

The present study conducted according to OECD guideline 425 revealed that the CrE did not produce any behavioral changes in both males and females during the study period. Furthermore, any mortality was recorded throughout two weeks. Therefore, this suggests that the LD50 is greater than 5000 mg/ kg according to the OECD guideline, thus the extract belongs to category 5 of the Global Harmonisation System of Chemical Substances considered as fairly toxic substance.

4.2. Body weight evolution

Every case of body weight loss was recorded, and the body weight increased gradually throughout the study period. Figure 22 shows that the weight curve similar to one reported by other animals (males and females) breeding institutions. Statistical analysis of body weight gain did not reveal any significant differences between treated and control groups (table 7).

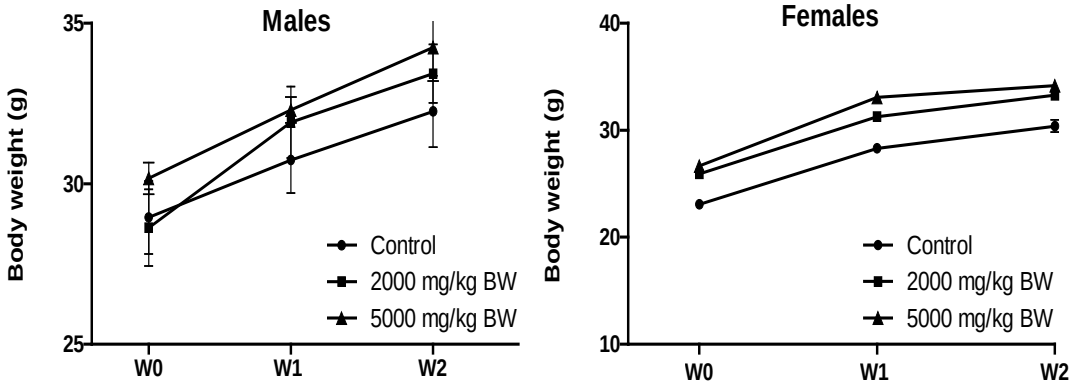

Figure 22. Body weight of mice treated orally with *R. picroides* CrE. Values expressed as mean ± SD, n= 5 animals/group.

Table 7. Body weight gain (g) of mice treated orally with *R. picroides* CrE.

Groups		**Control group**	**2000 mg/kg BW**	**5000 mg/kg BW**
Weight gain	Males	3.30 ± 0.35	3.78 ± 0.84^{ns}	4.08 ± 1.35^{ns}
(g)	Females	7.33 ± 1.18	7.37 ± 0.85^{ns}	7.51 ± 1.02^{ns}

Values expressed as mean ± SD, n= 5 animals /group. ns: non significant differences compared with control group ($p < 0.05$).

4.3. Relative organ weights

The oral administration of CrE caused no significant changes in relative weight of the organs (liver, spleen, kidneys, heart, lungs, brain and stomach) in the treated ones compared to the control mice of both sexes (table 8).

Table 8. Organs relative weight of mice treated with *R. picroides* CrE and control group.

Groups	Control group	2000 mg/kg BW	5000 mg/kg BW
Males			
Liver	68.17 ± 13.20	67.83 ± 7.85^{ns}	66.22 ± 9.68^{ns}
Kidneys	14.53 ± 1.08	15.28 ± 1.82^{ns}	14.61 ± 1.60^{ns}
Spleen	8.93 ± 1.52	9.84 ± 2.72^{ns}	8.71 ± 4.58^{ns}
Heart	5.28 ± 0.75	5.51 ± 1.12^{ns}	5.34 ± 0.54^{ns}
Lungs	9.51 ± 1.64	13.20 ± 4.77^{ns}	10.92 ± 3.71^{ns}
Brain	11.79 ± 1.20	10.99 ± 3.22^{ns}	12.00 ± 2.75^{ns}
Stomach	13.44 ± 2.87	10.98 ± 2.32^{ns}	11.34 ± 1.18^{ns}
Females			
Liver	62.92 ± 9.58	65.13 ± 4.94^{ns}	64.64 ± 10.23^{ns}
Kidneys	10.44 ± 1.00	11.98 ± 0.88^{ns}	11.17 ± 1.47^{ns}
Spleen	6.16 ± 1.64	6.30 ± 1.15^{ns}	5.72 ± 1.39^{ns}
Heart	4.04 ± 0.60	4.72 ± 0.54^{ns}	5.09 ± 0.77^{ns}
Lungs	9.73 ± 0.71	9.15 ± 4.03^{ns}	10.64 ± 3.54^{ns}
Brain	11.98 ± 1.97	11.33 ± 1.12^{ns}	11.00 ± 1.90^{ns}
Stomach	10.21 ± 1.88	10.86 ± 1.79^{ns}	10.78 ± 0.51^{ns}

Values expressed as mean ± SD, n= 5 animals /group. ns: non significant differences.

4.4. Biochemical parameters

Biochemical parameters analysis to investigate major toxic effects in tissues and, specifically, effects on liver and kidney, should be performed and may provide useful information.

Some enzymes such as ALT, AST and ALP can be used as sensitive indicative of hepatocellular effects (Ezeja *et al.*, 2014). In this study, AST and ALP did not present any significant variation both in males and females when compared to their respective controls. However, ALT in females treated with 2000 mg/kg BW showed a significant ($p < 0.05$) variation compared to the control group. When compared with control group, a relatively low ALT value for the experiment groups (males and females) was recorded (figure 23).

Consequently, the CrE did not alter ALT, AST and ALP activity of the treated groups. This is an indication of non-hepatotoxic potential of CrE.

