الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالي والبحث العلمي

Ferhat Abbas University
-Setif 1-

Faculty of Natural Sciences

جامعة فرحات عباس
سطيف 1

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Université Ferhat Abbas Sétif 1

DEPARTMENT OF MICROBIOLOGY

N°...../SNV/2022

THESIS

Presented by

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For the fulfillment of the requirements for the degree of

DOCTORATE OF SCIENCES

BIOLOGY

Specialty: MICROBIOLOGY

TOPIC

Therapeutic valorization of *Ononis angustissima* and *Genista saharae*: Biological activities

Presented publically in: 26/05/2022

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ACKNOWLEDGEMENTS

This work would not have been possible without the support of **ALLAH**, who gave me the will to face the challenge, the health, and the courage to finish this thesis.

I would like to express my appreciation to my supervisor, **Pr. HARZALLAH Daoud**, for giving me the opportunity to be part of his research team in the laboratory of applied microbiology. Thank you, Professor, for your attention, your availability and guidance.

I would like to express my thanks to the members of my doctoral examination board**.**

My sincere gratitude to **Pr. Jaroslava Švarc-Gajić**, head of laboratory of biotechnology, University of Novi Sad. Serbia. For providing all facilities to realize the major part of the experimental work in her laboratory, and for being always available in all stages of this work. It has been an honor working with you, and your amazing team.

Special thanks to **Mr. GUETTAF Sofiane**, who has been providing me with the moral and intellectual support throughout this enriching experience. Words can not thank you enough my brother.

I would like to express my gratitude to all family members, friends and colleagues who gave me their moral and intellectual support throughout this enriching experience.

Abstract

Ononis angustissima and *Genista saharae* are endemic Algerian plants with pharmacological potential, used for centuries in traditional medicine. Herein the efficiency of subcritical water extraction (SWE) of phenols and flavonoids from these plants was evaluated by spectrophotometric assays. Afterward, the optimal extracts were tested for its; biological activities (*in vitro)*, and toxicity (*in vivo*). The most important operational parameters of the technique (temperature, pressure, time) were optimized for each plant based on phenols yield. At defined optimal extraction conditions (155°C, 50 bars, 15min), maximum contents of phenols in the extracts were 18.33, and 21.12 mg GAE/g dry weight, for *O. angustissima* and *G. saharae*, respectively. The antioxidant activity of extracts was evaluated by different assays including, DPPH, and β-Carotene Bleaching Assays. The anti-diabetic effect of the extracts was investigated *in vitro* against α-amylase and α-glucosidase. Chemical profile of the tested extracts was defined by RP-HPLC-UV/Vis. Both chemical profiles and demonstrated bioactivities were comparable for *O. angustissima* and *G. saharae*. However, *G. saharae* exhibited slightly stronger antioxidant capacity and higher content of phenolic compounds. Three hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic) were the major phenolic compounds in the tested extracts. Two monomeric flavan-3-ols, namely catechin and epicatechin, were detected in *O. angustissima* extract with relatively high amount, but not in the extract of *G. saharae.* Tested extracts demonstrated inhibitory effects against both enzymes α-amylase and α-glucosidase. In both applied assays, *O.angustissima* exhibited stronger enzyme-inhibitory effects compared to *G.saharae*. The MTT assay to test the anti-proliferative activity of extracts was performed with three different cell lines. The most effective cell growth inhibition activity IC_{50} value of 18.34 µg/mL was observed in fibroblast cell line from murine (L2OB) treated with *G. saharae*. The lowest IC_{50} value (28.27 µg/mL) was calculated in the same cell line treated with *O. angustissima*. Slight higher IC_{50} values were calculated in the case of line derived from human rhabdomyosarcoma (RD) (29.45 and 32.45 µg/mL) for *G. saharae* and *O. angustissima* SW/extract, respectively, while in the case of Hep2c cells, the highest values of 31.25 and 39.79 µg/mL were seen for *G. saharae* and *O. angustissima* extracts, respectively. *G. saharae* seems to have better anti-proliferative activity, which could be explained by its higher and diverse content of phenolic compounds shown in the HPLC analysis. The antimicrobial activity of our extracts was studied against six bacterial strains, as well as two fungal strains. Both plant extracts were equally strong against the Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*) (MIC = 78.13 µg /mL). However, *G.saharae* SW/extract showed better activity against Gram-negative bacterial strains. Whereas, in the case of fungi *O.angustissima* extract had stronger activity against *Candida albicans* (MIC = 78.13 µg /mL). The acute toxicity of extracts was carried out using mice. The $LD_{50}$was found to be superior to 5000 mg/kg. Obtained results suggested that subcritical water extracts of *G. saharae* and *O. angustissima* could provide powerful natural; antioxidant, antimicrobial, and anti-proliferative agents, to be used in the management of different diseases.

Key words: *Ononis angustissima*, *Genista saharae*, subcritical water extraction, phenols, flavonoids, biological activities, Toxicity evaluation.

ملخص

تعتبر *Ononis angustissima* و *Genista Saharae* من النباتات الجزائرية المتوطنة ذات القيمة الدوائية، وتستخدم منذ قرون في الطب التقليدي. تم في هذه الدراسة تم تقييم كفاءة استخراج الفينولات والفلافونويد من هذه النباتات بواسطة الماء دون الحرج (SWE)، عن طريق قياسات طيفية. تم بعد ذلك، اختبار؛ الأنشطة البيولوجية (في المختبر)، والسمية (في الجسم الحي) للمستخلصات المثلى. تم تحديد اهم العوامل التشغيلية لهذه التقنية (درجة الحرارة، الضغط، والوقت) لكل مستخلص بناءً على مردود الفينولات. في ظروف الاستخلاص المثلى المحددة (155 درجة مئوية، 50 بار، 15 دقيقة)، كان الحد الأقصى لمحتوى الفينولات في المستخلصات 18.33، و21.1 مغ GAE / غ وزن جاف، لـ *O. angustissima*، و *G. Saharae*، على التوالي. تم تقييم النشاط المضاد للأكسدة للمستخلصات من خلال فحوصات مختلفة بما في ذلك، DPPH و β-Carotene Bleaching. تم دراسة تأثير المستخلصات المضاد لمرض السكر في المختبر ضد α-amylase و α-glucosidase. تم تحديد التركيب الكيميائي للمستخلصات المختبرة بواسطة RP-HPLC-UV / Vis. كانت كل من التركيبة الكيميائية والنشاطات الحيوية قابلة للمقارنة بين *O. angustissima* و *G. saharae*. ومع ذلك، أظهرت *G. Saharae* قدرة مضادة للأكسدة أقوى قليلاً ومحتوى أعلى من المركبات الفينولية. ثلاث أحماض هيدروكسي بنزويك (p-Hydroxybenzoic، protocatechuic ، syringic) كانت هي المركبات الفينولية الرئيسية في المستخلصات المختبرة. أظهرت المستخلصات التي تم اختبارها آثارًا مثبطة ضد كل من إنزيم ألفا الأميليز و ألفا جلوكوزيداز. في كلا الفحصين التطبيقيين، أظهرت *O.angustissima* تأثيرات تثبيط إنزيمية أقوى مقارنة بـ *G.saharae*. تم إجراء اختبار MTT لاختبار النشاط المضاد للتكاثر للمستخلصات بثلاثة نسائل خلوية مختلفة. لوحظ أن نشاط تثبيط نمو الخلايا الأكثر فعالية بقيمة IC50 بقيمة 18.34 ميكروغرام / مل في خلايا الأرومة الليفية من الفئران (L2OB) المعالج بـ *G. Saharae*. تم حساب أدنى قيمة (28.27 IC50 ميكروغرام / مل) في نفس الخلية المعالج بـ *O. angustissima*. تمت دراسة النشاط المضاد للميكروبات لمستخلصاتنا ضد ست سلالات بكتيرية، بالإضافة إلى سلالتين من الفطريات. كان كلا المستخلصين النباتيين قويين بشكل متساوٍ ضد السلالات البكتيرية إيجابية الجرام (*Staphylococcus aureus*و Bacillus subtilis ( )ميكروغرام / مل78.13 = MIC) ، ومع ذلك ، أظهرت *G.saharae* نشاطًا أفضل ضد السلالات البكتيرية سالبة الجرام. بينما، في حالة الفطريات، كان لمستخلص *O.angustissima* نشاط أقوى ضد *Candida albicans* (MIC = 78.13 ميكروغرام / مل)، ومتساوي ضد *Aspergillus niger* (MIC = 78.13 ميكروغرام / مل). تم فحص السمية الحادة للمستخلصات باستخدام الفئران. وجد أن الجرعة المميتة 50 أعلى من 5000 مغ / كغ. تشير النتائج التي تم الحصول عليها إلى أن مستخلصات الماء دون الحرج من *G. Saharae* و *O. angustissima* يمكن أن توفر؛ مضادات الأكسدة، مضادات ميكروبات، وعوامل مضادة للتكاثر، طبيعية وقوية لاستخدامها في علاج أمراض مختلفة.

الكلمات المفتاحية: *Ononis angustissima*، *Genista Saharae* ، استخلاص بواسطة الماء دون الحرج، الفينولات، الفلافونويد، السمية، التأثيرات البيولوجية.

