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Toxicity investigation of *Centaurea calcitrapa* extract

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In front of the jury composed of:

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Dedication

I dedicate this memoir to my dear parents for their constant sacrifice and care.

My friends who have immensely helped me throughout difficult times during this experience by their encouraging words, support and patience.

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Dedication

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List of abbreviation

BSA Eq: BSA equivalent

CMC : carboxy methyl cellulose

CP : cyclophosphamide

DNA: desoxyribose acid

FRAP : Ferric Reducing ability of plasma

GAE: gallic acid equivalent

HBSS : Hanks' Balanced Salt Solution

IP : intraperitoneal

MNi: Micronuclei

MOA: mode of action

NMRI: Naval Medical Research Institute

OECD : Organisation for Economic Co-operation and Development

QE : quercetin equivalent

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III. RESULTS and DISCUSSION

Introduction

Medicinal plants have been used by mankind as nutritional supplements and medicine for centuries. Their usage rate has grown more in recent years reaching as high as 80% in developing countries despite the availability of synthetic pharmaceuticals **(Zhang and Wang, 2023)**. This interest is often attributed to their abundance, the accustomed traditional use of plants in medicine, the belief that natural products are safer and researchers reporting that many of these plants have the ability to produce bioactive substances that are otherwise challenging to make by chemical synthesis (**Sponchiado *et al.*, 2016)**. They also possess a wide variety of compounds such as polyphenols, alkaloids, terpenoids that are associated with many beneficial effects including antioxidant, anti-inflammatory and antianalgesic properties **(Savithramma *et al.*, 2011)**.

Although the utilization of medicinal plants has proven to have healing effects, many studies reported that their bioactive compounds can also induce different kinds of toxicities **(Amorim *et al.*, 2013)**. The dual effect of these compounds emphasizes the importance to study the toxic potential and risk assessment of medicinal plants especially wild plants that are traditionally used in folk medicine as they are often understudied.

Centaurea calcitrapa is a wild plant that grows naturally in open landscapes and polluted areas. It is historically used in folklore medicine in multiple regions for its multiple therapeutic effects. Studies have shown that this plant is rich in sesquiterpene lactones which are known for their anti-inflammatory, anti-malarial and antipyretic proprieties which could explain the efficacy of this plant's usage in traditional medicine, but some studies also found that sesquiterpene lactones could cause different kinds of toxicity **(Amorim *et al.*, 2013; Moujir *et al.*, 2020)**. Despite this plant being rich in a substance with a dual effect characterized by therapeutic properties and toxicity risks, the toxic effects of *Centaurea calcitrapa* are not well known as there are very few studies about its toxicity *in vivo*.

The aim of our study is to test *in vivo* the acute and sub-chronic toxicity alongside the protective effect of *Centaurea calcitrapa* extract by evaluating different toxicity parameters including genotoxicity and associated oxidative stress markers in mice.

Chapter I
Bibliographic review

I.1. Medicinal plants in therapy

Plants have long been a crucial part of life for many indigenous communities worldwide, leading to extensive knowledge of their healing properties. The traditional use of plants or plant parts for medicinal purposes is known as herbalism or botanical medicine. Globally, there are an estimated 35,000 to 50,000 medicinal plants **(Shembo *et al.*, 2024)**.

Medicinal plants are plants that possess active substances or secondary metabolites that have a biological activity which can be found either in the entire plant or some parts of it like leaves, flowers and roots **(Abubakar and Haque, 2020)**. They have been traditionally used as nutritional supplements and medicine to treat and prevent a variety of diseases and relieve the symptoms they cause. In our present time of pharmacological advancement and synthetic production of pharmaceuticals, medicinal plants are still widely employed as many prescription drugs are made using substances found in plants in addition to their ability to produce molecules that are challenging to chemically synthesize **(Sponchiado *et al.*, 2016)**. Many researchers have also experimented on these plants performing many tests and assays on whole plants or plant parts to study their therapeutic effects. The results of these studies revealed beneficial proprieties: antifungal, antibacterial, antiviral, antioxidant, antimutagenic, antimicrobial, anti-inflammatory, anti-arthritic, anti-obesity, antidiabetic, anticarcinogenic, antinociceptive and analgesic activities **(Marrelli, 2021 ; Goo, 2022 ; Dincheva *et al.*, 2023)**.

Therapeutic effects of plants are mainly based on their metabolites. In fact, plants are living organisms that produce primary metabolites (eg. carbohydrates, lipids, proteins, nucleic acids) which are essential for cellular functions, growth and metabolisms. Plants also generate a variety of secondary metabolites (eg. phenolic compounds, alkaloids and terpenoids) which originate from primary metabolites and have different biological effects mainly in health maintenance and defence of plants against environmental stressors **(Elshafie *et al.*, 2023)**. Secondary metabolites are the plant substances that show bioactive effects, they exhibit beneficial proprieties that are found in medicinal plants and drugs (antimicrobial, antiviral, antifungal, antibacterial, etc) **(Hilal *et al.*, 2024)**. Some of plant secondary metabolites therapeutic benefits are reported in **Table I**.

Table I. The therapeutic benefits of some plant secondary metabolites

Secondary metabolites	Treatment	Plant example	Reference
Alkaloids	Coronary heart disease, chronic, heart failure, rheumatoidarthritis and neuropathic pain	*Aconitum* (Ranunculaceae)	**(Mi *et al.*, 2021)**
Flavonoids	Intestinal lesions in colitis,antilithic inflammation, pains,snakebite, antiulcerogenic activity, hypoglycaemic effect, hypolipidemic and antioxidant actions	*Musa paradisiaca*	**(Nisha and Mini, 2013)**
Terpenoids	Antitumor activity	*Artemisia L.*	**(Rabe *et al.*, 2011)**
Glycosides	Congestive heart failure, increase cardiac contractility	*Digitalis* L. (Foxglove, Plantaginaceae)	**(Verma *et al.*, 2016)**
Polyphenols	Antioxidant, antibacterial, antidiabetic, anti-inflammatory, antiaging as well as anticancer activities.	*Castanea sativa* Mill.	**(Jin *et al.*, 2024)**

I.2. *Centaurea* genus (Asteraceae)

I.2.1. Botanical description and taxonomic insights

Centaurea genus is part of the Asteraceae family. It is divided into three subgenera: *Lopholoma*, *Cyanus* and *Centaurea* and it contains nearly 500 species that are native to many areas mostly found in the Mediterranean region and Western Asia. In Algeria, 45 *Centaurea* species are present, 7 of them are found in the Sahara desert **(Ayad and Akkal, 2019)**. They grow annually, biennially or perennially and have different morphologies. Some of the species are edible plants better known in rural cuisine as it is less common for them to be incorporated in regular diets; others are used as traditional medicine due to their active compounds **(Petropoulos *et al.*, 2020)**.

Asteraceae is a large plant family that includes more than 1600 genera and 2500 species. It is found amongst vegetation and greenery in various parts of the world excluding Antarctica and is less common in tropical areas. It has diverse characteristics in appearance as species can range from trees that are over 30 m tall to smaller shrubs to perennial herb which are the most common kind of its species. Asteraceae family plants have a multitude of uses. A study reported that they have high nutritional values, rich in fibres and proteins and low in fat content. Essential minerals (Na, K, Ca and Mg) and vitamins (A, B, C and D) **(Rolnik and Olas, 2021)**. They also have therapeutic proprieties. They are the most cited family of medicinal plants used for wound healing in literature **(Gang *et al.*, 2024)**. Other pharmacological activities they possess are: anti-anxiety, antioxidant, antimicrobial, anti-inflammatory, antidiabetic and antiseptic activities which are shared across many species including well known ones such as chamomile (*Matricaria recutita*

L.), yarrow (*Achillea millefolium* L.), wormwood (*Artemisia absinthium* L.) and chicory (*Cichorium intybus*) **(Bessada *et al.*, 2015 ; Mihyaoui *et al.*, 2022)**.

Centaurea species are characterized by their unique floral shape that has apparent sterile peripheral florets without staminodes. Their flowers vary in colour: pink, purple, blue and yellow are the most common ones. They are also characterized by dry and membranous bract appendages that resemble an artichoke and are often spiny **(Hilpold *et al.*, 2014)**. Some *Centaurea* species are presented in **Figure 1**.

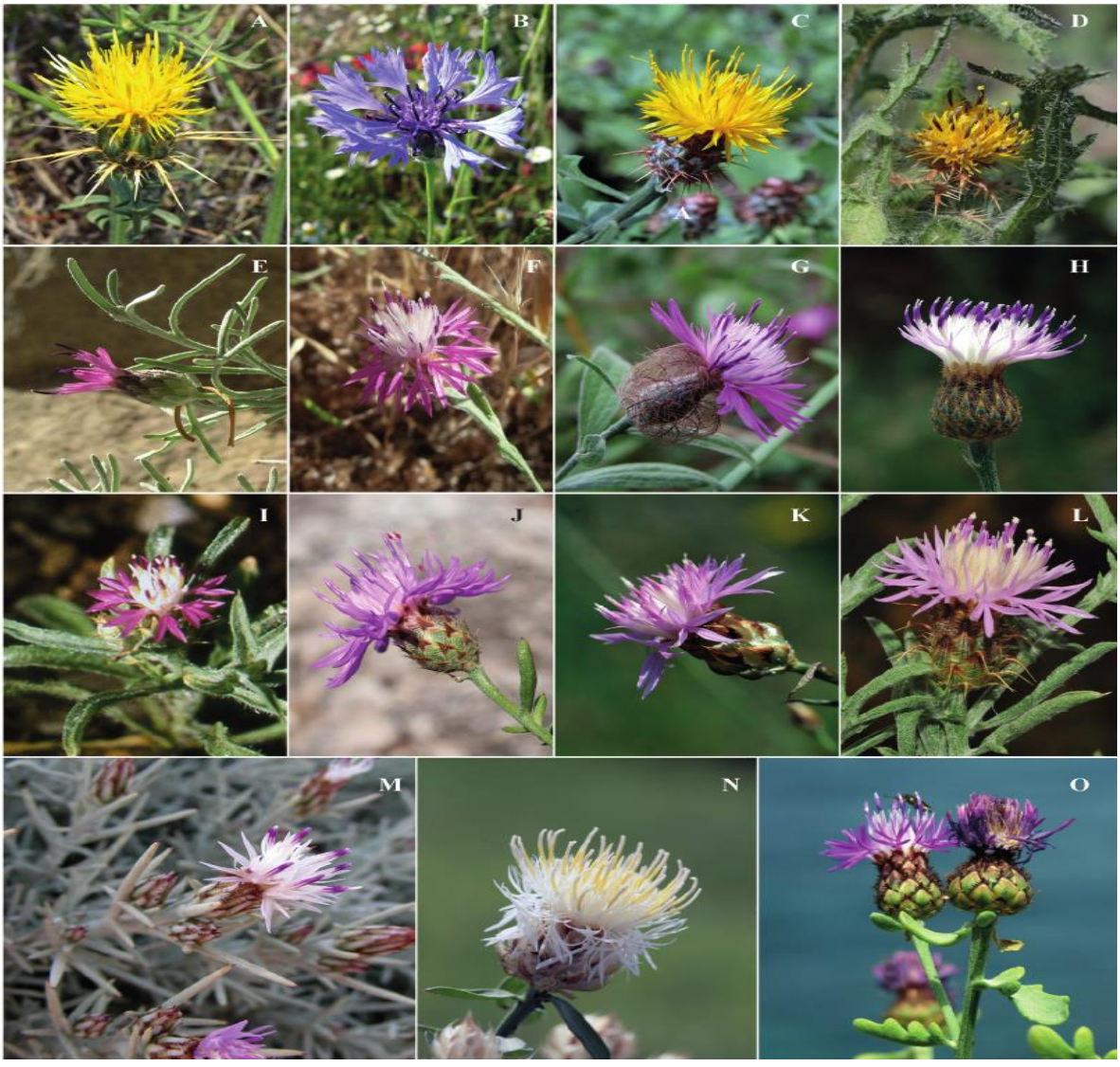

Figure 1: Photographs of the genus *Centaurea* , with special emphasis on subgenus *Centaurea* and sect. *Centaurea* . **(A)**Lopholoma : *C. ornata* , Soria, Spain ; **(B)** Cyanus : *Centaurea cyanus* , Soria, Spain. **(C)***Centaurea* : East-Mediterranean Clade (EMC): **(C)***C. lycopifolia* , Barcelona Botanical Garden. **(D–N)**, Circum-Mediterranean Clade (CMC). **(D)**, *C. benedicta* , Barcelona Botanical Garden (Photograph: A. Susanna); **(E)**, *C. akamantis* , Avakas gorge, Cyprus (Photograph: M. Galbany); **(F)**, *C. hierapolitana* , Afon lake, Turkey (Photogra - ph: A. Susanna); **(G)**,*C. hyrcanica* (Jacea-Phrygia group), Iran (Photograph: A. Pirani); **(H)**, *C. exarata* , Barcelona Botanical Gar- den ; **(I)**, *C. patula* , Barcelona Botanical Garden (Photograph: A. Susanna). **(J–N)**, Sect. *Centaurea* . **(J)**, *C. tenorei* , Minori, Italy (Photograph: A. Hilpold); **(K)**, *C. alba* , Sierra de Aracena, Spain (Photograph: L. Barres); **(L)**, *C. pulvinata* , Sierra de Abrucena, Spain (Photograph: G. Blanca); **(M)**, *C. horrida* , Sardinia (Photograph: S. Pisanu); **(N)**, *C. princeps* , Barcelona Botanical Garden (Photograph: A. Susanna); **(O)**, *C. panormitana*, Sferracavallo, Sicily (Photograph: A. Hilpold).