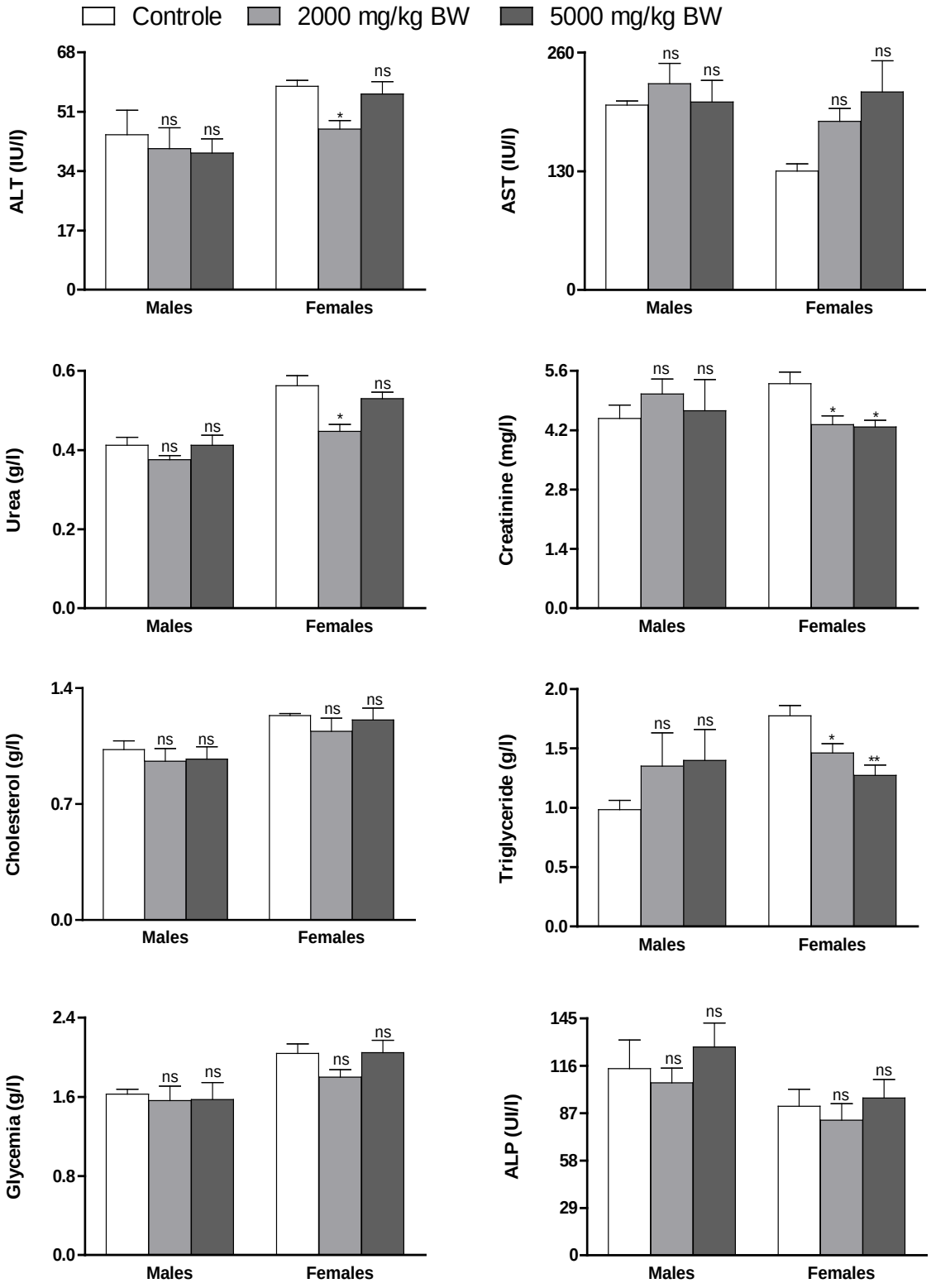

Figure 23. Biochemical parameters of control and mice treated with *R. picroides* CrE measured during the acute toxicity. Values were expressed as mean ± SEM (n = 5). ns : no significant, * : $p < 0.05$, ** : $p < 0.01$. ALT : alanine aminotransferase, AST : aspartate aminotransferase, ALP : alkaline phosphatase.

Creatinine and urea is considered as biomarkers of renal insufficiency (Lopez-Giacoman and Madero, 2015; Sinkala *et al.*, 2017). A significant ($p < 0.05$) dose-dependent decrease in creatinine occurred in females, which may indicate muscle wastage (Thongprayoon *et al.*, 2016) but is not consistent with the body weights since there was weight gain trends in all groups. Otherwise, females treated with 2000 mg /kg recorded a significant decrease, but not dose dependently, in blood urea level (figure 23). However, this last also depends on extra-renal factors as high proteins food or increased protein catabolism.

The level of triglycerides was significantly decreased, in dose dependant manner, in females treated with 2000 mg/kg BW (figure 23), and this may indicate that the extract stimulates oxidation of triglycerides to fatty acids by lipoprotein lipase (Kersten, 2014).

4.5. Liver and kidneys histopathology

The observation of histological sections of the liver and kidneys of treated mice showed a preservation of the cellular architecture (lobular and tubular) of the two organs compared with controls. However, some particularities have been considered. For males, a vascular congestion, inflammatory infiltrated and inflated hepatocytes were recorded in some hepatic tissues of males treated with 2000 mg/kg. At dose of 5000 mg/kg, some hepatic tissues showed an important congestion and a polymorphe inflammatory infiltrated (figure 24).

Althought enzymes levels indicated a non-hepatotoxic potential of CrE, the results of the liver histopathology however indicated some element of the extract's negative effect. The presence of vascular congestion in both males and females treated with 2000 and 5000 mg/kg BW could be due to the vasoconstriction action of CrE on the wall of blood vessels (Ashley, 2004). The leucocyte infiltration presented in liver of both males and females at dose 2000 and 5000 mg/kg BW could be the result of the liver inflammation. Therefore, further research to consider the possible hepatoprotective or hepatotoxic effect of the CrE is recommended.

In general, kidneys' histological section did not show any structure modifications compared

to control except the presence of glomerular amd medullar congestion in some tissues of both 2000 and 5000 mg/kg treated males (figure 24).

For females, a moderate congestion was recorded in some liver tissues treated with 2000 mg/kg. Furthemore, at dose of 5000 mg/kg, a medullar and cortical congestion with absence of nephrite were showed in some kidneys tissues.

