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TOPIC

Antioxidant activities and anti-inflammatory effects of *Ammodaucus leucotrichus* Coss. & Durieu extracts

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Dedication

To my family that's who and what I look forward to

My ultimate thanks are dedicated to my beloved parents; **Ahmed LAROUI** and **Soumia DRISS**; for their endless love, and prayers. I am truly thankful and proud to have you as my parents. I ask ALLAH to guards them and bless them with good health

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“AND **WHOEVER RELIES UPON ALLAH**

THEN **HE IS SUFFICIENT FOR HIM”**

Quran 65| Vol. 3

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Haifaa LAROUI

الملخص

يستخدم الكمون الصوفي (*Ammodaucus leucotrichus* Coss. & Durieu) (Apiaceae)) تقليديا في جنوب الجزائر لتخفيض مستوى السكر إلى جانب مجموعة واسعة من الاضطرابات الفسيولوجية بسبب خصائصه المعززة للصحة. تهدف هذه الدراسة إلى التحقق من صحة الاستعمالات التقليدية لهذا النوع من خلال التقييم المختبري وفي الجسم الحي للقدرات المضادة للأكسدة وللالتهاب لمستخلص ميثانولي للنبتة وأجزائه، بالإضافة إلى تقييم جانب الأمان في استخدام هذا النبات. نقعت بذور النبات في الميثانول، تم بعد ذلك فصل مكونات المستخلص الخام (CrE) باستعمال مذيبات عضوية مختلفة القطبية للحصول على: مستخلص الكلوروفورم (ChE)، مستخلص إيثيل الأسيتات (EAE) والمستخلص المائي (AqE). ضمن هذه المستخلصات، تم تسجيل أعلى محتوى من عديدات الفينول والفلافونويدات لدى المستخلص الخام (77.14 ± 0.01ميكروغرام مكافئ غرامي حمض الغاليك/مغ من المستخلص و19.59 ± 0.08 ميكروغرام مكافئ غرامي كارستين/مغ من المستخلص، على التوالي) .تم تحليل CrE ومستخلصاته، من الجانبين النوعي والكمي، فيما يتعلق بمركباتهم الفينولية بواسطة HPLC-ESI-MS/MS. أشارت النتائج إلى أن أحماض الكافييك والفيروليك والفانيليك هي الأحماض الفينولية الرئيسية إلى جانب كيمبفيرول كفلافونول. تم تقييم النشاط الإزاحي في المختبر باستخدام $DPPH^{\bullet}$، $ABTS^{\bullet+}$، H_2O_2، $OH^{\bullet}$، $O_2^{\bullet-}$ و$NO^{\bullet}$. تميز EAE بكونه الأكثر نشاطًا وفعالية تجاه الجذور الحرة. في الوقت نفسه، أظهر كلا من EAE وChE نسبة تثبيط عالية في مرحلة مبكرة من الأكسدة المقترنة بحمض البيتا كاروتين/حمض اللينوليك بنسبة 81.99 ± 2.942% و78.24 ± 1.46% على الترتيب. أظهر هذان المستخلصان حماية ملحوظة لحمض اللينوليك تجاه فوق أكسدة الدهون حتى بعد 96 ساعة، حيث كانت قيم امتصاصية MDA منخفضة جدًا. أظهر اختبار استخلاب المعادن أن AqE هو المستخلص الأكثر فعالية. في حين، و حسب اختبار القدرة الإرجاعية، تميزمستخلص EAE بأعلى نشاط مع EC_{50} قدره 0.12 ± 0.002 ملغم/مل. قامت جميع المستخلصات بحماية، بشكل فعال، كريات الدم الحمراء للفئران ضد انحلال الدم الناجم عن AAPH بطريقة مرتبطة بالتركيز. وبالمثل، عمل EAE وCrE على تفكيك الجلطة المتكونة بنسبة متقاربة 19.93% و19.92% على الترتيب، يعكس هذا نشاطهما المضاد للتخثر. بينما أظهر EAE وChE تأثيرا مضادا لتخثرالدم الجزئي حتى بعد 3 ساعات من الملاحظة. ثبطت جميع المستخلصات المختبرة إنزيم α-amylase بشكل ملحوظ. إذ أظهر EAE أفضل نشاط تثبيطي بـ 0.63 ± 0.003 ملغرام مكافئ اكربوز/غرام من المستخلص ضد α-amylase و 1.31 ± 0.037 ملغرام مكافئ اكربوز/غرام من المستخلص ضد α-glucosidase. قام CrE ومستخلصاته بحماية بنية HSA بشكل فعال في الظروف الحرارية حتى عند التركيز المنخفض (0.31 ملغرام/مل). سمح إعطاء 200 مغ/ كغ من المستخلص الخام عن طريق الفم بتثبيط الألم الناجم عن حمض الأسيتيك وتقليل تكوين الوذمة الناجم عن xylene وcarrageenan. كما بين الفحص المجهري للالتهاب الحاد الناجم عن croton oil أن تثبيط الوذمة مرتبط بالجرعة. من جانب آخر، إنخفضت مستويات النتريت (NO^{2-}) و MDA مع استعادة نشاط CAT، نتيجة لإعطاء CrE. وفقاً للمبادئ التوجيهية لمنظمة OECD، تم متابعة السمية الحادة باختبار جرعتين؛ 2 غ/كغ و5 غ/كغ حيث لم تلاحظ أي علامات تغير في سلوك الحيوانات أو الوفيات مما يشير إلى أن CrE مادة غير سامة. أكدت السمية تحت الحادة سلامة استخدام CrE بجرعات متكررة (100 و200 و400 مغ/كغ) يومياً بسبب عدم وجود علامات سمية فيما يتعلق بوزن جسم الحيوانات والوزن النسبي للأعضاء. بالإضافة إلى ذلك، أظهر التحليل النسيجي عدم وجود تغيرات مرتبطة بالعلاج في أقسام الكبد والكلى. أدى إعطاء CrE إلى خفض مستويات MDA و NO^{2-} وتحسين الحالة الخلوية لمضادات الأكسدة الأنزيمية من خلال تعزيز أنشطة SOD و CAT و GPx و GST في الكبد والكلى. وفي

الختام، أكدت هذه الدراسة اعتبارالمركبات النشطة بيولوجيا، خصوصا عديدات الفينول والفلافونويدات، لنبات *Ammodaucus leucotrichus* ، كعوامل مضادة للأكسدة ومضادة للالتهاب آمنة مدعمة بذلك استخدام هذا النبات في الطب الشعبي.

الكلمات المفتاحية: *Ammodaucus leucotrichus*؛ عديدات الفينول؛ HPLC؛ الاكسدة؛ مضادات الأكسدة؛ مضاد التهاب؛ السمية.

Abstract

Ammodaucus leucotrichus Coss. & Durieu (Apiaceae) is traditionally used in southern Algeria to decrease blood glucose levels along with a wide range of physiological disorders due to its health-promoting properties. This study aims to validate the traditional use of this species through *in vitro* and *in vivo* evaluation of antioxidant and anti-inflammatory potentials of plant methanolic extract and its fractions, together with assessment of the safety profile. Plant seeds were subjected to methanolic maceration followed by liquid-liquid scale extraction using solvents of different polarity to afford: crude extract (CrE), chloroform extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). Among these extracts, the highest contents of polyphenols and flavonoids were recorded in the crude extract (77.14 ± 0.01 µg GAE/mg E and 19.59 ± 0.08 µg QE/mg E, respectively). CrE and its fractions were qualitatively and quantitatively analyzed regarding their phenolic compounds by HPLC-ESI-MS/MS. Results indicated that caffeic, ferulic and vanillic acids were the major phenolic acids alongside with flavonol kaempferol. Anti-radical activity was assessed *in vitro* using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, $OH^{\bullet}$, $O_2^{\bullet-}$ and $NO^{\bullet}$. EAE was the most active and potent radical scavenger. Meanwhile, EAE and ChE showed a high inhibition percentage at an early stage of β-Carotene/linoleic acid coupled oxidation with 81.99 ± 2.942% and 78.24 ± 1.46 %, respectively. These two fractions showed remarkable protection of linoleic acid against lipid peroxidation even after 96 h, where MDA absorbance values were very low. Metal chelating showed that AqE was the effective extract. However, in ferric reducing ability, EAE performed the best reducing activity with EC_{50} of 0.12 ± 0.002 mg/ml. All extracts effectively protected mice erythrocytes against AAPH-induced hemolysis in a concentration-dependent manner. EAE and CrE similarly dissociated the formed clot with a comparable percentage of 19.93% and 19.92%, respectively, reflecting their antithrombotic activity. While EAE and ChE showed partial blood clotting even after 3 h of observation. All plant extracts performed a noticeable α-amylase inhibition. Where EAE showed the best inhibition activity with 0.63 ± 0.003 mg ACAE/g E against α-amylase and 1.31 ± 0.037 mg ACAE/g E against α-glucosidase. CrE and its fractions effectively protected the HSA structure in thermal conditions even at low concentration (0.31 mg/ml). Oral administration of 200 mg/kg of crude extract successfully inhibited acetic acid-induced nociception and reduced edema formation induced by xylene and carrageenan. However, a dose-dependent manner was observed to decrease ear edema by a microscopic examination in croton oil-induced acute inflammation. Nitrite (NO^{2-}) and MDA levels were decreased together with restoration of catalase activity by

CrE administration. According to OECD guidelines, acute toxicity was performed at two limit tests; 2 g/kg and 5 g/kg where no signs of changes in animals' behavior nor mortality were observed indicating CrE as a non-toxic substance. Sub-acute toxicity confirmed the safety use of CrE at repeated doses (100, 200 and 400 mg/kg) on a daily basis by lacking toxic signs regarding animals' body weight and organs relative weight. In addition, histological analysis showed the absence of treatment-related changes in livers and kidney sections. Administration of CrE decreased MDA and NO^{2-} levels and improved the cellular status of enzymatic antioxidants through enhancement of SOD, CAT, GPx and GST activities in the liver and kidney. To conclude, this study corroborated those bioactive compounds, mainly polyphenols and flavonoids, from this plant as antioxidant and anti-inflammatory agents with a safe profile and supports the use of this plant in folk medicine.

Keywords: *Ammodaucus leucotrichus*; polyphenols; HPLC; oxidative stress; antioxidant; anti-inflammatory; toxicity.

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List of abbreviations

2Cys-PRDX: 2-Cystiene-peroxyredoxin

AD: Alzheimer's disease

AlE: *Ammodaucus leucotrichus* extracts

ALS: Amyotrophic lateral sclerosis

Ammodaucus leucotrichus: *A. leucotrichus*

AqE: Aqueous extract

ARE: Antioxidant responsive element

AT: Antithrombotic

BCT: Blood clotting time

CAT: Catalase

ChE: Chlorofom extract

COX: Cyclooxygenase

CrE: Crude extract

DAMPs: Damage-associated molecular patterns

EAE: Ethyl acetate extract

EC: Electrophilic compound

EC-SOD: Extracellular superoxide dismutase

FIA: Flow injection analysis

FICA: Ferrous ion chelating assay

FRA: Ferric reducing ability

G^+: Guanine radical cation

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Glutathione disulfide

GST: Glutathione S transferase

HAT: Hydrogen atoms transfer

HD: Huntington's disease

HE: Hexane extract

HNE: 4-hydroxyalkenals

HPLC-ESI-MS: High Performance Liquid Chromatography-electrospray ionization coupled with mass spectrometry

ICAM-1: Intercellular adhesion molecule-1

iNOS: Inducible nitric oxide synthase

Keap1: Kelch-like erythroid CNC homologue (ECH)-associated protein 1

LAS EZ: Leica Application Suite EZ

LPO: Lipid peroxidation

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

MRM: Multiple reaction monitoring

NF-κB: Nuclear factor kappa B

NOSs: Nitric oxide synthases

NOXs: Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

Nrf2: Nuclear factor erythroid 2 p45 related factor

NSAIDs: Nonsteroidal anti-inflammatory drugs

OECD: Organization of Economic Co-operation and Development

PAMPs: Pathogen-associated molecular patterns

PD: Parkinson's disease

PHE: Public Health England

RBC: Red blood cells

RBrS: Reactive bromine species

RClS: Reactive chlorine species

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RSS: Reactive sulfur species

SET: Singlet electron transfer

SOD: Superoxide dismutase

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TBARS: Thiobarbituric acid-reactive substances

TFC: Total flavonoid content

TNF-α: Tumor necrosis factor α

TPC: Total polyphenol content

TRD/TRX: Thioredoxin reductase/thioredoxin

VCAM-2: Vascular cell adhesion molecule-1

XO: Xanthine oxidase

List of publication and conference contribution

Publication

1. **Laroui, H.**, Guemmaz T., Zerargui, F., Saffidine K., Guenifi S., Arrar L., Khennouf S., Zengin G., Baghiani A. (2024). Antioxidant and anti-inflammatory potentials of *Ammodaucus leucotrichus* Coss. & Durieu seeds' extracts: *In vitro* and *in vivo* studies. Journal of Ethnopharmacology, 336: 117964.
2. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Trabsa H., Arrar L., Baghiani A. (2023). Polyphenol content, antioxidant, antihemolytic and anticoagulant potentials of *Ammodaucus leucotrichus* seed extracts. Tropical Journal of Pharmaceutical Research, 22 (6): 1237-1246.

Conference contribution

International

1. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Baghiani A. Radical Scavenging Ability and Ferrous Ion Chelating Capacity of *Ammodaucus leucotrichus* Seeds Extracts: *In Vitro* Study. 5th international conference on life and engineering sciences (ICOLES). May 19-22, 2022. Antalya, turkey. Oral presentation.
2. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Baghiani A. Investigation of ferric reducing ability; antithrombotic and anticoagulant potentials of *Ammodaucus leucotrichus* seeds extracts. International conference on basic sciences and technology (ICBST). November 16-19, 2022. Antalya, Turkey. Poster presentation.
3. **Laroui, H.,** Guemmaz, T., Zerargui, F., Saffidine, K., Houchi, S., Azzi, N., Belabas, N.H., Douffa, Y., Zengin, G., Baghiani A. Antioxidant acitivity, enzyme inhibition and short term toxicity assessment of *Ammodaucus leucotrichus* seeds' extracts: *In vitro* and *in vivo* studies. 3rd International scientific conference on plant biodiversity and sustainability (ISCPB&S). May 21-22, 2024. Chieti, Italie. Oral presentation.

National

1. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Baghiani A. Antioxidant activity evaluation of *Ammodaucus leucotrichus* extracts. 1er séminaire national sur l'évaluation des activités biologiques des plantes médicinales et docking moléculaire (SNABPMDM-1). 22 March 2022. University Batna 2, Algeria. Poster presentation.
2. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Baghiani A. Hydroxyl radical scavenging activity and ferrous iron chelating capacity of *Ammodaucus leucotrichus* seeds extracts. 1er séminaire national sur la valorisation des ressources naturelles et de l'environnement (VRNE). 30 March 2022. University Setif 1 -Ferhat ABBAS. Setif, Algeria. Oral presentation.
3. **Laroui, H.**, Zerargui, F., Saffidine K., Guemmaz T., Baghiani A. Antioxidant and anti-hemolytic properties of *Ammodaucus leucotrichus* seed's extracts. National Seminar on Phytotherapy and Pharmacognosy (NSPP). March 14-15, 2023. Setif, Algeria. Poster presentation.

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References

Introduction

Secondary metabolites namely, radicals and non-radical species, generation takes place in many organs (mitochondria and endoplasmic reticulum) from physiological redox reactions. At low and moderate levels, these reactive species serve as critical molecules involved in the maintenance of several physiological functions; cell growth, proliferation, initiation and propagation of inflammation as signaling and effecter molecules. Paradoxically, their supraproduction alter the redox equilibrium and oxidative stress occurs. The latter makes a bridge connection with the propagation of inflammation.

Alterations that arise from oxidative stress or from inflammation occur in many ways. Damaging cell membrane via hydroxyl radical attack causing lipid peroxidation, and as consequence toxic breakdown products arise such as malondialdehyde (MDA) that reacts covalently with DNA and proteins causing more serious damage. Unregulated or overproduction of nitric oxide causing DNA damage, protein and lipid oxidation through attribution of peroxynitrite cytotoxicity thus leading to either cellular apoptosis or necrosis (Radi, 2018). To make matters worse, the reduction of antioxidant enzymes including superoxide dismutase and catalase to immobilize the protective defense system (Bezerra *et al.*, 2023). These scenarios make a substantial contribute to many physiological disorders, which leads to several pathological diseases including cancer, diabetes, arthritis rheumatoid, cardiovascular and neurodegenerative disorders (Ward, 2018; Wang *et al.*, 2021; Feng *et al.*, 2023).

Among the investigated therapeutics strategies in use to overcome the overwhelming oxidative injuries is the involvement of antioxidants. Natural antioxidants are the main targets in this field and have been investigated extensively up to date. Plants are considered as a major source of bioactive compounds. Plant-derived polyphenols and flavonoids have been

reported to exhibit wide range of biological potentials use as anti-inflammatory and antioxidant activities with fewer side effect and better tolerance.

Ammodaucus leucotrichus Coss. & Durieu, locally called "kammûn es-sofi", is small annual specie belonging to Apiaceae family, widely distributed in Sahara regions of North Africa including Algeria and tropical Africa (Abu Zarga *et al.*, 2013). Ethnobotanical research revealed that this plant is used by local population to treat diabetes mellitus and beside that rheumatism, asthma and gastro-intestinal pain, otitis media, excessive production of urine, and cardiac diseases (Abu Zarga *et al.*, 2013; Telli *et al.*, 2016; Idm'hand *et al.*, 2020).

This study fits in the context of improving human life and to corroborate the traditional claims of this species via the scientific validation: *in vitro* and *in vivo* evaluation of the antioxidant, anti-inflammatory activities and toxicity assessment.

Chapter 1: Literature Review

1. Oxidative stress

A basic principle of stress and stress responses were formulated by Selye (1936) and its biochemically link with health. In 1985, Sies coined the concept of oxidative stress as the disruption of redox equilibrium between the generation of oxidants within cells and tissues, and the antioxidant system in favor of oxidants, leading to signaling redox disruption and cellular damage (Zhang *et al.*, 2023a). Physiological low level of oxidants is significant for redox signaling, by targeting specific molecules for governing biological processes in living organisms and expressed by oxidative eustress (Sies, 2019). The oxidative burden due to production deviation of oxidants, excessive levels, illustrated by oxidative distress that causes biomolecules damage, as a consequence of higher exposures that overflows the specific targets during redox signaling (Sies, 2019). In both of these scenarios, redox homeostasis is achieved by countering oxidative challenge with adaptive stress response, which is the antioxidant defense systems (Sies, 2020). However, another tissue response may be expected during redox homeostasis failure, imbalance in favor of reactive species: tissue injury and cell death by triggering necrosis and apoptosis pathways thereby development of various diseases (Van lith and Ameer, 2016; Kumar *et al.*, 2023a).

1.1. Reactive species

Radical or nonradical chemical species are considered as reactive species with oxidant potential. These molecules contain one or more unpaired electrons in atomic or molecular orbitals, which endow a high reactivity to free radicals to abstract electrons from chemical species and oxidize them to attain their own stabilization (Milatovic *et al.*, 2019; Tafuri *et al.*, 2019). These molecules are classified according to their chemical structure and nature of the atom. Due to the widely various chemistries provided by biochemical precursors, such as oxygen and nitric oxide, there is a chemical diversity of reactive species: reactive oxygen

species (ROS), reactive nitrogen species (RNS), reactive chlorine species (RClS), reactive sulfur species (RSS) and reactive bromine species (RBrS) (**table 1**). However, both of ROS and RNS are considered as the most relevant ones (Böhm et al., 2023).

Table 1. Exemplary free and nonradicals of reactive oxygen, nitrogen, chlorine, sulfur and bromine species (Halliwell, 2006; Cortese-Krott *et al.*, 2020).

Free radicals		**Non-radicals**	
ROS		ROS	
	Superoxide anion, $O_2^{\cdot-}$ Hydroxyl radical, $OH^{\cdot}$ Peroxyl radical, $RO_2^{\cdot}$		Hydrogen peroxide, H_2O_2 Hydroperoxide, ROOH Singlet oxygen, $^1O_2$
RNS		RNS	
	Nitric oxide, $NO^{\cdot}$ Nitrogen dioxide, $NO_2^{\cdot}$ Nitrate radical, $NO_3^{\cdot}$		Peroxynitrite, $ONOO^-$ Nitryl chloride, NO_2Cl Nitrite, NO_2^-
RClS		RClS	
	Atomic chlorine, $Cl^{\cdot}$		Hypochlorous acid, HOCl Chlorine dioxide, ClO_2 Chlorine gas, Cl_2
RSS		RSS	
	Thiyl radical, $RS^{\cdot}$		Thiol, RSH Hydrogen sulfide, H_2S Disulfide, RSSR
RBrS		RBrS	
	Atomic bromine, $Br^{\cdot}$		Hypobromous acid, HOBr Bromine gas, Br_2 Bromine chloride, BrCl

1.1.1. Genesis of reactive oxygen and nitrogen species

Addition of one electron to the 5% of the molecular oxygen portion insides the cells, results in the formation of superoxide radical anion ($O_2^{\cdot-}$), which is an active source of free radicals, that is transformed into hydrogen peroxide (H_2O_2) by adding an extra electron and protons through

an enzymatic reaction (Rotariu *et al.*, 2022; Jomova *et al.*, 2023). Although, the majority of the superoxide radical is on its radical anion form, the protonated form, perhydroxyl radical ($HO_2^{\cdot}$), is present with 0.6% at physiological conditions (Jomova *et al.*, 2023). H_2O_2 can cross biological membranes, and its reactivity can be increased in the presence of transition metals species like Fe^{+2}/Cu^{+2} (Fenton reaction) or by the interaction with $O_2^{\cdot -}$ (Haber-Weiss reaction) to generates the most reactive radical, hydroxyl radical ($OH^{\cdot}$) (Rotariu *et al.*, 2022). Nitric oxide radical ($NO^{\cdot}$) is produced from the reaction of L-arginine and oxygen by the nitric oxide synthases (NOSs) family (Gupta *et al.*, 2016), and can easily combine spontaneously with the formed superoxide radical anion and slowly react with molecular oxygen, to generate highly reactive nitrogen specie peroxynitrite ($ONOO^-$) and nitrogen dioxide radical ($NO_2^{\cdot}$) (Gupta *et al.*, 2016; Milatovic *et al.*, 2019). H_2O_2, $O_2^{\cdot -}$ and $NO^{\cdot}$ react with few compounds, but participate in the generation of highly reactive species such as $OH^{\cdot}$ that reacts fast with almost everything in the organism, thus the deleterious effect is arised (**Figure 1**).

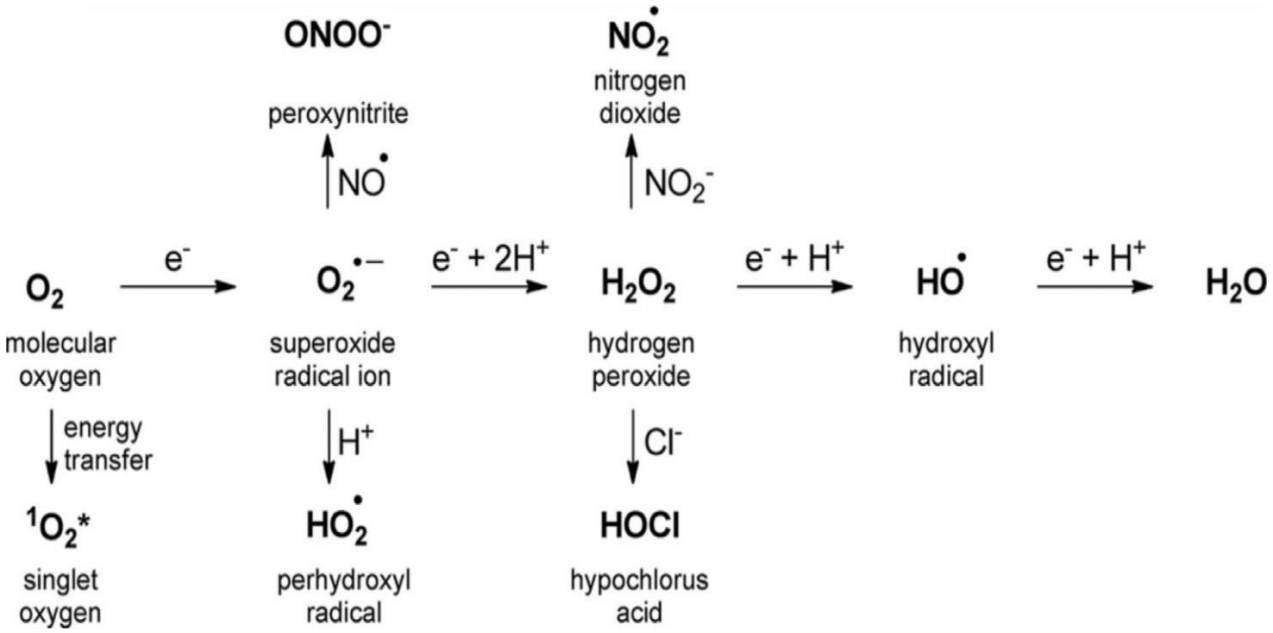


Figure 1. Electron transfer during reactive oxygen and nitrogen species generation (Mandal *et al.*, 2022).

1.1.2. Sources of reactive oxygen and nitrogen species

1.1.2.1. Exogenous sources

The total external triggers are denoted as Exposome (Sies, 2019), including a wide variety of oxidative challenges that participate directly or indirectly in the reactive species formation. Radiation, including Ionizing radiation, X-rays, neutrons, α, β and γ rays, and toxicity of chemotherapeutic agents (camptothecins, anthracyclines, epipodophyllotoxins and others) are responsible for the generation of high levels of ROS such as $OH^{\cdot}$ (Bhattacharyya *et al.*, 2014). Another significant generator of reactive species, cigarette smoke, which contains 10^{14}-10^{16} of various radicals ($^{\cdot}NO$, $RO_2^{\cdot}$…etc.) per puff, as well as more than 7000 chemical compounds and oxidative agents (Witschi, 2005; Yang *et al.*, 2008). Red meat contains a high level of iron, it consumption may induce the formation of ROS and RNS due to the increase of ferrous ion levels. In addition, high concentrations of alcohol (ethanol), generates ROS and induce NO synthesis (Padovan *et al.*, 2023).

1.1.2.2. Endogenous (Cellular) sources

Among the intracellular compartments, mitochondria, membrane-bound NADPH oxidases (NOXs) and peroxisomes are the major sites of ROS generations (Jomova *et al.*, 2023).

a. Mitochondrial respiratory chain

90% of ROS are produced through mitochondrial electron-chain transfer, where molecular oxygen is reduced by singlet electron transport to generate energy (adenosine triphosphate, ATP) via oxidative phosphorylation. In the inner membrane, enzymes based on cytochrome (complexes I-IV) and coenzymes that compose the mitochondrial respiratory chain, transfer electrons to reduce 95% of molecular oxygen without generation of reactive species (Tsutsui *et al.*, 2011; Kiran *et al.*, 2023). Electron leakage from transport carriers results in the reduction of 5% of molecular oxygen (O_2) thus the most crucial ROS is formed, $O_2^{\cdot -}$, which results in the rise of other reactive oxygen and nitrogen intermediates (Kiran *et al.*, 2023).

b. Membrane-bound NADPH oxidases (NOXs)

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is one of the main enzymes that generate ROS. Its various isoforms are present in different cell types such as colon epithelial cells and phagocytes (Böhm *et al.*, 2023). It is a multicomponent enzyme where after its activation (e.g. respiratory burst during phagocytosis); the Nox catalytic unit is denoted for the transfer of electron from NADPH to molecular oxygen to generate $O_2^{\cdot-}$ (Tsutsui *et al.*, 2011; Bhattacharyya *et al.*, 2014).

c. Peroxisomes

This cell organelle plays a key role in many of the metabolism pathways including pentose phosphate oxidative pathway, fatty acid oxidation, glyoxylate metabolism and amino acids catabolism (Wanders and waterham, 2006). During these events, ROS and RNS are generated through a chain of enzymes. Flavoproteins including acyl-CoA oxidases, polyamine oxidase and others (Fransen *et al.*, 2012) produce H_2O_2 and through Fenton reaction $OH^{\cdot}$ arises. Superoxide radical anion and nitric oxide are generated by two potential enzymes, xanthine oxidase (XO), using hypoxanthine and xanthine as substrates, and inducible nitric oxide synthase (iNOS), respectively (Fransen *et al.*, 2012).

Endoplasmic reticulum is another significant organelle contributor in ROS production (Ong and Logue, 2023). During disulfide bond formation and lipid metabolism, Endoplasmic reticulum oxidoreductase 1 and Cytochrome P450 generates $O_2^{\cdot-}$ and further H_2O_2 by using O_2 as an electron acceptor (Ong and Logue, 2023). Other cytosolic and plasma membrane enzymes contribute in simultaneous reactive species production in metabolic processes (lipid peroxyl radical, hypothiocyanite, $NO^{\cdot}$, $NO_2^{\cdot}$ HOCl, $O_2^{\cdot-}$, H_2O_2…etc.): monoamine oxidase, myeloperoxidase, lipooxygenase, cyclooxygenase and nitric oxide synthase family (neuronal, endothelial and inducible nitric oxide synthase) (Bhattacharyya *et al.*, 2014; Gupta *et al.*, 2016).

1.1.3. Physiological implications of reactive species

As previously mentioned in oxidative eustress, the low levels of reactive species participate as critical second messengers in the regulation of redox signaling pathways to initiate biological processes such as aging, apoptosis, pro-inflammatory signaling, cell growth and other range of physiological processes without triggering the damage of biomolecules (Checa and Aran, 2020). A wide range of redox-sensitive signaling pathways are modulated by reactive oxygen species, including the master regulators in eukaryotes for detoxification and inflammation, respectively: Nuclear factor erythroid 2 p45 related factor/Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Nrf2/Keap1) and nuclear factor kappa B (NF-κB) (Sies, 2020; Sies and Jones, 2020). For maintaining redox signaling, Nrf2/Keap1 master switch is activated in respond to various oxidants such as H_2O_2. Oxidative modification at $Cystein^{151}$ of Keap1 results in the release of the transcription factor Nrf2 from Keap1, allowing its translocation into the nucleus and binds to antioxidant responsive element (ARE) responsible for gene expression of antioxidant enzymes necessary for reactive oxygen detoxification (Sies and Jones, 2020). NF-κB is a critical switch that regulates inflammation. Its activation depends firstly on H_2O_2 and phosphorylation of the NF-κB inhibitor (IκB) kinase. Translocation of this transcription factor into the nucleus, results in the activation of pro-inflammatory mediators (cytokines, cyclo-oxygenase 2 and others) (Takada *et al.*, 2002; Checa and Aran, 2020).

1.1.4. Action of reactive species at supraphysiological level (imbalance)

Reactive species are two face characters, the first occurs with oxidative eustress and the second is their role in oxidative damage to biomolecules (DNA, proteins and lipids) when redox equilibrium is disrupted and characterized with high levels of radical species.

1.1.4.1. Oxidative damage to DNA

Nuclear and mitochondrial DNA is susceptible to reactive species attack at many levels: base oxidation, double stands breaks and repair system alteration and these modifications are involved in mitochondrial dysfunction, carcinogenesis, mutations, apoptosis and necrosis (Juan *et al.*, 2021). ROS reacts with deoxyribose and easily results in hydrogen abstraction from the carbon or methyl groups, resulting in ribose fragmentation (Cooke *et al.*, 2003). Guanine is the most susceptible to radical oxidation; loss of an electron generates guanine radical cation (G^{+}) (Zuo *et al.*, 2015). Furthermore, rupture of the nucleosomes alters the compaction and coiling of DNA within the chromatin (Juan *et al.*, 2021).

1.1.4.2. Oxidative damage to proteins and enzymes

Another critical damaging aspect of oxidative distress is alteration to the function and structural integrity of proteins, by oxidizing amino acids targets such as methionine and cysteine, causing the occurs of protein misfolding, fragmentation due to the peptide bond cleavage and aggregation (Böhm *et al.*, 2023). Thus, the loss of the catalytic activity of several enzymes is occurred and paralysis in the biological processes and metabolic pathways regulation.

1.1.4.3. Oxidative damage to lipids

Lipid peroxidation occurs when reactive species target lipids. Polyunsaturated fatty acids of lipid membranes are the most sensitive to oxidation, in reason of the higher number of double bonds and instability of hydrogen atom (Milatovic *et al.*, 2019). Abstraction of hydrogen atom from their methylene carbon in contact with a ROS oxidizing agent, results in the formation of numerous highly reactive intermediates such as malonaldehyde (MDA) and 4-hydroxyalkenals (HNE) (Kiran *et al.*, 2023). Lipid peroxidation alters a variety of biochemical pathways and results in a cellular damage due to the loss of membrane fluidity

and integrity (Juan *et al.*, 2021). As example, **figure 2** shows how many neurodegenerative diseases developed from the damage occur to DNA, proteins and lipids.