I.2.2. Phytochemical composition of *Centaurea* genus species.

Most of plant species that are part of the same family have similar chemical composition. Multiple studies focused on *Centaurea* plants report the presence of sugars, fatty acids, tocopherols. A study on the chemical composition of *Centaurea raphanina* subsp. *Mixta* leaves revealed the presence of glucose, sucrose and trehalose as the most abundant sugars, citric acid, mallic acid and oxalic acid for organic acids, α-tocopherols γ-tocopherols and α-linolenic, linoleic and palmitic acid **(Petropoulos *et al.*, 2020)**. Organic acids citric acid, succinic acid and quinic acid along with α-tocopherols γ-ocopherols have also been reported in flowers of *Centaurea cyanus* L **(Lockowandt *et al.*, 2019)**. The notable compounds that characterize the *Centaurea* genus are sesquiterpenes and flavonoids **(Ifantis *et al.*, 2013)**. The genus also has volatile oils as reported in **Table II**.

Table II. *Centaurea* genus secondary metabolites.

Plant	Sesquiterpenes	Flavonoids	Reference
Extracts			
C.papposa (Coss.) Greuter	Cnicin, eudesmanolide, elemanolides, elemane derivative	Eupatorin	**(Grafakou *et al.*, 2018)**
C. kilaea	Cnicin and dehydromelitensin	3′-O-methyl eupatorin, apigenin, cirsimaritin	**(Sen *et al.*, 2016)**
C.zuccariniana	Malacitenolide, cnicin, 4'-O-acetylcnicin , heliangolide and eudesmanolides,	apigenin , genkwanin , hispindulin	**(Ćirić *et al.*, 2012)**
C. tomorosii	Cnicin	santoflavone cirsimaritin hispidulin	**(Tesevic *et al.*, 2014)**
C. deusta Ten.	Cnicin and 4'-acetylcnicin		**(Karioti *et al.*, 2002)**
Volatile oils			
***C.scabiosa* L**	Caryophyllene oxide, alloaromadendrene epoxide,α-cyperone, and α-bisabolol		**(Carev *et al.*, 2022)**
***C.polymorpha* Lag.**	α-Cedrene, β-cedrene, β-curcumene and caryophyllene oxide		**(Forimsano *et al.*, 2006)**
C. sicana	Germacrene D and (E)-β-farnesene		**(Formisano *et al.*, 2008)**
C.giardinae	Caryophyllene oxide and germacrene D		

The chemical composition of *Centaurea* plants is very rich in secondary metabolites like sesquiterpenes which confers to them powerful bioactive proprieties but also makes them potentially toxic. Sesquiterpenes can have both these characteristics at the same time, the proprieties through which a compound causes a therapeutic effect against conditions or diseases might be the same proprieties that cause harmful toxic effects in healthy organisms **(Amorim *et al.*, 2013)**. Sesquiterpene lactones (STLs) are the most common sesquiterpenes amongst *Centaurea* species. They are strong alkylating agents, oxidative stress inducers, apoptotic inducers

and cell proliferation inhibitors in cancer cell lines which make them possess interesting anticancer and cytotoxic activities. Cnicin and dehydromelitensin are examples of STLs reported to have strong cytotoxicity against mammalian and prostate cell lines **(Gach *et al.*, 2015 ; Sen *et al.*, 2016)**. However, the mentioned characteristics are also found in genotoxic agents and *in vivo* and *in vitro* studies demonstrated that STLs can cause chromosomal aberrations (CA) **(Burim *et al.*, 2001)**. They also have anti-inflammatory activities that are mediated by prostaglandin synthesis inhibition. Because of this quality they are used to relieve inflammatory conditions like arthiritis and gastrointestinal distress **(Heinrich *et al.*, 1998)**.

I.3. *Centaurea calcitrapa* description and phytochemical composition

Centaurea calcitrapa also called purple starthistle is an herbaceous plant from the *Centaurea* genus. It grows biennially and is common throughout western Asia to North-western India, the western and southern regions of Europe and in North Africa. It can be found in fertile soils, rocky areas, waste places, alongside roads and between railroads **(Dimkić *et al.*, 2020).**

C. calcitrapa can reach 60cm in height. It has sessile flower heads similar to other *Centaurea* plants. They are characterized by multiple tubular vibrant purple-red flowers. Their involucre is made of several phyllaries that have long straw coloured spines attached to them which are unique to this species. Their leaves and stems are thin, green in colour and covered with fine hairs **(Dimkić *et al.*, 2020) (Figure 2)**.

Figure 2 : *Centaurea calcitrapa* L. (Village Sakule, Opovo municipality, the South Banat District, Vojvodina province, Serbia) **(Dimkić *et al.*, 2020)**

Sesquiterpene lacones and flavonoids are the major compounds identified in *C.calcitrapa* studies. A review by **Bruno et al.(2013)** compiled 9 different STLs and a review by **Formisano et al.(2012)** compiled 12 flavonoids. Eupatorin, cirsiliol, and jaceosidin are additional flavonoids

found by **Kitouni et al.(2015)** in Algerian *C.calcitrapa.* Lignans are another group of phenolic compounds found in this plant **(Marco *et al.*, 1992).** These findings are presented in **Table III**.

Table III. Main secondary metabolites found in *C. calcitrapa* **(Marco *et al.*, 1992; Formisano *et al.*, 2012 ; Bruno *et al.*, 2013 ; Kitouni *et al.*, 2015).**

Sesquiterpenes	
Germacanes	Cnicin, Cnicin4'-O-acetyl, Salonitenolide,11β,13-dihydrosalonitenolide 11α,13-dihydrosalonitenolide
Elemanes	8α-(3',4'-dihydroxy2'methylenebutanoyloxy)-dehydromelitensin; isocnicin and Melitensin
Bisabolanes	4,9-Dioxo-bisabol-2,7(14),10-triene 4,9-Dioxo-bisabol-2,7E,10-triene
Flavonoids	
Flavones	Apigenin, Luteolin, Eupatilin, Cirsiliol, Eupatorin andJaceosidin
Flavonols	Kaempferol, Quercetin
Flavanones	Naringenin
Flavone Glycosides	Cosmosiin, Apigenin-7-O-b-d-galacturonic acid methyl ester, Scutellarin
Flavonol Glycosides	Rutin, Chrysanthemin, Cyanin
Other Secondary Metabolites	
Lignans	Arctigenin, Pinoresinol and 7′(*S*)-hydroxyarctigenin

1.4. Medicinal plants toxicity and genotoxicity

Medicinal plants possess substances that induce toxicity *in vivo* by affecting different parts of organisms such as the organs, the mechanism of toxicity is caused by several principles affecting different systems **(Anywar *et al.*, 2021)**. In addition to systematic *in vivo* toxicity phytochemicals can also affect organisms on the cellular level by damaging macromolecules like proteins, lipids and DNA (desoxyribose acid). The toxicity targeting DNA in particular is called genotoxicity which is a crucial characteristic to test when investigating medicinal plants and their health risks **(Doak *et al.*, 2023)**.

1.4.1. Genotoxic studies on medicinal plants

Genotoxicity is one of the negative effects that plant substances have demonstrated in risk assessment studies. This led researchers to study the genotoxic potential of medicinal plants, especially plants that are traditionally used in folk medicine **(Oyeyemi *et al.*, 2015)**. Different plants show a variety of results when tested for their genotoxicity, a plant could also exhibit this trait only in specific parts (eg. roots, thorns, and seeds), specific concentrations and specific solvents. In contrast, some plants initially suspected to be genotoxic were found to be non-genotoxic. Furthermore, some studies also test the antigenotoxicity of plants to see if they have

preventative proprieties on genotoxins to reduce their ability to induce DNA damage. Some of these parameters are presented by few examples in **Table IV**.

Table IV: Medicinal plants studied for genotoxicity and protective effects.

Plant name (Plant part)	Extract Concentration (Extraction Solvent)	Genotoxicity test	Result	Reference
Zingiber officinale (Rhizum)	50 µg/ml (Ethanol)	Comet assay	Antigenotoxic	**(Jayakumar and Kanthimathi, 2012)**
Hibiscus sabdariffa (Fruits)	50, 100, 150 mg/kg (Aqueous)	Micronucleus assay (*in vivo*)	Antigenotoxic	**(Adetutu *et al.*, 2004)**
Hibiscus acetosella (Leaves)	50,100, 200 mg/kg (Ethanol)	Comet assay Micronucleus assay (*in vivo*)	Antigenotoxic	**(Vilela *et al.*, 2018)**
Papaver rhoeas (Flowers)	0.25,0.5, 25mg/ml (Methanol)	Comet assay	Genotoxic at 25mg/ml	**(Hašplová *et al.*, 2011)**
Parquetina nigrescens (Leaves)	1, 5, 10, 20, and 50% (Aqueous)	*Allium cepa* assay	Genotoxic at 10% and 20%	**(Alabi *et al.*, 2022)**
Portulaca oleracea L. (Aerial part)	1 and 2.5 mg/mL (Aqueous)	Comet assay	Antigenotoxic (aqueous extract)	**(Behravan *et al.*, 2011)**
Rosmarinus officinalis (Aerial part)	0.05, 0.1, 0.5, 1 and 2.5mg/mL (Aqueous)	Comet assay	Antigenotoxic (ethanol extract)	**(Razavi-Azarkhiavi *et al.*, 2014)**

I.4.2. Genotoxicity testing

Genotoxicity testing is a safety requirement by regulatory authorities, as many chemicals we're exposed to could create hazardous health problems due to potential genotoxic effects. Genotoxicity tests can be divided into *in vitro* and *in vivo* tests **(Doak *et al.*, 2023**). They are meant to identify genotoxic substances, the level of hazard they cause and predict their potential to induce damage to the genetic material **(Mohamed *et al.*, 2019).** For instance, some tests identify direct genotoxicity which means if the results of chemicals tested come out positive, they are DNA-reactive genotoxins with no safe threshold while other tests can detect indirect genotoxicity by identifying mechanisms other than DNA damage **(Nohmi, 2018**). The majority of genotoxicity testing studies employ an *in vitro* bacterial reverse mutation assay; they commonly add another *in vitro* test on mammalian cells and an *in vivo* assay while in other studies they omit the *in vitro* mammalian cells test. In some cases, additional tests may be used even if the three tests are negative **(Baldrick, 2021 ; Cimino, 2006).**

The Organisation for Economic Co-operation and Development (OECD) is a part of regulatory authorities that published a number of test guidelines (TG) for *in vitro* and *in vivo* test protocols that take into consideration different genotoxicological endpoints **(Azqueta and Dušinská, 2015).** The test guideline titles for genetic toxicology testing published on the OECD library website are mentioned in **Table V**.