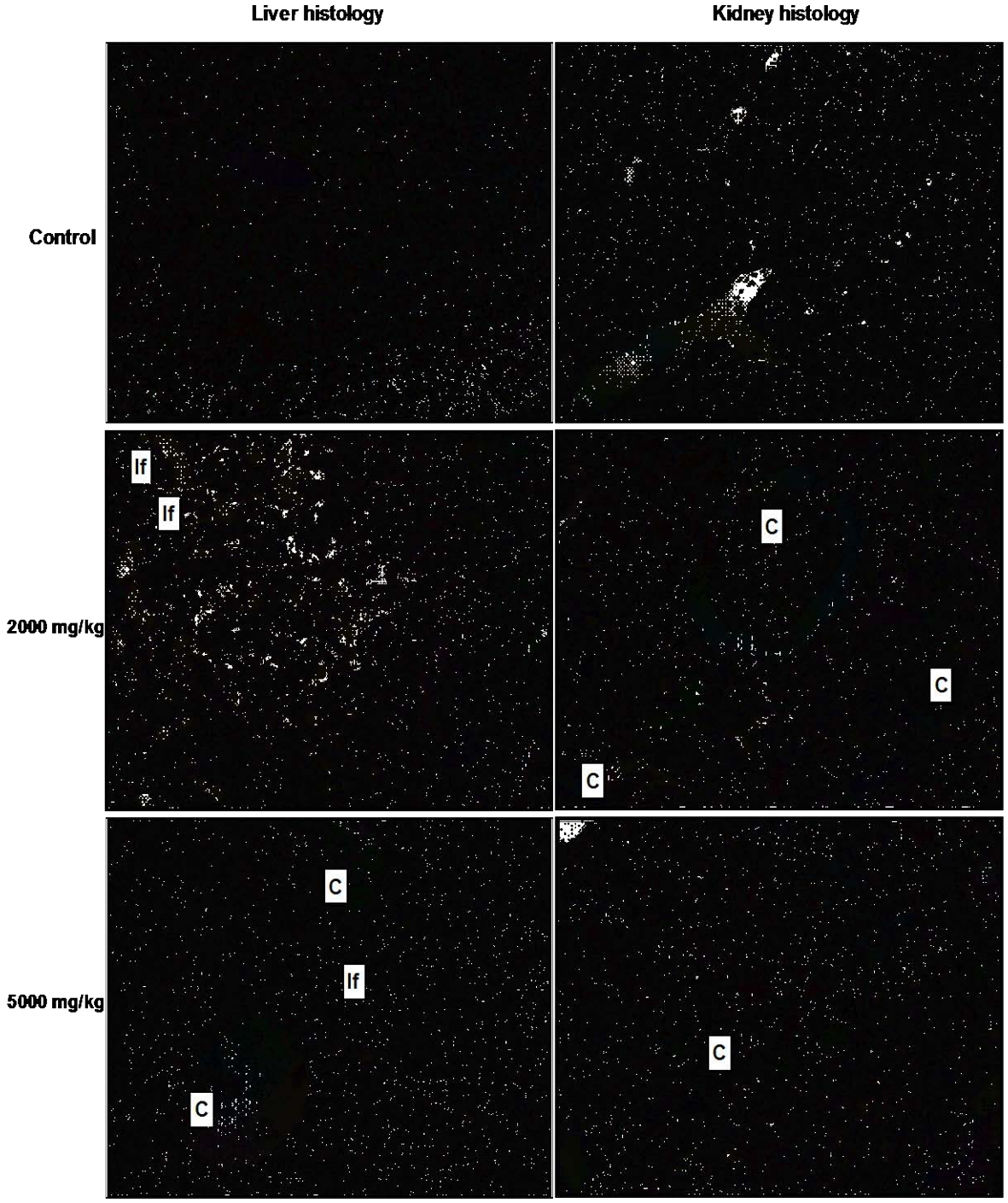

Figure 24. Histopathological analysis of males' liver and kidney treated with *R. picroides* CrE in acute oral toxicity (x 40). C: congestion, If: inflammatory infiltrate.

5. Subacute oral toxicity of crude extract and its *in vivo* antioxidant activity

In order to find out any possible effects of CrE on normal metabolic conditions, normal mice were used. The treatment was performed orally for 21 successive days in order to observe the subacute effects and the *in vivo* antioxidant activity of CrE.

5.1. Behavioral observations and mortality patterns

In the subacute toxicity tests, administration of 250, 500, and 1000 mg/kg doses of CrE to three groups of mice did not show any behavioral changes, visual symptoms of toxicity or mortality in animals during the entire 21-days observation period.

5.2. Body weight evolution

During the two first weeks, a non significant weight loss was noted in treated group with CrE at dose 250 mg/kg. Moreover, a weight loss was equally noted during the second week both in the treated groups with CrE (500 and 1000 mg/kg) and control group. However, an improvement was noted in the last week in all groups except control group (figure 25).

Since the weight loss was noted also in control group we cannot conclude that the intake of CrE was responsible for this effect. Therefore, further research to consider the possible weight loss effect of the CrE is recommended.

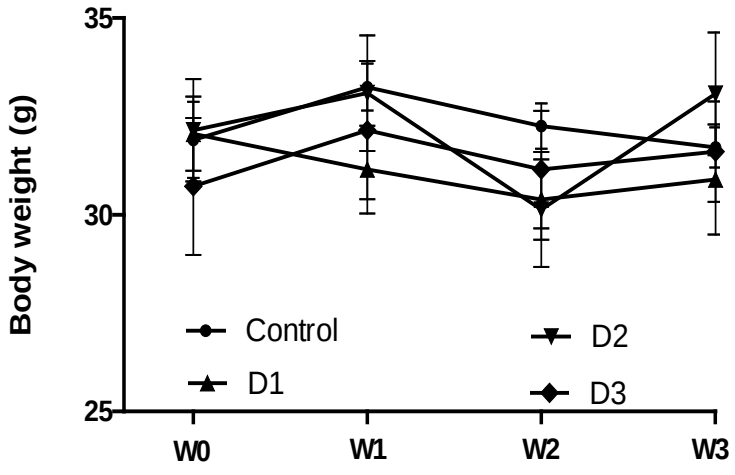

Figure 25. Body weight of mice treated orally with *R. picroides* CrE. Values expressed as mean ± SD, n = 6-7 animals/group.

5.3. Relative organ weights

The repeated oral administration of Vit C (100 mg/kg) and CrE (250, 500 and 1000 mg/kg) caused no significant changes in relative weight of the organs (kidneys, spleen, heart, lungs, brain and stomach) in the treated mice compared to the control mice (table 9). Such results indicated no toxic effect in both control and treated groups and there were statistically non-significant differences ($p > 0.05$).

Surprisingly, a significant increase in relative weight of liver was reported in all CrE doses treated groups (table 9). Extract is metabolized by the liver and the increase in its weight may be due to response to compensate for increase in demand of its metabolism (Sharma *et al.*, 2014).