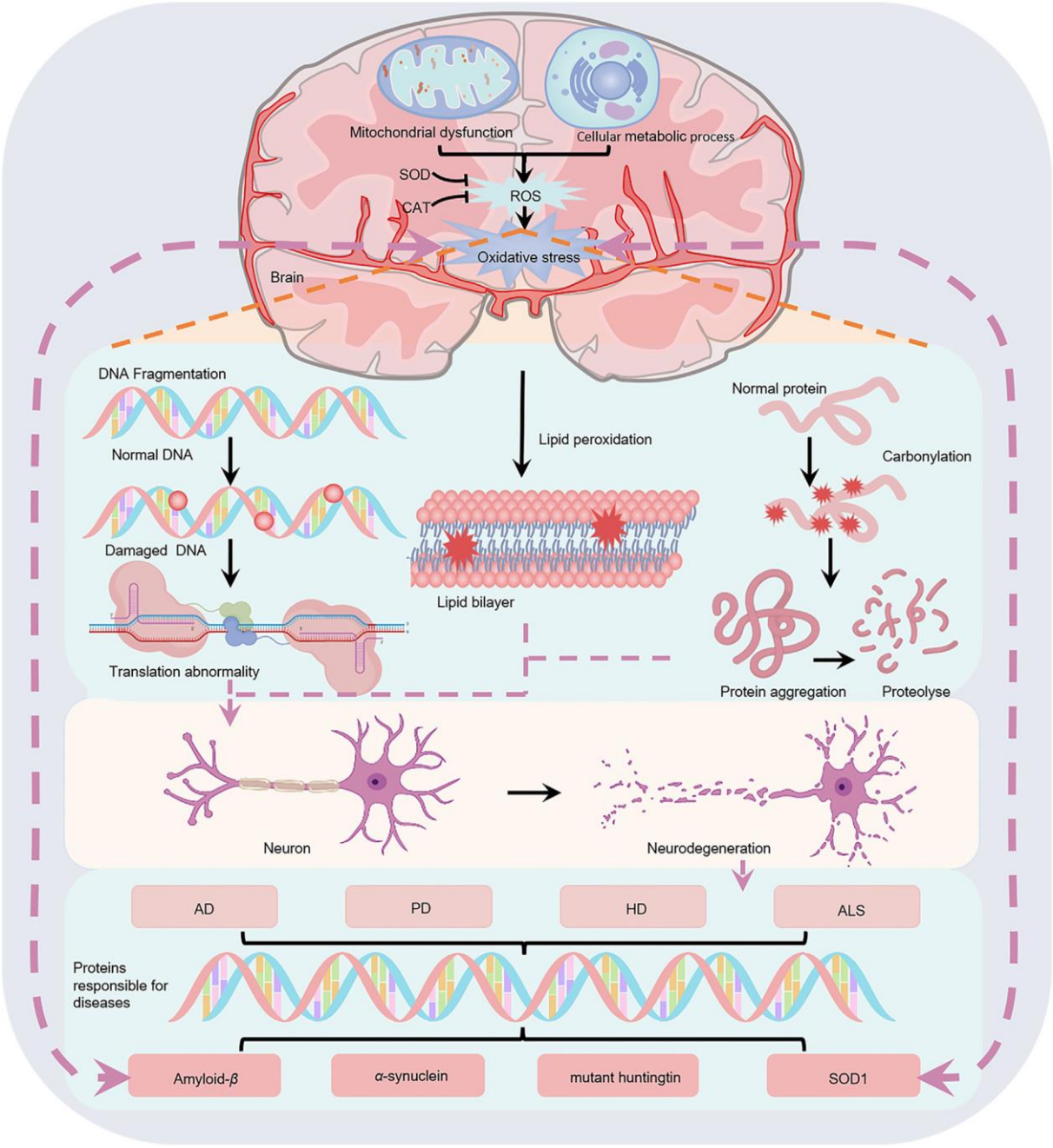


Figure 2. Important implication of oxidative stress in the pathogenesis of neurodegenerative illnesses. **CAT:** catalase; **SOD:** superoxide dismutase; Alzheimer's disease (**AD**), Parkinson's disease (**PD**), Huntington's disease (**HD**), amyotrophic lateral sclerosis (**ALS**) (Feng *et al.*, 2023).

2. Inflammation

The body uses inflammation as a defensive mechanism, a biological reaction of the immune system against cellular damage and numerous infectious agents and pathogenic insults, including bacteria and viruses as well as toxins and toxic substances (Chen *at al.*, 2018). The signal zeros: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) signal the presence of threat thereby triggers an inflammatory state (Burini *et al.*, 2020). The triggered inflammatory state by these harmful stimuli initiates a domino effect of different biochemical events. At tissue level, inflammation is characterized by the four classic symptoms: swelling, redness, heat, and pain (Takeuchi and Akira, 2010). However, at microscopic scale inflammation is orchestrated by intracellular signaling cascade that activates immune cells, which in turn generate and release inflammatory cytokines (Kiss, 2022). These cytokines are engaged in interactions and activation of various receptors, as consequence many signaling molecules, including NF-kB and mitogen-activated protein kinase (MAPK) are phosphorylated in response to receptor activation and signaling pathway regulated by ROS, thereby triggers the activation of variety of transcription factors needed for the maintenance of the inflammation (Chen *at al.*, 2018; Kiss, 2022). The amount of inflammatory mediators in resident tissue cells and the attracted inflammatory cells from bloodstream is controlled by this coordinated activation of signaling molecules. Inflammation is divided into two types based on the time course of inflammatory response: acute and chronic inflammation.

2.1. Acute inflammation

Acute inflammation is the immune system's early response to infections and tissue injury that lasts for few days, characterized by the four clinical features at tissue level (Sebire, 2010; Jain *et al.*, 2014). It is rapid and self-limiting process that contributes to the initiation of the healing process to restore the tissue homeostasis (Jain *et al.*, 2014; Kiss, 2022). This

biological process is mediated by three phases namely, vascular phase; cellular phase and resolution phase (Jain *et al.*, 2014).

2.1.1. Vascular phase

This first phase consists of vascular events include vasodilation which is responsible for heat and redness, together with the increase of vascular wall permeability, leading to elevation of blood flow and fluid penetration containing soluble mediators across the endothelium into the tissues leads to the formation of edema (Lordan *et al.*, 2019). All of these processes are triggered by lipid mediators such as prostaglandin and leukotriene, as well as vasoactive factors including histamines and bradykinin (Sansbury and Spite, 2016).

2.1.2. Cellular phase

The acute cellular response involves a highly coordinated network of many cell types. In this cellular event leukocytes marginate from the bloodstream and adherent to the endothelium due to the interaction with various expressed adherent molecules (selectins, integrins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-2) and others) (Jain *et al.*, 2014). In response to chemo attractants such as leukotrienes and cytokines, these immune cells migrate into the injured site resulting in the amplification and the sustaining of the acute inflammatory phase, through production of proinflammatory factors such as eicosanoids, interleukin-1 familly, tumor necrosis factor α (TNF-α) (Lordan *et al.*, 2019). Neutrophils are the first cells recruited and they are important mediators of the inflammatory response (Chen *et al.*, 2018). As the inflammation progress, macrophages and lymphocytes appear to assist in the neutralization of antigens. Platelets aggregate to the site of injury to stop the bleeding occurred from erythrocytes access by clot formation in rather to join tissue back together (Jain *et al.*, 2014).

2.1.3. Resolution phase

In the late phase of inflammation, tissue healing takes place to rectify tissue homeostasis and restore its architecture and prevent additional damage. During this event, chemokines and cytokines are counter-regulated and their gradients are diluted to eventually decrease the sensation of leukocytes thereby decreasing their recruitment to the injured site, apoptosis of spent immune cells and the formed clots are eliminated by fibrinolysis (Jain *et al.*, 2014; Chen *et al.*, 2018). In addition, capillaries are reduced and the collagen bundles increase in size to strength the wound (Jain *et al.*, 2014). Acute inflammation phases are illustrated in **figure 3** below.

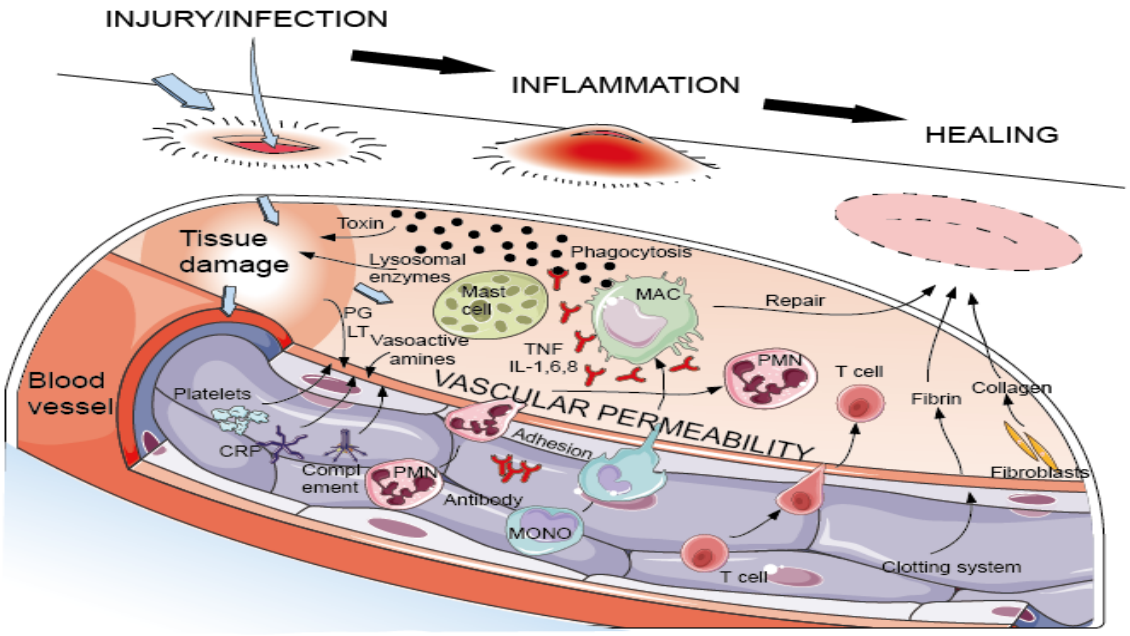


Figure 3. Schematic representation of acute inflammatory response mechanism.

2.2. Chronic inflammation

When acute inflammation is unable to stop the damage, innate and acquired immune responses persist in the engagement and inflammation becomes uncontrolled and reach the chronic state. Long-lasting, low-grade inflammation that lasts for several weeks, months, or even years is referred to as chronic inflammation (Wang *et al.*, 2021). It is morphologically

characterized by the presence of lymphocytes, macrophages, and plasma cells in tissues (Ward, 2018) (**figure 4**). It is believed that during this phase, macrophage activates fibroblasts to lay down collagen and activates other macrophages and lymphocytes. As results, an extensive scaring and collagenous scars and development of fibrosis take place as well as necrosis (Ward, 2018).

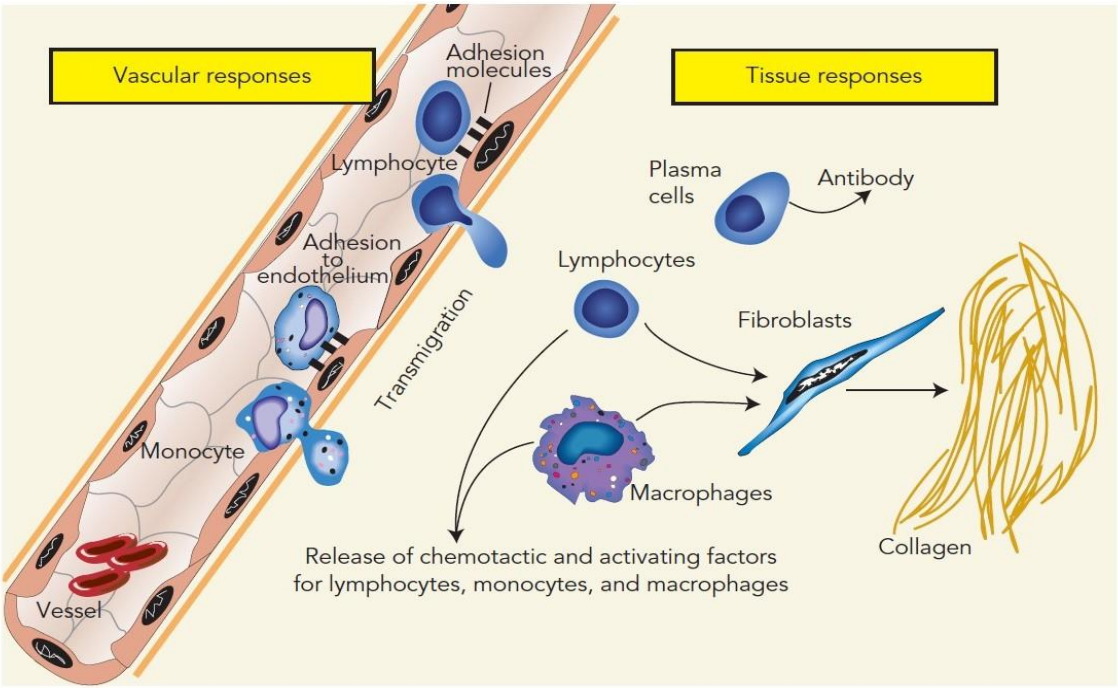


Figure 4. Mechanisms and cell types implicated during chronic inflammation (Ward, 2018).

An example of inflammation progression is that of *in vivo* introduction of biomaterials (Adjei *et al.*, 2016) as shown in **figure 5**; in which acute inflammation events take place on the biomaterial: coagulation cascade; complement activation and immune cells recruitment and adhesion to combat the foreign material. If the phagocytose of biomaterial is not achieved, macrophage fuse to form giant cell that produce ROS and proteolytic proteins to degrade the biomaterial and activates fibroblasts as consequence forms a fibrous capsule. These responses collectively induce pain and reduce the capacity and longevity of the biomaterial.

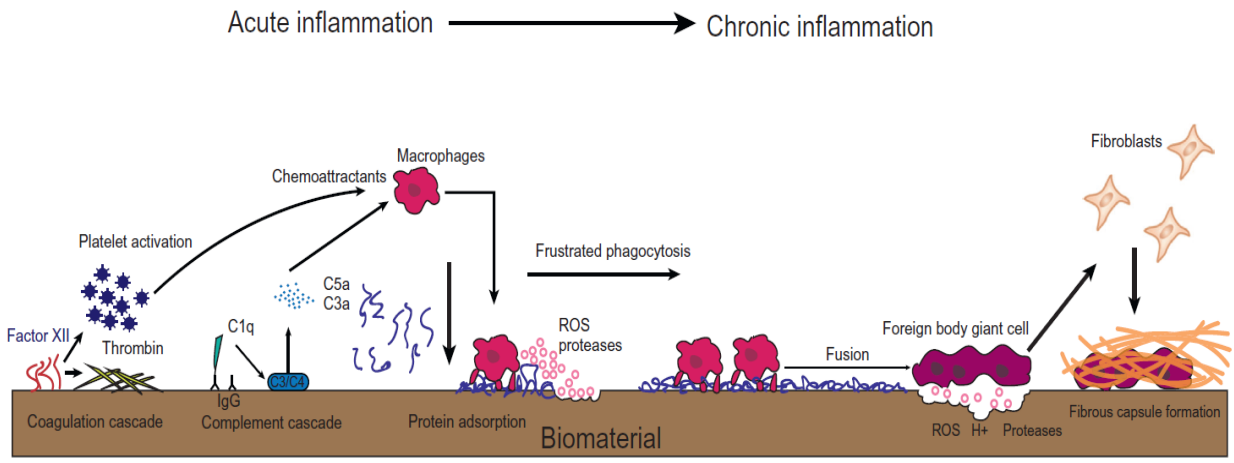


Figure 5. Schematic representation of immune response and progression of acute inflammation to chronic inflammatory state (Adjei *et al.*, 2016).

Inflammation is the most important parameter that alters the dynamic balance between health and illness. So far, chronic inflammation is linked with various ranges of diseases including cardiovascular diseases, atherosclerosis, rheumatoid arthritis, chronic allograft rejection, asthma, cancers and diabetes (Ward, 2018; Wang *et al.*, 2021).

2.3. Therapeutic interventions in inflammation

To suppress the inflammatory response in scenarios when it is desirable such as patient with bacterial meningitis or when inflammation represents a long-term threat (the case of biomaterial introduction or rejection of transplanted organ); it is mandatory to consider the principle of knowing the cause of inflammation or reactions causing serious functional problems to use the correct therapy. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the symptomatic treatment of certain acute or long-term conditions linked to pain and inflammation due to their cyclooxygenas (COX 1 and 2) inhibition (Ward, 2018). However, they poorly suppress the inflammation and have deleterious side effects such as acute renal dysfunction and necrosis and cardiovascular adverse effects (Harirforoosh *et al.*, 2013). Despite that corticosteroid therapy is significant effective in suppressing effects of the inflammatory response, their adverse effects are seriously damaging (osteoporosis, diabetes,

immune-suppression and others) (Liu *et al.*, 2013). In addition to molecular targeting therapy through blockage of proinflammatory mediators, inhibition of complement and adhesion-promoting molecules (Fattahi and Ward, 2016; Ward, 2018). Additionally, to the intervention of natural products including polyphenols due to their pharmacological capacities achieved by the engagement with board ranges of molecular targets (Wang *et al.*, 2021).

3. Diabetes mellitus

Glycaemic regulation is essential for balancing the energy demand by combining exogenous nutritional resources and internal storage. Long term low or elevated blood glucose levels are life threatening, leading to organs dysfunction. Diabetes is multifaceted metabolic disorder, being also chronic associated with oxidative stress and inflammation (Tsalamandris *et al.*, 2019; Rotariu *et al.*, 2022). It is characterized by chronic hyperglycemia caused by the deficiency of insulin resulting from the autoimmune destruction of pancreatic beta cells, the case of type 1 diabetes (T1D), or the insulin-resistance affected from environmental and genetic insults, as seen in type 2 diabetes (T2D) (Moini, 2019; Jomova *et al.*, 2023). The latter is the most common diabetes type that affects 90% of diabetic people according to the Public Health England (PHE) (PHE, 2014).

3.1. "Insulin resistance-hyperglycemia-oxidative stress" cycle

The state of oxidative stress in diabetic patients is responsible for the diabetic complications. Where, elevation of oxidative stress biomarkers such as MDA and the decrease in enzymatic antioxidants activity (SOD, CAT, glutathione peroxidase (GPx) and the non-enzymatic reduced glutathione (GSH) are correlated with diabetes mellitus (Zhang *et al.*, 2023b). The targeted attack of ROS to pancreatic β-cells results in insufficient insulin secretion (Zhang *et al.*, 2023b). Energy generation from glycolysis within mitochondria is accompanied with H_2O_2 production. Hyperglycemia that leads to excessive accumulation of glucose in the cells, puts mitochondrial antioxidants enzymes in encountering of high increase of $O_2^{\cdot-}$, $OH^{\cdot}$ and

H_2O_2 levels while these enzymes are not sufficient to neutralize them thus triggering oxidative stress (Rizwan *et al.*, 2019). Later, oxidative stress disrupts the function of mitochondria and provokes insulin-resistance (Black, 2022). The burden of the cumulative glucose alters mitochondria's gene expression and lead to oxidative stress and vice-versa. These scenarios form the deleterious "insulin resistance-hyperglycemia-oxidative stress" cycle (**figure 6**).

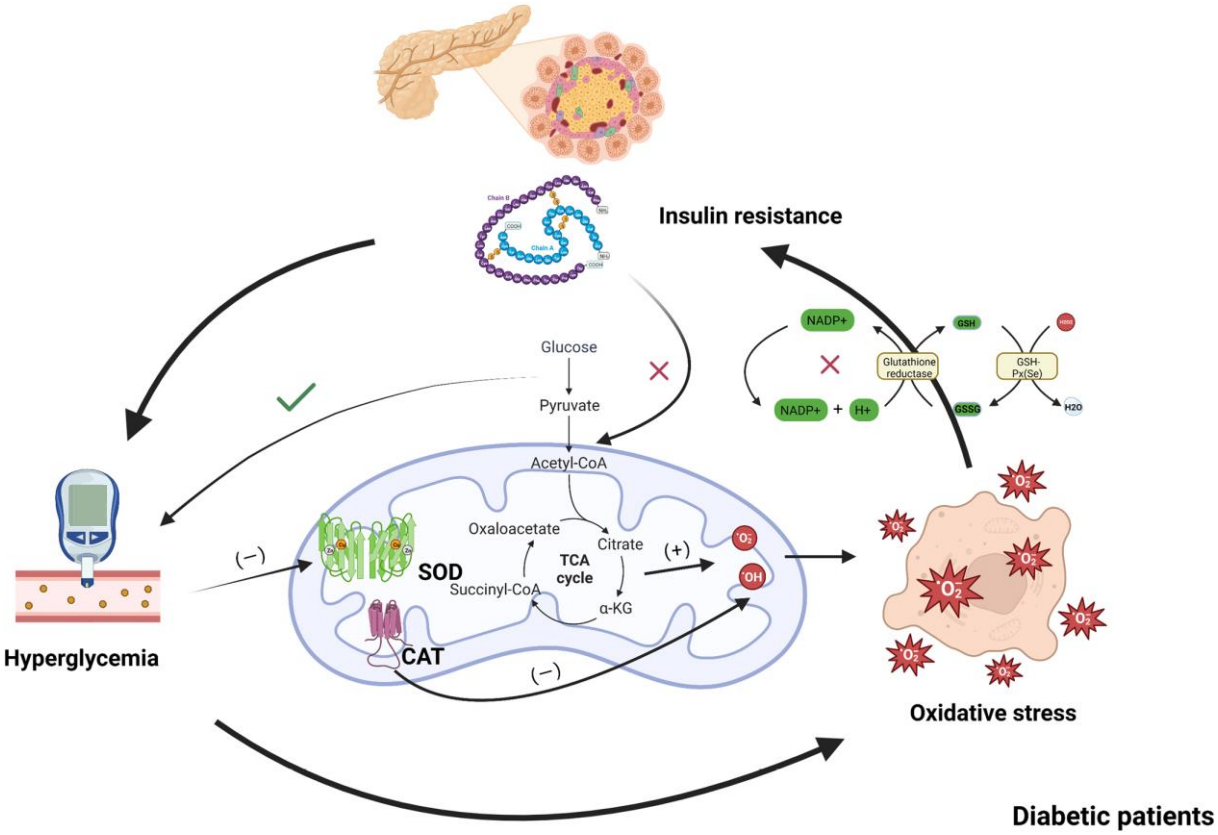


Figure 6. The "insulin resistance-hyperglycemia-oxidative stress" cycle implicated in diabetic patients (Zhang *et al.*, 2023).

The advancement of immunology field has been accompanied with understanding the pathophysiology of diabetes. Through the course of T1D and T2D, inflammation is highly related where many outlined studies found that inflamed β-cells known as insulitis occur, participation of effector-T cells in the development of T1D as they target β-cells related epitopes, implication of cytokines in the inflamed pancreatic cells and results in the regulation of iNOS and subsequent NO production (Tsalamandris *et al.*, 2019). Additionally, the

presence of immune cells (macrophage and lymphocytes) in pancreatic tissue contributes to the propagation of the diabetes (Tsalamandris *et al.*, 2019).

3.2. Therapeutic approaches to diabetes: case of carbohydrates-hydrolysis enzymes inhibition

In patients with T2D a practical approach is applied in rather to prevent carbohydrates absorption after food intake. Oral treatment with drugs such as acarbose and miglitol as targets to inhibit carbohydrates-hydrolysis enzymes including α-amylase and α-glucosidase, was found to be useful to decrease hyperglycemia. However, many side effects may occur such as flatulence, abdominal distension and diarrhea (Oboh *et al.*, 2015). Novel natural molecules without side effects from plants sources that are traditionally used to treat diabetes and scientifically validated are required to replace synthetic drugs and can be a holistic way to manage hyperglycemia.

4. Antioxidants

Within the cellular environment, the equilibrium between reactive species and antioxidant is maintained. Cells are equipped with antioxidant complex system for the maintenance of the equilibrium and to clear the overproduction of reactive species. This defense system encloses enzymatic and non-enzymatic antioxidants and the most import once are listed below.

4.1. Enzymatic antioxidants

4.1.1. Superoxide dismutase (SOD)

Superoxide dismutase is a metalloenzyme that exist in three isoforms present in mitochondria, cytosol and extracellular matrix: Cu, Zn-SOD (SOD1), Mn-SOD (SOD2) and extracellular (EC)-SOD (SOD3) (Jovoma *et al.*, 2023). It is one of the most effective enzymes to prevent cellular damage against oxygen radicals. This enzyme catalase a specific redox reaction:

dismutation or disproportionation of superoxide radical anion into reduced form H_2O_2 and O_2 (Gupta *et al.*, 2016).

4.1.2. Catalase (CAT)

Catalase is a tetrameric heme-contaning enzyme, located in the major sites of H_2O_2 generation (peroxisomes and mitochondria) (Sharma and Ahmad, 2014). There are three isoforms of this enzyme classified according to their structure and gene sequence: monofunctional catalase or typical catalase, catalase-peroxidase, and pseudocatalase or Mn-catalase (Nandi *et al.*, 2019). It plays a major role in oxidative stress-related conditions, through metabolizing millions of H_2O_2 molecules by one unit of CAT into H_2O and O_2 due to its high turnover rate (Jovoma *et al.*, 2023; Khandar *et al.*, 2023).

4.1.3. Glutathione peroxidase (GPx)

Glutathione peroxidase is a selenoprotein that has 1 to 8 isoforms, out of which isoform 1 is the most available in the cytosol and mitochondria of mammalians (Ursini and Maiorino, 2013). GPx breaks down H_2O_2 with higher affinity than CAT into water and lipid peroxides into alcohol, simultaneously by catalyzing a redox reaction with the electron donor, reduced glutathione (GSH) (Higuchi, 2014). It is implicated in the maintenance of mitochondria function and thiol redox balance.

4.1.4. Glutathione reductase (GR)

Glutathione reductase is a flavoprotein responsible for the recycling of GSH consumed during radicals and peroxides detoxification (Čolak and Žorić, 2019). After oxidation of GSH into the less active form for peroxide neutralization, glutathione disulfide (GSSG), GR reduces GSSG to form GSH, with the participation of NADPH as an electron donor (Higuch, 2014). The latter is regenerated through the pentose phosphate pathway.

4.1.5. Glutathione S transferase (GST)

Glutathione S transferase is a cytosolic and membrane bound enzyme that is classified into three subfamilies out of which canonical soluble GST is the predominant isoform in mammalians cells (Llavanera *et al.*, 2019). GST is a multifunctional protein that catalase glutathione conjugation-dependent reactions to detoxify the cells from electrophilic compounds (Llavanera *et al.*, 2019). The action of enzymes implicated in clearing reactive species and xenobiotics is well illustrated in the following **figure 7**.

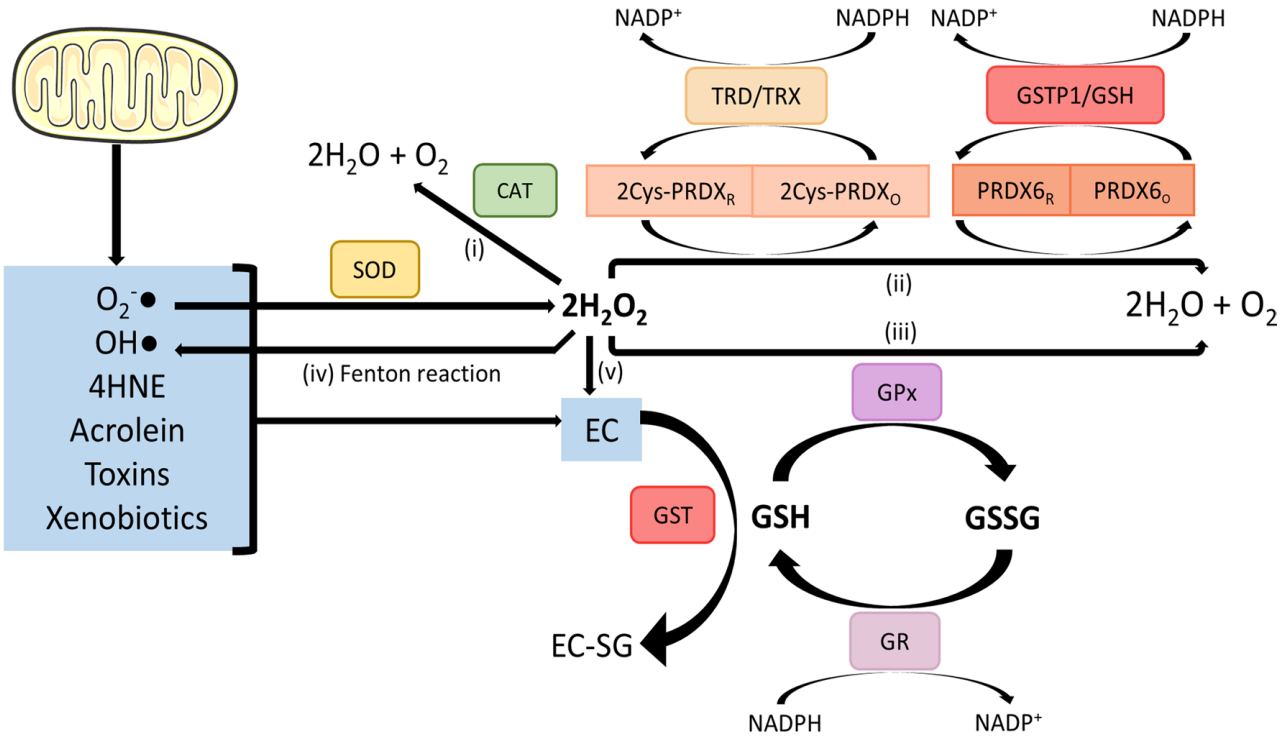


Figure 7. Concerted action of some important enzymes involved in cellular protection against reactive species (Llavanera *et al.*, 2019). **EC:** electrophilic compound; **TRD/TRX:** thioredoxin reductase/thioredoxin; **2Cys-PRDX:** 2-Cystiene-peroxyredoxin.

4.2. Non-enzymatic antioxidants

4.2.1. Reduced glutathione (GSH)

L-glutathione, reduced glutathione or simply glutathione a tripeptide includes glycine, L-cysteine, and L-glutamic acid (Asgari *et al.*, 2024). Due to its ability to transform to its oxidized form GSSG, is considered as major multifunctional thiol antioxidant that acts as

radical scavenger of a variety of ROS including H_2O_2 and $OH^{\cdot}$. Also, as a cofactor to both: GST and GPx. GSH participates in the regeneration of the active form (non-radical) of powerful antioxidant such as vitamins (C and E) (Jovoma *et al.*, 2023).

4.2.2. Coenzyme Q

Ubiquinones are lipid soluble antioxidants, present in all intracellular membranes. Their reduced form (ubiquinol) is the effective form for the antioxidant ability. This form inhibits at early stage lipid peroxidation. It is also involved in regeneration of vitamin E and functioning as chain-breaking antioxidant to inhibit proteins oxidation (Bentinger *et al.*, 2007).

4.2.2. Vitamins and carotenoids

Vitamin C (ascorbate, the active form) and vitamin E (α -Tocopherol, the most active isomer) are a class of water and fat-soluble vitamins, respectively. These two vitamins are mainly obtained from fruits, vegetables and vegetable oils. They play a major role in the reduction of oxidative damage. Vitamin E can increase GSH levels, enhance antioxidant enzymes activity and decrease lipid peroxidation (Feng *et al.*, 2023). While ascorbate neutralizes oxygen radicals including $O2^{\cdot-}$ and $OH^{\cdot}$ through radical scavenging activity (Böhm *et al.*, 2023). Both of these vitamins are previously reported to regenerates other antioxidants (Böhm *et al.*, 2023, Jovoma *et al.*, 2023). Carotenoids, tetraterpenoids, such as lycopene, astaxanthin, β-Carotene and lutein naturally endow plants and algae with red, orange and yellow pigments (Kabir *et al.*, 2022). These lipophilic compounds have a variety of biological activities to prevent development of diseases and indorsed in the decrease of oxidative damage (Kabir *et al.*, 2022). Their conjugated double bounds are responsible for the antioxidant ability and reflect their potential to inhibit lipid peroxidation and radical scavenging (Hacke *et al.*, 2022; Feng *et al.*, 2023).