Table V: OECD approved TG titles

In vitro	***In vivo***
• Test No. 471: Bacterial Reverse Mutation Test • Test No. 473: *In vitro* Mammalian Chromosomal Aberration Test • Test No. 476: *In vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes • Test No. 487: *In vitro* Mammalian Cell Micronucleus Test • Test No. 490:*In vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	• Test No. 474: Mammalian Erythrocyte Micronucleus Test • Test No. 475: Mammalian Bone Marrow Chromosomal Aberration Test • Test No. 478: Rodent Dominant Lethal Test • Test No. 483: Mammalian Spermatogonial Chromosomal Aberration Test • Test No. 485: Genetic toxicology, Mouse Heritable Translocation Assay • Test No. 486: Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo* • Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays • Test No. 489: *in vivo* Mammalian Alkaline Comet Assay.

I.4.2.1. *In vitro* genotoxicity tests

In vitro genotoxicity tests have the advantage of being quick, easy to reproduce and economic while still being reliable and useful at predicting the genotoxic risk that a substance may cause in whole organisms. They mostly use specific strains of bacteria or mammalian cell lines, peripheral mononuclear blood cell lines or lymphocytes **(Bardoloi and Soren, 2022)**.

These tests aim to assess the potential of chemicals to cause cancer and provide insights into DNA damage mechanisms. They do not definitively confirm carcinogenicity but are vital for understanding the likelihood of a substance inducing tumors or accelerating tumor development. Typically conducted in bacteria and mammalian cells, these assays involve tests for bacterial mutagenesis and mammalian chromosomal aberrations and mutagenesis. Chromosomal aberrations, indicative of carcinogenicity, are evaluated to measure a chemical's clastogenic potential. *in vitro* assays leverage the similarities in DNA across species and offer a cost-effective

and faster alternative to animal testing, aiding in reducing unnecessary animal experimentation **(Stearns 2007)**.

However, *in vitro* tests cannot be regarded as a replacement for the *in vivo* testing that safety assessment strategies rely on. This prompted researchers to improve them and enhance their performance in hopes of limiting animal testing. For example, it's possible to efficiently combine different *in vitro* tests or use other tests that would identify the substances' mechanisms of action in order to reach a conclusion about their genotoxic effects **(Corvi and Madia, 2017)**.

"ToxTracker is a unique *in vitro* genotoxicity assay that combines multiple biomarkers to get mechanistic insight into the MOA of genotoxic compounds. The assay not only includes markers for DNA damage as predictors for the standard genotoxicity assays but also includes markers for non-genotoxic MOA, including oxidative stress, protein misfolding and general cellular stress. All these types of cellular damage are associated with increased cancer risk. The ToxTracker assay showed a very high sensitivity (94%) and specificity (95%) for the detection of genotoxic compounds and has a strong correlation with the regulatory assays. Currently, an international interlaboratory validation study for ToxTracker according to OECD guidelines is ongoing. Seven laboratories with expertise in the field have tested a large selection of compounds. The first results of the study have been shared in March 2022. A publication on the results is currently in preparation"(directly from https://toxys.com/toxtracker/)

I.4.2.2. *In vivo* genotoxicity tests

In vivo tests are used to determine genotoxic effects on DNA and on chromosomes. Unlike *in vitro* tests, *in vivo* ones have the ability to take into consideration absorption, distribution, metabolism and excretion (ADME) processes along with cells' defense mechanisms and DNA repair **(Vugmeyster et al., 2012)**. When positive *in vitro* test results are obtained *in vivo* tests are used to verify them and detail their biological and physiological significance. Some substances that tested negative in *in vitro* tests can also be identified by *in vivo* tests. Thus, *in vivo* test results have more weight in the assessment of risk **(Kang *et al.*, 2013 ; Ren *et al.*, 2017)**. The specific endpoint identified in an *in vitro* test determines the choice of subsequent *in vivo* testing method. For instance, a positive outcome in the bacterial reverse gene mutation test typically warrants the transgenic rodent gene mutation assay as a follow-up. Additionally, in certain cases, an *in vivo* comet assay may also be conducted. Similarly, a positive result in the *in vitro* micronucleus test suggests the *in vivo* equivalent of this assay as the most appropriate follow-up method **(Van Bossuyt *et al.* 2020).**

Different methods and different models are used in *in vivo* testing since the goal is to have a model that is closest to humans. The most common ones are rodents and transgenic rodents **(Mišík *et al.*, 2022)**.

Zebrafish (*Danio rerio*) is a less known model used in *in vivo* tests. It has gained attention as an animal model for genotoxicity testing due to its many advantages: it is small in size making it easy to handle, has high productibility, its cardiovascular nervous and digestive systems are similar to mammals and there is a significant genetic resemblance between zebrafish and humans (around 75% similarity in genomes) **(Chakravarthy *et al.*, 2014; Chakraborty *et al.*, 2016)**.

Onion (*Allium cepa*) is a plant model that is commonly used for *in vivo* genotoxicity testing. The endpoint evaluated to detect genotoxic effects is chromosome aberration. *Allium cepa* is very easy to acquire, quick to grow and needs little maintenance **(Leme and Marin-Morales, 2009).** It also has the advantage of possessing chromosomes that are large in size >15000 Mbp (million base pairs), low in number (2n=16) and share morphological resemblance to mammalian cells **(Alias *et al.*, 2023)**. In addition, they display a rapid rate of root growth **(Marmiroli *et al.*, 2022)**. These roots highlight the effects in experiments as they visibly show abnormalities in their growth if the tested substance is genotoxic: the roots become significantly short in length as their growth is suppressed. When studying samples of these roots under a microscope chromosome abnormalities are observed **(Pakrashi *et al.*, 2014).**

I.4.2.3. Common genotoxicity tests

Multiple studies are carried with various tests to evaluate genotoxicity. However, as observed in bibliographic databases, some tests are employed more often than others. This may be due to common strategies developed by regulating authorities such as the OECD that base genotoxicity assessment on conventional tests that researchers routinely use and rely on for many years **(Türkez *et al.*, 2017)**. The most frequently used methods are presented in **Figure 3**. As observed the 3 most common methods are Ames test, Comet Assay and Micronucleus assay.

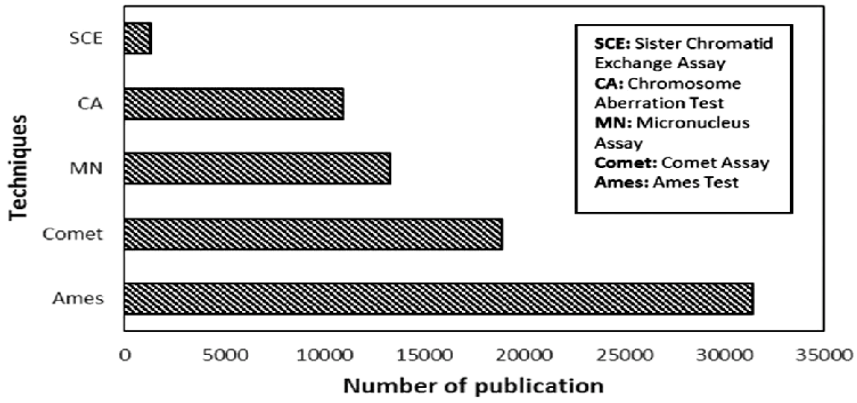

Figure 3: Frequency of some commonly used genotoxicity tests in last decade **(Türkez *et al.*, 2017)**

■ Micronucleus test

The micronucleus test, esteemed for its simplicity, sensitivity, reliability, and promptness in discerning genetic damage caused by chemical agents in a given environment, involves analyzing erythrocytes based on specific criteria, such as micronucleus diameter (ranging between 1/16 and 1/3 that of the principal nucleus), and their refringence and color, which should match those of the principal nucleus. Micronucleus analysis serves as a crucial marker for environmental biomonitoring, aiding in evaluating the initial effects of chronic exposure to xenobiotic compounds in the target species, whether in laboratory or field settings. Micronuclei (MNi), formed during cell division (anaphase) when pieces or entire chromosomes are lost, reflect various types of chromosome damage that lead to an increased amount of incorporated chromosomes in the cell which are covered with a nuclear envelope forming a small sized nucleus. These chromosome damages arise through mechanisms including chromatid or chromosome breakage, aberrations in anaphasic bridges, spindle malfunctions, or dysfunctions in chromosome-division-related organelles like kinetochores, as well as apoptosis. This method (**Figure 4**) is used in both *in vivo* and *in vitro* conditions and different types of cells and tissues are employed mainly: blood cells, bone marrow, liver tissue and lung tissue. The *in vitro* micronucleus test, preferred for investigating chromosome damaging potential, relies on detecting micronuclei, and a positive outcome may necessitate an *in vivo* follow-up study. Buccal micronucleus assessment, frequently employed in genetics, offers a minimally invasive means to gauge genomic damage, chromosome instability, and cell death, finding utility in studies concerning occupational and environmental genotoxin exposure, medical procedures, micronutrient deficiencies, lifestyle factors, pesticide exposure, genetic susceptibility, and polymorphisms of xenobiotic-metabolizing and DNA repair genes contributing to DNA damage **(Kang *et al.*, 2013 ; Benvindo-Souza *et al.*, 2020; Leonardi *et al.*, 2020; Van Bossuyt *et al.*, 2020).**

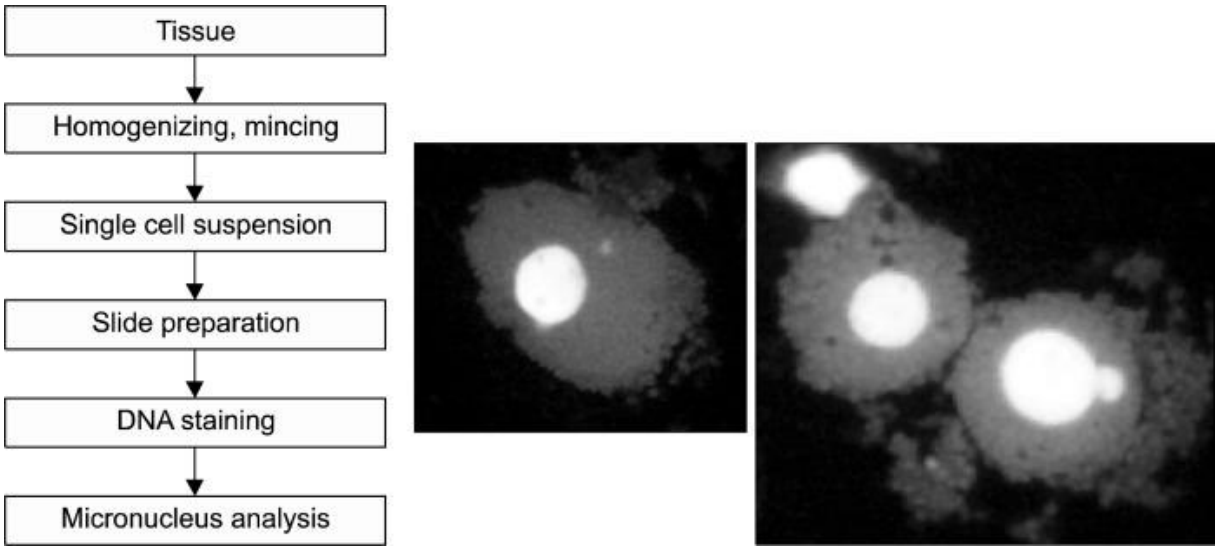

Figure 4: Scheme showing main procedure of *in vivo* micronucleus assay **(Kang *et al.*, 2013).**

Chapter II
Materials and methods

II.1. Plant material

II.1.1. Plant harvest

Centaurea calcitrapa (**Figure 5**) was harvested on April, 2023. The leaves were separated oven dried and grinded into a fine powder < 63µm.

Figure 5. Photographs of *Centaurea calcitrapa* (A) Leaves, (B) Aerial part.