Résumé

Ononis angustissima et *Genista saharae* sont des plantes endémiques algériennes à potentiel pharmacologique, utilisées depuis des siècles en médecine traditionnelle. Dans le présent travail, l'efficacité de l'extraction par l'eau sub-critique (EES) des phénols et des flavonoïdes de ces plantes a été évaluée par des tests spectrophotométriques. Ensuite, les extraits optimaux ont été testés pour leurs ; activités biologiques (*in vitro*) et toxicité (*in vivo*). Les paramètres opérationnels les plus importants de la technique (température, pression, temps) ont été optimisés pour chaque plante en fonction du rendement en phénols. Dans les conditions d'extraction optimales définies (155°C, 50 bars, 15 min), les teneurs maximales en phénols dans les extraits étaient de 18,33 et 21,12 mg de GAE/g de poids sec pour *O. angustissima* et *G. saharae*, respectivement. L'activité antioxydante des extraits a été évaluée par différents tests, à savoir ; pouvoir antioxydant total, DPPH, et blanchiment de la β-carotène. L'effet antidiabétique des extraits a été étudié *in vitro* contre l'α-amylase et l'α -glucosidase. Le profil chimique des extraits testés a été défini par RP-HPLC-UV/Vis. Tant les profils chimiques que les bioactivités démontrées étaient comparables pour *O. angustissima* et *G. saharae*. Cependant, *G. saharae* présentait une capacité antioxydante légèrement plus forte et une teneur plus élevée en composés phénoliques. Trois acides hydroxybenzoïques (p-hydroxybenzoïque, protocatéchique, syringique) étaient les principaux composés phénoliques dans les extraits testés. Les extraits testés ont démontré des effets inhibiteurs contre les deux enzymes α-amylase et α -glucosidase. Dans les deux tests appliqués, *O. angustissima* présentait des effets inhibiteurs enzymatiques plus forts que *G. saharae*. Le test MTT pour tester l'activité antiproliférative des extraits a été réalisé avec trois lignées cellulaires différentes. La valeur IC_{50} de l'activité d'inhibition de la croissance cellulaire la plus efficace de 18,34 µg/mL a été observée dans la lignée cellulaire de fibroblastes de souris (L2OB) traitée avec *G. saharae*. La valeur IC*50* la plus basse (28,27 µg/mL) a été calculée dans la même lignée cellulaire traitée avec *O. angustissima*. L'activité antimicrobienne de nos extraits a été étudiée contre six souches bactériennes, ainsi que deux souches fongiques. Les deux extraits de plantes étaient également puissants contre les souches bactériennes Gram-positives (*Staphylococcus aureus* et *Bacillus subtilis*) (CMI = 78,13 g/mL), cependant, *G.saharae* a montré une meilleure activité contre les souches bactériennes à Gram-négatives. Alors que, dans le cas des champignons, l'extrait d'*O.angustissima* avait une activité plus forte contre *Candida albicans* (CMI = 78,13 µg/mL), et égal contre *Aspergillus niger* (MIC = 78,13 µg /mL). La toxicité aiguë des extraits a été réalisée à l'aide de souris. La DL_{50} s'est avérée supérieure à 5000 mg/kg. Les résultats obtenus suggèrent que les extraits par l'eau sub-critique de *G. saharae* et *O. angustissima* pourraient fournir des agents ; antioxydants, antimicrobiens et antiprolifératifs, puissants naturels à utiliser dans la gestion de différentes maladies.

Mots clés : *Ononis angustissima*, *Genista saharae*, extraction par l'eau sub-critique, phénols, flavonoïdes, activités biologiques, Toxicité.

LIST OF ABBREVIATIONS

$AlCl_3$	Aluminium chloride
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AqE	Aqueous extract
AST	Aspartate aminotransferase
BHT	Butylated hydroxytoluene
CAT	Catalase
ChE	Chloroform extract
CrE	Crude extract
DPPH	1,1-diphenyl-2 picrylhydrazyl
DTNB	5,5 0- dithiobis-(2-nitrobenzoic acid)
EY	Extraction yield
EC_{50}	Effective concentration of 50%
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
BHA	Butylated hydroxyanisole
iNOS	Inducible nitric oxide synthase
LD_{50}	Lethal dose 50
SWE	Subcritical water extraction
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
•OH	Hydroxyl radical
O_2•-	Superoxide
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
ON^{OO-}	Peroxynitrite
QE	Quercetin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
MHB	Muller–Hinton broth
Vit C	Vitamin C
XO	Xanthine oxidase

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INTRODUCTION

Introduction

Medicinal plants have been used in *Algeria* for centuries for their valuable bioactive compounds to treat different ailments (Ziani *et al*., 2019). Besides its desertic and semi-desertic areas, Algeria is characterized by large pool of plants with high pharmacological potential (Ziani *et al*., 2018; Benarba, 2016). Many of plant sources still haven`t been sufficiently explored. *Ononis angustissima* and *Genista saharae* are Saharan endemic medicinal plants used for centuries by local population for various diseases.

Phenolic compounds are synthesized by plants as secondary metabolites necessary for their growth and physiology (Duthie *et al*., 2003). These chemicals are continuously in the focus of scientific research because of their potent effects and because they represent the most abundant phytochemicals in human diets. These phytochemicals exhibit an array of biological effects important for health protection, disease prevention, and overall wellbeing (Ozcan *et al.*, 2014). Phenolic compounds have protective role in diseases caused by oxidative damage (coronary heart disease, stroke, and cancers) (Carocho and Ferreira, 2013; Guimarães *et al*., 2014; Shahidi and Ambigaipalan, 2015). These molecules act against free radicals through antioxidant, redox, and metal chelation capacity, acting as reducing agents, hydrogen donators or singlet oxygen quenchers (Carocho and Ferreira, 2013).

Phenolic compounds have been extracted from plants sources by using various conventional extraction techniques such as maceration or Soxhlet extraction. (Kimbaris *et al*., 2006; Trochimczuk *et al*., 2004; Zhao *et al*., 2010). However, these methods have a number of obvious disadvantages, such as long duration, consumption of organic solvents, and limited efficiency towards different classes of phenolics (Zhang *et al*., 2019). A variety of innovative extraction techniques have been developed for the extraction of bioactive compounds from natural products, including subcritical water extraction (SWE) (Zhang *et al*., 2019; Zhang *et al*., 2019; Švarc-Gajić, 2012; Švarc-Gajić and Cvetanović, 2017; Švarc-Gajić, and Cvetanović, 2014), supercritical fluid extraction (SFE) (McHugh and Krukonis, 2013), ultrasound-assisted extraction (UAE) (Wen *et al*., 2018), microwave-assisted extraction (MAE) (Kaufmann and Christen, 2002), ultrahigh pressure-assisted extraction (UPE) (Prasad *et al*., 2010) and pulsed electric field extraction (PEF) (Corrales *et al*., 2008). In the recovery of bioactive compounds from plant sources, SWE captures more and more attention due to its safety, superior efficiency, selectivity, and environment-friendly nature. Literature search

reports the extraction of different bioactive ingredients such as polysaccharides, proteins, antioxidants, and polyphenols from plant sources by subcritical water (Herrero *et al*., 2016; Getachew and Chun, 2017). As an environmentally-friendly and efficient extraction technique, SWE has shown great potential for application in different fields considering the safety of obtained extracts, superior chemical composition, and compatibility of the extracts with food, pharmaceutical, and cosmetic products (Zhang *et al*., 2019).

According to available literature, there are no reports on the use of subcritical water for the recovery of polyphenols from *Ononis angustissima* and *Genista saharae*, neither the evaluation of its biological effects. Thus, the aim of this study was to evaluate the efficiency of SWE for obtaining *O. angustissima*, and *G. saharae* extracts with high content of polyphenols. Then evaluate the therapeutic effect of obtained extracts by testing different biological activities. Likewise, the antioxidant, anti-diabetic, anti-inflammatory, anti-proliferative, and antimicrobial activity. Finally, the *in vivo* acute toxicity of extracts was carried out.

Our thesis is consisted of three chapters, the first one comprises a literature review about medicinal plants and its secondary metabolites, the second chapter sited the material and methods used in this study, and the third chapter contains the obtained results and its discussion.

LITERATURE REVIEW

1. PLANTS AND MEDICINE

1.1. History

Medicinal plants have been used for centuries as a remedy for human illnesses because they contain components of therapeutic value. Recently, the development of resistance to antibiotics, anti-inflammatories, and other synthetic drugs, and the awareness of the negative side effects inflicted by modern drugs, has led to the acceptance of traditional medicine as an alternative form. Therefore, authors set out to study the biological activity of medicinal plants (Nostro *et al.,* 2000).

Plants have always been the major source of medicines, thanks to the richness of what is called secondary metabolism, however, man has only discovered the beneficial virtues of plants through a progressive approach, facilitated by organizational social relationships, in particular from the Neolithic (8000 BC). Observation linked to experience and the transmission of information collected over time means that some men become able to make a diagnosis, to find the plant that heals, and ultimately to cure the patient (Fouché *et al.,* 2000).

At the height of the Arab Empire (whose borders stretched from *India* to *Spain*), all written documents were collected in *Baghdad* in the largest library of the time (7th to 9th century). The Arabs had their specialists in medicine and pharmacy; *Abu Bakr al Razi* or *Rhazès* (865-925), was one of the great physicians of his time and also the precursor of psychotherapy. He was followed by *Ibn Sina* or *Avicenna* (980-1937) who wrote “The canon of medicine”. This book will serve as a basis for the teaching of medicine in the universities of Louvain and Montpellier until around 1650. Ibn al Baytar (1197-1248) wrote "the very complete sums of the simple"; this book contained a list of 1400 medicinal preparations and plants (Ati, 2010).