4.2.3. Phenolic compounds

Plants produce numerous chemical secondary metabolites fundamental for the survival and defense against stressful conditions. Ubiquitous diseases developed in mankind that involve inflammation and oxidative stress, are treated using a various range of natural secondary metabolites with functional characteristics defined through their remarkable biological properties and considered as bioactive compounds. Among these natural bioactive metabolites; the widely distributed group of phenolic compounds.

Phenolics are aromatic compounds characterized by the presence of multiple phenol structural units. The most distributed are benzoic and cinnamic acid derivatives and flavonoids within plants. In addition, polyphenols may occur polymerized with each other or associated with carbohydrates and organic acids. Due to their remarkable diversity and structure complexity, thousands of phenolics are identified and classified according to the source of origin, natural distribution, biological function, and chemical structure. Overall, as can be shown in **figure 8** polyphenols are classified with respect to the chemical structure, the number of phenol rings and their structural elements.

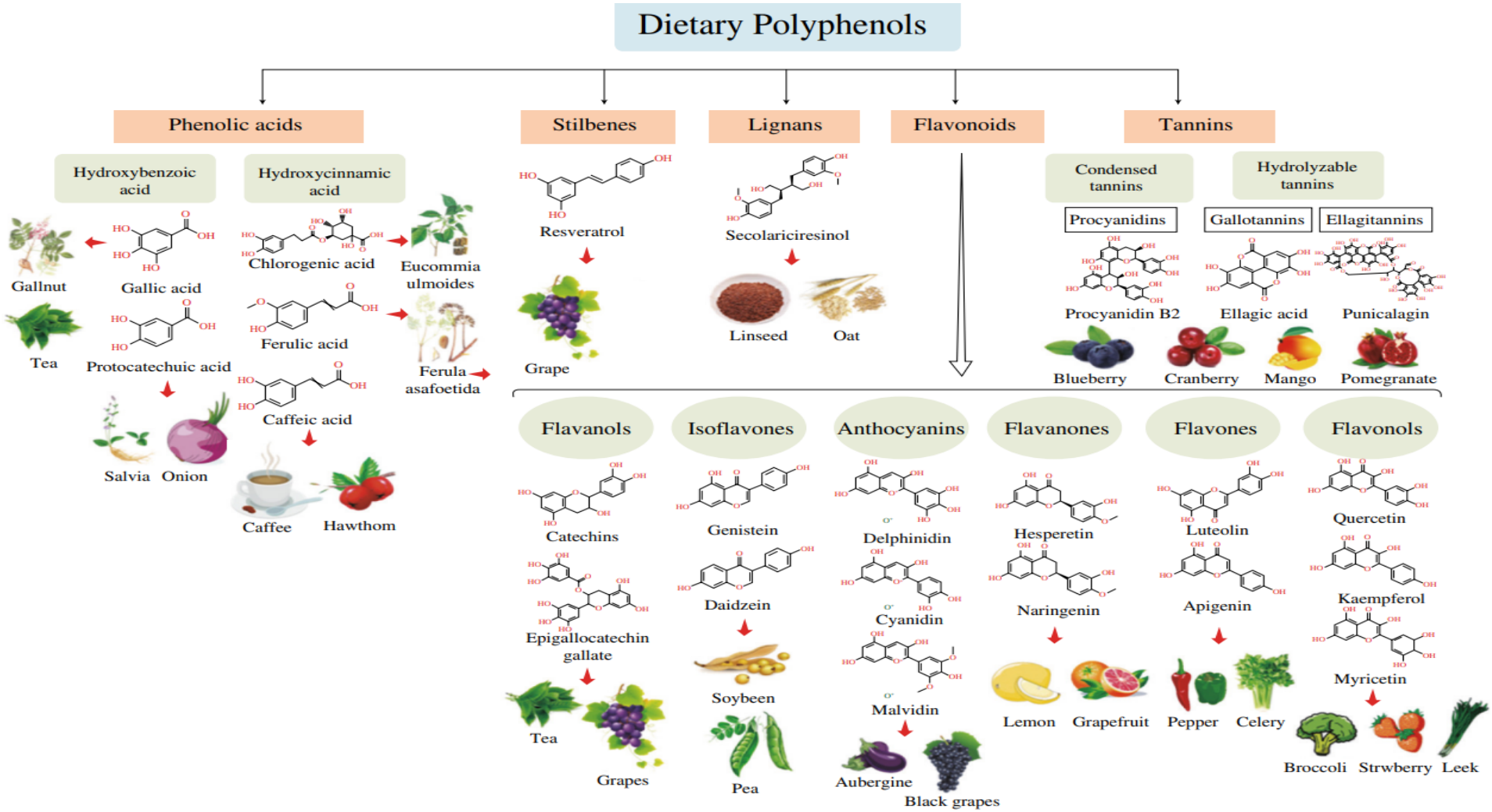


Figure 8. Phenolic compounds classification based on their chemical structure (Li *et al.*, 2023).

4.2.3.1. Structure-activity relationship

The fundamental feature of phenolic compounds is their antioxidant ability that is underlined by the reducing ability and binding properties (Cvitanović *et al.*, 2018). Mechanisms of the antioxidant ability of these compounds include scavenging of reactive radical and non-radical species, chelating or binding to metals to decrease the kinetics of Fenton reaction and metal reducing. In addition to enzymes inhibition (oxidant enzymes, e.g. COX) and stimulation (antioxidant enzymes, e.g. CAT), and regeneration of some antioxidants (Cvitanović *et al.*, 2018; Andrés *et al.*, 2023). All of these mechanisms are correlated with the compound chemical structure. According to Quideau *et al.* (2011), the basic phenyl ring of polyphenols that bears a hydroxyl group constitutes an amphiphilic moiety that combine the hydrophibicity of the aromatic ring with the hydrophilicity of the polar hydroxyl groups which endow the phenyl with ability to accept or donate hydrogen (**figure 9**). The hydrophobicity π-stacking (van der Waals) and formation of hydrogen bonds use it to bind physically with proteins, additionally; the metal chelating occurs with the presence of adjacent hydroxyl groups on the phenyl ring of polyphenols.

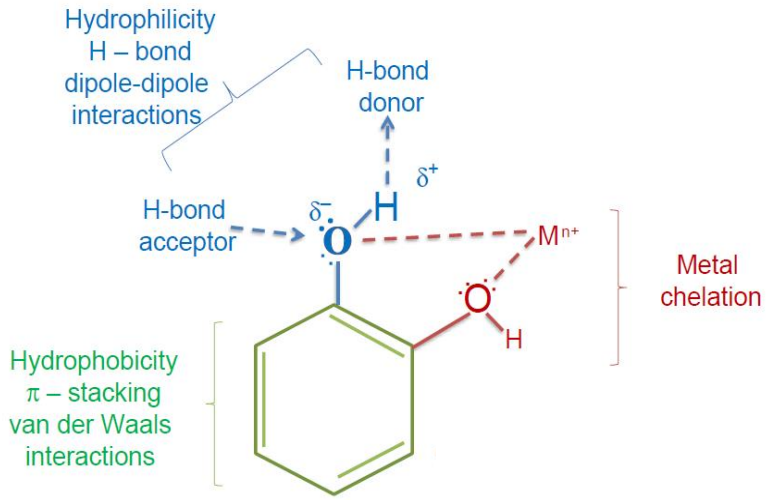


Figure 9. The fundamental physico-chemical properties of polyphenol according to Quideau *et al.* (2011).

Overall, number and position (e.g., *meta, ortho,* or *para*) of hydroxyl groups, the highly conjugated system (conjugated double bounds) and other hydrogen-donation groups such as amino and tiol determine the antioxidant activity (Leicach and Chludil, 2014). However, the degree of polymerization is a positive motivator of the antioxidant ability (Lang *et al.*, 2024).

It is worthy to highlight the electron delocalization capacity that endows polyphenols with the radical scavenging capability. During redox reaction, electron or proton donation, the formed radicals on the aromatic ring of polyphenols are delocalized (**figure 10**), resulting in greater electrochemical stability and enhancing the reversible redox-mediating capabilities of these compounds (Hsueh *et al.*, 2019).

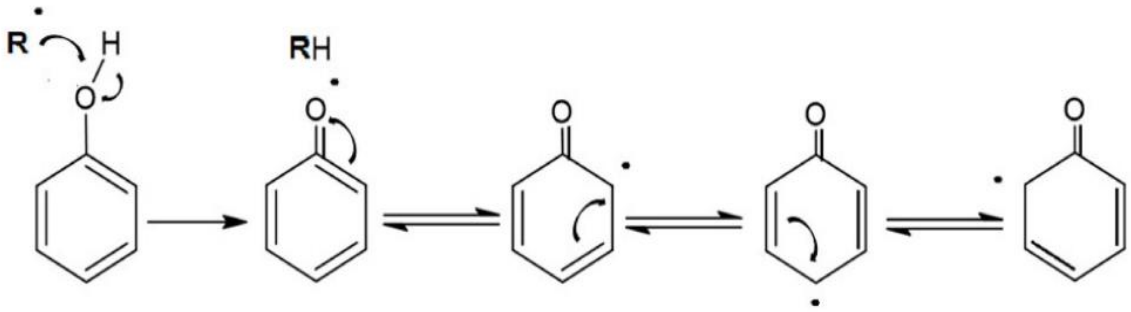


Figure 10. Illustration of electron delocalization within the aromatic ring of phenolic compounds (Flemming *et al.*, 2021).

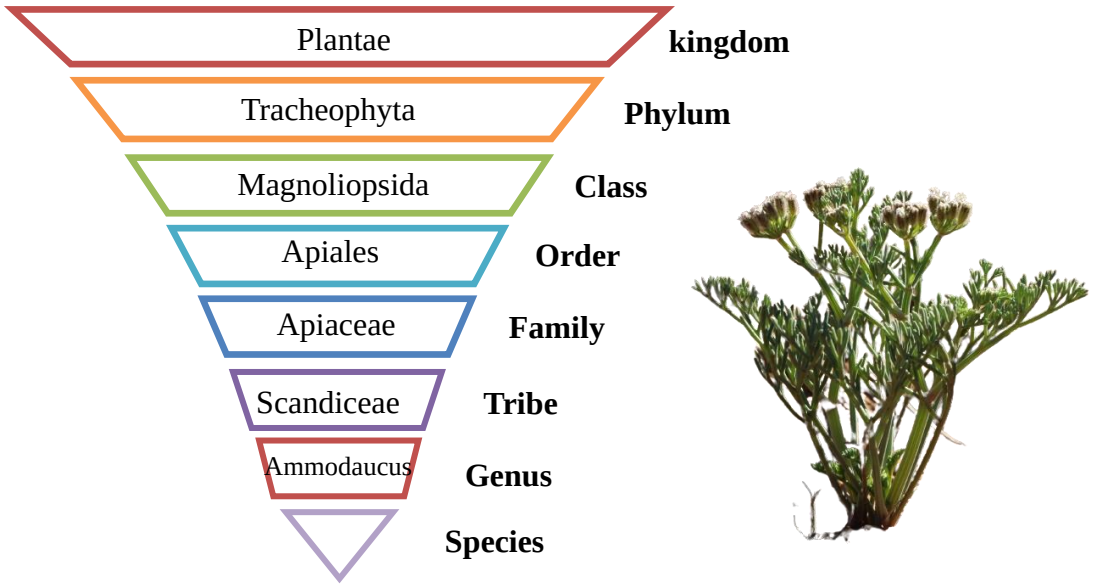
5. *Ammodaucus leucotrichus* Coss. & Durieu

5.1. Botanical description and classification

Among the plentiful plant diversity of the Sahara Desert, *Ammodaucus leucotrichus* Coss. & Durieu (*A. leucotrichus*) is the only species of the genus *Ammodaucus* in the *Apiaceae* family (Umbelliferae), it is commonly known as “kammûn es-sofi” in the most of North Africa countries, while in Algeria is referred as “Moudrayga, Oumdraiga or El massoufa” and in Tamahq (Touareg language) “akâman”, “Hairy Cumin” in English (Dahmane *et al.*, 2016; Idm’hand *et al.*, 2020). This species is geographically distributed in maritime sands of

Saharan and sub-Saharan countries of North Africa; Morocco, Algeria, Tunisia and extending to Egypt and Tropical Africa (Abu Zarga *et al.*, 2013).

The studied species is previously identified and classified in 1859 by the French botanical society as shown in the **figure 11** below.



Ammodaucus leucotrichus Coss. & Durieu

Figure 11. Botanical classification of *A. leucotrichus* Coss. & Durieu according to the French botanical society (1859).

Ammodaucus leucotrichus is a small glabrous annual plant, of 10 to 20 cm high and with finely striated stems (**figure 12: A** and **B**). The pinnatisect leaves are finely divided and slightly fleshy (**figure 12: C**). Its small white-rose flowers with five petals grouped in composed umbels and acting as dispersal units of 3 to 5 rayon (**figure 12: D** and **E**) of 1 to 3 cm long. The dry seeds (mericarps), 10-12 × 5-7 mm are covered with dense silky hairs 4-5 mm long (**figure 12: E** and **F**) (Dahmane *et al.*, 2016; Chatelain *et al.*, 2021). The plant has a strong smell of anise.

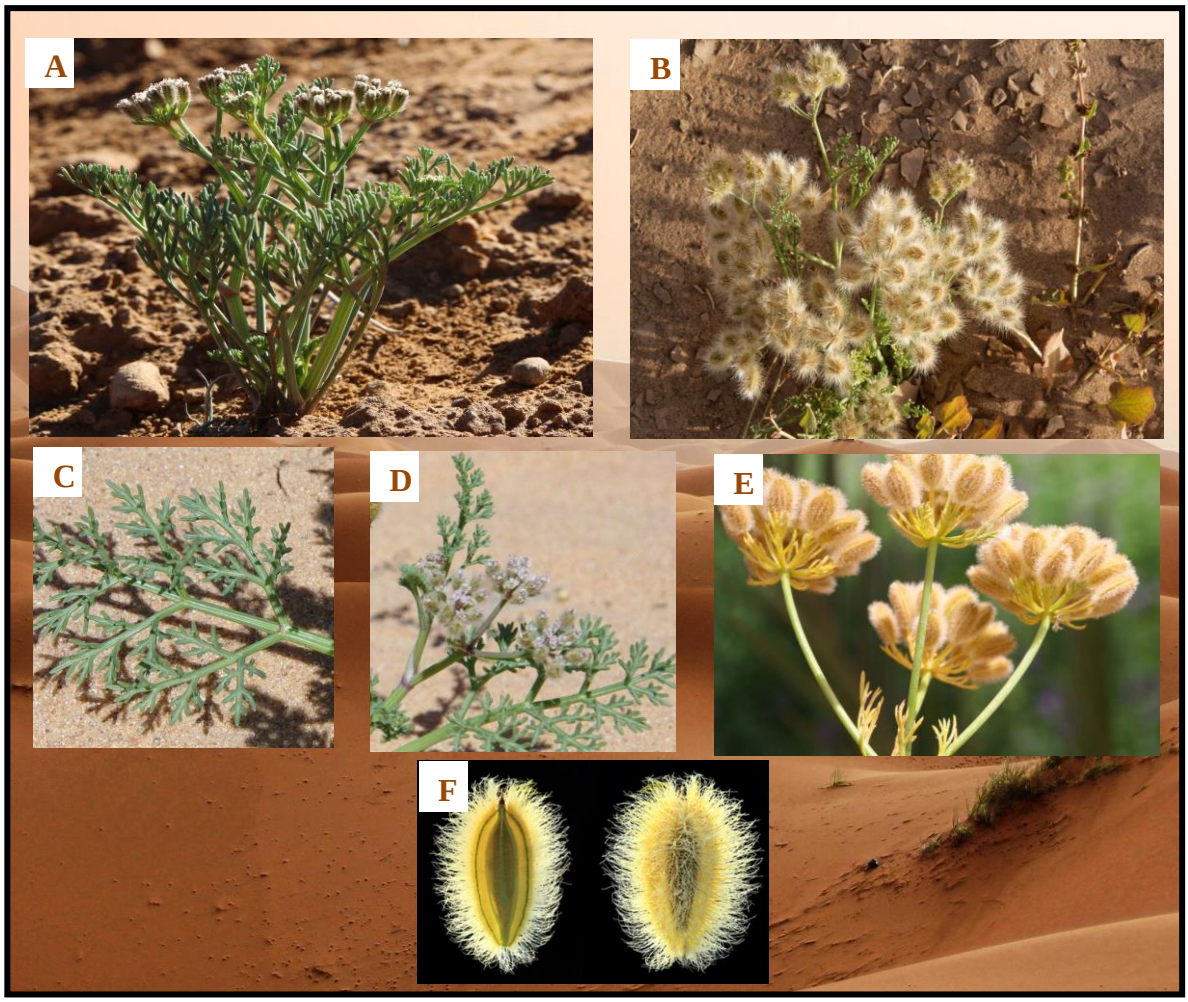


Figure 12. *Ammodaucus leucotrichus* Coss. & Durieu and its different parts (Atlas-Sahara last check on June 10th, 2024).

5.3. *Ammodaucus leucotrichus* and traditional medicine

The plant is widely used in various medical applications and with an extended culinary use. In the southern Algerian Sahara (Tassili), the leaves of this plant are used for their herbal flavoring characteristics in teas (Halla *et al.*, 2018). In the same region, milk mixed with crushed seeds is used to recover appetite (Mouderas *et al.*, 2019). However, seeds in powdered form are often used as a condiment during culinary preparation. Several ethnopharmacological investigations have noted the communally use of seeds, prepared as decoction and infusion, to treat many health problems and complications. Seeds are widely used as a sugar regular in diabetes and for stomach and colon diseases (Idm'hand *et al.*,

2020). In Ouargla region, this plant is among the most used by T1D patients to decrease blood glucose levels and for foot ulcer (Telli *et al.*, 2016). As infusion or decoction, seeds are used for abdominal and intestinal pain in children and adults, together against nausea and vomiting in newborns (Bouayyadi *et al.*, 2015). Seeds grinded with honey are taken orally to treat lung cancer (Aumeeruddy and Mahomoodally, 2021). Some Algerian healers prepare decoction of areal parts to treat thyroid gland complications (Abu Zarga *et al.*, 2013). Additionally, decocted seeds are good to use for diarrhea, cough, pulmonary diseases, anorexia, allergy, gastroenteritis and tachycardia (Abouri *et al.*, 2012). A remedy based on mixture of *A. leucotrichus* powdered seeds with *Petroselinum sativum* (vernacular name: parsley), in apple vinegar and honey are used against cystitis and nephritic colics (Idm'hand *et al.*, 2020). The plant is frequently used to treat arterial hypertension, intestinal worms, fever, wounds infections, and genital disorders, for scorpion stings, snakebites, kidney stones and liver diseases (Halla *et al.*, 2018; Mouderas *et al.*, 2019; Idm'hand *et al.*, 2020).

5.4. Phytochemistry and biological activities

A board range of bioactive compounds are identified from aqueous, n-butanol, ethyl acetate, methanolic, or ethanolic extracts and essential oils of the plant seeds. Through phytochemical screening using color reaction, compound groups found are: terpenes, tannins, anthracenes compounds, sterols, alkaloids, phenol acids, saponins, flavonoids and coumarins (Sebaa *et al.*, 2018). In addition, reports on the identification of individual chemical constituents through Gas Chromatography and High Performance Liquid Chromatography has revealed the presence of perillaldehyde and limonene as major compounds in the essential oils (Dahmane *et al.*, 2016), as well as polyphenols: p-coumaric, vanillic, ferulic and sinapic acids and flavonoids (naringenin and quercetin) were identified in ethyl acetate and n-butanol fractions (Mouderas *et al.*, 2019). Ziani *et al.* (2019) successfully identified seven phenolic compounds in hydroethanolic and aqueous extracts with the dominance of the flavone luteolin-7-O-

glucoside and luteolin-O-(malonyl-hexoside) isomer 2. The presence of such bioactive metabolites indicates the importance of plant extracts in pharmaceutical industries.

Several extracts and seeds essential oil from *A. leucotrichus* have been evaluated for their biological activities including antioxidant, anti-inflammatory, antibacterial, antidiabetic, and cytotoxicity activities.

a) Antioxidant activity

Essential oil and different extracts (hydromethanolic, methanolic, aqueous, ethyl acetate and n-butanol) were assessed for radical scavenging and ferric reducing ability (Sebaa *et al.*, 2018; Mouderas *et al.*, 2019). Results showed that all extracts and oil essential neutralized DPPH radicals and exhibited ferric reducing. However, methanolic and ethyl acetate extracts were the strongest. A cellular study showed that methanolic plant extract increased significantly NrF2 gene expression (Belbachir *et al.*, 2023).

b) Anti-inflammatory and anti-proliferative activities

The anti-edematogenic response of essential oils in carrageenan induced hind paw edema in mice was evaluated in the study of (Mohammedi *et al.*, 2018). The inhibitory activity of paw edema of essential oil was very weak compared to diclofenac group and showed similar values to that of control group received only distilled water. In addition, hydro-alcoholic extract from the plant seeds inhibited lipooxygenase (Bouyahya *et al.*, 2021). Ziani *et al.* (2019) revealed that the hydroethanolic extract reduced gene expression of iNOS through decreasing NO content, and exhibited anti-proliferative activity against many human tumor cell lines.

c) Antimicrobial activity

Previously reported that *A. leucotrichus* seeds methanolic, ethanolic and butanic extracts revealed a promising antibacterial activity against many bacteria namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsilla pneumonia*, *Staphylococcus aureus* and *Salmonella typhimurium* using disc diffusion methods (Sebaa *et al.*, 2018).

d) Anti-diabetic activity

Anti-diabetic activity of seeds aqueous extract was conducted by El-Ouady and Eddouks (2019) where they showed that plant extract lowered blood glucose levels. Also, Sebaa *et al.* (2021) confirmed that aqueous extract administration regulated hyperglycemia.

6. Aim and objectives

This study fits in the context of improving human life and to corroborate the traditional claims of this species via the scientific validation: *in vitro* and *in vivo* evaluation of the antioxidant, anti-inflammatory activities and toxicity assessment.

In this study three aspects are tackled in rather to achieve our objectives:

- The first part is dedicated to extraction, fractionation and quantitative and qualitative analysis of the extracted phytochemicals;
- The second one is devoted to *in vitro* antioxidant and anti-inflammatory activities through many systems (free radical scavenging, lipid peroxidation inhibition, ferrous chelating, ferric reducing, anti-hemolytic, antithrombotic and anticoagulant activities, enzyme inhibition and inhibition of human serum albumin heat denaturation);
- The final part is dedicated to *in vivo* anti-inflammatory activity using two edema induction models by application and injection of irritant agents. As well as the safety

profile assessment through acute and sub-acute toxicity together with estimation and quantification of oxidative stress-related markers in organs homogenates.

Chapter 2: Material and methods

1. Material

1.1. Plant material

The seeds of *Ammodaucus leucotrichus* (**figure 13**) were collected from western Algerian Sahara (W. Bachar) during summer 2021. The plant specimen was identified and authenticated by Professor Chermat Sabah, Setif 1 University. A voucher specimen was assigned (220/SNV/DA/UFAS/21) and deposited at the Applied Biochemistry Laboratory, Faculty of Nature and Life Sciences, Setif 1 University. Seeds were cleaned and sieved then left to dry at room temperature, in shadow for several weeks. Once dried, seeds were powdered using an electric grinder and stored until used for the extraction step.

Figure 13. *Ammodaucus leucotrichus* Coss. & Durieu (03/07/2022).

1.2. Experimental animals

The *in vivo* experiments were carried out on adult male Albino Wistar mice weighing between 25 g and 30 g, purchased from Algiers Pasteur Institute, Algeria. Animals were housed in

clean plastic cages for a period of seven days for acclimatization prior to experiments. Animals were maintained under standard laboratory conditions (26 ± 2 °C, natural light/dark cycle), with standard rodent diet and drinking water *ad libitum*. All experiments were conducted according to the Ethics Committee of Setif 1 University and performed following the internationally acceptable guidelines for evaluation the safety and efficacy of herbal medicines (Organization of Economic Co-operation and Development, OECD).

1.3. Chemicals

1.3.1. Main chemicals and solvents for *in vitro* and *in vivo* studies

Methanol, Ethanol, Hexane, Chloroform, Butanol, Ethyl acetate, Xylene, Acetic acid, Gallic acid, Quercetin, Kojic acid, Galantamine Hydrobromide, Acarbose, Ascorbic acid, Butylated hydroxytoluene (BHT), α-amylase (Pancreatic), α-glucosidase (Yeast), Acetylcholinesterase (Electrophorus electricus), Butyrylcholinesterase (Horse serum), Tyrosinase (Mushroom), Ethylenediamine tetraacetic acid disodium (EDTA-Na_2), Folin-Ciocalteau reagent, Aluminium chloride, 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$), azinobis ethylbenzothiazoline sulfonic acid (ABTS), 1-10 phenanthroline, ferrous sulfate ($FeSO_4$), Sodium nitroprusside, sulfanilamide, Nitrotetrazolium blue (NBT), β-Carotene, Linoleic acid, Thiobarbituric acid (TBA), Potassium ferricyanide [$K_3Fe(CN)_6$], 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-sulfonic acid (ferrozine), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), Human serum albumin (HAS), λ-Carrageenan, Croton oil, Glutathione, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Riboflavin, 1-Chloro-2,4-Dinitrobenzene (CDNB). All chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich chemicals, Fluka, Biochem Chemopharma and VWR International.

1.3.2. Reagents and standards for HPLC analysis

Cyanidin-3-glucoside chloride, delphinidin-3,5-diglucoside chloride, delphinidin-3-galactoside chloride, petunidin-3-glucoside chloride, malvidin-3-galactoside chloride, quercetin-3-glucoside and kaempferol-3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The remaining 31 analytical standards of the 38 phenolic compounds were supplied by Sigma-Aldrich (Milan, Italy). Formic acid (99%) was obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric acid (37%) was obtained from Carlo Erba Reagents (Milan, Italy). HPLC-grade methanol was supplied by Sigma-Aldrich (Milano, Italy). Deionized water (> 18 MΩ cm resistivity) was further purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 μm syringeless filter, Phenomenex (Castel Maggiore, BO, Italy).

2. Methods

2.1. Extraction and preparation of fractions

The methanolic crude extract was prepared according to Aouachria *et al.* (2017) with slight modifications. 5 kg of powdered dried seeds were macerated two times with 35 L of methanol. The mixture was left to stand for seven days at room temperature with occasional shaking and stirring. Afterwards, methanol mixture was filtered through filter cloth then through Whatman filter paper, and concentrated in Rotary evaporator (BÜCHI, Germany) at 45 °C to obtain methanolic crude extract.

During liquid-liquid fractionation (Markham, 1982), the selected solvent is added according to the order of increasing polarity to the methanolic crude extract as shown in **figure 14**, starting by hexane for defatting, then chloroform for extraction of aglycone flavonoids and finally ethyl acetate to extract glycosylated flavonoids. The obtained fractions were

concentrated by evaporation to afford: Hexane extract (HE), Crude extract (CrE), Chlorofom extract (ChE), Ethyl acetate extract (EAE) and the remaining aqueous phase (AqE). Extracts were preserved at 4 °C for further use.

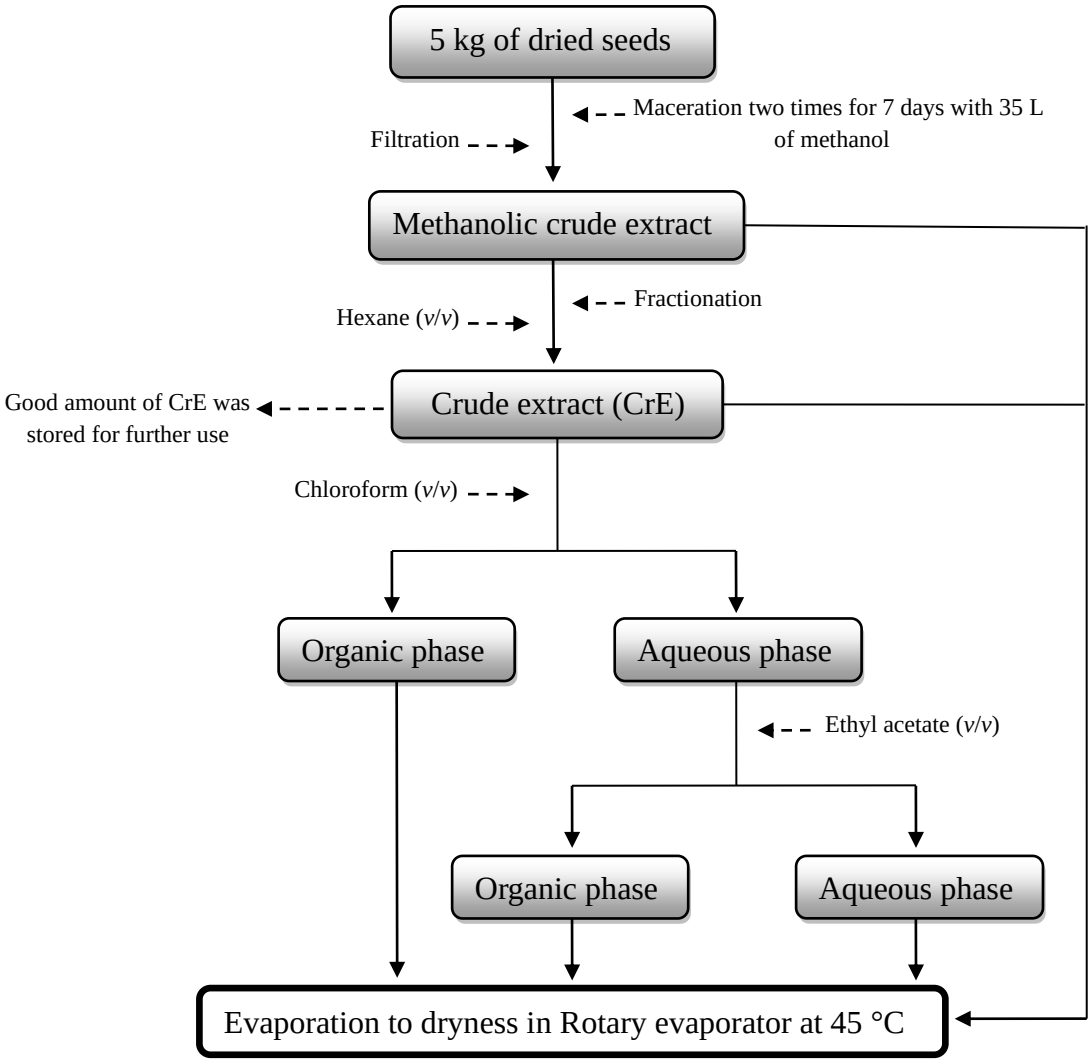


Figure 14. Preparation of CrE and its fractions from *Ammodaucus leucotrichus* seeds.

2.2. Phenolic content: quantitative and qualitative analysis

2.2.1. Total polyphenol content (TPC)

The Folin Ciocalteu reagent assay was applied for the quantification of total polyphenols as described by Trabsa *et al.* (2015). The method relies on the electron-transfer reaction in alkaline conditions, in which hydroxyl groups of polyphenols acts as electron donors to reduce the mixture of phosphotungstic and phosphomolybdic acids, resulting in the formation

of a blue chromophore (Pérez *et al.*, 2023). A mixture of 100 µl of plant extracts and 500 µl of diluted Folin Ciocalteu reagent (1:10, *v*/*v*) was incubated at room temperature for 4 min, and 400 µl of sodium carbonate (7.5%, *w*/*v*) was then added. The absorbance of the formed blue solution was measured at 765 nm after 2 h of incubation in the dark. Gallic acid at different concentrations (0.8-200 µg/ml) was used as standard (**figure 15**) and the results were expressed as microgram of Gallic acid equivalents per milligram of extracts (µg GAE/mg E).