II.1.2. Extraction

50 g of *Centaurea calcitrapa* leaf powder was macerated in 500 mL of a hydroethanolic solvent (Ethanol/Water; 80; 20; v/v). The mixture was agitated by a magnetic stirrer at 650 rpm for 24h at room temperature. The mixture was left to decant in a cylinder for 12h then the supernatant was poured into a flask. The maceration was repeated 4 times and the obtained supernatants were mixed and centrifuged for further purification at 3000 rpm for 10 min to get the final liquid extract (**Figure 6**) which was then evaporated.

Figure 6 : The resulted liquid extract after centrifugation.

The liquid extract was evaporated with a rotary vacuum evaporator (**Figure 7**) at 45°C and 135-139 rpm until the volume was significantly reduced. The remaining liquid was poured into labelled Petri dishes (empty weight) and kept in the oven at 40°C untill completely dried. The Petri dishes with the dried extract were then reweighed and stored in the fridge until further use.

Figure 7 : Evaportaing the ethanol from the extract using the rotary vacuum evaporator

The extraction yield is calculated using the equation used by **(Janaćković *et al.*, 2019)**.

Yield (%) = (Dried weight of extract x 100)/Dried weight plant material

II.2. Biochemical analysis of the extract

II.2.1. Determination of total phenolic compounds

Phenolic compounds are common metabolites found amongst plants. Studies have shown that regular consumption of foods with higher phenolic content leads to many health benefits and prevents chronic diseases **(Farasat *et al.*, 2014)**. To determine the total phenolic compounds (TPC) we used the Folin-Ciocalteu assay described by **(Singleton *et al.*, 1999)** with slight modifications.

The Folin-Ciocalteu assay is a spectrophotometric method that consists on the reduction of Folin-Ciocalteu reagent in the presence of phenolic compounds producing a blue colour complex that is measured by a spectrophotometer at 760 nm. The intensity of the blue colour increases proportionally with TPCs concentration **(Malta and Liu, 2014)**.

10 mg of dried extract were dissolved in 1mL of methanol with an ultrasonic bath and diluted to 1 mg/mL with ethanol. 200 µL of the diluted extract were mixed with 800 µL of 3.75% sodium carbonate (Na_2CO_3) and 1 mL of Folin-Ciocalteu reagent (1N). A blank was prepared by replacing the sample with ethanol. After 30 min of incubation at room temperature in a dark place the absorbances were measured at 760 nm.

The standard curve is done using different concentrations of gallic acid as a reference molecule following the same steps as the extract. We repeated all the concentrations 3 times and calculated the extract concentrations as gallic acid equivalent (GAE) using the gallic acid standard curve equation. The results are expressed in mg GAE/g of dried extract.

II.2.2. Determination of flavonoids

Flavonoids are vital natural bioactive compounds. The total flavonoid content (TFC) is commonly measured using the aluminum chloride colorimetric assay, which assumes a uniform response from all flavonoids when compared against a flavonoid standard. This quantification method relies on the specific flavonoid standard used, with the presence of flavonoids indicated by a yellowish color **(Shraim *et al.*, 2021)**.

The contents of flavonoids were determined by the method from **(Maksimović *et al.*, 2005)** with some small modifications. We mixed 1 mL of *Centaurea calcitrapa* extract solution with 0.5 mL of the reagent (133 mg crystalline aluminium chloride and 400 mg crystalline sodium acetate were dissolved in distilled water) and absorbances were recorded at 430 nm against the blank (1 mL of extract solution plus 0.5 mL of distilled water). The amount of flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions, and expressed as mg QE/g of dried extract).

II.3. *In vivo* toxicity testing

II.3.1. Animal model housing and experimental conditions

60 adult NMRI (Naval Medical Research Institute) male mice (6-8 weeks) were obtained from Pasteur Institute, Kouba, Algeria. They were housed in regular solid bottom cages under conventional conditions and had wood shavings as bedding. After a 15-day adaptation period in the animal facility, each mouse was identified by a specific motif on its tail using a marker (**Figure 8**) and each cage was labelled on the outside with its corresponding group number to easily identify them. The mice were given a standard diet (National Office of Livestock Feed, ONAB El Kseur) and regular tap water which were monitored throughout the experiment for any change in consumption after treatment.

The mice were randomly separated into 20 groups each group containing 3 mice. The groups were divided into two categories one for acute (A) toxicity and one for sub-chronic (SC) toxicity. Each category had one positive control group (C+), one control group (C-) four groups treated with extracts only (C1,C2,C3,C4) and four groups treated with the extract and a common mutagen (C1+,C2+,C3+,C4+). The three mice in each acute group were identified with the following motifs: (S*, S**, S***) and in each sub-chronic group with the following: (S, S-, S--). The toxicity tests were conducted by following the OECD 420 guideline.

Figure 8 : Identification of mice by marking

II.3.2. Acute test

Prior to treatment, the mice were fasted for 4 hours then weighed to determine the volume of treatment to administer. 0.8% carboxy methyl cellulose (CMC) was administered to the control groups (C- and C+) by gastric gavage along with an intraperitoneal (IP) injection of 0.9% NaCl for the negative control (C-) and cyclophosphamide (CP) prepared in a sterile environment for the positive control (C+). In labelled conical tubes four concentrations of *Centaurea calcitrapa* extracts: 250 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg were prepared by weighing the respective amounts of the dried extracts and dissolving them in CMC using an ultrasonic bath. The extracts were also administered by gastric gavage using a gavage needle along with an IP injection of 0.9% NaCl for the groups (C1,C2, C3, C4) and an IP injection of CP for the groups (C1+,C2+,C3+,C4+). 24h after the treatments the mice were anaesthetized with chloroform and sacrificed.

II.3.3. Sub-chronic test

The sub-chronic test was conducted in a similar manner as the acute test. The mice were fasted for 4 hours and weighed to determine the volume of treatment to administer prior to each treatment. The mice of C+ and C- were administered 0.8% CMC by gastric gavage and the groups (C1,C2, C3, C4) and (C1+,C2+,C3+,C4+) were administered *Centaurea calcitrapa* extracts dissolved in CMC in the following concentrations respectively 250 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg. The gastric gavage was done for 15 consecutive days. On the

15thday, the groups C- and (C1,C2, C3, C4) were administered 0.9% NaCl by IP injections and the groups C+ and (C1+,C2+,C3+,C4+) were administered CP by IP injections. 24 hours after treatment with NaCl/CP the mice were anaesthetized with chloroform and sacrificed.

II.3.4. Biosamples collection

- **Serum samples**

Blood samples were taken from each mouse using disposable surgical blades. The blood was collected in 1.5 mL labelled eppendorf tubes. The blood samples were left to sit in the fridge to separate the serums which were then pipetted in other labelled eppendorf tubes. The serum samples were kept in the freezer and later centrifuged at 3000 rpm for 10 minutes to eliminate any remaining cells. The serum samples were kept in the freezer for later use.

- **Viscera collection**

Each mouse was dissected and their vital organs (liver, heart, intestines, stomach, spleen, lungs and kidneys) were removed using dissection tools. The stomach and intestines were cleaned from any remaining food and the gallbladders were removed from the livers. Each organ was cleaned in chilled 0.9% NaCl solutions, weighed individually and a small piece of each was cut to preserve in 10% neutral formalin buffer in 5 mL labelled tubes and the rest was put in zip bags labelled for each mouse. The zip bags were put on ice to keep the organs cold during manipulation and preserved in the freezer for later use.

- **Bone marrow collection**

The thighs of each mouse was cut and the femur bones were removed. One side of each bone was cut and the inside was rinsed into 15 mL labelled conical tubes using a 5 mL syringe filled with 4 mL of Hanks' Balanced Salt Solution (HBSS) pre-incubated in the lab oven at 37°C.

II.4. Biological activities

II.4.1. Ferric Reducing ability of plasma (FRAP)

The FRAP assay is a colorimetric method used to assess antioxidant power. At a low pH, antioxidants present in tested samples reduce ferric-tripyridyltriazine (Fe^{III}-TPTZ) to form ferrous-tripyridyltriazine (Fe^{II}-TPTZ) producing a blue coloured solution that is measured at at 595nm **(Benzie and Strain, 1996)**.

The FRAP reagent was prepared with the method described by **(Benzie and Strain, 1996)**. An acetate buffer (300 mM, pH 3.6) was prepared by dissolving 8.16 g of sodium acetate in 200 mL of distilled water and adjusting the solution's pH with glacial acetic acid and HCl. 40 mM of HCl by diluting 0.07 mL of 35% HCl in 19.93 mL of distilled water. Tripyridyltriazine (10 mM) by dissolving 62.5 mg TPTZ in 20 mL of 40 mM HCl. Ferric chloride (20 mM) by dissolving 108.1 mg ferric chloride hexahydrate in 20 mL of distilled water. The FRAP reagent was

prepared by mixing all the prepared solutions and incubated in the lab oven at 37°C. In eppendorf tubes, 30 µL of serum sample and 1 mL of FRAP reagent were mixed and incubated at 37°C for 8 minutes, the absorbance was measured at 595 nm. The concentrations were done in triplicates. The absorbances were plotted against a FeSO4 standard curve. The results are expressed in µM FeSO4.

II.4.2. Catalase activity

Catalase is an enzyme that breaks down two hydrogen peroxyde (H_2O_2) into oxygen (O_2) and water (H_2O). The kinetics assay is a spectrophotometric method that is based on measuring the decrease in absorbance of hydrogen peroxide as it gets decomposed by catalase **(Li and Schellhorn, 2007)**.

A phosphate buffer (0.1 M pH 6.8) was prepared with K_2HPO_4/KH_2PO_4. An H_2O_2 solution by diluting H_2O_2 in distilled water. In a quartz cuvette 350 µL of the buffer were mixed with 1600 µL of the H_2O_2 solution and 50 µL of liver homogenate sample were added and mixed thoroughly to start the reaction. The reaction was followed by using a spectrophotometer and the UVProbe software for 60 seconds at 240 nm.

- **Determination of protein content**

In order to calculate the catalase activity, the protein concentration in liver samples was measured with a modified Bradford method **(Bradford, 1976)**.

10% (w/v) liver homogenates were prepared by adding 1 mL of cold phosphate buffer (PB) (0.1 M, pH7.4) to 100 mg of liver of each mouse in eppendorf tubes and homogenizing with a sonicator. The obtained homogenates were centrifuged at 10 000 g for 10 min.

The Bradford method uses Coomassie Brilliant Blue G-250 dye, which changes colour from red/brown to blue when it binds to specific amino acids in proteins **(Miranda, 2024)**. Firstly, we prepared a stock solution by mixing 20 µL of liver homogenate supernatant with 380 µL of PB, and from this mixture, 20 µL was taken and mixed with 80 µL of PB. The Bradford reagent was then prepared using 100 mg of Coomassie Brilliant Blue G-250, 50 mL of 50% ethanol, 100 mL of 85% phosphoric acid, and 850 mL of distilled water. For sample preparation, 100 µL of the prepared supernatant were mixed with 1 mL of the Bradford reagent, then incubated it for 5 minutes. Finally, the absorbance was measured at 595 nm against a blank consisting of 1 mL of Bradford reagent and 100 µL of PBS, and protein concentrations were determined using a BSA standard calibration curve. The results are expressed in BSA equivalent (BSA Eq).

II.4.3. Genotoxicity test (*In vivo* Micronuleus test)

The collected tubes of bone marrow were centrifuged at 1000 rpm for 10 minutes. The pellets of the centrifuged tubes were used to prepare smears on slides that were labelled for each mouse. The smears were done in triplicates. After air drying the slides overnight, they were fixed with methanol absolute for 10 minutes. The fixed slides were stained using May Grunwald first for 2 minutes and Giemsa for 10 minutes then rinsed with distilled water for 2 minutes. One slide per group was observed under a microscope using the 100x objective lens with immersion oil.

II.5. Statistical analysis

The results are expressed as mean ± SD or SEM. The data were subjected to one-way analysis of variance (ANOVA) and used Dunnett's Multiple Comparison Test and Tukey's Multiple Comparison Test using GraphPad Prism 5 program. $P < 0,05$ was considered significant.