1.2 The natural products of plants and their biological activities

Plants produce a large number of compounds. They have multiple interests, which are used in industry, food, cosmetology and dermopharmacy. They have especially distinguished themselves in therapy. Natural plant products can be classified into two categories, primary metabolites and secondary metabolites.

- ***Primary metabolites*:** they have an essential role for metabolism and plant development, are found in all species.
- ***Secondary metabolites*:** they are not produced directly during photosynthesis but result from subsequent chemical reactions. They are molecules that do not participate directly in the development of plants but rather intervene in relations with biotic, abiotic stresses, pathogenic microorganisms, etc... It is therefore conceivable that the plant can develop a particular metabolism allowing it to synthesize the most diverse substances in order to defend itself. They are different in different species. Among them: terpenes, flavonoids, tannins, saponosides, alkaloids, and coumarins (Collin, 2007). **Table 1** summarizes the various plant metabolites with a biological effect.

Table 1. Some examples of the main medicines of plant origin of natural products (De *et al.*, 1999; Pietta, 2000; Ogunleye *et al* 2003; Okwu *et al.*, 2004; Kappers *et al.*, 2005; Ayind *et al*, 2007; Bahar *et al*, 2008; Tiwari *et al*, 2011).

Compound	**Example of a botanical source**	**Therapeutic category / use**
Phenols	*Bryophyllum pinnatum*	Antioxidant, anti-inflammatory, antibacterial
Flavonoids	*Ficus palmate*	Antioxidant, cardiotonic, anti-inflammatory, antiulcer, anti-allergic, anticancer, antiviral, and antimicrobial
Tannins	*Xanthium strumarium*	Antioxidant, anti-inflammatory and analgesic
Alkaloids	*Catharanthus roseus*	Anticancer, antibacterial, cough suppressant, local anesthetic, and narcotic
Terpenoids	*Taxus brevifolia*	Anticancer, anti-inflammatory, antiviral and antibacterial
Steroids	*Digitalis purpurea*	Cardiotonics, antimicrobial, and insecticide
Reducing sugars	*Acacia nilotica*	Antidiarrhoeal, defense mechanism against microorganisms, insects and herbivores
Saponosids	*Terminalia bellerica*	Antibacterial, antifungal and antidiarrhoeal

1.2.1. Polyphenols

There is a very wide variety of phenols, from simple compounds like salicylic acid, a molecule that synthetically produces aspirin, with more complex substances like phenolic compounds to which glucosides are attached (Ati, 2010). They are widely distributed in the plant kingdom. Approximately 10 000 naturally occurring compounds belong to the category of "phenolics", all of which possess a common structural feature: an aromatic ring bearing at least one hydroxyl substituent (Goleniowski *et al.*, 2013). Polyphenols are biosynthesized by several different routes which two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway (Mandal *et al.*, 2010).

It is assumed that plants always seek to protect themselves against infections and phytophagous insects; phenolic acids, such as rosmarinic acid, are strong antioxidants and anti-inflammatories and may have antiviral properties (**Figure 1**) (Zitka *et al*., 2011).

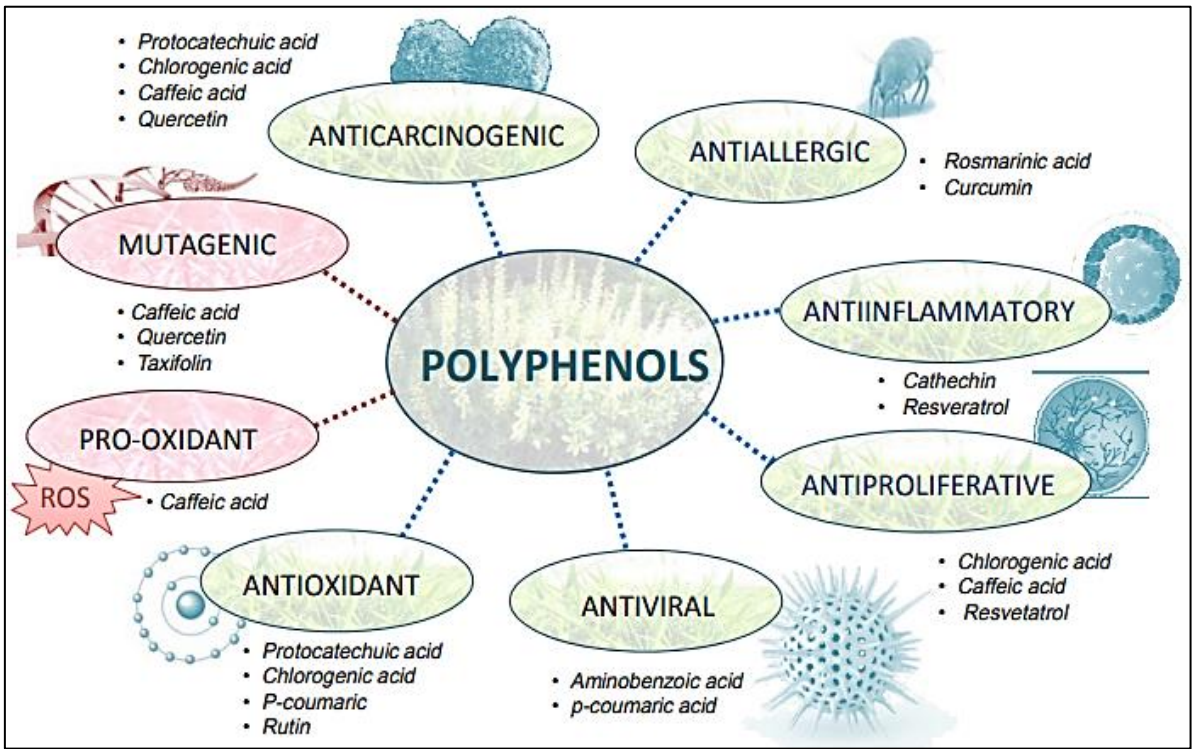


Figure1. Polyphenols and their biological properties (Zitka *et al*., 2011).

;Polyphenols have a great variety of beneficial effects like anticarcinogenic (e.g., quercetin, protocatechuic acid) antiallergic (e.g., rosmarinic acid, curcumin), antiinflammatory (e.g., cathechin, resveratrol), antiproliferative (e.g., chlorgenic acid, caffeic acid, resveratrol), antiviral (e.g., aminobenzoic acid, p-coumaric) and antioxidant (e.g., rutin, chlorgenic acid, quercetin on human health. Their antioxidant properties and abilities to modulate several enzymes are also important. Some flavonoids have also mutagenic (e.g., quercetin) and/or prooxidant effects (e.g., caffeic acid) and they may interfere with essential biochemical pathways.

Phenols include among others:

- **Flavonoids:** The term flavonoid designates a very wide range of natural compounds belonging to the family of polyphenols. Which can be found in a wide variety of fruits and vegetables consumed daily by humans. In addition to their role in plant pigmentation, some of these compounds exhibit biological activities of interest, such as antioxidant, anti-inflammatory, antihypertension, anti-influenza, antifungal, and antiviral actions (Achille, 1980; Kambouche *et al.,* 2008; Claisse, 1993).
- **Tannins:** Tannins are polar polyphenols very abundant in angiosperms, gymnosperms, and dicotyledons, exist in almost every part of the plant: leaves, fruits and roots, they are characterized by their antioxidant capacity and their therapeutic property. Tannins help stop bleeding and fight infections (Kechkar, 2008; Sablonnière, 2002 ; Meyer *et al.,* 2004).

1.2.2. Terpenes

Terpenes are a widely represented group of natural products. They are formed by the polymerization of units with 5 carbon atoms. They constitute the odoriferous principle of plants. This odor is due to the release of very volatile molecules.

Studies done on animals have shown that certain classes of terpenes such as: β-sitosterol, like its glucoside, have anti-inflammatory, antipyretic, antineoplastic and immunomodulatory properties. Also, terpenes are widely used in the human nutrition sector (flavor, preservative) and in the perfume industry (Allen, 2006).

1.2.3. Saponosids

From the Latin word "sapon" which means soap grass. They are heterosidic secondary metabolites found in many plants and some marine organisms. Amphiphilic in nature, these molecules are known for their surface-active property or their ability to lyse red blood cells (hemolysis) (Marfak, 2003).

1.2.4. Alkaloids

Alkaloids are molecules of natural origin. They are found mainly in plants, but also in animals and in certain microorganisms. They have a basic nature, contain nitrogen and are pharmacologically active, they are also used as major analgesics (morphine), to combat excess uric acid (colchicine), as a paralyzing / stimulating substance (curare and caffeine) , as

an anticancer (vinblastine, vincristine). They are also strong antimicrobial agents (Milane, 2004).

2. FABACEAE (legumes)

Fabaceae (legumes) are considered to be improver plants, their roots have nodules, bacteria that fix atmospheric nitrogen (Messali, 1995). This family contains more than 18,000 species distributed among three sub-families;*Mimosaceae*, *Caesalpiniaceae* and *Papilionaceae*. The latter is cosmopolitan, but the *Mimosoideae* and *Caesalpinioideae* are rather tropical. Among the 750 genera of the *Fabaceae* family, in Algeria there are about 53 genera and 337 species (Quezel and Santa, 1963). The legume family is extremely rich in flavonoids. The Papilionaceae subfamily is characterized by the presence of isoflavones, anthocyanin rotenoids and glycosylated flavonols (Harborne, 1967).