Table 9. Organs relative weight of mice treated with *R. picroides* CrE and control group.

Groups	Control group	Vit C 100 mg/kg BW	CrE 250 mg/kg BW	CrE 500 mg/kg BW	CrE 1000mg/kg BW
Liver	53.90 ± 3.69	59.40 ± 5.58^{ns}	68.40 ± 8.67**	62.85 ± 4.73*	65.07 ± 6.03*
Kidneys	13.46 ± 1.00	13.67 ± 2.30^{ns}	15.60 ± 1.12^{ns}	12.80 ± 1.70^{ns}	13.92 ± 2.14^{ns}
Spleen	03.70 ± 0.46	05.40 ± 1.04^{ns}	06.06 ± 1.62*	05.19 ± 1.55^{ns}	04.00 ± 0.58^{ns}
Heart	04.80 ± 0.57	04.85 ± 0.33^{ns}	05.43 ± 0.70^{ns}	04.99 ± 0.89^{ns}	04.58 ± 0.46^{ns}
Lungs	06.47 ± 1.00	06.96 ± 1.27^{ns}	07.18 ± 0.43^{ns}	06.65 ± 1.78^{ns}	06.31 ± 1.54^{ns}
Brain	10.96 ± 1.19	12.73 ± 2.70^{ns}	12.35 ± 1.50^{ns}	12.07 ± 2.74^{ns}	11.52 ± 2.37^{ns}
Stomach	07.52 ± 1.75	09.10 ± 1.22^{ns}	09.98 ± 1.42^{ns}	08.59 ± 2.09^{ns}	08.46 ± 1.19^{ns}

Values expressed as mean ± SD (n = 6-7). ns: no significant, * : $p < 0.05$, ** : $p < 0.01$.

5.4. Biochemical parameters

Results (figure 26) showed that the statistical analysis evaluated biochemical parameters indicated that there were no significant differences between the treated mice and the control group. However, a significant ($p < 0.05$) decrease in dose non-dependent regarding urea was recorded in treated mice with CrE (250 and 1000 mg/kg) and Vit C (100 mg/ml).

Interestingly, these results of studied biochemical parameters showed any evidence of adverse effects in all doses treated groups of mice.

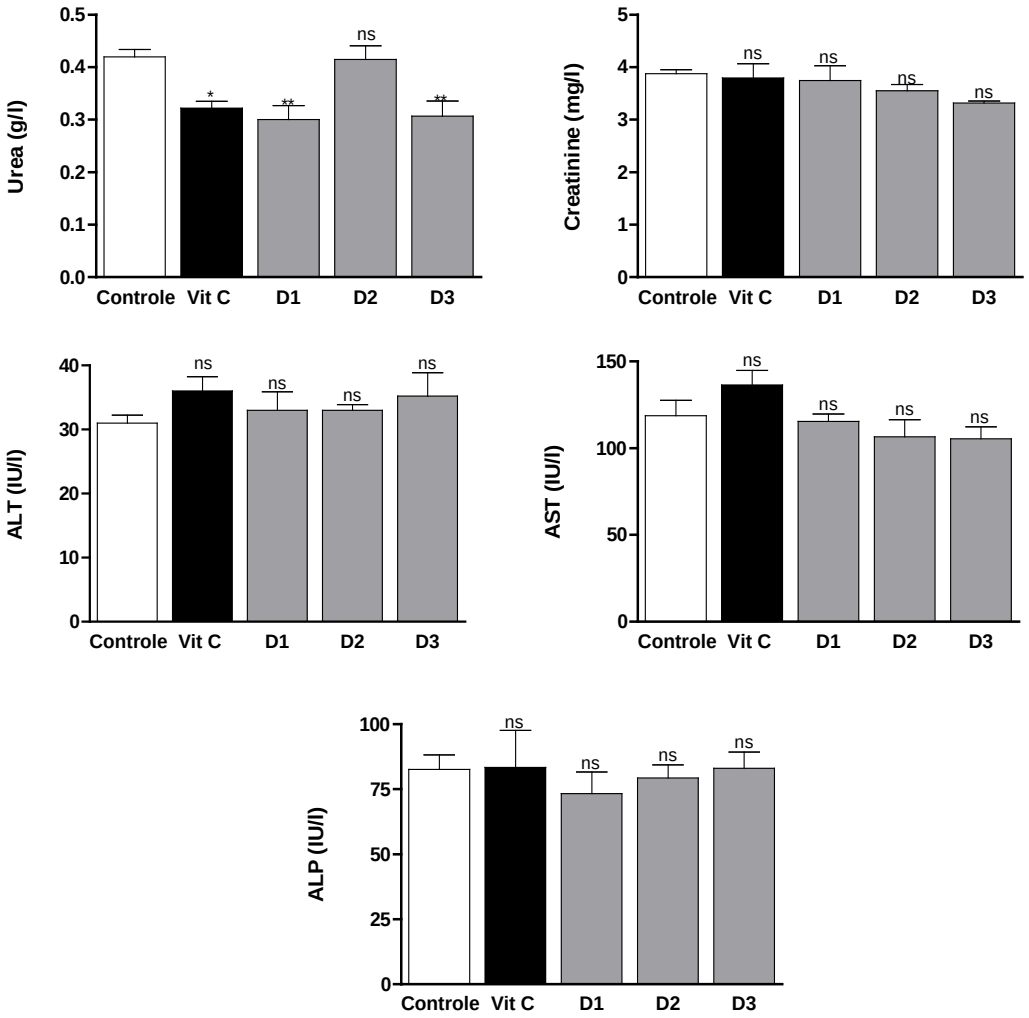

Figure 26. Biochemical parameters of control and mice treated with *R. Picroides* CrE and Vit C measured during the subacute toxicity. Values were expressed as mean ± SEM (n = 6-7). ns : no significant, * : $p < 0.05$, **: $p < 0.01$. ALT : alanine aminotransferase, AST : aspartate aminotransferase, ALP: alkaline phosphatas, Vit C : vitamin C, D : dose.

