الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالي والبحث العلمي

Ferhat Abbas University, Setif 1
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جامعة فرحات عباس ، سطيف 1
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DEPARTMENT OF BIOCHEMISTRY

N°……SNV/2023

THESIS

Presented by

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For the fulfillment of the requirements for the degree of

DOCTORATE OF SCIENCES

BIOLOGY

Special filed: BIOCHEMISTRY

TOPIC

In vitro and *In vivo* anti-inflammatory and hepatoprotector effects of Algerian *Echium plantagineum* and *Brassica rapa* extracts

Presented publically in: **21 / 12 / 2023**

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ACKNOWLEDGEMENTS

*First of all, I praise **ALLAH** for letting this work see light. Alhamdou li LEH RAB Al-Alamine.*

*I would like to express the most sincere and grateful thanks to my supervisor **Pr. Abderrahmane SENATOR** for making all the steps of this work easy, for spending precious time and efforts on reading and correcting my manuscript, for his advices and guidance, and most importantly for his unlimited patience towards my interruptions during my doctoral journey.*

*I would like to thank deeply and cordially the president of my jury **Pr. Hamama BOURICHE,** who has always been so comprehensive. I thank her for her enriching advices, encouragements, support and for bringing me energy and hope every time, since my first time in the lab. Thank you very much madame.*

*I would also like to show my gratitude for my committee for their patience and willingness to read my thesis and give critiques, comments and opinions in order to make it a better work. Thank you **Dr. Fatima BENCHEIKH**, **Pr. Widad SOBHI** and **Dr. Sahra Amel BELAMBRI** for honoring my work by your presence.*

*Special thanks and gratitude go to **Dr. Hichem MEZITI** for his availability, and for the help he provided in the laboratory during all the period of our scientific journey. Thank you **Hichem**, it has been a pleasure to have you as a teammate.*

*I would not forget to express my deep thanks to **Dr. Abdelali BOUSSIF** for the help he brought to me especially at the first steps of this work. I also thank very deeply **Dr. Seoussen KADA** for her unlimited help and support, encouragements and precious advices. Thank you **Seoussen**, I owe you so much.*

*A lot of thanks go to my colleague **Abir REZZAGUI** who shared with me the details of the beautiful and the hard moments of this journey. Also, I'm thankful to all the other members of the lab for providing an amicable and lively working environment. Finally, I thank all those who directly or indirectly helped in the achievement of this work.*

ملخص

تتمثل هذه الدراســة في تحــديد التركيب الكيميائي النباتي و تقييم التــأثيرات البيولوجية للمستخلص المائي و الميثانولي لكل من نبتتي *Echium plantagineum* و *Brassica rapa*. أوضحت الدراسة الكيميائية النباتية للمستخلصات أنها تحتوي على نسبة معتبرة من المركبات الفينولية، الفلافونويدات و كذا الدباغ مع سيادة هذه الأخيرة في جميع المستخلصات. أظهر تحليل HPLC وجود عــشرمركبات فينـولية في المـستخلص المـيثانولي و سبعة في المــستخلص المـــــائي لنبتـــة *E. plantagineum* و التي من أهمها الكارسيتين 3-او جالاكتوزيد و حمض القاليك بالترتيب. بينما يحتوي كل من المستخلص المائي و الميثانولي لنبتة *B. rapa* على ستة مركبات فينولية أهمها حمض القاليك. أظهر استكشاف النشاطية المضادة للأكسدة أن المستخلص المائي و الميثانولي لنبتة *E. plantagineum* يملكان قدرة عالية على كسح جذور·ABTS، ·OH، $O_2^{\cdot}$ وH_2O_2، إرجاع و إستخلاب المعادن و كذا تثبيط أكسدة البيطا كاروتين بنسبة 93 % و 81 % بالترتيب. كما أظهرت مستخلصات نبتة *B. rapa* قدرات لا بأس بها في الاختبارات المذكورة سابقا و لكن بفعالية اقل من فعالية النبتة الأولى. كل المستخلصات المدروسة أظهرت تأثير وقائي كبير مشابه لتأثير المركب المرجعي (Trolox) ضد تحلل كريات الدم الحمراء الناتج عن AAPH. من ناحية أخرى أظهرت معالجة الجرذان بـ : 200 و 400 مغ/كلغ من المستخلصات المدروسة قدرة معتبرة في تثبيط وذمة الرجل المحفزة بالكاراجينين بنسب تراوحت بين 45.27 % و 90.66%. كما تم تقييم النشاطية المضادة للالتهاب للمستخلصات باستعمال اختباري إتلاف بروتين الألبومين و تحلل الكريات الدموية بفعل محلول منخفض التركيز. أظهرت كل المستخلصات قدرة معتبرة على تثبيط إتلاف بروتين الألبومين بنسب تراوحت ما بين 22.53 % و 70.10%. بالإضافة إلى قدرة المستخلصين الميثانوليين لكلتا النبتتين على تثبيط تحلل الكريات الدموية بنسبة 79.05 % و 87% بالترتيب. وجد أن هذه الفعالية أعلى من فعالية المستخلصات المائية لكلتا النبتتين و بنفس التراكيز. أظهرت دراسة النشاطية الوقائية للكبد أن المستخلصات المائية و الميثانولية للنبتتين المدروستين قامت بحماية الكبد ضد سمية مركب رباعي كلورير الكربون (CCL_4)، فقد تسببت المستخلصات في خفض مستويات AST بنسب تتراوح بين 54% و 89.5%, ALT (62.6%-87.4%) وALP (40.7%- 68.3%). كما قامت المستخلصات برفع تركيز CAT و خفض تركيز MDA. كما أثرت المستخلصات بطرق مختلفة على النسيج الكبدي بحيث أظهرت نسب متفاوتة و أنواع مختلفة من الاصابات النسيجية الكبدية. كخلاصة، وجد أن المستخلصات المائية و الميثانولية لكل من نبتتي *E. plantagineum* و *B. rapa* تمتلك تأثيرات معتبرة مضادة للأكسدة، مضادة للتحلل الدموي، مضادة للالتهاب و واقية للكبد كما تمثل مصدرا للمركبات النشطة بيولوجيا و التي من الممكن استعمالها في تطبيقات علاجية مهمة.

الكلمات المفتاحية : *Echium plantagineum* ، *Brassica rapa* ، مضادات الالتهاب، مضادات الأكسدة، مضادات التحلل، حماية الكبد.

Abstract

This study consists of the determination of phytochemical composition and the evaluation of biological effects of methanolic (Met E) and aqueous (Aq E) extracts of *Echium plantagineum* and *Brassica rapa*. The phytochemical study has shown that all extracts present considerable content of phenolic compounds, flavonoids and tannins with predominance of these lasts. The HPLC analysis allowed the identification of ten compounds in Met E and seven in Aq E of *E. plantagineum,* whose majority compounds are, respectively, quercetin 3-O- galactoside and gallic acid. While both Aq E and Met E of *B. rapa* present six phenolic compounds whose gallic acid is the most dominant. The *in vitro* exploration of antioxidant activity has shown that Aq and Met extracts of *E. plantagineum* scavenge very effectively $ABTS^{\cdot}$**,** $OH^{\cdot}$**,** $O_2^{\cdot}$ and H_2O_2 species, chelate and reduce strongly ferrous ions, and inhibit B-carotene oxidation with 93% and 81%, respectively. Aq and Met extracts of *Brassica rapa* exhibited important effects in previous tests, but with less efficiency than the first plant. However, all studied extracts have shown a very important protective effect, similar to that of Trolox, against AAPH induced hemolysis. *In vivo*, the pretreatment of rats with 200 and 400mg/Kg has shown inhibition effects of carrageenan induced paw edema with percentages going from 45.27% to 90.66%. The anti-inflammatory activity was also evaluated *in vitro* using the test of bovine serum albumin denaturation and the test of erythrocytes lysis induced by hypotonic medium. All the studied extracts inhibited the BSA denaturation with percentages included between 22.53% and 70.10%. Moreover, Met E of *E. plantagineum* and Met E of *B. rapa* prevented the hypotonic induced hemolysis with 79.05% and 87%, respectively. These effects are more pronounced than that of the aqueous extracts at the same concentrations. The study of the hepatoprotective activity showed that Aq and Met extracts of the studied plants protected liver from damage caused by CCL_4 intoxication. They reduced significantly the level of AST with percentages going from 54% to 89,5% , of ALT (62,6% - 87,4%) and of ALP (40,7% - 68,3%). They also increased significantly the concentration of CAT and reduced that of MDA. However, the studied extracts showed different effects on the histology of liver presenting different levels and types of hepatic lesions. In conclusion, extracts of *E. plantagineum* and *B. rapa* posses antioxidant, anti-hemolytic, anti-inflammatory and

hepatoprotector effects, and represent an important source of bioactive compounds with possible interesting therapeutic applications.

Key words: *Echium plantagineum*, *Brassica rapa*, antioxidant, anti-inflammatory, anti-hemolytic, hepatoprotection.

Résumé

La présente étude consiste en la détermination de la composition phytochimique ainsi que l'évaluation des effets biologiques des extraits aqueux (E Aq) et méthanolique (E Met) de *Echium plantagineum* et *Brassica rapa*. L'étude phytochimique a montré que tous les extraits présentent une teneur considérable en composés phénoliques, en flavonoïdes et en tannins avec la prédominance de ces derniers. L'analyse par HPLC a permis d'identifier dix composés phénoliques dans l'E Met et sept composés phénoliques dans l'E Aq de *E. plantagineum* dont les composés majoritaires sont, respectivement, la quercetine 3-O- galactoside et l'acide gallique. Tandis que les extraits aqueux et méthanolique de *B. rapa* présentent six composés phénoliques dont l'acide gallique est le plus dominant. L'exploration *in vitro* de l'activité antioxydante a montré que les extraits Aq et Met de *E. plantagineum* scavengent très efficacement les espèces $ABTS^{\cdot}$, $OH^{\cdot}$, $O_2^{\cdot}$ et H_2O_2, chélatent et réduisent fortement les ions ferreux, et inhibent l'oxydation du B-carotène avec des pourcentages de 93% et 81%, respectivement. Les extraits Aq et Met de *B. rapa* ont exercé des effets importants dans les tests précédents, mais avec moins d'efficacité que la première plante. Cependant, tous les extraits étudiés ont montré un effet protecteur très important contre la lyse érythrocytaire induite par l'AAPH, similaire à celui obtenu avec le Trolox. *In vivo,* le prétraitement des rats avec les doses 200 et 400 mg/Kg avec les extraits étudiés a montré un effet inhibiteur de l'œdème de la patte induit par la carragénine avec des taux d'inhibition compris entre 45.27% et 90.66%. L'activité anti-inflammatoire a été évaluée aussi i*n vitro,* par le test de dénaturation du BSA et le test de la lyse érythrocytaire induite par un milieu hypotonique. Tous les extraits étudiés ont inhibé la dénaturation du BSA avec des pourcentages allant de 22.53% à 70.10%. De plus, L'E Met de *E. plantagineum* et celui de *B. rapa* ont prévenu l'hémolyse avec des pourcentages de 79.05% et 87% respectivement. Ces effets sont beaucoup plus prononcés que ceux des extraits aqueux aux mêmes concentrations. L'étude de l'activité hépatoprotectrice a montré que les extraits Aq et Met des plantes étudiées ont protégé le foie vis-à-vis de la toxicité causé par le CCL_4. Ils ont diminué le taux des AST avec des pourcentages allant de 54% à 89.5%, des ALT (62.6% - 87.4%) et des PAL (40.7% - 68.3%). Ils ont aussi augmenté significativement la concentration de CAT et diminué celle de MDA. Cependant, les extraits étudiés ont montré des effets différents sur l'histologie du foie présentant différents niveaux et

types de lésions tissulaires. En conclusion, les extraits de *E. plantagineum* et *B. rapa* possèdent des effets antioxydant, anti-hémolytique, anti-inflammatoire et hépatoprotecteur et représentent une importante source de composés bioactifs pouvant avoir des applications thérapeutiques intéressantes.

Mots clés : *Echium plantagineum*, *Brassica rapa*, anti-oxydant, anti-inflammatoire, anti-hémolytique, hépatoprotection.

List of abbreviations

AAPH:	2,2 azobis (2-amidinopropane) dihydrochloride
ABTS:	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ALP:	alkaline phosphatase
ALT:	alanine transaminases
AST:	aspartate transaminase
BHA:	butylated hydroxyanisole
BHT:	butylated hydroxytoluene
BSA:	bovine serum albumine
CAT:	catalase
COX:	cyclooxygenase
$CCl_3^{\cdot}$:	trichloromethyl free radicals
$CCl_3OO^{\cdot}$:	trichloromethylperoxy radical
CCl_4:	carbon tetrachloride
CYP2E1 **:**	cytochrome P450 family 2 subfamily E member 1
DTNB:	5,5′-dithiobis-(2-nitrobenzoic acid)
EC_{50} :	Effective concentration corresponding to a 0.5 absorbance
ECM:	extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
GAE:	gallic acid equivalents
GSH:	glutathione
GSHpx:	glutathione peroxidase
HPLC-	*High-Performance Liquid Chromatography Electrospray Ionization*
ESI-MS:	*Mass Spectrometry*
HSCs:	hepatic stellate cells
HT_{50}:	Half hemolysis time.
IC_{50}:	Inhibitor concentration of 50% of radicals
ICAM-1:	intercellular adhesion molecule-1
IFN-γ :	interferon gamma
IGF-1:	insulin-like growth factor-1
KCs:	Kuppfer Cells
MDA:	malondialdehyde

MMPs: matrix metalloproteinases

NADPH: nicotinamide adenine dinucleotide phosphate oxidase

NAFLD: non-alcoholic fatty liver disease

NAPQI: N-acetyl-p-benzoquinone imine

NBT: Nitro blue tetrazolium

NF-κB : kappa-B nucleus factor

NKT : natural killer T cells

NOS: Nitric oxide synthase

NSAIDs: Non-steroidal anti-inflammatory drugs

OECD: Organization of Economic Co-operation and Development guideline

PAF: platelet-activating factor

PBS: phosphate buffered saline

PDGF: Platelet-derived growth factor

PGE: prostaglandin

PECAM-1: platelet–endothelial cell adhesion molecule-1

PMS: phenazinemethosulfate

QE: quercetin equivalents

RNS: reactive nitrogen species

ROS: reactive oxygen species

SOD: superoxide dismutase

TAE: tannic acid equivalent

TBA: thiobarbituric acid

TCA: trichloroacetic acid

TGF-β1: *Transforming growth factor* -β1

TNB: thionitrobenzoic acid

TNF: tumor necrosis factor

VEGF-α: vascular endothelial growth factor-α

XO: xanthine oxidase

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INTRODUCTION

Introduction

Reactive oxygen species (ROS) are produced during normal cellular metabolism, and participate in physiological processes at moderate concentrations. However, uncontrolled production of these species when, combined with a failure of the antioxidant system of the organism, produces an imbalance between oxidants and antioxidants called oxidative stress. ROS can alter bio-molecules as proteins, lipids and nucleic acids, being able to start or aggravate several diseases.

On the other hand, the inflammatory process is a vital response to injury, infection, trauma and many other insults. This response consists of elaborating a cascade of both pro-inflammatory and anti-inflammatory mediators, acting in balance. If anti-inflammatory response successes, it leads to cell regeneration and wounds healing. However, when it escapes from control and regulation, it participates in the development of several diseases.

Furthermore, liver is a vital organ of huge importance since it is implicated in a variety of metabolic activities including detoxification of the body from toxins. The liver injuries, whose oxidative stress is the major reason, alter its functions resulting in metabolic dysfunction as immediate consequence, and lead to many complications. Thus, the development of liver protective agents is of paramount importance in the protection from liver damage.

In order to fight against this huge number of disorders touching the totality of the human body, researches are increasingly oriented towards finding natural molecules endowed with biological activities. These molecules could represent a medication that is cheaper, more available and having fewer side effects than synthetic one.

This study aims to evaluate certain biological effects namely antioxidant, antihemolytic, anti-inflammatory as well as hepatoprotective effects of aqueous and methanolic extracts of *Echium plantagineum* aerial part, used in Algerian common medicine, and *Brassica rapa* roots, used in folk medicine and consumed as a vegetable.

BIBLIOGRAPHIC REVIEW

I. Oxidative stress

Oxidative stress results from an imbalance between the formation of reactive oxygen/nitrogen species (ROS/RNS) in an organism, and its ability to eliminate them or to repair their resulting damage by deficiency of enzymatic and nonenzymatic antioxidants (Rahal *et al*., 2014; Poprac *et al.*, 2017).

I.2. ROS/RNS formation

Living organisms produce ROS during normal cellular metabolism at an appropriate concentration for physiological cell processes. However, high concentrations of ROS cause adverse modifications to cell components, such as lipids, proteins and nucleic acids (Al-Dalaen and Al-Qtaitat, 2014). During oxidative phosphorylation (ATP production process), electrons undergo an oxidation-reduction reactions chain. The final destination in this chain is an oxygen molecule that is converted to water in normal conditions. If O_2 is incompletely reduced, superoxide radical ($O_2^{\cdot-}$) is produced (Al-Dalaen and Al-Qtaitat, 2014). This $O_2^{\cdot-}$ undergoes a dismutation reaction catalyzed by the superoxide dismutase (SOD) to form H_2O_2 (Ahsan *et al*., 2003), which is less reactive than $O_2^{\bullet-}$, but in the presence of transition metals such as Fe^{2+}, it breaks down to highly toxic hydroxyl radicals ($OH^{\cdot}$), responsible for damaging all types of biomolecules, namely DNA, proteins, lipids and carbohydrates (Gupta, 2015; Keshari *et al*, 2015). The H_2O_2 can also react with chlorine to form one of the most damaging ROS, hypochlorite (HOCl) (Keshari *et al*., 2015). Furthermore, $O_2^{\bullet-}$ can react with NO, an important cell signaling molecule, to form reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), a cytotoxic molecule that can oxidize a number of target molecules like DNA and proteins (Gupta, 2015). Other ROS can be formed by oxidative deterioration of lipids and proteins named peroxyl ($ROO^{\cdot}$) and alkoxy ($RO^{\cdot}$) radicals, highly oxidizing molecules which can cause DNA mutations and apoptosis (Gupta, 2015).

I.3. ROS/RNS sources

ROS and RNS are continually produced in the cell by different mechanisms and can have either endogenous or exogenous sources.

I.3.1. Endogenous sources

The superoxide radical anion appears to play a central role in ROS formation since other reactive intermediates are formed in reaction sequences starting with $O_2^{\cdot -}$ (Gulcin*et al*., 2012). Thus, mitochondrion is considered as the major organelle responsible for ROS production, being the site of transferring one single electron to O_2 resulting in $O_2^{\cdot -}$ formation (Roede and Jones, 2010; Holmström and Finkel, 2014). Peroxisomes are also involved in the metabolism of ROS as they are one of the major sites of intracellular H_2O_2 production. They produce $O^{\cdot}{}_2^{-}$ and $NO^{\cdot}$ as a consequence of their normal metabolism (Del Río and López-Huertas, 2016). ROS are also produced in the organism as a part of the primary immune defense. Phagocytic cells such as neutrophils and macrophages synthesize large amounts of $O_2^{\cdot -}$, via the action of NADPH oxidase on molecular oxygen, during their defense mechanism against foreign organisms (Gulcin *et al*., 2012). Immune cells produce also $NO^{\cdot}$, the precursor of an even more reactive species, peroxinitrite ($ONOO^-$), in terms of inflammatory response (Rodrigo *et al*., 2011).

Enzymes are other endogenous producers of ROS either directly or as by-products of their activity (Kohen and Niska, 2002). Nitric oxide synthase (NOS) is destined to produce $NO^{\cdot}$ from conversion of L-arginine to L-citruline (Guzik *et al*., 2003). While cytochrome p-450 is responsible for $O_2^{\cdot -}$ formation by direct O_2 reduction (Ahsan *et al.*, 2003; Lee *et al.*, 2004). Furthermore, xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine, and can further catalyze the oxidation of xanthine to uric acid resulting in the $O_2^{\cdot -}$ formation (Halliwell and Gutteridge 2015)..

I.3.2. Exogenous sources

ROS can also be generated from external sources. Exposure of living organisms to UV radiations leads to cleavage of H_2O_2 into $OH^{\cdot}$ radicals. Air pollutants such as cigarette smoke and industrial contaminants constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung. Drugs, xenobiotics (such as toxins, pesticides and herbicides) and chemicals (such as alcohol) contribute to ROS formation as by-products of their metabolism *in vivo* (Kohen and Nyska, 2002).

I.4. ROS/RNS cell targets

At physiological concentrations, ROS may be required for normal cell function. However, high concentrations are able to damage crucial biomolecules such as lipids, proteins and carbohydrates (figure 1). Also, they may cause DNA damage that can lead to mutations (Gulcin, 2012).

I.4.1. Lipids

The polyunsaturated fatty acids, major constituents of cell membranes, are one of the favored oxidation targets for ROS particularly $O_2^{\cdot -}$ and $OH^{\cdot -}$, potent initiators of lipid peroxidation (figure 1). Abstraction of a hydrogen atom from a molecule of polyunsaturated fatty acid initiates a propagation of chain reactions, and a hydrogen atom is abstracted from a second molecule, leading to a new free radical. At the end of this chain of reactions, end-products of lipid peroxidation, such as malondialdehyde (MDA), are produced and accumulated in biological systems (Rahal *et al*., 2014).

I.4.2. Proteins

Proteins, major constituents of cell membranes, are significant targets for ROS attack (figure 1), especially the $OH^{\cdot}$, $RO^{\cdot}$ and RNS. Protein damages can be direct or indirect and they include peroxidation, damage to specific amino-acid residues, changes in their tertiary structure, degradation and fragmentation. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins (Kohen and Nyska, 2002).

I.4.3. Carbohydrates

Glucose can be oxidized in presence of metallic ions leading to cetoaldehydes liberation as well as H_2O_2 and $OH^{\cdot}$ formation, which can cause cleavage of proteins and their glycation by attaching that cetoaldehyde. This glycosoxydation phenomenon concern diabetes patients and contributes to the fragility of their vessels walls and retins (Favier, 2003).

I.4.4. Nucleic acids

Reactive oxygen species, especially $OH^{\cdot}$, can interact with DNA and cause several types of damage (figure 1) including modification of DNA bases, single- and double-DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system (Kohen and Nyska, 2002).

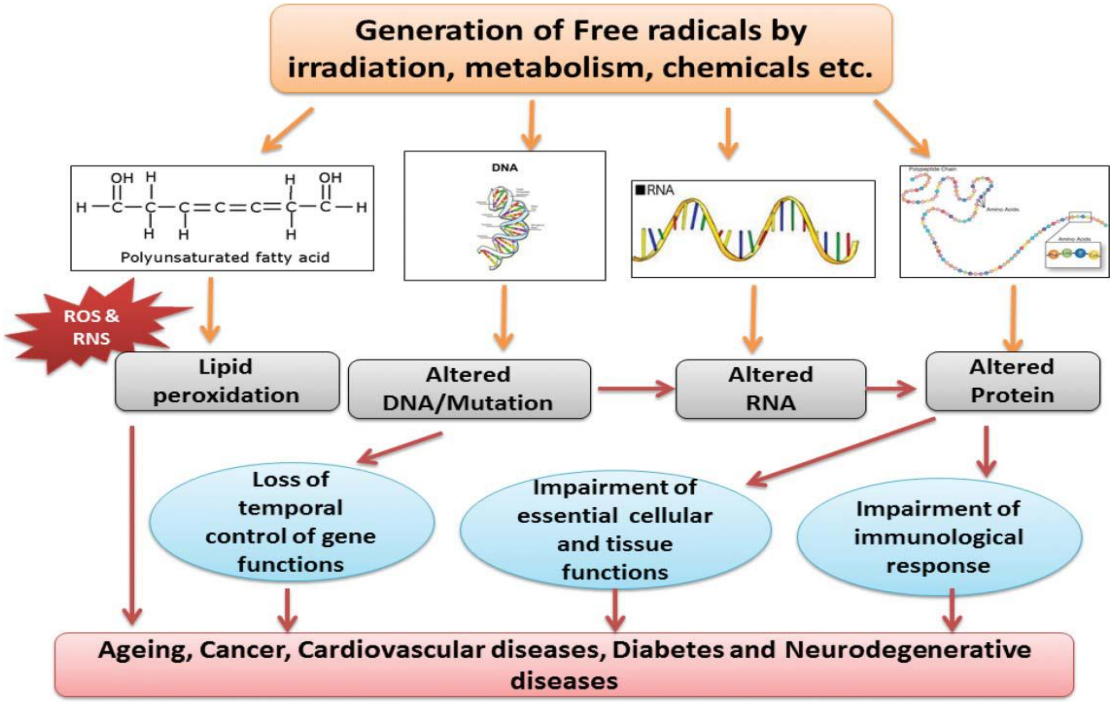

Figure 1. Reactive oxygen species targets and their associated pathologies (Ganesan *et al.*, 2017).

I.5. Oxidative stress associated pathologies

Oxidative stress is implicated in so many human diseases (figure 1) such as cancer, arteriosclerosis, aging, arthritis, Parkinson syndrome, ischemia and liver injury (Willcox *et al.*, 2004; Gunalan *et al.*, 2012), as a primary factor or associated with complications (Favier, 2003; Gunalan *et al.*, 2012). Gunalan *et al.* (2012) have reported that about 95% of pathological conditions associated with oxidative stress were observed in individuals who are older than 35 years old of age. This can be explained by the fact that aging both reduces antioxidant defenses and raises ROS production and accumulation in the organism (Favier, 2003).

I.6. Antioxidants

Antioxidants are substances capable of delaying or inhibiting the oxidation of a substrate when present at a lower concentration than this substrate (Godic*et al.*, 2014). These antioxidants can be enzymatic or non-enzymatic.

I.6.1. Enzymatic antioxidants

The enzymatic antioxidants remove dangerous oxidative products by converting them into hydrogen peroxide, then into water, in a multi-step process that requires a number of trace metal elements. These enzymatic antioxidants cannot be supplemented orally but must be produced in our body (Gupta, 2015), and they include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx).

I.6.1.1. Superoxide dismutase (SOD)

The superoxide dismutase breaks down the superoxide into oxygen and hydrogen peroxide (as represented in the equation bellow), with assistance of copper, zinc, manganese and iron. SOD is present in nearly all aerobic cells and extracellular fluids (Gupta, 2015).

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

I.6.1.2. Catalase (CAT)

The catalase enzyme converts hydrogen peroxide into water and oxygen (using iron and manganese as cofactors), hence finishing up the detoxification process that SOD started (Gupta, 2015). The reaction is represented by the following equation:

$$2\ H_2O_2 \xrightarrow{Mn^{2+}\text{- CAT}} O_2 + 2\ H_2O$$

I.6.1.3. Glutathione peroxidase and glutathione reductase

The glutathione peroxidase and the glutathione reductase are a group of selenoproteins (particularly abundant in liver) that require selenium, an essential trace element, for their structural and enzymatic functions. The glutathione peroxidase helps in breaking down hydrogen peroxide (equation 1) and organic peroxides, ROOH (equation 2) into water and alcohols by oxidation of glutathione (Gupta,

2015). The glutathione reductase reduces, then, the oxidized glutathione (equation 3), as demonstrated in the following equations:

$$2\ GSH + H_2O_2 \longrightarrow GS–SG + 2\ H_2O \quad ... \ (1)$$

$$2\ GSH + R\text{-}OOH \longrightarrow GS\text{-}SG + ROH + H_2O \quad \dots \ (2)$$

$$GS–SG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+ \quad \dots \ (3)$$

I.6.2. Non enzymatic antioxidants

The non-enzymatic antioxidants include vitamin C, vitamin E, carotenoids, selenium, glutathione (GSH) and plant polyphenols. They act by interrupting free radical chain reactions.

I.6.2.1. Vitamin C

Vitamin C is a water-soluble dietary supplement that acts in the aqueous environments of the body, by scavenging free radicals such as $O_2^{\cdot -}$, $OH^{\cdot}$ and lipid hydroperoxides. It cooperates with vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins. It also plays an important role in raising intracellular GSH levels by protection of protein thiol groups against oxidation (Im*et al.*, 2014).

I.6.2.2. Vitamin E

Vitamin E is a lipid-soluble component and represents the first line of defense against lipid peroxidation. It protects the cell membranes from free radicals attack by inhibiting ROS production and breaking their propagation chain of reactions when fat undergoes oxidation (Rivzi *et al.*, 2014). During the antioxidant reaction, α-tocopherol donates a hydrogen atom to a lipid peroxyl radical (figure 2), becoming radical it's self. Thus, the α-tocopherol radical can be reduced to the original α-tocopherol form by ascorbic acid (Im *et al.*, 2014).

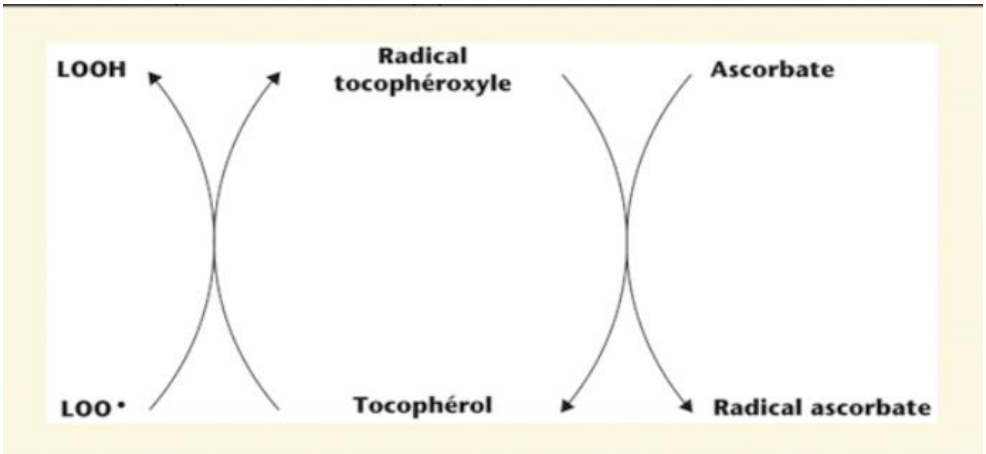

Figure 2. Antioxidant action of α-tocopherol and its regeneration by ascorbic acid (Guilland, 2011).

I.6.2.3. Carotenoids

Carotenoids are among the most common natural pigments with B-carotene being the most prominent. They are responsible for many of the red, orange and yellow hues of plant leaves, fruits and flowers. Carotenoids are lipophilic molecules which tend to accumulate in lipophilic compartments like membranes or lipoproteins. Among their various antioxidant defense strategies, carotenoids are most likely involved in the scavenging of singlet molecular oxygen ($1O_2$) and peroxyl radicals (Stahl and Sies, 2003).

I.6.2.4. Glutathione

Glutathione is a tripeptide found in every single cell of our body. It has been called the “master antioxidant” since it maximizes the activity of all the other antioxidants. Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, GSH is able to donate a reducing equivalent from the thiol group of cysteine to another unstable molecule, becoming reactive itself. However, it readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). GSH can be regenerated from GSSG by the glutathione reductase enzyme. In healthy cells and tissues, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress (Gupta, 2015).

I.6.2.5. Polyphenols

Polyphenols are secondary plants metabolites that occur in all parts of the plant. Among them, flavonoids are the most abundant and the best phytochemicals acting as antioxidants due to the –OH groups in their flavan nucleus B ring. This last is responsible of donating hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to relatively stable flavonoids radicals (Karak, 2019). Mechanisms of antioxidant action of flavonoids include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS and upregulation or protection of antioxidant defenses (Kumar and Pandey, 2013). Quercetin, epicatechin and rutin are examples of flavonoids having strong antioxidant activity (Kumar and Pandey, 2013).

Tannins have also been demonstrated to exert antioxidant activity. This property is related to their chemical structure as they possess phenolic rings able to bind a wide range of molecules and act as electron scavengers to trap ions and radicals. Their high molecular weight and high degree of hydroxylation of aromatic rings explain the high antioxidant potential observed in different tests (Fraga-Corral *et al*., 2021).

II. Inflammation

Inflammation is a protective strategy adopted by the organism in response to harmful stimuli such as pathogen infection, damaged cells, tissue injury and chemical irritation (Karak, 2019). It is a complex biological response that involves immune cells, blood vessels and molecular mediators (Karak, 2019), and aims to eradicate the harmful agent and to potentiate tissue repair (Sherwood and Toliver-Kinsky, 2004). However, excessive inflammation may lead to tissue injury and can cause physiological decompensation, organ dysfunction and death. Based on timing and pathological features, inflammation is either acute or chronic (Sherwood and Toliver-Kinsky, 2004).

II.1. Acute inflammation

Acute inflammation is the early response of a tissue to injury. It is nonspecific and relatively of short duration (hours to days). It is characterized by vasodilatation,

exudation of protein-rich fluid (plasma) and migration of cells (primarily neutrophils) into the site of injury (Sherwood and Toliver-Kinsky, 2004).

II.1.1. Vasodilatation

Vasodilatation is mediated primarily by nitric oxide (NO) and vasodilatory prostaglandins. NO is produced from L-arginine through the action of nitric oxide synthase (NOS), by activated leukocytes after exposure to microbial products or pro-inflammatory cytokines (Vallance and Chan, 2001). Prostaglandins are lipid mediators produced from arachadonic acid through the action of cyclo-oxygenase (Tabernero *et al*., 2003). The NO produced causes subsequent smooth muscle relaxation resulting in vascular expansion, in order to facilitate the local delivery of soluble mediators and inflammatory cells (Sherwood and Toliver-Kinsky, 2004). Vasodilatation is clinically characterized by redness (erythema) and warmth at the site of injury (Kumar *et al*., 2007).

II.1.2. Exudation of fluid

Exudation of fluid is the passage of protein-rich fluid from the microcirculation into the interstitium as a result of the increased vascular permeability caused by the action of histamine, bradykinin, leukotrienes, complement components (C3a, C5a), substance P and platelet-activating factor (PAF). In response to these factors, the barrier functions of small blood vessels are altered and the permeability of capillaries and venules is increased for both water and proteins. This, lead to a transvascular flux of fluid and protein into the inflamed interstitium in order to allow the delivery of soluble factors such as antibodies and acute-phase proteins to the site of injury (Sherwood and Toliver-Kinsky, 2004). This exudation is responsible of swelling (edema), one of the major features of the acute inflammation (Mc Graw, 2023).

II.1.3. Leucocyte recruitment

The leucocytes recruitment consists of the delivery of leucocytes to the site of infection or inflammation. Neutrophils dominate the early phase (first 24 hours) and remain predominant for several days, then other inflammatory cells like phagocytic cells (macrophages) and lymphocytes enter the area (Mc Gray, 2023).