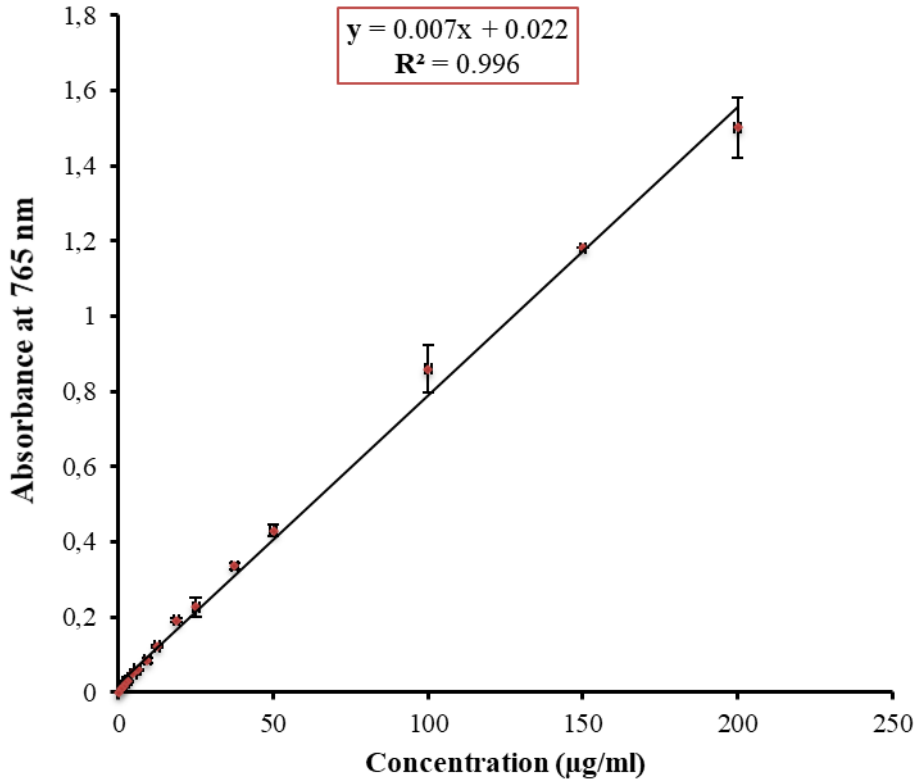


Figure 15. Standard curve of gallic acid. Values are the mean of triplicates ± SD.

2.2.2. Total flavonoid content (TFC)

Quantification of total flavonoid content was performed using the Aluminium chloride colorimetric assay (Mayouf *et al.*, 2019). It is based on the complexation of Aluminium with specific groups of flavonoids due to their great affinity to bind metal ions, resulting in a stable yellow complex. To 1 ml of plant extracts an equal volume of 2% (*w*/*v*) Aluminium chloride was added and the mixture was incubated at room temperature for 10 min before absorbance was read at 430 nm. Quercetin at different concentrations (1.6-50 µg/ml) was used for the

standard calibration curve (figure 16). Total flavonoid content was expressed as microgram of Quercetin equivalents per milligram of extracts (µg QE/mg E).

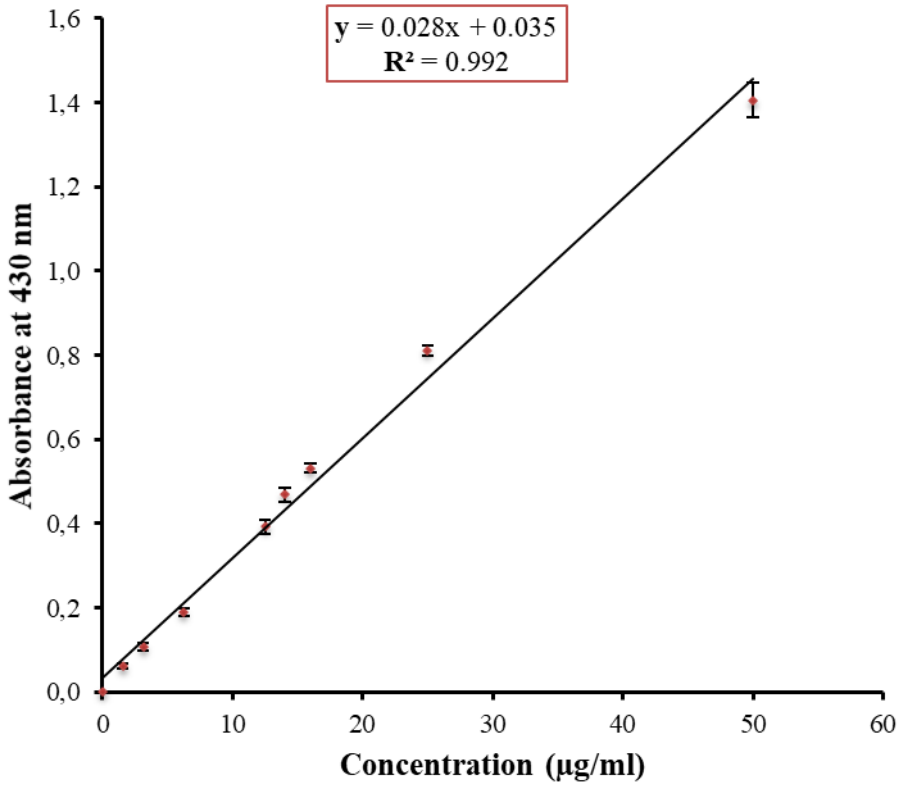


Figure 16. Standard curve of quercetin. Results are the mean of triplicates ± SD.

2.2.3. HPLC-ESI-MS/MS triple quadrupole analysis

High Performance Liquid Chromatography coupled with mass spectrometry (HPLC-MS/MS) studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with electrospray ionization (ESI) source operating in negative and positive ionization modes. In fact, the instrument allowed performing a single run with polarity switching without any problems. MS/MS parameters of each analyte were optimized in flow injection analysis (FIA) (1 µl of a 10 mg l^{-1} individual standard solution) by using Optimizer Software (Agilent). The separation of target compounds was achieved on a Synergi Polar–RP C18 analytical column (250 mm x 4.6 mm, 4 µm) from Phenomenex (Chesire, UK). The column was preceded by a Polar RP security

guard cartridge (4 mm x 3 mm ID). The mobile phase was a mixture of (A) water and (B) methanol, both with formic acid 0.1%, at a flow rate of 0.8 ml min^{-1} in gradient elution mode. The composition of the mobile phase varied as follows: 0–1 min, isocratic condition, 20% B; 1–25 min, 20–85% B; 25–26 min, isocratic condition, 85% B; 26–32 min, 85–20% B. All solvents and solutions were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 µl. The temperature of the column was 30 °C, and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 12 l/min, the nebulizer pressure was 55 psi, and the capillary voltage was 4000 V. Detection was performed in the dynamic-multiple reaction monitoring (dynamic-MRM) mode, and the dynamic-MRM peak areas were integrated for quantification. The most abundant product ion was used for quantitation, and the others for qualification. The specific time window for each compound (Δ retention time) was set at 2 min (Yagi *et al.*, 2023). The chromatogram for standard compounds is given in **figure 17**.

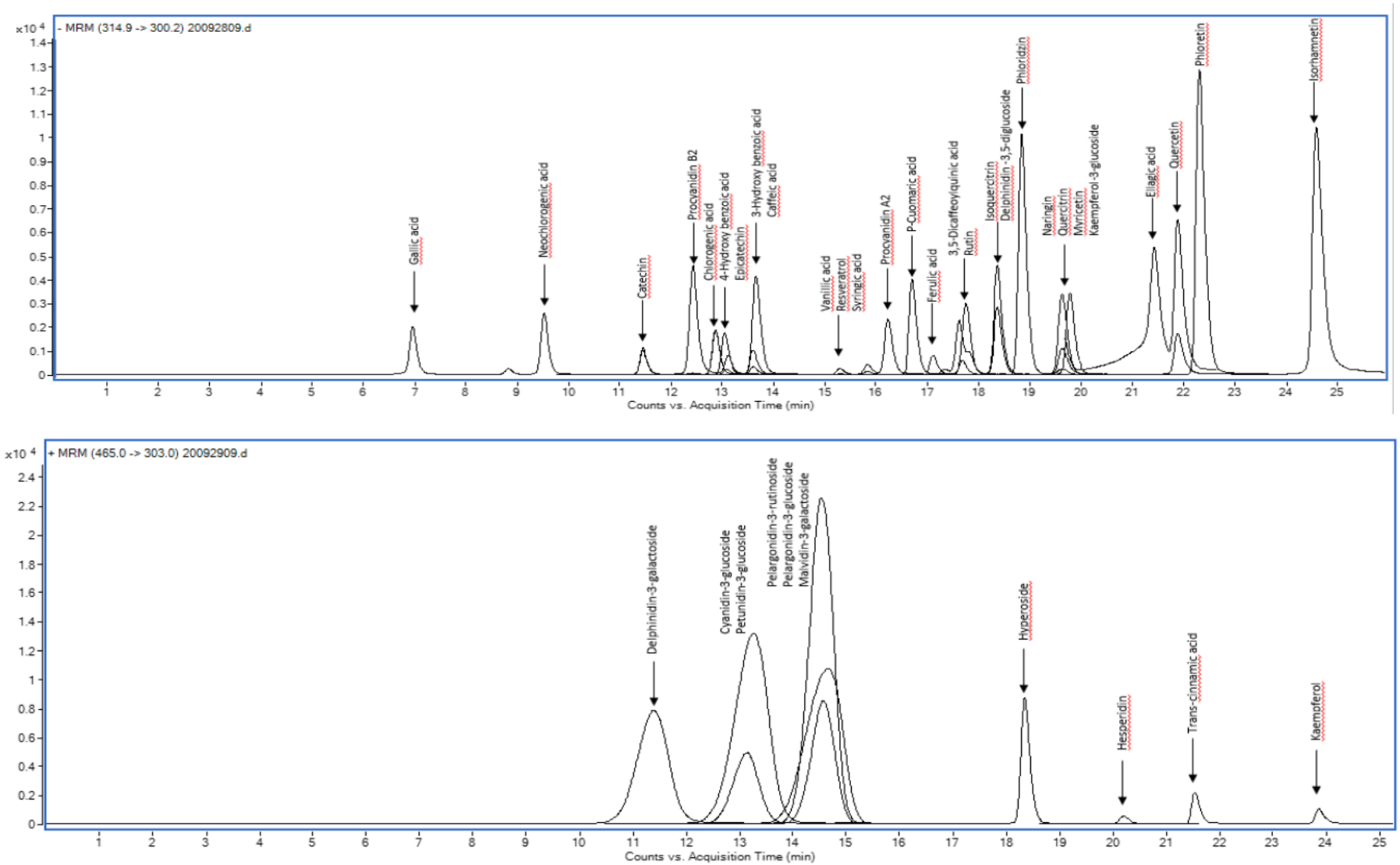


Figure 17. Chromatogram of 38 phenolic standard compounds used in HPLC-ESI-MS/MS analysis (Yagi *et al.*, 2023).

2.3. *In vitro* antioxidant activity evaluation of *Ammodaucus leucotrichus* extracts (AlE)

2.3.1. Radical scavenging activity

2.3.1.1. DPPH radical scavenging activity

The first indication of radical scavenging activity of *A. leucotrichus* extracts was assessed using DPPH stable radical as mentioned by Guemmaz *et al.* (2020). When $DPPH^{\cdot}$ undergoes a mechanism of hydrogen donating and/or electron transfer in the presence of antioxidant is reduced, thereby its free radical property is lost and its color is reduced from violet to yellow. Aliquot of 50 µl of various concentrations of plant extracts/BHT was mixed with 1250 µl of purple-colored methanol solution of $DPPH^{\cdot}$ (0.004%, *w*/*v*). Absorbance was determined at 517 nm after 30 min incubation in darkness. The percentage of radical inhibition was calculated using the following formula:

$$\textbf{Inhibition of DPPH}^{\cdot}\textbf{ (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.1.2. ABTS cation radical ($ABTS^{\bullet+}$) scavenging activity

The $ABTS^{\bullet+}$ scavenging method is another common and easy-to-apply assay for the overall screening of the antioxidant activity of phenolic compounds. In the current study, the evaluation of radical scavenging activity of plant extracts was applied as described by Abed and Belattar (2023), where ABTS cation radicals were generated in 16 h incubation from a mixture consisting of 7 mM of ABTS and potent oxidizing agent, potassium persulphate at 2.45 mM. Afterwards, the stock solution of $ABTS^{\bullet+}$ was diluted by a ratio of 1:75 (*v*/*v*) to achieve an absorbance of 0.7 at 734 nm. To 975 µl of the working solution, 25 µl of AlE and standard at different concentrations were added, followed by 10 min incubation at room temperature. Reduction of the intense blue color of the working solution is monitored spectrophotometrically at 734 nm and the percentage of radical inhibition was calculated using the below equation:

$$\textbf{Inhibition of ABTS}^{\bullet+}\textbf{ (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.1.3. Hydrogen peroxide (H_2O_2) scavenging assay

The method used in our current study to determine the H_2O_2 scavenging potential of the plant extracts (Laroui *et al.*, 2023) is based on the capability of ferrous ions to form with 1,10-phenanthroline an orange complex, hence indicating a successful radical scavenging capacity of H_2O_2 in the media. Briefly, 1.5 ml of AlE/standard with various concentrations were mixed with 250 µl of ferrous ammonium sulfate (1 mM) and 62.5 µl of H_2O_2 (5 mM) and thereafter incubated for 5 min in darkness. A volume of 1.5 ml of 1,10-phenanthroline (1 mM) was added to the mixture. Finally, absorbance was taken after 10 min incubation at 510 nm. The percentage of Hydrogen peroxide scavenging potential was calculated using the following formula:

$$\textbf{H}_2\textbf{O}_2\textbf{ scavenging activity (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.1.4. Hydroxyl radical ($OH^{\cdot}$) scavenging assay

The present assay described by Mayouf *et al.* (2019) is used to evaluate the $OH^{\cdot}$ scavenging activity of bioactive compounds present in our extracts, by generating hydroxyl radicals in a Fenton reaction. Radicals were generated at pH 2.3 in a mixture of 9 mM ferrous sulfate and 0.3% H_2O_2. 300 µl of this mixture were added to 150 µl of plant extracts/ascorbic acid at various concentrations, followed by 15 min incubation at 32 °C. A volume of 75 µl of sodium salicylate (20 mM) was added and the final mixture was incubated for another 15 min at 32 °C. Absorbance was measured at 562 nm and the percentage of radical scavenging capacity was calculated according to the below formula:

$$\textbf{OH}^{\cdot}\textbf{ scavenging activity (\%)} = \frac{1 - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.1.5. Superoxide anion ($O_2^{\bullet-}$) scavenging capacity

Superoxide anion scavenging capacity was performed by measuring the reduction of nitrotetrazolium blue into blue formazan via $O_2^{\bullet-}$ generated in a non-enzymatic system (Laouicha *et al.,* 2018). The assay mixture (pH 7.4) contained an equal volume (500 µl) of nicotinamide adenine dinucleotide at 468 µM and 156 µM of NBT were added to 50 µl of various concentrations of the plant extracts. The reducing reaction was initiated by adding 50 µl of phenazine methosulphate (60 µM). The mixture was incubated for 5 min at room temperature before the absorbance was measured at 560 nm. The percentage of radical scavenging capacity was calculated as follows:

$$O_2^{\bullet-}\ \textbf{scavenging capacity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.1.6. Nitric oxide radical ($NO^{\bullet}$) scavenging capacity

The protocol of Awah and Verla (2010) was adopted to evaluate the capacity of plant extracts to inhibit nitric oxide radicals with slight modifications. Nitric oxide generated at physiological and aerobic conditions is converted into nitrite ions (NO^{2-}) and further a pink chromophore finally results during the diazotization and coupling of these ions. A mixture containing 500 µl of sodium nitroprusside solution (5 mM, pH 7.4) and an equal volume of samples at various concentrations was incubated for 90 min to generate $NO^{\bullet}$ under a light source. Additionally, 500 µl of 1% sulfanilamide in 5% phosphoric acid was added and followed by 10 min incubation in darkness. The final reaction of NO^{2-} coupling was initiated by adding 500 µl of 0.1% naphthyl ethylenediamine dihydrochloride (NEDD) and absorbance was monitored at 543 nm after 20 min incubation. To calculate the percentage of nitric oxide radical scavenging activity, the below formula was used:

$$\textbf{NO}^{\bullet}\ \textbf{scavenging capacity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.2. β-Carotene/Linoleic acid bleaching assay

The assay is used as previously mentioned by Laroui *et al.* (2023) to screen plant extracts for the potential to inhibit the initiation and propagation phases of lipid peroxidation. The ROS produced by the saturated water with oxygen triggers oxidation of linoleic acid, lipid peroxidation product will eventually initiate β-Carotene oxidation thereby losing its yellowish color. A quantity (0.5 mg) of β-Carotene was dissolved in 1 ml of chloroform and followed by adding 200 mg of tween 40 and 25 µl of linoleic acid to the mixture. After the evaporation of chloroform, 100 ml of water saturated with oxygen was added to the mixture to form an emulsion. An aliquot of 350 µl of extracts/BHT at 2 mg/ml was mixed with 2.5 ml of the emulsion and absorbance was read immediately (Time zero) at 490 nm. The reaction mixture was incubated at room temperature in the dark for 24 h, and the absorbance was monitored at different times ranging 1, 2, 4, 6 and 24 h. Distilled water and methanol were used as negative standards. The percentage of antioxidant activity was calculated with the equation below:

$$\textbf{Antioxidant activity (\%)} = \frac{\text{Absorbance of sample } (T_x)}{\text{Absorbance of sample } (T_0)} \times 100$$

Whereas, T_x represents the measured absorbance after 1, 2, 4, 6 and 24 h (T_0 is the absorbance at time zero).

2.3.3. Lipid peroxidation inhibition

The method of Yen *et al.* (2001) was used to measure the ability of bioactive compounds present in the plant extracts to inhibit linoleic acid peroxidation at an early stage. An emulsion was prepared by adding 0.280 g of linoleic acid, 0.280 g tween 20 and 50 ml phosphate buffer 0.02 M (pH 7). A volume of 2.5 ml of this emulsion was mixed with 0.5 ml of the different extracts at 0.1 mg/ml, then the mixture was incubated at 37 °C in the water bath for 5 days. Linoleic acid is easily oxidized, as its auto-oxidation initiates during incubation lipid

peroxides are formed, the estimation of these peroxides at 24 h intervals involve adding 0.1 ml of the reaction mixture to 0.1 ml of ferrous chloride (0.02 M) dissolved in 3.5% HCl; 0.1 ml ammonium thiocyanate (30%, *w*/*v*) and 4.7 ml of 75% ethanol. The absorbance of the resulting red chromophore is measured at 500 nm and the percentage of lipid peroxidation inhibition was calculated following the formula:

$$\textbf{Lipid peroxidation inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.3.1. Thiobarbituric acid-reactive substances (TBARS)

The assay involves the reaction of thiobarbituric acid (TBA) with the final product malondialdehyde (MDA), resulting from linoleic acid autoxidation during 5 days of incubation, in acidic conditions by the end to form a pink-colored complex MDA-TBA (Bentahar *et al.*, 2016). Briefly, 250 µl of the incubated mixture was added to 500 µl TBA (0.67%, *w*/*v*) and an equal volume of 20% (*w*/*v*) of trichloroacetic acid (TCA). The mixture was incubated in a water bath at 95 °C for 10 min, followed by 20 min centrifugation at 3000 rpm. The absorbance of the supernatant was measured at 532 nm.

2.3.4. Ferrous ion chelating assay

The used method to evaluate the ferrous ion chelating ability of the plant extracts is based on the measurement of the pink chromophore resulting from ferrous ion-ferrozine complex formation (Aouachria *et al.*, 2017). The reaction medium contains 250 µl of different concentrations of samples/EDTA, 50 µl ferrous chloride (0.6 mM) and 450 µl of methanol. The solution was left to stand for 5 min at room temperature. Afterwards, an aliquot of 50 µl of ferrozine (5 mM) was added to the reaction mixture and absorbance was measured at 562 nm after 10 min incubation. The percentage of plant extracts chelating activity was calculated using the below equation:

$$\textbf{Ferrous chelating activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3.5. Ferric reducing antioxidant power

The potential of bioactive compounds presents in our extracts to reduce ferric ions is evaluated according to Zerargui *et al.* (2016) with minor modifications. A volume of 100 µl of phosphate buffer (0.2 M, pH 6.6) was mixed with an equal volume of potassium ferricyanide and plant extracts at various concentrations, the reaction mixture was then incubated for 20 min at 50 °C. 250 µl of trichloroacetic acid (10%, *w*/*v*) was added to stop the reaction followed by centrifugation at 3000 rpm for 10 min. The supernatant (250 µl) was transferred to a mixture containing 250 µl distilled water and 500 µl of ferric chloride (0.1%, *w*/*v*) and the absorbance of the formed blue solution was monitored at 700 nm. The concentration providing 0.5 absorbance was deduced from a linear regression curve of each extract and standard.

2.3.6. Free radical-induced erythrocyte hemolysis assay

The *in vitro* oxidative hemolysis of mice erythrocytes was used to investigate the antioxidant potential of *A. leucotrichus* extracts against free radical-induced damage to red blood cell membrane as mentioned by Zerargui *et al.* (2023). Erythrocytes were freshly prepared from mice blood, by washing three times the collected blood with cold phosphate buffer saline (PBS) (10 mM, pH 7.4), followed by centrifugation at 3000 rpm for 10 min. A 2% erythrocytes suspension was prepared and used as an oxidizable target and 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was used as a pro-oxidant agent. The reaction mixture contained 20 µl of AlE and ascorbic acid (0.025, 0.05, 0.1 and 1 mg/ml), 80 µl of 2% erythrocytes suspension and 136 µl of ice cold AAPH (300 mM). The free radical-induced hemolysis was monitored continuously at 630 nm using a 96-well microplate reader (BioTEK

instruments, USA) and results were expressed as the time corresponding to 50% maximum erythrocyte hemolysis (half-hemolysis time, HT_{50}).

2.3.7. Antithrombotic activity

The antithrombotic activity of *A. leucotrichus* extracts was conducted using the freshly venous blood of healthy human volunteers as previously reported by Laroui *et al.* (2023). For the clot formation, 500 µl of the blood was transferred to pre-weighted microtubes and incubated for 45 min at 37 °C. After, the serum was removed without disturbing the clot and microtubes were re-weighted. Plant extracts were screened at 50 mg/ml and an aliquot of 100 µl was added to the microtubes, followed by 90 min incubation at 37 °C. After clot lysis, the fluid was removed and microtubes were weighted again to calculate the difference after clot disruption. Citrate was used as positive control. The percentage of antithrombotic (AT) was calculated as follows:

$$\textbf{AT (\%)} = \frac{\text{Weight}_{\text{clot}} - \text{Weight}_{\text{lysed clot}}}{\text{Weight}_{\text{clot}}} \times 100$$

2.3.8. Blood clotting time effect

The ability of AlE to inhibit blood clotting was evaluated by the described method of Saffidine *et al.* (2023). In this protocol, a volume (370 µl) of freshly donated blood from healthy volunteers was added immediately to microtubes containing 74 µl of each plant extracts at 50 mg/ml. Citrate and distilled water were used as positive and negative controls, respectively. Microtubes were incubated at room temperature and monitored every 30 sec for complete clotting.

2.4. Enzyme inhibition

2.4.1. α-amylase inhibition assay

Inhibition of α-amylase of plant extracts was performed according to Uysal *et al.* (2015). It is based on the measurement of starch protection in the presence of enzyme inhibitors. In a 96-well microplate, a volume of 50 µl of plant extracts at 2 mg/ml and standard inhibitor acarbose at various concentrations were mixed with 100 µl of 0.05% potato starch and 50 µl of α-amylase solution (10 U/ml) prepared in cold PBS (20 mM, pH 6.9) with 6 mM NaCl. After 10 min incubation at room temperature, the reaction was stopped by adding 25 µl of working indicator containing potassium iodide, diode and 1 M HCl. After this, the mixture was incubated for 5 min at room temperature and the absorbance was recorded at 630 nm in Multiskan GO microplate reader (Thermo scientific, Finland). Results were expressed as microgram equivalents of acarbose per milligram of extracts (mg ACA/g E).

2.4.2. α-glucosidase inhibition assay

Inhibition of α-glucosidase activity was determined using colorimetric substrate 4-Nitrophenyl-α-D-glucopyranoside (PNPG) that upon the enzymatic activity, a dark yellow chromophore is resulted indicating a *p*-nitrophenol releasing (Zengin *et al.*, 2016). A volume of 50 µl of α-glucosidase (0.2 U/ml) prepared in phosphate buffer (0.1 M, pH 6.9) was mixed with an equal volume of CrE and its fractions (2 mg/ml) and acarbose at various concentrations, in addition to 10 mM of PNPG in a 96-well microplate. The mixture was left to stand for 10 min at room temperature, then the reaction was stopped by adding 50 µl sodium carbonate (2%, w/v). Finally, after 5 min incubation, absorbance was measured at 405 nm against a blank (without enzyme) and the inhibitory activity of AlE was expressed as microgram equivalents of acarbose per milligram of extracts (mg ACA/g E).

2.5. Evaluation of *in vitro* anti-inflammatory activity of AlE

Plant extracts anti-inflammatory activity was determined using albumin heat-induced denaturation method according to Hamoudi *et al.* (2021) with slight modification. Test solutions (500 µl) containing 1.25 and 0.31 mg/ml of plant extracts and aspirin were mixed separately with 500 µl of human serum albumin (HSA) (0.2%, *w*/*v*) dissolved in 20 mM Tris-HCl buffer (pH 6.8). The reaction mixture was incubated for 20 min at 37 °C, and later, was heated at 65 °C in a water bath for 10 min. After cooling, the supernatant was aspirated and the absorbance was read at 660 nm. The protective effect of HSA was calculated in percentage according to the following formula:

$$\textbf{Protective effect (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6. *In vivo* evaluation of crude extract anti-inflammatory activity and its toxicity

2.6.1. Evaluation of CrE anti-inflammatory activity

2.6.1.1. Experimental animal design

This experiment involved five groups of 5 to 8 mice and prior to the day of experiments; mice were fasted overnight but had access to water. All the substances used were diluted in distilled water (DW) and administrated orally except for the test extract; it was dissolved in 5% DMSO and then diluted in DW. Group 1 received distilled water and served as the control group (CG), Group 2 administrated standard drugs: 150 mg/kg of Aspirin in Analgesic effect and 50 mg/kg of Indomethacin in Carrageenan, xylene and croton oil-induced edema and this group was allocated to positive group (PG). Groups 3, 4 and 5 were served as test groups and received respectively: 100 mg/kg (Dose 1 group; D1 G); 200 mg/kg (Dose 2 group; D2 G) and 400 mg/kg (Dose 3 group; D3 G) of CrE. A digital caliper was used to measure the induced edema in animals' soft material (ears and paws) in millimeters.

2.6.1.2. Analgesic effect by the acetic acid- induced writhing method

Analgesic effect of CrE was determined using the acetic acid-induced writhing method (Zerargui *et al.*, 2022). Five animal groups received respectively distilled water, the analgesic drug Aspirin at 150 mg/kg and CrE at varying doses (100, 200 and 400 mg/kg). After 1 h from administration, acetic acid (0.9%, *v*/*v*) was injected intraperitoneally (10 ml/kg) to create a pain sensation. Animals were divided separately in clear cages and the number of writhing episodes was counted at 5 min intervals for 30 min. The percentage of analgesic effect was calculated as follows:

$$\textbf{Analgesic effect (\%)} = \frac{\text{Mean writhing times of CG} - \text{Mean writhing times in test group}}{\text{Mean writhing times of CG}} \times 100$$

2.6.1.3. Carrageenan-induced paw edema

Anti-edematous effect of plant extract on carrageenan-induced paw edema model was performed as described by Laroui *et al.* (2024). Prior to the experiment, the thickness of mice's right hind paws was measured with a digital caliper. In this test, CG was given distilled water and PG administrated Indomethacin at 50 mg/kg, whereas the animals in test groups were given CrE at various doses. After 1 h, paw edema was induced by the injection (20 µl) of freshly made 1% (*w*/*v*) λ-carrageenan into the right subplantar surface of mice paw. Paw thickness was measured again at 1, 2, 3 and 4 h after edema induction and the percentage of edema inhibition was calculated as the following equation:

$$\textbf{Edema inhibition (\%)} = \frac{\text{Paw edema of CG} - \text{Paw edema of test group}}{\text{Paw edema of CG}} \times 100$$

2.6.1.4. Xylene-induced ear edema

Anti-inflammatory activity of various doses of *A. leucotrichus* CrE was investigated using irritant agent in the acute inflammation model (Mayouf *et al.*, 2019). Animal groups received the vehicle, 50 mg/kg Indomethacin and CrE (100, 200 and 400 mg/kg) 1 h prior to the

experiment and ear thickness was measured. Acute inflammation was induced by applying to the anterior surface of the right ear 30 µl of xylene. After 45 min, ear edema was measured and the following formula was used to calculate the percentage of auricular edema reduction:

$$\textbf{Auricular edema reduction (\%)} = \frac{\text{CG ear edema} - \text{experimental group ear edema}}{\text{CG ear edema}} \times 100$$

2.6.1.5. Croton oil-induced ear edema

This method is a widely used animal model for studying topical acute inflammation. According to the adopted protocol of Cheniti *et al.* (2022), ear edema is induced by topical application (20 µl) in the inner surface of mice's right ears of a freshly prepared croton oil (109 µg/ear) in acetone-water (1:1, *v*/*v*) after 1 h of vehicle, indomethacin (50 mg/kg) and various doses of CrE were administrated orally. The left ear remained untreated. After 6 h, the increase in ear thickness was measured, and then animals were sacrificed by cervical dislocation. Three of the edematous ears per group were excised immediately together with the left ones (untreated ears, UE), trimmed to remove hair excess, and finally fixed in 10% formaldehyde to preserve the native structure of tissue for histological examination. Meanwhile, the rest of the samples were collected in tubes and stored at -20 °C for the measurement of oxidative stress-related markers: NO^{2-}, malondialdehyde (MDA) and catalase (CAT) activity. Auricular edema reduction of the tested extract in percentage was determined by following the below formula:

$$\textbf{Auricular edema reduction (\%)} = \frac{\text{CG ear edema} - \text{experimental group ear edema}}{\text{CG ear edema}} \times 100$$

2.6.2. Acute toxicity of CrE

The acute oral toxicity of CrE was conducted as mentioned in the 'Up-and-Down-Procedure' of testing chemicals in animals according to the OECD guideline 425 (OECD, 2022). The limit test at 2000 mg/kg and 5000 mg/kg were used on mice males. The animals were

randomly divided into three groups, each contain 5 mice, weighed and marked for identification. The first group received orally distilled water and considered as control group (CG), the second and third groups were served as tested groups and administrated orally CrE at dose 2000 mg/kg (Dose 2g/kg group; D2g/kg G) and 5000 mg/kg (Dose 5g/kg group; D5g/kg G), respectively. Test extract was dissolved in non-toxic substance 5% DMSO and diluted in distilled water. For the limit test at 2000 mg/kg, two animals of group 1 and 2 were separated, received distilled water and CrE. Animals were observed for 5, 10, 15 and 30 min then after 1, 2, 24 and 48 h for any toxic sings according to the Hippocratic screening of Malone (1982); changes in hair, eyes and respiration rate, hyperemia of ear, tail response, grooming, aggressivity, ataxia, sleep, food and water intake and lethality. Furthermore, the rest of animals in group 2 were administrated CrE after 48 h and observed for a total of 14 days. In addition, the limit test at 5000 mg/kg was carried out on group 3 the same. Body weight of mice was taken on day 0, 2, 7 and after 14 days.

2.6.3. Sub-Acute toxicity of CrE

The oral Sub-Acute toxicity was carried out according to OECD guideline 407 on oral repeated toxicity testing of chemicals in rodents (OECD, 2008). Healthy mice males were randomly divided into five experimental groups with 8 animals each; weighed, marked for identification and placed under standard conditions of the laboratory. The first group assigned to the control group of toxicity (CGT) which received DW, the second group administrated Ascorbic acid at 100 mg/kg diluted in DW and considered as positive group of toxicity (PGT), treatment groups were assigned to groups 3, 4 and 5 which received respectively CrE, dissolved in 5% DMSO and then diluted in DW, at: 100 mg/kg (Dose 1 group of toxicity; D1 GT), 200 mg/kg (Dose 2 group of toxicity; D2 GT) and 400 mg/kg (Dose 3 group of toxicity; D3 GT). The tested substances in addition to the vehicle were administrated per gavage daily for 21 consecutive days. Body weight was measured once a week throughout the experiment.

At the end of the procedure (day 21) animals were fasted overnight but had unlimited supply to water and on day 22 animals were sacrificed, followed by exsanguinations. Blood samples were collected from the jugular vein using tubes containing anticoagulant EDTA for free radical-induced erythrocyte hemolysis assay. In addition, vital organs: livers, kidneys, spleen, heart, lungs and stomach were carefully dissected, washed with cold sodium chloride (0.9%, *w*/*v*) and their weight was recorded. The relative organ weight of each mouse was determined using the following formula:

$$\textbf{Relative organ weight (\%)} = \frac{\text{Organ weight}}{\text{Body weight (day 22)}} \times 100$$

Livers and kidneys were sectioned; a small section of each organ was fixed in buffered formaldehyde (10%, *v*/*v*) for histopathological inspection, and the rest of organ was collected in tubes and stored in -20 °C for the assessment of oxidative stress-related markers: NO^{2-}, MDA, glutathione (GSH), CAT, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S transferase (GST).