5.5. Liver and kidneys histopathology

Histopathological evaluation of the vital organs; liver and kidney (figure 27) displayed no relevant macroscopic or histological changes in animals that received Vit C and CrE (250 mg/kg). The liver showed normal architecture and no evidence of lesion ; the kidney showed adequate glomeruli and normal tubules. However, histological cutting of liver of mice treated with CrE (500 and 1000 mg/kg) showed in a dose dependent vascular congestions and hepatocyte lysis for the higher dose (1000 mg/kg). Vascular congestion and inflammatory zones with mononuclear cell infiltrates were also observed on kidney sections at 500 and 1000 mg/kg.

The presence of vascular congestions on both liver and kidney sections could be due to the vasoconstriction action of CrE on the walls of blood vessels (Ashley, 2004). Therefore, this finding suggests that administration of the extract at 500 and 1000 mg/kg doses for three weeks duration induces damage to the liver and kidneys of treated animals.

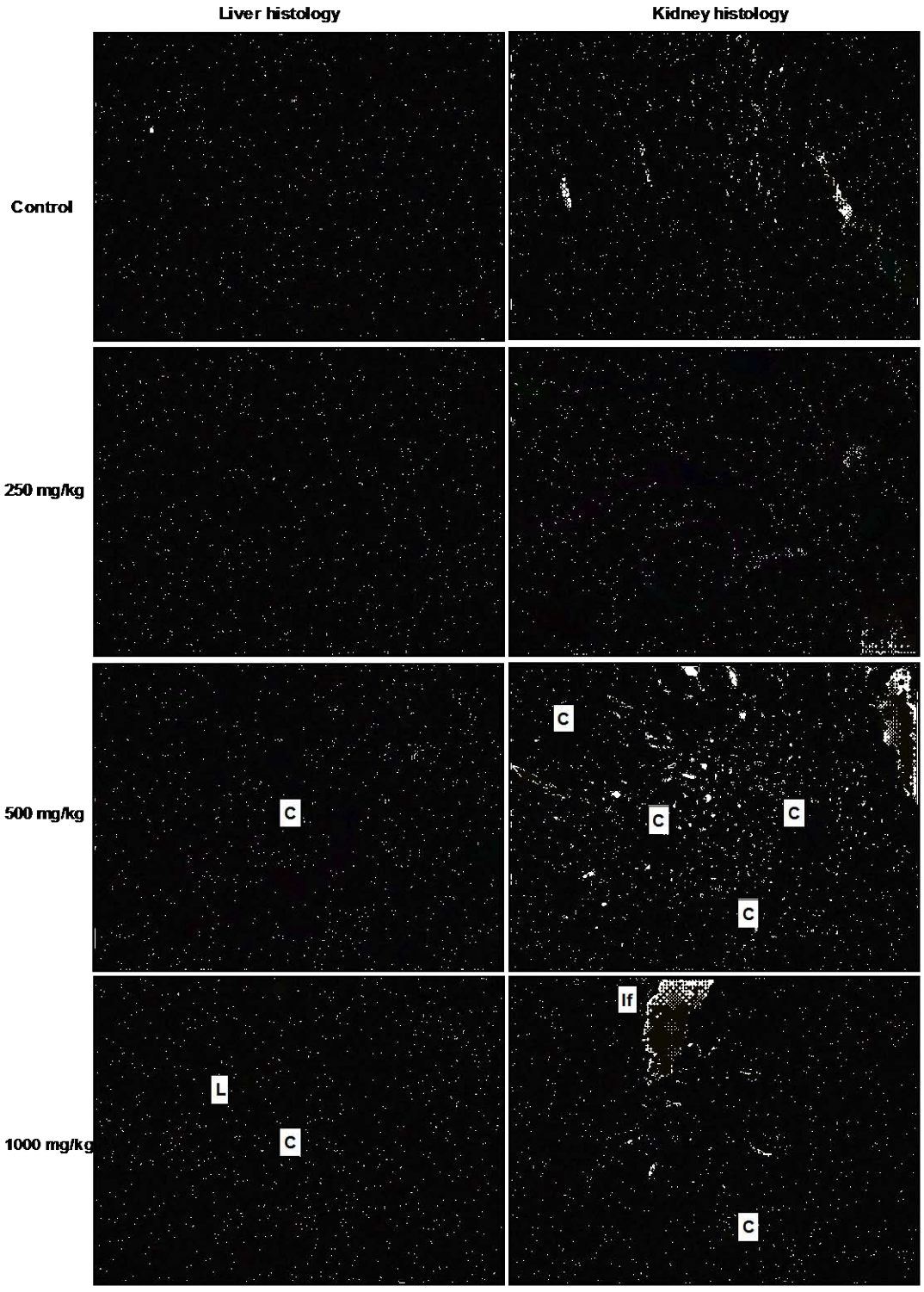

Figure 27. Histopathological analysis of mice'liver and kidney treated with *R. Picroides* CrE in subacute oral toxicity (x40). C: congestion, If: inflammatory infiltrate, L: hepatocyte lysis.

5.6. Oxidative hemolysis inhibition assay and DPPH radical-scavenging activity

The effect of CrE and Vit C oral administration on mice erythrocytes was investigated. It appeared from the obtained hemolysis sigmoid curves that the treatment with CrE and Vit C lead to hemolysis delay which is evident as displacement of the curves to the right. Even though not significant compared to control group (HT 50 = 96.58 ± 7.25 min), the HT50 values revealed an extension of half time of hemolysis in CrE: 250, 500 and 1000 mg /kg (110.91 ± 5.23, 111.64 ± 5.24 and 104. 11 ± 3. 13 min, respectively) and Vit C : 100 mg/kg (HT50 = 113.40 ± 5.90) treated group.

Evaluated by DPPH scavenging assay, it appeared that the plasma scavenging ability was not significantly improved following the administration of CrE and Vit C. The results showed that the group of mice treated with CrE: 250, 500 and 1000 mg/kg (scavenging % = 32.71 ± 1.86, 41.30 ± 10.32 and 20.00 ± 1.95 %, respectively) and that of Vit C (scavenging % = 30.05 ± 5.96 %) were not significantly different compared to the control group (29.24 ± 1.54%).

The results indicate that the CrE did not improve the antihemolytic and free radical scavenging activities, in contrast to the importance of these activities of the CrE determined *in vitro*. Or, the anti-hemolytic and free radical scavenging activities have been attributed to their polyphenolic and flavonoid contents. Thus, the main reason behind these results lies on the bioavailability of active compounds, as well as their metabolism in the body. In fact, the absorption of polyphenols in organism is rather low.