Chapter III
Results and discussion

III.1- Extraction yield

Centaurea calcitrapa leaf extract was prepared with ethanol: water (80:20) using four successive macerations with no heat. The resulting dried extract (**Figure 9**) is dark green in color and has a flaky texture.

Figure 9: Dried hydroethanolic extract of *Centaurea calcitrapa*

The yield obtained after calculating the dried extract is 25.46 %. An 80% ethanol solvent was used to extract *Centaurea calictrapa* leaves through four successive macerations, yielding a result of 25.46%. Our result is in agreement with a study by **Dimkić et al.(2020)** that reported a similar yield of 23.3% using a 70% ethanol solvent. Their study also suggested that water-alcohol mixture solvents extract the most metabolites. The high yield obtained could also be explained by the prolonged submersion of our plant material in the solvent during the four repeated macerations along with the controlled temperature conditions when drying the leaves and in the process of eliminating the solvent, as high uncontrolled heat degrades metabolites. Our finding suggests that our plant extract is rich in metabolites.

III.2. Phytochemical investigation of *Centaurea calcitrapa* extract

III.2.1. Results of TPC and TFC determination

The TPC was determined by the Folin-Ciocalteu method using gallic acid as a reference and calculated using the gallic acid standard curve equation ($y = 6.390x – 0.317$, $R^2 = 0.968$) (Annex 1) while the TFC was determined with the aluminium chloride colometric assay using quercitin as a reference and calculated with the quercitin standard curve equation ($y= 31.15x –$

0.163 ,R^2 = 0.977) (Annex 2). Our extract demonstrated a TPC of 487.536 mg GAE/g of dried extract and a TFC of 29.146 mg QE/g of dried extract (**Table VI**).

Surprisingly, our finding of the TPC is very high compared to a study by **Aboul-Soud et al.(2022)** reporting a TPC of 44.72 mg GAE/g of dry weight. However, they only used ethanol as a solvent which might explain the difference in content as water can extract the metabolites that are more polar **(Abubakar and Haque, 2020)**. In comparison with the same study, our finding of the TFC is higher but still in line with their result of 22.65 mg QE/g of dry weight. The high presence of polyphenols and flavonoids in *Centaurea calcitrapa* is also in agreement with the literature as many of these compounds were identified such as the following flavonoids: kaempferol, luteolin, quercitin, rutin and apigenin **(Formisano *et al.*, 2012)**. Our results indicate the potential antioxidant proprieties of our extract associated with high polyphenols and flavonoids content.

Table VI: Total polyphenol and flavonoid content on *Centaurea calcitrapa* extract. The data is presented as mean ± SD of triplicate measures.

Total polyphenol content mg GAE/g of dried extract	**Total flavonoid content mg QE/g of dried extract**
487.536 ± 5.131	29.146 ± 0.343

III.3. *In vivo* toxicity investigation of *Centaurea calcitrapa* crude extract

III.3.1. Behaviour changes of mice

The mice of the acute test were observed for 4 hours after treatments, particular attention was directed to any change in their moving ability, energy level and anxiety symptoms such as tremors which are visible signs of toxicity **(Nigatu *et al.*, 2017)**. We observed no changes in behaviour and no signs of toxicity in all the treated groups compared to the control groups.

An average of 2 hours every day was spent with the mice of the sub-chronic test, we also observed them for any toxicity signs. We noticed that some mice exhibited hyperactivity and climbing. Hyperactivity is reported as a possible toxicity symptom in a study by **Almança et al. (2011)**. However, in our study, this sign was also observed in the negative control groups suggesting that it was not caused by the treatment.

III.3.2. Body weights, food and water consumptions

In the acute group, no significant changes in water consumption, food consumption, and body weight (b.w) were observed, indicating that the short-term administration of the test substance did not impact these parameters. Similarly, in the sub-chronic group, there were no significant differences between the test groups and the control group. This demonstrates that the plant extract (*Centaurea calcitrapa*) do not influence body weight, water, and food consumption over both short and long periods. The findings are summarized in **Table VII** below, which provides a detailed comparison of the measured parameters across the different groups.

Table VII: Results showing the changes of body weight, water and food consumption across of mice test groups

Group	Treatment	Δ Body weight(g)	Water consumption(ml)	Food consumption(g)
C-	CMC (0.8%) + NaCl (0.9 %)	2.33	30	28.925
C+	Cyclophosphamide (50 mg /kg b.w)	3.33	30	43.363
C1	*C. calcitrapa* extract (250 mg /kg b.w)	1.00	30	52.225
C2	*C. calcitrapa* extract (500 mg /kg b.w)	-1.33	25	18.288
C3	*C. calcitrapa* extract (1000 mg /kg b.w)	0.67	32.5	43.375
C4	*C. calcitrapa* extract (2000 mg /kg b.w)	-0.50	27.5	73.013
C1 +	*C. calcitrapa* extract (250 mg /kg b.w) + CP (50 mg /kg b.w)	-0.33	5	30.575
C2+	*C. calcitrapa* extract (500 mg /kg b.w) + CP (50 mg /kg b.w)	1.50	15	18.313
C3+	*C. calcitrapa* extract (1000 mg /kg b.w) + CP (50 mg /kg b.w)	0.33	15	53.6
C4+	*C. calcitrapa* extract (2000 mg /kg b.w) + CP (50 mg /kg b.w)	2.33	10	24.925

III.3. Viscera organs

III.3.1. Macroscopic observations

The visceral organs of the acute and sub-chronic groups were analysed under a macroscope to determine if there were any changes in the test groups that might indicate toxicity. However, no significant changes were observed compared to the control groups. Some of the visceral organs

from different groups are shown below in **Figure 10** which, upon observation, are all similar and show no differences.

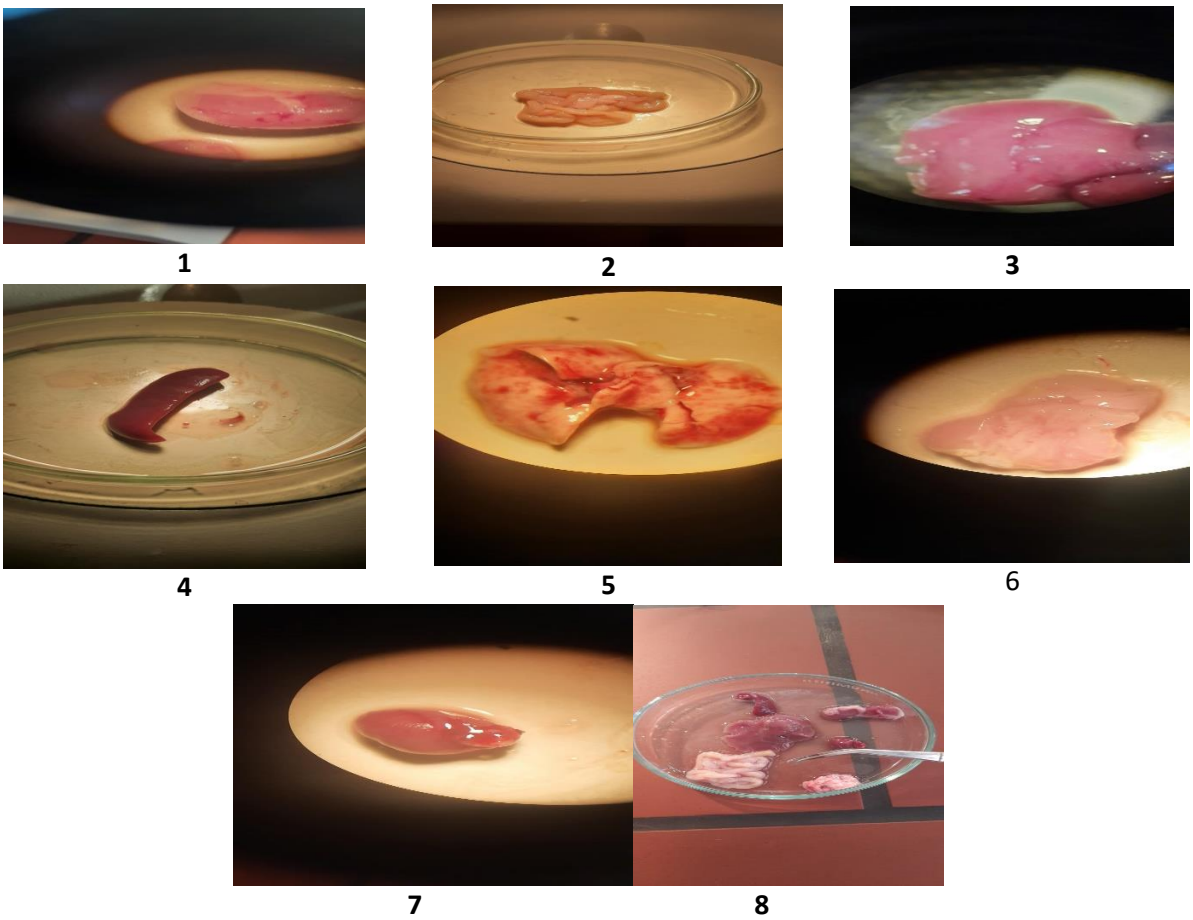

Figure 10: Illustrative Original photos of macroscopic aspect of some collected viscera**.** 1.kidney, 2. Intestines, 3. Liver, 4. Spleen, 5. Lungs, 6. Stomach, 7. Heart, 8. all organs.

III.3.2. Organ weight changes

The liver, heart, intestine, stomach, spleen, lungs, and kidneys of each mouse were weighed after dissection to assess the presence of organ-specific toxicity related to changes (increase/decrease) in organ sizes. The change is expressed in (organ/body) weights for each group.

The results of the organ weight changes in acute groups (**Figure 11**) show no significant increase or decrease in organ weights of all the treated groups except for the treatment groups C2 at 0.032556 ± 0.001424 and C2+ at 0.031858 ± 0.005486 which were treated with the extract at 500mg/kg b.w that showed a significant decrease in the weight of intestines compared to the positive control at 0.055145 ± 0.001789. This particular observation could be due to the variability in length of intestines, technical errors during dissecting and cutting may have led to this particular observation.