2.1. Botanical aspect of Fabaceae

The origin of this family comes from the fruit, a pod called by the first botanists legumes, hence the name of the family (Guigard, 1994). The plants of this family have simple or compound leaves, usually alternate and stipulate sometimes more or less entirely transformed into tendrils. They typically have pulvini at the base of the main and secondary spines involved in more or less important nasties. The flowers generally regular or irregular hermaphroditic, hypogynous to perigynous, usually pentacyclic and pentameric except at the level of the gynoecium which is unicarpellate. The five sepals are connected in a lobed tube often bilabiate. In Europe, the corolla is typically papilionaceous and consists of five petals. The superior, in an adaxial position, called the standard, is generally the widest. In some cases, it can be found in an abaxial position by resupination of the flower. The lateral petals are called wings, free and identical to each other or sometimes slightly adherent to the lower petals. These last petals, the most internal, are similar and often connate with each other to form the keel surrounding the stamens and the pistil. The stamens are usually ten in number, nine of which have filaments very often connected with each other, the tenth stamen being free. The stamens are then diadelphous and surround the pistil; they are sometimes monadelphous (all connected with each other). Philadelphia can also be obtained by forming two groups of five stamens each. In rare cases, all of the stamens may be free. The superior ovary consists of a single carpel with normally several campylotropic, biteguminous ova,

sometimes only one (Trifolium). The fruit is a variable pod most often dry and capsular, follicle with ventral and dorsal dehiscence, sometimes an achene, rarely a samara or a drupe. The pod may be lomentaceous, that is to say without longitudinal dehiscence but with transverse ruptures creating inseminated sections. The seeds have a hard seed coat and contain a curved embryo with little or no albumen (Spichiger *et al*., 2002).

2.2. Systemic position of Fabaceae

The botanical classification given by Quezel and Santa, (1963) is as follows:

- Kingdom: Planta
- Branch: Spermatophytes
- Sub-branch: Angiosperms
- Class: Dicotyledons
- Order: Fabales
- Family: Fabaceae

2.3. Geographical distribution of Fabaceae

The two subfamilies *Mimosoideae* and *Caesalpinioideae* are found in tropical areas. On the other hand, the *Papilionaceae* subfamily to which the two studied species belongs form a cosmopolitan subfamily with 2/3 of all genera and species of the legume family, with 440 genera and 12000 species. This subfamily contains most of the legume species used for food such as: soybean (*Glycine maximum*), chickpea (*Arietinumcicer*), green bean (*Phaseolus vulgaris*), lentil (*Leusculinaris*), and peanut (*Hypogaeaarachis*) (Polhill, 1981).

2.4. Previous chemical studies on Fabaceae

Phytochemical studies carried out on the legume family with regard to bibliographic data have shown the presence of flavonoids, triterpenes, and isoflavonoids which are almost characteristic of this family and especially aglycone isoflavonoids (Aaku *et al*., 1998).

2.5. Use of Fabaceae

- Ornamental plants (*Acacia*, *Albizzia*, *Bauhinia*, *Cercis*, *Erythrina*, *Paraserianthes*, *Wistartia*, etc.)

- Food plants such as pea (*Pisum Sativum*), Bean (*Phaseolus Vulgaris*), lentil (*Lens Culinaris*), Bean (*Vicia Faba*), chickpea (*Cicer Arietinum*), peanut (*Arachis Hypogea*), soybean (*Glycine Max*) etc.
- Forage plants (*Trifolium, Medicago, Hedysarum* etc.). The root of *Glycirhiza Glabra* gives licorice.
- Medicinal plants (*Genista Numedica* and *Ononis Spinosa* etc.)

2.6. Toxicity of Fabaceae

A significant number of *Papilionaceae* are poisonous, and it is important to note that their order contains more than 16,000 dangerous species (Bruneton, 2001). The parts of plants most often implicated in poisonings are the seeds where toxic principles are accumulated (Marstom *et al*., 1984).

3. PRESENTATION OF THE GENUS GENISTA

The *Genista* genus has about 150 species distributed in Europe and the Mediterranean region (Bruneton, 1999). According to the bibliography, this genus shows a richness in phenolic compounds, in particular isoflavonoids are known for their various biological activities. The *Genista* genus was first described by Linne in 1753, it belongs to the *Fabaceae* family, a subfamily of the *Papilionaceae*, and to the *Genisteae* tribe *Quezel* and *Santa* (1963). In *Algeria*, there are 23 species of which 11 are endemic (Maire, 1987). The *Genista* genus constitutes an interesting material that deserves to be better known in order to highlight its advantages and potentialities.

3.1. Isolated metabolites of the *Genista* genus

Plants of the *Genista* genus have been the subject of numerous phytochemical and pharmacological investigations. These investigations show that the main secondary metabolites of the genus consist of alkaloids and flavonoids (Lograda, 2010). Indeed, previous chemical studies on the *Genista* genus indicate the presence of alkaloids (Kirch *et al*., 1995; Pistelli *et al*., 2001; Martins *et al*., 2005; Zellagui *et al*., 2005); flavonoids and isoflavonoids (Pistelli *et al*., 1998; 2000; Giachi *et al*., 2002; Tosun *et al*., 2009), which are markers of the chemotaxonomic genus (Harborne, 1994), and afterward saponins (Boutaghane *et al*., 2013).

3.2. The species *Genista Saharae Coss*. and *Dur*. (Pomel).

3.2.1. Botanical aspect and systemic classification

According to *Quezel and Santa* (1963), *Genista saharae* Coss. and Dur. (Pomel) is an endemic plant of the Sahara with a campanulate calyx with 5 short subequal teeth, almost glabrous. Plant with long, cylindrical and erect branches, 1-2 meters high. Inflorescence in a simple or compound raceme, with widely spaced flowers. Pod polysper, compressed, stipulated, 25-50 mm long (**Figure 2**). Its vernacular name is "*Tellegit*".

- Flowering: December-April.
- Geographical area: Endemic to the Sahara.
- Geographical distribution: Quite rare in the Northern Sahara.
- Ecology: Foot of dunes, nebkas and desert and sub-desert sandy pastures.
- Classification of the species *Genista Saharae Coss*. & *Dur*.

  kingdom: *Plantae*

  Division: *Magnoliophyta* Cronquist

  Subdivision: *Magnoliophytina* Frohne & U. Jensen

  Class: *Rosopsida* Batsch

  Subclass: *Rosidae* Takht.

  Superorder: *Fabanae* R. Dahlgren

  Order: *Fabales* Bromhead

  Family: *Fabaceae* Lindl

  Tribe: *Genisteae* (Adans.) Benth.

  Genus: *Spartidium* Pomel

  Species: *Genista Saharae Coss*. and *Dur*. (Pomel).

3.2.2. Traditional use

Several species of the genus *Genista L*. have medicinal properties, for example, *Genista microcephala* is used to treat food poisoning and microbial infections (El-Rhaffari *et al*., 1999), *Genista balansae* is used as an analgesic and anti-inflammatory (Agelet and Vallès, 2003) and *Genista tenera* is used in traditional medicine for the control of diabetes (Rauter *et al*., 2009).

In Algeria, the aerial parts of *Genista saharae* are traditionally used for the treatment of respiratory diseases and have a diuretic property (Bouchouka *et al*., 2012). In Morocco in the *Tafilalet* region, *Genista saharae* is recommended for digestive disorders (El-Rhaffari *et al*., 1999). In addition, this plant is appreciated by camels for its retention properties (Cuenod, 1954; Le Floc'h, 1983).

3.2.3. Phytochemical study

The species *Genista Saharae* has been the subject of few chemical studies; the presence of isoflavones, flavones, and flavonols has been demonstrated in the hydroalcoholic extract (Mekkiou *et al*., 2005). The essential oils of the aerial parts were analyzed and a total of 58 volatile compounds were identified. Palmitic acid was the major constituent followed by mystic acid, then lauric acid and linoleic acid (Lograda *et al*., 2009).

3.2.4. Recognized biological properties

The *Genista* genus has lately attracted the attention of various authors for its biological properties, in particular, antidiabetic (Rauter *et al*., 2005; 2009), anti-inflammatory, antiulcer (Ilarionov *et al*., 1979; Rainova *et al*., 1988), spasmolytics, antioxidants, and phytoestrogens (Luczkiewicz and Glod, 2003; Kaouter *et al*., 2014; Garritano *et al*., 2005). In addition, some species of the *Genista* genus have shown cytotoxic activity against different human cancer cell lines (Scarpato *et al*., 2008; Rigano *et al*., 2009; 2010), effects on the thyroid gland (Korpachov *et al*., 1995), acetylcholinesterase inhibition, as well as genotoxicity (Rauters *et al*., 2009). Several other reports have indicated that the genus *Genista* contains a variety of secondary metabolites of various types, including isoflavones, which have been shown to be biologically active (Dixon *et al*., 1995).

The *Genista Saharae* species has been the subject of some studies on biological effects *in vitro*. According to Bouchouka *et al,* (2012), the ethyl acetate fraction of *Genista Saharae* showed remarkable antioxidant activity in scavenging the DPPH radical of the order of 25.6 mg of vitamin C equivalent per g of extract. The combination of HPLC-MS with the DPPH trapping test of the methanolic extract of *Genista Saharae* also shows a strong antioxidant activity with an IC50 = 8.27 µg/ml (Meriane *et al*., 2014).

The evaluation of the antibacterial activity of the ethyl acetate fraction on *Staphylococcus Aureus* shows a strong antibacterial activity (Bouchouka *et al*., 2012). In

contrast, the effect of essential oils on *Escherichia Coli*, *Staphylococcus Aureus*, and *Pseudomonas Aeruginosa* shows low antibacterial activity. (Lograda *et al*., 2009).