II.1.3.1. Leucocytes emigration

The emigration of leucocytes (figure 3) starts by margination, movement of neutrophil from the central bloodstream to the periphery of the vessel, in contact with the

endothelium. Then, weak adhesive interactions develop between neutrophils and vascular endothelial cells allowing the rolling of neutrophils over the vascular endothelium. This rolling is facilitated by selectins, a family of surface glycoproteins expressed in leucocytes (L-selectins) and endothelial cells (E-selectins). As rolling progresses, a high-affinity adhesive interaction known as adherence develops. This adherence occurs between integrins, expressed on the surface of neutrophils, and ligands, particularly intercellular adhesion molecule-1 (ICAM-1), that are present on the endothelial cell membrane. These interactions cause tight adherence of the neutrophil to the endothelium and facilitate diapedesis and chemotaxis. Diapedesis is the passage of neutrophil through endothelial cell junctions to the extravascular inflammatory environment, a process that is facilitated by adhesion molecules such as platelet–endothelial cell adhesion molecule-1 (PECAM-1), present on the surface of both the endothelial cells and the neutrophils. The binding of PECAM-1 decreases the neutrophil adhesion to ICAM-1, resulting in the inhibition of adherence and the promotion of diapedesis. Finally, chemotaxis ensures the migration of neutrophils and other leucocytes to the site of infection in response to chemoattractants (bacterial byproducts, complement components and chemokines), that are produced following an invasion or a tissue damage (Kumar *et al.*, 2007). The inflammatory infiltrate within injured tissue is determined by the profile of chemokine secretion induced by a particular disease or inflammatory process (Sherwood and Toliver-Kinsky, 2004).

II.1.3.2. Leucocytes immune response

Once leucocytes are in the site of infection (figure 3), they are activated by microbial products or mediators of inflammation to induce a number of responses including phagocytosis of particles (an early step in the elimination of harmful substances), production of lysosomal enzymes and ROS/RNS species that destroy phagocytosed microbes and remove dead tissues, and production of mediators that amplify the inflammatory reaction (Kumar *et al.*, 2007).

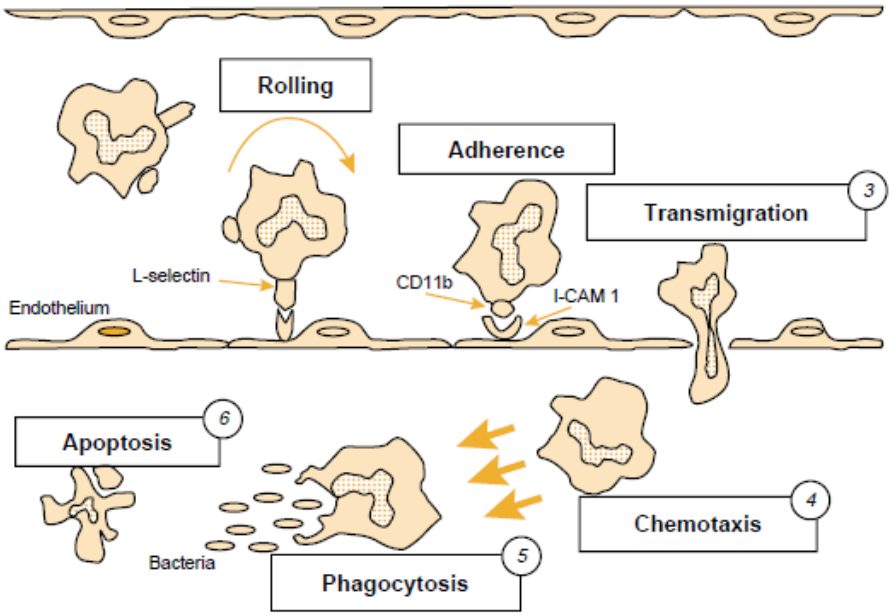

Figure 3. Mechanisms of neutrophil emigration to the site of infection (Sherwood and Toliver-Kinsky, 2004).

II.1.4. Inflammation resolution

For a successful resolution of the acute inflammation, removal of the inciting agent, shutdown of pro-inflammatory signaling and clearance of pro-inflammatory mediators are expected in order to attenuate and then to stop leucocytes recruitment to inflammatory sites. This includes down regulation of pro-inflammatory molecules like cytokines, chemokines, eicosanoids and cell adhesion molecules (Eming *et al*., 2007), and upregulation of anti-inflammatory molecules like IL-1 receptor antagonist or soluble TNF receptor (Teder *et al*., 2002; Jiang *et al*., 2005).

As the acute inflammation is characterized by the accumulation of neutrophils in the affected tissue, the key in the resolution is the removal of these cells from the local inflamed sites. One of the mechanisms of neutrophils removal is caspase-mediated apoptosis, followed by macrophage phagocytic clearance of the apoptotic neutrophils, a mechanism called efferocytosis (Sugimoto *et al*., 2019).

Moreover, macrophages are key components of the repair of inflamed or injured tissues. During the resolving phase of inflammation, macrophages produce anti-inflammatory mediators, such as IL-10 and TGF-β1 (*Transforming growth factor* -β1), and multiple growth factors, including PDGF, IGF-1 and VEGF-α. These mediators can promote fibroblasts differentiation into myofibroblasts, regulate

remodeling of extracellular matrix components and stimulate collagen synthesis. They also promote angiogenesis and restore oxygen supply in healing wounds, resulting in a complete structural and functional tissue recovery (Saclier *et al.*, 2013; Eming *et al.*, 2017).

II.2. Chronic inflammation

Chronic inflammation lasts several months to years depending on the cause of the injury and the ability of the body to repair the damage. It persists for multiple reasons including failure of eliminating the agent causing an acute inflammation; long period exposure to a low level of a particular irritant that cannot be eliminated by enzymatic breakdown or phagocytosis; an autoimmune disorder in which the immune system attacks its own components; and the increased production of noxious components by the inflammatory inducers (free radicals, advanced glycation end products, uric acid crystals, oxidized lipoproteins…etc.) causing oxidative stress and mitochondrial dysfunction (Pahwa *et al.*, 2022).

As the features of the acute inflammation continue, including vasodilation, capillary permeability and diapedesis, the inflammation becomes chronic. However, the composition of the infiltrate changes soon and the macrophages and lymphocytes replace short-lived neutrophils. These cells produce inflammatory cytokines, growth factors and enzymes, and hence contribute to the progression of tissue damage and secondary repair including fibrosis and granuloma formation (Cutolo *et al.*, 2019; Milenkovic *et al.*, 2019).

II.3. Inflammation associated pathologies

Inflammation has long been recognized as a major cause of diseases. Tissue injuries associated to acute and chronic inflammation are observed in many organ systems including heart, pancreas, liver, kidney, lung, brain and intestinal tract. Hence, inflammation leads to the development of several diseases, such as cardiovascular diseases, atherosclerosis, type 2 diabetes, rheumatoid arthritis and cancers (Chen *et al.*, 2018; Pahwa *et al.*, 2022). Persistent pro-inflammatory cytokines production results in continue activation and infiltration of leucocytes into the inflammatory site (Lowe *et al.*, 2011). These cytokines are responsible of amplifying the inflammatory response including cellular hyperproliferation and tissue degradation (Noack *et al.*, 2018).

II.4. Anti-inflammatory drugs

II.4.1. Non steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) represent one of the most common classes of medications used worldwide for inflammation and its related disorders, acting as inhibitors of the cyclooxygenase (COX). COX is involved in the metabolism of arachidonic acid and the synthesis of prostanoids including potent pro-inflammatory prostaglandins as PGE2 and PGF2a (Cao *et al.*, 2010). The COX exists in two isoforms: COX-1 and COX-2. COX-1 is expressed constitutively in almost all cell types, including platelets, stomach, kidney, vascular endothelium, forebrain and uterine epithelium and is responsible for various physiological functions, whereas COX-2 is inducible and expressed during tissue damage or inflammation in response to pro-inflammatory cytokines such as IL-1b, IFN-γ and TNF-α (tumor necrosis factor -α) (Scott *et al.*, 2010). Most of the NSAIDs are organic acid containing drugs including salicylate derivatives (aspirin), carboxylic and heterocyclic acid derivatives (indomethacin), propionic acid derivatives (ibuprofen, ketoprofen, flurbiprofen) and phenyl acetic acid derivatives (diclofenac). These drugs prevent the access of arachidonic acid into the active site of the COX and stop its pathway (Marnett *et al*., 2009). Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal ulceration, perforation, obstruction and bleeding has limited their therapeutic usage. NSAIDs are metabolized in the liver, and they have been implicated in hepatocellular damage and hepatic failure in several species (Clark-Price, 2014). The fact that NSAIDs are not selective toward inhibition of COX-2 but influence the normal physiological functions of COX-1 correlates with their ability to cause these side effects. Thus, the research for anti-inflammatory agents that present selective COX-2 inhibition but less reactivity towards COX-1 is important (Shaikh *et al*., 2015).

II.4.2. Steroidal anti-inflammatory drugs

Steroidal anti-inflammatory drugs **(**glucocorticoids) are synthetic analogs of the natural steroid hormones produced by the adrenal cortex. They are involved in the metabolism and have immunosuppressive, anti-inflammatory and vaso-constrictive effects (Hodgens and Sharman, 2022). Glucocorticoids inhibit phospholipase A2, which is critical for producing inflammatory cytokines, impairing release of arachidonic acid and regulation of apoptosis in thymocytes (Ericson-Neilsen and

Kaye, 2014). They also inhibit the production of B cells and T cells at high concentrations (Ericson-Neilsen and Kaye, 2014). Furthermore, they can inhibit the gene expression and translation for inflammatory leukocytes and structural cells such as epithelium leading to a reduction in pro-inflammatory cytokines, chemokines, cell adhesion molecules and other proteins involved in the inflammatory response (Liu *et al*., 2013).

In the same way as NSAIDs, inappropriate use of corticosteroids (dose and duration) can be associated with adverse effects including diabetes, glaucoma, cataracts, immune-suppression, cardiovascular and gastrointestinal echoes, dermatological problems and even psychiatric disturbance (Hodgens and Sharman, 2022).

II.4.3. Natural anti-inflammatory substances

Polyphenols act in several levels of the inflammatory response including obstruction of the cyclooxygenase and lipoxygenase paths and hence inhibition of the arachidonic acid metabolism (Yoon and Baek, 2005), signal transduction mechanisms implicated in inflammatory cells activation (Capiralla *et al*., 2012), pro-inflammatory cytokines synthesis, adhesion molecules expression, NF-κB activation as well as reactive oxygen species production (Zeinalia *et al*., 2017). Hesperidin, luteolin and quercetin are known to possess such types of anti-inflammatory properties. They mainly affect the enzyme systems involved in the generation of inflammatory processes. Flavonoids also inhibit phospho-di-esterases involved in cell activation (Karak *et al*., 2019).

III. Liver

III.1. Anatomy and Histology

Liver, the most significant and chief metabolic organ, is located in the upper right-hand side of the abdomen, and represents 2% of the total body weight (Ganesan *et al*., 2017). It has a dark reddish-brown color due to the large quantity of blood flowing through it. It is composed of two main lobes, right and left separated by the falciform ligament, with the right lobe being the larger (Qin and Crawford, 2018). Each lobe is made up of thousands of functional units named lobules, which are held together by a fine dense irregular fibro elastic connective tissue layer which extends into the structure of the liver, by accompanying the vessels (veins and arteries), ducts and nerves through the hepatic portal. Each lobule is made up of rows of different types of cells: hepatocytes, Kupffer cells, sinusoidal cells and stell cells (Malarkay *et al*.,

2005) perforated by specialized blood capillaries called sinusoids (Abdelmisih and Bloomston, 2010). The liver is irrigated by two large blood vessels: the hepatic artery and the portal vein, which deliver oxygen and intestinal nutrients, respectively (Dooley *et al*., 2011). Outflow also involves two routes: hepatic veins which drain evacuated blood into the inferior vena cava (Dooley *et al*., 2011) and bile canaliculi, which transport secreted bile to the common hepatic duct (Ozougwu, 2017). The hepatic artery, the portal vein and the common hepatic duct form together a structure named portal triad, which resides in every corner of the lobule hexagon (figure 4).

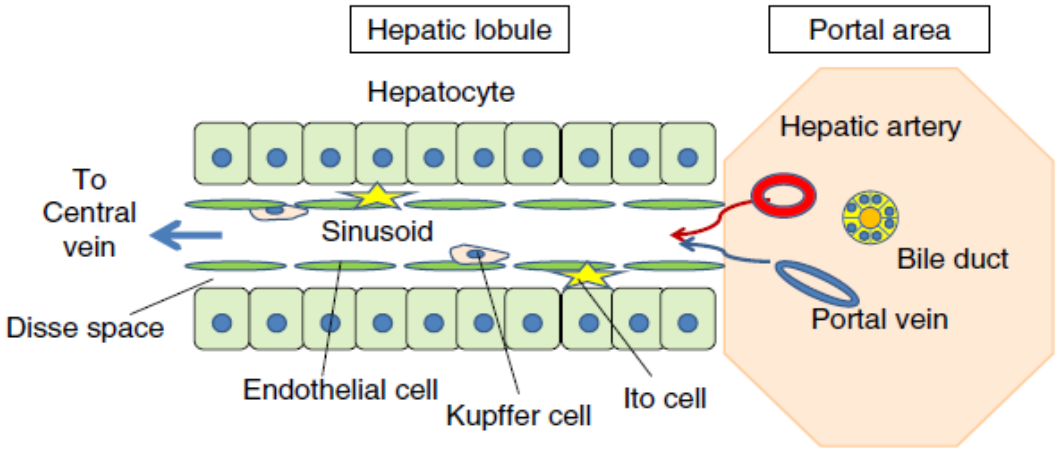

Figure 4. Architecture of the liver (Ozougwu, 2017).

III.2. Liver cells

The liver lobule consists mainly of plates of hepatocytes (parenchymal cells) and sinusoids, with a collagen light matrix between the two. The hepatic sinusoid walls are lined by three other different types of cells (non parenchymal cells): the sinusoidal endothelial cells, kupffer cells and stellate cells (figure 4). Additionally, pit cells and the liver specific natural killer T cells (NKT) are often present in the sinusoidal lumen (Ozougwu, 2017).

III.2.1. Hepatocytes

Hepatocytes represent 60 % of the liver‘s cells and about 80 % of the liver‘s total cell mass. Hepatocytes are large and rich in organelles such as endoplasmic reticulum and Golgi apparatuses. They contain many and large mitochondria, as well as lysosomes and peroxisomes (Ozougwu, 2017). The main function of hepatocytes is to participate in lipid, carbohydrate and protein metabolism. They also produce serum proteins such

as albumin and coagulation factors. Furthermore, hepatocytes produce and secrete bile as well as detoxify and excrete cholesterol, steroid hormones and xenobiotic drugs. Numerous xenobiotics are metabolized by the mixed-functions of monooxidases found in hepatocytes (Ozougwu, 2017).

III.2.2. Endothelial cells

The sinusoidal endothelial cells line the walls of the hepatic sinusoids and perform a function of filtration due to the presence of fenestrae. These cells also demonstrate large endocytic capacity for extracellular matrix components and immune complexes. In general, they engulf smaller size particles and may play a role in clearance of viruses, but do not possess phagocytic function. They may also function as antigen presenting cells and secrete certain cytokines and eicosanoids (Ozougwu, 2017).

III.2.3. Kupffer Cells

Kupffer cells are resident macrophages of the liver and represent the largest tissue resident macrophage population of the body (80-90%). They reside within the sinusoid lumen and are in constant contact with materials absorbed from the gastrointestinal tract (Ishibashi *et al.*, 2009). Upon activation by pathogenic agents, they are able to secrete a vast range of inflammatory mediators such as cytokines, reactive oxygen species, eicosanoids and nitric oxide. Kupffer cells have receptors that enable them to bind cells covered with immunoglobulins or bind to complement receptors and subsequently phagocytose cell (Ozougwu, 2017).

III.2.4. Stellate cells

Stellate cells, fat storing cells of the liver, are located in the space of disse in close contact with hepatocytes and sinusoidal endothelial cells (Stalnikowitz, 2003). They are the main vitamin A storing cells harboring large amounts of retinol and retinyl palmitate in lipid droplets within their cell cytoplasm (Ozougwu, 2017). Thus, the liver plays a central role in uptake and storage of vitamins A, storing about 95 % of retinoids found in the body. Stellate cells also control the turnover of extracellular matrix and regulate sinusoid contractility. They may become activated under stressful conditions and transformed into myofibroblast, playing a key role in inflammatory fibrotic response (Ozougwu, 2017).

II.2.5. Pit cells

Pit cells are defined morphologically as large granular lymphocytes and functionally as liver-associated natural killers (NK) and natural killers T cells (NKT). They are situated inside the sinusoidal lumen, adhering to the endothelial and Kupffer cells and exert antitumor functions by exocytosis of perforin/granzyme-containing granules, induction of death receptor-mediated apoptosis in target cells, and production of various cytokines that augment the activities of other immune cells (Nakatani *et al*., 2004).

III.3. Liver functions

The liver is a crucial and indispensable organ, having a wide range of physiological functions. It is involved in the metabolism of nutrients such as lipids, proteins and carbohydrates (Refaey *et al*., 2015). It is also involved in the breakdown of toxins and different foreign substances such as drugs, alcohol, chemicals, heavy metals, infectious organisms, pesticides, herbicides, fats, food additives and dead cells, and removing them from the blood. It serves as a storage compartment for numerous substances such as glycogen, vitamins and minerals (Okaiyeto *et al*., 2018). It helps in regulating the normal glucose concentrations during fasting by playing a fundamental role in glycogen metabolism, which consists of hepatic glucose production by glycogenolysis (Ganesan, 2017). The liver performs its digestive role by producing bile, which is needed to digest and emulsify fats and oils as well as other substances, such as vitamins A, D, E, and K. It is also implicated in manufacturing some proteins such as blood proteins, enzymes, hormones, immune factors as well as the blood-clotting factors (Ganesan, 2017). Finally, the liver has the ability to produce cholesterol that is capable of transporting energy supplying fats needed for the ATP generation in the body whenever there is depletion in the blood sugar level (Okaiyeto *et al*., 2018).

III.4. Liver pathologies

The term of liver disease describes any condition or disorder that interferes with the normal functioning of the liver which may eventually leads to its dysfunction or total loss of function (Govind, 2011). Considering the duration, liver diseases are either acute or chronic diseases. The acute liver disease occurs rapidly and lasts for a very short duration. The chronic liver diseases are typically long term, generally over 6

months, cause periodical destruction and regeneration of liver parenchyma leading to fibrosis, cirrhosis, chronic hepatitis and even liver carcinoma (Ganesan *et al*., 2017). The majority of the hepatic diseases causing agents include microorganisms, metabolic diseases, inherited related hepatic diseases, autoimmune diseases and liver malignancies (Ganesan, 2017). Toxicity by xenobiotics (alcohol, drugs and chemicals) is another causing agent and the most common risk factor of hepatic pathologies which causes liver damage through the free radical-mediated inflammatory process (Khan *et al*., 2020). The liver injury occurs due to the activation of xenobiotics to chemically active metabolites which have a tendency to react with cellular macromolecules leading to protein dysfunction, lipid peroxidation and DNA damage (Gulati, 2018). Azab and Albasha (2018) as well as Okaiyeto and collaborators (2018) classified liver diseases into inflammatory liver diseases (acute or chronic hepatitis), non-inflammatory diseases (hepatosis) and degenerative disorders (resulting in liver fibrosis and cirrhosis).

III.4.1. Hepatitis

Hepatitis is a medical condition which is defined by the inflammation of the liver and characterized by the presence of inflammatory cells in the tissue of the liver which can be self-limiting or progress to fibrosis and cirrhosis (Ifeanyi *et al*., 2018). It can be caused by immune cells in the body attacking the liver and causing autoimmune hepatitis, infections from viruses (such as hepatitis A, B, C, D and E), bacteria or parasites, liver damage from alcohol, poisons or medications (Sivakrishnan, 2019). It can be acute, lasting for less than six months, or chronic when persisting for longer period of time (Ifeanyi *et al*., 2018).

III.4.2. Fibrosis

Hepatic fibrosis occurs in response to inflammation or direct toxic insult to the liver. It is a wound healing process characterized by increase and accumulation of extracellular matrix (ECM) proteins especially collagen types I and III, proteoglycans, fibronectin and laminin in response to liver injury (Stalnikowitz, 2003). This ECM allows the migrating cells to contain the injury agent reaching a successful conclusion with dismantling of the repair apparatus. The ECM is taken down by matrix proteinases while activated stellate cells undergo apoptosis, restoring the normal tissue structure. The extracellular degradation of matrix proteins is regulated by matrix metalloproteinases (MMPs) produced by hepatic stellate cells. When the injury is recurrent or chronic, an imbalance between fibrogenesis and fibrolysis occurs

resulting in deposition of collagen and leading to scar formation. As scarring progresses from bridging fibrosis to the formation of complete nodules, it results an architectural distortion and ultimately liver cirrhosis (Stalnikowitz, 2003). The deposition of collagen has consequences on hepatic pattern of blood flow and perfusion of heapatocytes. As capillarized sinusoids are characterized by accumulation of fibrillar extracellular matrix in the space of disse, there will be impairment in metabolic exchange between blood and hepatocytes (Stalnikowitz, 2003). Fibrosis is generally considered as an irreversible consequence of hepatic damage.

III.4.3. Cirrhosis

Liver cirrhosis, one of the most serious alterations, is a complication of many liver diseases that is characterized by abnormal structure and function of the liver. It consists of progressive hepatocytes injury and nodular parenchyma followed by regeneration and widespread fibrosis, with hepatocellular necrosis leading to disorganization of lobular architecture (Schuppan and Afdhal, 2008). Angiogenesis accompanies scar production which results in the formation of abnormal channels between the central hepatic veins and the portal vessels. This in-turn causes shunting of blood around the regenerating parenchyma and hence restoring intrahepatic circulatory pathways (Ifeanyi *et al*., 2018). Such interconnecting vessels provide relatively low-volume, high-pressure venous drainage and as a result, portal vein pressure increase. Thus, it contributes to portal hypertension, the most common complication of cirrhosis (Ishibashi *et al*., 2009).

III.4.4. Steatosis

Steatosis consists of the retention of fat within hepatocytes and results from an imbalance between uptake and synthesis of fats and their oxidation and export (Ahmed *et al*., 2017).It is further subdivided into microsteatosis and macrosteatosis (Fromenty *et al*., 2003). Macrosteatosis is characterized by a single bulky fat vacuole in hepatocytes, displacing the nucleus to the edge of the cell. In microsteatosis, the cytoplasm of the hepatocytes contains tiny lipid vesicles without nuclear dislocation (Ahmed *et al*., 2017).

III.4.5. Necrosis

Necrosis, also called cell death, is a process in which viable cells become nonviable, resulting in dissolution of their cell contents. Under physiological conditions, it is a well-regulated process that leads to replacement of individual effete cells. However, under pathological conditions, necrosis is often the end result of various forms of tissue injury. Necrosis is a common finding in acute and chronic liver diseases, and with persistence of the underlying cause, it is followed by progressive fibrosis. Viral hepatitis, autoimmune diseases, ischemic liver injury, drugs induced injury and transplantation are the important causes for liver necrosis (Krishna, 2017).

III.5. Hepatotoxins

Hepatotoxin is a toxic chemical substance which damages the liver. Toxic liver damage produced by means of drugs and chemical compounds may virtually mimic any shape of the liver's affections. Several chemicals were largely used to induce experimental hepatotoxicity including carbon tetrachloride (CCl_4), galactosamine, thioacetamide, anti-tubercular drugs, paracetamol, alcohol and some antibiotics. Most of the inorganic compounds producing hepatotoxicity are phosphorus, copper and iron. The natural substances may also serve as hepatotoxins encompassing plant toxins such as pyrrolizidine alkaloids, mycotoxins and bacterial toxins (Hassan *et al.*, 2019).

III.6. Mechanism of hepatotoxicity caused by carbon tetra-chloride

Carbon tetrachloride is widely used as an industrial solvent, degreasing and cleaning agent (Weber *et al.*, 2003). It is also a very active chemical capable of causing injury to hepatocytes resulting in hepatic necrosis. Thus, it is widely used, as hepatotoxin, in the animal acute liver injury model to study hepatoprotective effect of drugs (Refaey *et al.*, 2015; Gulati, 2018). CCl_4 is metabolized, in hepatocytes, by CYP2E1 (enzyme from the cytochrome P450 family located in the endoplasmic reticulum) to highly reactive trichloromethyl free radicals ($CCl^{\cdot}_3$). The $CCl^{\cdot}_3$ radicals can bind to cellular molecules such as lipids, proteins and nucleic acid impairing crucial cellular processing (Weber *et al.*, 2003). The $CCl^{\cdot}_3$ can also react with oxygen to form the trichloromethylperoxy radical ($CCl_3OO^{\cdot}$**)**, even more reactive species (Weber *et al.*, 2003; Refaey *et al.*, 2015). The $CCl_3OO^{\cdot}$ initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular

those associated with phospholipids. This affects the permeability of mitochondrial, endoplasmic reticulum and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, and hence cell damage (Weber *et al*., 2003). Toxic levels of CCl_4 in the system results in fatty accumulation in the liver due to the blockage in the synthesis of lipoproteins responsible for transporting triglycerides away (Lima *et al*., 2010), thus leading to alteration of normal metabolic functions of the liver (Bhuvaneswari *et al*., 2014). A single dose of CCl_4 when administrated to a rat produces, within 24 h, a centrilobular necrosis and fatty changes, and reaches it most awareness in the liver within 3h. The development of necrosis is associated with the leakage of hepatic enzymes into the serum (Hassan *et al*., 2019).

III.7. Treatment of hepatic diseases

III.7.1. Classic treatment

The treatment of liver diseases depends on the type and the degree of progression of this disease. The use of antiviral medicines is helpful in liver infections like hepatitis B and C. Steroids and immunosupressor drugs can be adopted in liver autoimmune hepatitis. In the case of an intoxication induced by drugs like paracetamol, it is necessary to use N- acetylcysteine, the precursor of glutathione able to conjugate the active metabolite: N-acetyl-p-benzoquinone imine (NAPQI). The change of lifestyle is a strategy adopted in the treatment of some liver ailments. Abstinence from the alcohol consummation in the alcoholic steatosis as well as weight loss and adopting a healthy diet in steatosis and non alcoholic liver pathologies, are recommended (Sahu, 2007; Dooley *et al*., 2011).

III.7.2. Alternate treatment

Many natural products such as flavanoids, coumarins, glycosides, organic acids as well as plant crude extracts have been reported to have hepatoprotective activity and thus, used for the treatment of liver disorders (Ravi Kiran *et al*., 2012). Catechin, apigenin, quercetin, naringenin and rutin are reported for their hapatoprotective activities (Tapas *et al*., 2008). Zhu *et al*. (2012) have demonstrated that the anthocyanin: cyanidin-3-O-β-glucoside increases hepatic glutamate-cysteine ligase expression, resulting in a decrease in hepatic ROS levels and proapoptotic signaling. Furthermore, the treatment with this anthocyanin lowers hepatic lipid peroxidation,

inhibits the release of pro-inflammatory cytokines and protects against the development of hepatic steatosis.

Silymarin is a flavonoid having three structural components (silibinin, silydianine and silychristine) extracted from the seeds and fruits of milk thistle (*Silybum marianum*). It has clinical applications in the treatment of cirrhosis, ischemic injury and toxic hepatitis induced by various toxins such as acetaminophen and toxic mushroom (Saller *et al.*, 2001). The pharmacological properties of silymarin involve the regulation of cell membrane permeability and integrity, inhibition of leukotriene, ROS scavenging, suppression of NF-κB activity, depression of protein kinases and collagen production. Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 resulting in DNA biosynthesis and cell proliferation leading to liver regeneration in damaged livers (Kumar and Pandey, 2013).

III.8. Correlation between oxidative stress, inflammation and pathologies

The oxidative stress is a common basis in all types of hepatic injury, being responsible of the alteration of main biological molecules such as DNA, proteins and lipids (Andrade *et al*., 2015). These biomolecules oxidation-resulting products are involved in the modulation of signaling pathways associated with gene transcription, protein expression, apoptosis and hepatic stellate cell activation, leading to both the onset and progression of liver fibrosis (Pomacu *et al*., 2023). Main liver sources of ROS are the mitochondria and the cytochrome P450 in the hepatocyte, Kuppfer cells and neutrophils. The aldehydic products such as malondialdehyde (MDA), are generated by lipid peroxidation in cell membranes and organelles due to damage by ROS in polyunsaturated fatty acids, and are able to diffuse from their site of origin to other places, amplifying the effects of oxidative stress (Rolo *et al*., 2012). Activated hepatic phagocytes, and Kuppfer Cells (KCs), also known as stellate macrophages are one of the liver resident innate immune cell populations. They are involved in all forms of chronic inflammatory liver diseases as a tissue response to oxidative stress (Andrade *et al.*, 2015). Activated KCs, in a defensive mechanism against microbes, produce NAPH oxidase responsible of producing ROS in hepatocytes that cause DNA damage, induce apoptosis and the expression of genes involved in the synthesis of pro-inflammatory cytokines. ROS released by KCs activate the hepatic stellate cells

(HSCs) leading to an increase in the proliferation and the synthesis of extracellular matrix (ECM), contributing to fibrosis (figure 5) and cirrhosis (Diesen and Kuo, 2011). Oxidative stress associated with inflammation causes focal or zonal necrosis, destruction of hepatocytes and architectural disarray (Chatterjee and Mitra, 2015).

Inflammation involvement in liver injuries is manifested as an infiltration of the inflammatory cells to the site of injury that may produce more ROS and RNS, able to increase the expression of genes coding pro-inflammatory cytokines (Pomacu *et al.*, 2023). Inflammation can destroy hepatic parenchymal cells, increasing the risk of chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD) or viral hepatitis (Chen *et al.*, 2018). Excessive inflammation in liver may leads to extensive loss of hepatocytes, ischemia-reperfusion injury, metabolic alterations and eventually permanent hepatic damage (Takaki and Yamamoto, 2015). As the inflammation progress to chronic stage, there is activation of the immune system innate and adaptive, with polymorphonuclear leukocytes (PMN) infiltration, inducible nitric oxide synthase (iNOS) upregulation and recruitment of lymphocytes. Leukocytes and Kupffer cells produce large amounts of nitric oxide ($NO^{\cdot}$) and cytokines, particularly TGF-β and TNF-α, potent profibrogenic cytokine and inflammation modulator, respectively (Pomacu *et al.*, 2023). The oxidative stress and inflammation are tightly correlated and create a vicious cycle which is involved in the progression to cirrhosis and ultimately hepatocellular carcinoma of liver diseases (Pomacu *et al.*, 2023).

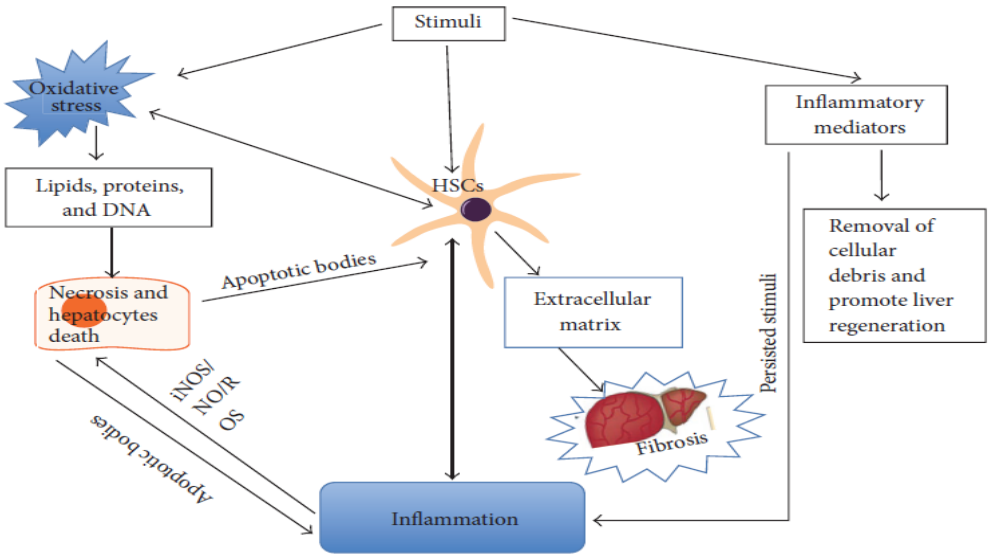

Figure 5. Interaction of oxidative stress and inflammation promoting fibrogenesis (Li *et al.*, 2016).

IV. Studied plants

In the present study, we have selected two plants, *Echium plantagineum* and *Brassica rapa* based on the facts that they both are obtained from Setif region, used in common medicine and/or alimentary consummation and the lack in bibliographic researches concerning their biological activities.

IV.1. *Echium plantagineum*

IV.1.1. Botanical description

Echium plantagineum L., is an annual herbaceous species which belongs to the Boraginaceae family, native from Europe and Northern Africa and indicated for ornamental, melliferous and medicinal purposes (Berti *et al*., 2007; Comunian *et al*., 2016). It's known as viper's bugloss, blueweed and snake's flower and called "Hachichat al-afaa" in the region of Setif, Algeria. It has spread widely throughout the world and has become an invasive weed in Australia and South America (Berti *et al*., 2007). These plants form a rosette with oval leaves at the beginning of the season. Several stems are produced at the base of the rosette. Stems have long, white trichomes or hairs (figure 6). Flowers uncurl on an inflorescence called a cyme that has as many as 30 flowers. Flowers are perfect, blue, purple, sometimes white, and pink, trumpet-shaped and sessile (Berti *et al*., 2007). Seeds are dark brown or grey, small only 3 mm long, and have a triangular shape with three sides. This plant grows to about 70 to 120 cm in height (Berti *et al*., 2007).

Figure 6. Photography of *Echium plantagineum* from Babor region (Setif, Algeria).

IV.1.2. Traditional use

Species of Echium have been used for thousands of years in the Mediterranean area as folk medicines possessing depurative, diaphoretic, diuretic and mood-enhancing properties. Also, many species have been to treat ailments including fissures of the hands, general abrasions and even snakebites (Jin *et al*., 2020).

IV.1.3. Phytochemistry and biological activities

To date, the promising benefits and medicinal use of the Echium genus have been largely neglected (Jin *et al*., 2020). Yet, a variety of the biologically active constituents have been isolated from Echium species, such as naphthoquinones, flavonoids, terpenoids and phenols, which exert anxiolytic, antioxidant, anti-inflammatory, antibacterial and antiviral effects (Jin *et al*., 2020). Phenolic compounds from *E.plantagineum* bee pollen and seed oil are extensively studied, however, very few studies regarding flavonoids or other phenolic compounds (table 1) from the aerial part extracts of this species are available until now (Jin *et al*., 2022).

Table 1. Phytochemical profile of *E. plantagineum* crude extracts established by LC-ESI-HRMS (Jin *et al*., 2022).