2.6.3.2. *In vivo* free radical-induced erythrocyte hemolysis assay

Collected blood was centrifuged at 3000 rpm for 10 min and the plasma was removed. Then, the blood was subjected to 3 times washing with PBS and 2% erythrocytes suspension was prepared. The assay was conducted as previously mentioned *in vitro* (Aouchria *et al.*, 2017); 136 µl of AAPH (300 mM) solution in ice cold PBS was added to the microplate wells already containing 80 µl of 2% erythrocytes suspension and 20 µl of PBS. The absorbance was recorded every 15 min for 4 h at 630 nm and the incubation temperature was maintained at 37 °C. Results were expressed as the time corresponding to 50% maximum hemolysis (half-hemolysis time, HT_{50}).

2.6.4. Histological analysis

The fixed tissues (ears, livers and kidneys) are firstly transferred into cassette, and placed for tissue processing in an automated machine that pumps chemicals (ethanol with increasing concentration and xylene) in and out the tissue specimens to remove the water. Secondly, in the embedding station, specimens were removed from the cassette and placed into a mold, the high temperature in this station keeps the paraffin wax as a liquid to be easier to manipulate the tissue samples, and form paraffin blocks. Thirdly, the cooled blocks were transferred to sectioning by using microtome to obtain very thing sections of tissue with a thickness of approximately 5 µm. After this, the sections were picked up from a worm water bath and placed into a glass slide. Finally, to visualize the tissues' structure histological stains were used, Hematoxylin-Eosin, in which nucleus of cells is shown in blue and cytoplasm in pink. Slides were analyzed qualitatively under a high-quality LEICA DM1000 Microscop (Leica Microsystems, Germany) and the taken images were treated with Leica Application Suite EZ (LAS EZ) software.

2.6.5. Tissue homogenates preparation

To form a suspension of tissue, 4.5 ml of cold potassium chloride (1.15%) is added to previously weighted ears, livers and kidneys (0.5 g). The suspension was subjected to mechanical disruption on ice using an electric homogenizer then centrifuged two times at 4000 rpm for 10 min at 4 °C to remove cell debris. Supernatant was divided into aliquots, transferred to microtubes and stored at -20 °C for further assess of oxidative stress-related markers. Total protein in tissue homogenates was quantified using a commercial Kit (DiaScan) based on biuret colorimetric method, with bovine serum albumin as standard.

2.6.5.1. Estimation of MDA in tissue homogenates

Tissues MDA content was quantified according to the method described by Bentahar *et al.* (2016). In microtubes, 250 µl of 0.67% TBA and an equal volume (125 µl) of 20% TCA in

addition to tissue homogenates were mixed. The tubes were heated in a boiling water bath (100 °C) for 20 min, then ice-cooled before adding 1 ml of butanol to extract the MDA-TBA complex. Centrifugation was performed for 15 min at 3000 rpm, and absorbance of the supernatant was measured at 532 nm. Malondialdehyde content was estimated using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and the results are expressed as nmol/g of tissue.

2.6.5.2. Estimation of $NO^{\cdot}$ in tissue homogenates

The measurement of nitrite ion levels is an index for nitric oxide radical production. According to the adopted protocol of Abdelouhab *et al.* (2012), NO^{2-} is estimated by adding to 100 µl of tissue homogenates an equal volume (50 µl) of 1% sulfanilamide diluted in 5% phosphoric acid and 0.1% of the NEDD solution. The mixture was incubated at room temperature for 25 min in darkness, and absorbance was read at 543 nm. A standard curve of nitrite was prepared the same (**figure 18**), the unknown concentration of NO^{2-} in tissue homogenates was calculated using the linear regression equation and the results are expressed in µM.

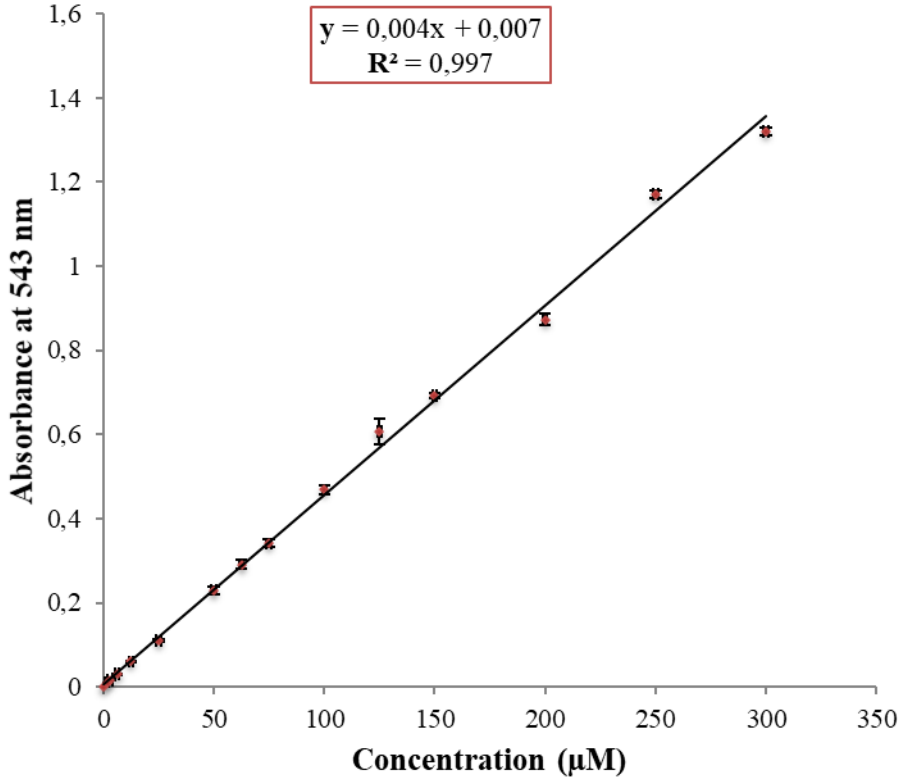


Figure 18. Standard curve of sodium nitrite. Values are the mean of triplicates ± SD.

2.6.5.3. Estimation of GSH in tissue homogenates

Reduced GSH levels were estimated using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic), DTNB) following Aouchria *et al.* (2017) method. 25 µl of tissue homogenates were diluted by adding 5 ml of 0.1 M phosphate buffer (pH 8). A volume of 1.5 ml of this diluted mixture was added to 10 µl of 0.01 M of DTNB solution, followed by 5 min incubation at room temperature. Oxidation of GSH yields a yellow derivate 5′-thio-2-nitrobenzoic acid (TNB) measurable at 412 nm. Levels of GSH are calculated using its extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} and results are presented as nmol/mg of protein.

2.6.5.4. Determination of CAT activity

The standard UV spectrophotometric kinetic method used to determine CAT activity is based on the rate of H_2O_2 decomposition at 240 nm (Righi *et al.*, 2021). The 30% H_2O_2 was diluted with 50 mM phosphate buffer (pH 7.4) to achieve a concentration of 19 mM. An aliquot of 17 µl of tissue homogenates was added immediately to 983 µl of buffered H_2O_2, and the decrease of absorbance was monitored for 30 sec at 240 nm against a blank (buffer) using UV-1800 Spectrophotometer (SHIMADZO, USA). Enzymatic activity was calculated using the extinction coefficient of H_2O_2 and expressed as µmol/min/mg of protein.

2.6.5.5. Determination of SOD activity

In order to estimate superoxide dismutase activity, an indirect method using NBT was applied according to Chouala *et al.* (2024). The method employs a system containing riboflavin, methionine and NBT, illumination of this system and under aerobic conditions; generates superoxide anion and this acts as an electron-carrying intermediate to reduce NBT to blue formazan. The used concentrations of riboflavin, methionine and NBT were 0.26 mM, 39 mM and 2.64 mM, respectively, and all solutions were prepared using 50 mM phosphate buffer at pH 7.8. The reaction mixture containing 1 ml of methionine in 0.3 mM EDTA, 85.2 µl of NBT and 22.6 µl of riboflavin, was mixed with 50 µl of diluted tissue homogenates (1:5, *v*/*v*).

The final volume was adjusted to 3 ml with buffer and the final mixture was incubated in front of a light source and under aerobic conditions for 20 min. The inhibition of NBT reduction in the presence of SOD was followed at 580 nm. The rate of NBT reduction in the absence of enzyme sample was used as the reference rate. Enzymatic activity was calculated following the below equation and results are expressed as U/mg of protein, where one unit defines the amount of SOD that inhibits 50% of NBT reduction.

$$\textbf{SOD (U/mg of protein)} = \frac{\left(\frac{\left(\frac{\text{Absorbance}_{\text{reference rate}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{reference rate}}}\right)\times 100}{50} \times 3 \times \frac{1}{V} \times D\right)}{\text{mg of protein}}$$

Where: reference rate is where the total reduction of NBT; 3 is the total volume of the reaction mixture in ml; V is the volume of enzyme sample in ml; D is the dilution factor of the tissue homogenates.

2.6.5.6. Determination of GPx activity

Determination of glutathione peroxidase activity is based on a modified protocol of Chenna *et al.* (2024) that measures in a fixed time GSH consumption by the enzyme in the presence of Ellman's reagent. A reaction mixture containing 200 µl of tissue homogenates, 200 µl of 0.1 M buffer phosphate (pH 7.4) in 1 mM EDTA and 400 µl of 0.1 mM GSH, was pre-incubated in water bath at 37 °C for 5 min. The reaction was initiated by adding 200 µl of 1.3 mM H_2O_2. After 10 min incubation at 37 °C, the reaction was arrested with acidification by adding 1 ml of 1% TCA and centrifugation was performed at 3000 rpm during 10 min. Finally, aliquot of 480 µl of the supernatant was mixed with 2.2 ml of disodium hydrogen phosphate (0.32 M) and 320 µl of 1 mM DTNB. The final mixture was incubated for 5 min and absorbance of the resulted yellow chromophore was measured at 412 nm. Control tube not containing tissue homogenates was included. Enzyme activity was calculated using the following equation and

results are expressed as nmol GSH oxidized per minute per milligram of protein (nmol/min/mg of protein).

$$\textbf{GPx (nmol/min/mg of protein)} = \left(\frac{\text{Absorbance sample} - \text{Absorbance control}}{\text{Absorbance control}}\right) \times \left(\frac{0.04 \times \left(1 \times \frac{1}{V}\right)}{\text{mg of protein} \times 10}\right)$$

Where: 0.04 is the inner concentration of GSH; 1 is the volume of the enzymatic reaction in ml; V is the volume of tissue homogenates in ml; 10 is the enzymatic reaction time.

2.6.5.7. Determination of GST activity

Glutathione S transferase activity was assayed kinetically according to Shultz *et al.* (2002), by monitoring spectrophotometrically the conjugation of reduced GSH to 1-chloro-2,4-dinitrobenzene (CDNB) results in increase of the absorbance at 340 nm. To analyze samples, 30 µl of 33 mM GSH diluted in 0.1 M phosphate buffer (pH 8.5), was mixed with an equal volume of 33 mM CDNB diluted in absolute ethanol, the volume was adjusted to 995 µl by adding phosphate buffer (0.1 M, pH 6.5). Afterwards, 5 µl of tissue homogenates was added immediately, mixed and the increase of absorbance was recorded every minute for 5 min. A blank was prepared without enzyme preparation. Enzyme activity was calculated using the extinction coefficient of CDNB as follows and results are expressed as µmol/min/mg of protein:

$$\textbf{GST (µmol/min/mg of protein)} = \frac{\left(\frac{\Delta \text{ Absorbance/min}_{\text{sample}} - \Delta \text{ Absorbance/min}_{\text{blank}}}{9.6}\right) \times 1 \times \frac{1}{V}}{\text{mg of protein}}$$

Where: 1 is the total volume in ml; V is the volume of enzyme preparation in ml.

3. Statistical analysis

In this study, experiments were performed in triplicates, and data are expressed as mean ± standard variation (SD) or standard error of the mean (SEM) for *in vitro* and *in vivo*

experiments, respectively. Statistical comparison between extracts or animal groups was determined by one or two-way analysis of variance (ANOVA) followed by *Tukey* and *Bonferroni* post-tests. $p \leq 0,05$ was considered to indicate a statistically significant difference. The statistical software GraphPad Prism version 5.0 (San Diego, CA., USA) was used for graphing and analysis and Microsoft Excel was used for calculation and linear graphing. All the EC_{50} and IC_{50} were calculated from linear regression equations.

Chapter 3: Results and discussion

1. Extraction and phytochemical analysis

1.1. Extraction

Most biological studies done on *A. leucotrichus* are related to the phytochemical composition of the essential oils, and few scientific approaches have been carried out towards the phenolic content and their bioactive properties. In this current study, a crude methanolic extract comprising various classes of polyphenols and other components was obtained through the methanolic maceration. Liquid-liquid scale extraction with solvents of increasing polarity yielded in: hexane extract (HE) which contains lipids and resulting in deffated crude extract (CrE), chloroform extract (ChE) rich in aglycone flavonoids, ethyl acetate extract (EAE) containing glycosylated flavonoids and the remaining compounds (polysaccharides, tannin…etc.) are present in aqueous extract (AqE). Total phenolics (TPC) and flavonoids content (TFC) together with the yield of the crude extract and its fraction are presented in **table 2**.

The solid-liquid extraction of phytochemicals from *A. leucotrichus* seeds using organic solvent, methanol, showed a considerable amount of 8%. However, the use of solvents of increasing polarity yielded a low amount of flavonoids which are comparable to each other in term of quantity: ChE (4.14%) and EAE (4.62%). The total amounts of polyphenols and flavonoids were estimated by the Folin Ciocalteu and Aluminum chloride methods, expressed in µg of gallic acid equivalents and quercetin equivalents per mg of the extract, respectively. Results showed that CrE carry an important content of total phenolic and flavonoids (77.14 ± 0.010 µg GAE/mg of E and 19.59 ± 0.084 µg EQ/mg of E, respectively). It is worthy to highlight that EAE was the most abundant fraction with flavonoids (16.92 ± 0.105 µg EQ/mg of E), compared to ChE and AqE.

Table 2. Extraction yield, total phenolics (TFC) and flavonoids content (TFC) of CrE and its fractions.

Extracts	Yield (%)	Total phenolics content (mg GAE/g of extracts)	Total flavonoids content (mg QE/g of extracts)
CrE	08%	77.14 ± 0.010	19.59 ± 0.084
ChE	04.14%	69.83 ± 0.163	06.06 ± 0.039
EAE	04.62%	64.57 ± 0.045	16.92 ± 0.105
AqE	89.37%	31.74 ± 0.010	05.27 ± 0.009

Values are the mean ± SD (n = 3). CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract. GAE: gallic acid equivalent; QE: quercetin equivalent. ChE, EAE and AqE were obtained from an amount of 51 g of CrE.

Solid-liquid extraction is the classic and most commonly used method in recovering phenolic compounds; it is based on the diffusion of target analytes contained in a solid matrix into the extraction solvent (Tomaz *et al.*, 2019). There are many variables; solvent type, organ used, composition in secondary metabolites, duration, affinity of the solvent towards the analyte, and polyphenols solubility in which the yield, diffusion and efficiency of extraction are influenced (Tomaz *et al.*, 2019; Laroui *et al.*, 2023). However, the type and ratio of the used solvent are the main influencers on the quantity of extracted phenolic compounds (Sepahpour *et al.*, 2018; Hellal *et al.*, 2020). Previously reported that methanol is the best-used solvent in extracting phenolic compounds from medicinal plants (El Atki *et al.*, 2019). In the current study, the divergence of the extracted bioactive compounds observed from liquid-liquid scale extraction results is related to the difference in the polarity of phenolic compounds towards the used solvents, in addition, this could explain that a large range of polyphenols classes present in the plant do have a considerable affinity toward methanol. This organic solvent successfully increased the membrane permeability thereby extracting the target bioactive compounds.

1.2. Phytochemical analysis

Quantitative analysis of 35 phenolic compounds from *A. leucotrichus* different extracts were performed following the described analytical method HPLC-ESI-MS/MS. The results of

analysis and the bioactive compounds concentration are summarized in **table 3**. The concentration of total identified polyphenols and flavonoids was in the following decreasing order: EAE (10040.78 µg/g) > ChE (8390.67 µg/g) > CrE (7057.06 µg/g) > AqE (1116.91 µg/g). A total of 13 compounds were identified in CrE including hydroxycinnamic and hydrobenzoic acids: caffeic acid, the most abundant phenolic acid (5196.65 µg/g), followed by neochlorogenic acid (488.06 µg/g), vanillic acid, 4-hydroxybenzoic acid, ferulic acid, syringic acid, chlorogenic acid, p-coumaric, gallic acid. Additionally, low quantities of hydrolysable tannin, ellagic acid, was detected (97.86 µg/g) along with flavanol catechin, flavonol isoquercitrin and anthocyanidin delphindin 3,5 diglucoside. 9 compounds were detected in ChE and it was characterized by the high concentration of caffeic, vanillic and ferulic acids with 5010, 1499.01 and 1033.88 µg/g, respectively. Isorhamnetin, as flavonol, was present in this extract with very low concentration. Flavonoids were quite present in a limited number in EAE with a total of 19 compounds identified, including flavonol class with kaempferol presenting the major contribution (7459.28 µg/g), quercetin, rutin, isoquercetrin, quercetrin, isorhamnetin and hyperoside. Phloridzin, a chalcone member, had the lowest concentration in this extract (0.60 µg/g). Although the aqueous extract displayed the least number (4 compounds) and concentration of bioactive compounds, AqE had considerable amounts of kaempferol (685.86 µg/g) and neochlorogenic acid (300.06 µg/g) together with low concentration of hydrolysable tannin and chlorogenic acid. Most of these phenolic compounds previously reported in many studies to have beneficial effect on human health, due to their antioxidant, anticancer, anti-inflammatory activities and considered as effective enzymes inhibitors. These results support that methanol used in maceration, successfully recovered diversity of phenolic compounds with potent activities.

Table 3. Phenolic compounds profile of *Ammodaucus leucotrichus* seeds' extracts characterized by HPLC-ESI-MS/MS analysis.

Compound	**CrE**	**ChE**	**EAE**	**AqE**
Gallic acid	12.95	n.d.	190.94	n.d
Neochlorogenic acid	488.06	n.d	86.68	300.06
Delphindin-3-galactoside	n.d.	n.d.	n.d.	n.d.
Catechin	85,35	n.d.	n.d.	n.d.
Procyanidin B2	n.d.	n.d.	n.d.	n.d.
Chlorogenic acid	117.83	7.38	142.34	53.89
4-Hydroxybenzoic acid	216.87	190.67	530.78	n.d.
Epicatechin	n.d.	n.d.	n.d.	n.d.
Cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.
Petunidin-3-glucoside	n.d.	n.d.	n.d.	n.d.
3-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.
Caffeic acid	5196.65	5010.00	750.75	n.d.
Vanillic acid	376.60	1499.01	230.57	n.d.
Pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.
Pelargonidin-3-rutinoside	n.d.	n.d.	n.d.	n.d.
Malvidin-3-galactoside	n.d.	n.d.	n.d.	n.d.
Syringic acid	167.52	402.07	36.83	n.d.
Procyanidin A2	n.d.	n.d.	n.d.	n.d.
P-Coumaric acid	66.66	108.14	192.40	n.d.
Ferulic acid	179.85	1033.88	60.07	n.d.
Rutin	n.d.	n.d.	4.12	n.d.
Hyperoside	n.d.	n.d.	179.20	n.d.
Isoquercitrin	20.30	n.d.	77.65	n.d.
Delphindin 3,5 diglucoside	30.58	n.d.	81.40	n.d.
Phloridzin	n.d.	n.d.	0.60	n.d.
Quercitrin	n.d.	n.d.	2.97	n.d.
Myricetin	n.d.	n.d.	n.d.	n.d.
Naringin	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-glucoside	n.d.	n.d.	n.d.	n.d.
Hesperidin	n.d.	n.d.	n.d.	n.d.
Ellagic acid	97.86	133.89	9.66	77.11
Quercetin	n.d.	n.d.	3,66	n.d.
Phloretin	n.d.	n.d.	n.d.	n.d.
Kaempferol	n.d.	n.d.	7459.28	685.86
Isorhamnetin	n.d.	5.62	0.89	n.d.
Total phenolic compounds	**7057.06**	**8390.67**	**10040.78**	**1116.91**

Concentrations were expressed in μg/g of extract. n.d. means "not detected".

2. Antioxidant activities

2.1. Radical scavenging activity

The antioxidant activity of *A. leucotrichus* extracts was evaluated using six radical scavenging assays: $DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, $OH^{\bullet}$, $O_2^{\bullet-}$ and $NO^{\bullet}$. IC_{50} was calculated and shown in **table 4**, in which the higher activity is achieved with lower IC_{50} values. According to the results represented, EAE was the potent scavenger in all of the above mentioned six tested systems with a remarkable IC_{50} values equal to 0.02 ± 0.0003 ($DPPH^{\bullet}$), 0.02 ± 0.0002 ($ABTS^{\bullet+}$), 0.04 ± 0.001 (H_2O_2), 0.30 ± 0.012 ($OH^{\bullet}$), 0.21 ± 0.01 ($O_2^{\bullet-}$) and 0.19 ± 0.01 mg/ml ($NO^{\bullet}$). It is noteworthy to mention the non-significance difference ($p > 0.05$) observed with this extract compared to the standards: BHT and ascorbic acid in $DPPH^{\bullet}$, H_2O_2 and $OH^{\bullet}$ tests. In all the assays the order of decreasing scavenging activity was found to be EAE > ChE > CrE > AqE except for $NO^{\bullet}$ scavenging, CrE was the weakest scavenger.

Table 4. $DPPH^{\bullet}$, $ABTS^{\bullet+}$, ROS and RNS scavenging activity of CrE and its fractions.

Extracts	IC_{50} values (mg/ml)					
	$DPPH^{\bullet}$	$ABTS^{\bullet+}$	H_2O_2	$OH^{\bullet}$	$O_2^{\bullet-}$	$NO^{\bullet}$
CrE	$0.10 \pm 0.002^{***}$	$0.05 \pm 0.0003^{***}$	$0.15 \pm 0.003^{***}$	$2.39 \pm 0.007^{***}$	$0.62 \pm 0.07^{***}$	$0.73 \pm 0.01^{***}$
ChE	$0.07 \pm 0.003^{***}$	$0.04 \pm 0.0008^{***}$	$0.12 \pm 0.001^{***}$	0.71 ± 0.049^{ns}	$0.46 \pm 0.03^{***}$	$0.28 \pm 0.06^{**}$
EAE	0.02 ± 0.0003^{ns}	$0.02 \pm 0.0002^{***}$	0.04 ± 0.001^{ns}	0.30 ± 0.012^{ns}	$0.21 \pm 0.01^{**}$	$0.19 \pm 0.01^{*}$
AqE	$0.30 \pm 0.006^{***}$	$0.11 \pm 0.002^{***}$	$0.67 \pm 0.023^{***}$	$12.88 \pm 0.245^{***}$	$0.89 \pm 0.05^{***}$	$0.66 \pm 0.03^{***}$
BHT	0.02 ± 0.0001	0.01 ± 0.0006	n.t.	0.62 ± 0.004	n.t.	n.t.
Ascorbic acid	n.t.	n.t.	0.02 ± 0.0002	n.t.	n.t.	0.01 ± 0.0001
Gallic acid	n.t.	n.t.	n.t.	n.t.	0.04 ± 0.001	n.t.

Values are mean of triplicates ± SD. ns: $p > 0.05$; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. Comparison was against standards (BHT, Ascorbic acid, Gallic acid). n.t.: not tested. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

Many studies show that polyphenols exhibit many biological activities. However, most interest has been devoted to their action as antioxidants due to their ability to scavenge free radicals. Indeed, phenolic and flavonoid compounds are responsible for neutralizing free radicals by hydrogen atoms transfer (HAT) or singlet electron transfer (SET) or the combination of both (Cvitanović *et al.*, 2018). The first one is based on the donation of a hydrogen to the free radical and the second one depends on the antioxidants to reduce radicals through singlet electron transfer and at the end of these reactions, antioxidant its self becomes relatively stable radical with absence of reactive positions towards O_2 (Liang and Kitts, 2014; Andrés *et al.*, 2023). The presence of polyphenols and flavonoids in the CrE and its fractions could to some extent justify their contribution in the observed radical scavenging activity, and such reactions HAT and SET did take place during the neutralization of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, ROS and RNS, which some of them are responsible for oxidative damage in the living cells. As shown in the beneath schematic representation (**figure 19**):

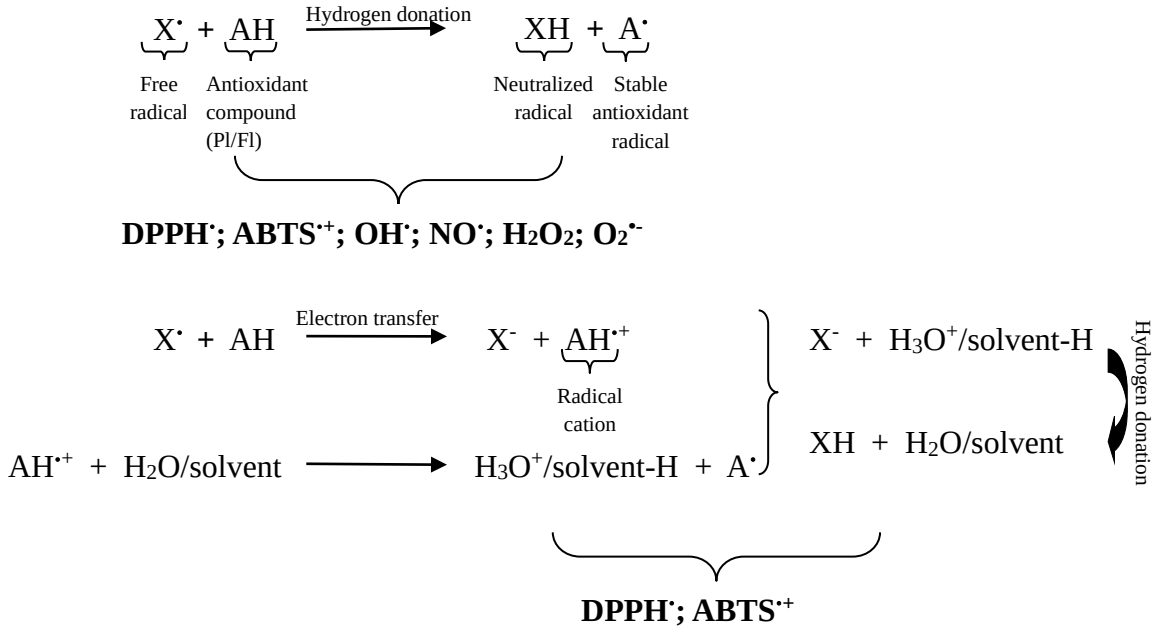


Figure 19. Reaction mechanisms (HAT and SET) of bioactive compounds present in the CrE and its fractions involved in $DPPH^{\bullet}$; $ABTS^{\bullet+}$; $O_2^{\bullet-}$; H_2O_2; $NO^{\bullet}$ and $OH^{\bullet}$ neutralization. Pl: polyphenols. Fl: flavonoids (Sideeg *et al.*, 2020; Echegaray *et al.*, 2021).

Antioxidant activity of polyphenols is governed by the type of compound and the chemical structure including some of the parameters: degree of methoxylation and number of hydroxyl groups, which indorse them with the ability to act as free radical scavengers (Cvitanović *et al.*, 2018; Gao *et al.*, 2022). Results of correlation showed a large range from strong to very week negative correlation between TPC [R = (-0.918; -0.261)], TFC [R = (-0.918; 0.058)] and radical scavenging methods ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, $OH^{\bullet}$, $O_2^{\bullet-}$ and $NO^{\bullet}$). However, phenolic compounds present in the plant extracts are previously studied and considered as bioactive molecules with remarkable radical scavenging activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and highly reactive species (including $OH^{\bullet}$ and $O_2^{\bullet-}$) and in some cases are more potent than BHT and Butylated hydroxyanisole (BHA); through having the ability to donate hydrogen or electron as consequence to neutralize free radicals. Among these bioactive molecules: caffeic, vanillic, ferulic, p-hydroxybenzoic, p-coumaric, chlorogenic and neochlorogenic acids (Gao *et al.*, 2022; Andrés *et al.*, 2023). Scientific evidence has shown that flavonoids are excellent antioxidants because they are highly reactive as electron or hydrogen donors to stabilize free radicals (Seyoum *et al.*, 2006). Bors *et al.* (1990) proposed the favorable chemical structure for effective radical scavenging function of flavonoids; the presence of 3',4'-dihydroxy occupied B ring, a C_2-C_3 double bond and the presence of 3-hydroxyl group and 5-hydroxyl group for maximal anti-radical activity. Favonoids (kaempferol, quercetin, quercetrin and rutin) of ethyl acetate fraction are potent radical scavengers (Patil *et al.*, 2013; Sivasothy *et al.*, 2013; Panat *et al.*, 2015) and probably elevated EAE activity with polyphenols content. In addition, the difference in the scavenging potential observed by the extracts could be due to the difference in the number of various radicals trapped per polyphenol molecule, i.e. stoichiometry of reaction (Sahreen *et al.*, 2017).

2.2. β-Carotene/Linoleic acid bleaching assay

Inhibition capacity of linoleic acid and β-Carotene coupled oxidation in the presence of plant extracts was monitored spectrophotometrically during 24 h and the results are represented in **figure 20**. All the extracts inhibited the bleaching of β-Carotene similarly to BHT which showed a stable potent activity (98.16 ± 0.754%). The highest inhibition activity was observed with EAE (81.99 ± 2.942%), followed by ChE, CrE and AqE.

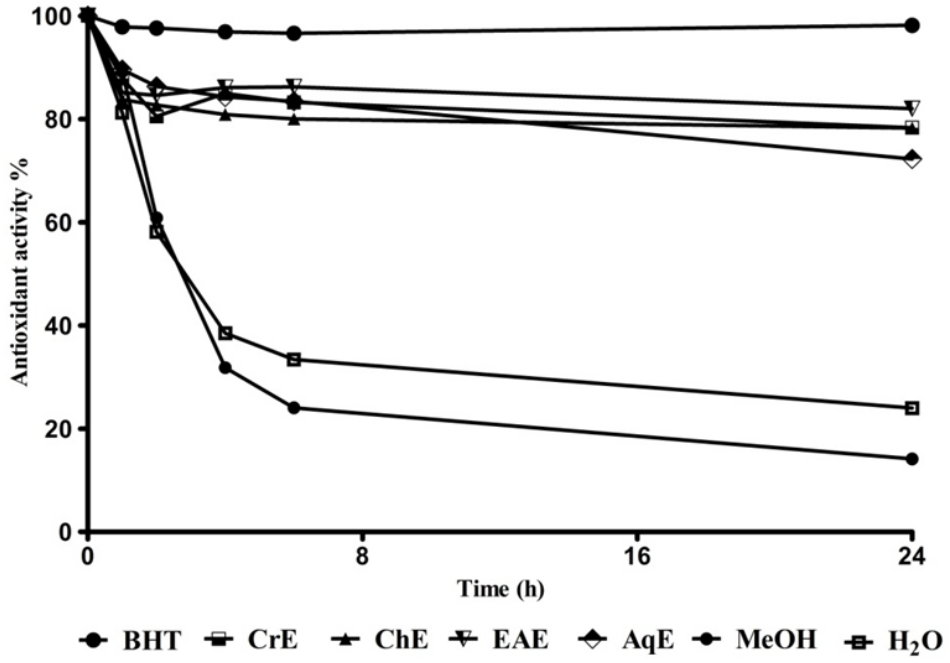


Figure 20. Time course of β-Carotene bleaching during 24 h in the presence of AlE, BHT, water and methanol. Results are the mean of three experiments. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

The lipid bilayer of membrane is rich in unsaturated fatty acids especially linoleic acid and arachidonic acid, that are most susceptible targets to lipid peroxidation (Ordonez *et al.*, 2006). β-Carotene bleaching assay is a good model for assessing the early stage of lipid peroxidation (initiation and propagation). The oxidation of the coupled β-Carotene/Linoleic acid is a free-radical mediated phenomenon. The arising of a conjugated diene hydroperoxides upon the abstraction of a hydrogen atom from linoleic acid (Schaich *et al.*, 2013), attacks double bonds

of β-carotene therefore, the compound oxidizes and loses its characteristic orange color. The correlation performed between this assay with TPC (R= 0.782) and TFC (R= 0.668) exhibited a strong positive relationship. Therefore, the strong inhibition values obtained from the studied extracts (CrE, ChE, EAE and AqE) can be ascribed to their polyphenols and flavonoids content, relying on hydrogen atom transfer mechanism, to neutralize at early stage and convert free radicals formed in the system into more stable non-reactive species and terminate the chain of radical reaction (Pisoschi *et al.*, 2016; Sahreen *et al.*, 2017). This is in consistence with Pandey *et al.* (2018) who reported that phenolic compounds are known to scavenge free radicals generated in case of β-Carotene bleaching assay. In similar study of Sivasothy *et al.* (2013) and Sevgi *et al.* (2015), kaempferol, caffeic acid, ferulic acid, vanillic acid were considered as a potent reducing agents to neutralize the free radical-mediated β-Carotene/Linoleic acid oxidation, related to the presence of the required antioxidant functional groups as mentioned in the section results and discussion (radical scavenging activity). Thus, these potent antioxidants together with other phenolics attribution are responsible for the high inhibition activity of the tested extracts.