Figure 2. Morphological appearance of the *Genista Saharae* plant
(Personal photos, 2017)

4. PRESENTATION OF THE GENUS ONONIS

The plants of the genus *Ononis* formerly called Anonis by Tournefort have about 75 species distributed in Europe, Asia and the Mediterranean region. There are more than 34 species that exist in Algeria, most of which are poorly understood phytochemically and pharmacologically (Quezel and Santa, 1963). The genus is difficult to decide whether a plant of the large legume family belongs to it, and yet its characters are extremely ambiguous, i.e. they merge with several related genus such as *Spartidium*. (Drapiez, 1837).

4.1. Isolated metabolites of the genus *Ononis*

The tribe of the species *Ononis (Trifoleae)* belongs to the *Papilionaceae* subfamily and contains the anthocyanins 3-glucosid or 3,5-diglucosidae (Haborne *et al*., 1967). Several research works on the chemical composition of the genus *Ononis* have been carried out. In several studies, it has been shown that resorcinol derivatives are very common in this kind of plant as well as isoflavonoids, terpenoids, sterols, and phenolic acids (Bouheroum, 2011). Mediterranean *Ononis* species: O. *Fruticosa*, O. *Natrix* subsp. *Ramosissima*, and O. *Tridentata* were analyzed for flavonoids in their exudate. More than 20 aglycon flavonoids were identified (Wollenwebera *et al*., 2007). In addition, the species *Ononis Speciosa* contains

known compounds, such as: α-sitosterol and ononitol, derived from resorcinol and flavonoids (quercetin and two isoflavonoids) (Barrero *et al*., 1989), terpenoids, sterols and phenolic acids (Rowan *et al*., 1972). The study of the n-hexane extract of the aerial parts of *Ononis Natrix*, a species of the same genus as *Ononis Angustissima*, has described nine compounds, five of which are derived from resorcinol (Canedo *et al*., 1997).

4.2. The species *Ononis Angustissima Lam*. (Sirdj)

4.2.1. Botanical aspect and systemic classification

From Quezel and Santa (1963), *Ononis Angustissima* Lam. is an endemic plant to the northern Sahara (*Guardaia, Bechar, Biskra, Boussaâda*, up to *Beni Abbas*). *Ononis Angustissima* is a plant with yellow flowers too, on elongated peduncles and united in loose clusters; narrow, almost linear leaflets with little or no toothing; small bushes with very branched stems at the base, with persistent branches after flowering and becoming somewhat thorny (**Figure 3**). Its vernacular name is "*Tfiza*" (Chehma *et al*., 2015).

- Flowering: April -September.
- Geographical area: Endemic to the Sahara.
- Geographical distribution: Quite rare in the Northern Sahara.
- Ecology: nebkas and sub-desert pastures.
- Classification of the species *Ononis Angustissima* Lam. (Ozenda, 1958)

  kingdom: *Plantae*
  Division: *Magnoliophyta* Cronquist
  Subdivision: *Magnoliophytina* Frohne & U. Jensen
  Class: *Rosopsida* Batsch
  Subclass: *Rosidae* Takht.
  Superorder: *Fabanae* R. Dahlgren
  Order: *Fabales* Bromhead
  Family: *Fabaceae* Lindl
  Tribe: *Trifoleae*
  Genus: *Ononis* Sirdj
  Species: *Ononis Angustissima Lam*.

4.2.2. Traditional use

The use of this species for medical purposes is poorly documented. The genus *Ononis* in general is represented in Egypt by eight species. Several plants of this genus have been used in the treatment of jaundice, urinary inflammations, and kidney stones (Bouheroum, 2007). The species *Ononis Natrix* has been used to treat diarrhea and some urinary and rheumatic disorders (Barrero *et al*., 1989). *Ononis Hirta* is used to treat skin cancer, necrosis, and herpes (Talib *et al*., 2010).

Only Chehma *et al*. (2015) reported the traditional use of the aerial part of the species *Ononis Angustissima* for its hemostatic properties.

4.2.3. Phytochemical study

Only the chloroform extract of the *Ononis Angustissima* plant has been the subject of a phytochemical study, providing five compounds, four of which have been identified as flavones (Bouheroum, 2007).

4.2.4. Recognized biological properties

The antioxidant activity of *Ononis Spinosa* ethanolic extract was evaluated by examining its ability to inhibit anion formation (superoxide test). The results were encouraging (IC50 1.35 mg / ml) (Çoban *et al*., 2003). The studies by Tawaha *et al*. (2007) have shown that the species *Ononis Natrix* has remarkable total antioxidant activity.

The anti-inflammatory activity *in vitro* of the species *Ononis Ramosissima* was tested by Bremner *et al*. (2009). The results showed that the different extracts (alcoholic) were active with regard to the inhibition of TNF-α mediators (10 µg/ml). The anti-inflammatory activity *in vivo* has shown that the aerial parts of *Ononis Macrosperma* promote remarkable wound healing induced by acetic acid (Süntar *et al*., 2011). Talib *et al*. (2010) showed that *Ononis Hirta* and *Ononis Sicula* have bacteriostatic properties against *Salmonella Typhimurium* and *Bacillus Cereus*. In addition, the butanolic extracts of *Ononis Spinosa* had strong antifungal activity against *Aspergillus Flavus, Fusarium Moniliforme* and *Candida Albicans* (Mahasneh and El-Oqlah, 1999).

The species *Ononis Vaginalis* allowed the isolation of seven flavonoids including eupatilin which has a cytotoxic activity (Abdel-Kader Maged and Braz, 1997).

The species *Ononis Angustissima* has been the subject of few studies for biological effects *in vitro*. According to Ghribi *et al*, (2015), the ethanolic extract of *Ononis Angustissima* showed remarkable antioxidant activity of scavenging the DPPH radical with an IC50 = 24.48 µg/ml. Likewise, the same study reports significant cytotoxic activity of some phenolic compounds isolated from *Ononis Angustissima*. Another study on the hydromethanolic extract of *Ononis Angustissima* reveals remarkable antioxidant activity in scavenging the DPPH radical with an EC50 = 20.83 µg / ml (Djeridane *et al*., 2010).

Figure 3. Morphological appearance of the *Ononis Angustissima* plant (Personal photos, 2018).

5. OXIDATIVE STRESS

Oxidative stress is the alteration of cellular redox homeostasis induced either by an excessive production of reactive oxygen species (ROS) or nitrogen (RNS) or by a depletion of the capacities of antioxidant defenses by a deficiency of intake and/or production of antioxidants (Kirschvink *et al*., 2008).

At low concentrations, ROS exert physiological effects and play important roles in the body. Indeed, ROS are the mediators of multiple signaling and transcription functions essential for the normal functioning and survival of cells, as well as the programming of their elimination. Under pathological circumstances, or under the action of certain exogenous

factors, an overproduction of these species is possible. In this case, ROS can damage the structure of macromolecules (nucleic acids, proteins, lipids, and carbohydrates), generate new oxidizing products and cause cytotoxicity (Sayre *et al*., 2005). The intensity of this damage is proportional to the rate of ROS production, their duration of action as well as the specific defense tools present in the attacked tissues (Bloomer and Fisher-Wellman, 2008).

5.1. Reactive oxygen species

The reactive oxygen species ROS (**Table 2**) are chemicals (atoms, molecules) including free radicals that have at least one free electron on the outer layer.

5.1.1 Main Reactive Oxygen Species

Oxygen is normally converted into water molecules in the respiratory chain (mitochondria). This process is not perfect, however, because 2 to 5% of the oxygen is consumed and transformed into O_2•- by a univalent reduction, and therefore the inevitable production of very reactive intermediates results (Pincemail *et al*., 2001; Finaud *et al*., 2006);

- Superoxyde Radical (O_2•-)
- Hydrogen peroxide (H_2O_2)
- Hydroxyle Radical (OH•)

Table2. Reactive oxygen species (Kohen and Nyska, 2002).

Name	Symbol
Radical species	
Oxygen (bi-radical)	O_2
Superoxide anion	$O_2^{\bullet -}$
Hydroxyl radical	$OH^{\bullet}$
Peroxyl radical	$ROO^{\bullet}$
Alkoxyl radical	$RO^{\bullet}$
Nitrogen monoxide	$NO^{\bullet}$
Non-radical species	

Hydrogen peroxide	H_2O_2
Organic peroxide	ROOH
Hypochloric acid	HOCl
Ozone	O_3
Aldehydes	HCOR
Singlet oxygen	$^1O_2$
Peroxynitrite	$ONOO^-$

5.1.2. Main sources of reactive oxygen species

ROS are continually produced inside and outside the eukaryotic cell by various mechanisms. Endogenous production is considered to be significantly greater than that of exogenous origin. (Madamanchi *et al.*, 2005).