Another factor is the short half-life of polyphenols in plasma, which are usually in the range of a couple of hours. In addition to poor absorption, polyphenols and especially flavonoids are extensively metabolized in liver and intestine when ingested (Cvejić *et al.*, 2017).

5.7. Estimation of CAT activity, GSH and MDA

Changes in the activity of CAT, content of GSH and MDA in both livers and kidneys of mice were investigated.

Changes in the activity of CAT were investigated since it was one of the first lines of defence against ROS in the organism by detoxifies H_2O_2 into water. The CAT activity in both livers and kidneys homogenates has not undergone any significant changes during treatment with CrE (250, 500 and 1000 mg/kg). Also, no change was observed in positive control group (table 10). This consequence was somehow expected since the consumption of the studied extract did not induce the synthesis of this enzyme in normal mice. Otherwise, this would be an intervention of the body homeostasis. These results are in accordance with the study conducted by Celep and their collaborators (2013) investigating the antioxidant capacity of chery leaves in rats after 21 days of oral administration. They reported that no changes in the activity of CAT were observed in the liver homogenates of normal rats.

Table 10. Estimation of CAT activity (µmole/min/mg of protein), GSH (nmole/g of tissue) and MDA (nmole/g of tissue) in liver and kidney of mice.

Groups		Control group	Vit C 100 mg/kg BW	CrE 250 mg/kg BW	CrE 500 mg/kg BW	CrE 1000mg/kg BW
Liver	**CAT**	31.55 ± 4.55	32.84 ± 2.71	32.03 ± 4.28	33.85 ± 6.28	34.26 ± 1.90
	GSH	56.14 ± 2.26	58.06 ± 2.35	83.42 ± 2.48[a,b]	96.27 ± 4.91[a,b]	89.84 ± 1.65[a,b]
	MDA	235.48 ± 5.05	153.55 ± 22.87[c]	178.04 ± 7.66[c]	186.78 ± 14.04	200.23 ± 3.45
Kidney	**CAT**	29.70 ± 3.14	30.11 ± 3.38	31.90 ± 2.65	32.59 ± 1.90	32.28 ± 3.34
	GSH	55.51 ± 2.61	81.25 ± 2.21[a]	69.96 ± 2.40[a]	82.05 ± 5.61[a]	80.78 ± 2.50[a]
	MDA	117.46 ±11.44	83.4 ± 2.67[c]	83.38 ± 7.88[c]	95.81 ± 5.94	80.03 ± 4.35[c]

Values were expressed as means ± SEM (n = 6) and evaluated by one-way ANOVA. CrE : crude extract, Vit C : vitamin C, CAT : catalase, GSH : glutathion, MDA : malondialdehyde.
[a] $p < 0.0001$, compared with control group.
[b] $p < 0.0001$, compared with group treated with Vit C.
[c] $p < 0.01$, compared with control group.

The GSH is the most prevalent and abundant low-molecular weight antioxidant within cells (present in mM concentrations). It serves as significant first lines of defence against oxidative stress, and it plays an important role in maintaining the integrity of cells (Aquilano *et al.*, 2014). The GSH and its related enzymes play key roles in protecting the cell against the effects of ROS (Kıvrak *et al.*, 2017).

Following the analysis of the table 10, we notice that the administration of CrE (250, 500 and 1000 mg/kg) caused a significant increase in GSH level in livers (48.59%, 71.48% and

60.03%, respectively) and kidneys (26. 02%, 47.81% and 45.51%, respectively). However, Vit C (100 mg/kg) produced a significant increase only in kidneys GSH (46.36%). Therefore, such significant increase of GSH level was possibly due to an improvement in the GSH status which could be brought about by the constituents of CrE. We have reported that the CrE contains the antioxidant constituents: total polyphenols and flavonoids. These phytochemicals could have induced the phase antioxidant enzymes and thus increased the biosynthesis of cellular antioxidants, especially GSH (Upadhyay and Dixit, 2015).

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in cell membranes that generates a number of degradation products, resulting in oxidative stress (Najeeb *et al.*, 2012). Hence, the measurement of lipid peroxidation is an important indicator in the assessment of antioxidant potential. The MDA, which is an end product of lipid peroxidation, is also one of the most frequently used biomarkers to evaluate the antioxidant activity *in vivo* (Reddy *et al.*, 2017).

According to the results (table 10), a significant decrease of MDA level was observed in livers of mice treated with Vit C (100 mg/kg) and CrE (250 mg/ml). Even though not significant, the administration of CrE (500 and 1000 mg/kg) decrease the MDA level. In terms of kidneys MDA level, both 250 and 1000 mg/kg administration caused statistical significant decrease. It was stated that decrease in the levels of lipid peroxidation could be related to the ability of CrE to scavenge ROS, thus preventing further damage to membrane lipids (Pradeep *et al.*, 2007). It should be reminded that CrE also showed strong activity against free radical and a remarkable reducing power according to our *in vitro* assay results. Another important point about lipid peroxidation that should be highlighted is the correlation between β-carotene bleaching assay and MDA levels. β-carotene bleaching assay is, as mentioned previously, used as a screening tool for the inhibition of lipid peroxidation *in vitro*.

We also noticed that CrE showed good activity in β-carotene bleaching assay, and it induced decrease in the levels of MDA in normal mice.

Notably, CrE exhibited similar or strong GSH level and similar or higher lipid peroxidation inhibition effect than Vit C *in vivo*, probably due to the differences in the affecting factors of antioxidant activities between *in vitro* and *in vivo* assays (Liu *et al.*, 2010).

CONCLUSION

Conclusion

It has been known for a long time that phenolic compounds occurring naturally in plants present a broad spectrum of health-promoting properties resulting from their biological activity, especially antioxidant activity. Extracting them from plants in mild and effective conditions assess their toxicity profile and better understand their mechanisms of antioxidant activity constitute scientific issues which are the basis of our study.

In the first part, the work focused on the extraction of polyphenols. Yield relative to the weight of the vegetable powder was more or less important. The extraction yields vary according to the plant studied, the nature and the physicochemical characteristics of the solvents used and in particular their polarity. Results obtained from the phytochemical analysis of CrE and various fractions of *R. picroides* revealed the presence of phenolics compounds and the most important amount of both total phenols and flavonoids was found in EAE.