2.3. Lipid peroxidation

Lipid peroxidation inhibition by plant extracts at 0.1 mg/ml using ferric thiocyanate to measure peroxide levels at early stage of linoleic acid autoxidation, and TBARS methods to evaluate the final product of peroxidation are presented in **figures 21** and **22**, respectively. The used concentration 0.1 mg/ml of plant extracts and BHT was noticeably effective to inhibit linoleic acid peroxidation (**figure 21**). Among the extracts used, EAE and ChE performed a high inhibition rate of the polyunsaturated acid peroxidation after 96 h with values equal to 69.71 ± 0.79% and 66.17 ± 0.23%, respectively. Whereas, BHT as standard inhibited lipid peroxidaion by 77.49 ± 1.39%. The inhibition activity of CrE and AqE

decreased rapidly after 48 h to achieve values of 37.61 ± 0.70% and 48.61 ± 1.16% after 96 h, respectively.

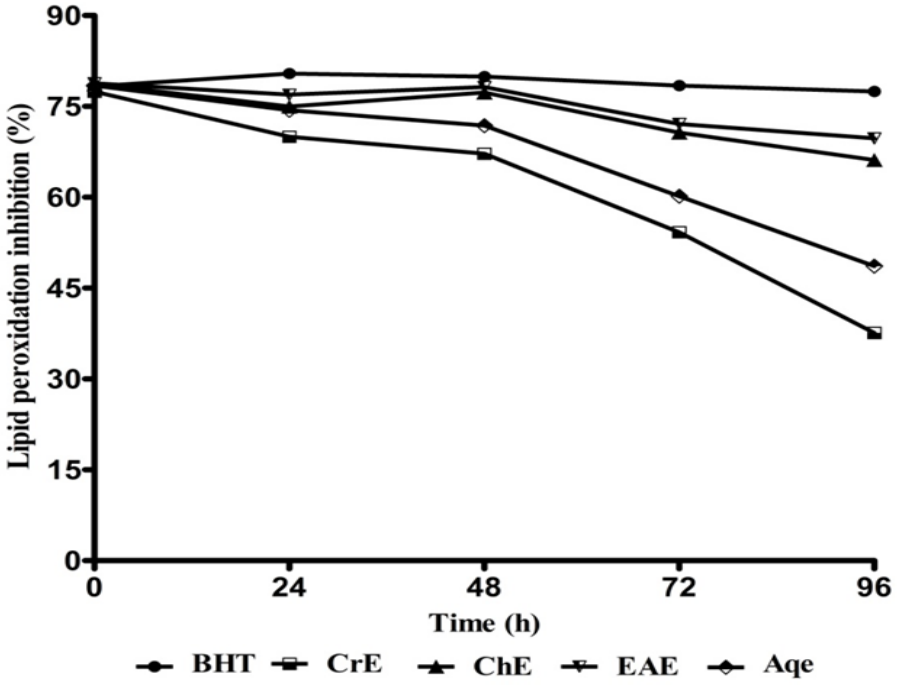


Figure 21. Lipid peroxidation inhibition kinetic of plant extracts and standard BHT during 96 h incubation. Results are mean of triplicates. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

After incubation time (**figure 22**), based on the very low absorbance values in TBARS method observed with EAE (0.06 ± 0.003), ChE (0.06 ± 0.004) and AqE (0.07 ± 0.002), all of these extracts delayed the oxidation of linoleic acid as much as BHT (0.05 ± 0.004) (p > 0.05). Interestingly, in the presence of CrE; complex of thiobarbituric acid (TBA) with malondialdehyde (MDA) levels were low by giving an absorbance of 0.08 ± 0.003.

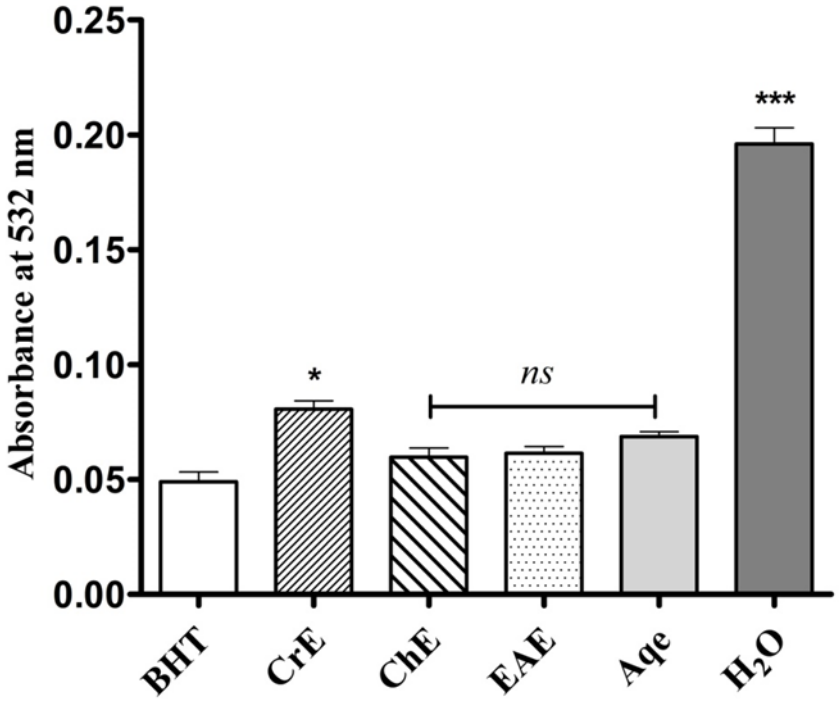


Figure 22. Absorbance values of TBA-MDA complex as index of lipid peroxidation after 96 h incubation of *A. leucotrichus* different extracts, standard and control (H_2O). Results are expressed the mean of triplicates ± SD. ns: $p > 0.05$; *$p \leq 0.05$; ***$p \leq 0.001$ compared to the standard (BHT). CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

Measurement of lipid peroxidation (LPO) indices by ferric thiocyanate and TBARS reflects an oxidative stress statue in the used system, and it is another model for the screening of bioactive agents that can inhibit the early stage of lipid peroxidation. The advantage of employing a lipid substrate solution system for extent comes to the reduced composition, thus less interference, but without losing the sensitivity of the reaction (Li *et al.*, 2023). The polyunsaturated acid omega 6, linoleic acid, is very susceptible to LPO, its autoxidation generates primary oxidation products at the initiation phase: peroxyl radicals, considered being lipids chain degradation initiators by abstracting a proton from another lipid molecule to generate at the end secondary products such as MDA, and the cycle restart until the total degradation of lipids is achieved (Guéraud *et al.*, 2010; Félix *et al.*, 2020). From the lipid

inhibition results and MDA levels, most of the extracts successfully neutralized the peroxidation at the early stage of LPO and by acting as chain-breaking antioxidants to stop the propagation of lipid radicals thereby inhibition MDA formation. Results of correlation showed a very week positive relation between lipid peroxidation, MDA levels and TPC (0.063 and 0.145, respectively). However, a negative correlation was observed with TFC (-0.249) and lipid peroxidation; in addition to week positive correlation registered with MDA levels (0.490). However, from previous studies of Saija *et al.* (1995) to newer ones of Yüksel *et al.* (2021), advocated the presence of polyphenols and flavonoids for preventing LPO damage and through a mechanism of HAT. Findings of radicals scavenging capacity of extracts presented previously mainly attributed to the capacity of neutralizing lipid peroxyl radicals. According to Dangles *et al.* (2000) and Vellosa *et al.* (2011), AqE with abundant kaempferol content together with the divergence in flavonoids content of EAE including kaempferol, quercetin, rutin and isoquercitrin maybe are the responsible for the successful neutralization of lipid peroxides. As mentioned previously in the section of results and discussion (radical scavenging activity), some of the abundant phenolic compounds with potent antioxidant activity, including caffeic acid, vanillic acid and ferulic acid, reported to neutralize lipid peroxides (Khan *et al.*, 2016; Taqvi *et al.*, 2021; Kumar *et al.*, 2023b). This could justify the noticeable ability of ChE and enhanced the activity of EAE in harmony with its potent flavonoid content.

2.4. Ferrous ion chelating assay and ferric reducing capacity

Chelating of Fe^{+2} and reduction of Fe^{+3} using plant extracts and standards are graphically presented in **figure 23** and **figure 24**, respectively. Results in **figure 23** showed that AqE performed the best iron bound among the extracts studied with an EC_{50} equal to 0.88 ± 0.019 mg/ml. CrE and ChE showed a considerable metal chelating activity (1.24 ± 0.023 and 1.56 ± 0.021 mg/ml, respectively).

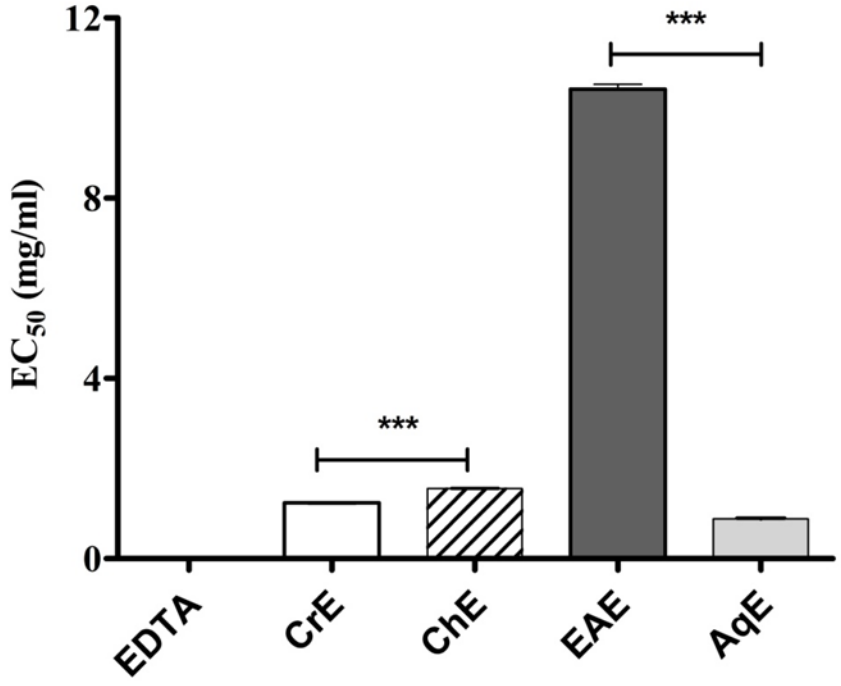


Figure 23. Graphical representation of metal chelating activity of plant extracts and EDTA. Values are the mean of triplicates ± SD. Comparison was against EDTA where ***$p \leq 0.001$. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

However, in ferric reducing capacity (**figure 24**), EAE was the most active extract with EC_{50} equal to 0.12 ± 0.002 mg/ml. The significant data observed by CrE and ChE was 0.26 ± 0.009 and 0.37 ± 0.005 mg/ml, respectively.

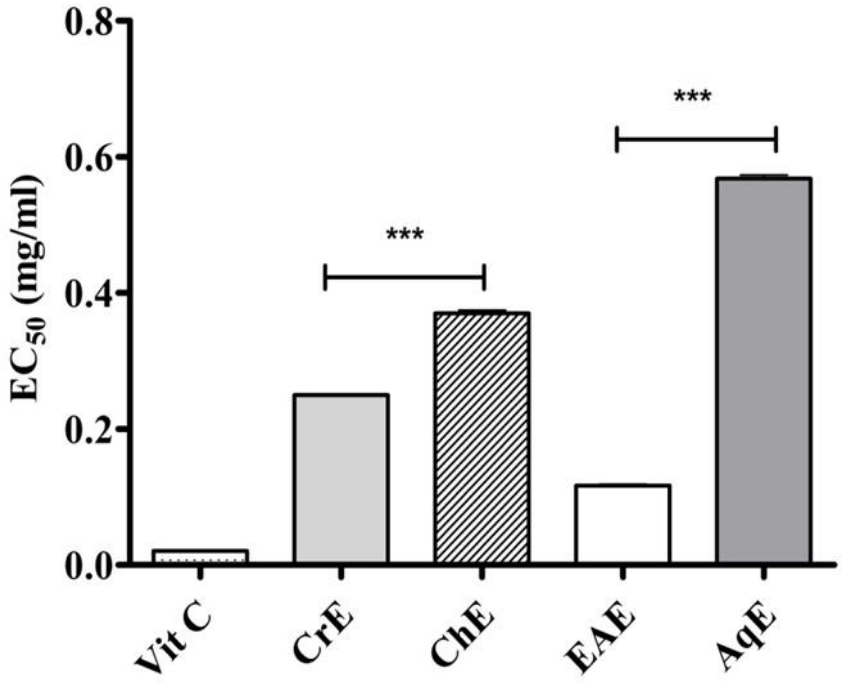


Figure 24. Reducing power capacity of *A. leucotrichus* and ascorbic acid. Results are expressed as mean ± SD (n=3). ***$p \leq 0.001$ compared to ascorbic acid. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

The capacity to chelate transition metals is a critical mechanism of antioxidant activity. Interestingly, AqE with significantly lower polyphenol levels as well as weaker scavenging activities exhibited a remarkable chelating effect; this activity is possibly contributed to the presence of other components. Correlation between this assay and TPC (R= 0.172) and TFC (R= 0.453) showed a weak positive relation. The study conducted by Wang *et al.* (2009) and Wu *et al.* (2024) showed that there are other molecules (e.g. polysaccharides as well as proteins) more effective in chelating transition metals. Guemmaz *et al.* (2020) reported that iron bounding of plant extracts is more efficient in aqueous medium; thereby the ferrous chelating capacity is directly proportional to the polarity of their solvents. In consequence, these two studies might be more important for the observed chelating effects of AqE. Likewise, CrE and ChE remarkable ferrous chelating is probably relayed to the presence of specific type of phenolic compounds. Mazzone (2019) and Scarano *et al.* (2023) showed that caffeic acid had a potent activity in sequestering ferrous ions and inhibiting Fenton reaction

together with chlorogenic acid. Scarano *et al.* (2023) mentioned that the carboxylate groups of caffeic and chlorogenic acid are more efficient in chelating metals more than methoxy groups of vanillic, ferulic and syringic acid. Besides, the third hydroxyl group in the structure of flavonoids enhance radical scavenging activity but hinder the chelating ability, along with the glycosylation at all of the crucial hydroxyl positions can influence the antioxidant activity (Rice-Evans *et al.*, 1997; Scarano *et al.*, 2023). Therefore, this could justify the observed EAE low activity.

A significant indicator of antioxidant capacity of the studied samples is their iron-reducing ability; from ferric (III) iron reduction form to ferrous (II) iron which is an indicator of electron-donating ability and linked in free radical chain breaking; considered as an important mechanism of phenolic compounds as antioxidants (Benchikh *et al.*, 2016). Correlation results showed that ferric reducing ability was negative with the amount of TPC (-0.751) and TFC (-0.827). As part of the antioxidant activity, reducing ability of some of the major and potent phenolic compounds present in the extracts (caffeic, vanillic and ferulic acids) was previously examined in Karamac *et al.* (2017); Hayun *et al.* (2020) and Gao *et al.* (2021) studies. Results showed a strong reducing capability of these compounds, suggesting that extracts were relaying mainly on these compounds with their mono and dihydroxylation structure to successfully reduce ferric ions. Additionally, to the mentioned phenolic compounds, flavonoids such as kaempferol, quercetin, isoquercitrin and quercitrin participated in ferric reducing through their hydroxyl groups (Li *et al.*, 2016; Tian *et al.*, 2021).

2.5. Free radical-induced erythrocyte hemolysis assay

The oxidative hemolysis of erythrocyte has been proposed as a valuable in vitro model to study the relationship between free radical-induced damage of biological membranes and the effects of *A. leucotrichus* extracts. The HT_{50} values obtained by the extracts studied are

presented in **figure 25**. In the case of red blood cells (RBC) incubated with AAPH, a marked decrease in hemolysis time was observed compared to once treated with *A. leucotrichus* extracts in which cells had the ability to withstand the free radical attack. CrE and its fraction showed a concentration dependent manner of erythrocyte protection. All the extracts showed a comparable HT_{50} values ranging from 51.50 to 63.69 min at 0.025 mg/ml and 57.26 to 85.34 min at 0.05 mg/ml where two-way ANOVA performed between these two concentrations showed no significant difference ($p > 0.05$). Increasing the concentration up to 0.1 mg/ml significantly increased antihemolytic effect especially those of ChE and EAE (HT_{50} = 172.07 ± 12.771 min and 161.87 ± 20.265 min, respectively) more than two folds of ascorbic acid (where $p \leq 0.01$). However, CrE and AqE showed a comparable activity to that of ascorbic acid ($p > 0.05$) with HT_{50} value equal to 94.70 ± 22.1 min and 78.04 ± 4.48 min, respectively. Among the extracts, EAE at 1 mg/ml performed the best activity against free radical-induced hemolysis (HT_{50} = 201.7 ± 8.3 min).

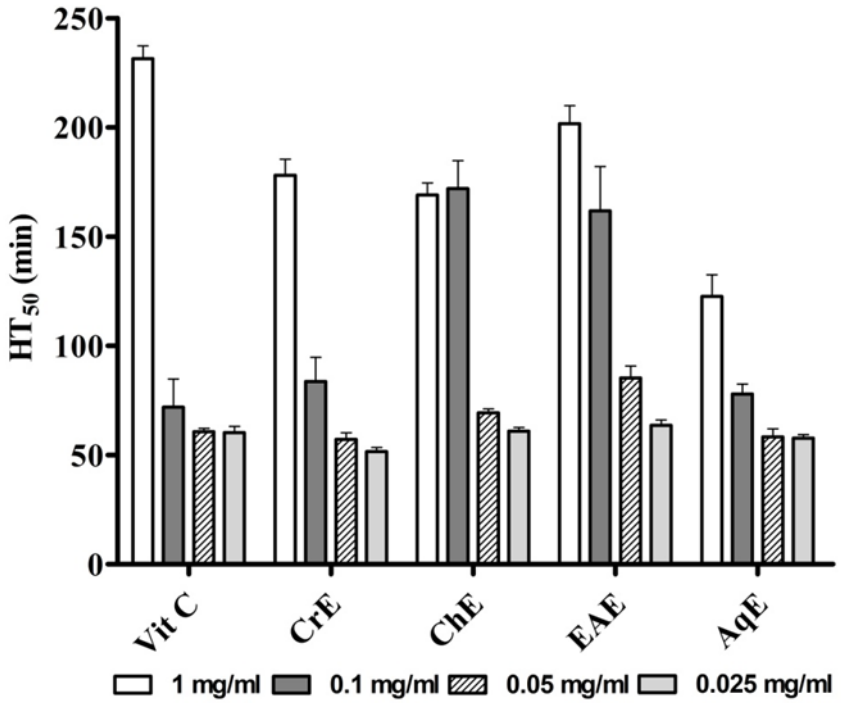


Figure 25. Graphical representation of various concentrations of the studied extracts and vitamin C (0.025; 0.05; 0.1; 1 mg/ml) on AAPH-induced hemolysis in mice RBC. Values are the mean ± SEM (n = 4). CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

The generated peroxyl radicals (ROO•) by AAPH, induce chain oxidation reaction of erythrocyte phospholipid and proteins leading to quick damage and structure failure thereby hemolysis of the cells (Costa *et al.*, 2009; Wu *et al.*, 2018). Antioxidative and antihemolytic properties of the plant extracts may be linked up to the presence of flavonoids and polyphenols. Evidence of red blood cells sedimentation in the microplate wells strongly suggest the binding of phenolic compounds to the cells' membrane and protect its architecture from AAPH-induced damage and this is in concordance with the results of Costa *et al.* (2009). Relying on the previous anti-radical activity and LPO inhibition results, the plant phenolic compounds were capable of carrying out similar function in this test; in which the protection of RBC against hemolysis is due to the radical scavenging and chain breaking in lipids chain oxidation, through hydrogen atom transfer to trap ROO· and forming more stable and less reactive species. Kaempferol, quercetin, caffeic, vanillic and ferulic acids present in the tested extracts were potent inhibitors of AAPH-induced hemolysis through radical scavenging (Tai *et al.*, 2012; Lopez *et al.*, 2017; Wu *et al.*, 2018; Lopes *et al.*, 2021). Additionally to their redox reactions, it is well documented that lipophilicity of phenolic compounds elevates the protective effect against free radical-induced damage (Tai *et al.*, 2012; Lopes *et al.*, 2021). Lipophilicity attributes to the deep incorporation of phenolic compounds into the lipid bilayer, causing changes to the membrane's fluidity to hinder the diffusion of ROO· and improve the membrane stability by the increase of van der Waals contacts within the lipid membrane bilayer (Oliveira *et al.*, 2012; Ramchoun *et al.*, 2015; Lopes *et al.*, 2021). It is worthy to note that the obtained results do not reflect the activity of the mentioned phenols only, however, it may be attributed to the synergism of bioactive molecules present in the extracts with low quantity such as quercetin and p-coumaric (Dai *et al.*, 2006; Lopez *et al.*, 2017), and this could explain the high activity registered by EAE and ChE.

2.6. Antithrombotic and blood clotting time

Effect of the plant extracts on clot lysis and platelet aggregation is represented at concentration of 50 mg/ml in **table 5**. Clot lysis was observed with all of the tested samples. However, CrE and EAE recorded a similar trend with 19.92% and 19.93%, respectively. It was the highest antithrombotic activity observed compared to citrate. A partial blood clotting was observed only with ChE and EAE, both of these extracts had a noticeable anticoagulant activity even after 3 h of observation.

Table 5. Antithrombotic activity (AT) and Blood clotting time (BCT) of CrE and its fractions, Citrate and distilled water.

Parameter	Citrate	H2O	CrE	ChE	EAE	AqE
AT (%)	25.36	n.t.	19.92	17.76	19.93	10.66
BCT (min)	n.c.	7 ± 1	27 ± 10	0	0	10 ± 2

Values are mean of triplicates ± SD. n.c.: no observed clotting; n.t.: not tested. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

From the current results, the remarkable antithrombotic and anticoagulant activities of plant extracts may be endorsed to the presence of polyphenols and flavonoids, especially those of EAE and ChE, which did reduce platelet functions, resulting in antithrombotic and anticoagulant effect (Kubatka *et al.*, 2022). Platelet aggregation can be modulated in the presence of polyphenols through platelet membrane stabilization, activation of the anticoagulant members (heparin cofactor II), interaction and inhibition of proteins involved in the aggregation network such as cyclo-oxygenase (COX) and phospholipase C, and modulation of many factors of the platelet activation signaling pathway (Natella *et al.*, 2006; Han *et al.*, 2012; Bojic *et al*., 2019). In addition, many previous studies advocate the presence of phenolic compounds in plants are nominated to have antithrombotic activity (Alamgeer *et al.*, 2018). Han *et al.* (2012) reported that phenolics-rich plant can affect clot lysis because they are selectively bound to platelet thrombi, maybe due to their lipophilicity involved in

platelet stabilization (Bojic *et al*., 2019). Accumulating evidence showed some of the phenolic compounds already present in the extracts, either in low or abundant quantities exerts potent anticoagulant and antithrombotic activities, through: modulating integrin αIIbβ3 (fibrinogen receptors) activity; decreasing of coagulant factors enzymatic activity including thrombin; decreasing thromboxane 2 generation (implicated in platelet aggregation) and inhibition of COX; and other downstream regulation of signaling factors (Linglei *et al.*, 2020; Kubatka *et al.*, 2022). This could reflect the potent molecular effect of the extracts observed *in vitro* and could be carried *in vivo*. Among these compounds there is: caffeic, ferulic and vanillic acids, kaempferol, quercetin, rutin and quercitrin.

2.7. Enzyme inhibition

The investigated α-amylase and α-glucosidase inhibition of the studied samples is presented in **table 6**. All plant extracts performed a noticeable α-amylase inhibition through starch protection. However, EAE showed the highest inhibition activity on this enzyme (0.63 ± 0.003 mg ACAE/g E). Also, EAE performed the best inhibition activity against α-glucosidase with 1.31 ± 0.037 mg ACAE/g E. Whereas CrE displayed the lowest activity (0.27 ± 0.048 mg ACAE/g E). ChE and AqE fractions on the other hand did not perform any inhibition activity on α-glucosidase.

Table 6. The effect of *A. leucotrichus* extracts on α-amylase and α-glucosidase inhibition.

Plant extracts	α-amylase (mg ACAE/g E)	α-glucosidase (mg ACAE/g E)
CrE	0.39 ± 0.049	0.27 ± 0.048
ChE	$0.52 \pm 0.061^{*}$	n.i.
EAE	$0.63 \pm 0.003^{**}$	$1.31 \pm 0.037^{***}$
AqE	0.37 ± 0.06^{ns}	n.i.

Results are the mean of triplicates ± SD. ns: $p > 0.05$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ compared to CrE. n.i.: no inhibition. ACA: acarbose equivalent. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

The purpose of this study was to determine the effectiveness of *A. leucotrichus* extracts to alleviate postprandial hyperglycemia through strategy of inhibiting starch-hydrolyzing enzymes, α-amylase and α-glucosidase, considered as a potential therapeutic approach to type 2 Diabetes. α-amylase hydrolyses α-1,4-glucan polysaccharides, such as starch, to produce primarily maltose and oligosaccharides that subsequently hydrolyzed by α-glucosidase to release glucose from a non-reducing ends (Terra and Ferreira, 2005; Ahmed *et al.*, 2022). A weak positive relationship between TPC (0.363), TFC (0.208) and α-amylase was observed, along with the respectively obtained weak relation and strong one with TPC (0.248), TFC (0.622), respectively and α-glucosidase. This shows that the observed enzyme inhibition activities by the plant extracts is correlated with the selective inhibition profile of phenolic compounds. Probably, kaempferol, and even the low quantity of quercetin and rutin stood out in inhibiting α-amylase and α-glucosidase similarly to the obtained results of Hendra *et al.* (2024). Caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, and vanillic acid are among the phenolic compounds found in CrE and ChE that have been shown to have a significant inhibitory potential against α-amylase and α-glucosidase (Oboh *et al.*, 2015; Rasouli *et al.*, 2017; Zheng *et al.*, 2020; Khan *et al.*, 2022). The potential mechanism is through hydrogen bonding of hydroxyl groups with amino acids residues of both enzymes, additionally to van der walls and hydrophobic interactions resulting in the retardation of enzyme activity (Sun *et al.*, 2019; Ćorković *et al.*, 2022; Hendra *et al.*, 2024).

The observed antioxidant activities (free radical scavenging, β-Carotene/Linoleic acid bleaching assay, lipid peroxidation, TBARS, ferrous chelating and ferric reducing potentials as well as enzyme inhibition) are supported by Pearson correlation analysis where results are depicted in **table 7**.

Table 7. Pearson correlation coefficients (R) of TPC, TFC and *in vitro* antioxidant activities.

Anti O	**TPC**	**TFC**	**DPPH$^{\cdot}$**	**ABTS$^{\cdot-}$**	**H_2O_2**	**OH$^{\cdot}$**	**$O_2^{\cdot-}$**	**NO$^{\cdot}$**	**β-CBA**	**LPO**	**TBARS**	**FICA**	**FRA**	**α-amy**	**α-glu**
TPC	1.000														
TFC	0.643	1.000													
DPPH$^{\cdot}$	-0.857	-0.575	1.000												
ABTS$^{\cdot-}$	-0.847	-0.628	0.997	1.000											
H_2O_2	-0.918	-0.609	0.991	0.986	1.000										
OH$^{\cdot}$	-0.917	-0.535	0.986	0.974	0.996	1.000									
$O_2^{\cdot-}$	-0.631	-0.460	0.941	0.944	0.886	0.878	1.000								
NO$^{\cdot}$	-0.261	0.058	0.687	0.667	0.595	0.620	0.854	1.000							
β-CBA	0.782	-0.668	-0.978	-0.990	-0.954	-0.933	-0.961	-0.678	1.000						
LPO	0.063	-0.249	-0.517	-0.493	-0.413	-0.445	-0.726	-0.977	0.508	1.000					
TBARS	0.145	0.490	0.293	0.260	0.187	0.232	0.523	0.890	-0.268	-0.965	1.000				
FICA	0.172	0.453	-0.614	-0.651	-0.516	-0.474	-0.819	-0.720	0.745	0.653	-0.484	1.000			
FRA	-0.751	-0.872	0.907	0.937	0.892	0.849	0.880	0.508	-0.769	-0.324	0.067	-0.755	1.000		
α-amy	0.363	0.208	-0.789	-0.788	-0.700	-0.701	-0.946	-0.960	0.821	0.895	-0.750	0.865	-0.708	1.000	
α-glu	0.248	0.622	-0.622	-0.670	-0.542	-0.486	-0.785	-0.583	0.768	0.487	-0.290	0.978	-0.824	0.776	1.000

Anti O: antioxidant test; **TPC**: total polyphenolics content; **TFC**: total flavonoids content; **DPPH$^{\cdot}$/ABTS$^{\cdot+}$/ H_2O_2/OH$^{\cdot}$/O2$^{\cdot-}$/$^{\cdot}$NO:** free radical scavenging methods; **β-CBA**: β-Carotene bleaching assay; **LPO:** lipid peroxidation; **TBARS:** thiobarbituric acid reactive substances; **FICA**: ferrous ion chelating assay; **FRA**: ferric reducing ability; **α-amy:** α-amylase; **α-glu:** α-glucosidase.

3. *In vitro* anti-inflammatory activity

Protective effect of *A. leucotrichus* extracts against human serum albumin (HSA) heat denaturation was investigated and results are represented in **figure 26**. Plant extracts were evaluated at two concentration, 1.25 and 0.31 mg/ml and were compared to aspirin as standard. Results showed that protective effect of HSA was increased in concentration dependent manner with most of extracts. At 0.31 mg/ml, EAE and AqE performed a potent anti-inflammatory activity with percentage equal to 83.81 ± 2.90% and 73.88 ± 3.25%, respectively. However, the increase in concentration (1.25 mg/ml) showed similar values of HAS protection; 77.95 ± 4%, 76.36%, 80.03 ± 2.79% and 80.11 ± 0.81% observed with CrE, ChE, EAE and AqE, respectively.

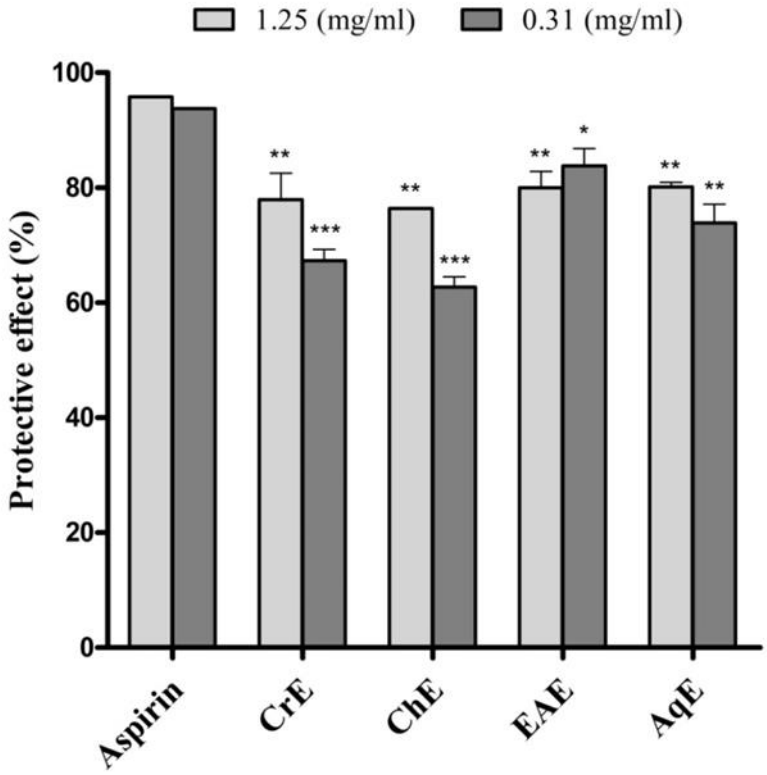


Figure 26. Protective effect of *A. leucotrichus* extracts and aspirin on heat-induced albumin denaturation at 1.25 and 0.31 mg/ml. Results are expressed as mean ± SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to standard aspirin. CrE: crude extract; ChE: chloroform extract; EAE: ethyl acetate extract; AqE: aqueous extract.