- **Endogenous sources**

a) Mitochondria

Mitochondria are thought to be the largest contributors to intracellular oxidant production (Holmström and Finkel, 2014). It contains numerous redox enzymes capable of transferring single electron to the O2 generating the O•-2 through the tricarboxylic cycle enzymes, electron-transport chain complexes I, II and III, among others enzymes (Murphy, 2009).

b) *Endoplasmic reticulum*

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

c) *Peroxysomes*

The presence of enzymes that produce ROS in peroxysomes indicates that peroxisomes are involved with the metabolism of ROS. Peroxisomes are one of the major sites of intracellular H2O2 production since they contain numerous enzymes producing H2O2, glycolate oxidase, urate oxidase, aspartate oxidase, XO, NOS and acyl CoA oxidases

(del Río and López Huertas, 2016). A part from H2O2, it has been demonstrated that peroxisomes also produce O•2- and NO• as a consequence of their normal metabolism. Currently, there is no evidence that mammalian peroxisomes contain enzymes that produce •OH or ONOO- (Lismont *et al.*, 2015). However, H2O2 inside peroxisomes may give rise to •OH through the Fenton reaction. In addition, as these organelles contain enzymatic sources of O•2- and NO•, and the reaction of NO• with O•2-to form ONOO- is kinetically and thermodynamically favored, it is very likely that peroxisomes also generate ONOO- (Lismont *et al.*, 2015).

d) *NADPH oxidase*

The NADPH oxidases comprise a family of proteins able to transfer electrons from NADPH across cellular membranes; electrons react with O2 generating O•-2 and attendant other downstream ROS (Hansen *et al.*, 2018). Several physiological functions have been related with ROS generation by the NADPH oxidases family, including cell signalling, host defence, control of ventilation, smooth muscle relaxation, control of erythropoietin production and other hypoxia-inducible functions (Holmström and Finkel, 2014).

e) Xanthine oxidase (XO)

The XO catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid and is a well-known producer of $O^{\bullet 2-}$ (Halliwell and Gutteridge 2015). Xanthine oxidoreductases are present in two forms, depending on their electron acceptor. Xanthine dehydrogenase uses NAD^+, and XO uses O_2 to produce $O^{\bullet 2-}$. Under pathological conditions, such as tissue ischemia, xanthine dehydrogenase can be converted to XO (Toro and Rodrigo, 2009).

f) Nitric oxide synthase (NOS)

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

g) Arachidonate cascade enzymes

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

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

- **Exogenous sources**

The ROS are also produced in the biological systems by various exogenous sources. Cigarette smoke contains many oxidants, free radicals and organic compounds, such as O•2- and NO•. In addition, inhalation of cigarette smoke into the lung also activates some endogenous mechanisms, such as accumulation of neutrophils and macrophages, which further increase the oxidant injury (Briben *et al.*, 2012).

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

Hyperoxia refers to conditions of higher oxygen levels than normal partial pressure of O2 in the lungs or other body tissues. It leads to greater production of ROS and RNS (Berkelhamer *et al.*, 2013).

Ionizing radiation, in the presence of O2, converts •OH, O•-2, and organic radicals to H2O2 and organic hydroperoxides. These hydroperoxide species react with redox active metal ions, such as iron and copper, via Fenton reactions and thus induce oxidative stress (Azzam *et al.*, 2012). Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, can induce generation of reactive radicals via a Fenton-type reaction (Shahid *et al.*, 2014).

5. 2. Biological consequences of oxidative stress

Lipids, proteins and nucleic acids represent the main targets of ROS. The attack of circulating lipids results in the formation of oxidized LDL which are taken up by macrophages, will form the lipid depot of atherosclerotic plaque of cardiovascular disease. While the attack of membrane phospholipids modifies the fluidity of the membrane and therefore the functioning of many receptors and transporters and signal transduction (Favier, 2003). The unsaturated fatty acids (FAs) of membrane phospholipids are the preferred targets of ROS. Their peroxidation generates primary products in the form of conjugated polyenes which can combine with oxygen to form peroxyl radicals (Spiteller, 2006). These peroxyl radicals (LOO •) extract a hydrogen atom from another fatty acid to form lipid hydroperoxides (LOOH) and new FA radicals, which will propagate peroxidation. Malonedialdehyde (MDA) and 4-hydroxynonenal (HNE) are examples of aldehydes resulting from lipid peroxidation and can be used as markers in detecting lipid peroxidation in patients (Pincemail *et al.*, 1999). The LOO • formed will be able, thanks to their high reactivity, to extract hydrogens from other adjacent molecules such as sugars and proteins (Spiteller, 2006).

The proteins most sensitive to radical attacks are those which contain a sulfhydril group (SH). It has been estimated that proteins can trap 50-75% of ROS. They can undergo either crosslinks by formation of bi-tyrosine bridges, or cuts in the event of strong aggression or modifications of certain amino acids in the event of moderate aggression (Favier, 2003). The oxidation of amino acids involves an attack on one of the methyl groups bonded to a nitrogen atom to form an amino acid radical which will react with oxygen to form an aldehyde with the expulsion of a hydrogen peroxide radical or hydrogen peroxide (Spiteller, 2006). Oxidized proteins lose their biological properties and become more sensitive to the action of proteases. They also become very hydrophobic, either by removing ionizable amine groups, or by exteriorizing the central hydrophobic zones and will then form abnormal clusters in or around the cells (Favier, 2003). Oxidative damage to proteins can even generate new antigens that elicit immune responses (Aruoma, 1999; Favier, 2003). Likewise, this oxidative damage can contribute to secondary damage such as the activation of DNA repair enzymes and loss of fidelity of DNA polymerases (Aruoma, 1999).

The mitochondrial genome has a susceptibility to oxidative stress 10 times greater than that of the nuclear genome (Richter et al., 1988). As the mitochondrial genome codes for a

few subunits of proteins involved in oxidative phosphorylation, their lack of expression could exacerbate the leakage of electrons from the respiratory chain for the production of ROS. Thus, the greater the electron leakage, the more the formation of ROS causing numerous mitochondrial mutations would worsen this phenomenon (Beckman and Ames, 1998).

Both in the mitochondria and in the nucleus of cells, oxidative damage on the bases of RNA or DNA can occur by Fenton reactions via the action of OH • radicals, under the effect of aldehydes of peroxidation lipid such as HNEs or by peroxynitrites (Sayre et al., 2005; Lyn Patrick, 2006). These alterations can lead to DNA splitting and be mutagenic (Sayre et al., 2005; Bloomer and Fisher-Wellman., 2008). Lipid peroxidation is also able to affect cell proliferation by forming intra and intermolecular bonds between sulfurized amino acids in RNAs and DNAs (El-Mesery et al., 2009). Guanines are preferentially attacked and the product of their degradation can be detected by measuring 8-hydroxydeoxyguanosine in the blood and in the urine (Finaud et al., 2006).

Sugars are attacked by ROS via the abstraction of hydrogen to form a carbonyl and expel a hydroperoxide radical (• OOH). The process is continued until a dicarbonyl compound is formed (Spiteller, 2006). By auto-oxidation, sugars such as glucose form dicarbonyl compounds, the best known of which are glyoxals and glycolaldehydes, which may bind to proteins and alter their chemical properties (Wells-Knecht *et al*., 1995). This has been demonstrated in diabetics and has been found to correlate with the severity of the disease (Glomb and Monnier, 1995) and contributes to the fragility of their vascular walls and their retina (Favier, 2003). The glyco-oxidation of sugars and the glycation of proteins have been demonstrated in agglomerations.

5. 3. Pathological implications of oxidative stress

Oxidative stress is involved in a large number of pathologies (**Figure 4**) as a triggering or promoting factor. It is therefore the initial cause of several diseases: cancer, cataracts, amyotrophic lateral sclerosis, accelerated aging (Favier, 2003).

It is one of the genesis factors of multifactorial diseases such as Alzheimer's disease, rheumatism and cardiovascular disease (Evans and Goldfine, 2000).

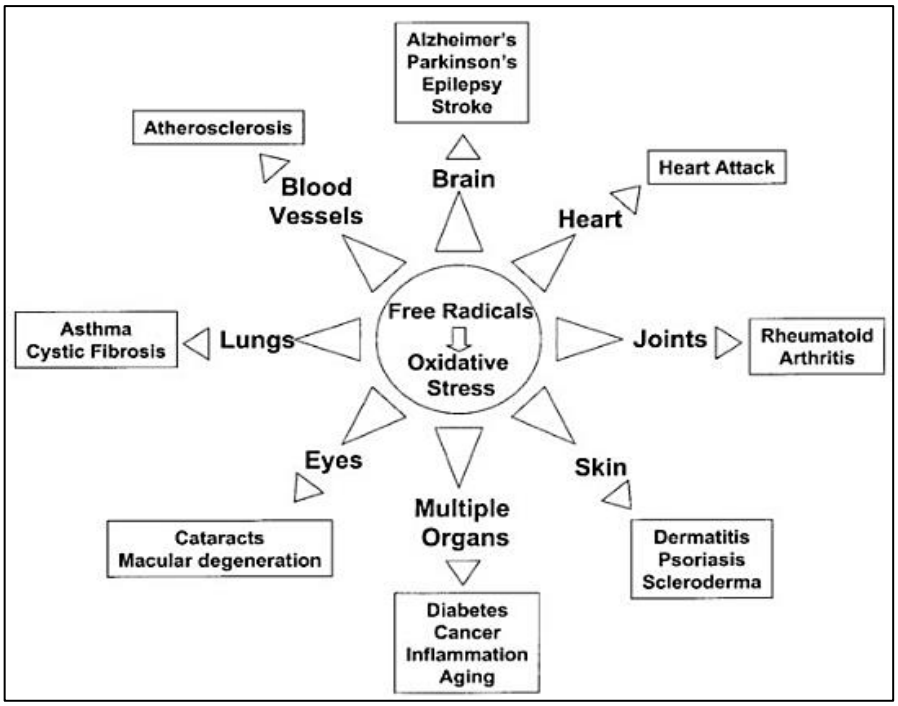


Figure 4. Main pathological circumstances accompanying oxidative stress (Evans and Goldfine,2000).

5.3.1 Cancer

Cancer is a multifactorial disease that is the disorderly and uncontrolled multiplication of certain normal cells in the body. These cells escape the normal mechanisms of differentiation and regulation of their multiplication and resist programmed cell death. For cancer to develop, the cell must accumulate several mutations in its genome (Macdonald *et al*., 2003).