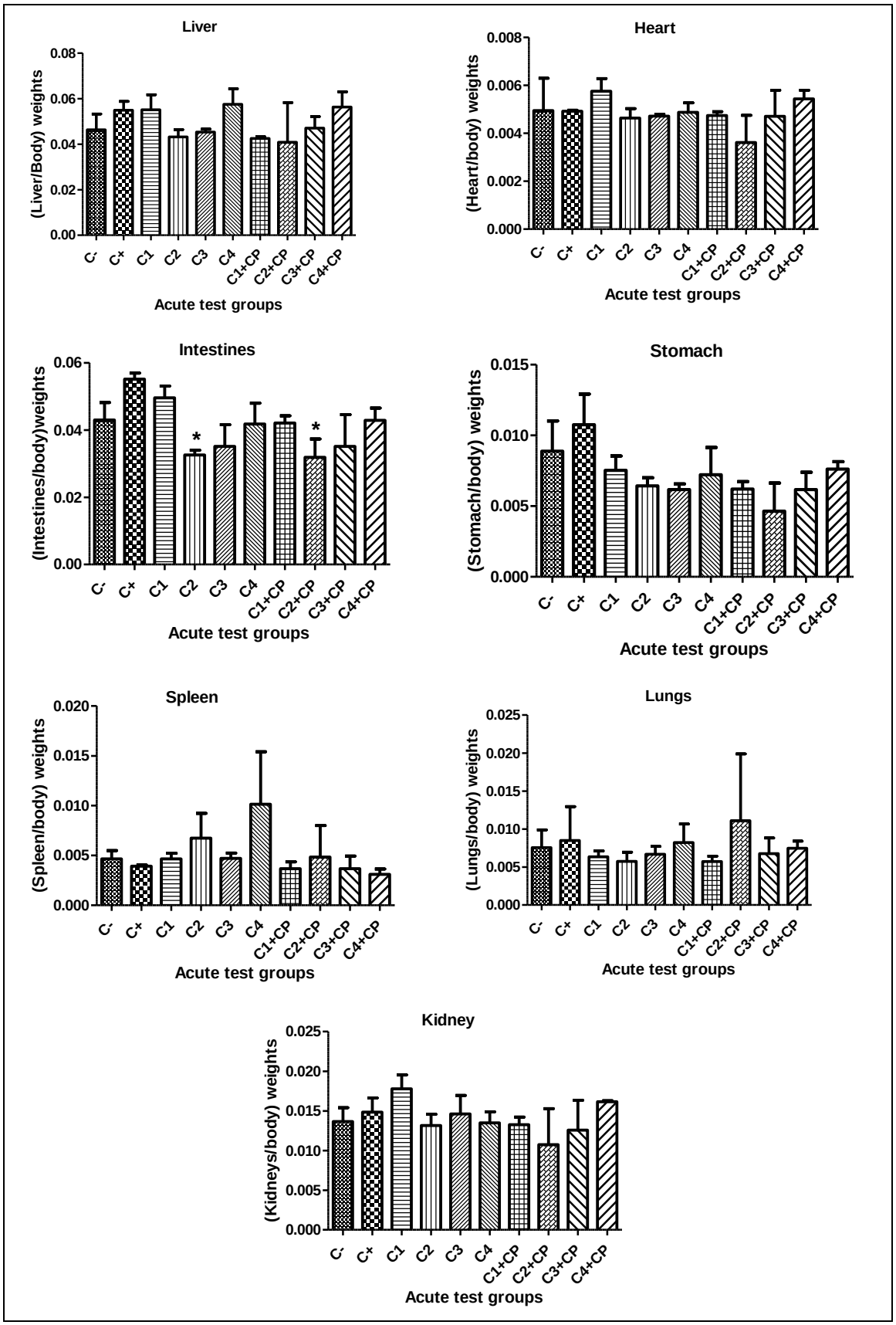

Figure 11: The changes of organ weights expressed in (organ/body) weights of acute test groups. Values are expressed in mean ± SD (n= 2-3). Comparisons are made with C+. C- : saline 0.9% C+ : CP 50 mg/kg b.w C1 :250 mg/kg b.w C2 :500 mg/kg b.w C3 : 1000 mg/kg b.w C4 : 2000 mg/kg b.w.

Our results, for organ size changes in sub-chronic test groups (**Figure 12**), show that all concentrations of the *C. calcitrapa* extract in all treatment groups have no significant effect on organ weight changes compared to the control groups, except for the groups treated with the second concentration (500 mg/kg b.w), which show a significant decrease in intestine size. The intestines' size for the groups C2 was 0.035116 ± 0.000688 and C2+ was 0.030955 ± 0.000020, compared to the positive control group at 0.041111 ± 0.000544 and the negative control group at 0.043148 ± 0.001356. This might suggest that *C.calcitrapa* hydroethanolic extract at 500 mg/ kg b.w can reduce intestinal size and might be useful in relieving enlarged intestines in mice which is in agreement with the therapeutic effects of STLs that are found in this plant and were found to relieve gastrointestinal distress and inflammation **(Heinrich *et al.*, 1998)**. Taking in consideration all the results, our findings suggest that *C.calcitrapa* hydroethanolic extract does not cause toxicity related to organs in mice.

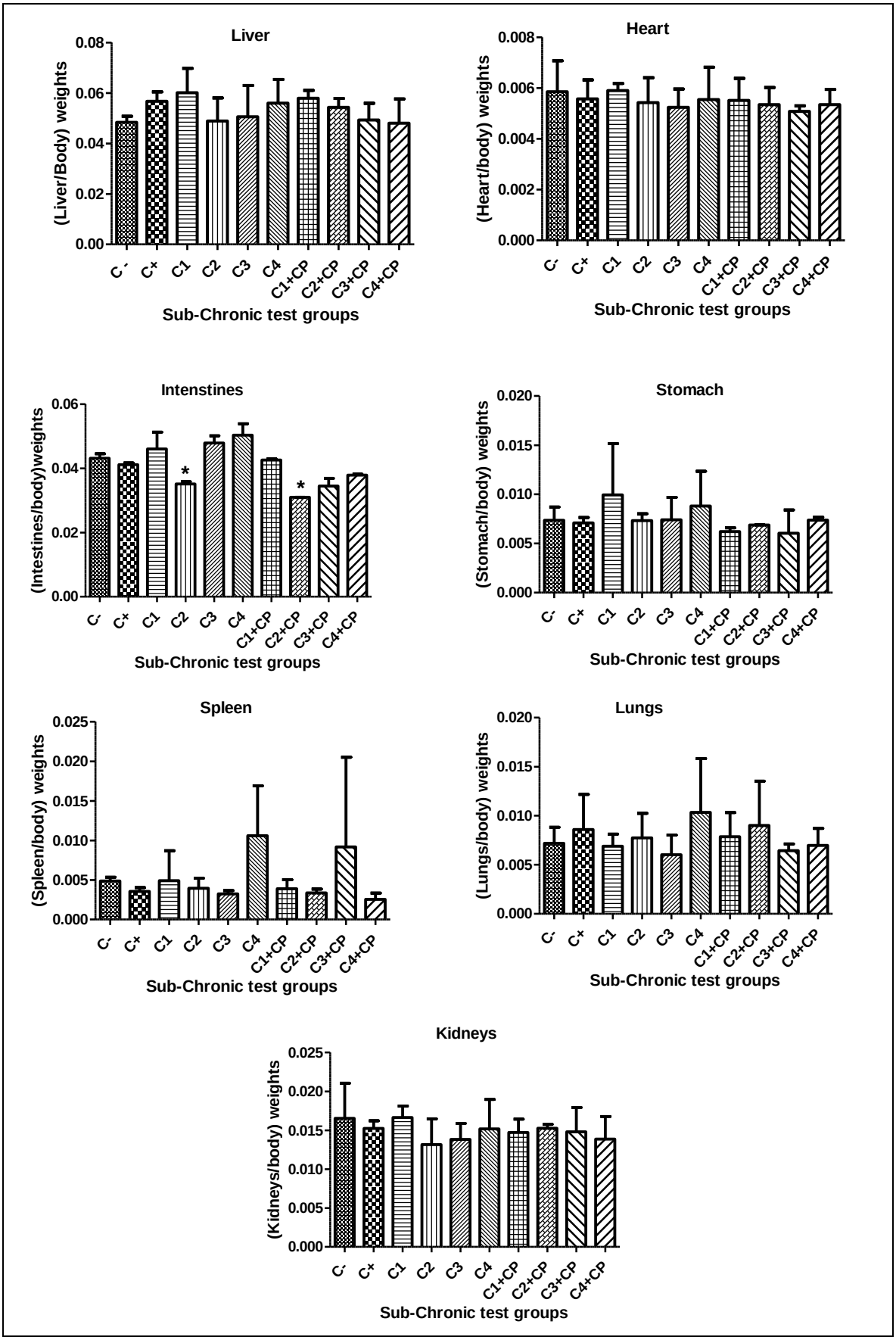

Figure 12: The changes of organ weights expressed in organ/body weights of sub-chronic groups. Values are mean± SD (n= 2-3). Comparisons are made with C- and C+ groups. C- : saline 0.9% C+ : CP 50 mg/kg b.w C1 :250 mg/kg b.w C2 :500 mg/kg b.w C3 : 1000 mg/kg b.w C4 : 2000 mg/kg b.w.

III.3.3. Ferric reducing ability of plasma (FRAP) results

The antioxidant capacity of plasma was measured using the FRAP assay with a FeSO4 standard curve (y= 0.7382x + 0.0375) (Annex 3). No significant changes were found in the treatment groups compared to the control groups (**Figure 13**). Unexpectedly, no significant change was observed between the negative and positive groups in both the acute and sub-chronic tests. According to literature research, no studies have been conducted on *C. calcitrapa's* antioxidant abilities in vivo using the FRAP assay employed, making it impossible to compare these results with other studies. These results could be explained by the lack of bioavailability of the extract's antioxidant substances. The extract is rich in polyphenols and flavonoids, which have powerful antioxidant abilities in vitro. However, studies have shown that these compounds get degraded by the intestinal microflora and are poorly absorbed **(Fernández-Ochoa et al., 2022)**. The mice were treated by gastric gavage, suggesting that the antioxidants in the extract may have been largely degraded and unable to produce a significant effect in the FRAP assay. These findings suggest that *C. calcitrapa* hydroethanolic extract has a very small influence on the induction and reduction of oxidative stress in mice.

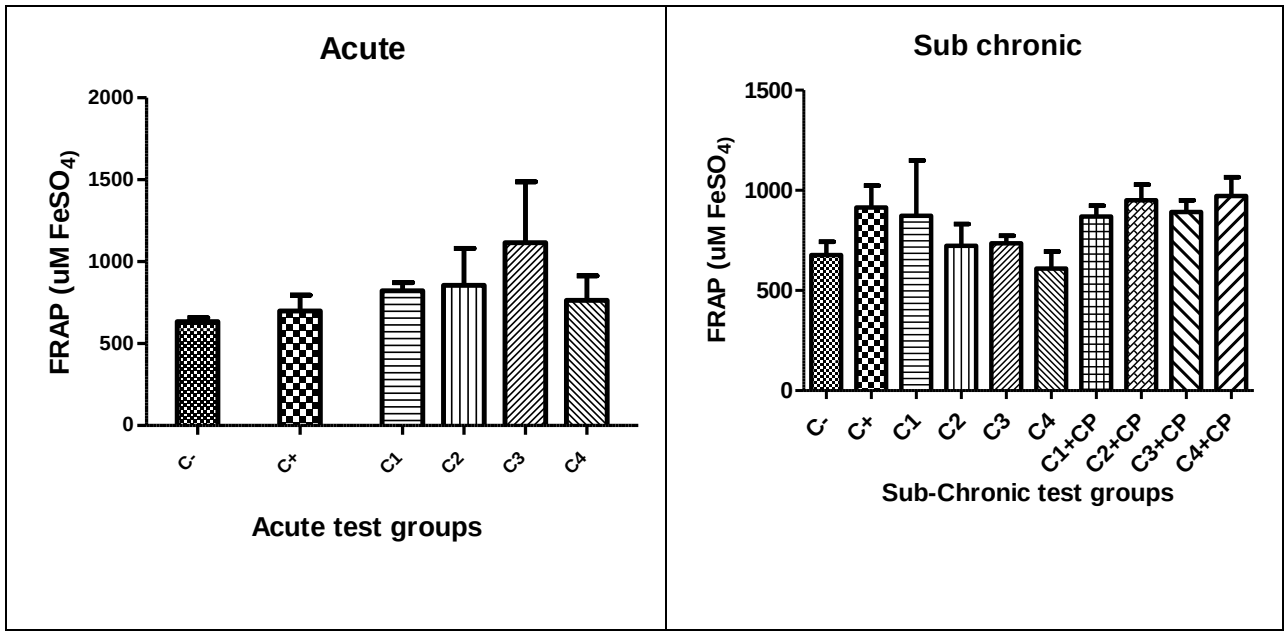

Figure 13: Ferric reducing ability of plasma in acute and sub-chronic test groups. Values are expressed in mean mean ± SEM (n= 2-3). C- (saline 0.9%) C+ (CP 50 mg/kg b.w). C1 :250 mg/kg b.w C2 :500 mg/kg b.w C3 : 1000 mg/kg b.w C4 : 2000 mg/kg b.w.

III.3.4. Catalase activity results

The catalase activity was determined by a kinetic method following the degradation of H2O2, and the results were expressed in [H2O2] mM/mg protein BSA E. In the acute test groups, treatment with extracts only (E) (**Figure 14**) at concentrations of 250, 500, and 2000 mg/kg b.w

significantly enhanced catalase activity compared to the positive control, suggesting they did not induce oxidative stress. It was also noted that the group treated with 1000 mg/kg b.w appeared similar to the positive control, although not significantly, suggesting it could have caused oxidative stress. In addition, the groups treated with extract and CP (E+CP) at higher concentrations (500, 1000 and 2000 mg/kg b.w) also showed highly significant and very highly significant increases in catalase activity compared to the positive control. The concentration of 500mg/ kg b.w in particular has shown very high significant increases in both treatment groups (C2 and C2+CP). These findings suggest that acute administration of *C.calcitrapa* extract does not induce oxidative stress and prevents it at higher concentrations possibly by preventing reactive oxygen species **(Barzilai and Yamamoto, 2004)**.