Class of phenolic compounds	Phenolic compounds	Plant's part
Flavonoids	kaempferol-3-O-(3″/4″-acetyl)neohesperidoside	R
	quercetin-3-Oneohesperidoside	L,F, S
	kaempferol-3-Oneohesperidoside	L, F, S
	kaempferol-3-O-(4″-rhamnosyl)neohesperidoside	S
	kaempferol-3-Oneohesperidoside-7-Orhamnoside	R
	kaempferol-3-O-rutinoside	L, F, R, S
	quercetin-3-O-sophoroside	L, F, S, R
Anthocyanins	Delphinidin-3-O-glucoside	L, S
	Cyanidin-3-O-rutinoside	L, F, S
	Petunidin 3-glucoside	F
	Petunidin 3-rutinoside	L
	Malvidin 3-rutinoside	L, F, S
Other phenolic compounds	Caffeicacid	L, F, S, R
	Trigonotin A	R
	salvianolicacid A	L, F, S, R

	Viridifloricacid	L, F, S, R
	p-coumaricacid	F
	Globoidnan B	L, F, S, R
	Lithospermicacid	L, F, S, R
	rabdosiin	L, F, S, R
	Lithospermicacid B	L, F, S, R
	Rosmarinicacid	L, F, S, R
	Sagerinicacid	L, F, S, R
	Echiumin B	L, F, S, R
	Globoidnan A (eritrichin)	L, F, S, R
	RosmarinicacidmethylEster	F
	Echiumin D	L, S
	Echiumin C	L, F

L: leaf; **F:** flower; **S:** stem; **R:** root.

IV.2. *Brassica rapa subsp. rapa*

IV.2.1. Botanical description

Brassica, the most important genus in *Brassicaceae* (also called *Cruciferae*) family, consists of about 350 genera and almost 3,500 species (Dejanovic *et al*., 2021). *Brassica rapa subsp. rapa* (turnip) is a biennial, out-crossing and mesopolyploid herbaceous plant. Turnip is indigenous to Europe, Russia, Central Asia, and the Near East, and is now widely cultivated as vegetable and oil source throughout the world (Paul *et al*., 2019). Turnip, known locally in Algeria as "Lifft Siidi" (figure 7), is one of the oldest cultivated vegetables that have been used for human consumption since prehistoric times (Fernandes *et al*., 2007). It grows well in cold climates and can be stored several months after harvest. Turnip leaves are usually light green, thin and sparsely pubescent. A white-fleshed, large global or tapered root develops at the base of the leaf petioles. Branched flowering stems are also produced. The flowers are clustered at the top of the raceme, and are usually raised above the terminal buds. Bolting occurs in late winter followed by the formation of flower buds, which are also consumed before opening and while still green (Fernandes *et al*., 2007).

Figure 7. Photography of *Brassica rapa* roots from Remada region (Setif, Algeria).

IV.2.2. Traditional use

Plants of Brassicaceae include several of the most commonly consumed vegetables all over the world. Turnip edible parts are commonly consumed as raw, boiled and/or fermented vegetables in the preparation of soups and stews (Beltagy, 2014). Young turnip roots are commonly consumed raw in salads, while, the turnip greens (leaves) and tops (stems, buds and flowers) are usually served cooked or steamed (Dejanovic *et al*., 2021). The flower buds are eaten sauteed, with a mixture of hot olive oil and garlic, and with rice (Fernandes *et al*., 2007). Further to its use as a vegetable, turnip has been used to treat chronic gastritis, cholecystitis and cholecystolithiasis. It is one of the oldest cultivated vegetables in high-altitude localities in Asian countries where its roots have been widely consumed for fatigue relief and hypoxia prevention (Cao *et al*., 2021). Turnip roots have also long been purposed in traditionally cure for common cold due to their antibacterial properties. According to Wu *et al*. (2013), this species is well known for its ability to alleviate jaundice, combat liver illnesses, relieve hangover and improve chronic constipation and kidney function.

IV.2.3. Phytochemical content and biological activities

The *Brassica* plants are very rich in several nutritional (carbohydrates, lipids, proteins, vitamins, minerals) and phytochemical (glucosinolates, isothiocyanates, flavonoids and phenolics) components of medicinal value (Dejanovic *et al*., 2021).

The chemical composition analysis of *Brassica rapa* roots showed their richness in multiple nutritional components including carbohydrates, proteins, dietary fibers, vitamin C, essential aminoacids and mineral elements, but fewer fats (Cao *et al*., 2021). Carbohydrate derivatives often mean modification of sugar molecules by the addition of substituents other than hydroxyl groups, such as amino sugars, acidic sugars, deoxy sugars, sugar alcohols, glycosylamines, sugar phosphates and sucrose esters. They are good examples of amphiphilic derivatives in which both the hydrophilic and hydrophobic moieties come from vegetal resources (Wu *et al*., 2013). A variety of pharmacological effects of carbohydrate compounds are reported as enzyme-activated irreversible inhibitors, modulator of inflammatory states, skin barriers, hepatoprotective agents and inhibitors of glycosidase as potential therapeutic agents against HIV, diabetes and cancers (Wu *et al*., 2013).

Glucosinolates are characteristic constituents of *Brassica rapa* roots (Dejanovic *et al*., 2021). They are a diverse group of nitrogen- and sulfur-rich secondary metabolites, classified into three major groups, namely, aliphatic, aromatic and indole glucosinolates (Thiruvengadam *et al*., 2016).

B. rapa also presents a profile rich in phenolic compounds and organic acids, known for their antioxidant, anti-inflammatory, anti-diabetes and neuroprotective activities (Cao *et al*., 2021). Studies reported 35 flavonoids in this plant that occur in glycosides (Paul *et al*., 2019). Among them, kaempferol 3-O-sophoroside-7 Oglucoside, kaempferol 3-O-(feruloyl/caffeoyl)-sophoroside-7-Oglucoside, isorhamnetin 3,7-O-diglucoside and isorhamnetin3-O-glucoside are the main ones (Cao *et al*., 2021). Liquiritin, liquiritigenin, 4,4’-dihydroxy-3’-methoxychalcone, isoliquiritin and licochalcone A are flavonoids identified in turnip roots (Jeong *et al*., 2013).The organic acids in turnip include malic acid,aconitic acid, citric acid, ketoglutaric acid, shikimic acid andfumaric acid. Malic acid is the dominant one, and it is more abundant in the roots than in the leaves, stems and flower buds. The other organic

acids are, however, predominant in the turnip aerial part. Ferulic acid and synaptic acid are only detected in turnip roots (Fernandes *et al*., 2007).

Some chemical constituents of *B. rapa* such as indole alkaloids (Wu *et al*., 2012), sterols (Kim *et al*., 2004) and fatty acids (Bang *et al*., 2007) as well as the ethanolic extract from the roots have been reported to modulate the deleterious effects of diabetes (An *et al*., 2010), prevent high-fat diet-induced obesity (Jung *et al*., 2008) and protect against cisplatin-induced nephrotoxicity (Kim *et al*., 2006). The ethanolic extract, also, showed an inhibition effect on reactive oxygen species levels and glutamate-induced cell death in HT-22 cells (Wu *et al*., 2013).

MATERIALS AND METHODS

I. Materials

I.1. Plant material

Echium plantagineum L. was collected from Babor region (Northern of Setif), at the East of Algeria. It was identified by Pr. Houssine Laouar, University of Setif. The aerial part (leaves and flowers) was cleaned up, air-dried at room temperature and then reduced to powder using electrical grinder. The powder was conserved in clean dried glass bottle at room temperature and protected from light until use.

Brassica rapa L roots were purchased from local market of Setif (Algeria). Roots were cleaned with water and then cut into thin washers. All samples were put on absorbent paper and heat dried before they got reduced to powder using a mortar.

I.2. Experimental animals

Adult non-pregnant female *Albino Wistar* rats weighing 150-180 g were obtained from Pasteur institute of Algiers (Algeria). They were housed in polypropylene cages with free access to water and food and acclimatized under the laboratory conditions at least for 1 week before use. All procedures were performed following European Union Guidelines for Animals Experimentation. Animalswere fasted overnight before treatment and then divided into groups of 5animals each.

The toxicity study was performed using young adult non-pregnant female *Swiss albinos* mice weighing 20-30 g. They were obtained from Pasteur institute (Algeria) and kept, for 7 days of acclimatization, in plastic cages under normal laboratory conditions (12 h light/dark cycle, 25 °C) prior to the experiments. All the animals were given food and water *ad libitum*. The experiments were conducted following the Organization of Economic Co-operation and Development guideline (OECD, 2001) for chemical testing.

I.3. Human and bovin blood

Fresh human blood was obtained from healthy voluntary donors. The bovine blood was obtained by sampling from healthy animals in the slaughterhouse of the city of Setif.

II. Methods

II.1. Preparation of plant extracts

Aqueous extracts (AqE) of *Echium plantagineum* and *Brassica rapa* were prepared according to the method described by Ljubuncic *et al*. (2005). Dried material (50 g) was boiled in 1000mL of distilled water for 25 min followed by filtration through Whatman filter paper N°3. The filtrate was dried and the yield of the obtained powder was calculated (40% and 58%, respectively). The powder was stored at -32°C until required.

Methanolic extracts (Met E) of the two plants were prepared as described by Motamed and Naghibi (2010). Plant material (50 g) was macerated twice for 24h with 500mL of 80% methanol and then with 50% methanol at room temperature with frequent agitation. After filtration through Whatman filter paper N°3, the filtrate was concentrated under reduced pressure at 40 °C with a rotavapor (Buchi, Flawil, Switzerland) and then dried. The obtained powder (yields: 21,9% and 55,5%, respectively) was stored at -32 °C until use.

II.2. Phytochemical analysis

II.2.1. Determination of total polyphenols

The content of total polyphenolic compounds in the extracts was determined using Folin-Ciocalteu assay (Li *et al*., 2007). Samples (100 µl) or gallic acid (standard) were mixed with 500µL of 1:10 diluted Folin-Ciocalteu reagent. After 4min, 400µL of 7.5% sodium carbonate (Na_2CO_3) solution were added. After incubation at room temperature for 90min, the absorbance was measured at 760nm. The concentration of total polyphenol compounds was determined as mg of gallic acid equivalents per 1 g of extract (GAE/mg extract) from the calibration curve represented in figure 8.

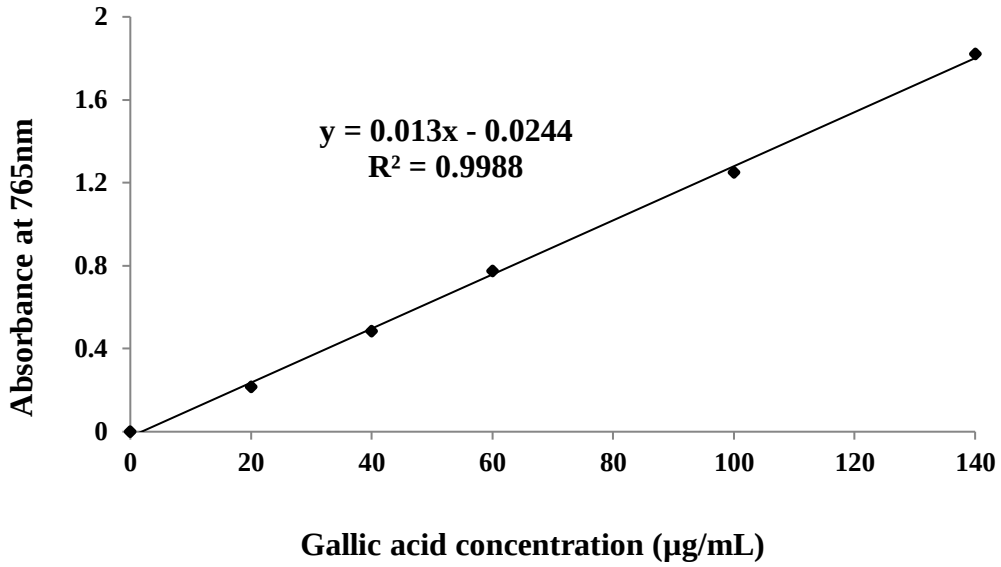

Figure 8. Standard curve of gallic acid for determination of total polyphenols. Each value represents mean ± SD (n = 3).

II.2.2. Determination of total flavonoids

The total flavonoid content was determined by the aluminum chloride method as described by Bahorun *et al*. (1996). Briefly, 1 mL of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of extracts. After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used for the standard calibration curve (figure 9). The data were expressed as milligram quercetin equivalents (QE)/g extract.

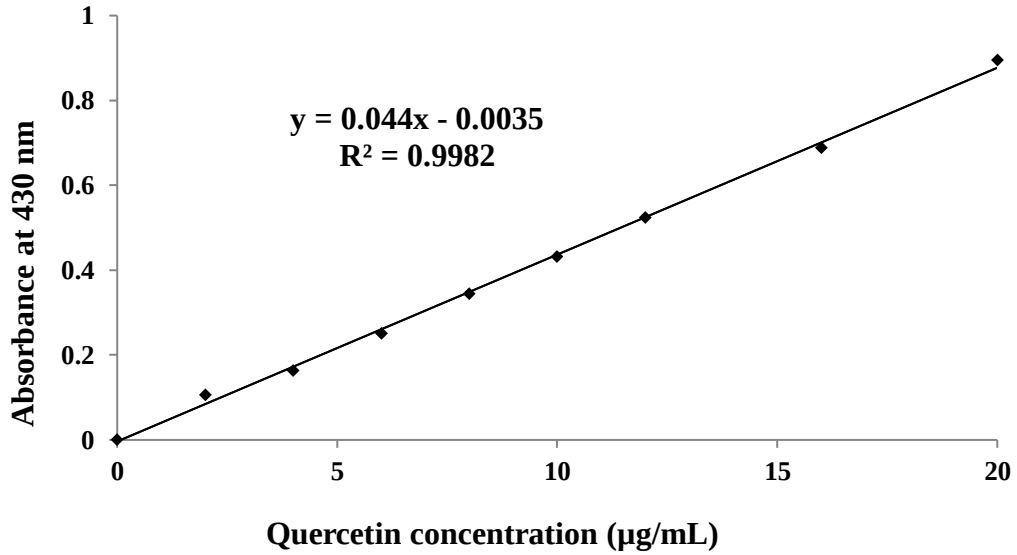

Figure 9. Standard curve of quercetin for determination of total flavonoids. Each value represents mean ± SD (n = 3).

II.2.3. Determination of total condensed tannins

Tannin content of the used extracts was determined using the hemoglobin precipitation assay according to Bate-Smith (1973). A volume of 1mL of each extract or tannic acid (standard) was mixed with 1mL of hemolyzed bovine blood (absorbance = 1.6). The mixture was incubated for 20 min, then centrifuged at 4000rpm for 10min. the absorbance of the supernatant was measured at 756nm and tannin content was expressed as mg tannic acid equivalent per g of extract (TAE/g extract) using the calibration curve of tannic acid (figure 10).

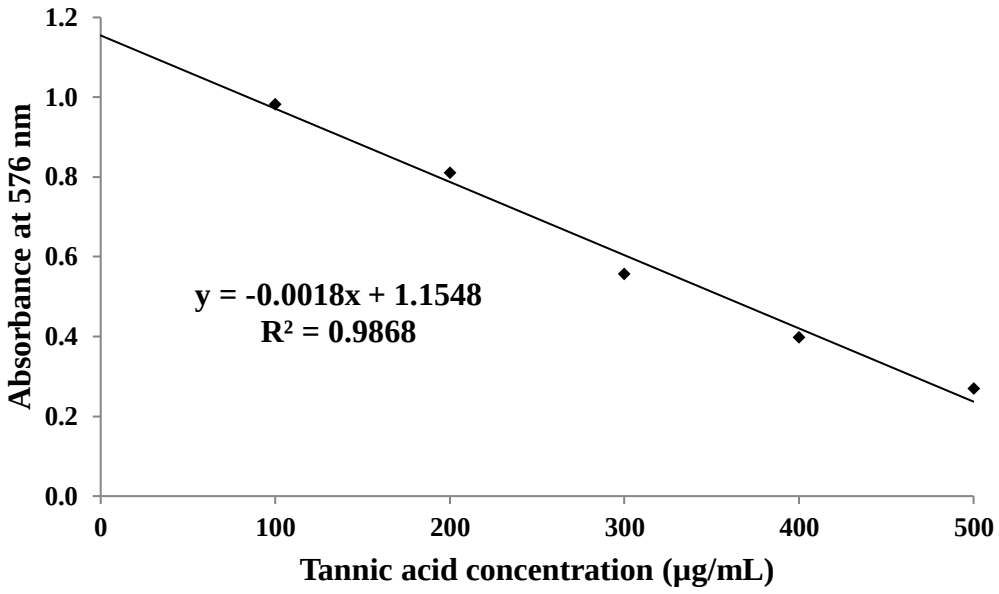

Figure 10. Standard curve of tannic acid for determination of total tannins. Each value represents mean ± SD (n = 3).

II.2.4. HPLC-ESI-MS analysis

The phenolic compounds of the aqueous and methanolic extracts of *E. Plantagineum* and *B. rapa* were identified using HPLC-ESI-MS according to the method described by Sulaiman *et al*. (2014). The used analytic system consisted of an Agilent 1200 Series HPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with a mass detector API-3200 and a ZORBAX SB-C18 (4.6 × 150 mm, 3.5 µm) column. Injection volume was 20 µL. Gradient elution was applied by using a binary solvent system with solvent A (0.1% formic acid in water, v/v) and solvent B (acetonitrile) at a flow rate of 1mL/min. The mass spectra data were acquired using an ESI capillary voltage of 4500 V in the negative ion mode which conditions were as follows: the cone-voltage was 70 V, the collision energy was 35 eV, and the nitrogen drying gas

was set at a flow rate of 4L/min with the drying gas and vaporizer temperature maintained at 650 °C. Analyst (Version 6.0, AB Sciex, USA) was used to analyse the mass spectra.

II.3. Acute oral toxicity

The acute toxicity study was conducted to determine the adverse effects of aqueous and methanolic extracts of *E. plantagineum* and *B. rapa* on the liver and the biochemical parameters. It was conducted according to the guideline of the Organization for Economic Co-operation and Development (OECD, 2001). Mice were divided into 9 groups of five animals each. The treated groups were orally given the Aq or Met extracts of either *E. plantagineum* or *B. rapa* in a single dose of 2000 mg/kg and 5000 mg/kg body weight, while the negative control group received only water vehicle. The animals were observed intensively during the first 4 hours after administering the extracts and then once a day for 14 days in order to detect any behavioral changes or mortality.

II.4. Antioxidant activity

II.4.1. ABTS radical scavenging assay

The ability of *E. plantagineum* and *B. rapa* extracts to scavenge the ABTS radical was evaluated according to Re *et al.* (1998). A volume of 2.5mL of ABTS (7 mM) was mixed to 2.5mL of potassium persulfate (2.45 mM) and the mixture was allowed to stand in the dark at room temperature for 12–16 h to produce ABTS radical cations ($ABTS^{\bullet+}$). The $ABTS^{\bullet+}$ solution was diluted with ethanol until obtaining an absorbance of 0.70 (±0.02) at 734 nm. The scavenging activity was estimated by mixing 50µL of diluted extracts or Trolox (standard) with 1mLof $ABTS^{\bullet+}$ solution. The absorbance was read at 734nm after 30min of incubation at room temperature. The antioxidant activity was calculated as followed:

$$ABTS^{\bullet+}\text{scavenging activity (\%)} = (A_0 - A_1 / A_0) \times 100$$

IC_{50} values, concentrations that inhibit 50% of ABTS radicals, are determined.

II.4.2. Hydroxyl radical scavenging assay

The scavenging ability of hydroxyl radicals was evaluated as described by Smirnoff and Cumbes (1989). A volume of 1mL of ferrous sulfate (1.5mM) was added to 700µL of hydrogen peroxide (6mM) and 300µL of sodium salicylate (20mM). Then,

1mL of different concentrations of Aq or Met extracts of *E. plantagineum* and *B. rapa* or ascorbic acid (standard), was added. The mixture (3mL) was incubated at 37°C for 1h before measuring absorbance at 562nm.The antioxidant activity was calculated as following:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A_S - A_B/A_C)] \times 100$$

A_C: absorbance without sample.**A_B:** absorbance of the blank (without sodium salicylate).
A_S: absorbance of the sample.

II.4.3. Superoxide anion scavenging assay

The superoxide anion scavenging activity of Aq and Met extracts of the two plants was measured as described by Robak and Gryglewski (1988). The superoxide anions generating system contains 0,5mL of Tris–HCl buffer (16mM, pH 8.0), 1 mL of NBT (0,3mM) solution, 0.5mL of NADH (0.936mM) solution and 1 mL of samples (extracts and vitamin C) at different concentrations. A volume of 0.5mL of phenazinemethosulfate (PMS) solution (0.12mM) was added to start the reaction. After 5min of incubation at 25°C, the absorbance was measured at 560nm.The antioxidant activity was calculated as follows:

$$\text{Superoxide anion radical scavenging activity (\%)} = (A_C - A_S / A_C) \times 100$$

A_C: absorbance ofthe control .**A_S:** absorbance of the sample.

II.4.4. Hydrogen peroxide scavenging assay

This method was carried out according to Mukhopadhyay *et al*. (2016). A volume of 750 µL of either plants extracts or vitamin c were added to 125 µL of ferrous ammonium sulfate (1mM) and 35 µL of H_2O_2 (5mM). The mixture was incubated at room temperature for 5min. Then, a volume of 750 µL of 1,10phenanthroline (1mM) was added and the absorbance was read at 510nm. The ability of compounds having hydrogen peroxide scavenging activity was calculated using the following formula:

$$\%\ H_2O_2 \text{ scavenging activity} = (A_{test}/ A_{blank}) \times 100\%$$

Where A_{blank} is the absorbance of solution containing only ferrous ammonium sulphate and 1,10phenanthroline and A_{test} is the absorbance of the solution containing ferrous ammonium sulphate, hydrogen peroxide along with test compound and 1,10phenanthroline.

II.4.5. Ferrous ions chelating assay

The chelating ability of ferrous ions by methanolic and aqueous extracts of *E. plantagineum* and *B. rapa* was estimated by the method described by Le *et al*. (2007). The reaction mixture contained 250 µL of test materialor EDTA (standard chelator) at different concentrations, 50 µL of $FeCl_2$ (0.6 mM) and 450 µL of methanol. After well shaking and incubation for 5 min at room temperature, 50 µL of ferrozine (5 mM) were added. The mixture shaken again, followed by a second incubation for 10 min at room temperature before reading absorbance at 562nm.

The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Ferrous ions chelating activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

A_0= absorbance of the control

A_1= absorbance of the test compound.

II.4.6. Reducing power assay

The reducing power of *E. plantagineum* and *B. rapa* extracts was determined according to Topçu *et al*. (2007). A volume of 2.5 mL of sample solutions at different concentrations was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After 20 min of incubation at 50 C°, 2.5 mL of TCA (10%) were added and the mixture was centrifuged at 3000 rpm for 10min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Data were expressed as EC_{50} (Effective concentration that corresponds to a 0.5 absorbance at 700nm).

II.4.7. Antioxidant assay using β-carotene bleaching method

This method was realized as described by Bougatef et al. (2009).β-carotene (0.5 mg) was added to 1 mL of chloroform, 25 µL of linoleic acid and 200 mg of tween-40. The chloroform was evaporated under vacuum at 45°C then 100 ml of distilled water saturated with oxygen were added. The resulting mixture was vigorously stirred to obtain stable emulsion. A volume of 2,5 mL of this mixture was placed in test tubes containing 0,5mL of samples (200µg/mL), then incubated in water bath at 50 °C for 2 h. The absorbance was measured at 470nm at time intervals of 15min. BHT and BHA

were used as standards. The extracts capacity to prevent the oxidation of β-carotene was determined according to the following formula:

$$\text{Antioxidant activity \%} = [1- (At_0 – At_{120}) \text{ test} / (At_0 - At_{120}) \text{ control}] \times 100$$

At_0: absorbance at time t = 0.

At_{120}: absorbance at time t = 120 min.

II.4.8. AAPH-induced hemolysis in red blood cells assay

The peroxidation of erythrocytes membranes was provoked with AAPH according to the method described by Takebayashi *et al*. (2010). Heparinized rat blood was centrifuged at 6000 rpm for 10min and washed three times with PBS buffer (pH 7.4). Then, 100µL of the red blood cells were suspended in 4.9mL of PBS to obtain a 2% dilution. Moreover, 120µL of the 2% blood cells solution was pre-incubated for 15min at 37°C on microplates with 60µL of extracts or Trolox (used as standard antioxidant). Finally, 120µL of AAPH solution (300mM) were added and the reaction mixtures were then incubated at 37°C for 4 to 5h.The degree of hemolysis was determined spectrophotometically by measuring absorbance every 30min at 620nm. The inhibition percent was calculated as following:

$$\text{Hemolysis Inhibition (\%)} = (A_0 – A_X / A_0 – A_{final}) \times 100$$

A_0: absorbance at t_0. **A_X:** absorbance at a specific time. **A_{final}**: absorbance at the end of the experience.

Data are also represented by HT_{50}: necessary time for 50% of hemolysis. The presence of an antioxidant (Trolox or extracts) is supposed to increase thehalf time of hemolysis which influences a good resistance of erythrocytes.

II.5. Anti-inflammatory activity

II.5.1. Carrageenan-induced rat paw edema test

Carrageenan-induced rat paw edema test is an acute inflammation model that was used to evaluate the anti-inflammatory activity of aqueous and methanolic extracts of *E. plantagineum* and *B. rapa*, according to the method described by Winter *et al*. (1962). All extracts at two doses (200mg/kg and 400mg/kg) as well as standard anti-inflammatory, aspirin (at 100mg/kg) were administered orally to rats 1h prior the subcutaneously injection of 0.1 ml of carrageenan 1 % (w/v) into the subplantar

region of the right hind paw. The negative control group was injected by 0.1 ml of carrageenan, and received orally the vehicle. The volume of the injected paw was measured at 0, 1, 2, 3, 4, 5, and 6 h after carrageenan injection, using a Plethysmometer (UGO Basile, Germany). The volume of edema was expressed for each animal as percentage change in rat paw volume after carrageenan injection, compared to placebo group set to 100%.

% of swelling increase= [paw volume at T (V_t)-initial paw volume (V_0)/initial paw volume (V_0)] * 100 (Ndiaye *et al*., 2016).

Anti-inflammatory activity was measured as fallowing:

% of inhibition of edema = % of swelling increase (control)-% of swelling increase (test)/% of swelling increase (control)*100.

II.5.2. Anti-denaturation effect on Bovine Serum Albumin evaluation

The *in vitro* anti-inflammatory activity of all studied extracts was evaluated by the method of proteins denaturation inhibition described by Sakat *et al*. (2009). A volume of 500 µl of 0,2% aqueous solution of bovine serum albumine (BSA) was added to 500 µl of either AqE or MetE of the two used plants, diclofenac was used as standard anti-inflammatory. All solutions were incubated at 37°C for 20 min, and then the heat was elevated to 65°C for 10 min before they finally got cooled. Absorbance was read at 660nm and the results are expressed as percentage of inhibition of proteins denaturation, calculated following the formula:

$$\text{Inhibition } \% = (Ac - As) / (Ac) \times 100$$

Ac= absorbance of the control.
As= absorbance of the sample (extract or Diclofenac)

II.5.3. Membrane stabilization of human erythrocytes

The ability of aqueous and methanolic extracts of *E. planatgineum* and *B. rapa* to protect human erythrocytes membrane from hemolytic effect of hypotonic solution was evaluated following the method described by Debnath *et al*. (2013). Briefly, a volume of 0,5ml of erythrocytes suspension diluted at 40% (v/v) in isotonic PBS solution (10mM, pH=7.4, 150mM NaCl), was added to 5 ml of hypotonic solutions (PBS 10mM, pH=7,4, 50mM NaCl) of extracts or diclofenac at different

concentrations. The negative control which represents 100% of hemolysis contains only erythrocytes and hypotonic solution. After 10 min of incubation at room temperature, a centrifugation was done for 10min, at 3000 rpm and 4°C. Then, absorbance of the supernatant was read at 540 nm, and the percentage of hemolysis inhibition was calculated as following:

$$\text{Inhibition } \% = 100 \times (A_c - A_t)/ A_c$$

A_c= absorbance of the negative control.

As= absorbance of the sample (extract or diclofenac)

II.6. Hepatoprotective Activity Determination

Hepatoprotective effects of *E. plantagineum* and *B. rapa* extracts were evaluated according to the method described by Kamisan *et al.* (2013). Briefly, rats were divided into 14 groups of five rats each. The first group of animals (negative control) was pre-treated orally with water (10 ml/kg) once daily for 7 consecutive days followed by intraperitoneal injection with olive oil (10 ml/kg). The animals of the positive group control received orally 100mg/kg of silymarin once daily for 7 consecutive days prior to CCl_4 intoxication. Six groups of animals received orally either aqueous or methanolic extracts of *E. plantagineum* at the doses 50, 100 and 200mg/kg, and six other groups were pretreated orally by either aqueous or methanolic extracts of *B. rapa* (100, 200 and 400 mg/kg), once daily for 7 consecutive days prior to CCl_4 intoxication. Three hours after the last treatment, 1mL/kg of CCl_4 (50% in olive oil) was administered intraperitoneally. At the eighth day, rats are sacrificed, and both of blood and livers are recovered for subsequent analysis mentioned, in details, in the following sections.

II.6.1. Determination of biochemical parameters

After 24h of the hepatotoxic agent administration, blood samples were collected in heparinized tubes and the plasma was separated by centrifugation at 2500 rpm for 10 min for determination of biochemical parameters (ALT, AST, ALP, cholesterol, triglycerides, urea and creatinine). Biochemical parameters were measured using the COBAS INTEGRA Automatic Chemical Analyzer. Liver was quickly removed, cleaned and washed in ice-cold saline solution. Each liver specimen was dissected into 2 pieces. One piece was fixed in10% formalin for histopathological examination,

whereas, the second one was stored in freezer for antioxidant markers evaluation (CAT, MDA and GSH).

II.6.2. Determination of antioxidant parameters

A 0.5g of each liver tissue was homogenized, in 5mL cold phosphate buffer (0.1 M, pH 7.4), and centrifuged at 4000rpm for 20 min at 4°C. Supernatants were collected and then used for the CAT, MDA and GSH measurements.

II.6.2.1. Catalase activity

Catalase activity was determined by the method described by Aebi (1984). The method consists of mixing 34 µl of liversupernatant with 966 µl of H_2O_2 (19.5mM) prepared in phosphate buffer (50 mM, pH 7).Changes in absorbance were recorded at 240nm using spectrophotometer UV/VIS (SPECORD210 PLUS) against a blank containing onlyliversupernatant and phosphate buffer. Catalase activity was calculated in terms of µmol H_2O_2 consumed/min/mg of protein.

II.6.2.2. Malondialdehyde content

Concentrations of MDA in the liver were determined according to Mihara and Uchiyama (1978). A volume of 125 µl of liver supernatant was added to 125 µl of trichloroacetic acid (20%) and 250 µl of thiobarbituric acid (0.67%).The reaction mixtures were incubated at 100°C for 15 min. The tubes were cooled, and 1 ml of n-butanol was added. After centrifugation at 3000 rpm for 15 min, the supernatants were collected and the absorbance was read at 530 nm. MDA content was calculated using MDA - TBA extinction coefficient ($\varepsilon = 1.56 \times 10^5$ $mol^{-1}.L.cm^{-1}$).

II.6.2.3. GSH content

The method described by Beutler *et al*. (1993) for GSH dosage is based on fractionation of DTNB molecule by GSH in an alkaline pH (8-9) into thionitrobenzoic acid (TNB) which absorbs at 412 nm. Briefly, 25 µL of supernatant were diluted in 5 mL of phosphate buffer (0.1 M, pH 8), then, 3 mL of this solution were mixed to 20 µl of DTNB (0.01 M). The mixture was incubated in ambient temperature for 5min. The absorbance was measured at 412 nm against a phosphate buffer blank. The concentration of GSH was measured using the coefficient of extinction $14150 M^{-1}cm^{-1}$ and the results were expressed as nmol/g of tissu.

II.6.2.4. Total proteins content

Total proteins of liver supernatants were measured according to the method of Biuret using the Kit (Spinreact BSIS30-1). The Biuret method is based on the fact that proteins react with copper salts to form a blue-purple complex in alkaline pH (Koller, 1984). A volume of 1 mL of the reactive solution was added to 25µL of liver supernatant. After incubation at 37 °C for 10 min, the absorbance was read at 540 nm. The color intensity is proportional to the quantity of proteins in the sample.

II.6.3. Histopathological assessment of hepatic tissue damage

Livers were excised immediately after the animals were sacrificed and cleaned in normal saline. Each fresh tissue sample was divided into pieces, and each piece was fixed in 10% natural formalin during 48h. After fixation and dehydration using a series of ethanol solutions, tissue specimens were embedded in paraffin, and from each block, 5 µm-thick sections were cut and stained with hematoxylin and eosin for the estimation of morphological changes, hepatocyte necrosis, steatosis and inflammatory cell infiltration. The slides were examined and photographed under a Leica DM1000 Microscope with Leica DFC495 Digital Camera and PC System with Leica LAS Software (V 3.8).

II.7. Statistical analysis

All data were expressed as means ± SD for *in vitro* tests, or SEM for *in vivo* tests. IC_{50} values were calculated by regression analysis. Statistical analysis was conducted using the GraphPad Prism 5 Demo software. One-way analysis of variance (ANOVA) test was used for multiple comparisons and signification determination. The difference was considered significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

I. Phytochemical analysis

I.1. Total phenolics, flavonoids and tannins contents

Phenolic compounds are the major contributors to the antioxidant capacity of plants (Li *et al*., 2007). They are, also, qualified of other biological activities that are related to the antioxidant ability, such as, anti-inflammatory (Gulcin *et al*., 2010) and hepatoprotective activities (Igarashi *et al*., 2008). Thus, total contents of phenolic compounds of *E. plantagineum* aerial part extracts and *B. rapa* roots extracts have been evaluated, and the results are presented in table 2.

Met E of *E. plantagineum* contains the highest amounts of polyphenols, flavonoids and tannins compared to Aq E, with tannins being the major compounds in both extracts. The obtained values are higher than those obtained by Bekhradnia and Ebrahimzadeh (2016) who have reported that *Echium amoenum* contains 41,69µgGAE/mg extract of polyphenols and 4,14 µg QE/mg extract of flavonoids. However, Aouadi *et al*. (2021) have reported higher values concerning the phenolic composition of another Boraginaceae belonging species, *Echium humile*. They found that methanolic and aqueous extracts of this species present values of polyphenols of 443,05 µg GAE/mg extract and 440,59 µg GAE/mg extract, and flavonoids contents of 14.48µg QE/mg and 14.96 µg QE/mg, respectively. The same study showed that methanolic and aqueous extracts of *E. humile* contain low amount of tannins (47.97 and 6.89µg CE/mg extract, respectively) compared with our values. The ethanolic extract of *E. vulgare* showed comparable amount of polyphenols (100, 21µg GAE/mg), higher quantity of flavonoids (35,16 µg QE/mg) and less amount of tannins (65,39µg TAE/mg extract) than methanolic extract of *E. plantagineum* extracts (Bošković *et al*., 2022).