Twelve different fractions (F1-F12) from CrE were obtained by column chromatography on silica gel. From the F10, a flavonoid compound was successfully isolated. Using ^{1}H-NMR, and ^{13}C-NMR, the compound was characterized as luteolin 7-O-β- glucoside isolated for the first time in the entire R. *picroides* L. plant.

Due to the diversity of antioxidants and the complexity of oxidation process, the antioxidants capacities of plant extracts differ from one method to another, making it impossible to compare between methods. For this reason, we applied several antioxidant assays that would provide a better insight into the true antioxidant potential of *R. picroides* extract and fractions.

The antihemolytic property of *R. picroides* against AAPH-induced oxidative hemolysis was studied. Results showed that CrE and fractions had a very important antihemolytic activity where EAE exhibited the greatest protective effect against hemolysis of erythrocytes. The

antihemolytic assay proves the protective activity of the cell membrane, in particular the erythrocyte membrane which represents an important medical challenge particularly against certain genetic diseases.

The AqE, F1, F2, F3 and F12 were found to have a strong ion chelating activity significantly similar to EDTA. However, CrE and other fractions exhibited a lower ferrous ion chelating activity. On the other hand, EAE appeared to have the strongest reducing power comparable to Vit C.

The CrE, ChE and EAE had a strong H_2O_2 scavenging effect higher than BHT. F5 found to have the strongest effect in this case. In addition, the DPPH scavenging assay revealed that EAE, ChE, and F12 possessed a very high radical scavenging effect which was similar to that of quercetin.

The oxidation of β-carotene is highly reduced during the 24 hours in the presence of EAE, ChE, F1, F10 and F11. The obtained results showed that EAE appeared to possess the greatest antioxidant activities, the matter that gives a very good correlation between its antioxidant activities and its polyphenols and flavonoids contents.

It is known that antioxidant activities increase proportionally with the phenolics content. Our results showed that antihemolytic, reduction of β-carotene oxidation and DPPH scavenging activities presented a significant correlation with phenolics content. However, no significant correlation was recorded between phenolics content and the remained activities (chelating, reducing and H_2O_2 scavenging activities). Thus, is possibly due to a specific structure, synergetic or additive effect, which may exist between phenolic compounds and other components.

From the *in vitro* results, the CrE and its fractions seem to be of real and potential interest due to their strong antioxydant activities.

Regarding to the toxicity assessment, this study provides valuable data on the acute and sub-acute oral toxicity profiles of *R. picroides* that should be very useful for any future *in vivo* and clinical study of this plant medicine. Results allow us to conclude that the CrE is fairly toxic; with oral LD50 higher than 5000 mg /kg BW on both male and female mice. Moreover, daily administration extract at doses ranged from 500 to 1000 mg/kg could result in alteration of liver and kidney histology. Therefore, the use of *R. picroides* CrE is safe at doses ≤ 250 mg/kg.

To confirm the antioxidant potential of the CrE, *in vivo* antioxidant properties were investigated. An approach was performed by administration to male mice three doses 250, 500 and 1000 mg/kg. The analysis of antioxidant potential parameters revealed that the CrE administration increased significantly the level of GSH and decreased the level of MDA in both liver and kidney. In contrast, no significant change in the activity of CAT was observed in both the liver and kidney homogenates of traited mice. The CrE supplementation improved slightly the plasma antioxidant status and the anti-hemolytic protective effect which remained non significant compared to the control group.

The results indicate that the CrE did not improve the antihemolytic and free radical scavenging activities, in contrast to the importance of these activities of the CrE determined *in vitro*. Or, the anti-hemolytic and free radical scavenging activities have been attributed to their polyphenolic and flavonoid contents. Thus, the main reason behind these results lies on the bioavailability of active compounds, as well as their metabolism in the body. In fact, the absorption of polyphenols in organism is rather low. Another factor is the short half-life of polyphenols in plasma, which are usually in the range of a couple of hours. In addition to poor absorption, polyphenols and especially flavonoids are extensively metabolized in liver and intestine when ingested.

In conclusion, this work allowed highlighting:

- ✓ The isolated and characterized compound from *R. picroides* CrE belongs to the flavone group: luteolin 7-O-β-glucoside.
- ✓ Strong antioxidant capacity of the CrE and its derived fractions for different *in vitro* antioxidant assays.
- ✓ Antioxidant capacity may be related with the antioxidant constituents such as polyphenols and flavonoids and may be the result of possible synergism with other compounds.
- ✓ These results suggested the use of ethyl acetate fraction as primary antioxidant therapeutic source.
- ✓ The LD50 of *R. picroide* CrE was higher than 5000 mg/kg.
- ✓ The use of *R. picroide* CrE is safe at doses ≤ 250 mg/kg.
- ✓ The administration of CrE to the mice acted favorably on tissue antioxidant potential.

The present study is the first report on the toxicity profile and antioxidants activity of *R. picroides*. Therefore, more researches and studies are required as there is a large untapped reservoir waiting to be investigated.

- ✓ The future research should include further research aimed to identify, isolate and characterize more active constituents responsible for the strong observed antioxidant activity in order to determine the exact mechanism of the antioxidant activity.
- ✓ Other *in vivo* kinds of antioxidant assays are needed.
- ✓ More work should be carried on both pharmacological and biological activities.
- ✓ Chronic toxicity of the plant should be carried out.
- ✓ The plant should be exploited as natural antioxidants in real food systems.
- ✓ Studies on the pharmacokinetics and pharmacodynamics of plant extracts would be useful before considering further investigations as to their therapeutic use.