In the sub-chronic test groups, treatment with extracts only (E) at concentrations of 250 and 500 mg/kg b.w significantly enhanced catalase activity compared to the positive control, while treatment with the concentration of 1000 mg/kg b.w significantly decreased catalase activity compared to the positive control. It was noted that the lowest concentration of 250 mg/kg b.w became similar to the negative control when administered regularly (15 days).

Surprisingly, the mentioned concentrations (250, 500 and 1000 mg/kg b.w) exhibited the opposite effects in the groups E+ CP compared to groups treated with extract only. A sudden significant decrease in catalase activity was observed with the concentration of 250 mg/kg b.w and a very significant decrease with 500 mg/kg b.w compared to the positive group, suggesting that the CP effect was higher. In contrast, concentration 1000 mg/ kg b.w shows very high significant increase in catalase activity compared to the positive control and seems to be similar to the negative control suggesting that repeated administration of concentration 1000 mg/kg b.w prevents oxidative stress.

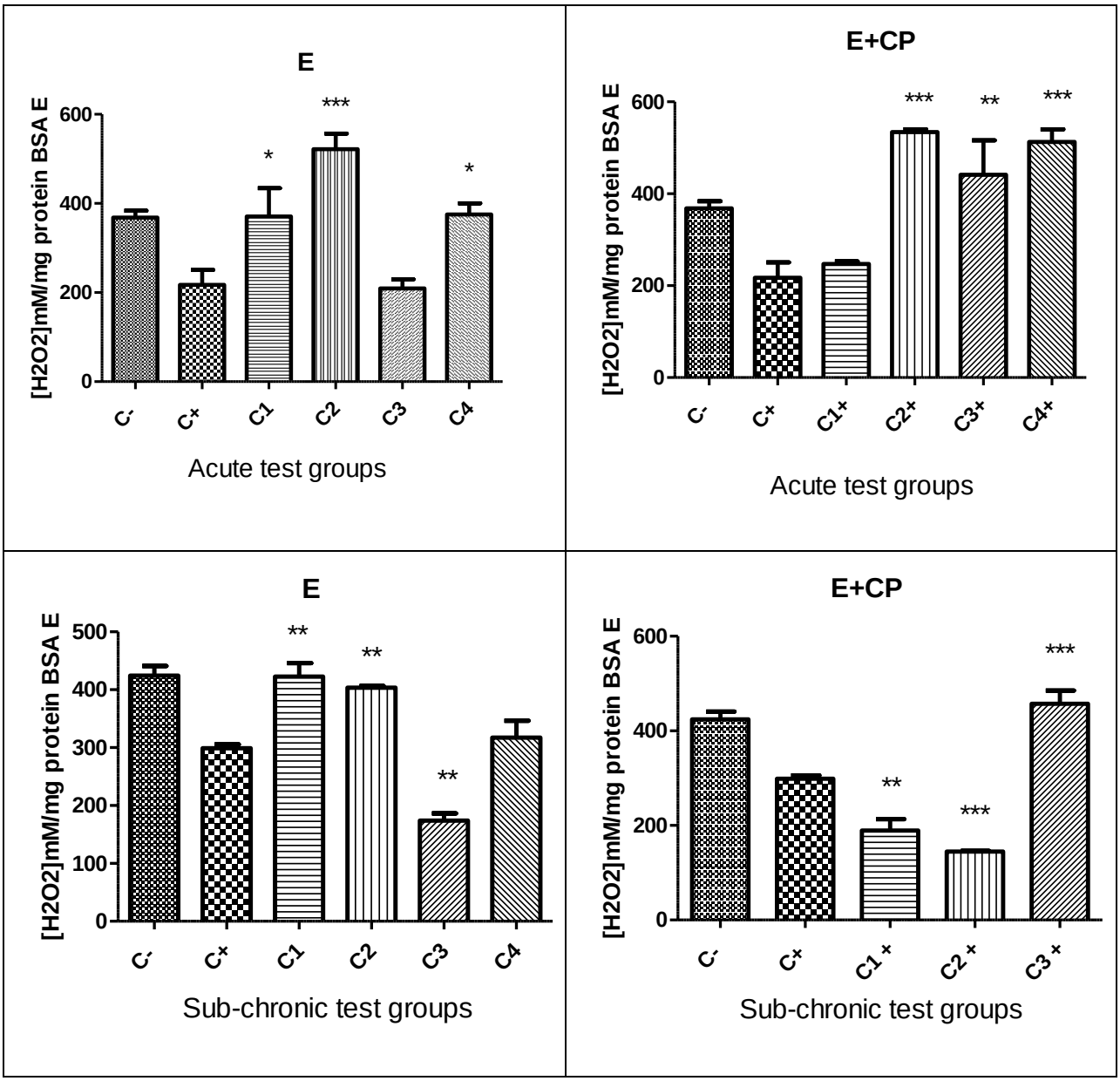

Figure 14: Catalase activity results in acute and sub-chronic groups treated with extract only (E), extract with CP (E+CP). Values are expressed in mean mean ± SEM (n= 3). Comparisons are made with C+. C- : saline 0.9% C+ : CP 50 mg /kg b.w C1 :250 mg/kg b.w C2 :500 mg/kg b.w C3 : 1000 mg/kg b.w C4 : 2000 mg/kg b.w. C4+ (Sub-chronic) not shown due to technical errors.

III.3.4. Genotoxicity results

An *in vivo* micronucleus test was conducted to observe micronuclei formation and tissular organization (**Figure 15**) induced by treatments in bone marrow. The microscopic observations are summarized in **Table VIII.**

Table VIII : Summary of microscopic observations of acute and sub-chronic toxicity studied groups.

Group	Received treatment	Bone Marrow Tissu	Abundance of micronuclei
Acute toxicity groups			
C-	CMC 0.8 %	Normal cells and tissu organization	Rare (-)
C+	CMC 0.8% + CP (50mg/kg b.w.)	Disturbed Cells division	Frequent (++)
C1	*C.calcitrapa* extract (250 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C2	*C.calcitrapa* extract (500 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C3	*C.calcitrapa* extract (1000 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C4	*C.calcitrapa* extract (2000 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C1+	*C.calcitrapa* extract (250 mg/kg b.w) + CP (50mg/kg b.w.)	Slightly disturbed Cells division	Rare (+)
C2+	*C.calcitrapa* extract (500 mg/kg b.w) + CP (50mg/kg b.w.)	Slightly Disturbed Cells division	Rare (+)
C3+	*C.calcitrapa* extract (1000 mg/kg b.w) + CP (50mg/kg b.w.)	Slightly Disturbed Cells division	Rare (+)
C4+	*C.calcitrapa* extract (2000 mg/kg b.w) + CP (50mg/kg b.w.)	Slightly Disturbed Cells division	Rare (+)
Sub chronic toxicity groups			
C-	CMC 0.8 %	Normal cells and tissu organization	Rare (-)
C+	CMC 0.8% +CP (50mg/kg b.w.)	Disturbed Cells division and tissu organization	Frequent (++)
C1	*C.calcitrapa* extract (250 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C2	*C.calcitrapa* extract (500 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C3	*C.calcitrapa* extract (1000 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C4	*C.calcitrapa* extract (2000 mg/kg b.w)	Normal cells and tissu organization	Rare (-)
C1+	*C.calcitrapa* extract (250 mg/kg b.w) + CP (50mg/kg b.w.)	Disturbed Cells division and tissu organization	/
C2+	*C.calcitrapa* extract (500 mg/kg b.w) + CP (50mg/kg b.w.)	Disturbed Cells division and tissu organization	/
C3+	*C.calcitrapa* extract (1000 mg/kg b.w) + CP (50mg/kg b.w.)	Disturbed Cells division and tissu organization	/
C4+	*C.calcitrapa* extract (2000 mg/kg b.w) + CP (50mg/kg b.w.)	Disturbed Cells division and tissu organization	/

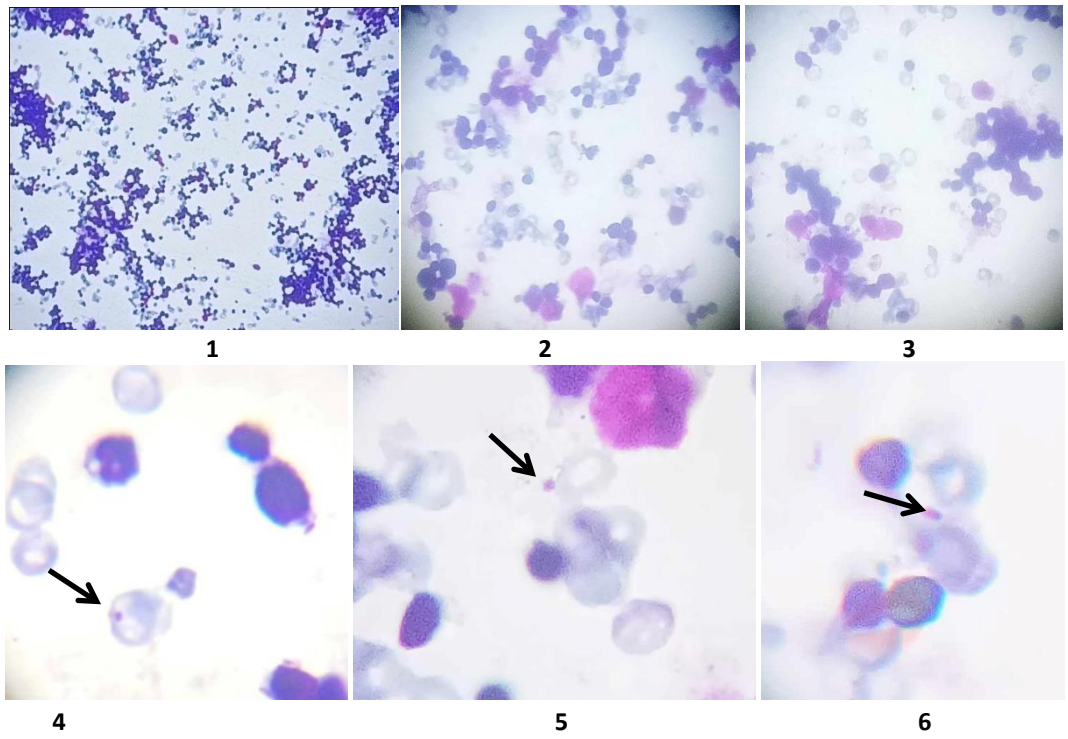

Figure 15 : Illustrative photos of microscopic observations of the tissular organization (1,2,3) and the micronulei (4, 5, 6) in the bone marrow smears.

Through observations, it was noticed that cyclophosphamide disturbs bone marrow tissues and cell divisions and induces micronuclei formation (C+) compared to the negative control group. The C.*calcitrapa* extract showed no genotoxic effects compared to the control groups in both acute and sub-chronic tests. In addition, the extract was able to minimize the genotoxic effects of CP in acute all tested concentrations in the acute test groups. Furtheremore, it induced disturbed cell division and tissu organization in sub-chronic test groups.

Genotoxins harm the genetic material causing abnormalities such as DNA mutations, structural and numerical chromosome aberrations and accumulation of DNA fragments which lead to the formation of micronuclei. **(Brandsma *et al.*, 2020)**. They can cause damages by direct interactions with DNA. Some substances manage to break through cell membranes and enter the nucleus to then interact with DNA directly **(Wu *et al.*, 2021).** They also can cause damage to proteins and lipids by generating reactive oxygen species (ROS) to attack them and disrupt their function **(Barzilai and Yamamoto, 2004)**. Cyclophosphamide (CP) is an alkylating agent, the most commonly used anticancer and immunosuppressant drug. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas and rheumatic arthritis and also in the preparation for bone marrow transplantation. The major limitation of cancer chemotherapy is the injury of normal tissue, leading to multiple organ toxicity. The important

factor for the therapeutic and the toxic effects of cyclophosphamide is the requirement of the metabolic activation by the hepatic microsomal cytochrome P 450 mixed functional oxidase system. Phosphoramide mustard and acrolein are the two active metabolites of cyclophosphamide. Cyclophosphamide's antineo-plastic effects are associated with the phosphoramide mustard, while the acrolein is linked with its toxic side effects. Acrolein interferes with the tissue antioxi-dant defense system, produces highly reactive oxygen free radicals and are mutagenic to mammalian cells. Due to the highly reactive nature, free radicals can readily combine with other molecules, such as enzymes, receptors and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal functions. During various myocardial stress, the activities of enzymatic antioxidants were also decreased **(Senthilkumar *et al.*, 2006)**.