In the other hand, aqueous extract of *B. rapa* is similar to methanolic extract in total phenolics and flavonoids, but richer in tannins composition. Similarly to *E. plantagineum*, values show that tannins represent the major constituents of both *B. rapa* extracts (Table 2). The obtained results with turnip don't concord with those found by Semwal *et al*. (2021), who reported values of 102.28 µg GAE/mg and 22.25 µg QE/mg extract, of polyphenolic and flavonoid contents respectively, of the ethanol extract of *B. rapa*.

In fact, several factors including geographical location, the growth stage of the plant and the difference between the used assays can explain the obtained differences (Tolić *et al.*, 2017). The extraction method also represents an important criterion responsible of the difference in quantitative and qualitative composition between plant extracts. It has been reported that methanol and water as well as their mixture at different ratios are very efficient and mostly used for high recuperation of phenolic compounds (Sahreen *et al.*, 2010; Xia *et al.*, 2010; Bouzid *et al.*, 2011), and obtaining better antioxidant activity (Barros *et al.*, 2010).

Water is more efficient in extracting molecules when it is combined to high temperature, leading to cells disturbance, and hence facilitating the penetration of the solvent and the solubilization of molecules (Albano and Miguel, 2011). However, the high temperature can deteriorate some thermolabile molecules, thus, decoction must occur in a short time (Seidel, 2005). The hydroalcoholic maceration in an ambient temperature for a long duration and with re-extraction of the plant debris via replacing the exhausted solvent by a new one, allows the obtaining of the maximum of molecules, without altering or modifying them.

Table 2. Total polyphenols, flavonoids and tannins contents of *E. plantagineum* and *B. rapa* extracts.

Plants	Extracts	Yields	Polyphenols (µgGAE/mg extract)[a]	Flavonoids (µg QE/mg extract) [a]	Tannins (µg TAE/mg extract)[a]
E. plantagineum (aerial part)	Aq E	40%	61,89	7,11	240,02
	Met E	21,94%	122	12,14	317,93
B. rapa (roots)	Aq E	58%	19,41	1,29	376,34
	Met E	55,48%	18,21	1,23	316,89

GAE: Galic Acid Equivalent; QE: Quercetine Equivalent. TAE: Tannic Acid Equivalent ([a]mean ± SD, *n*= 3).

I.2. HPLC analysis

The figures 11 and 12 represent the chemical profiles of polyphenolic composition of *E. plantagineum* and *B. rapa* extracts, respectively. The comparison with retention times of the used standards (Table 3) shows that Met E of *E. plantagineum* presents 10 phenolic compounds, whose major components are Quercetin 3-O- galactoside

followed by rutin and then rosmarinic acid. Aq E, however, contains 7 phenolic compounds with gallic acid being the major one, followed by catechol. Hydrobenzoic acid and ferulic acid are minor compounds in both extracts.

As far as the bibliographic research provided, phenolic compounds reported in *E. plantagineum* are limited to flavonoids identified in honey and bee pollen of this plant. A part the study carried on by Jin *et al*. (2020) on an Australian species, there are no studies regarding flavonoids or other phenolic compounds in extracts of *E. plantagineum*. In the limit of the standards used in the HPLC analysis of the present study, the obtained results don't concord with the findings of Jin *et al*. (2020). The kaempferol-3-O-rutinoside and the quercetin-3-O-sophoroside and other derivatives of quercetin and kaempferol are among the flavonoids found by these authors in *E. plantagineum*. Rosmarinic acid in addition to other phenolic acids like coumaric and caffeic acids were also identified.

However, the results of the present study agree with some ones found with other species of the Boraginacea family. Aouadi *et al*., (2021) have identified nine phenolic acids in *E. humile* including ferulic acid and rosmarinic acid, and ten flavonoids including quercetin-3-O-galactoside and rutin. They also reported the presence of two flavonols (catechin and epicatechin). Bošković *et al.* (2022) have identified chlorogenic, ferulic and rosmarinic acids, in addition to rutin and quercitin in the ethanolic extract of *E. vulgare.* These results are in concordance with those obtained in this study. In fact, literature survey revealed the existence of phenolic acids, flavonoids and tannins in Echium genera. *E. arenarium* extracts have been shown to contain four flavonoids, namely, luteolin-7-O-glucoside, myricitrin, myricetin and quercetin (Kefi*et al*., 2018). Furthermore, Radwan *et al*. (2007) have reported the presence of four flavonoids, including luteolin-7-O-rutinoside, apigenin, apigenin- 7-O-rhamnoside and quercetin-3-O-rhamnoside in *E. sericeum*. Rosmarinic acid is widely distributed in Echium species such as *E. amoenum, E. russicum* and *E. vulgare* (Jin *et al*., 2020).

On the other hand, Met E of *B. rapa* presents 6 phenolic compounds, with gallic acid being the major compound followed by ferulic acid. Aq E contains gallic acid as major compound followed by epigallocatechin. Hydroxybenzoic acid and epicatechin are minor compounds in Met E and Aq E, respectively (table 4).

The studies reported 35 flavonoids in this plant that occur in glycosides (Paul *et al.*, 2019). Among them, kaempferol, isorhamnetin and their glycosylated derivatives are the most important (Cao *et al.*, 2021). In fact, the use of alcohol, water or a mixture of the two in the extraction of flavonoids lets obtain glycosides as well as polar aglycones, such as hydroxylated ones (Dias *et al.*, 2021). Liquiritin, liquiritigenin, 4,4'-dihydroxy-3'-methoxychalcone, isoliquiritin and licochalcone A are flavonoids identified in turnip roots (Jeong *et al.*, 2013). Furtheremore, among the phenolic acids reported in the litterature on turnip roots, ferulic acid has been identified in this study. This identification is confirmed by Fernandes *et al*. (2007).

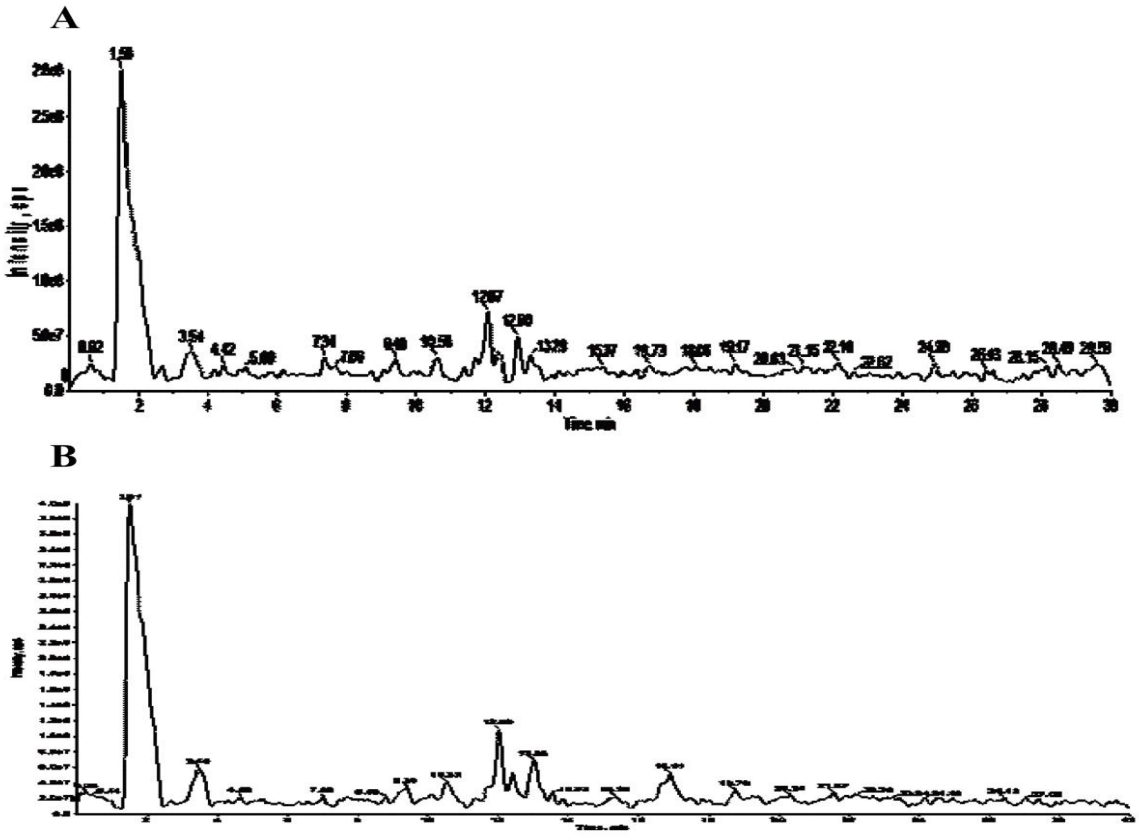

Figure 11. HPLC chromatograms for aqueous extract (A) and methanolic extract (B) of *E. plantagineum.*

Table 3. Phenolic compounds determined by HPLC-ESI-MS in aqueous and methanolic extracts of *E. plantagineum*.

Phenolic compound	Retention time (min)	Aqueous extract (mg/Kg)	Methanolic extract (mg/Kg)
Gallic acid	1.61	27.5	30.2
Catechol	5.70	17.5	17.0
Procyanidin B2	6.23	-	-
Epigallocatechin	6.67	-	8.47
Hydroxybenzoic acid	6.71	4.10	4.35
P-Coumaric acid	6.92	-	-
Ferulic acid	7.25	4.37	4.47
Chlorogenic acid	7.45	5.08	69.5
Transcinamaldehyde acid	7.87	-	-
Rutin	8.55	12.9	191
Quercetin 3-O- galactoside	8.71	-	196.5
Isoquercitrin	8.73	-	18.2
Quercetin	8.74	-	-
Caffeicacid	9.35	-	-
Rosmarinic acid	9.37	6.65	137.5

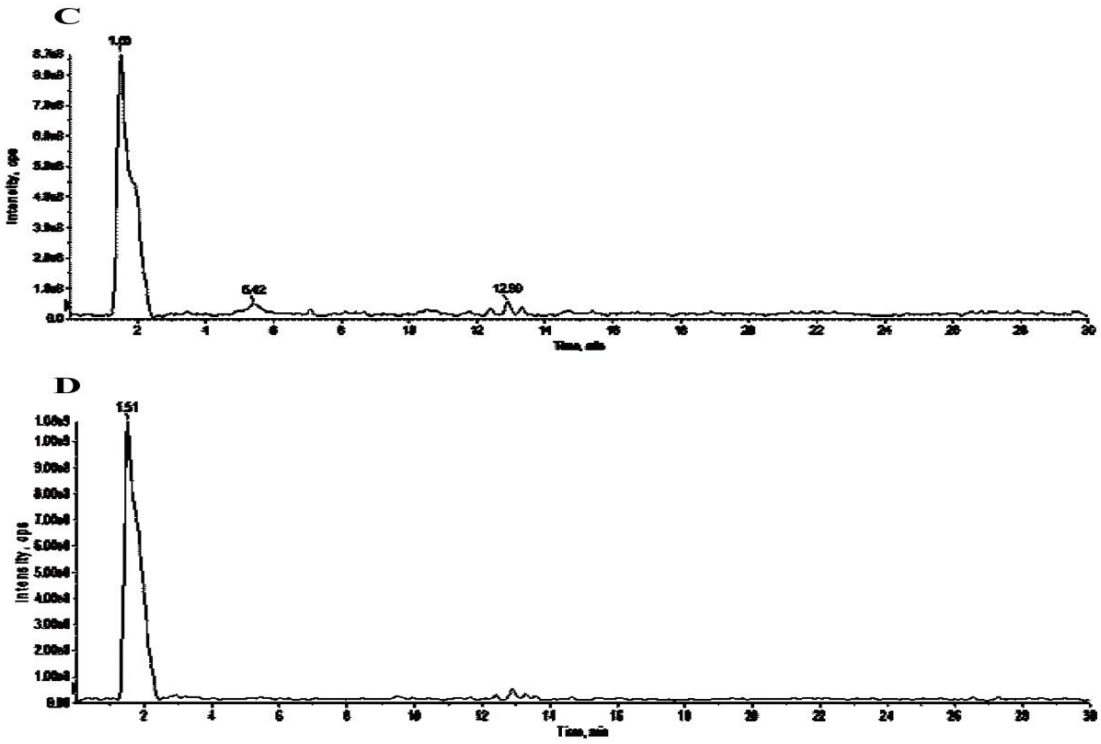

Figure 11. HPLC chromatograms for aqueous extract (C) and methanolic extract (D) of *B. rapa.*

Table 4. Phenolic compounds determined by HPLC-ESI-MS in aqueous and methanolic extracts of *B. rapa*.

Phenolic compound	Retention time (min)	Aqueous extract (mg/mL)	Methanolic extract (mg/Kg)
Gallic acid	1.61	27.2	27.2
Catechol	5.70	-	15.4
Chlorogenic acid	6.07	5.08	5.08
Caffeic acid	6.28	-	-
Syrengic acid	6.30	-	-
Epicatechin	6.51	0.346	-
Epigallocatechin	6.67	8.47	-
Hydroxybenzoic acid	6.68	4.10	3.88
Rutin	6.79	-	4.49
P-Coumaric acid	7.03	-	-
Quercetin 3-O- galactoside	7. 16	-	-
Ferulic acid	7.41	4.58	24.8
Transcinamaldehydeacid	7.87	-	-
Quercetin	7.89	-	-

II. Extracts acute toxicity

Following the oral administration of 2g/Kg and 5g/Kg of Aq E and Met E of the selected plants, the female mice are observed over four hours and then regularly for the next 14 days. Mice have shown neither mortality nor toxicity symptoms during the period of observation. Therefore, the tested plants extracts seem to be safe until the dose of 5g/Kg.

The bibliographic research has not shown any study concerning the acute toxicity of *E. plantagineum* on rats. However, in a study carried out by Peterson and Jago (1984) on the toxic effect of pyrrolizidine alkaloids from *E. plantagineum*, it has been reported that the young rats fed on pellets providing a 20-40% level of *Echium* for up to 28 days presented 70% of mortality from acute hemorrhagic necrosis of the liver between days 22 and 35. Rats that received *Echium* continuously or intermittently over extended periods died with a mixture of acute necrosis and chronic liver damage typical of pyrrolizidine alkaloid poisoning. Young rats fed on 40% *Echium* for only the first 14 days, and having consumed the equivalent of 0.7 g total alkaloid per kg live weight, developed extensive chronic liver damage, leading to deaths from the

35th day after the start of feeding. This study demonstrates that this plant has a sub-acute toxicity related to high consummation of the plant material over an extended period.

These findings don't confirm those of Culvenor *et al.* (1984), who found that the prolonged feeding of dried pelleted *Echium* to sheep caused only a very low level of pyrrolizidine alkaloid poisoning. The activity of liver microsomes in the production of pyrrolic metabolites from *Echium* alkaloids was 5-7 times greater in rats than in sheep.

III. Antioxidant activity of *E. plantagineum* and *B. rapa* extracts

Oxidative stress plays a major role in the development of chronic diseases such as cancer, arthritis, aging, cardiovascular and neurodegenerative diseases (Akinwunmi *et al*., 2016). Epidemiological studies indicate that plant-based diets rich in fruits and vegetables are inversely related to these illnesses by contribution in reducing the risk of their development (Bahorun *et al*., 2004). This has been related to their high content of phenolic compounds such as phenolic acids, flavonoids and tannins, qualified of antioxidant properties. The antioxidant activity of phenolic compounds is mainly attributed to their redox properties making them reducing agents, hydrogen donors, quenchers of singlet oxygen and chelators of metal ions (Liyana-Pathirana and Shahidi, 2006). Consequently to these acts, radicals are converted to more stable products resulting in termination of the initiated chain reactions (Akinwunmi *et al*., 2016). Thus, the antioxidant capacity of the selected plants extracts was explored in this study by several tests.

III.1. ABTS radical scavenging activity

The potential of the selected plants extracts to scavenge free radicals was assessed by their ability to quench ABTS radicals. The ABTS assay is based on the production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. The presence of an antioxidant eliminates ABTS radicals by reducing them, resulting in discoloration of the radical solution (Re *et al*., 1999). ABTS assay is an excellent tool for determining the hydrogen-donating ability of chain-breaking antioxidants. Its advantage is that measuring antioxidant capacity of plant extracts at wavelength maximum absorption of 734 nm eliminates color interference (Awika *et al*., 2003).

Both *E. plantagineum* extracts showed a concentration dependent scavenging activity of the ABTS radicals (Figure 13). However, Aq E was more powerful (IC_{50} = 167.03µg/mL) than Met E (IC_{50} = 261.45µg/mL). These results are not concurring with the amounts of polyphenolic compounds present in each extract. This is probably due to the fact that the active principals responsible for this activity are more available in Aq E than in Met E. In fact, a study carried on by Pilerood and Prakash (2014) has shown that the hot water extract (100°C) of *E. amoenum* presented the highest antioxidant activity in DPPH and reducing power tests than hydroalcoholic extracts. The same species was found, in another study (Asghari *et al*., 2018), to afford ABTS radical scavenging activity by its aqueous and methanolic extracts represented by IC_{50} values of 17.1 µg/mL and 51.6 µg/mL, respectively. Our results reflect a less powerful activity than the last ones, but are more powerful than those found by Aoudi *et al*. (2021) with Aq E and Met E of *E. humile*. It can be concluded that the difference of antioxidant activity of plants belonging to the same family reflects the variety in the bioactive molecules composition.

In the same way, the scavenging activities of Aq E and Met E of *B. rapa* were increased in a concentration-dependant manner (Figure 13). Met E (IC_{50} = 1,867mg/mL) was significantly more effective than Aq E (IC_{50} =3,042mg/mL), but the two were less effective than BHT (IC_{50} = 0,033 mg/mL) and Trolox (IC_{50} = 0,04 mg/mL). This activity is much stronger than that found by Beltagy (2014), who reported IC_{50} values of 5,39mg/mL and 19,27mg/mL, in DPPH test, for Aq E and Met E of turnip roots, respectively. However, the same author has found that these two extracts exhibited a better activity than the other solvents fractions (light petroleum, ethyl acetate and chloroform).

III.2. Hydroxyl radical scavenging activity

The hydroxyl radical is the most dangerous free radical formed in biological systems due to its ability to damage almost every kind of molecule found in living cells (Saravanakumar *et al*., 2015). $OH^{\cdot}$ scavenging activity of studied extracts was evaluated by their ability to compete with salicylic acid for $OH^{\cdot}$ radicals in the $OH^{\cdot}$ generating/detecting system (Sudha *et al*., 2011). *E. plantagineum* extracts showed concentration dependent scavenging activity towards hydroxyl radicals as represented in figure 14. Aq E is more effective (IC_{50} = 103.44µg/mL) than both methanolic

extract (IC_{50} = 311.96 µg/mL) and vitamin C (IC_{50} = 261.86µg/mL). Bošković *et al*. (2022) have reported an IC_{50} value of 68,58 µg/mL of the ethanolic extract of *E. vulgare* in the $OH^{\cdot}$ assay. In fact, this species showed higher quantity of flavonoids than *E. plantagineum*, which may explain the higher $OH^{\cdot}$ scavenging ability. However, the results found in the present study are comparable to those found with Aq and Met extracts of *E. ameonum* (Asghari *et al*., 2018).

Both aqueous and methanolic extracts of *B. rapa* showed statistically similar activities which were less pronounced than that of vitamin C. At the same concentration (2,5 mg/mL), the Aq E reached 64,57% of activity and the Met E achieved 70,94%. Hence, *B. rapa* extracts can be considered as good scavengers of hydroxyl radicals.
The methanolic extract of *Brassica oleracea* (broccoli), belonging to Brassicaceae, showed a maximum of scavenging activity of 51,44% towards OH radical, reflecting a weaker activity compared to that found in this study. This can be explained by the fact that the same extract showed a less content of polyphenols (6,45µgGAE/mg of extract), as reported by Borowski *et al*. (2008).

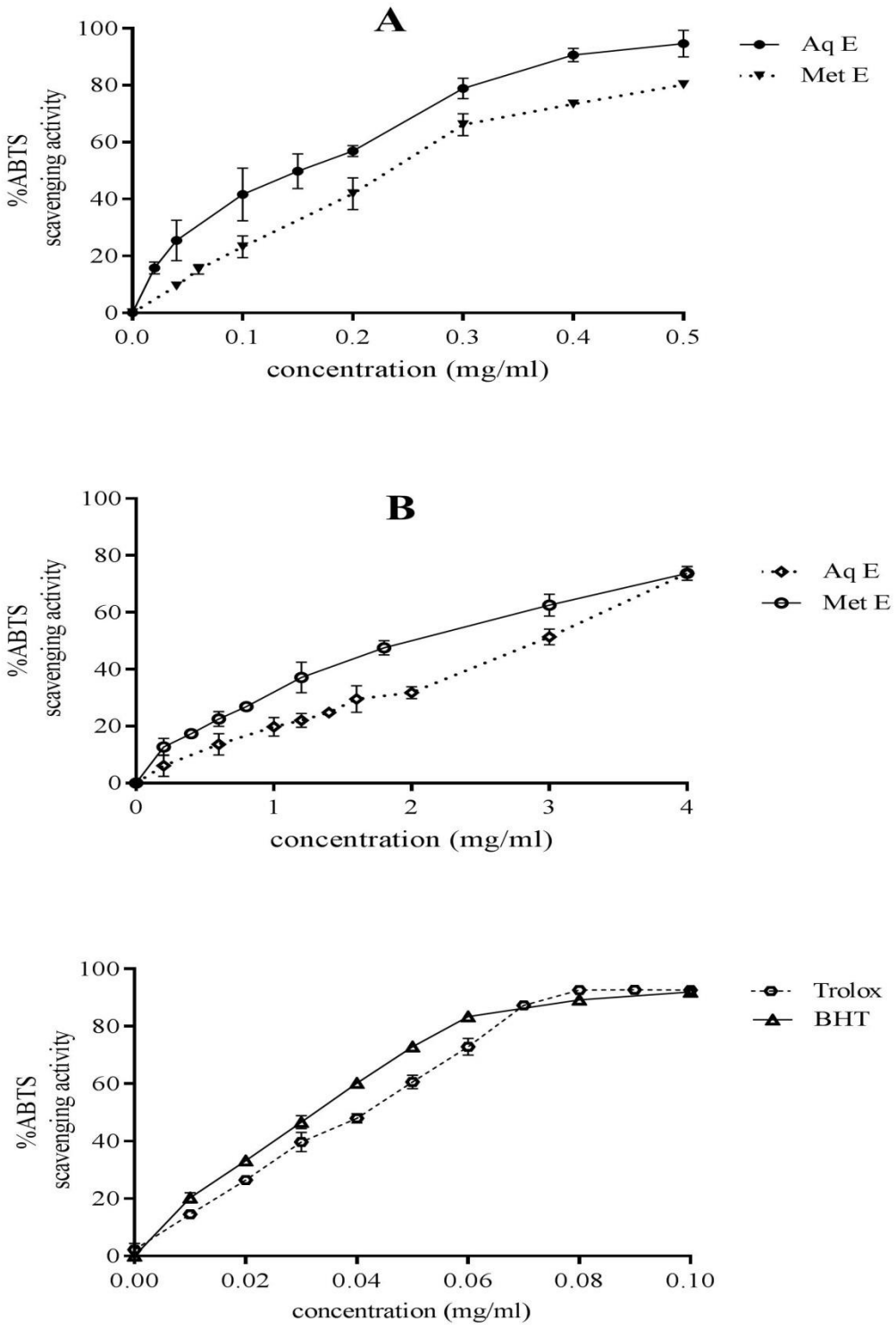

Figure 13. ABTS scavenging activity of *E.plantagineum* (A) and *Brassica rapa* (B) extracts, as well as standards (Trolox and BHT). The values are means±SD (n=3).

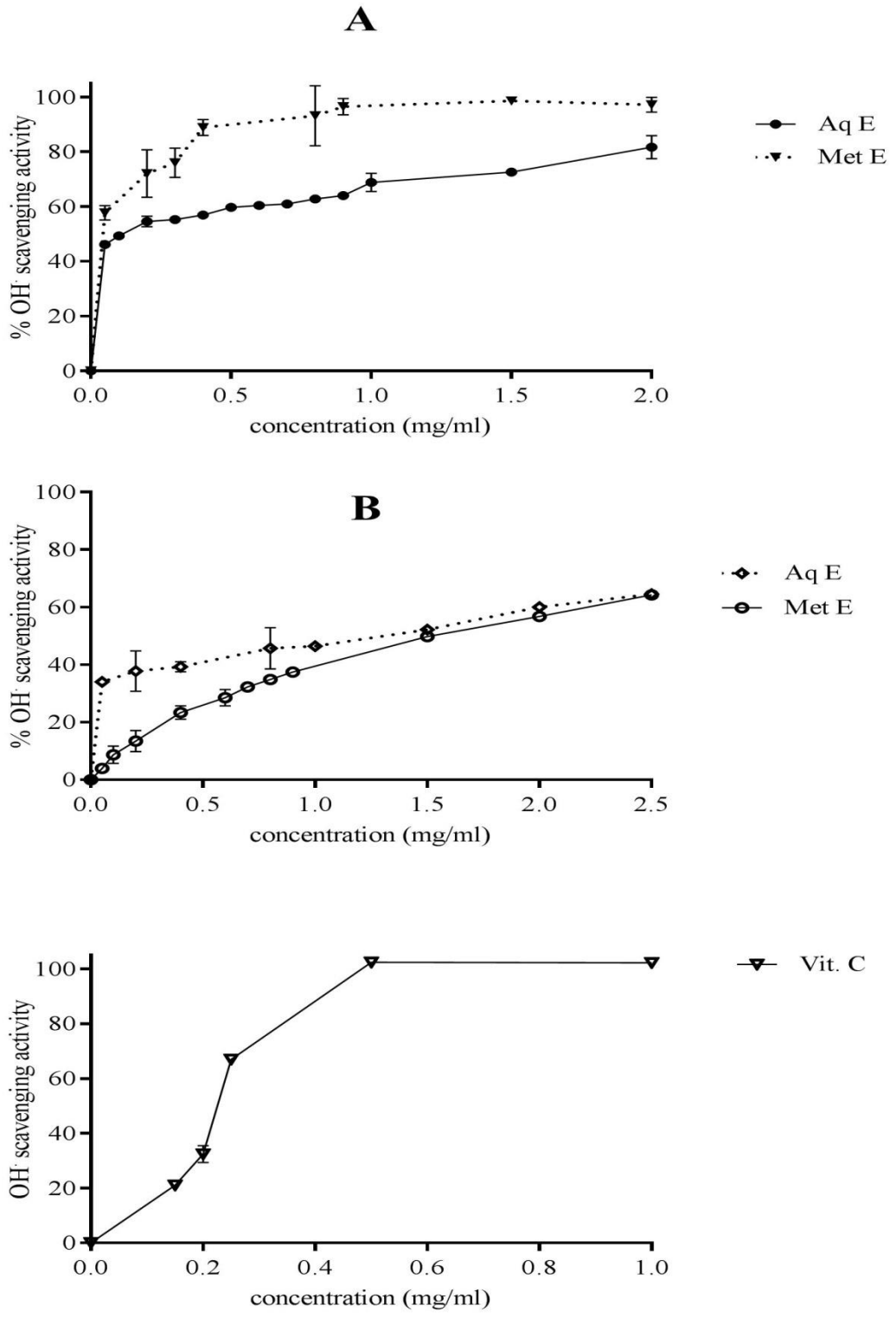

Figure 14. OH· scavenging activities of *E. plantagineum* (A), *Brassica rapa* (B) extracts and vitamin C (Vit. C).Values are means±SD (n=3).

III.3. Superoxide anion scavenging activity

Although the superoxide anion is a weak oxidant by itself, its scavenging is important because it is one of singlet oxygen and hydroxyl radical's precursors (Saravanakumar *et al.*, 2015). Aq and Met extracts of *E. plantagineum* acted against $O_2^{\cdot}$ in concentration dependent manner (Figure 3). The values of IC_{50} show that Aq E was more efficient (IC_{50}=563.78 µg/mL) than Met E (IC_{50}=830.825 µg/mL), but both of them were less powerful than ascorbic acid (IC_{50}=24.34µg/mL).

The scavenging activity of *B. rapa* Met E has increased as the concentration increased until it reached 49,94% of superoxide anion scavenging activity at a concentration of 20 mg/mL (figure 15). At this range of concentration, Aq E didn't show any activity. In fact, Bahorun and his collaborators (2004) have reported in their study on vegetables that the low antioxidant activities of tomato and carrot may be attributed to the extraction process resulting in more water-soluble molecules. Besides, medicinal plants demonstrated much stronger antioxidant activity and contained significantly more phenolics than common vegetables and fruits according to Cai *et al*. (2004). Yet, *Brassica oleracea* has efficiently scavenged the superoxide anion in a concentration-dependent manner with IC_{50} values of 0.93 mg/mL and 0.25 mg/mL corresponding to aqueous and ethanolic extracts, respectively (Bidchol *et al*., 2009).

In contrary to the other tests established in the present study, *B. rapa* extracts showed a very weak or no activity in the superoxide scavenging test. Thus, it is always useful to assess the antioxidant activity of a product by several different tests.

III.4. Hydrogen peroxide scavenging activity

Hydrogen peroxide is a non-radical speies capable of crossing the cell membrane and oxidizing its compounds. It can inactivate enzymes by the oxidation of thiol groups (Saravanakumar *et al*., 2015). It's also the precursor of highly toxic hydroxyl radicals by reaction with ferrous ions (Bozin *et al*., 2008). The principle of the used test is based on the formation of a red-orange ferrous ions (Fe^{+2})-1,10-phenanthroline complex. The addition of H_2O_2 causes oxidation of all ferrous ions to ferric ions (Fe^{3+}) unable of forming the red-orange complex. However, the addition of hydrogen peroxide scavenger to ferrous ions before adding H_2O_2 itself forbids the ferrous to ferric conversion, and the addition of phenanthroline yields the chromophore

complex. Therefore, higher absorbance indicates higher hydrogen peroxide scavenging activity.

Figure 16 shows that the H_2O_2 scavenging activity of *E. plantagineum* Met E increases with increasing concentration until it reaches maximum of activity (69%) at 500 µg/mL, and then stabilizes. In the same way, the Aq E activity increases with increasing concentration until it reaches maximum of activity (55,43%) at 400 µg/mL, and then decreases to 45% and stabilizes. IC_{50} values shows that Met E (IC_{50}=193.87µg/mL) acted more effectively than Aq E (IC_{50} =262.46µg/mL). Standard antioxidant (ascorbic acid) was better than both of them (IC_{50}= 51.93µg/mL).

Quercetin-3-0-galactoside, the major component of Met E of *E. planatgineum*, has been reported to have, *in vitro*, protective effects towards cells against cytotoxicity caused by H_2O_2. It could attenuate PC12 cells apoptosis caused by oxidative stress, by permeating the cell membrane and then inhibiting the formation of free radicals and their propagation chain reactions by chelating transition metal-ions in the cell (Liu *et al*., 2005).

Aq E of *B. rapa* showed concentration dependent activity in scavenging H_2O_2 (Figure 9). It reached 62,15% of inhibition at 5mg/mL and presented an IC_{50} value of 3,73mg/mL, while methanolic extract afforded only 46,10% of inhibition at the same concentration, and the curve stabilized before it reached 50% of activity. This activity is much weaker than standard antioxidant. Jaiswal *et al*. (2012) have reported better H_2O_2 scavenging activities of the methanolic extracts of two varieties of *Brassica oleracea* (Brassicaceae) with IC_{50} values of 1.3 mg/mL (Broccoli) and 2.8 mg/mL (white cabbage).

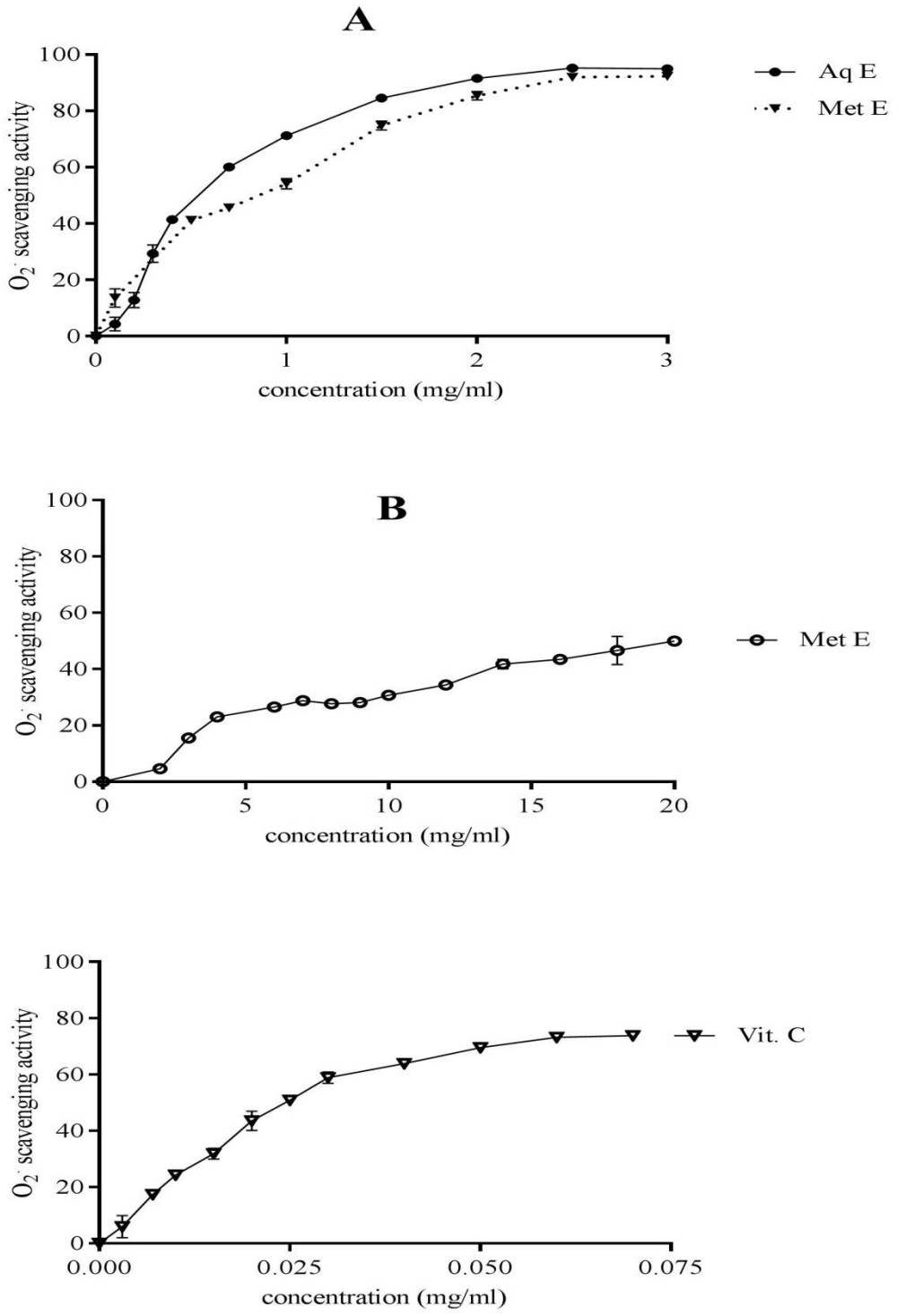

Figure 15. $O_2^{\cdot}$ scavenging activity of *E. plantagineum extracts* (A), *B. rapa* methanolic extract (B) and ascorbic acid. The values are means±SD (n=3).