- **Cancer treatment**

The goal of cancer treatment is the elimination of the cancerous tumor as well as the prevention of the appearance of other localized or metastatic tumors. In general, there are two methods of cancer treatment: Classic methods such as surgery, chemotherapy, radiotherapy, etc. are known for their compromising side effects. On the other hand, alternative methods such as herbal medicine remain by far a quality choice, especially when it comes to vulnerable patients (Rakhi *et al*., 2011).

- **Herbal medicine**

Herbal medicine is the use of plants as medicine to restore and keep the body in good condition. Over 80% of the world's population use it for various health problems (Farnsworth

and Kass, 1986). Several scientific studies have confirmed the beneficial effects of herbal remedies in curing or relieving several diseases including cancer. Indeed, a large number of in vitro studies have shown that the crude extracts, aqueous or hydro-alcoholic, of some medicinal plants exert a cytotoxic effect on various cancer cell lines (Jo *et al*., 2008 and Ray *et al*., 2010) Flavonoids are considered among the most important bioactive molecules.

5.3.2 Diabetes

Diabetes mellitus is an important endocrine metabolic disease (disorder) of multiple etiologies caused by alternating carbohydrate metabolism, which is characterized by abnormally high levels of plasma glucose which is termed hyperglycemia and glucose intolerance, with disruption of lipid and protein (Betu, 2018). The World Health Organization (WHO) defined Diabetes mellitus as a chronic state of hyperglycemia with fasting glycemia greater than 1.26 g/l (Makan, 2006). This disease is the consequence of an interaction of hereditary (acquired) and environmental factors (Murea *et al.,* 2012).

- **Main types of diabetes**

a) Type I diabetes (TID)

Type1 diabetes (TID) is represented by 5 - 10% of diabetics. Children and teenagers are frequently affected, but it can also occur at any age, including older people (ADA, 2014). TID is characterized by the more or less rapid destruction of the β cells of the Langerhans (Concannon *et al.,* 2009).

b) Type II diabetes (TIID)

Type II diabetes (TIID) is more prevalent, accounting for 90% to 95% of people with diabetes (ADA, 2014). TIID which is linked to genetic and environmental factors, is characterized by insulin resistance and a relative deficit in pancreatic β-cell insulin secretion (ADA, 2014; Stumvoll *et al.,* 2005).

- **Diabetes treatment**

a) Synthetic treatment

Insulin remains to be the most effective way to achieve natural and well-regulated blood sugar. The function of the insulin administered for the patient is to replace the body's Insulin. The key expected effects are to enhance the use of peripheral glucose and to activate

glycolysis, glycogenesis, lipogenesis, and protein synthesis. In addition, Insulin attempts to prevent lipolysis and gluconeogenesis (Bailey, 2019; Kelley and Good paster, 2001).

However, the continued use of these synthetic agents should be reduced, as these inhibitors are known to cause many side effects, such as unwanted gastrointestinal symptoms (gas, bloating, abdominal cramps, vomiting, abdominal distension and diarrhea). In addition to that, some of them may increase the incidence of kidney tumors, liver disorders (liver damage and acute hepatitis). These side effects may have been caused by excessive inhibition of pancreatic α-amylase, leading to bacterial fermentation of undigested carbohydrates in the colon. These drugs have other drawbacks such as liver toxicity and increase symptoms and risk factors for heart disease (Arulselvan *et al.,* 2014).

The World Health Organization Committee Expert in diabetes recommended the traditional medicinal plants to be further investigated, as they are frequently considered to be free from toxic and side effects. Therefore, the search for safer and more effective bioactive agents has continued to be an important biomedical drug development research (Arulselvan *et al.,* 2014).

b) **Natural treatment**

The use of medicinal plants to manage diabetes is one of the applications of natural medicine. The community, therefore, uses various plants to keep the level of blood glucose within standards. This practice has attracted researchers to perform experiments to clarify the mechanism of action of these natural remedies (Senyange *et al,*. 2015).

Plants have three ways function to treat diabetes mellitus. The first way is decreasing the supply of glucose in the blood by inhibiting hepatic glucose production (endogenous sources of glucose). Alternatively, by reducing intestinal glucose absorption (exogenous origin of glucose). The second strategy is increasing glucose intake by the peripheral tissues, particularly muscles. The third strategy involves insulin secretion stimulation (Hui *et al.,* 2009).

5. 4. Antioxidants

Antioxidants are compounds of endogenous or exogenous origin that serve to control the level of reactive species to neutralize them and minimize oxidative damage (Tang and Halliwell, 2010). The antioxidant defence mechanisms can be divided into enzymatic and non-enzymatic defences.

5.4.1. Enzymatic antioxidants:

- *Superoxide dismutase (SOD)*

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

- *Catalase (CAT)*

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

- *Glutathione peroxidase (GPx)*

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

5.4.2. Non-enzymatic antioxidants

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

- *Glutathione (GSH)*

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

- *Vitamin C (Vit C)*

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

- *Vitamin E*

Vitamin E is the major lipid-soluble component in the cell antioxidant defence system and is exclusively obtained from the diet. It has numerous important roles within the body

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

- Carotenoids

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

- *Polyphenols*

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

6. INFLAMMATION

The inflammatory reaction is the body's nonspecific defense response to aggression. The main function of inflammation is to eliminate the offending agent and allow tissue repair. Short-term (acute) inflammation is a beneficial phenomenon for the body which aims to restore its physiological integrity. While the negative aspect of inflammation occurs when the latter persists and becomes chronic (Weill *et al.*, 2003).

6.1. Acute inflammation

The acute inflammation lasts from a few days to a few weeks. Acute inflammation occurs in several phases (**Figure 5**).

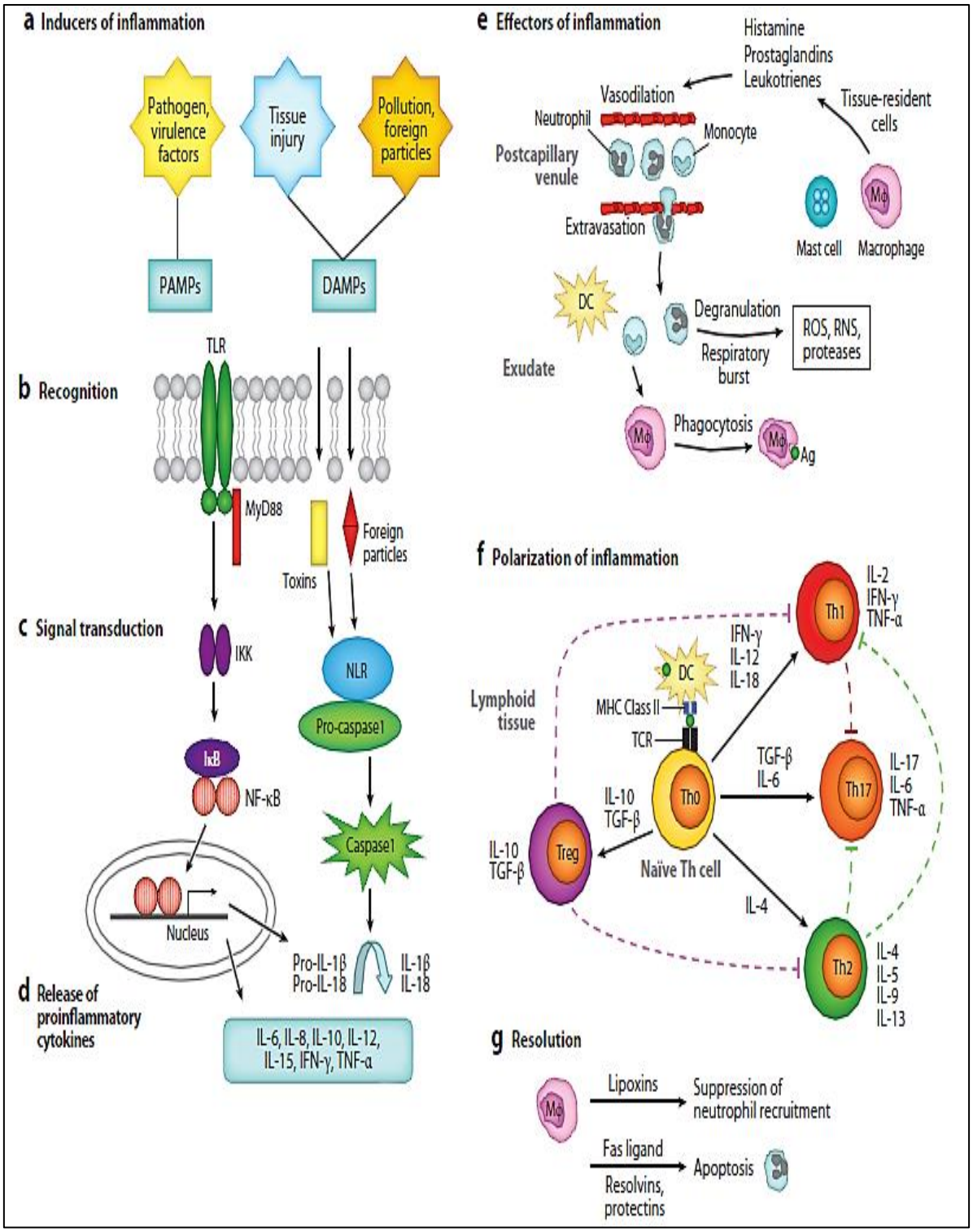


Figure 5. Stages of acute inflammation (Ashley *et al*., 2012).