It is well-documented that inflammatory response to harmful stimuli involves protein denaturation and this could be the initial step that leads to chronic inflammatory disorders such as rheumatoid arthritis (Cherbal *et al.*, 2023). Alteration of hydrogen, electrostatic and disulfide bonds of proteins caused by thermal heat results in loss of their structure together with biological function (Kpemissi *et al.*, 2023). In this study, the *in vitro* anti-inflammatory activity was used for screening of new anti-inflammatory drugs and indeed, results indicated an effective protein protection was obtained by the studied extracts even at low concentration. The highly nominated bioactive compounds extracted from *A. leucotrichus* seeds, phenols and flavonoids, have a great anti-denaturant activity. It is well documented that the chemistry of the most protein-phenolic interaction is through non-covalent interactions (Yilmaz *et al.*, 2022). Hydrogen donation capability of phenolics to produce hydrogen bonds and combination with hydrophobic forces are the primary forces participate in the interactions (Yilmaz *et al.*, 2022). The anti-denaturant activity observed by CrE and ChE was highly related to ferulic acid, caffeic acid and vanillic acid binding ability to HSA (Zhang *et al.*, 2008; Myint *et al.*, 2021; Yang *et al.*, 2024). In addition to the synergic action of other phenolic content such as the 4-hydroxy benzoic acid (Myint *et al.*, 2021), that could not be negligible. The abundance and co-operation of kaempferol and caffeic acid in EAE. As well as kaempferol and the low content of chlorogenic acid in AqE, may be the reason for the observed high anti-denaturant activity among the extracts studied (Kang *et al.*, 2004; Xiao *et al.*, 2008; Zhang *et al.*, 2008). However, Xiao *et al.* (2008) and Liu *et al.* (2010) showed that rutin, quercitrin and quercetin have a binding affinity towards bovine serum albumin, it means that other phenolic compounds, even with low concentration in *A. leucotrichus* extracts, shared this activity by co-enhancing the interaction.

4. *In vivo* analgesic effect and anti-inflammatory activity of CrE

4.1. Analgesic effect

Analgesic effect of CrE at 100 mg/kg, 200 mg/kg and 400 mg/kg, in addition to aspirin (150 mg/kg) on acetic acid induced writhing in Wistar mice was evaluated and results are presented in **figure 27**. The decrease in the frequency of writhing was in dose dependent manner in animal groups received 100 mg/kg and 200 mg/kg of CrE, reaching a similar activity ($p > 0.05$) to that of aspirin (58.67 ± 3.14%) with the middle dose (54.56 ± 6.49%). However, the increase of CrE dose to 400 mg/kg diminished the activity to 28.05 ± 2.71%.

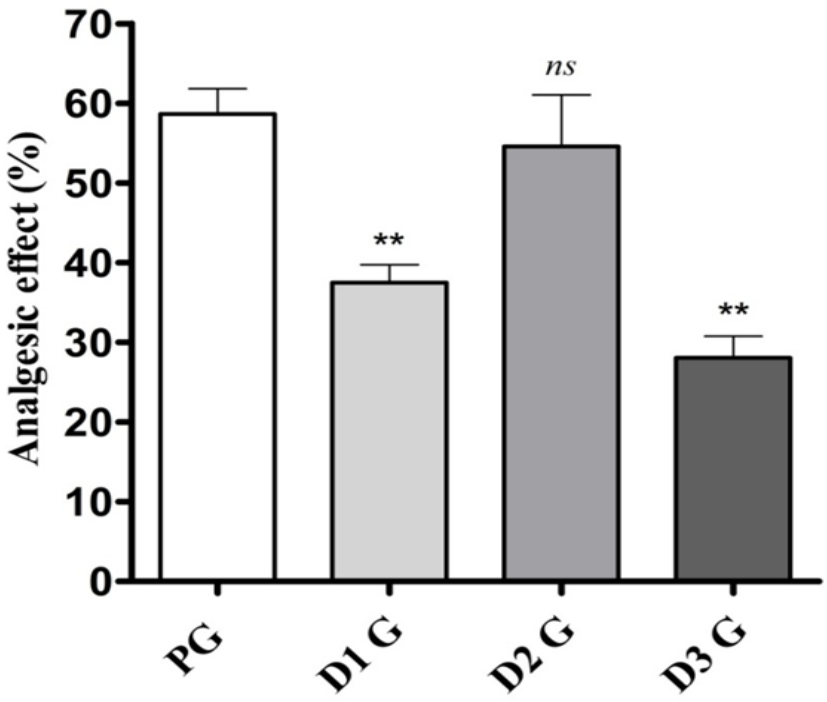


Figure 27. Anti-nociceptive activity of CrE at various doses on acetic acid induced writhing in mice. Values are expressed as mean of five mice in each group ± SEM. Comparison was against aspirin, where: ns: $p > 0.05$; **$p \leq 0.01$. PG: positive group; D1 G: dose 1 group; D2 G: dose 2 group; D3 G: dose 3 group.

Analgesic effect using acetic acid is a preferred test for its sensitivity to examine anti-nociceptive activity of the tested sample. Episodes of muscles contraction, the expansion of

the hind limbs, and the lengthening of the body, are the hallmarks of the acute intense pain censored via nociceptors by peritoneal injection of acetic acid, in which a pro-inflammatory pain marker in animal models, prostaglandins, is synthesized via cyclooxygenase (COX) pathway (Ashagrie *et al.*, 2023; Hmamou *et al.*, 2023) in addition of the release of many endogenous inflammatory and nociceptive mediators in the peritoneal fluid including the vaso-active amines: serotonin and histamine, tumor necrosis factor alpha (TNF-α), IL-1β, etc., and all of these mediators increase sensitivity to nociceptors (Campos *et al.*, 2014; Hamoudi *et al.*, 2021). *A. leucotrichus* crude extract successfully relieved the pain symptoms in mice and performed an anti-nociceptive effect comparable to aspirin, which is considered as inhibitor of COX (Voilley *et al.*, 2001). The presence of phenolic compounds in the crude extract could justify the analgesic effect performed especially with the similar trend observed to that of aspirin at dose 200 mg/kg. Polyphenols as caffeic, vanillic and ferulic acids exhibited anti-nociceptive activity in visceral inflammatory pain models; through a mechanism of COX inhibition thereby prostaglandin production and inhibition of inflammatory cytokines (Gamaro *et al.*, 2011; Park *et al.*, 2011; Campos *et al.*, 2014; Kasik *et al.*, 2019). Therefore, the results suggest that the observed anti-nociceptive activity is relayed on these natural products that do exist in CrE.

4.2. Carrageenan induced-paw edema model

As presented in **table 8**, anti-edematous activity of CrE was in dose-related and time-dependent manner at doses of 100 and 200 mg/kg. Oral administration of CrE at 200 mg showed the highest reduction in paw edema after 3 h of carrageenan injection with maximum inhibition activity of 74.05 ± 8.56% not significantly different from indomethacin at dose of 50 mg/kg (62.45 ± 7.1%). Whereas, increase of CrE dose to 400 mg/kg registered a decrease in paw edema inhibition after 2, 3 and 4 h of carrageenan injection with values 39.95 ± 5.27%, 12.03 ± 3.16%, 44.44 ± 4.29%, respectively.

Table 8. The effect of plant CrE at different doses and indomethacin on carrageenan induced paw edema.

Groups	Paw edema inhibition (%)			
	1 h	**2 h**	**3 h**	**4 h**
PG	25.93 ± 5.66	43.26 ± 2.24	62.45 ± 7.1	53.01 ± 9.57
D1 G	27.31 ± 2.05^{ns}	$24.82 \pm 2.95^{**}$	$30.06 \pm 4.02^{**}$	$26.32 \pm 5.87^{**}$
D2 G	$44.44 \pm 8.69^{**}$	$61.35 \pm 6.14^{**}$	74.05 ± 8.56^{ns}	59.98 ± 5.82^{ns}
D3 G	$43.83 \pm 1.63^{**}$	39.95 ± 5.27^{ns}	$12.03 \pm 3.16^{***}$	44.44 ± 4.29^{ns}

Results are expressed as mean of five mice ± SEM. *ns*: $p > 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to PG. PG: positive group; D1 G: dose 1 group; D2 G: dose 2 group; D3 G: dose 3 group.

Carrageenan-induced paw edema model is one of the models used in this study to examine a possible use of plant-derived bioactive compounds as anti-inflammatory alternative. The acute inflammation reaction coupled with carrageenan paw injection consisted of 2 phases; the first one is up to 1 h of carrageenan injection and characterized by the realize of histamine, serotonin and bradikinin (Hossain *et al.*, 2020; Hmamou *et al.*, 2023). The second is prostaglandin-mediated phase that promote inflammation, derived from arachidonic acid and synthesized by COX (Morris, 2003; Hossain *et al.*, 2020). Following the injection of λ-carrageenan into the subplanter region of mice's paw an increase of paw diameter was observed as edema formation and reached its maximum after 3 h in CG (from our data), indicating an acute inflammation. Results showed that oral administration of CrE decreased edema formation with a best effect after 3 h of post injection indicating that bioactive compounds of the plant performed anti-edematous activity at the first stage and persisted after 4 h. The anti-inflammatory activity of CrE may be the results of the combined action of several phenolic compounds. In line with similar studies, several researches have reported that in carrageenan acute inflammation model, caffeic acid and vanilic acid inhibited the inflammatory signaling pathway of kinins (histamine and bradykinin), pro-inflammatory cytokine production (TNF-α and IL-1β) (Campos *et al.*, 2014; Santos *et al.*, 2022) and inhibited neutrophil recruitment. Furthermore, activation of the major transcription factor of

pro-inflammatory mediators; nuclear factor kappa B (NF-κB); was suppressed due to vanillic acid oral administration (Campos *et al.*, 2014).

4.3. Ear model edema induced by xylene and croton oil

Ear edema induced by xylene application was reduced after CrE oral administration as shown in **figure 28** and not significantly different than indomethacin at 50 mg/kg ($p > 0.05$) with an anti-edematous activity of 72.38 ± 5.99%. Doses 100, 200 and 400 mg/kg of the tested CrE showed an auricular edema reduction with respective values: 68.02 ± 2.91%, 68.60 ± 4.72 and 53.49 ± 8.48%.

Compared with croton oil-induced positive group (indomethacin at 50 mg/kg) that performed anti-edematous activity of 60.87 ± 7.57%, treatment with CrE reduced ear edema dose-dependently with a similar trend of activity ($p > 0.05$). It is worthy to highlight that oral administration of CrE at dose 400 mg/kg showed a high reduction of croton oil-induced edema with a percentage of 71.27 ± 3.70 (**figure 28**).

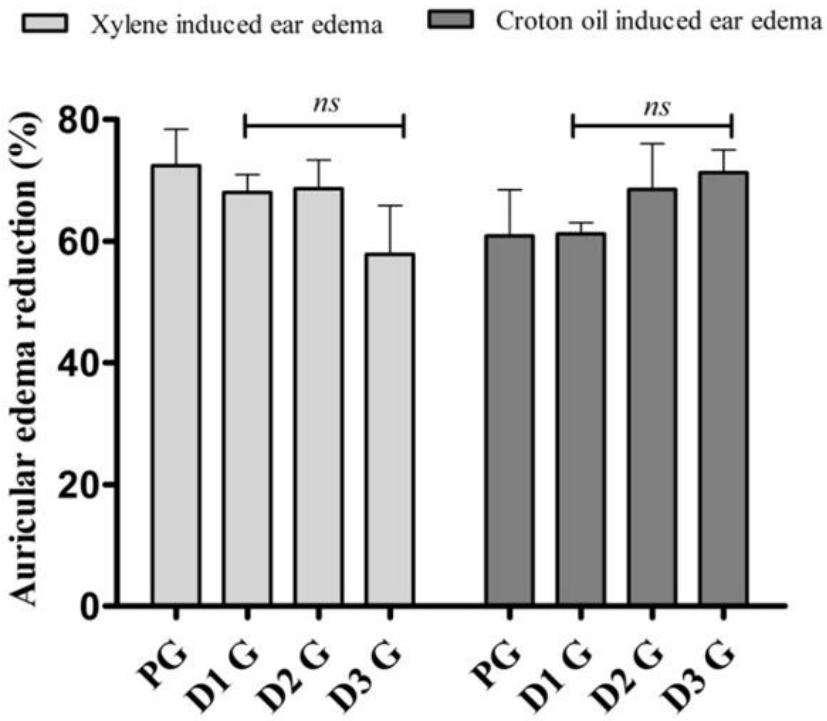


Figure 28. Reduction of auricular edema induced by xylene and croton oil application of the tested extract and indomethacin. Values are expressed as the mean ± SEM. Comparison was against PG where, ns: $p > 0.05$. PG: positive group; D1 G: dose 1 group; D2 G: dose 2 group; D3 G: dose 3 group.

Histological analysis of mice ears edema induced by croton oil application is shown in **figure 29** with hematoxylin and eosin staining. Croton oil application resulted in an obvious acute inflammation characterized by an increase in ear thickness (vasodilatation), with an edematous dermis, its volume is represented with red line, and cellular infiltration observed clearly in CG compared to the untreated ears. Histological examination emphasized that administration of CrE reduced considerably hallmarks of inflammation, inflammatory cells infiltration, vasodilatation and vascular permeability, dose-dependently, and successfully recovered ear tissue damage resulted from irritant agent application to normal at 400 mg/kg as similar as indomethacin (50 mg/kg) effect.

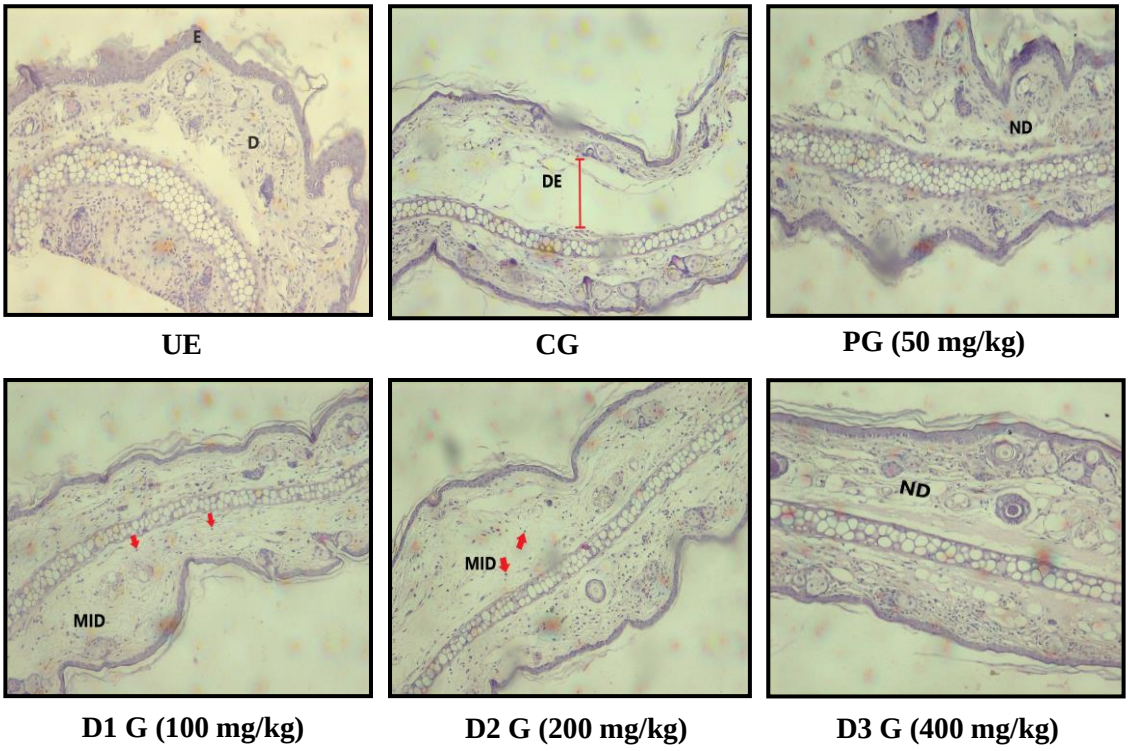


Figure 29. Histological analysis (10× magnification, hematoxylin and eosin staining) of mice ear biopsies after 6 h of topical croton oil application. **UE:** untreated ears; **CG:** control group; **PG:** positive group; **D1 G:** dose 1 group; **D2 G:** dose 2 group; **D3 G:** dose 3 group. **E:** epidermis; **D:** dermis; **DE:** dermal edema; **ND:** normal dermis; **MID:** mild inflammatory dermis. Red line indicates volume of edema and red arrow indicates cellular infiltration in the ear tissue.

The acute inflammation induced by xylene in the current study is associated with a neurogenic inflammation phase characterized by the release of substance P which cause increase in vascular permeability and stimulate immune cells (Vigna, 2004; Barry *et al.*, 2022) thereby allowing fluid accumulation and edema formation. Followed by inflammatory phase that pro-inflammatory mediators such as histamine and eicosanoids (prostaglandin) take part (Barry *et al.*, 2022; Cheniti *et al.*, 2022), thereby causing an increase in cell infiltration, capillarity permeability and edematous changes (Cheniti *et al.*, 2022). The main irritant constituent in the used croton oil, 12-o-tetracanoilphorbol-13-acetate (TPA), that stimulates a several different inflammatory pathways leading the production of interleukins including IL-6

and TNF-α, activation of COX-2 and synthesis of eicosanoids, leukocyte migration, in addition to the increase in radicals flow (Camponogara *et al.*, 2020; Santos *et al.*, 2022). The hallmarks of inflammation in response to the applied irritants cytotoxicity were accurate in CG, whereas the tested groups treated with CrE showed decreasing ear thickness and pathological associated parameters even 6 h after edema induction and preservation of tissue architecture as observed from the histological observation. Bioactive molecules extracted from *A. leucotrichus* had a promising anti-inflammatory activity, and possibly advocated to phenolic compounds. Highlighting caffeic acid, kaempferol, ferulic acid and vanillic acid according to their high content, through attenuation of substance P, selective gene expression inhibition of pro-inflammatory mediator COX-2, inhibition of mRNA expression of TNF-α and interleukins in addition to the inhibition of NF-κB activation (Park *et al.*, 2011; Kadioglu *et al.*, 2015; Nile *et al.*, 2016; Hu *et al.*, 2022; Adeyi *et al.*, 2023). May be some of these mechanisms are responsible for the observed anti-inflammatory activity, however, we cannot base only on the previous mentioned phenolic compounds, since CrE contains a variety of active compounds that can act synergistically to enhance the anti-inflammatory effect.

4.3.1. Nitrite, MDA levels and catalase (CAT) activity in ears homogenates treated with croton oil

The estimated contents of MDA and NO^{2-} in addition to the determined CAT activity in ears homogenates after croton oil-induced edema is shown in **table 9**. Irritant agent application increased MDA and NO^{2-} levels (68.19 ± 0.49 nmol/g of tissue and 06.25 µM, respectively) in ears homogenates of the control group while significantly lowered CAT activity to 02.49 ± 0.29 µmol/min/mg of protein. Administration of CrE at 100, 200 and 400 mg/kg successfully restored the enzyme activity into normal condition with a maximum activity observed with 400 mg/kg administration (07.73 ± 0.02 µmole/min/mg of protein) and it was more effective than indomethacin ($p \leq 0.001$). MDA levels were decreased dose-dependently to 39.46 ± 2.92

nmol/g of tissue lower than that of untreated ears ($p \leq 0.001$) with 400 mg/kg administration of the tested extract. A significant reduction in nitrite levels observed with all CrE doses compared to control group until reached values not significantly different than that of untreated ears ($p > 0.05$).

Table 9. MDA and NO^{2-} levels and CAT activity in ear tissue homogenates of croton oil-induced edema model.

Groups	Doses	CAT (µmol/min/mg of protein)	MDA (nmol/g of tissue)	NO^{2-} (µM)
UE	-	06.55 ± 0.01	60.58 ± 2.45	01.25
CG	DW	$02.49 \pm 0.29^{***b}$	$68.19 \pm 0.49^{ns,c}$	06.25^{***d}
PG	50 mg/kg	$03.75 \pm 0.20^{***}$	$48.45 \pm 2.11^{**}$	01.88 ± 0.88^{ns}
D1 G	100 mg/kg	$07.73 \pm 0.02^{*d}$	$61.96 \pm 0.48^{ns,b}$	$01.63 \pm 0.18^{ns,a}$
D2 G	200 mg/kg	$06.33 \pm 0.032^{ns,d}$	$46.38 \pm 3.92^{**a}$	$01.87 \pm 0.53^{ns,a}$
D3 G	400 mg/kg	$07.48 \pm 0.12^{*d}$	$39.46 \pm 2.94^{***a}$	$01.75 \pm 0.71^{ns,a}$

Results are the mean of triplicates SD. *ns*: $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, compared to untreated ears. a, b, c, d indicates $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, compared to positive group. DW: distilled water. PG: positive group; D1 G: dose 1 group; D2 G: dose 2 group; D3 G: dose 3 group.

From our previous results, histological analysis revealed an obvious state of acute inflammation with croton oil application, thus this inflammatory process is associated with oxidative stress as previously shown in the study of Santos *et al.* (2022), which leaded to the reduction in cellular antioxidant enzyme (CAT) activity, overproduction of reactive nitrogen species (NO) and increase in the levels of lipids oxidation secondary metabolite, MDA. Plant extract was capable of carrying a similar function from *in vitro* to the living organism, hence proved that bioactive compounds present in *A. leucotrichus* are a potent antioxidant and anti-inflammatory agents against cytotoxicity of damaging agents and cellular oxidative stress damage. Catalase is the first line antioxidant of defense against ROS; administration of CrE restored its lowered activity to normal caused by the triggered state of oxidative stress from croton oil-induced acute inflammation, thus antioxidant activity of polyphenols present in the CrE could be responsible for this action. In the first hand directly, by increasing enzyme

activity, which ferulic acid and p-coumaric acid present in the plant extract previously reported to elevate CAT activity (Alam, 2019). In the other hand indirectly, through enhancing catalase gene expression, polyphenols can recognize components of signal transduction pathways, nuclear factor-erythroid-2-related factor 2 (Nrf2) that following its activation and nuclear translocation, genes encoding antioxidant detoxifying enzymes including catalase are activated (Surh, 2008; Hu, 2011). Kaempferol, ferulic acid and vanillic acid present in CrE in a good amount, regulated positively Nrf2 signaling pathway and increased mRNA and protein expression of antioxidant detoxifying enzymes as reported by Alam (2019); Alam (2020) and Ahmadi *et al.* (2021). Nitric oxide has a major function in signaling pathways that are implicated in physiological processes including, inflammation, vasodilation, apoptosis and others (Andrabi *et al.*, 2023). Its overproduction has toxic consequences related with various diseases such as cancer and diabetes (Adeyi *et al.*, 2023). Inflammatory stimuli induce inducible nitric oxide synthase (iNOS) to produce a high amount of NO which was reflected by the levels of nitrite in the control group of animals and as a consequence, it causes a state of nitrosative stress that leads to cell alteration (Ansari *et al.*, 2020). The presence of polyphenols as ferulic acid, caffeic acid and flavonoids as kaempferol in the tested extract could justify the inhibition of reactive nitrogen specie, through carrying the same scavenging *in vitro* activity to trap NO, suppression of iNOS gene transcription or inhibition of NO production (Mattos *et al.*, 2015; Alam, 2019). During lipid peroxidation, MDA levels arise and since it is a highly reactive and mutagenic product, MDA is the most studied metabolic product during peroxidative damage (Banc *et al.*, 2022). A high content of MDA was obtained suggesting that during the inflammatory condition and oxidative stress state, an overproduction of free radicals leaded to lipid peroxidation in cells membrane (Mehrotra *et al.*, 2011). Caffeic acid and vanillic acid antioxidative acting is highly related with the diminishing of MDA levels in the tested groups orally administrated CrE, through

their radical scavenging activity to prevent the deleterious action of radicals against lipid membrane (Mattos *et al.*, 2015; Ma *et al.*, 2019).

5. Acute toxicity

The acute toxicity experiment was assessed using a limit test dose of 2000 mg/kg and 5000 mg/kg. Following oral administration of CrE at dose 2000 mg/kg and 5000 mg/kg, no significant signs of neither changes in animals' behavior nor acute toxicity, i.e., Hippocratic screening, were observed during the observation trial spanned from a short period (minutes), extended to hours and continued on a daily basis for 14 days. In addition, no mortality was marked in Swiss Albino mice used in this experiment. As shown in **figure 30**, body weight of animals administrated CrE at given doses (2000 and 5000 mg/kg) increased with a steady state (32.36 ± 0.792 and 31.96 ± 0.824 g, respectively) and similarly ($p > 0.05$) to the control group (32.94 ± 1.051 g), therefore the plant did not acutely impact the appetite and food intake. As no fatality was registered among the animal, the lethal dose (LD_{50}) of CrE is thus greater than 5000 mg/kg.

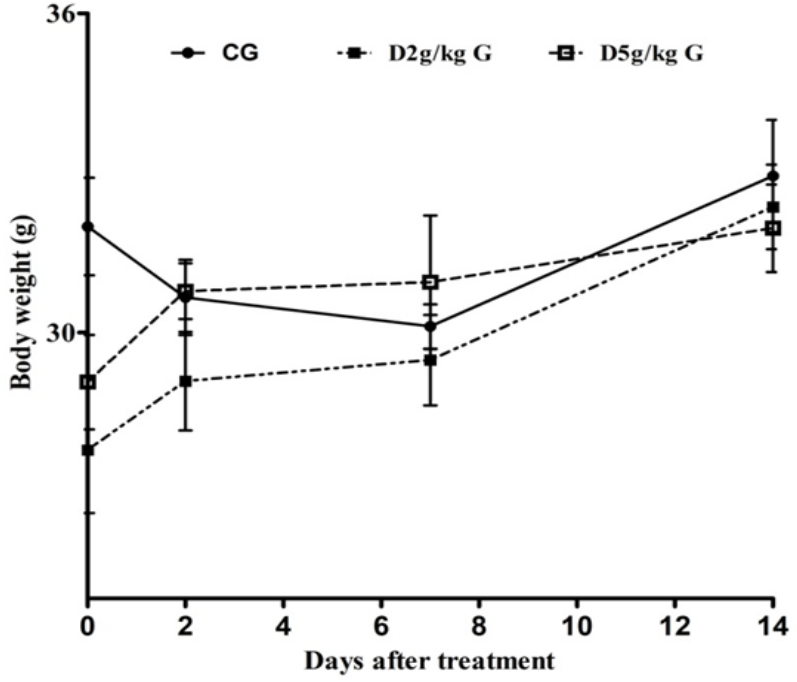


Figure 30. Body weight of Swiss albino male mice 14 days after treatment with plant CrE at dose 2 g/kg and 5 g/kg. Results are the mean of 5 mice ± SEM. CG: control group; D2g/kg G: dose 2 g/kg group; D5g/kg G: dose 5 g/kg group.

Medicinal plants preparations are well-known for their major part of disease treatment across Africa and all over the world (Teke and Kuete, 2014). However, levels of toxicity could arise from the potent compounds of plants extracts especially in high doses, accurate knowledge of their toxicity is essential to identify safe doses and research for any clinical signs. In this study, the main used type of toxicological evaluation is acute toxicity, where the extract at fixed doses 2 g/kg and 5 g/kg did not have any impact on general condition nor causing side effects (aggressivity, ataxia, respiration rate, lethality and others), reflecting the safe use of methanolic CrE even when facing the high intake. Changes in body weight of animals reflect the status wellness. In this experiment, mice experienced a steady increase in body weight, confirming the non-influence of the plant on animals' normal metabolism process. LD_{50} of CrE is the outcome of the experience and indicated the safety profile of this tested plant; the obtained findings (value higher than 5 g/kg) categorize *A. leucotrichus* methanolic CrE as practically non-toxic substance according to Loomis and Hayes (1996) classification and according to the OECD guidelines 425 (2022), this extract is classified as low toxic substance by falling into category 5.

6. Sub-acute toxicity

6.1. Impact on animal behavior, body weight and organ relative weight

Another model for toxicological evaluation of plant extracts was conducted in this study according to the OECD guideline 407. The daily oral administration at short term (21 days) of ascorbic acid at 100 mg/kg and CrE at increasing doses (100, 200 and 400 mg/kg) did not produce any toxic signs and no mortality among the animal groups was recorded during the time of observation. After 21 days, body weight of the treated-animal groups: ascorbic acid and CrE at increasing doses, increased to achieve: 33.91 ± 0.519, 31.60 ± 0.411, 31.69 ± 0.893 and 32.85 ± 0.348 g, respectively, similarly ($p > 0.05$) to the control group (32.99 ± 0.443 g) as shown in **figure 31**. During the experiment, relative organ weight (liver, kidneys,

spleen, heart, lungs and stomach) of the tested substances did not change compared to the control group ($p > 0.05$) (**table 10**).

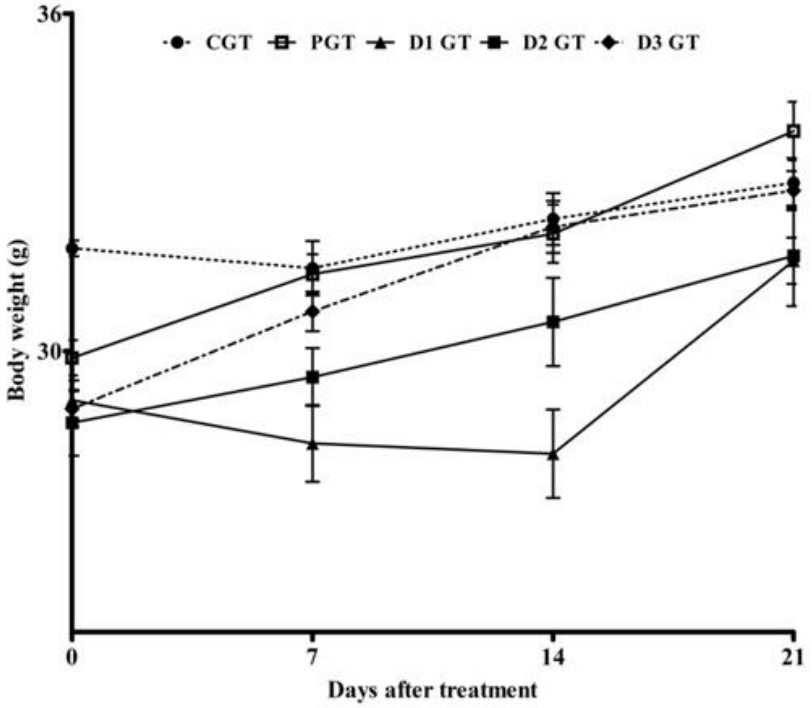


Figure 31. Impact of CrE at doses of 100, 200 and 400 mg/kg on body weight of male mice after 21 days of daily oral administration. All values are expressed as the mean of 8 animals ± SEM. CGT: control group of toxicity, PGT: positive group of toxicity; D1 GT: dose 1 group of toxicity; D2 GT: dose 2 group of toxicity; D3 GT: dose 3 group of toxicity.

Table 10. Relative organ weight after sub-acute toxicity in mice administrated daily with increasing doses of CrE (100, 200 and 400 mg/kg).

Groups	CGT	PGT	D1 GT (100 mg/kg)	D2 GT (200 mg/kg)	D3 GT (400 mg/kg)
Liver	5.61 ± 0.064	5.92 ± 0.285^{ns}	5.11 ± 0.514^{ns}	5.52 ± 0.153^{ns}	5.51 ± 0.178^{ns}
Kidneys	1.59 ± 0.061	1.65 ± 0.058^{ns}	1.41 ± 0.056^{ns}	1.44 ± 0.035^{ns}	1.63 ± 0.049^{ns}
Spleen	0.70 ± 0.068	0.72 ± 0.109^{ns}	0.72 ± 0.065^{ns}	0.64 ± 0.061^{ns}	0.58 ± 0.052^{ns}
Heart	0.58 ± 0.018	0.53 ± 0.023^{ns}	0.54 ± 0.040^{ns}	0.54 ± 0.019^{ns}	0.54 ± 0.011^{ns}
Lungs	0.69 ± 0.040	0.62 ± 0.027^{ns}	0.69 ± 0.027^{ns}	0.68 ± 0.027^{ns}	0.71 ± 0.038^{ns}
Stomach	0.96 ± 0.055	1.00 ± 0.070^{ns}	1.00 ± 0.098^{ns}	1.03 ± 0.049^{ns}	1.07 ± 0.092^{ns}

All data are expressed as the mean SEM. ns: $p > 0.05$ compared to the control group (CGT). CGT: control group of toxicity, PGT: positive group of toxicity; D1 GT: dose 1 group of toxicity; D2 GT: dose 2 group of toxicity; D3 GT: dose 3 group of toxicity.