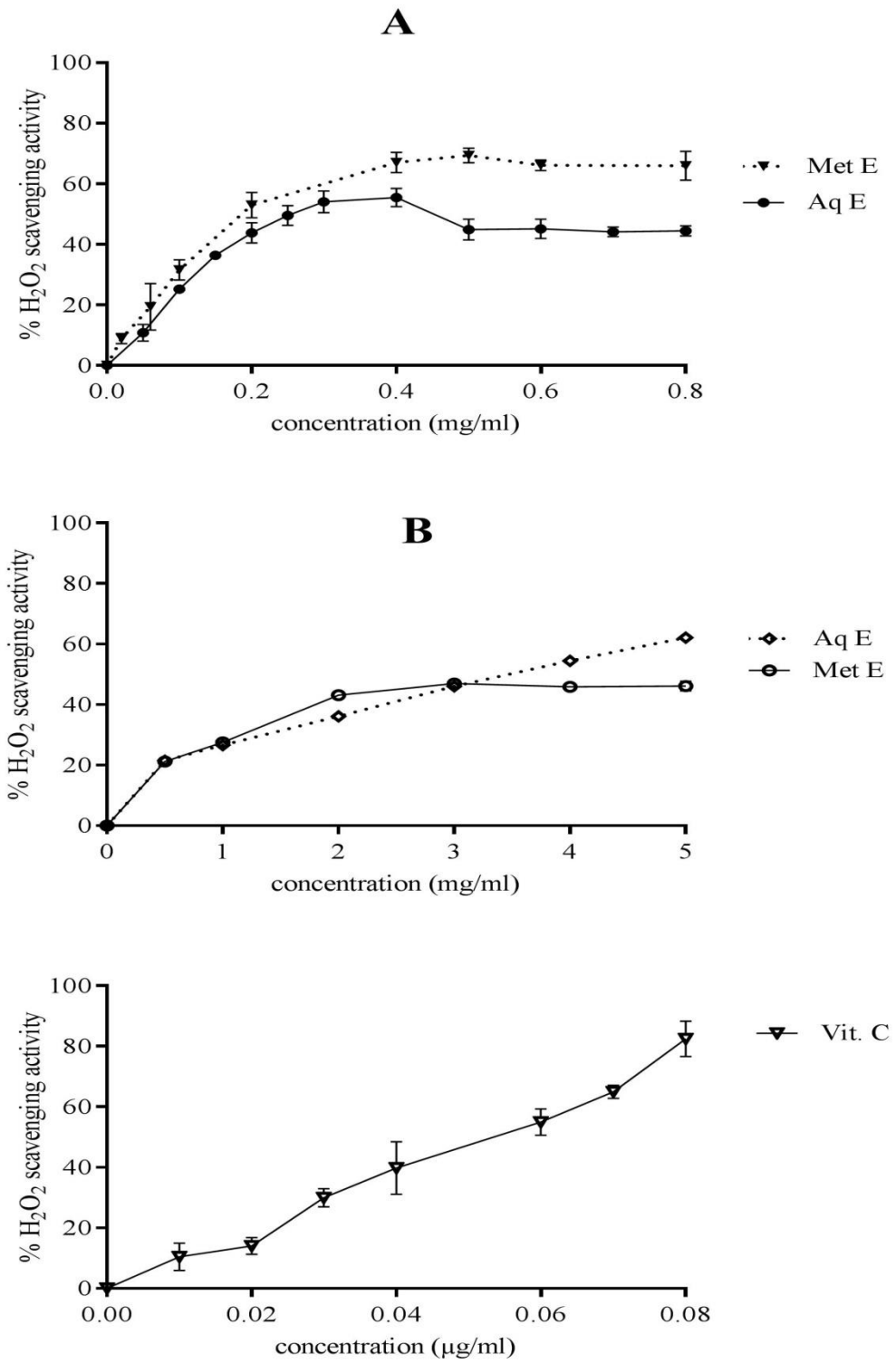

Figure 16. H_2O_2 scavenging activity of *E. plantagineum* extracts (A), *B. rapa* extracts (B) and vitamin C. The values are means±SD (n=3).

III.5. Ferrous ions chelating activity

Bivalent ferrous ions can be very harmful by the catalysis of superoxide and hydroxyl radicals formation *via* Fenton reaction (Saravanakumar *et al*., 2015). Fe^{2+} ions are also responsible for lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl (Bidchol *et al*., 2009). Ferrous ions chelating test is based on the capacity of ferrozine to form red complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted and hence the red color of the complex is decreased (Le *et al*., 2007). The importance of the chelation property lies in the interference with the aggravation of some metal ions associated pathologies such as neurodegenerative ones (Dusek *et al*., 2016).

Both of *E. plantagineum* extracts inhibited the formation of the red Fe^{2+}-ferrosine complex as efficiently as their concentrations increased (figure 17). However, Aq E showed more activity than Met E with IC_{50} values of 164.91µg/mL and 247.57µg/mL, respectively. These values are lower than that obtained with EDTA. These results are much more powerful than those found by Eruygur *et al*. (2012) with four species belonging to the family of Boraginaceae. In fact, they reported chelation percentages of 46.4%, 48%, 49.4% and 65.3% provided by the aerial parts ethanol extracts of *Echium vulgare*, *E. parviflorum*, *E. italicum* and *E. angustifolium*, at a concentration of 400µg/mL. At this concentration, Aqueous and Methanolic extracts of *E. plantagineum* afforded 80% and 74,7% of chelation activity, respectively. The efficiency of the aqueous extract in comparison with methanolic one indicates that the active molecules responsible for the catch of ferrous ions have water soluble nature. This has been confirmed by certain studies reporting that hydrophilic molecules practice the chelating activity through their high nucleophile character, and that the plant extracts induce chelating effects which increase along with increasing solvents polarity (Sahreen *et al*., 2010; Ozen *et al*., 2011).

On the other hand, Met E and Aq E of *B. rapa* exerted chelating activity which increased with concentration (figure 17). The IC_{50} measurements indicate that Met E (IC_{50}=0,223mg/mL) was much stronger in chelating ferrous ions than Aq E (IC_{50}=1,232mg/mL). However, this chelating activity was weak compared to EDTA. These results are more efficient than those reported by Jaiswal *et al*. (2012) on methanolic extracts of *Brassica oleracea* vegetables namely Broccoli (IC_{50}=4.66

mg/mL) and white cabbage (IC_{50}=5.85 mg/mL). Bidchol *et al*. (2009) have also reported that both of aqueous and ethanolic extracts of another variety of *B. oleracea* exhibited ferrous ions chelating activity with IC_{50} value of 0.35 mg/ml. This activity is comparable to that of Met E, but more pronounced than that of the aqueous extract of *B. rapa*.

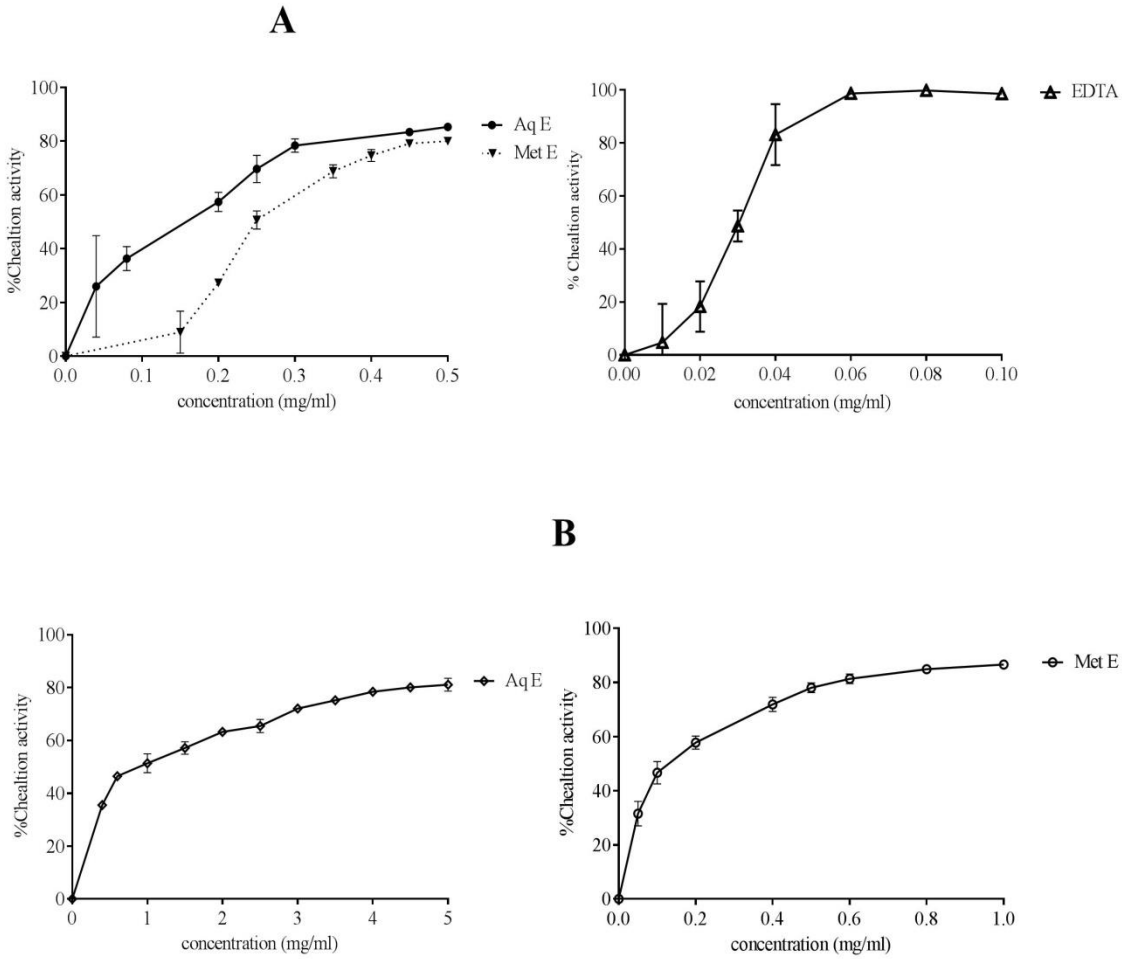

Figure 17. Chelation activity of *E. plantagineum*(A), *B. rapa* (B) extracts and EDTA.The values are means±SD (n=3).

III.6. Reducing power

The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method (Chaouche *et al*., 2013). In this test, the presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form (Bourgou *et al*., 2008). The reductive activity of Aq E and Met E of both *E. plantagineum* and *B. rapa* compared to BHT has been illustrated in figure 18.

Met E and Aq E of *E. plantagineum* exerted similar and remarkable reducing activity with IC_{50}values of 117µg/mL and 128µg/mL, respectively. This reductive activity remains less important than that of BHT (IC_{50}= of 61.29µg/mL). The results of the reducing power test as well as those of iron chelating test indicate that both *E. plantagineum* extracts can deal with ferrous ions either by binding (chelating) them or reducing them.

Concerning *B. rapa*, Met E was significantly more effective than Aq E (EC_{50} = 224µg/mL and 481,667µg/mL, respectively), but both of them showed a strong reductive concentration-dependent capacity (Figure 18), even though lower than that of BHT. These results suggest that *B. rapa* extracts contain electron donors reacting with free radicals to convert them into more stable products and to terminate radical chain reactions. Bidchol *et al*. (2009) have shown that the reducing power of *Brassica oleracea* correlated well with increasing extract concentrations. However, reducing power of aqueous extract was higher than that of the ethanolic extract. In another study carried out by Jaiswal *et al*. (2012) on methanolic extracts of three varieties of *B. oleracea*, the EC_{50} values were much higher than those found in this study (1.6-4.4 mg/mL), reflecting weaker reducing abilities. The reducing properties of antioxidants are generally associated with the presence of reductones, which have been shown to exert antioxidant action by donating a hydrogen atom and breaking the free radical chain. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Bidchol *et al*., 2009).

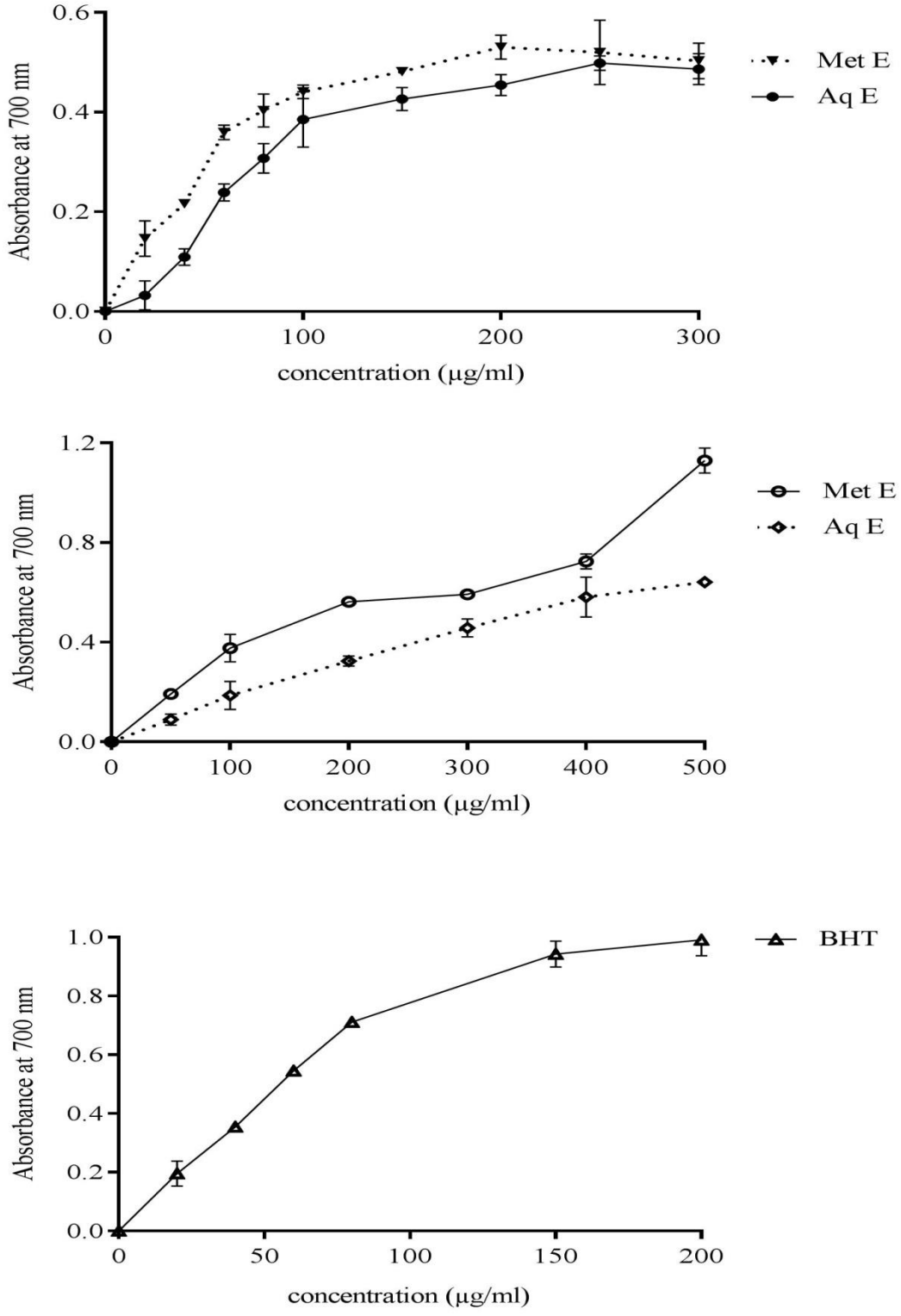

Figure 18. Reducing power of *E. plantagineum* (A), *Brassica rapa* (B) extracts and BHT. The values are means±SD (n=3).

III.7. β-carotene bleaching assay

In this test, oxidation of linoleic acid produces hydroperoxide derived free radicals that attack β-carotene double bonds. This attack results in a bleaching of the linoleic acid/β-carotene emulsion, which is supposed to be retarded in the presence of an antioxidant (Chouche *et al*., 2013). The absorbance at 470nm during 2 hours reflects the ability of plant extracts to delay oxidation of β-carotene (Figure 19A). Both Aq E and Met E of *E. plantagineum* afforded very strong protective activity against β-carotene bleaching. At 2 mg/mL, Aq E and Met E exerted 93% and 81% of lipid peroxidation inhibition, respectively, which is so comparable to standard antioxidant BHT at the same concentration (figure 19B).

At 2mg/mL, Aq E and Met E of *B. rapa* inhibited lipid peroxidation with 58,55% and 51% respectively (figure 19B), which remains less effective than BHT (100%). According to Liyana- Pathirana and Shahidi (2006), an extract capable of retarding or inhibiting the oxidation of β-carotene may be described as a free radical scavenger and primary antioxidant. More powerful activities of inhibiting β-carotene bleaching were found with three species of Brassica namely *Brassica oleracea* (red cabbage) which afforded 97% of inhibition, *B. juncea* (mustard cabbage) with a percentage of 85% and *B. rapa* (Chinese white cabbage) with 79% of bleaching inhibitor effect (Yee *et al*., 2007).

Polyphenols and flavonoids are able to retard the lipid auto-oxidation through their radical scavenging property (Ayala *et al*., 2014). Therefore, they act at an early stage by scavenging superoxide and hydroxyl radicals or by terminating the propagation of the radical reaction chain giving hydrogen (Sandhar *et al*., 2011). Besides, the lipophilic structure of polyphenols allows them to integrate in the lipidic phase of the cell membrane, and hence facilitating their protective action against lipid peroxidation (Djeridane *et al*., 2006; Amessis-Ouchemoukh *et al*., 2014).

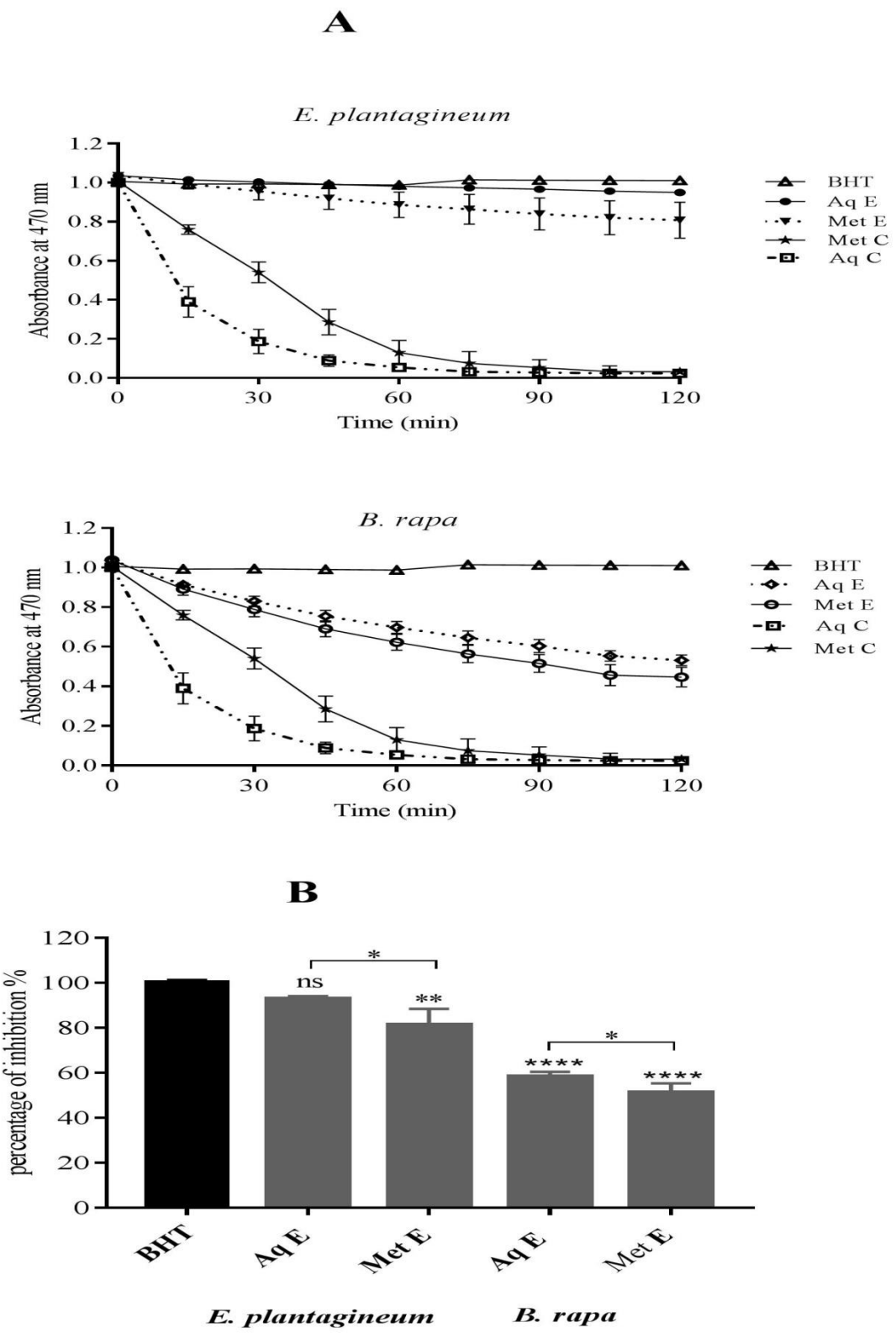

Figure 19. (A) Kinetics of B-carotene bleaching in the presence and the absence of *E. plantagineum* and *B. rapa* extracts and BHT. The values are means±SD (n=3). **(B)** Percentage of inhibition of B-carotene bleaching of the different samples.

III.8. AAPH-induced hemolysis in RBC assay

The ability of the extracts to protect biological membranes against radical attacks was elucidated in the present study using the AAPH test. The decomposition of AAPH in physiological environment to alkyl radicals ($R^{\cdot}$) can form peroxyl radicals ($ROO^{\cdot}$) in presence of oxygen. These lasts will attack erythrocytes lipids and proteins to induce the oxidation chain, disturbing membranes organization and eventually leading to hemolysis (Cheung, 2003; Banerjee *et al.*, 2008). Kinetics of the red blood cells hemolysis in the presence of extracts (figure 20A) show that there is an important antihemolytic activity at the tested concentrations, similar to that of Trolox. Aq E of *E. plantagineum* doesn't show antihemolytic activity at 60µg/mL, but afforded very important effect at 150 and 300 µg/mL with HT_{50} of 161.62 min and 145.73 min, respectively (figure 20B). Met E showed very powerful and similar antihemolytic effects at all the tested concentrations. However, Aq E and Met E of *B. rapa* protected red blood cells from radical attack more efficiently at 60 µg/mL and 300 µg/mL than at 150µg/mL (figure 20B).

Hemolysis of human red blood cells is a very good model for studying free radical induced oxidative damage to membranes and to evaluate the antioxidant activity of new compounds (Kunwar *et al.*, 2007). Free radicals attack erythrocyte membrane components, such as proteins and lipids, and cause changes in the structure and function of membranes, resulting in hemolysis. The raison behind the use of AAPH as a free radical initiator is that, unlike other oxidants, it does not easily penetrate into the cell (Zou *et al.*, 2001). In fact, Zou *et al.* (2001) have demonstrated that in this model, the hemolysis begins by depletion in GSH that provides the first line of defense during oxidative insult. Subsequently, lipid peroxidation begins the process of oxidative damage to erythrocytes mediated by peroxyl radicals. Finally, the degradation of membrane proteins occurs, and the hemolysis ensues. These results support the idea that hemolysis is the consequence both of lipid peroxidation and the degradation of membrane proteins. Hence, it is presumed that a good protective effect of the cell membrane can be provided by a mixture of hydrophilic and hydrophobic antioxidants, as showed a study carried on by Miki *et al.* (1987) on a mixture of ascorbic acid and α-tocopherol. The α-tocopherol protects phospholipids from peroxidation, when ascorbic acid may react directly with lipid peroxyl radicals at the lipid-water interface, and inhibit the oxidation of surface thiols of the extrinsic

proteins exposed to the aqueous phase. These data allow presuming that the anti-hemolytic activity of the methanolic extracts of *E. plantagineum* and *B. rapa* observed in this study can be interpreted by the presence of both hydrophilic and hydrophobic components acting synergistically to protect the membrane of erythrocytes from the radical attack.

Additionally, *B. rapa* extracts showed mean values of bioactive compounds much lower than *E. plantagineum* extracts, and yet, they showed similar anti-haemolytic activity. Oancea *et al*. (2018), in their study on the anti-hemolytic effect of 70% hydroethanolic extracts of different samples of *Brassica oleracea*, have recorded no correlation between the content of polyphenols and the antihemolytic activity. They found that the most significant inhibition of AAPH-induced oxidative hemolysis of erythrocytes was afforded by the extract presenting the lowest bioactive content. They have referred this to the contribution of otheri mportant water-soluble bioactive compounds existing in the red cabbage hydrophilic extracts, such as ascorbic acid, glucosinolates and minerals which may act synergistically and could explain the mechanism of interaction with erythrocytes membranes.

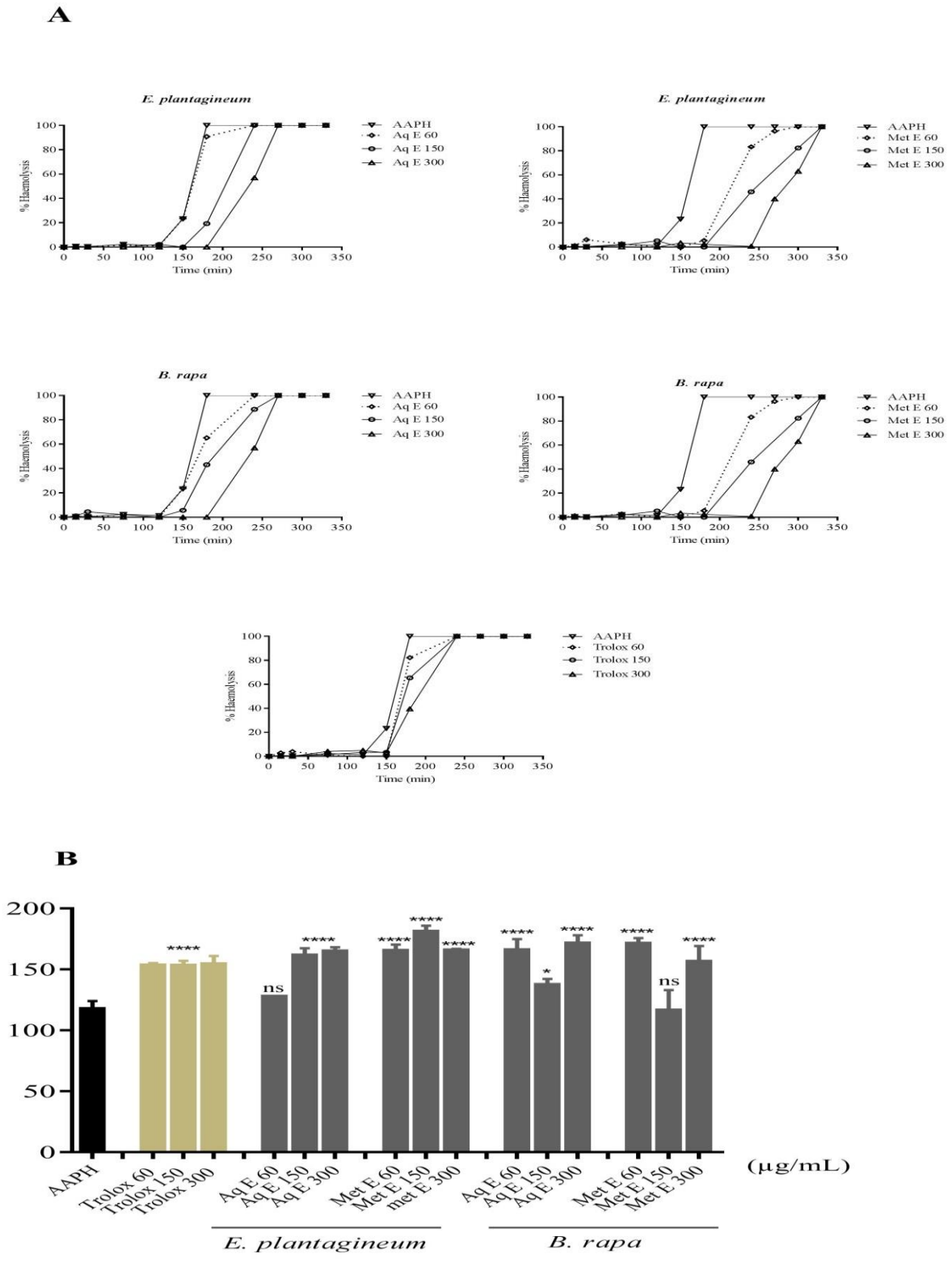

Figure 20. Inhibitor effects of E. plantagineum extracts and Brassica rapa extracts on AAPH induced RBC haemolysis. (A) Kinetics of the red blood cells hemolysis caused by AAPH and followed by measurement of absorption at 630nm. (B) Half-Hemolysis Time (HT50) of different samples.

To conclude, the antioxidant activity of the selected plants extracts, evaluated in the present study by several assays, can be referred to their polyphenolic compounds that have been identified by the HPLC analysis. This last showed that all the extracts share the presence of phenolic acids, such as ferulic and gallic acids, and flavonoids as rutin, compounds that have important antioxidant capacities.

Ferulic acid is a powerful antioxidant known as "multifunctional antioxidant" since it acts through multiple mechanisms. Thus, it is the most representative compound of its subclass, presenting a wide range of biological activities (Damasceno *et al*., 2017). It has an unusual effectiveness in inhibiting the generation of ROS and RNS in oxidative processes, and prevents peroxidation caused by UV (Damasceno *et al*., 2013; Damasceno *et al*., 2017).

Moreover, Merkl *et al.* (2010) reported that phenolic acids including p-hydroxybenzoic acid, ferulic acid and gallic acid possess good antioxidant effects as reveratol and trolox, with the gallic acid being the most powerful. This last provides efficient protection for biological systems against oxidative damage caused by reactive species including the radical hydroxyl ($HO^{\cdot}$), superoxide ($O_2^{\cdot}$) and peroxyl ($ROO^{\cdot}$) and the non-radicals, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). It's also a strong chelating agent (Badhani *et al*., 2015).

Chlorogenic acid, also present in all studied extracts, has been reported for its antioxidant activity manifested in several mechanisms, namely, the direct free radicals scavenging, implicating its polyhydroxyl structure; the activation of the anti-oxidant signaling pathway and the regulation of the expression level of related genes resulting in enhancement of the anti-oxidant capacity; direct regulation of the activity of endogenous oxidase system and associated proteins (Wang *et al*., 2022).

Furethermore, rutin, among the major compounds of Aq E and Met E of *E. plantagineum*, has been reported to have powerful antioxidant capacity. This activity has been demonstrated by different antioxidant assays. It neutralizes or sequesters free radicals such as $OH^{\cdot}$ and $O_2^{\cdot}$, prevents lipid peroxidation (Yang *et al*., 2008) and chelate transition metals (Pulido *et al*., 2000).

IV. Anti-inflammatory activities

IV.1. Carrageenan induced paw edema

The inflammatory response induced by carrageenan in rats is characterized by a marked edema formation resulting from the release of several mediators (histamine, serotonin and bradykinin), which is sustained by the release of prostaglandins and nitric oxide produced by inducible isoforms of cyclooxigenase (COX- 2) and nitric oxide synthase (iNOS), respectively (Passos *et al*., 2007). In addition, myeloperoxidase (MPO) is another important feature of this inflammatory model. It is an enzyme found primarily in azurophilic granules of neutrophils commonly used as a marker for tissue neutrophil content, and its inhibition is an indicator of anti-inflammatory activity (Passos *et al*., 2007).This evidence allows us to suggest that the anti-inflammatory activity of an extract is related to the inhibition of one or more of the intracellular signaling pathways involved in the generation of these mediators.

The subplantar injection of carrageenan has provoked maximum of edema of 0.96 mL after 4 hours of inflammation induction, which corresponds to 71.93% of edema rate (Figure 21A). Aq and Met extracts of selected plants have exerted very powerful anti-edematous effects similar to that of aspirin. In fact, pretreatment of rats with 200 and 400mg/Kg of Aq E of *E. plantagineum* reduced significantly edema with 51.57% and 61.37%, while Met E decreased edema with 45.27% and 54.17%, respectively (figure 21B). The pretreatment of rats with the same doses of Aq E of *B. rapa* reduced edema with 67.08% and 64.73%. These effects are statistically similar. The inhibition effects exerted by Met E of *B. rapa* are 90.66% and 88.76% for 200 and 400 mg/Kg, respectively. Aspirin, used as standard anti-inflammatory has shown 65.86% of edema inhibition at 100mg/Kg.

In a study carried on by Moita *et al*. (2013), using a cellular model of lipopolysaccharide (LPS)-stimulated macrophages, the hydromethanolic extract of *E. plantagineum* bee pollen decreased the levels of arachidonic acid metabolites derived from COX-2, demonstrating that it has an anti-inflammatory activity. In another study explorating the anti-allergic activity (which involves anti-inflammatory mechanisms), the same extract was effective in inhibiting basophils degranulation under an allergic stimulus, as ascertained by the levels of β-hexosaminidase released, ameliorating the symptoms of allergy (Moita *et al*., 2014). In addition, Gupta and Kaur (2014) have

reported that the methanolic extract of *Cordia dichotoma* (Boraginaceae) inhibited significantly the carrageenan induced edema after 4 hours of its induction in rats. On the other hand, Shin *et al*. (2011) have reported that the pretreatment of rats with 100 mg/kg of the n-hexane ethyl acetate extract of *B. rapa* roots reduced the carrageenan induced paw edema by 31.9% after 5 hours of carrageenan treatment.