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ملخص

تستخدم نبتة (*Reichardia picroides* (*R. picroides* أساسا لأغراض غذائية، و تستعمل تقليديا كمخفضة لنسبة السكر في الدم و مدرة للبول و للحليب و منشطة. و على حد علمنا، لم تتم الإشارة إلى أي دراسات تتمحور حول سمية النبتة و نشاطها المضاد للأكسدة. تهدف دراستنا إلى معرفة التركيب الكيميائي للنبتة و تقييم سميتها وكذا نشاطها المضاد للأكسدة مخبريا و في الحي. تم استخلاص متعدد الفينول متبوعا بفصل أولي اعتمادا على قطبية المذيب المستخدم لإعطاء المستخلص الخام (CrE) و مستخلص الكلوروفورم (ChE) ومستخلص الإيثيل أسيتات (EAE) و المستخلص المائي (AqE). سمح الفصل على كروماتوغرفيا العمود على هلام السيليكا بالحصول على 12 جزء (F1-F12)، كما خضعت كل تجزئة إلى التحليل على كروماتوغرفيا الطبقة الرقيقة. تم عزل المركب النقي lutéoline 7-O-β-glucoside، الذي ينتمي إلى الفلافون، من F10 وتم تحديد بنيته الكيميائية بواسطة جهاز القياس الطيفي للرنين المغناطيسي النووي. أظهرت نتائج التحليل الكمي أن أعلى كمية من الفينول الكلي والفلافونويد سجلت في EAE (331.64 ± 3.11 ميكروغ مكافئ غرامي حمض الغاليك/ مغ من المستخلص و 48.14 ± 0.70 ميكروغ مكافئ غرامي كارستين/ مغ من المستخلص، على الترتيب). وقد تم إجراء عدة تجارب مخبريا بغية تحديد النشاط المضاد للأكسدة لـ CrE و أجزائه. كشف CrE و أجزاؤه عن نشاط جد معتبر مضاد للانحلال الدموي وذلك بتسجيل زيادة في قيم HT 50 التي تراوحت من 76.92 ± 3.59 دقيقة بالنسبة للشاهد و 188.15 ± 1.91 دقيقة بالنسبة لـ EAE. وُجد أن F12 يملك أقوى نشاط قابط للأيون مساو لحد كبير لنشاط EDTA. أبدى EAE أقوى قدرة إرجاعية مساوية لقدرة Vit C. سجل F5 أعلى قدرة إزاحية لـ H_2O_2 والتي تفوق لحد كبير قدرة BHT. سُجل تثبيط لابيضاض البيتاكاروتين خلال 24 ساعة في وجود كلا من ChE و EAE و F1 و F10 و F11.كشف اختبار إزاحة جذر DPPH أن EAE و ChE و F12 يملكون قدرة إزاحية جد عالية. تم تقييم السمية الحادةللنبتة على الفئران، وذلك لتسجيل الأعراض المصاحبة للسمية و عدد الوفيات المحتملة للحيوانات لمدة أسبوعين لحساب متوسط الجرعة المميتة (LD50) لـ CrE. أظهرت النتائج أن الجرعات المعطاة لم تتسبب في حدوث أي وفيات أو تغيرات في السلوك العام للفئران الذكور و الإناث، كما وجد أن LD50 يفوق 5000 مغ / كغ. وفيما يخص دراسة السمية فوق الحادة، فقد تم إعطاء CrE بجرعات 250 و 500 و1000 مغ / كغ / يوم لمدة 21 يوما متتالية. و تبيّن أن الجرعات بين 500 و 1000 مع / كغ تسبب تغيرات في أنسجة الكبد والكلى. أجريت دراسة على الفئران الذكور من أجل تقييم القدرة المضادة للأكسدة، حيث تم معاملتها بـ CrE بجرعات 250 و 500 و1000 مع / كغ / يوم لمدة 21 يوما متتالية. أثبتت النتائج زيادة ملحوظة في مستوى GSH و انخفاضا في مستوى MDA في كل من الكبد والكلى، فيما لوحظ تحسن طفيف للقدرة المضادة للأكسدة للبلازما و للتأثير المضاد للانحلال الدموي. و عليه نستنتج أن *R. picroides* تملك قدرة مضادة للأكسدة جد عالية سواءا مخبريا أو في الحي، بالإضافة إلى أنها غير سامة و آمنة عند استعمال جرعات ≥ 250 مغ/كغ.

ABSTRACT

Reichardia picroides (*R. picroides*) is a species mainly used for daily source; it is used in the traditional medicine as hypoglycemiant, diuretic, depurative, galactagogue and tonic agent. To our knowledge, there are no studies on the antioxidant effect and the toxicity of this plant. The objective of the present study was, therefore, to evaluate the antioxidant activity and the toxicity of crude extract (CrE) and its fractions *in vitro* and *in vivo*. Polyphenols extraction and fractionation from plant material was performed using solvent of different polarity giving the following phases: CrE, chloroform extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). The CrE was then subjected to silica gel column chromatography which results in 12 fractions (F1-F12). Each fraction was subjected to TLC analysis. The pure compound was isolated from F10 and its structure was established by NMR spectroscopy as luteolin 7-O-β-glucoside. Results of polyphenols estimation showed that the highest amount of both total phenols and flavonoids was found in EAE (331.64 ± 3.11 µg gallic acid equivalent (GAE) / mg of extract and 48.14 ± 0.70 µg quercetin equivalent (QE) / mg of extract, respectively). To determine the antioxidant potential of CrE and its fractions, several *in vitro* assays were carried out. The CrE and its fractions had a very important ($p < 0.0001$) antihemolytic activity and revealed an extension of HT 50 from 76.92 ± 3.59 min of control to 188.15 ± 1.91 min of EAE. The F12 was found to have the strongest ion chelating activity comparable to that of EDTA. The EAE appeared to have the strongest reducing power closer to that of Vit C. The F5 found to have the strongest H_2O_2 scavenging effect higher than that of BHT. The bleaching of β-carotene is highly inhibited in the presence of ChE, EAE, F1, F10 and F11.The DPPH scavenging assay revealed that EAE, ChE and F12 possessed a very strong radical scavenging effect. The acute toxicity of CrE was carried out using mice. Signs accompanying toxicity and possible death of animals were monitored for two weeks to ascertain the median lethal dose (LD_{50}) of the CrE. The administrated doses did not resulted mortality or changes in general behaviors. The LD_{50} was found to be superior to 5000 mg/kg. In subacute toxicity study, the CrE was administered by gavage at the doses of 250, 500 and 1000 mg/kg/day for 21 consecutive days. Daily administration of CrE at doses ranged from 500 to 1000 mg/kg resulted in alteration of liver and kidney tisssues. *In vivo* approach was performed by administration three doses 250, 500 and 1000 mg/kg to male mice. The analysis of antioxidant potential parameters revealed that the CrE administration increased significantly the level of GSH and decreased the level of MDA in both liver and kidney. The CrE supplementation improved slightly the plasma antioxidant status and the anti-hemolytic protective effect which remained non significant compared to the control group. It can therefore be sugges
ted that *R. picroides* had potent antioxidant activities both *in vitro* and *in vivo*. The use of this extract is safe at doses ≤ 250 mg/kg.