As continuity of the previous toxicological evaluation, this scientific validation is useful for the determination of the safer dose ranges of *A. leucotrichus* preparation that can be administrated even for a long period. The selection of doses levels was according to the previous data of acute toxicity, where no impact of the treatment on animal behavior nor body weight were recorded at high doses (5 g/kg), in addition to the common drug-intake where low doses are administrated on daily basis in order to achieve high effect and without side effects. From OECD guidelines 407 (2008), difference sequence between doses should be two to four folds intervals. From these findings, the selected doses: 100, 200 and 400 mg/kg of CrE indicates that daily oral administration did not show any side effects either on animal compartment, toxic-related signs or mortality and food intake. According to Raina *et al.* (2015), organs weight is a useful hallmark of physiological and pathological wellness of animals. The targeted organs such as liver and kidneys are toxicity-sensored organs because of their vital roles in toxins removal (Unuofin *et al.* 2017). Under sub-acute toxicity assessment, CrE did not reveal significance changes in the targeted organs indicating that this extract was not toxic at the tested doses range and without any adverse effects.

6.2. Free radical-induced erythrocyte hemolysis assay

Daily oral administration of CrE and ascorbic acid, at varying doses, effect on mice red blood cells was investigated and the results are presented in **figure 32**. CrE successfully delayed the radical-induced erythrocyte hemolysis compared to the control group. In the absence of AAPH, erythrocytes of each mouse were stable and only a little hemolysis was observed within 4 hours of the assay. In the opposite, addition of APPH induced a fast hemolysis. Ascorbic acid administration displayed an HT_{50} equal to 62.10 ± 2.235 min, which is not significantly different ($p > 0.05$) from that of CG (HT_{50} = 61.84 ± 2.32 min). A significant extension of half-time hemolysis ($p \leq 0.05$) was obtained in the presence of *A. leucotrichus* extract at doses 100 and 200 mg/kg (HT_{50} = 77.66 ± 2.95; 77.51 ± 2.865 min, respectively)

when compared to CGT. However, increasing CrE to 400 mg/kg remarkably diminished the HT_{50} to 70.26 ± 1.571 min.

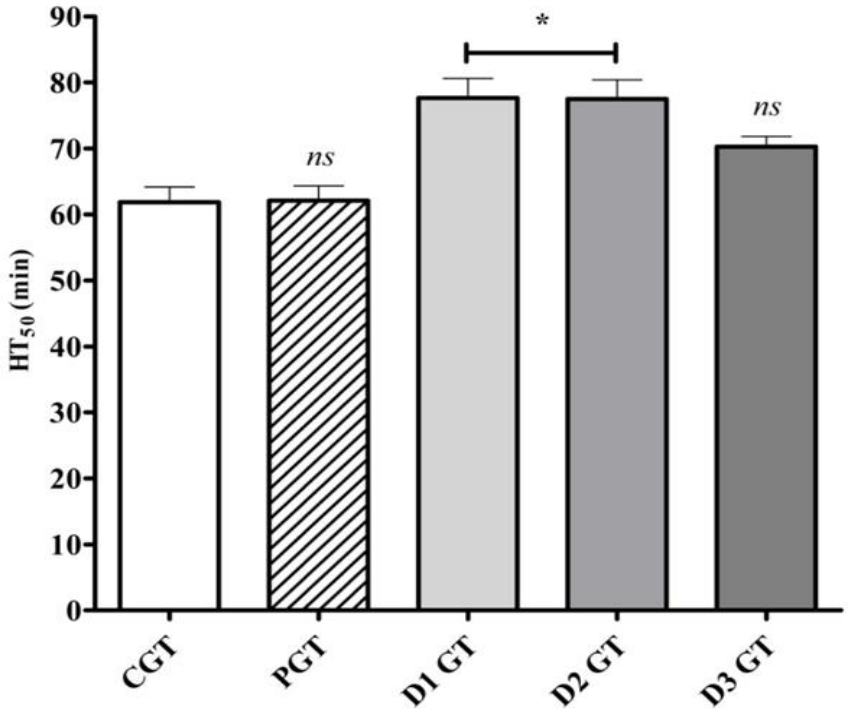


Figure 32. Graphical representation of HT_{50} values of the tested substances during obtained during erythrocytes hemolysis by radicals originated from AAPH. All data are presented as mean ± SEM. CGT: control group of toxicity, PGT: positive group of toxicity; D1 GT: dose 1 group of toxicity; D2 GT: dose 2 group of toxicity; D3 GT: dose 3 group of toxicity.

A variety of chronic diseases including cancer are related with free radical induced oxidation of membranes, herein a good model of oxidative damage of biomolecules is used through thermal generation of radicals from an oxidant compound, AAPH, in rather to induce mice erythrocyte hemolysis. Using mice red blood cells to imitate oxidative damage is in line with similar human hemolysis model (Aouchria *et al.*, 2017). Treatment with plant CrE delayed significantly hemolysis where 100 and 200 mg/kg performed the best activity compared to the higher one. It is worth noting that a similar function from *in vitro* lipid peroxidation and free radical-induced erythrocyte hemolysis assays conducted previously was carried by CrE antioxidant agents, mostly phenolic compounds, suggesting that due to the scavenging activity of these compounds, the generated radicals were trapped thus inhibiting lipid

peroxidation in erythrocyte membrane. The abundant phenolic acids in CrE namely, caffeic, neochlorogenic and vanillic acids could be the dominant contributors to the obtained results. Previous studies reported that these bioactive compounds are potent antioxidants, having the ability to inhibit lipid peroxidation and showed a strong intracellular ability to protect membrane structure and scavenge reactive species (Ul Amin *et al.*, 2017; Via and Mechanick, 2020; Lopes *et al.*, 2021).

6.3. Histological analysis and oxidative stress related markers

Histological examination was carried out to identify any morphological alterations in the control and treated groups. **Figure 33** illustrates the microscopic examination of mice livers and kidneys with hematoxylin and eosin staining for sub-acute toxicity study. Liver sections of control and treated groups with ascorbic acid (100 mg/kg) and CrE at 100, 200 and 400 mg/kg showed a normal histological structure of hepatocytes sheets separated by sinusoids. Kidney sections revealed absence of tissue alteration in all animal groups, showing nephron cells with visible nucleoli, in addition to intact renal glomeruli surrounded by Bowman's capsule. No degeneration or necrosis was observed in both of the sections of the treated groups compared to control group. However, vascular congestion was observed in liver and kidney sections of all animal groups with a vasodilatation of hepatic and nephron veins.

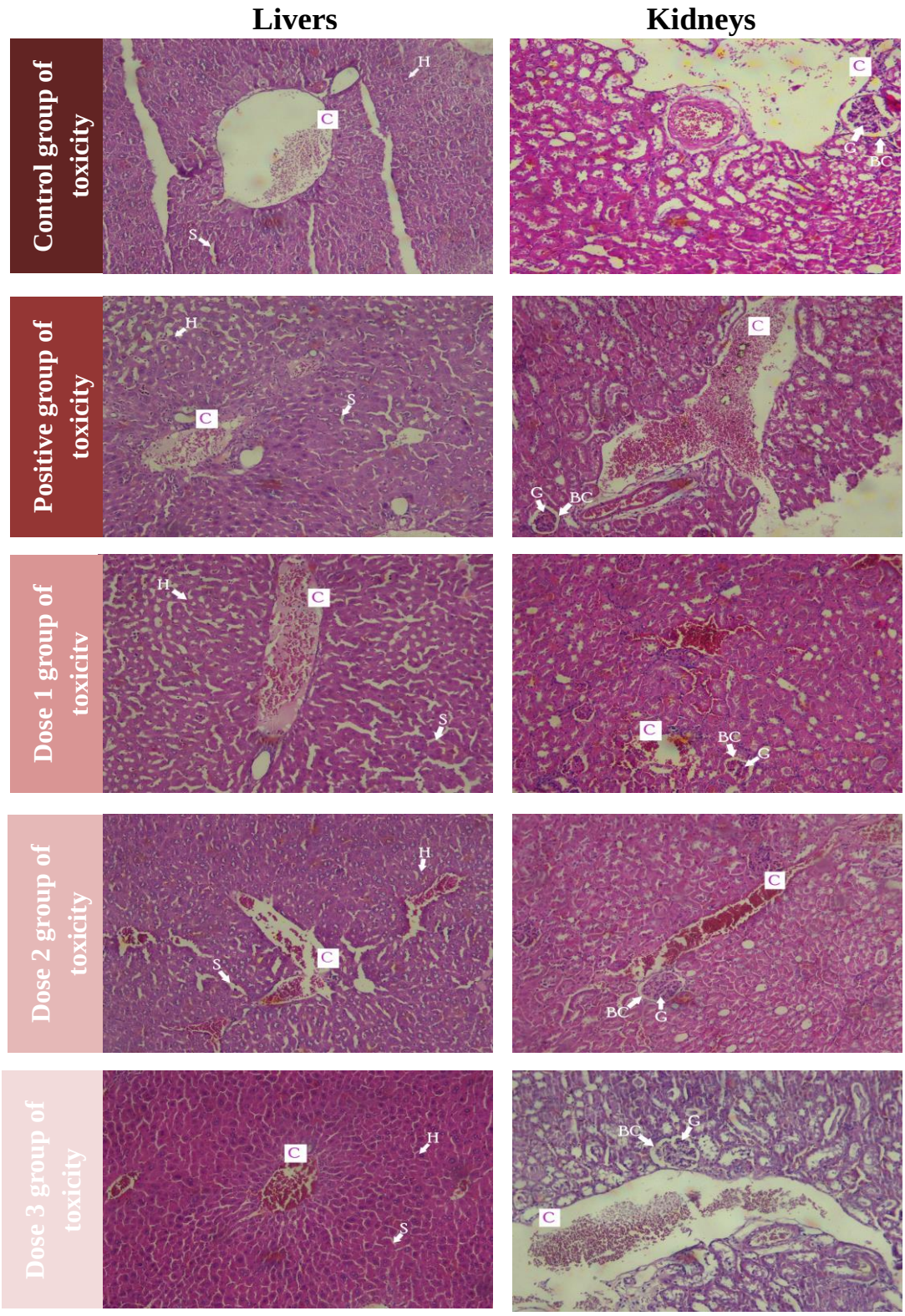


Figure 33. Histological sections of male mice livers and kidneys (40× magnification, hematoxylin and eosin staining) of control and treated groups with ascorbic acid (100 mg/kg) and CrE (100, 200 and 400 mg/kg). **C:** congestion; **BC:** Bowman's capsule; **G:** renal glomeruli; **H:** hepatocytes; **S:** sinusoids.

The levels of MDA, NO^{2-} and GSH were estimated in liver and kidney homogenates of mice of the sub-acute toxicity study. The measurement of enzymatic antioxidants activity: CAT, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S transferase (GST) was conducted and the results are summarized in **table 11**. Significant decrease in MDA levels were observed for the treated groups (ascorbic acid and CrE) compared to the control group in the livers. CrE treatment at dose 200 mg/kg showed the least MDA levels in kidneys (116.31 ± 11.64 nmol/g of tissue, $p \leq 0.001$). The effect of CrE at dose of 200 mg/kg on NO^{2-} content in liver homogenates was noticeable by decreasing its levels to 2.17 ± 0.411 µM compared to control group. Also, kidneys' NO^{2-} levels were diminished almost to the half in positive and dose 1 groups. However, increasing CrE doses did not show any effect on nitrite levels in both of organs. There was no significance difference ($p > 0.05$) of GSH levels in livers and kidneys among animals treated with ascorbic acid and CrE and the control group. CAT activity was increased slightly to 46.72 ± 2.266 µmol/min/mg of protein by the daily administration of CrE at 200 mg/kg compared to the control group ($p \leq 0.05$). Remarkably, SOD activity in animals treated with ascorbic acid and CrE was found to be more than two folds higher than that of control group, with doses 200 and 400 mg/kg showing the best effect (433.77 ± 19.96, 420.1 ± 3.91 U/mg of protein, respectively). CAT and SOD activity in the kidneys of the tested groups did not differ from the control group. In addition, 200 mg/kg of CrE increased liver GPx activity to 29.63 ± 3.244 nmol/min/mg of protein and in dose-dependently of plant extract GST activity was elevated. Moreover, kidneys' GPx activity was restored by increasing CrE dose where 100 mg/kg diminished its activity to 5.42 ± 0.810 nmol/min/mg compared to control group ($p \leq 0.01$). An important elevation (6.01 ± 0.515 mmol/min/mg of protein) in kidneys GST activity was observed with the daily administration of 200 mg/kg of CrE.

Table 11. Oxidative stress related markers of mice treated with CrE at 100, 200 and 400 mg/kg and ascorbic acid (100 mg/kg) during 21 days.

Organs	Markers	CGT	PGT	D1 GT	D2 GT	D3 GT
Liver	**MDA (nmol/g of tissue)**	148.15 ± 7.603	97.44 ± 9.661*	103.27 ± 6.362*	88.62 ± 12.094*	99 ± 15.392*
	NO^{2-} (µM)	10.07 ± 0.560	7.63 ± 0.302^{ns}	10.46 ± 0.382^{ns}	2.17 ± 0.411***	7.79 ± 0.507^{ns}
	GSH (nmol/mg of protein)	35.07 ± 7.143	32.53 ± 5.161^{ns}	31.41 ± 4.223^{ns}	39.19 ± 2.486^{ns}	43.52 ± 4.577^{ns}
	CAT (µmol/min/mg of protein)	34.71 ± 3.870	37.21 ± 2.503^{ns}	38.53 ± 2.755^{ns}	46.72 ± 2.266*	33.58 ± 1.316^{ns}
	SOD (U/mg of protein)	167.61 ± 10.878	354.96 ± 3.545***	358.31 ± 12.325***	433.77 ± 19.96***	420.1 ± 3.91***
	GPx (nmol/min/mg of protein)	18.23 ± 2.530	19.73 ± 2.418^{ns}	24.35 ± 2.957^{ns}	29.63 ± 3.244*	13.73 ± 1.405^{ns}
	GST (mmol/min/mg of protein)	9 ± 1.169	12.92 ± 0.792^{ns}	14.50 ± 1.643*	14.61 ± 1.600*	15.07 ± 0.408*
Kidney	**MDA (nmol/g of tissue)**	284.82 ± 13.529	186.92 ± 6.923*	306.69 ± 39.94^{ns}	116.31 ± 11.64***	131.08 ± 0.61***
	NO^{2-} (µM)	11.27 ± 1.732	6.60 ± 1.658*	5.23 ± 0.603**	9.73 ± 1.304^{ns}	10.28 ± 0.690^{ns}
	GSH (nmol/mg of protein)	19.48 ± 0.545	17.83 ± 0.462^{ns}	18.29 ± 0.751^{ns}	20.95 ± 0.544^{ns}	20.69 ± 0.850^{ns}
	CAT (µmol/min/mg of protein)	35.67 ± 2.805	37.03 ± 3.094^{ns}	38.78 ± 1.554^{ns}	38.49 ± 2.176^{ns}	38.20 ± 2.591^{ns}
	SOD (U/mg of protein)	94.55 ± 2.223	88.65 ± 2.918^{ns}	99.80 ± 2.635^{ns}	89.90 ± 2.21^{ns}	83 .75 ± 4.406^{ns}
	GPx (nmol/min/mg of protein)	11.88 ± 0.979	9.17 ± 1.159^{ns}	5.42 ± 0.810**	10.79 ± 0.448^{ns}	11.712 ± 0.67^{ns}
	GST (mmol/min/mg of protein)	2.07 ± 0.186	4.39 ± 0.264**	3.96 ± 0.255**	6.01 ± 0.515***	2.96 ± 0.117^{ns}

All data are the mean of 8 mice ± SEM. ns: $p > 0.05$;*$p \leq 0.05$;**$p \leq 0.01$;***$p \leq 0.001$ compared to the control group. CGT: control group of toxicity, PGT: positive group of toxicity; D1 GT: dose 1 group of toxicity; D2 GT: dose 2 group of toxicity; D3 GT: dose 3 group of toxicity.

The antioxidant activity of the plant extract was confirmed through *in vivo* short term toxicity assessment and it is a strategy to reveal the effect of the bioactive compounds of the tested extract on the prevention from oxidative stress-caused damage. Histological examination proved that CrE administration at increasing doses on daily basis did not show any treatment-related changes. Level estimation of the metabolic product of lipid peroxidation (MDA), NO^{2-}, GSH as well as the enzymatic measurement of the primary endogenous antioxidant enzymes (CAT, SOD, GPx and GST) in organ homogenates, that have a vital function of detoxification, are indicative of the ultimately damage that cells are undergoing or the degree of resistance to oxidative stress. The experimental data showed that the plant extract diminished significantly MDA and NO^{2-}, indicating that the bioactive compounds of CrE exhibited a similar function from *in vitro* and *in vivo* studies, already discussed in section results and discussion (radical scavenging, lipid peroxidation and nitrite, MDA levels and CAT activity in croton oil ears homogenates), to trap nitric oxide and inhibit lipid peroxidation thereby the formation of MDA. As enzymatic antioxidant defense system against reactive species, SOD catalyze the dismutation of superoxide anion into H_2O_2, further CAT and GPx reduce the generated H_2O_2 into water (Hassimoto and Lajolo, 2010) and GST associate SH groups with H_2O_2 to finally neutralize it (Llavanera *et al.*, 2019). SOD enzymatic activity was elevated more than two folds by administration of CrE. Nevertheless, this enhancement may arise of H_2O_2 levels in the cells into deleterious levels. The balance among the enzymatic antioxidant defense system is important, thus CAT, GPx and GST enzymatic activity was increased significantly compared to control group by the CrE. Even though, the reduced GSH is a cofactor to GST and is oxidized by the GPx to neutralize H_2O_2, its levels were maintained at physiological equilibrium as to control group, suggesting that the CrE did not affect its regeneration process. CrE daily administration successfully improved cellular status of enzymatic antioxidants. It is likely due to phenolic and flavonoids

compounds. Activation of Nrf2 to sequester into the nucleus, thereby activation of genes responsible for the expression of antioxidant and detoxification enzymes could for extent be the molecular action of phenolic compounds to enhance SOD, CAT, GPx and GST levels (Cañas *et al.*, 2023). Belbachir *et al*. (2023) found that methanolic extract of *A. leucotrichus* seeds increased significantly NrF2 gene expression. Among the phenolic compounds identified in CrE, the abundant caffeic acid is previously mentioned as an activator of Nrf2 due to its electrophilic moiety that oxidizes Kelch-like erythroid associated protein 1 (keap1) and induced Nrf2-targeting antioxidant gene expression (Sirota *et al.*, 2015; Bovilla *et al.*, 2021). In addition to the powerful antioxidant activity of vanillic acid that it is previously reported to alleviate oxidative stress through activation of Nrf2 and restore SOD, CAT and GPx activity (Ma *et al.*, 2019).

Conclusion and future perspectives

This thesis aimed to validate the traditional use of *Ammodaucus leucotrichus* Coss. & Durieu related to a wide range of diseases and complications through scientific evidence, *in vitro* and *in vivo* antioxidant and anti-inflammatory activities as well as toxicity assessment, regarding its secondary metabolite compounds.

In the first part, methanolic maceration afforded a considerable amount of total phenolics and flavonoids which reflects the remarkable selectivity of methanol in extracting the targeted anylates. Qualitative and quantitative analyses by HPLC-ESI-MS/MS showed the predominant presence of phenolic acids namely caffeic, ferulic and vanillic acids together with flavonol class including kaempferol.

In the second part of this study, in an attempt to link *A. leucotrichus* seeds' extracts activities to pharmacology; treatments strategies are related to the antioxidant and anti-inflammatory systems. A board range of reactive species, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, $OH^{\bullet}$, $O_2^{\bullet-}$ and $NO^{\bullet}$, were targeted to assess *A. leucotrichus* seeds' extracts radical scavenging capacity. Results showed that EAE had the best and potent activity in all of the tested systems. Among the extracts, EAE and ChE proved to be the most efficient inhibitors of lipid peroxidation in early phase of linoleic acid autoxidation and β-Carotene/linoleic acid coupled oxidation. Furthermore, in the case of dealing with metals, ferrous chelating capacity was performed weakly by the CrE, ChE and strongly by AqE. Ferric reducing is an extrinsic property of polyphenols and flavonoids, where EAE showed a good reducing ability among the extracts. In radical induced erythrocytes hemolysis, mice erythrocytes incubated with plant extracts had the ability to resist to free radical attack even at low concentration of 0.025 mg/ml extracts. Extracts at 50 mg/ml delayed blood coagulation and had the ability to dissociate the formed clot reflecting their promising antithrombotic activity. The approach used in the treatment of diabetes

mellitus through inhibition of α-amylase and α-glucosidase implicated in carbohydrates hydrolysis highlighted the good capability of the bioactive compounds present in EAE for enzyme inhibition. Inflammatory diseases related to protein denaturation are life threatening, the model used in this research, human serum albumin heat denaturation, reflected the anti-inflammatory activity of the plant extracts even at low concentration.

Finely, *in vivo* induced edema models using irritant agents, carrageenan, xylene and croton oil, revealed that CrE at dose of 200 mg/kg had a remarkable anti-edematous activity and this was confirmed by histological analysis. In addition, anti-nociceptive activity was noticed at the same dose used previously. The obvious inflammatory state linked with croton oil application leaded to increase oxidative stress markers (MDA and NO^{-2}) levels and decrease CAT activity in ear homogenates. Successfully, CrE administration at dose 200 mg/kg decreased MDA and NO^{-2} levels and restored CAT activity. Acute toxicity assessment results lead to conclude that the methanolic CrE is non-toxic extract and it could be used safely. Also, results of sub-acute toxicity confirmed the safe use of the plant at low repeated doses without altering body weight or organs relative weight comparing to the control group. Evaluation of oxidative stress-related markers in liver and kidney homogenates showed that CrE bioactive compounds increased CAT, SOD, GST and GPx activities and decreased MDA and NO^{-2} content. In addition to the absence of treatment-related changes on livers and kidney histological sections.

Evidence gathered in this study supports the promising use of this medicinal plant in the treatment of diabetes, and maybe in other physiological disorders, that reflects a good image on the capacity of its bioactive compounds existing in this plant extracts. As antioxidant agents dealing with wide spectrum of reactive oxygen species, transition metals and enzyme inhibitors to regulate the hyperglycemia and also as anti-inflammatory compounds. Therefore,

this plant could be used where antioxidant and/or anti-inflammatory activities were warranted. More studies are required regarding the active principles of this plant:

- Isolation, identification and characterization of the active constituents responsible for the remarkable antioxidant and anti-inflammatory activities in order to determine the exact mechanism behind the observed activities;
- Expand the research biological activities scope, whether *in vitro* or *in vivo*;
- Anti-diabetic activity should be conducted to corroborate the actual traditional claims;
- Toxicity of biological active compounds (purified from this plant) should be assessed to verify their safety profile;
- In depth studies on the pharmacokinetics and pharmacodynamics of plant compounds would be useful to determine the appropriate dosage.

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الملخص

يستخدم الكمون الصوفي (Apiaceae) *Ammodaucus leucotrichus* Coss. & Durieu)) تقليديا في جنوب الجزائر لتخفيض مستوى السكر إلى جانب مجموعة واسعة من الاضطرابات الفسيولوجية بسبب خصائصه المعززة للصحة. تهدف هذه الدراسة إلى التحقق من صحة الاستعمالات التقليدية لهذا النوع من خلال التقييم المختبري وفي الجسم الحي للقدرات المضادة للأكسدة وللالتهاب لمستخلص ميثانولي للنبتة وأجزائه، بالإضافة إلى تقييم جانب الأمان في استخدام هذا النبات. نقعت بذور النبات في الميثانول، تم بعد ذلك فصل مكونات المستخلص الخام (CrE) باستعمال مذيبات عضوية مختلفة القطبية للحصول على: مستخلص الكلوروفورم (ChE)، مستخلص إيثيل الأسيتات (EAE) والمستخلص المائي (AqE). ضمن هذه المستخلصات، تم تسجيل أعلى محتوى من عديدات الفينول والفلافونويدات لدى المستخلص الخام ± 0.01 (77.14ميكروغرام مكافئ غرامي حمض الغاليك/مغ من المستخلص و19.59 ± 0.08 ميكروغرام مكافئ غرامي كارستين/مغ من المستخلص، على التوالي) .تم تحليل CrE ومستخلصاته، من الجانبين النوعي والكمي، فيما يتعلق بمركباتهم الفينولية بواسطة HPLC-ESI-MS/MS. أشارت النتائج إلى أن أحماض الكافييك والفيروليك والفانيليك هي الأحماض الفينولية الرئيسية إلى جانب كيمبفيرول كفلافونول. تم تقييم النشاط الإزاحي في المختبر باستخدام $DPPH^{\bullet}$، $ABTS^{\bullet+}$، H_2O_2، $OH^{\bullet}$، $O_2^{\bullet-}$ و $NO^{\bullet}$.تميز EAE بكونه الأكثر نشاطًا وفعالية تجاه الجذور الحرة. في الوقت نفسه، أظهر كلا من EAE وChE نسبة تثبيط عالية في مرحلة مبكرة من الأكسدة المقترنة بحمض البيتا كاروتين/حمض اللينوليك بنسبة 81.99 ± 2.942% و78.24 ± 1.46% على الترتيب. أظهر هذان المستخلصان حماية ملحوظة لحمض اللينوليك تجاه فوق أكسدة الدهون حتى بعد 96 ساعة، حيث كانت قيم امتصاصية MDA منخفضة جدًا. أظهر اختبار استخلاب المعادن أن AqE هو المستخلص الأكثر فعالية. في حين، و حسب اختبار القدرة الإرجاعية ، تميزمستخلص EAE بأعلى نشاط معEC_{50} قدره 0.12 ± 0.002 ملغم/مل. قامت جميع المستخلصات بحماية، بشكل فعال، كريات الدم الحمراء للفئران ضد انحلال الدم الناجم عن AAPH بطريقة مرتبطة بالتركيز. وبالمثل، عمل EAE وCrE على تفكيك الجلطة المتكونة بنسبة متقاربة 19.93% و19.92% على الترتيب، يعكس هذا نشاطهما المضاد للتخثر. بينما أظهر EAE وChE تأثيرا مضادا لتخثرالدم الجزئي حتى بعد 3 ساعات من الملاحظة. ثبطت جميع المستخلصات المختبرة إنزيم α-amylase بشكل ملحوظ. إذ أظهر EAE أفضل نشاط تثبيطي بـ 0.63 ± 0.003 ملغرام مكافئ اكربوز/غرام من المستخلص ضد-α amylase و 1.31 ± 0.037 ملغرام مكافئ اكربوز/غرام من المستخلص ضد α-glucosidase .قام CrE و مستخلصاته بحماية بنية HSA بشكل فعال في الظروف الحرارية حتى عند التركيز المنخفض (0.31 ملغرام/مل). سمح إعطاء 200 مغ/ كغ من المستخلص الخام عن طريق الفم بتثبيط الألم الناجم عن حمض الأسيتيك وتقليل تكوين الوذمة الناجم عن xylene وcarrageenan. كما بين الفحص المجهري للالتهاب الحاد الناجم عن croton oil أن تثبيط الوذمة مرتبط بالجرعة. من جانب آخر، إنخفضت مستويات النتريت (NO^{2-}) و MDA مع استعادة نشاط CAT، نتيجة لإعطاء CrE. وفقاً للمبادئ التوجيهية لمنظمة OECD، تم متابعة السمية الحادة باختبار جرعتين؛ 2 غ/كغ و5 غ/كغ حيث لم تلاحظ أي علامات تغير في سلوك الحيوانات أو الوفيات مما يشير إلى أن CrE مادة غير سامة. أكدت السمية تحت الحادة سلامة استخدام CrE بجرعات متكررة (100 و200 و400 مغ/كغ) يومياً بسبب عدم وجود علامات سمية فيما يتعلق بوزن جسم الحيوانات والوزن النسبي للأعضاء. بالإضافة إلى ذلك، أظهر التحليل النسيجي عدم وجود تغيرات مرتبطة بالعلاج في أقسام الكبد والكلى. أدى إعطاء CrE إلى خفض مستويات MDA و NO^{2-} وتحسين الحالة الخلوية لمضادات الأكسدة الأنزيمية من خلال تعزيز أنشطة SOD و CAT و GPx و GST في الكبد والكلى. وفي الختام، أكدت هذه الدراسة اعتبارالمركبات النشطة بيولوجيا، خصوصا عديدات الفينول والفلافونويدات، لنبات *Ammodaucus leucotrichus* ، كعوامل مضادة للأكسدة ومضادة للالتهاب آمنة مدعمة بذلك استخدام هذا النبات في الطب الشعبي.

الكلمات المفتاحية: *Ammodaucus leucotrichus*؛ عديدات الفينول؛ HPLC؛ الاكسدة؛ مضادات الأكسدة؛ مضاد التهاب؛ السمية.

Abstract

Ammodaucus leucotrichus Coss. & Durieu (Apiaceae) is traditionally used in southern Algeria to decrease blood glucose levels along with a wide range of physiological disorders due to its health-promoting properties. This study aims to validate the traditional claims of this species through *in vitro* and *in vivo* evaluation of antioxidant and anti-inflammatory potentials of plant methanolic extract and its fractions, together with assessment of the safety profile. Plant seeds were subjected to methanolic maceration followed by liquid-liquid scale extraction using solvents of different polarity to afford: crude extract (CrE), chloroform extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). Among these extracts, the highest contents of polyphenols and flavonoids were recorded in the crude extract (77.14 ± 0.01 µg GAE/mg E and 19.59 ± 0.08 µg QE/mg E, respectively). CrE and its fraction were qualitatively and quantitatively analyzed regarding their phenolic compounds by HPLC-ESI-MS/MS. Results indicated that caffeic, ferulic and vanillic acids were the major phenolic acids alongside with flavonol kaempferol. Anti-radical activity was assessed *in vitro* using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, $OH^{\bullet}$, $O_2^{\bullet-}$ and $NO^{\bullet}$. EAE was the most active and potent radical scavenger. Meanwhile, EAE and ChE showed a high inhibition percentage at an early stage of β-Carotene/linoleic acid coupled oxidation with 81.99 ± 2.942% and 78.24 ± 1.46 %, respectively. These two fractions showed remarkable protection of linoleic acid against lipid peroxidation even after 96 h, where MDA absorbance values were very low. Metal chelating showed that AqE was the effective extract. However, in ferric reducing ability, EAE performed the best reducing activity with EC_{50} of 0.12 ± 0.002 mg/ml. All extracts effectively protected mice erythrocytes against AAPH-induced hemolysis in a concentration-dependent manner. EAE and CrE similarly dissociated the formed clot with a comparable percentage of 19.93% and 19.92%, respectively, reflecting their antithrombotic activity. While EAE and ChE showed partial blood clotting even after 3 h of observation. All plant extracts performed a noticeable α-amylase inhibition. Where EAE showed the best inhibition activity with 0.63 ± 0.003 mg ACAE/g E against α-amylase and 1.31 ± 0.037 mg ACAE/g E against α-glucosidase. CrE and its fractions effectively protected the HSA structure in thermal conditions even at low concentration (0.31 mg/ml). Oral administration of 200 mg/kg of crude extract successfully inhibited acetic acid-induced nociception and reduced edema formation induced by xylene and carrageenan. However, a dose-dependent manner was observed to decrease ear edema by a microscopic examination in croton oil-induced acute inflammation. Nitrite (NO^{2-}) and MDA levels were decreased together with restoration of catalase activity by CrE administration. According to OECD guidelines, acute toxicity was performed at two limit tests; 2 g/kg and 5 g/kg where no signs of changes in animals' behavior nor mortality were observed indicating CrE as a non-toxic substance. Sub-acute toxicity confirmed the safety use of CrE at repeated doses (100, 200 and 400 mg/kg) on a daily basis by lacking toxic signs regarding animals' body weight and organs relative weight. In addition, histological analysis showed the absence of treatment-related changes in livers and kidney sections. Administration of CrE decreased MDA and NO^{2-} levels and improved the cellular status of enzymatic antioxidants through enhancement of SOD, CAT, GPx and GST activities in the liver and kidney. To conclude, this study corroborated those bioactive compounds, mainly polyphenols and flavonoids, from this plant as antioxidant and anti-inflammatory agents with a safe profile and supports the use of this plant in folk medicine.

Keywords: *Ammodaucus leucotrichus*; polyphenols; HPLC; oxidative stress; antioxidant; anti-inflammatory; toxicity.