Some compounds present in the studied extracts may be responsible for their anti-inflamatory activity. Indeed, chlorogenic acid has been reported to act directly on the NF-kB signaling pathway to control the expression of pro-inflammatory and anti-inflammatory factors (Wang *et al*., 2023). Also, phenolic acids exhibit strong inhibition effect against MPO, a key enzyme in infection and inflammation processes that converts hydrogen peroxide and chloride to HOCl. For instance, gallic acid and its derivatives demonstrated the anti-inflammatory activity by two mechanisms: strong inhibition of MPO activity, hence directly inhibiting the production of HOCl, and scavenging the harmful reactive species produced by this enzyme (Rosso *et al*., 2006).Various flavonoids (quercetin, apigenin and catechins) have also been shown to have anti-inflammatory activity by inhibiting COX-2 and iNOS (marchand *et al*., 2002). Additionally, they inhibit cytosolic tyrosine kinase as well as neutrophil degranulation (Middleton *et al*., 2000). Among the anti-inflammatory mechanisms of rutin, it has been found to alleviate ROS induced oxidative stress and inflammation in rats via targeting p38-MAPK, NFκB, COX-2, i-NOS, TNF-α and IL-6 (Nafees *et al*., 2015).

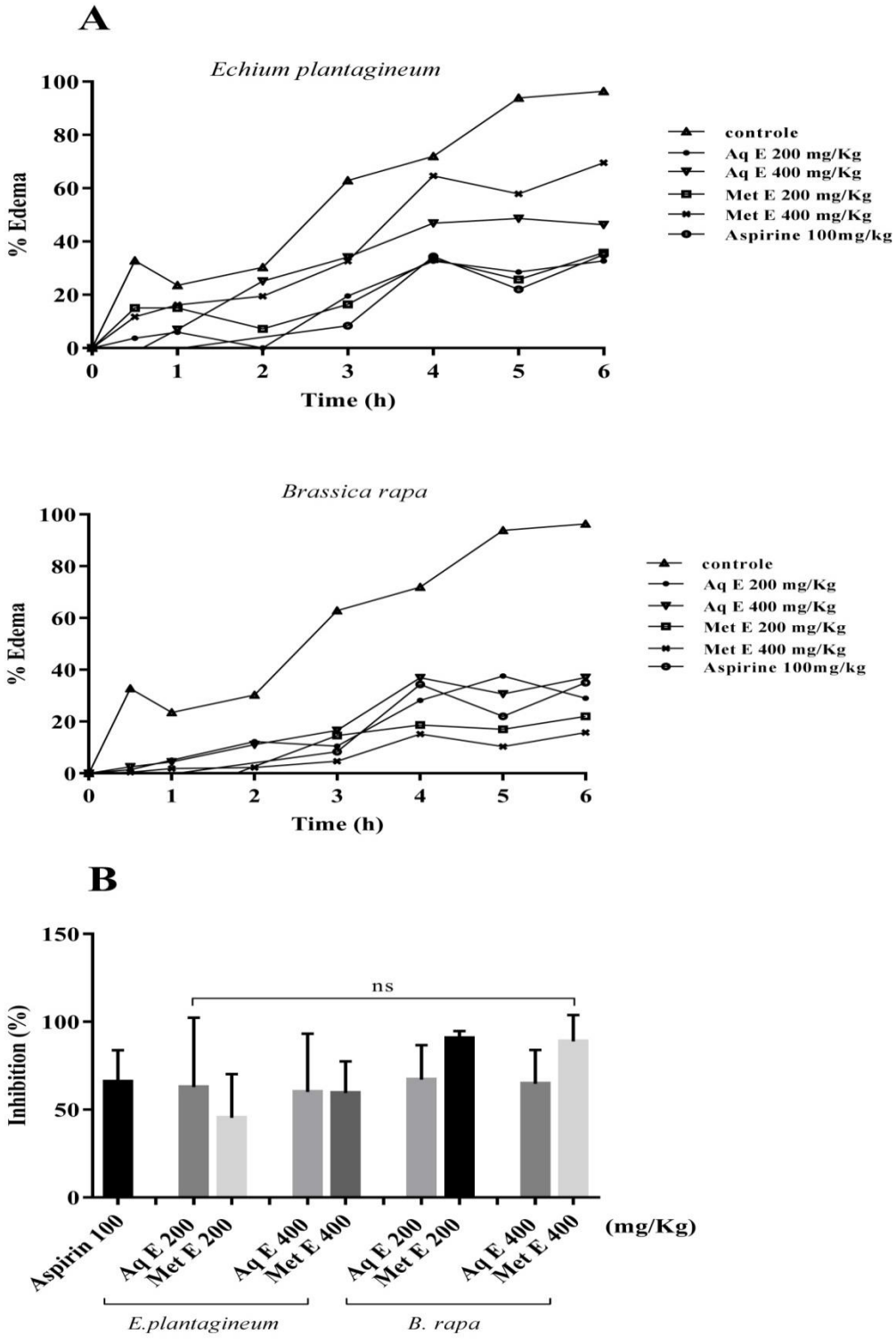

Figure 21. Effects of Aq E and Met E of *E. plantagineum*, *B. rapa* and aspirin on carrageenan induced paw edema in rats. **(A):** Kinetics of the edema development caused by carrageenan injection. **(B):** percentage of edema inhibition of different samples. Edema is induced by injection of 0.1 ml of carrageenan (1%) into the subplantar region of the right hind paw, after oral treatment with aspirin (100mg/Kg), Aq and Met extracts (200 and 400mg/Kg). Rats of control group are only injected with carrageenan. Values are mean±SEM (n=5).

IV. 2. Proteins denaturation

The denaturation of proteins is a well-documented cause of inflammation. It occurs when the protein loses its secondary or three-dimensional structure following the exposition to heat, infectious agents or chemicals (Adarsh *et al*., 2011). This denaturation exposes certain sites in the protein that become auto-antigens (Lanneau, 2010) implicated in certain autoimmune diseases like arthritis (Umapathy *et al*., 2010).

Our results show that all extracts inhibit the denaturation of BSA at 50 and 150 µg/ml, with percentages going from 22.53% to 70.10% (figure 22). The most powerful inhibitor effects were obtained with Aq E (70.10%) and Met E (57.80%) of *E. plantagineum* at 150 µg/ml. These activities are statistically similar to that of standard anti-inflammatory (Diclofenac) at a concentration of 25 µg/ml. Williams *et al*. (2008) have reported that substances that inhibit the protein denaturation with a percentage superior to 20% are qualified of anti-inflammatory properties. According to that, the results of the present test confirm that all the studied extracts are endowed with anti-inflammatory activity. The researches presume that this activity can be explained by the interaction of the bioactive molecules with plasmatic proteins at specific sites rich in three amino acids (tyrosine, threonine and lysine), and that this is the way the polyphenols act in order to protect the proteins against the thermic denaturation (Duganath *et al*., 2010).

Durairaj *et al*. (2018) found that the hydroethanolic extract of *Cordia Sebestena*, specie from Boraginaecea family, shows a maximum of inhibition activity of protein denaturation of 90.97 % at 500µg/ml. Another study conducted by Venkatachalam (2021) on another Boraginaceae belonging species, *Coldenia Procumbens,* has shown that the ethanol extract had a percentage of protein denaturation inhibition of 82% at a concentration of 250µg/ml, when the aqueous extract showed 73% of inhibition at the same concentration.

On the other hand, Semwal *et al*. (2021) have reported that ethanol extract of *B. rapa* exhibited the maximum of protection against the BSA denaturation (93.10%) at 800 µg/mL.

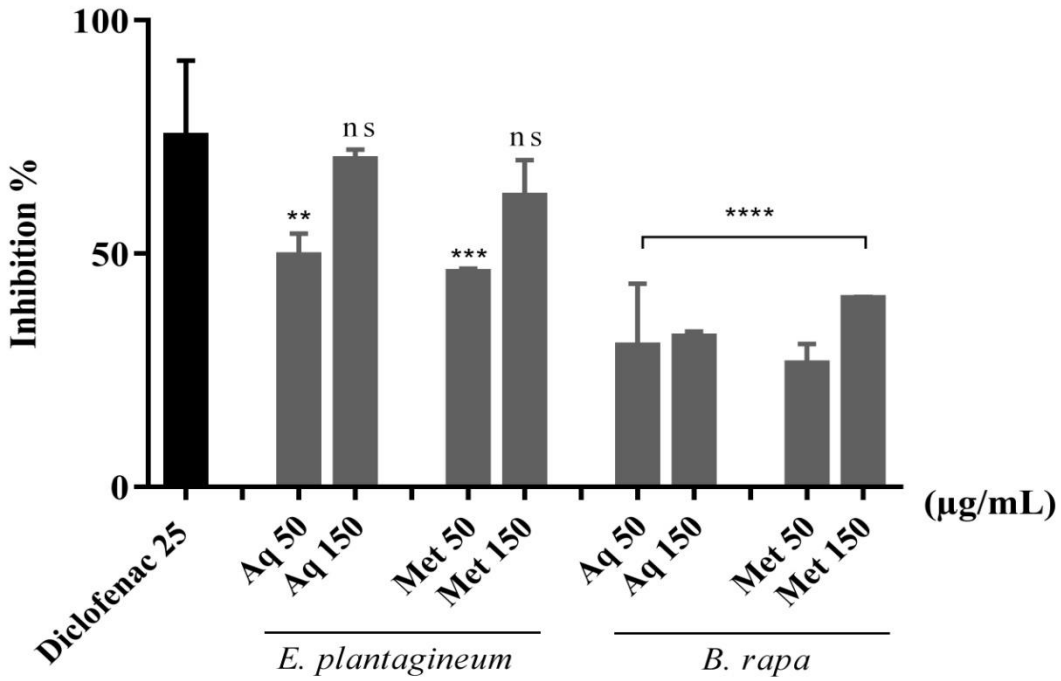

Figure 22. Effect of Aq and Met extracts of *E. plantagineum* and *B. rapa* on the denaturation of BSA. Values are mean ± SD (n=3). ***p < 0.001, **p <0.01,*p < 0.05 compared with Diclofenac.

IV.3. Effects of extracts on human erythrocytes stability

The study of erythrocytes is a good model for evaluation of the protective effect of compounds toward cells by both their antioxidant and anti-inflammatory abilities. First, the exposure of red blood cells to hypotonic medium leads to membrane lysis accompanied with lipid peroxidation and hence, a secondary damage through lipid peroxides resulting in hemoglobin oxidation (Hallowe and Whiteman, 2004; Mohamed Saleem *et al.*, 2011). Secondly, the membranes of erythrocytes resemble lysosomal membranes, and their stabilization reflects the ability to stabilize lysosomal membranes (Sumathi and Anuradha, 2016). Considering that the lysosomal enzymes released during inflammation cause damages to tissues and assist a variety of disorders, and then the protective effect of an extract toward lysosomal membrane reflects its anti-inflammatory property. In fact, this is the way the non-steroidal drugs exert their anti-inflammatory effect, as indicated by Hasan *et al*. (2018). Additionally, compounds with membrane-stabilizing properties can prevent the release of phospholipases that initiate the formation of inflammatory mediators (Jahan *et al.*, 2014).

According to the results of the present study, methanolic extracts of the selected plants showed more activity than their aqueous extracts at the same concentrations. Met E of *E. plantagineum* protected human erythrocytes from hemolysis with 79.05% against 22.7% of inhibitor effect realized by Aq E at the concentration of 1000 µg/ml (figure 23). In the same way, Met E of *B. rapa* inhibited hemolysis with 87% at 2000µg/ml, however, Aq E exhibited 33.23% of inhibition at the same concentration. These effects were powerful but remain less important than the effect obtained with Diclofenac (99%). Durairaj *et al*. (2018) have reported that the hydroethanolic extract of *Cordia sebestena* (Boraginaceae) showed a maximum of hemolysis inhibition activity of 81.16 % at 500µg/ml. While, Venkatachalam (2021) showed that the ethanolic and aqueous extracts of another Boraginaceae (*Coldenia procumbens*), afforded a protective effect towards erythrocytes against hypotonic lysis with a percentage of 98% and 95% at 1000µg/ml, respectively.

On the other hand, Jahan *et al*. (2014) have reported that the methanolic extract of *B. rapa* has afforded 78% of membrane stabilizing activity at the same concentration tested in the present study, reflecting a less powerful effect. Besides, the methanolic extract was more efficient than aqueous one, confirming the results found in the present study. Ruiz-Ruiz *et al*. (2017) reported that polyphenols and flavonoids have an important *in vivo* and *in vitro* stabilizing effect on the lysosomal membrane.

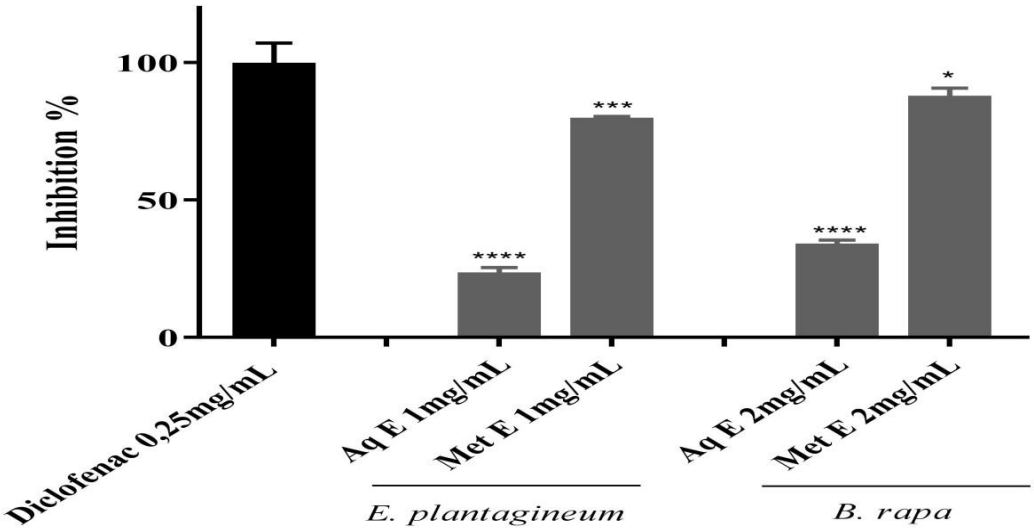

Figure 23. Effect of Aq and Met extracts of *E. plantagineum* and *B. rapa* on the hypotonic induced lysis of human erythrocytes. Values are mean ± SD (n=3). ****p < 0.0001,***p < 0.001, **p <0.01,*p < 0.05 compared with Diclofenac.

V. Hepatoprotective activity

In order to evaluate the hepatoprotective properties of *E. plantagineum* and *B. rapa*, rats receive orally the plant extracts, daily for 7 days. Then, hepatotoxicity is induced with intraperitoneal injection of CCl_4. This model is one of the best-characterized systems of xenobiotic induced hepatotoxicity in experimental animals (Ben Hsouna *et al*., 2018).

CCl_4 is metabolized into free radicals that attack biomolecules (proteins, membrane lipids and DNA), leading to acute and chronic hepatic toxicity, resulting in all forms of liver disorders namely steatosis, necrosis, fibrosis and cirrhosis (Weber *et al.*, 2003). Excess of these radicals, in particular CCl_3•, cause peroxidation of membrane lipids leading to the leakage of transaminases (ALT and AST), alkaline phosphatase (ALP), bilirubin and LDH into the blood (Kumawat *et al.*, 2012; Hurkadale *et al.*, 2012). This is associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. The generated radicals also reduce the level of antioxidant enzymes resulting in an oxidative stress, and contributing to acute and chronic lesions in different tissues (Singh *et al.*, 2015).

V.1. Effects of extracts on biochemical parameters

Intraperitoneal injection of CCl_4 increases significantly the rate of seric transaminases (AST and ALT) and alkaline phosphatase (ALP) compared with animals of the negative control group, because of the lesion of the hepatic tissue. During hepatic injury, hepatocytes function is disturbed due to the increase of their membranes fragility, leading to the release of intracellular enzymes in the blood (Lin *et al.*, 2008). Thus, the level of ALT and AST is a good indicator of hepatotxicity. ALT is sensitive specifically towards acute hepatic lesions, and hence represents the best parameter for hepatic injury detection (Fiedman and Keefe, 2004; Nkosi *et al.*, 2005). The increase in the seric level of ALP, normally released in the bile, can results in a damage (Farida *et al.*, 2012) or an obstruction in the bile ducts (Girish and Pradhan, 2012).

However, the results of the present study have shown that the treatment of rats with different concentrations: 50, 100 and 200mg/Kg of Aq E and Met E of *E. plantagineum*, and 100, 200 and 400 mg/Kg of Aq E and Met E of *B. rapa*, reduced significantly the rate of these biochemical parameters.

V.1.1. Effects on aspartate transaminases (AST)

The obtained results show that Aq E and Met E of *E. plantagineum* didn't reduce but elevated the seric level of AST compared with the control group (treated only with CCL_4). In fact, in contrary to ALT enzyme which is specific to liver injuries, AST reflects mostly the lesions in other tissues such as muscle affections and myocardial infarction.

On the other hand, Aq E and Met E of *B. rapa* have reduced, very effectively, the seric AST levels (figure 24). Aq E provided a reducer effect in a very significant way ($p<0.001$), with all tested concentrations (100, 200 and 400 mg/Kg). This activity (80.27% at 100mg/Kg) was more pronounced than that obtained with silymarin (43% of inhibition), used as standard hepatoprotector. Met E exhibited the most powerful effect with 89.46% at 200 mg/Kg but all the doses are still more powerful than silymarin (table 5).

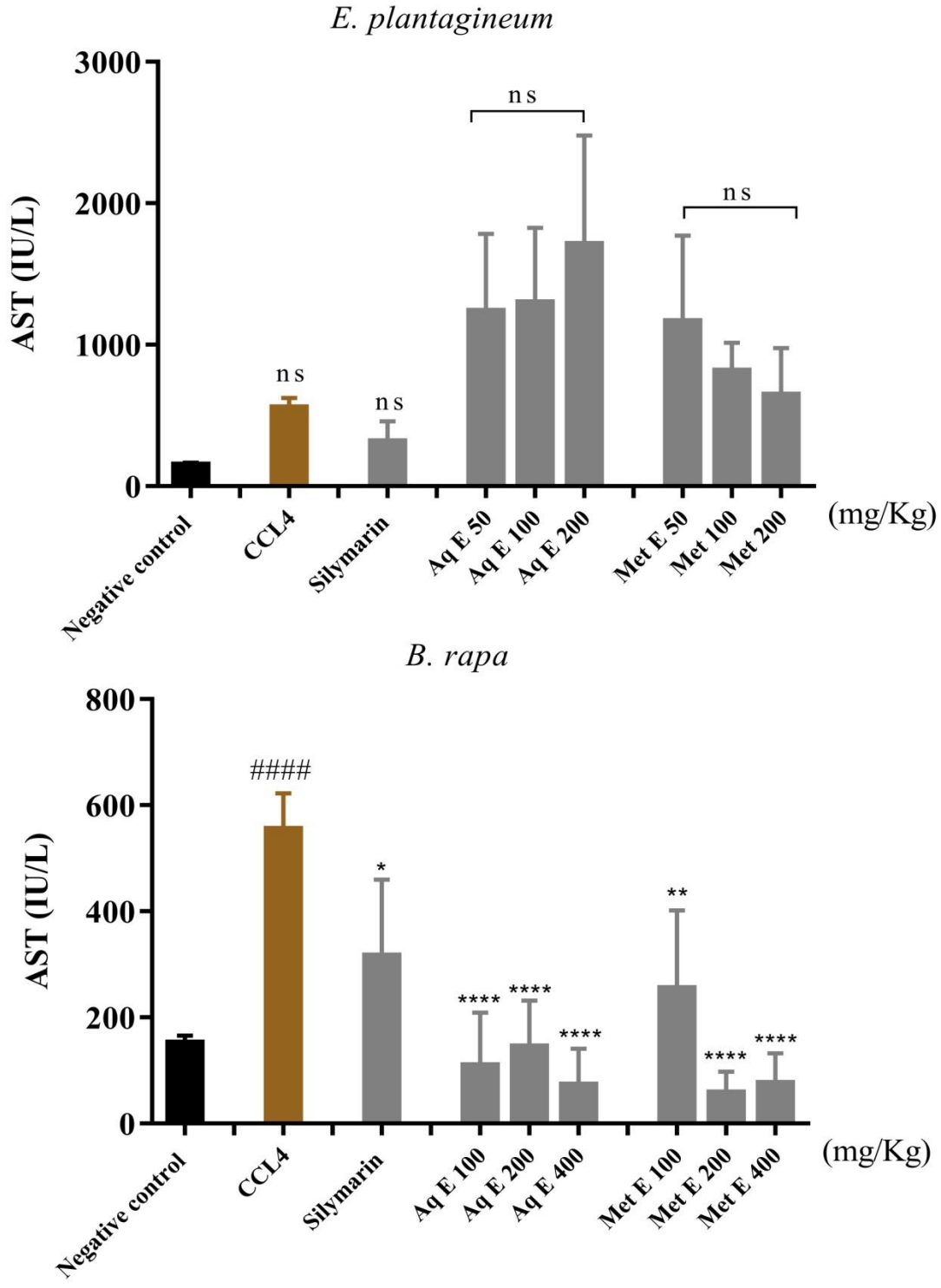

Figure 24. Effects of *E. plantagineum* and *B. rapa* extracts on seric levels of AST. Hepatotoxicity is induced with 1ml/Kg of CCl_4 50%. Silymarin (100 mg/Kg) is used as standard hepatoprotector.Values are mean ± SEM (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$vs control (treated with CCl_4). # vs normal group (negative control).

V.1.2. Effects on alanine transaminases (ALT)

E. plantagineum extracts showed very strong reducer effects, at all doses, on ALT seric level in dose-independent way. In fact, the dose of 100mg/Kg was the most effective ($p<0.01$) among the three tested doses for both extracts (figure 25). Aq E reduced ALT level with a percentage of 74.76%, when Met E produced a reducer effect with 82.65% (table 5). These effects are statistically similar, and comparable to the effect obtained with silymarin.

B. rapa Aq E reduced very effectively the seric levels of ALT at almost all the tested concentrations (figure 25). Aq E produced the most powerful effect ($p<0.0001$) at the dose of 400mg/Kg with 84.19% of inhibition, against 57.16% of inhibition produced by silymarin. The other two doses were less effective than the mentioned dose, but still stronger than silymarin. However, Met E didn't show activity at the dose of 100mg/Kg, but was very powerful ($p<0.0001$) at the two other doses, with 87.37% and 83.49% (table 5).

V.1.3. Effects on alkaline phosphatase (ALP)

Both of Aq E and Met E of *E. plantagineum* reduced the level of ALP effectively and in concentration-independent way (figure 26). Aq E acted at the doses of 50 and 100mg/Kg in similar way than silymarin, but didn't show activity at the dose of 200mg/Kg. In the same way, Met E acted very effectively ($p<0.001$) at the dose of 50mg/Kg (63.56%), even more efficiently than silymarin, but showed less activity at 100 mg/Kg (40.73%) and no activity at 200 mg/Kg (table 5).

Aq E and Met E of *B. rapa* also showed an activity independent of concentration. Aq E was more powerful at 100 and 200 mg/Kg than at 400mg/Kg. However, Met E was more effective at the doses of 100 and 400mg/Kg than at the dose of 200mg/Kg, all the doses being more effective than the standard.

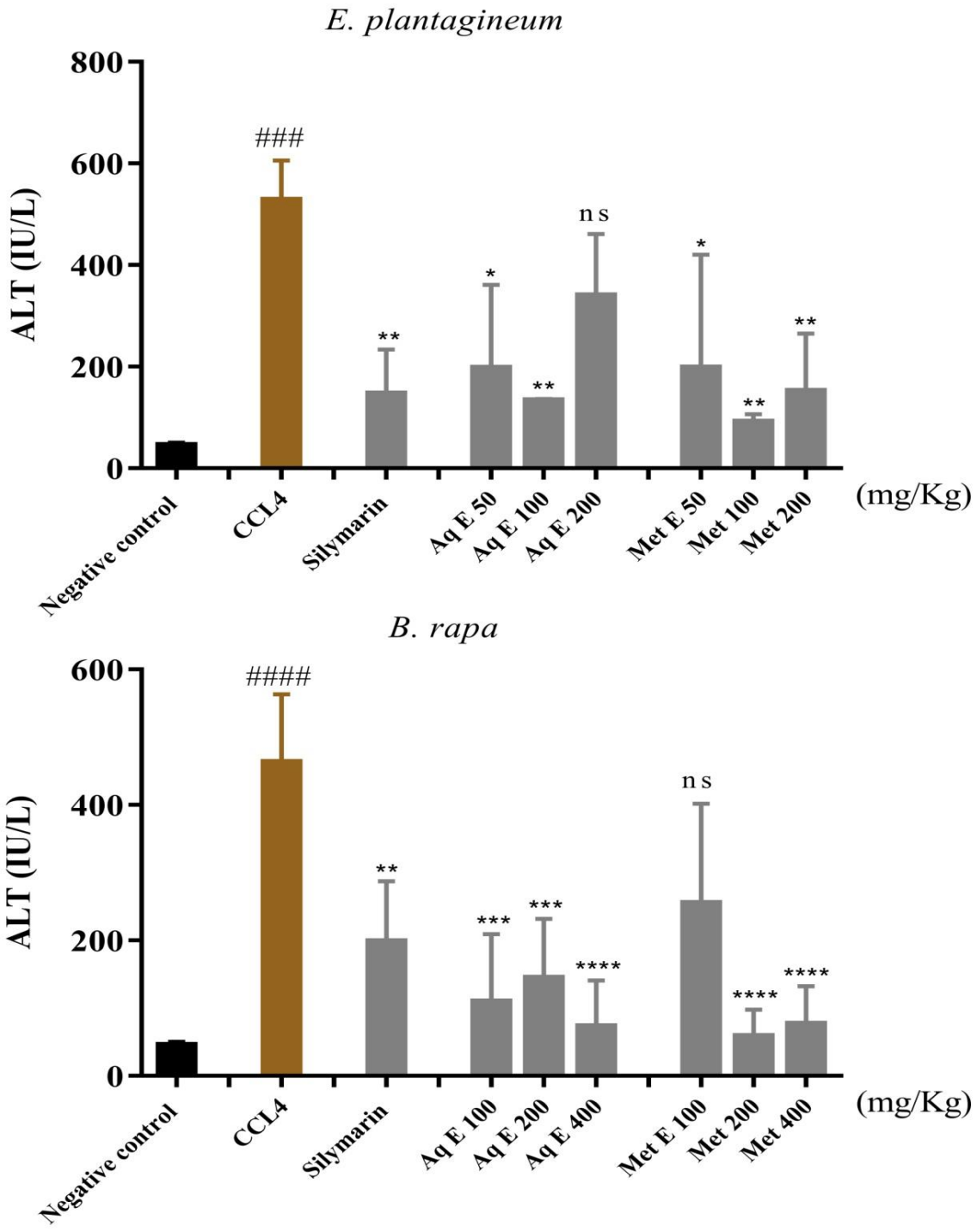

Figure 25. Effects of *E. plantagineum* and *B. rapa* extracts on seric levels of ALT. Hepatotoxicity is induced with 1ml/Kg of CCl_4 50%. Silymarin (100 mg/Kg) is used as standard hepatoprotector. Values are mean ± SEM (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$vs control (treated with CCl_4). # vs normal group (negative control).

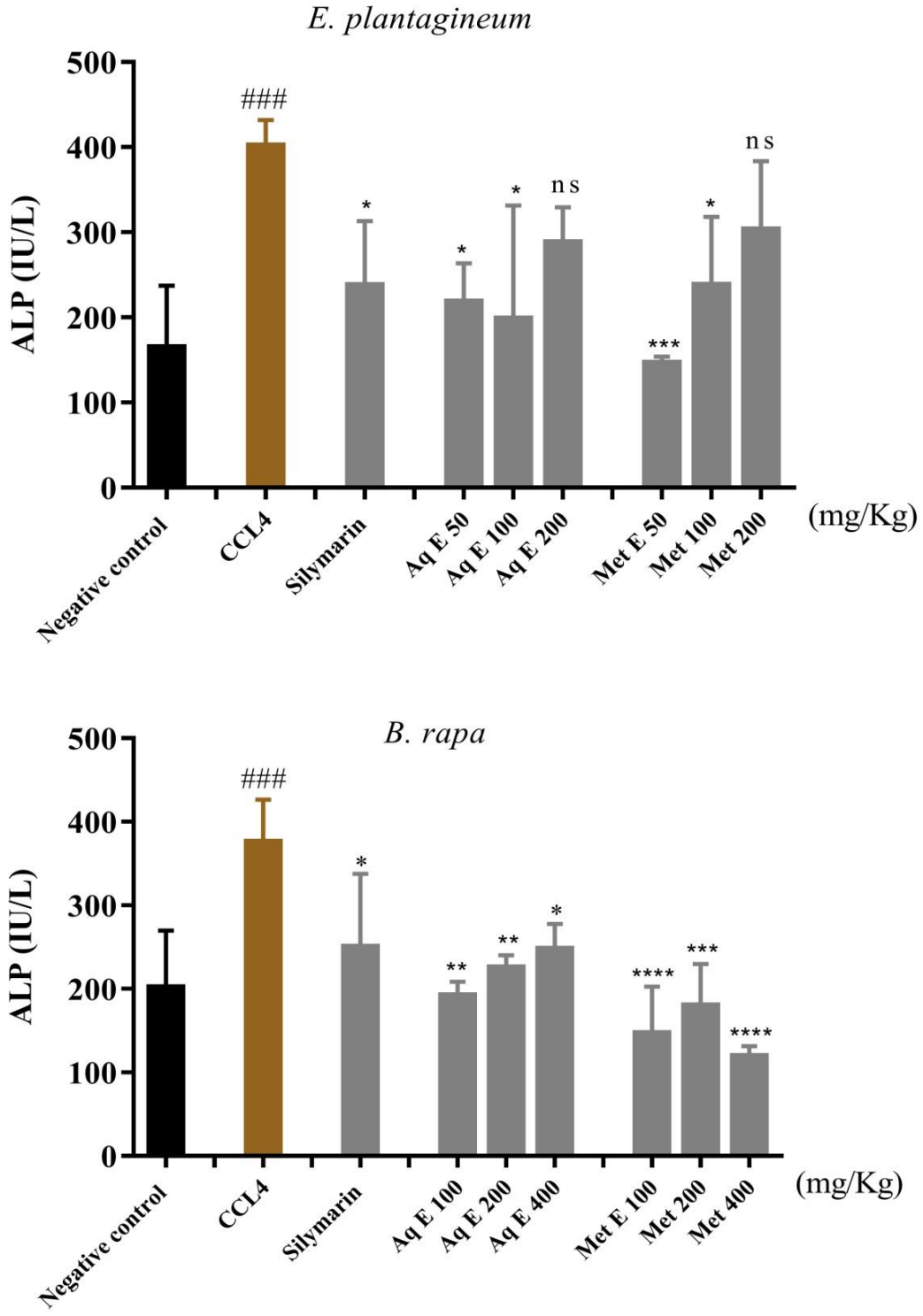

Figure 26. Effects of *E. plantagineum* and *B. rapa* extracts on seric levels of ALP. Hepatotoxicity is induced with 1ml/Kg of CCl_4 50%. Silymarin (100 mg/Kg) is used as standard hepatoprotector. Values are mean ± SEM (n = 5). **: $p<0.05$, **: $p<0.01$, ***: $p<0.001$*vs control (treated with CCl_4). # vs normal group (negative control).

Table 5. Effects of Aq E and Met E of *E. plantagineum* and *B. rapa* on AST, ALT and ALP.

	AST (%)	**ALT (%)**	**ALP (%)**
CCl_4	/	/	/
Silymarin + CCl_4	43,00±14,90*	57,16±9,55**	33,42±13,39*
E. plantagineum			
Aq E 50 mg/Kg + CCl_4	-	62,62±15,46*	45,69±6,48*
Aq E 100 mg/Kg + CCl_4	-	74,76±0,32**	50,66±19,14*
Aq E 200 mg/Kg + CCl_4	-	35,65±11,47ns	26,00±4,60ns
Met E 50 mg/Kg + CCl_4	-	62,46±24,29*	63,56±0,93***
Met E 100 mg/Kg + CCl_4	-	82,65±1,58**	40,73±9,92*
Met E 200 mg/Kg + CCl_4	-	71,14±10,65**	24,56±10,00ns
B. rapa			
Aq E 100 mg/Kg + CCl_4	80,27±8,99****	76,37±10,77***	48,85±2,47**
Aq E 200 mg/Kg + CCl_4	73,91±7,01****	68,75±8,40***	40,03±1,98**
Aq E 400 mg/Kg + CCl_4	86,80±6,08****	84,19±7,28****	34,11±4,00*
Met E 100 mg/Kg + CCl_4	54,05±13,21**	44,96±15,82ns	60,90±7,42****
Met E 200 mg/Kg + CCl_4	89,46±3,52****	87,37±4,22****	52,13±6,60***
Met E 400 mg/Kg + CCl_4	86,22±5,03****	83,49±6,03****	68,22±1,58****

Values are means ± SEM (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, **** : $p<0.0001$ vs the control group intoxicated by carbon tetra-chloride (CCL_4).

V.1.4. Effects on cholesterol and trigycerides

CCl_4 caused a significant increase in triglycerides and cholesterol levels, probably due to disturbance in mechanisms of association of triglycerides with appropriate apoprotein to form the carrier lipoprotein molecule (Tiwari *et al.*, 2014; Shanmugam *et al.*, 2016).The metabolism of CCl_4 results in free radicals formation, that attack lipids membrane of endoplasmic reticulum leading to decrease in metabolic functions like proteins synthesis and cholesterol storage (Verma *et al.*, 2015).

Our results show that the injection of CCL_4 induces an important elevation of the level of cholesterol and triglycerides. Treatment of rats with Aq E of *E. plantagineum* has reduced very significantly ($p<0.001$) the level of cholesterol at the dose of 50mg/Kg, similarly to silymarin, but no effect was noticed at 100 and 200mg/Kg (figure 27). Met E acted more effectively at the doses of 50 and 100mg/Kg than at the dose of 200mg/Kg. However, no effect towards the cholesterol level was noticed with all the doses of Aq E and met E of *B. rapa*.

V.1.5. Effects on creatinine and urea

Results show that treatment of rats with CCL_4 didn't increase creatinine and urea levels (figure 28). In the same way, treatment with different doses of Aq and Met extracts of the selected plants have not shown any effect on the levels of kidney markers.