Conclusion and perspectives

Our work's main objective is to assess the toxicity and toxicity preventative effects of *Centaurea calcitrapa* leaf extract through acute and sub-chronic tests.

The *Centaurea calcitrapa* extract has demonstrated a great potential for antioxidant activities with the in vitro tests showing its very high content in phenolic compounds and flavonoids. Our *in vivo* observations have demonstrated that the extract shows no severe toxicity and is safe to ingest even at high doses for a long period of time in mice. The treated mice exhibited no treatment-related toxicity effects during our study. Furthermore, the organ-size related toxicity and FRAP tests with the treatment showed no severe toxicity. The catalase activity determination and micronucleus test also showed no severe toxicity and even exhibited preventative effects against oxidative stress, micronuclei formation and cytotoxicity caused by CP in acute tests but not in sub-chronic test. We can therefore suggest that *C.calcitrapa* leaf extracts are non-toxic in acute and sub-chronic treatments and possess antioxidant, anticytotoxic, antigenotoxic and tissue protective properties making it a potentially great substance for preventing toxicities like cyclophosphamide-chemotherapy-induced toxicity.

To our knowledge, this work is the first *in vivo* toxicity assessment of hydroethanolic *C.calcitrapa* leaf extract. We would like to note that it was conducted using conserved dried powdered leaf extract. It would be interesting to study the fresh plant leaf extracts for a longer period of time. Further studies are also needed to investigate its different concentration-related effects and broaden our understanding of toxic and therapeutic properties of *C.calcitrapa*.

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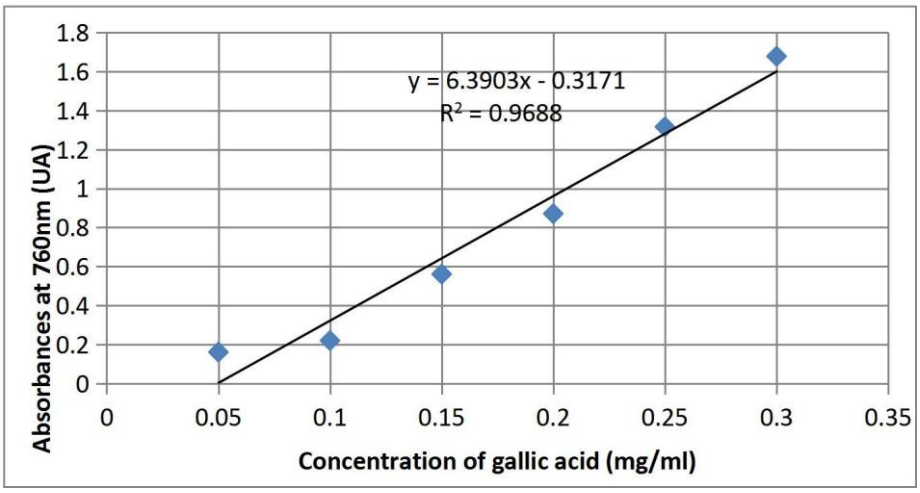
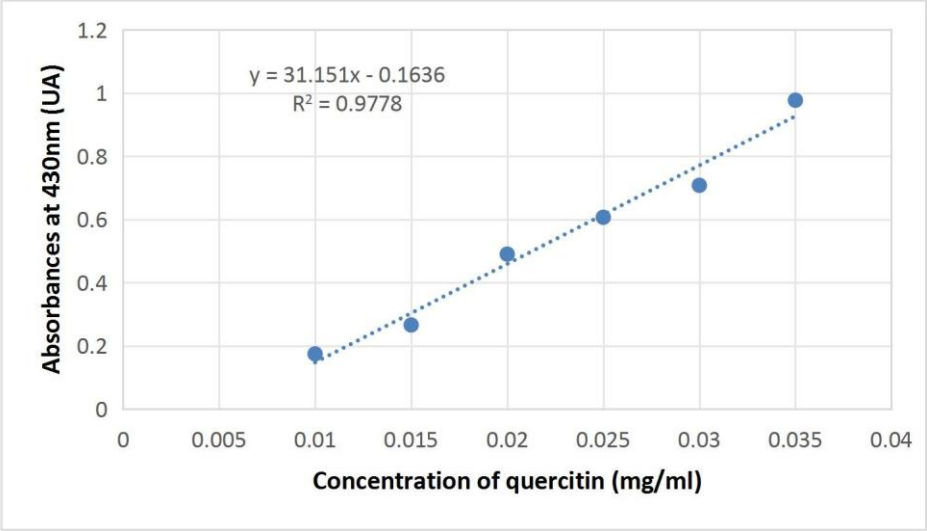
Annexes

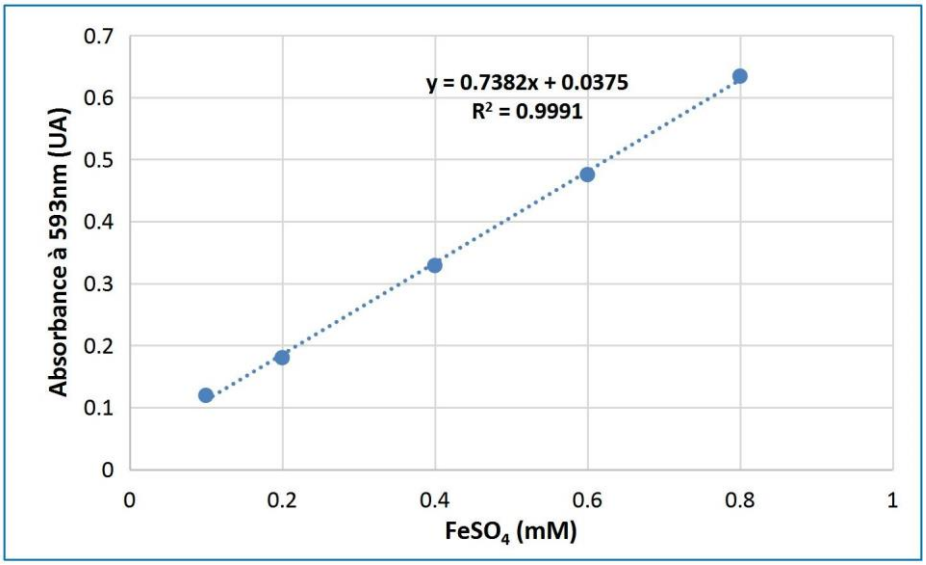
Annex 1 : Gallic acid standard curve for total polyphenol content determination.



Annex 2 : Quercitin standard curve for total flavonoid content determination.



Annex 3 : FeSO4 standard curve for FRAP (*Ferric Reducing Ability of Plasma*)



Abstracts

Abstract

Centaurea calcitrapa is a medicinal plant that is traditionally used for its benefits but little research is conducted about its toxicity *in vivo*. This study aimed to investigate *C.calcitrapa* leaf extract's acute and sub-chronic toxicity and its prevention in mice. The extract's polyphenol and flavonoid contents were determined by spectrophotometry. 16 mice groups were treated by gastric gavage with extract at 250, 500, 1000, 2000 mg/kg per day for 24h or 15 days and a single intraperitoneal injection of either saline or cyclophosphamide (CP) 24h before sacrifice. Organ size related toxicity, FRAP, catalase activity and genotoxicity by micronucleus test were determined for each group. *In vitro* analysis showed high polyphenol 487.536 ± 5.131 mg GAE/g and flavonoid content 29.146 ± 0.343 mg QE/g. Overall, *in vivo* biological activity tests with all extract concentrations showed no severe toxicity and significant reduction in CP-induced oxidative stress, cytotoxicity and micronuclei formation in acute tests but not in sub-chronic test. Our work suggests that *C.calcitrapa* extract is safe for *in vivo* testing and has great potential in antioxidant activities and antigenotoxicity effects.

Keywords: *Centaurea calcitrapa*, toxicity, medicinal plants, *in vivo* testing

Resumé

La *Centaurea calcitrapa* est une plante médicinale traditionnellement utilisée pour ses bienfaits mais peu de recherches sont menées sur sa toxicité *in vivo*. Cette étude visait à étudier la toxicité aiguë et subchronique de l'extrait de feuilles de *C.calcitrapa* et sa prévention chez la souris. Les teneurs en polyphénols et en flavonoïdes de l'extrait ont été déterminées par spectrophotométrie. 16 groupes de souris ont été traités par gavage gastrique avec un extrait à raison de 250, 500, 1 000, 2 000 mg/kg par jour pendant 24 h ou 15 jours et une seule injection intrapéritonéale de solution saline ou de cyclophosphamide (CP) 24 h avant le sacrifice. La toxicité liée à la taille des organes, le FRAP, l'activité catalase et la génotoxicité par test du micronoyau ont été déterminés pour chaque groupe. L'analyse *in vitro* a montré une teneur élevée en polyphénols de 487,536 ± 5,131 mg GAE/g et en flavonoïdes de 29,146 ± 0,343 mg QE/g. Dans l'ensemble, les tests d'activité biologique *in vivo* avec toutes les concentrations d'extrait n'ont montré aucune toxicité grave et une réduction significative du stress oxydatif, de la cytotoxicité et de la formation de micronoyaux induits par la CP dans les tests aigus, mais pas dans les tests subchroniques. Nos travaux suggèrent que l'extrait de *C. calcitrapa* est sans danger pour les tests *in vivo* et présente un grand potentiel en termes d'activités antioxydantes et d'effets antigénotoxicité.

Mots clés: *Centaurea calcitrapa*, toxicité, plantes médicinales, tests in *vivo*

الملخص

يعتبر نبات *Centaurea calcitrapa* من النباتات الطبية التي تُستخدم تقليدياً لفوائدها ولكن لم تُجرَ أبحاث كثيرة حول سميتها في الجسم الحي. هدفت هذه الدراسة إلى التحقق من السمية الحادة وشبه المزمنة لمستخلص أوراق الكالسيترابا والوقاية منها في الفئران. تم تحديد محتويات المستخلص من البوليفينول والفلافونويد بواسطة القياس الطيفي. عولجت 16 مجموعة من الفئران عن طريق التجرع المعدي بمستخلص المستخلص بجرعات 250، 500، 1000، 2000 ملجم/كجم يومياً لمدة 24 ساعة أو 15 يوماً وحقن واحد داخل الصفاق إما بمحلول ملحي أو سيكلوفوسفاميد (CP) قبل 24 ساعة من التضحية. تم تحديد السمية المتعلقة بحجم العضو، والسمية المرتبطة بحجم العضو، ونشاط الكاتلاز والسمية الجينية عن طريق اختبار النواة الدقيقة لكل مجموعة. أظهر التحليل في المختبر ارتفاع نسبة البوليفينول 487.536 ± 5.131 مجم مكافئ غازي/غرام ومحتوى الفلافونويد 29.146 ± 0.343 مجم مكافئ كمي/غرام. وعموماً، لم تظهر اختبارات النشاط البيولوجي في الجسم الحي مع جميع تركيزات المستخلص أي سمية شديدة وانخفاضاً كبيراً في الإجهاد التأكسدي الناجم عن الفوسفات والسمية الخلوية وتكوين النوى الدقيقة في الاختبارات الحادة ولكن ليس في الاختبار شبه المزمن. يشير عملنا إلى أن مستخلص الكالسيترابا آمن للاختبار في الجسم الحي ولديه إمكانات كبيرة في الخصائص المضادة للأكسدة وتأثيرات في مضادات السمية.

الكلمات المفتاحية: *Centaurea calcitrapa*، السمية، النباتات الطبية، الاختبار في الجسم الحي