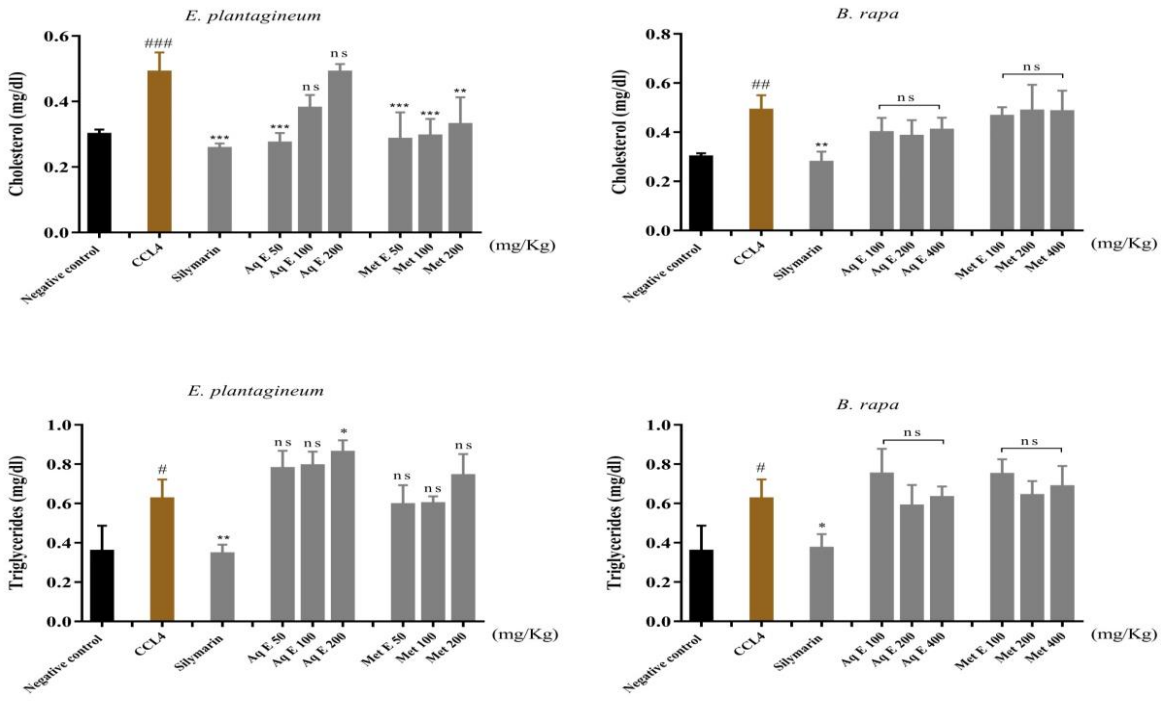

Figure 27. Effects of *E. plantagineum* and *B. rapa* extracts on seric levels of cholesterol and triglycerides. Hepatotoxicity is induced with 1mL/Kg of CCl_4 50%. Silymarin (100 mg/Kg) is used as standard hepatoprotector. Values are mean ± SEM (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$vs control (treated with CCl_4). # vs normal group (negative control).

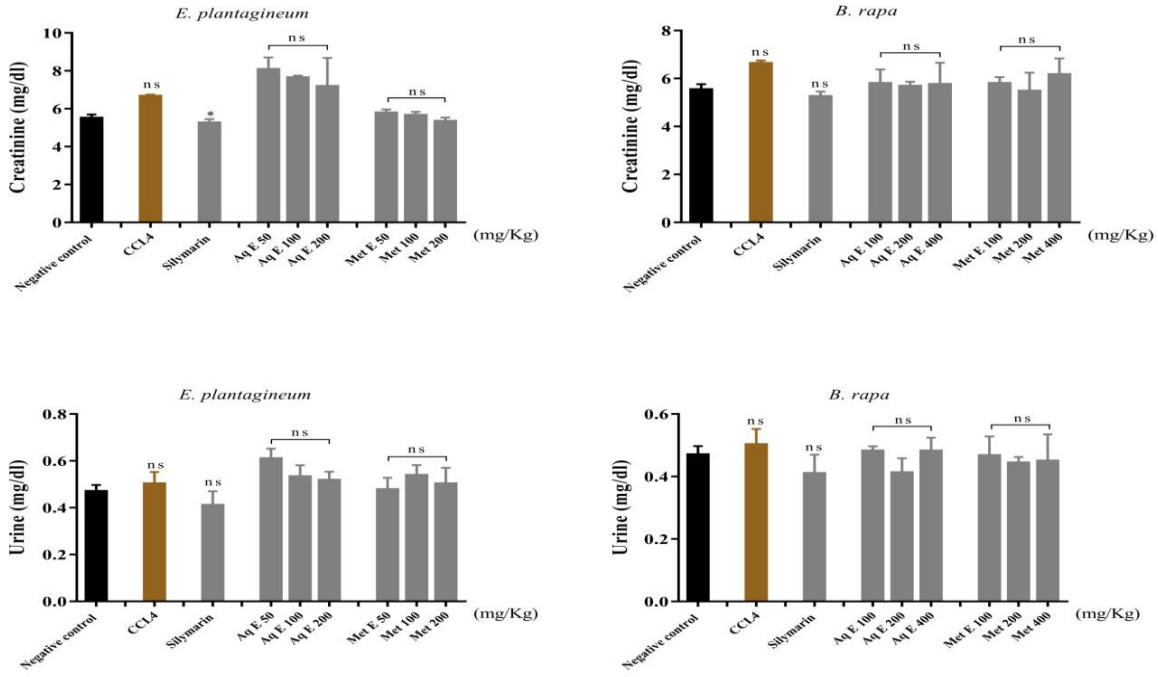

Figure 28. Effects of *E. plantagineum* and *B. rapa* extracts on seric levels of creatinine and urea. Hepatotoxicity is induced with 1mL/Kg of CCl_4 50%. Silymarin (100 mg/Kg) is used as standard hepatoprotector. Values are mean ± SEM (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$vs control (treated with CCl_4). # vs normal group (negative control).

V. 2. Effect of extracts on antioxidant parameters

V.2.1. Effect on Catalase

The intraperitoneal injection of CCl_4 reduces very significantly ($p<0.0001$) the concentration of catalase. The level of CAT in the liver supernatant of intoxicated rats is 61,88 ± 19,18 U/mg of proteins (table 6). All extracts of *E. plantagineum* increase significantly the level of CAT. The pretreatment with Aq E of *E. plantagineum* increases very significantly the CAT level (186,75±23,52 U/mg of proteins) at the dose 200mg/Kg. The doses 50 and 100mg/Kg produce a significant effect on CAT, but steel less pronounced. Met E increases very effectively ($p<0.0001$) the CAT level with the obtained concentrations of 188,69±63,61 and 166,52±27,48 U/mg of proteins at the doses 50 and 200 mg/Kg, respectively. These effects were more pronounced than that of the dose 100mg/Kg.

Concerning *B. rapa*, only the Aq E, at the dose of 400 mg/Kg, showed a significant effect in increasing the CAT level, with a concentration of 122,39±37,32 U/mg (table 6).

V.2.2. Effect on MDA

The increase in the level of MDA after intoxication with CCl_4 reflects an important oxidative stress that causes tissue lesions and a failure in antioxidant mechanisms. MDA is an indicator of lipid peroxidation. It can penetrate the membrane lipid belayer leading to damage and durcicity and consequently loss of membrane function. This results in reduction of the fluidity and rise of the permeability of the membrane, leading to swelling and cellular necrosis (Su *et al.*, 2016). The effects of the extracts on MDA prove that they can protect the liver from damage by inhibition of the lipid peroxidation and the inhibition of chain oxidation reaction (Goel *et al.*, 2005).

Results show that intraperitoneal injection of CCl_4 increases very significantly ($p<0.0001$) the concentration of MDA. The level of MDA in the liver supernatant of intoxicated rats is 152,91±53,63 nmole/mg of proteins. The pretreatment with Aq E of *E. plantagineum* at the doses 50 and 100 mg/Kg reduces very significantly the MDA level with values of 60,16 nmole/mg and 55,24 nmole/mg of proteins, respectively (table 6). These effects are similar to that of silymarin (76,83 nmole/mg of proteins). Met E exhibited the best effect, as efficiently as silymarin, at the doses 50 and 200mg/Kg with values of 79,88 and 63,27 nmole/mg of proteins, respectively.

Aq E and Met extracts of *B. rapa* reduced effectively the concentration of MDA at 100, 200 and 400mg/Kg with percentages of 22,89 - 56,70 nmole/mg of proteins (table 6).

V.2.3. Effect on GSH

The obtained results show that CCL_4 intoxication did not produce any significant change in the GSH level. In the same way, pretreatment with silymarin, aqueous and methanolic extracts of *E. plantagineum* and *B. rapa* didn't show significant effects on GSH level (table 6).

Table 6. Effects of Aq E and Met E of *E. plantagineum* and *B. rapa* on CAT, MDA and GSH.

	CAT (U/mg of proteins)	**MDA** (nmole/mg of proteins)	**GSH** (nmole/g of tissue)
Negative control	166,17 ± 37,71	43,17±10,40	165,47±32,73
CCl_4	61,88± 19,18$^{\#\#\#\#}$	152,91±53,63$^{\#\#\#\#}$	226,31±77,52^{ns}
Silymarin + CCl_4	…….	76,83±18,13****	168,35±51,27
E. plantagineum			
Aq E 50 mg/Kg + CCl_4	130,57±20,02*	60,16±11,26****	150,45±12,65
Aq E 100 mg/Kg + CCl_4	135,45±11,12*	55,24±55,24****	222,88±59,07
Aq E 200 mg/Kg + CCl_4	186,75±23,52***	85,32±38,78***	190,47±35,67
Met E 50 mg/Kg + CCl_4	188,69±63,61****	79,88±6,07****	199,89±64,61
Met E 100 mg/Kg + CCl_4	158,64±22,75***	86,72±2,57***	181,17±14,15
Met E 200 mg/Kg + CCl_4	166,52±27,48****	63,27±11,63****	177,53±26,11
B. rapa			
Aq E 100 mg/Kg + CCl_4	58,61±17,46^{ns}	56,70±19,59****	66,59±6,53^{ns}
Aq E 200 mg/Kg + CCl_4	83,02±14,90^{ns}	25,51±7,50****	63,70±7,20^{ns}
Aq E 400 mg/Kg + CCl_4	122,39±37,32*	34,44±4,55****	65,58±9,78^{ns}
Met E 100 mg/Kg + CCl_4	78,75±9,58^{ns}	28,86±4,19****	64,70±9,69^{ns}
Met E 200 mg/Kg + CCl_4	82,09±21,40^{ns}	22,89±2,74****	74,25±17,17^{ns}
Met E 400 mg/Kg + CCl_4	39,52±11,05^{n}	28,42±4,66****	61,31±9,68^{ns}

Values are mean± SEM (n = 6). **: p<0.05, **: p<0.01, ***: p<0.001, **** : p<0.0001* vs the control (treated only with CCl_4). # = comparison with negative control.

V. 3. Effects of the extracts on liver histology

The histological examination showed that the CCl_4 treated rats showed a hepatic tissue with important lesions namely hepatocyte necrosis, centrilobular infiltration, steatosis as well as swelling and clarification of hepatocytes. Silymarin, at a dose of 100 mg/Kg, showed almost complete normalization of the liver tissue and significant recovery of hepatocytes in different sections of the liver (figure 29). However, the livers of rats treated with different doses of aqueous and methanolic extracts of *Echium plantagineum* and *Brassica rapa* present different levels of lesions. In fact, hepatic tissues of rats treated with Aq E of *E. plantagineum* (at the doses 50, 100 and 200 mg/Kg) showed, mostly, swelling and clarification of hepatocytes as well as lymphocyte infiltration (figures 29D, E, F). The rats treated with the Met E presented mostly steatosis at the dose of 50 mg/Kg (figure 29G), while lymphocyte infiltration and necrosis are the lesions characterizing livers of rats treated with the doses of 100 and 200 mg/Kg (figures 29H, I).

On the other hand, the rats pre-treated with Aq E presented centrilobular congestion, steatosis and lymphocyte infiltration (figure 30), however the highest dose of 400 mg/Kg seems to be the most effective in enhancing these hepatic lesions compared with the doses of 100 and 200 mg/Kg. The methanolic extract, in contrary, has shown the most powerful effect with the lowest dose (100 mg/Kg), presenting a normal hepatic parenchyma (figure 30M), compared with the two other doses 200 and 400 mg/Kg presenting, respectively, an important steatosis and lymphocyte infiltration (figures 30N, O).

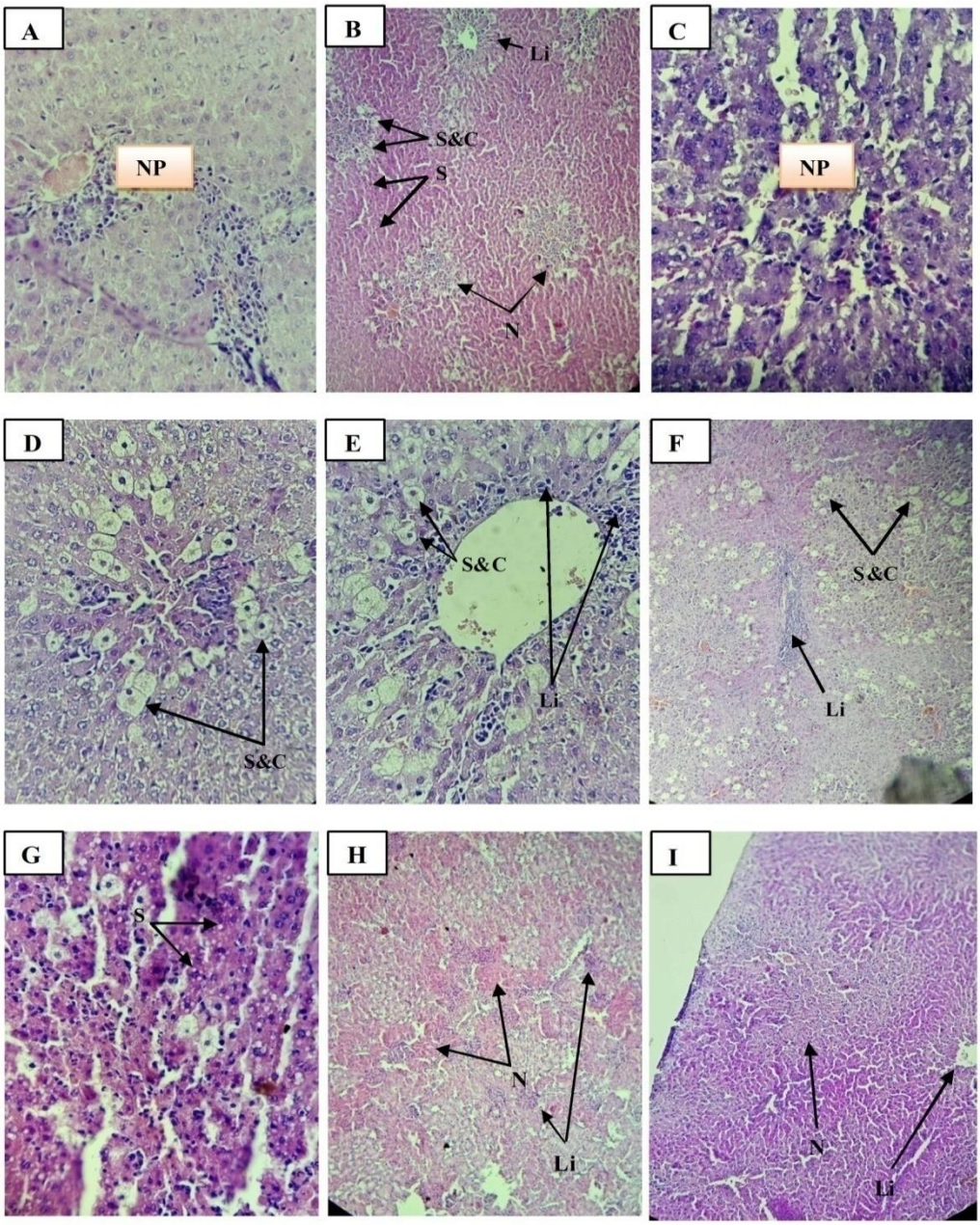

Figure 29. Liver histological sections (x 100) of the hepatoprotective activity of *E. plantagineum* extracts. A: Hepatic parenchyma of normal rats. B: Hepatic parenchyma of CCL_4 pre-treated rats. C: Hepatic parenchyma of 100 mg/Kg Sylimarin pre-treated rats. D: Hepatic parenchyma of 50 mg/Kg *E. plantagineum* Aq E pre-treated rats. E: Hepatic parenchyma of 100 mg/Kg *E. plantagineum* Aq E pre-treated rats. F: Hepatic parenchyma of 200 mg/Kg *E. plantagineum* Aq E pre-treated rats. G: Hepatic parenchyma of 50 mg/Kg *E. plantagineum* Met E pre-treated rats. H: Hepatic parenchyma of 100 mg/Kg *E. plantagineum* Met E pre-treated rats. I: Hepatic parenchyma of 200 mg/Kg *E. plantagineum* Met E pre-treated rats.

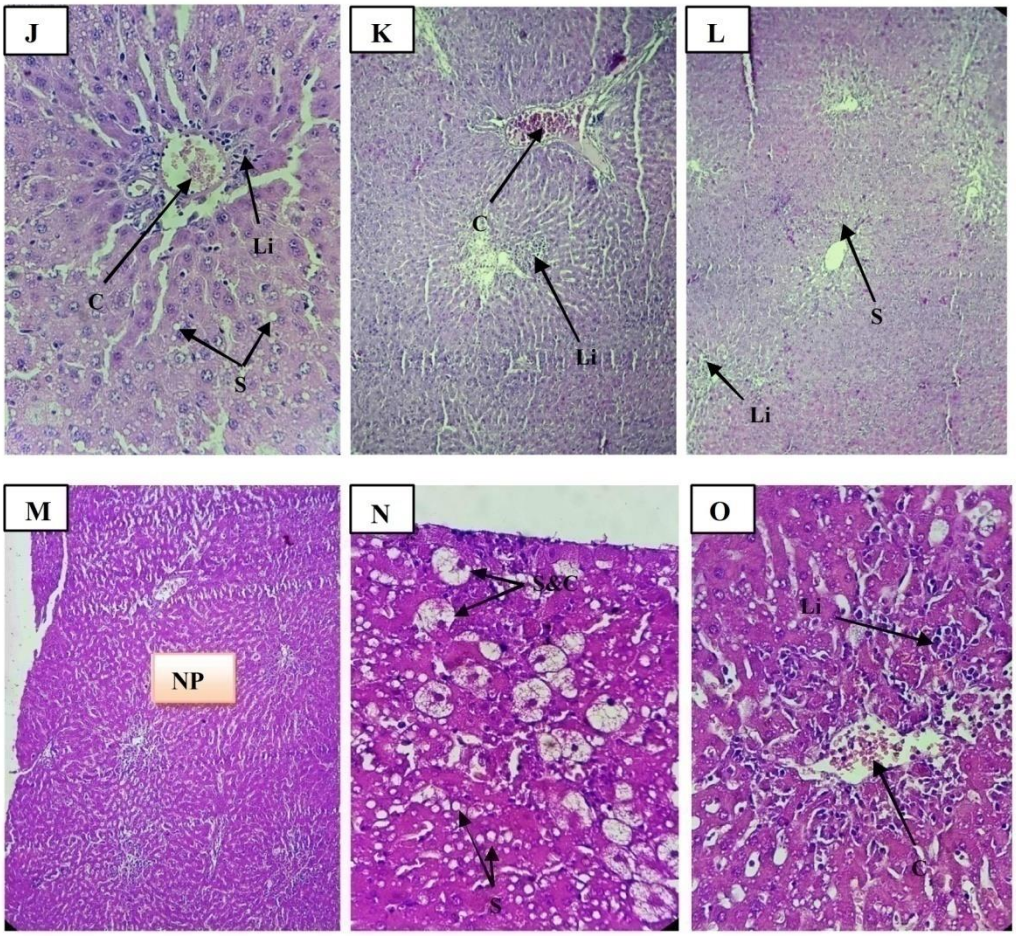

Figure 30. Liver histological sections (x 100) of the hepatoprotective activity of *B. rapa* extracts. J: Hepatic parenchyma of 100 mg/Kg *B. rapa* Aq E pre-treated rats. K: Hepatic parenchyma of 200 mg/Kg *B. rapa* Aq E pre-treated rats. L: Hepatic parenchyma of 400 mg/Kg *B. rapa* Aq E pre-treated rats. M: Hepatic parenchyma of 100 mg/Kg *B. rapa* Met E pre-treated rats. N: Hepatic parenchyma of 200 mg/Kg *B. rapa* Met E pre-treated rats. O: Hepatic parenchyma of 400 mg/Kg *B. rapa* Met E pre-treated rats. (C: congestion, N: necrosis, S: steatosis, Li: Lymphocyte infiltration, S&C: swelling and clarification).

Several studies carried out on our selected plants or species belonging to the same families have shown their protective effect of the liver against chemicals induced hepatotoxicity. Kumar *et al*. (2020) have reported inhibiting effects on the CCL_4 induced hepatic damage of hydroethanolic extract of *Onosma bracteata* (Boraginaceae). The extract reduced significantly the level of the serum marker enzymes (AST, ALT and ALP), decreased MDA and increased the CAT function compared with the control group.

The pre-treatment of rats with *B. rapa* juice, at a dose of 16 mL/ kg, protected the rats against CCl_4-induced hepatotoxicity as proved by significant reduction of the serum

ALT, AST, ALP and bilirubin levels (Al-Snafi, 2015). Moreover, the protective effect of turnip roots ethanolic extract on early hepatic injuries was studied in alloxan-induced diabetic rats. The turnip roots extract at a dose of 200 mg/kg decreased significantly the levels of serum biomarkers of hepatic injury. Furthermore, it significantly decreased the lipid peroxidation and elevated the levels of antioxidant enzymes in diabetic rats. The study also showed that histopathological changes were in agreement with biochemical findings (Al-Snafi, 2016).

The decrease effect of polyphenolic compounds towards biochemical parameters (AST, ALT and ALP) has been demonstrated by several studies. Gallic acid has the capacity to restore the physiological integrity of hepatocytes, there by reducing the elevated values of serum AST, ALT and ALP, as well as increasing CAT levels and reducing the lipid peroxidation products levels in lindane hepatotoxicity model (Padma *et al*., 2011). Additionally, Touiss *et al*. (2021) have reported good protective effects of a rosmarinic acid-rich extract from *Ocimum basilicum* against CCL_4 liver toxicity. At the dose of 200mg/Kg, the rosmarinic acid-rich extract reduced significantly the levels of plasma marker enzymes (AST, ALT and ALP), as well as the concentration of MDA. Furthermore, chlorogenic acid is known for its good protective effect on the liver against the accumulation of exogenous chemicals. Its hepatoprotective character is related to its antioxidant and anti-inflammatory properties. It acts on the expression of enzymes and proteins related to the oxidative system and hence inhibit the liver damage caused by oxidative stress. It acts also on the regulation of the expression level of the genes promoting the apoptosis of necrotic cells and direct or indirect inhibition of the expression of pro-inflammatory factors and related signal pathways (Wang *et al*., 2022).

Several flavonoids such as catechin, apigenin, quercetin, naringenin and rutin are reported for their hepatoprotective activities (Tapas *et al*., 2008). The total flavonoids extract, containing quercetin and luteolin derivatives among other flavonoids, improved cell viability and inhibited cellular leakage of hepatocyte AST and ALT, at a concentration range of 1-100 μg/mL.Similarly, flavonoids at oral doses of 50, 100, and 200mg/kg significantly reduced the serum levels of AST, ALT, total proteins and albumin. The histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoids (Wu *et al*., 2006).

CONCLUSION AND PROSPECTS

Conclusion and prospects

The present study aimed to establish the phenolic profile of aqueous and methanolic extracts of the aerial part of *Echium plantagineum* and the roots of *Brassica rapa*, and evaluate their antioxidant, anti-hemolytic, anti-inflammatory and hepatoprotective effects.

The hydroalcoholic extraction was established for her known effectiveness in the extraction of both hydrophilic and hydrophobic phenolic compounds. The aqueous extraction represents a way to benefit from plants when preparing decoctions. Thus, methanolic and aqueous extracts of both *Echium plantagineum* and *Brassica rapa* were realized by maceration and decoction, respectively. The phytochemical study has shown that all extracts present considerable content of phenolic compounds, flavonoids and tannins. The HPLC analysis allowed the identification of ten compounds in Met E and seven in Aq E of *E. plantagineum*, whose majority compounds are, respectively, quercetin 3-O- galactoside and gallic acid. While both Aq E and Met E of *B. rapa* present six phenolic compounds whose gallic acid is the most dominant one.

The *in vitro* exploration of the antioxidant activity has shown that Aq and Met extracts of *E. plantagineum* scavenge very effectively $ABTS^{\cdot}$, $OH^{\cdot}$, $O_2^{\cdot}$ and H_2O_2 species, chelate and reduce strongly ferrous ions and inhibit the B-carotene oxidation. Aq and Met extracts of *B. rapa* exhibited important effects in the previous tests, but with less efficiency than the first plant. All studied extracts have shown a very important protective effect against AAPH induced hemolysis.

In the anti-inflammatory study, the extracts have shown *in vivo*, important inhibition effects of carrageenan induced paw edema. Also, they inhibited, *in vitro*, the BSA denaturation and prevented the hypotonic induced hemolysis.

The hepatoprotective activity of the extracts was evaluated using the CCL_4 model. The results showed that the extracts reduced the levels of the biochemical parameters AST, ALT and ALP that have been increased following the treatment with CCL_4. Extracts had also good effects on the markers of the oxidative stress. They increased significantly the CAT level, and decreased the concentration of MDA.

In conclusion, extracts of *E. plantagineum* and *B. rapa* possess antioxidant, antihemolytic and anti-inflammatory effects, that can probably explain their hepatoprotective activity. These biological properties are due to their content of phenolic compounds. Hence, these plants represent an important source of bioactive compounds with possible interesting therapeutic applications.

However, further studies must be conducted mainly on the sub-acute toxicity of *E. plantagineum* as well as on the isolation, the purification and the identification of molecules responsible for the activities explored in this study. The exploration of mechanisms of action of the identified bioactive molecules on inflammatory mediators, antioxidant systems *in vivo* and enzymes implicated in production of ROS, as well as their direct effects on hepatocytes and enzymes implicated in xenobiotics metabolism, are objectives for necessary subsequent studies.

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Annals of R.S.C.B., ISSN: 1583-6258, Vol. 26, Issue 1, 2022, Pages. 3964 - 3981
Received 24 November 2022; Accepted 15 December 2022.

Phenolic content and Antioxidant activities of Algerian *Echium plantagineum* Extracts

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ABSTRACT

Objective: This study aims to explore the phenolic composition of aqueous (Aq E) and methanolic (Met E) extracts of Algerian *Echium plantagineum* aerial part and evaluate their antioxidant capacity. **Methods**: Total phenolics, flavonoids and tannins contents were determined by spectrophotometric methods using Folin-Ciocalteu, aluminium chloride and hemoglobin precipitation assays, respectively. The antioxidant activities were evaluated using several *in vitro* tests including reactive oxygen species ($ABTS^{\cdot}$, $OH^{\cdot}$, $O_2^{\cdot}$ and H_2O_2) scavenging assays, ferrous ions chelating test, reducing power and anti-hemolytic assays. **Results**: The phytochemical study revealed a predominance of tannins, total polyphenols and flavonoids in both extracts. Aq E showed more efficiency to scavenge $ABTS^{\cdot}$, $O_2^{\cdot}$ and $OH^{\cdot}$ radicals and to chelate ferrous ions. However, Met E was more efficient (IC_{50}=193.87µg/mL) in scavenging H_2O_2 than Aq E (IC_{50}=262.46µg/mL). In the same way, Met E inhibited AAPH induced hemolysis (HT_{50}=179.79±5.82 min) more than Aq E (HT_{50}=161.62±5.87 min). On the other hand, both Met E and Aq E exhibited very high reducing power (IC_{50}=117µg/mL and 128µg/mL, respectively). **Conclusion**: This study shows that *Echium plantagineum* extracts possess strong antioxidant activity, which is probably due to their phenolic composition. Therefore, this plant can constitute a promising source of natural antioxidants.

Key words: Antioxidant, *Echium plantagineum*, Flavonoids, Phenolic compounds, ROS.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes, chronic inflammation, neurodegenerative disorders and even certain types of cancer [1, 2]. Many synthetic antioxidants, such as butylatedhydroxytoluene (BHT), are used as additives in food industry to prevent deterioration. However, there is concern about their safety on human health [3]. Medicinal plants seem to be one of the main sources of safe antioxidants and healthcare supplements. It has been reported that polyphenolic compounds are among the active substances in these plants having multiple biological activities.

Echium plantagineum, known as purple flower, is an annual herbaceous species which belongs to the Boraginaceae family. Native from Europe and Northern Africa, it is indicated for ornamental,

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 26, Issue 1, 2022, Pages. 3964 - 3981
Received 24 November 2022; Accepted 15 December 2022.

melliferous and medicinal purposes [4]. Findings showed that the various parts of *Echium* species could be used for their antibacterial, anti-inflammatory, anti-proliferative, antidepressant, antioxidant, antiviral, anxiolytic and cytotoxic properties [5, 6]. *E. plantagineum* bee pollen as well as its seeds oil have been extensively studied, however, very few studies on phenolic composition and antioxidant activities of the aerial part of this plant are available. Therefore, the present study was designed to explore the phenolic content as well as the *in vitro* antioxidant activities of *E. plantagineum* aerial part extracts.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteau reagent, gallic acid, quercetin, tannic acid, Butylatedhydroxtoluene (BHT), 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$), EDTA, $FeCl_2$, ferrosine, potassium persulfate, 2,2′azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt (ABTS), Trolox, potassium ferricyanide, tricholoroacetic acid, $FeCl_3$, hydrogen peroxide, ferrous sulfate, ascorbic acid, phenazinemethosulfate (PMS), nitro blue tetrazolium (NBT), NADH, ferrous ammonium sulfate, 1, 10-phenanthroline and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma (Germany). Sodium bicarbonate, Aluminum chloride and sodium salicylate were purchased from Prolabo (France). All other chemicals used in this study were of analytical grade.

Plant material

Echium plantagineum L. was collected in June 2017 from Babor region (Northern of Setif, Algeria). It was identified and authenticated taxonomically by Pr. Laouar H., Laboratory of Valorization of Natural Biological Resources, University of Setif 1, Algeria. A specimen was conserved at the same laboratory. The aerial part was cleaned, air-dried at room temperature and then reduced to powder.

Preparation of the plant extracts

E. plantagineum aqueous extract (Aq E) was prepared by boiling 50 g of powdered plant in 1000mL of distilled water for 25 min followed by filtration through Whatman filter paper N°3. The filtrate was dried and the obtained powder (yield 40%) was stored at -32°C until required.
Methanolic extract (Met E) was prepared by maceration of 50g of the powdered plant material, twice for 24h,with 500mL of 80% methanol and then with 50% methanol at room temperature, with frequent agitation. After filtration, the filtrate was concentrated under reduced pressure at 40 °C (Buchi, Flawil, Switzerland) and then dried. The obtained powder (yield 22%) was stored at -32 °C until use.

Determination of total polyphenols

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 26, Issue 1, 2022, Pages. 3964 - 3981
Received 24 November 2022; Accepted 15 December 2022.

The content of total polyphenolic compounds in *E. plantagineum* extracts was determined using Folin-Ciocalteu assay [7]. Samples (100 µl) were mixed with 500 µL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min., 400 µL of 7.5% sodium carbonate (Na_2CO_3) solution were added. After 90 min. of incubation at room temperature, the absorbance was measured at 765 nm. Gallic acid was used as astandard. The concentration of total polyphenolic compounds was determined as mg of Gallic acid equivalents per 1 g of extract (mg GAE/g extract).

Determination of total flavonoids

The total flavonoid content was determined by the aluminum chloride method [8]. Briefly, 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of extracts. After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used for the standard calibration curve. The concentration of total flavonoid compounds was determined as mg of quercetin equivalents per 1 g of extract (mg QE/g extract).

Determination of total condensed tannins

Tannins content of *E. plantagineum* extracts was determined using the hemoglobin precipitation assay [9]. Tannic acid was used as standard. A volume of 1mL of each extract was mixed with 1mL of hemolyzed bovine blood (absorbance = 1.6). The mixture was incubated for 20 min, and then centrifuged at 4000rpm for 10min. The absorbance of the supernatant was measured at 756nm and tannins content was expressed as mg tannic acid equivalent per 1 g of extract (mg TAE/g extract).

ABTS radical scavenging activity

The ability of *E. plantagineum* extracts to scavenge the ABTS radical was evaluated according to Re et al. [10]. A volume of 2.5 mL of ABTS (7 mM) was mixed with 2.5mL of potassium persulfate (2.45 mM) and the mixture was allowed to stand in the dark at room temperature for 12–16 h to produce ABTS radical cations ($ABTS^{\bullet+}$). The $ABTS^{\bullet+}$ solution was diluted with ethanol to obtain an absorbance of 0.70 (±0.02) at 734 nm. The scavenging activity was estimated by mixing 50µL of diluted *E. plantagineum* extracts (0-500µg/mL) or Trolox (0-100µg/mL) with 1mL of $ABTS^{\bullet+}$ solution. The absorbance was read at 734 nm after 30min. of incubation at room temperature. The antioxidant activity was calculated as followed:

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of the control without test sample and A_1 is the absorbance of the test sample.

Hydroxyl radical scavenging activity

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Received 24 November 2022; Accepted 15 December 2022.

The scavenging ability of hydroxyl radicals was evaluated as described by Smirnoff and Cumbes [11]. A volume of 1 mL of ferrous sulfate (1.5 mM) was added to 700 µL of hydrogen peroxide (6 mM) and 300 µL of sodium salicylate (20 mM). Then, 1 mL of *E. plantagineum* extracts, at different concentrations (0-2000µg/mL), or ascorbic acid (standard antioxidant) was added. The mixture was incubated at 37°C for 1h before measuring absorbance at 562 nm. The antioxidant activity was calculated as following:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1– (A_S – A_B/ A_C)] \times 100$$

A_C: absorbance without sample. **A_B:** absorbance of the blank (without sodium salicylate).
A_S: absorbance of the sample.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski [12]. The superoxide anions generating system contains 0.5mL of Tris–HCl buffer (16mM, pH 8.0), 1 mL of NBT (0.3mM) solution, 0.5mL of NADH (0.936mM) solution and 1 mL of *E. plantagineum* extracts at different concentrations (0-3000µg/mL) or ascorbic acid. A volume of 0.5 mL of phenazinemethosulfate (PMS) solution (0.12 mM) was added to start the reaction. After 5 min. of incubation at 25°C, the absorbance was measured at 560 nm and the antioxidant activity was calculated using the following equation:

$$\text{Superoxide anion radical scavenging activity (\%)} = (A_C – A_S / A_C) \times 100$$

A_C: absorbance of the control. **A_S:** absorbance of the sample.

Hydrogen peroxide scavenging activity

This method was carried out according to Mukhopadhyay et al. [13]. A volume of 750 µL of *E. plantagineum* extracts at different concentrations (0-800µg/mL) was added to 125 µL of ferrous ammonium sulfate (1mM) and 35 µL of H_2O_2 (5mM). The mixture was incubated at room temperature for 5min. Then, a volume of 750 µL of 1,10-phenanthroline (1mM) was added and the absorbance was read at λ =510nm. The ability of the extracts to scavenge hydrogen peroxide was calculated using the following formula:

$$H_2O_2 \text{ scavenging activity (\%)} = (A_{Test} / A_{Blank}) \times 100\%$$

A_{blank}= absorbance of the solution containing only ferrous ammonium sulphate and 1,10-phenanthroline.
A_{test}= absorbance of the solution containing ferrous ammonium sulphate, hydrogen peroxide along with test compound and 1,10-phenanthroline.

Ferrous ions chelating activity

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The chelating ability of ferrous ions by *E. plantagineum* extracts was evaluated by the method described by Le et al. [14]. The reaction mixture contained 250 µLof the extracts at different concentrations (0-1200µg/mL) or EDTA (standard chelator), 50 µL of $FeCl_2$ (0.6 mM) and 450 µL of methanol. After well shaking and incubation for 5 min at room temperature, 50 µL of ferrozine (5 mM) were added. The mixture was shaken and incubated again for 10 min at room temperature before reading absorbance at 562 nm.
The inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Ferrous ions chelating activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the test compound.

Reducing power

The reducing power of *E. plantagineum* extracts was determined according to Topçu et al. [15]. A volume of 2.5 mL of *E. plantagineum* extracts solutions at different concentrations (0-300µg/mL) was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After 20 min of incubation at50 C°, 2.5 mL of TCA (10%) were added and the mixture was centrifuged at 3000 rpm for 10min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Data were expressed as Effective Concentration corresponding to a 0.5 absorbance (EC_{50}).

Blood total antioxidant capacity

The peroxidation of erythrocytes membranes was induced with AAPH according to Takebayashi et al. [16]. Heparinized male rat blood was centrifuged at 6000rpm for 10min and washed three times with PBS buffer (pH 7.4). Then, 100µL of the red blood cells were suspended in 4.9mL of PBS to obtain a 2% dilution. On the other hand, 120µL of the 2% blood cells solution was pre-incubated for 15min at 37°C on microplates with 60µL of *E. plantagineum* extracts (150µg/mL) or Trolox, used as standard antioxidant. Finally, 120µL of AAPH solution (300mM) were added and the reaction mixtures were then incubated at 37°C for 4 - 5h.The degree of hemolysis was determined spectrophotometically by measuring absorbance every 30min at 620nm. The inhibition percent of hemolysis was calculated as following:

$$\text{Hemolysis inhibition (\%)} = (A_0 - A_X \,/\, A_0 - A_{Final}) \times 100$$

A_0: absorbance at t_0. **A_X:** absorbance at a specific time. **A_{Final}**: absorbance at the end of the experience.

Data are also represented by the necessary time for 50% of hemolysis (HT_{50}): The presence of an antioxidant (Trolox or extracts) is supposed to increase the half time of hemolysis which influences a good resistance of erythrocytes.

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Statistical analysis

All data were expressed as means ± SD of three replications. IC_{50} values were calculated by regression analysis. One-way ANOVA test was used for statistical analysis. The values were considered to be significantly different when the p value was less than 0.05.

RESULTS ANS DISCUSSION

Total polyphenols, flavonoids and tannins contents

Phenolic compounds such as phenolic acids, flavonoids and tannins are considered to be major contributors to the antioxidant capacity of plants [7]. Results showed that both *E. plantagineum* extracts contained phenolic compounds. However, methanolic extract contains the highest amount of polyphenols, flavonoids and tannins compared to aqueous extract (Table 1). Tannins represent the major compounds in both extracts.

Table 1. Polyphenols, flavonoids and tannins contents of *E. plantagineum* extracts

Extracts	Polyphenols (mg GAE/g extract)[a]	Flavonoids (mg QE/g extract)[a]	Tannins (mg TAE/g extract)[a]
Aq E	61.89 ± 1.89	7.11 ± 0.65	173.36 ± 62.37
Met E	122 ± 11.39	12.14 ± 0.67	239.47 ± 68.99

GAE: Galic Acid Equivalent; **QE:** Quercetine Equivalent. **TAE:** Tannic acid Equivalent,[a]mean±SD,*n* = 3.

Arumugam et al. [17] have reported that Met E of *Echium angustifolium* aerial part, a species from Boraginaceae family, presents an amount of flavonoids close to that of *E. plantagineum*, but lower amount of polyphenols. Another study, carried on by Aouadi et al. [18], showed that Met E and Aq E of *Echium humile* (Boraginaceae) presented higher amounts of polyphenols and flavonoids than *E. plantagineum* extracts, but lower amounts of tannins.

ABTS radical scavenging activity

The ABTS assay is based on the production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. The presence of an antioxidant eliminates ABTS radicals by reducing them, resulting in discoloration of the radical solution [10]. The advantage of this method is that measuring antioxidant capacity of plant extracts at long

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wavelength maximum absorption of 734 nm eliminates color interference [19]. Both *E. plantagineum* extracts showed a concentration dependant scavenging activity of the ABTS radicals (Figure 1). However, Aq E was more powerful (IC_{50} = 167.03µg/mL) than Met E (IC_{50} = 261.45µg/mL). These results are not concurring with the amounts of polyphenolic compounds present in each extract. This is probably due to the fact that the active principals responsible for this activity are more available in Aq E than Met E. These results are very powerful comparing to those found with Aq E and Met E of *Echium humile* (Boraginaceae) [18].

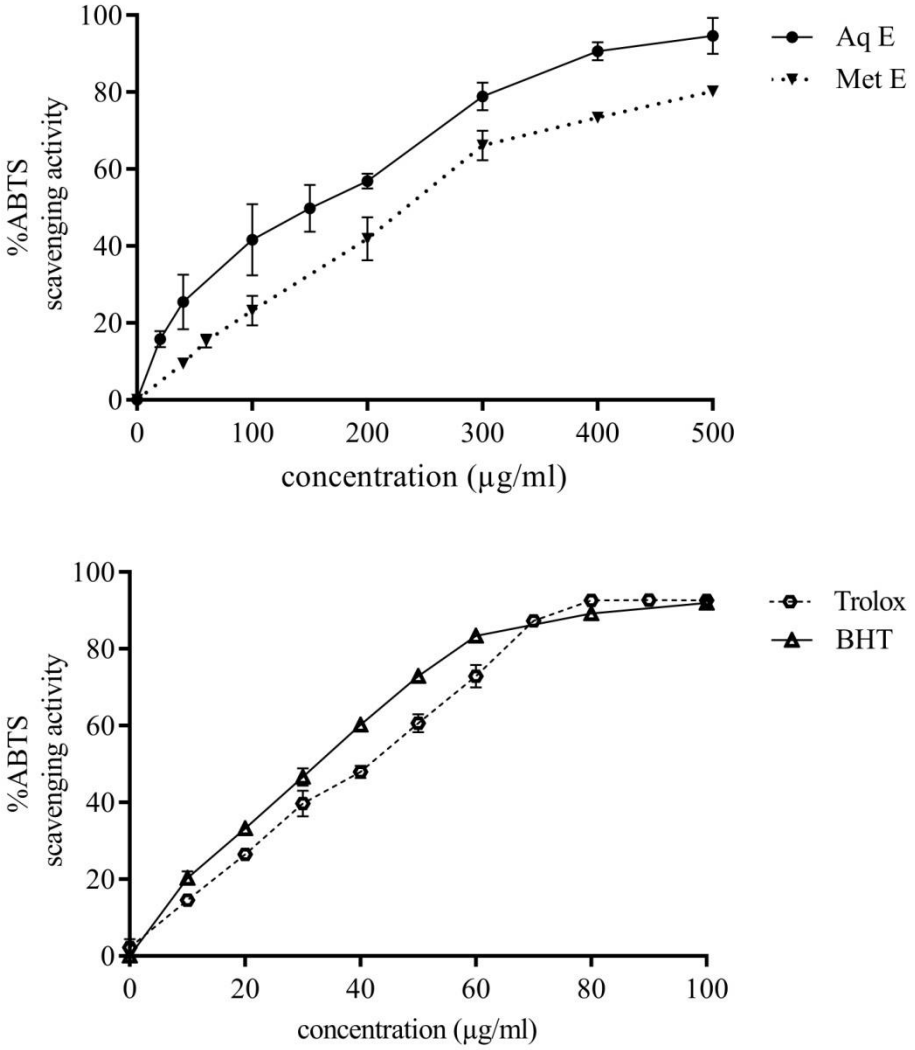

Figure 1. ABTS scavenging activity of *E. plantagineum* extracts, Trolox and BHT. The values are means ± SD (n=3).

Hydroxyl radical scavenging activity

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Hydroxyl radical is generated both *in vivo* and in foods and can undergo several reactions, including dismutation to give H_2O_2. Hydroxyl radical is the most damaging molecule among ROS, being highly reactive, able to damage almost any organic molecule and cannot be eliminated by an enzymatic reaction [20]. *E. plantagineum* extracts showed concentration dependant scavenging activity towards hydroxyl radicals as represented in figure 2. Aqueous extract is more effective (IC_{50} = 103.44µg/mL) than both methanolic extract (IC_{50} = 261.86µg/mL) and vitamin C (IC_{50} = 311.96 µg/mL). Phenolic compounds may be responsible for this antioxidant potential. In fact, the antioxidant activities of phenolic compounds are mainly due to their redox properties, making them free radical quenchers, hydrogen donators and reducing agents [21].

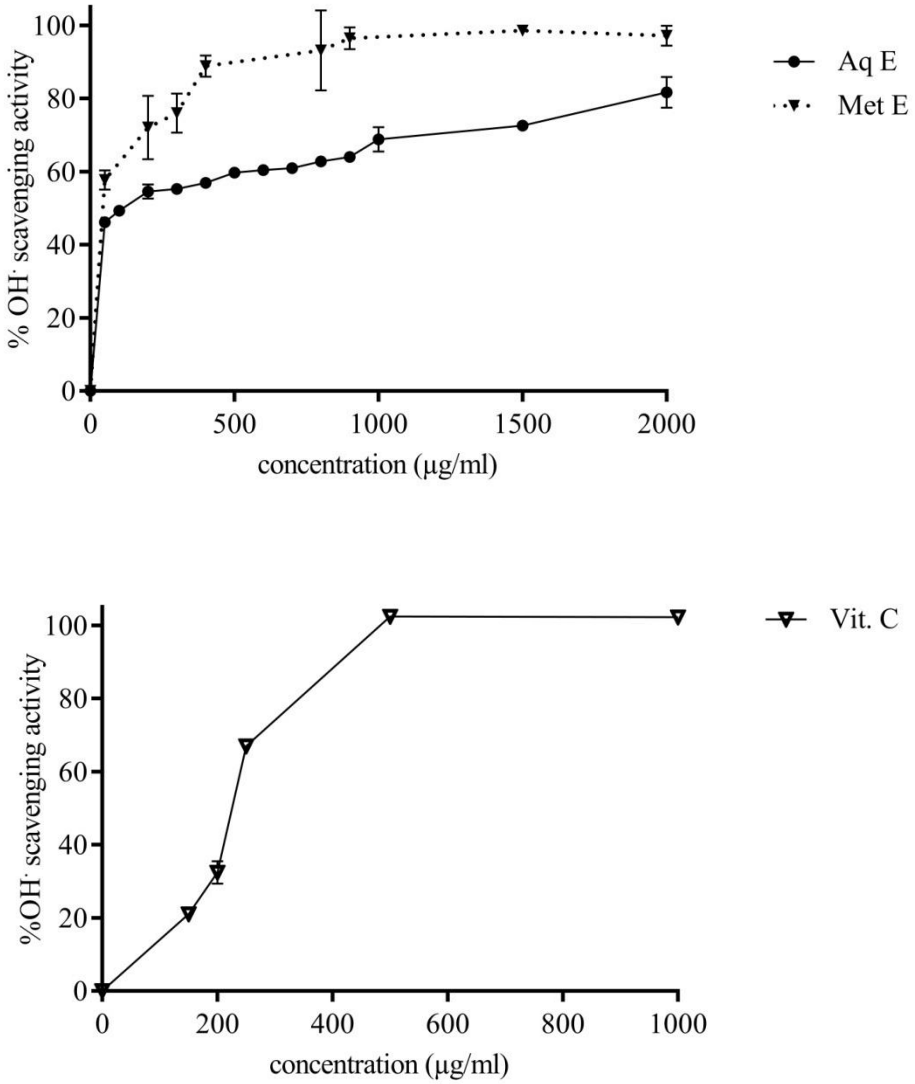

Figure 2. OH· scavenging activities of *E. plantagineum extracts* and vitamin c (Vit. C).Values are means±SD (n=3).

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Superoxide anion scavenging activity

The aqueous and methanolic extracts of *E. plantagineum* act against $O_2^{\cdot}$ in concentration dependent manner (Figure 3). Results show that Aq E was more efficient (IC_{50}=563.78 µg/mL) than Met E (IC50=830.825 µg/mL), but both of them were less powerful than ascorbic acid (IC_{50}=24.34µg/mL). Zemmouri et al. [22] showed that *Borago officinalis* (Boraginaceae) extracts were more efficient in scavenging superoxide anion than *E. plantagineum* extracts, even though these lasts are richer in phenolic compounds. This may be explained by a difference in the nature of the phenolic composition between the two plants. Another study carried on by Amudha and Rani [23] showed that ethanolic extract of *Cordia retusa* (Boraginaceae) exhibited a superoxide anion scavenging activity compared to that found in the present study. In fact, studies concerning the phenolic composition and antioxidant activities of *E. plantagineum* aerial part are very scanty. Thus, it was necessary to compare our results with those found with species that belong to the same family.

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Received 24 November 2022; Accepted 15 December 2022.

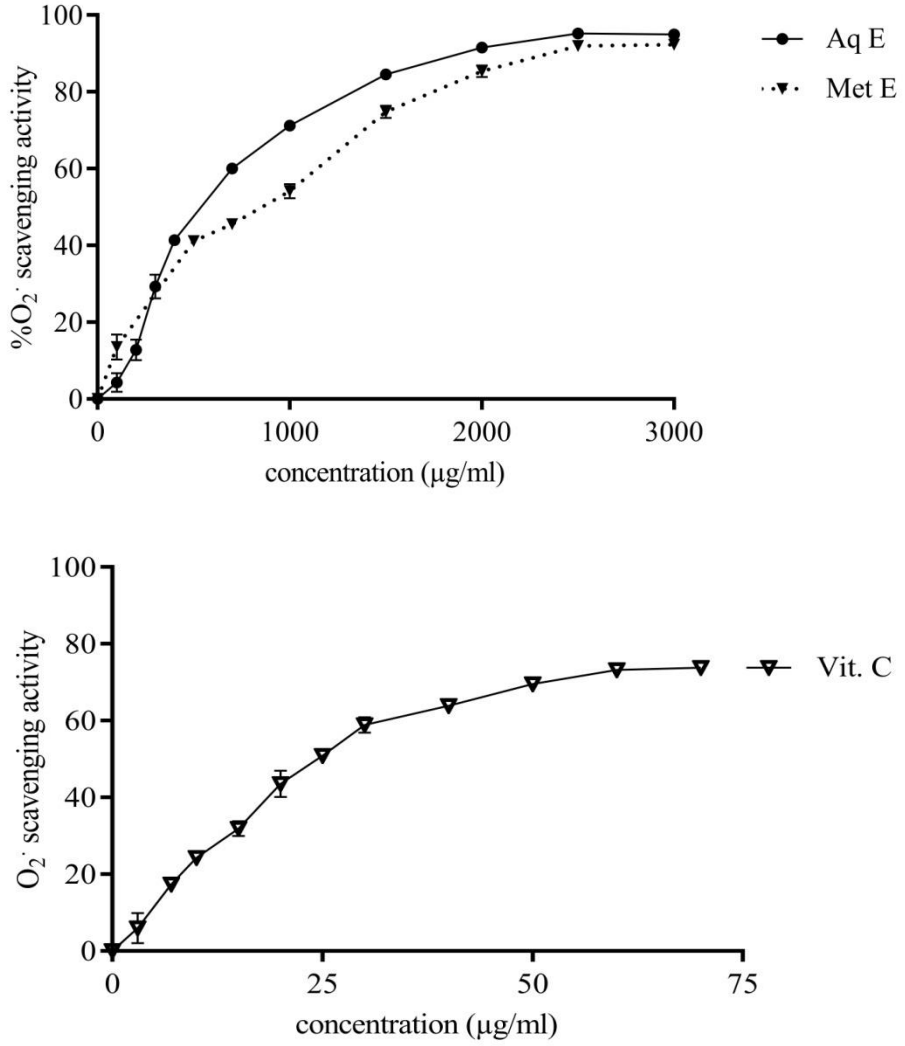

Figure 3. $O_2^{\cdot}$ scavenging activity of *E. plantagineum extracts* and ascorbic acid. The values are means±SD (n=3).

Hydrogen peroxide scavenging activity

Hydrogen peroxide is not a free radical itself, but leads to free radicals formation like $OH^{\cdot}$, which then do the damage [20]. The principle of the used test is based on the formation of a red-orange ferrous ions (Fe^{+2})-1,10-phenanthroline complex. The addition of H_2O_2 causes oxidation of all ferrous ions to ferric ions (Fe^{3+}) unable of forming the red-orange complex. However, the addition of hydrogen peroxide scavenger to ferrous ions before adding H_2O_2 itself forbids the ferrous to ferric conversion, and the addition of phenanthroline yields the chromophore complex. Therefore, higher absorbance indicates higher hydrogen peroxide scavenging activity. Figure 4 represents the peroxide hydrogen scavenging activity of Aq E and Met E of *E. plantagineum*.

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Both extracts exerted a good hydrogen peroxide scavenging activity. However, the effect of Met E (IC_{50}=193.87µg/mL) was better than the effect of Aq E (IC_{50} =262.46µg/mL). Standard antioxidant (ascorbic acid) was better than both of them (IC_{50} = 51.93µg/mL). In a study carried on by Bekhradnia and Ebrahimzadeh [24], polyphenolic extract of *Echium amoenum* petals exhibited better hydrogen peroxide scavenging activity (IC_{50}= 110.5 µg/mL), but still close to that of *E. plantagineum* extracts. Methanolic extract of another member of Boraginaceae family, *Trichodesma zeylanicumm*, showed a scavenging activity of hydrogen peroxide also close to that found with *E. plantagineum* extracts [25].

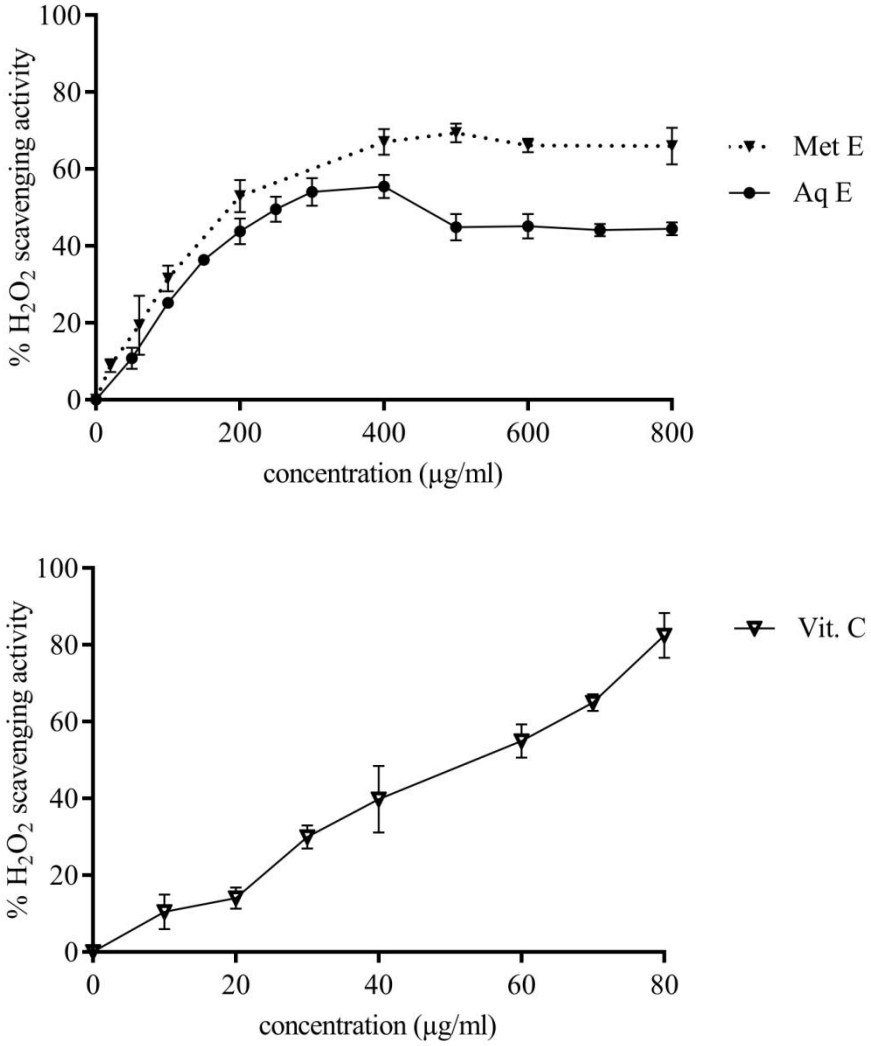

Figure 4. H_2O_2 scavenging activity of *E. plantagineum* extracts and vitamin C. The values are means±SD (n=3).

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Ferrous ions chelating activity

Ferrous ions chelating test is based on the ability to form a complex with Fe^{2+} leading to a decrease in the amount of Fe^{2+}-ferrosine red complex, therefore a decrease in absorbance [14]. Fe^{2+} catalyzes the decomposition of hydrogen or lipid peroxide into the highly reactive and biologically damaging hydroxyl radical. Thus, the ability of an antioxidant to chelate Fe^{2+} or other transition metal in human body is such important property to measure [26]. Methanolic and aqueous extracts of *E. plantagineum* exerted a ferrous ions chelating activity which increased with increasing concentration (Fig. 5). However, Aq E showed more activity than Met E with IC_{50} values of 164.91µg/mL and 247.57µg/mL, respectively. These values are lower than that obtained with EDTA, known to be a very powerful chelator. Bekhradnia and Ebrahimzadeh [24] have reported that polyphenolic extract of *Echium amoenum,* from Boraginaceae, showed more activity in iron chelating assay. However, the ethanolic extract of another Boraginaceae belonging species, *Cordia retusa,* showed less activity [23].

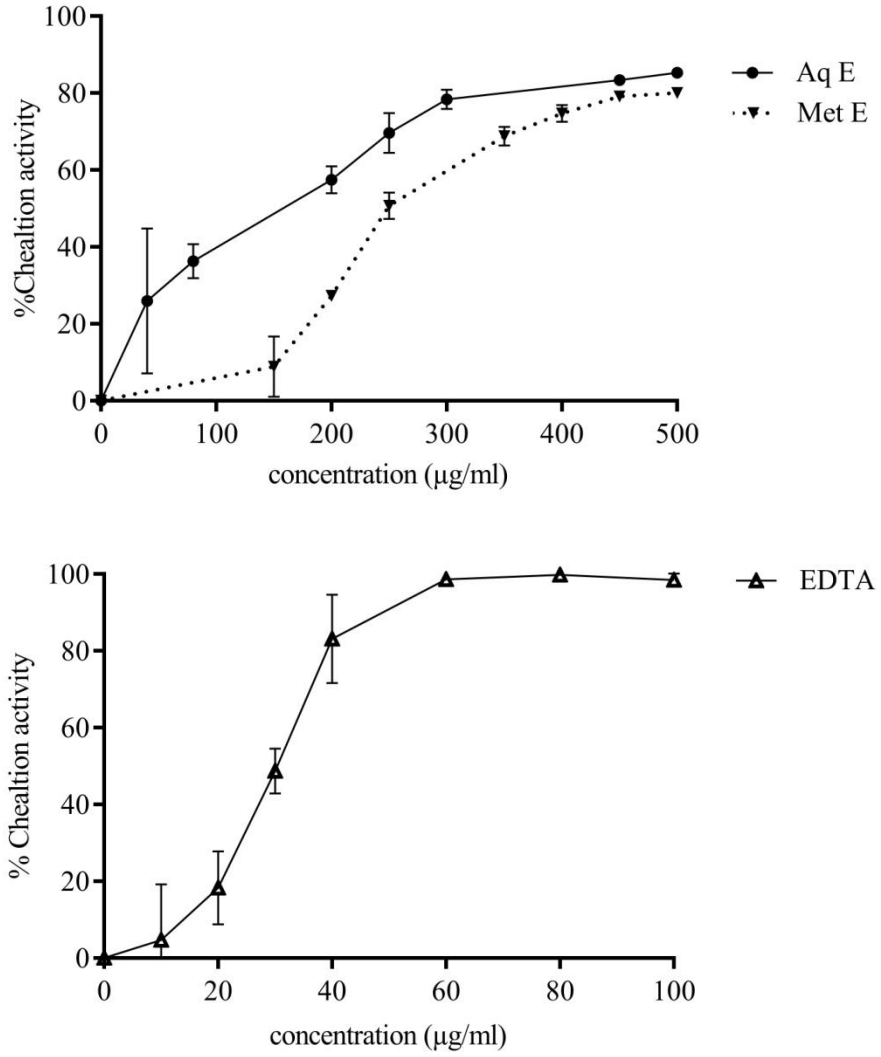

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Figure 5. Chelation activity of *E. plantagineum* extracts and chelator standard (EDTA).The values are means±SD (n=3).

Reducing power

Reducing power test consists of direct reduction of $Fe^{3+}(CN^{-})^{6}$to $Fe^{2+}(CN^{-})^{6}$ leading to an increase in the amount of the red complex $Fe_4^{3+}[Fe^{2+}(CN^{-})_6]^{3+}$, and consequently an increase in absorbance [14]. The reductive activity of aqueous and methanolic extracts of *E. plantagineum* compared with BHT has been illustrated in Figure 6. Met E and Aq E exerted similar and remarkable reducing activity with IC_{50} values of 117µg/mL and 128 µg/mL, respectively. This reductive activity remains less important than that of BHT (IC_{50}= of 61.29µg/mL), but much stronger than that of Aq E and Met E of *Echium humile* aerial part [18]. The results of the reducing power test as well as those of iron chelating test indicate that both *E. plantagineum* extracts can deal with ferrous ions either by binding (chelating) them or reducing them.

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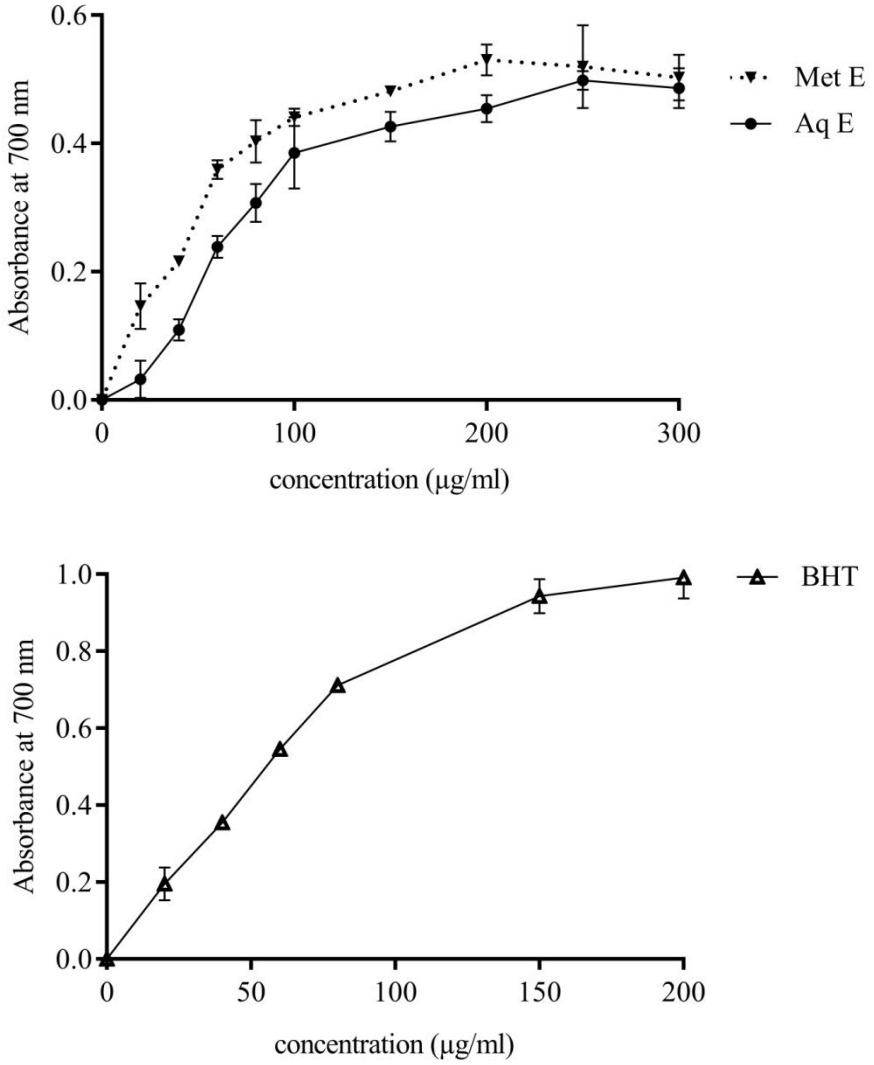

Figure 6. Reducing power of *E. plantagineum* extracts and BHT. The values are means ± SD (n=3).

Blood total antioxidant capacity

The ability of the extracts to protect biological membranes against radical attacks was elucidated in the present study using the AAPH test. The decomposition of AAPH in physiological environment to alkyl radicals (R·) can form peroxyl radicals (ROO·) in presence of oxygen. These lasts will attack erythrocytes lipids and proteins to induce the oxidation chain, disturbing membranes organization and eventually leading to hemolysis [26, 27]. The obtained sigmoid hemolysis curves (figure 7) show that both of Aq E and Met E could be the raison of a delay of red blood cells hemolysis, which appears in a shift of the curves to the right. The HT_{50} values (Figure 8) show that Aq E exhibited a strong anti-hemolytic activity (HT_{50}=161.62±5.87 min) at

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150 µg/mL, statistically similar to that of Trolox ($HT_{50=}$ 153.17±3.95 min), when Met E was more efficient ($HT_{50=}$179.79±5.82 min) than both of Trolox and Aq E at the same concentration. This can be explained by the fact that the extracts may contain molecules such as polyphenolic compounds able to either inhibit AAPH radical formation or penetrate the lipid bilayer of the red blood cells enhancing their integrity, and consequently their resistance against radicals aggression.

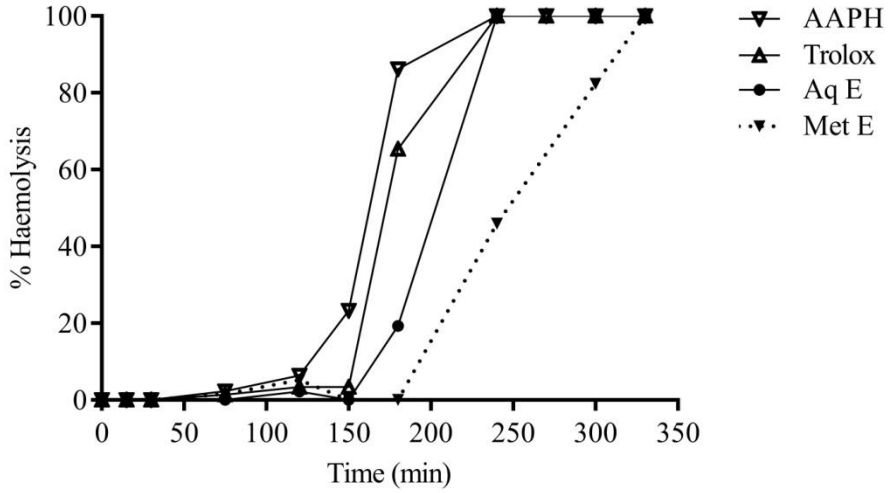

Figure 7. Kinetics of the red blood cells hemolysis caused by AAPH. The tested concentration of aqueous and methanolic extracts of *E. plantagineum* as well as Trolox is 150µg/mL. The negative control contains only AAPH. The values are means ± SD (n= 3**).**

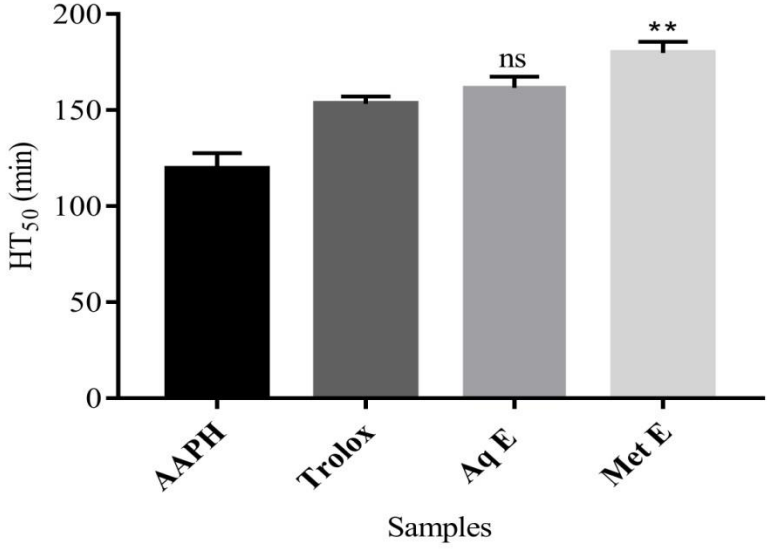

Figure 8. Half-Hemolysis Time (HT50) of *E. plantagineum* extracts and Trolox (150µg/mL). Values are expressed as means±SD. **P<0.01, ns: not significant versus the standard (Trolox).

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CONCLUSION

In the present study, the antioxidant activity of aqueous and methanolic extracts of *E. plantagineum* was evaluated through several antioxidant tests. Results revealed that both extracts acted as good antioxidants in different ways, which can be explained by their important phenolic content. Therefore, this plant could be a promising source of natural antioxidants considered as good alternatives for synthetic ones.

ACKNOWLEDGEMENTS

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MESRS) [grant number D01N01UN190120190004 Project].

STATEMENT OF CONFLICT OF INTEREST

Authors declare no conflict of interest.

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