6.1.1. Vascular phase

Activation of platelets is the first step in the vascular phase (Steinhubl, 2007). Resident mast cells are also likely to initiate the inflammatory response (Botting and Botting, 2000). Once these two types of cells are activated, several mediators such as serotonin, histamine and arachidonic acid derivatives are released. This induces vasodilation of the blood vessels with an increase in local flow and a change in vascular permeability, which allows extravasation of plasma proteins to the tissues (exudation). It is accepted that sensory neurons contain cyclooxygenases capable of synthesizing prostaglandins (Richardson and Vasko, 2002). The increased microcirculatory flow at the inflamed site explains the sensations of swelling and pain (Weill *et al*., 2003).

6.1.2. Cell phase

Plasma exudation allows the appearance of several substances in extravascular spaces (antibodies, bactericides, coagulation factors, complement components, interleukins, interferons, etc.) in addition to leukocytes (Steinhubl, 2007).

6.1.3. Resolution phase

Stopping the inflammatory response involves several mediators such as anti-inflammatory cytokines (IL-10 and TGF-b1, etc.), the expression of soluble receptors such as TNF-α and apoptosis of inflammatory cells. Tissue repair involves macrophages, endothelial cells and fibroblasts (Eming *et al*., 2007). The return to a physiological state consists first of all in the repair of the endothelium by the endothelial cells themselves (Weill *et al*., 2003).

6.2. Chronic inflammation

Chronic inflammation is characterized by a duration of months or years that can extend throughout an individual's life. Unlike what happens in acute inflammation, the vascular and cellular phases do not follow one another but coexist throughout the course of the inflammation. Phenomena of tissue destruction and repair attempts are also present (Fauve and Hevin, 1998).

6.3. Cardinal signs of inflammation

Inflammation usually manifests itself in four cardinal symptoms: heat, pain, redness, and swelling.

- **Pain**

Pain is defined by the WHO (World Health Organization) in 1979 as an unpleasant sensory and emotional experience related to actual or potential tissue damage or described as such. Bi-directional communications between the central nervous system and the immune system will be presented. Communications that are involved in the body's defense reactions which help increase the sensation of pain. The central nervous system constantly receives information about the condition of peripheral tissues and in return sends information to peripheral tissues via chemical messengers. These chemical messengers are mainly pro-inflammatory cytokines and chemical mediators (Calvino and Bouhassina, 2009).

- **Chemical mediators and pain**

During peripheral inflammation, molecules synthesized and released in the inflammatory focus, such as prostaglandins, bradykinin, serotonin, certain cytokines and NGF (nerve growth factor), act directly on the terminations of primary sensory neurons to stimulate them (Calvino and Bouhassina, 2009).

- **Edema**

Edema is the result of intravascular fluid passing through connective tissue. This passage is made possible by the increase in the permeability of the vessel wall which allows the leakage of a liquid rich in proteins such as vasoactive mediators including calcitonin gene-related peptide (CGRP), substance P (SP), bradykinin, and nitric oxide "NO" released by injured axons after synergistic neuro-immune interactions (diebold *et al*., 1995).

This inflammatory edema has beneficial effects on the body such as the dilution of toxic products, and the local supply of high molecular weight substances such as immunoglobulins and complement elements. On the other hand, inflammatory edema can be harmful to the organism, because of its abundance, its sudden onset as well as its site of installation (edema of the larynx, or of the brain) (diebold et al., 1995).

- **Heat**

Heat (locally) or fever (generally) corresponds to a situation of hyperthermia of the whole organism. The underlying mechanism is the local release of vasodilating factors, allowing blood to flow to the pathological area. Heat is considered an important clinical sign for the diagnosis of inflammatory syndromes (Lapointe, 2008).

- **Redness**

The redness or erythema appears quickly soon after the initial attack, initially, there is an intense release of cytokines as a result of the activation of macrophages and dendritic cells, these cytokines trigger the inflammatory reaction. Mainly They act on the wall of blood capillaries, causing them to dilate (vasodilate) (Lapointe, 2008).

6.4. Inflammatory cells

Several cell lines participate in the inflammatory process ;

- Polynuclear neutrophils
- Mast cells
- Monocytes
- Platelets
- Polynuclear basophils
- Eosinophilic polymorphonuclear cells

6.5. Inflammatory mediators

The inflammatory reaction is characterized by a succession of events allowing the delivery of leukocytes to the inflammatory focus. These steps are controlled by numerous humoral and cellular mediators (**Figure 6**) (Amulic *et al*., 2012). The main mediators are:

- Cytokines
- Neuropeptides
- Lipid mediators
- Complement fractions
- Coagulation factors
- Activated forms of oxygen and nitrogen

- Metalloproteases

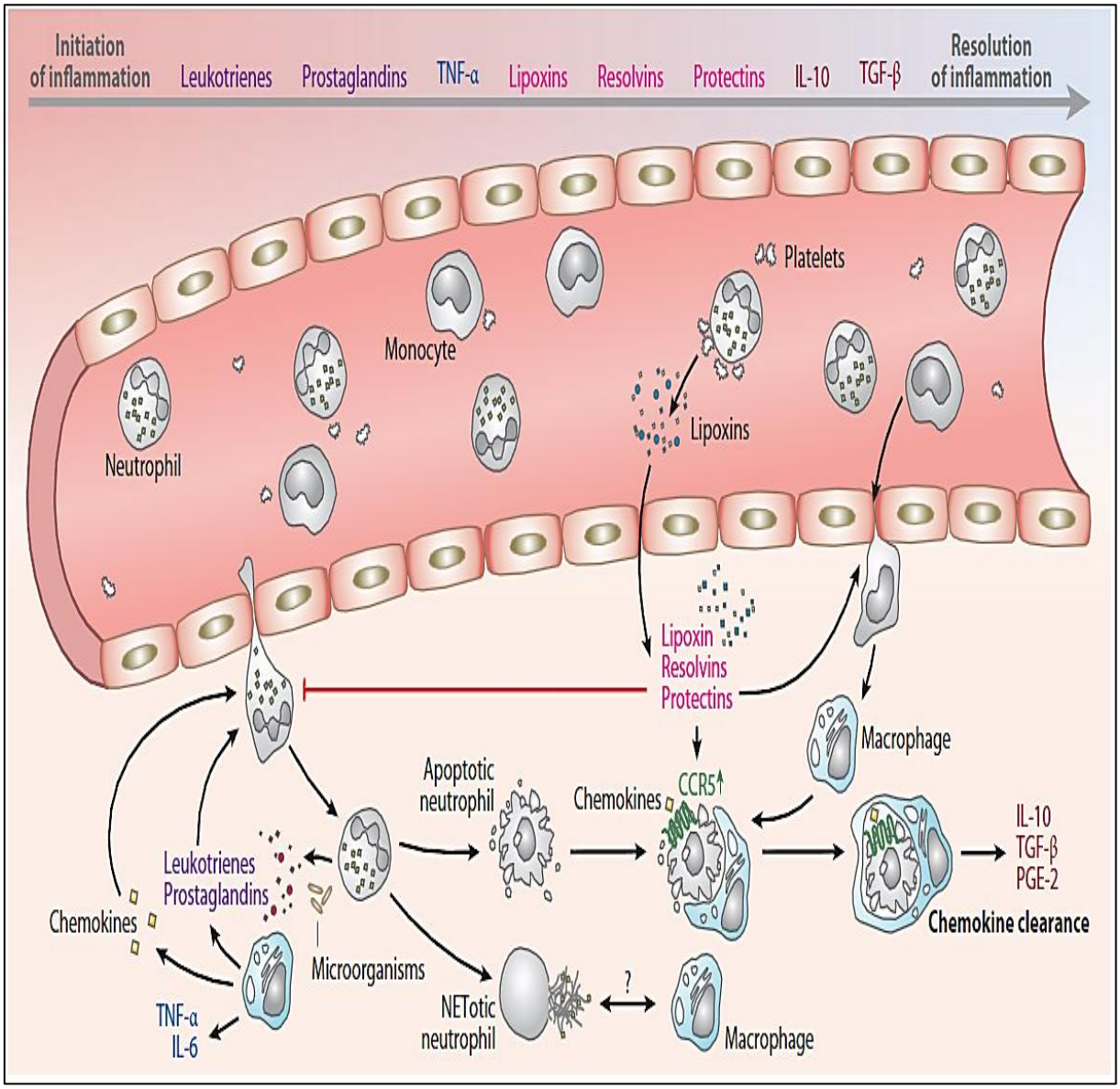


Figure 6. Cellular and biochemical mediators of inflammation (Amulic *et al*., 2012).

6.6. Anti-inflammatory drugs

An anti-inflammatory therapy intends to control the excess of nonspecific tissue reactions and to prevent the transformation of the acute phase of inflammation into a chronic phase.

6.6.1. Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used successfully for the relief of pain and fever. These are drugs with anti-inflammatory, antipyretic, and analgesic properties.

They share the non-selective inhibition of cyclooxygenase (Bidaut-Russel, 2008). However, the use of NSAIDs is associated with many adverse effects (Bidaut-Russell, 2008), attributed to their non-selective inhibition of cyclooxygenase isoforms (**Figure 7**). COX-1, which is found constitutively in most human tissues. Its inhibition by NSAIDs is responsible for the adverse effects. For this reason, pharmaceutical industry laboratories have tried to develop selective inhibitors of COX-2, an enzyme induced after the body is exposed to inflammatory stimuli (Bidaut-Russell, 2008).

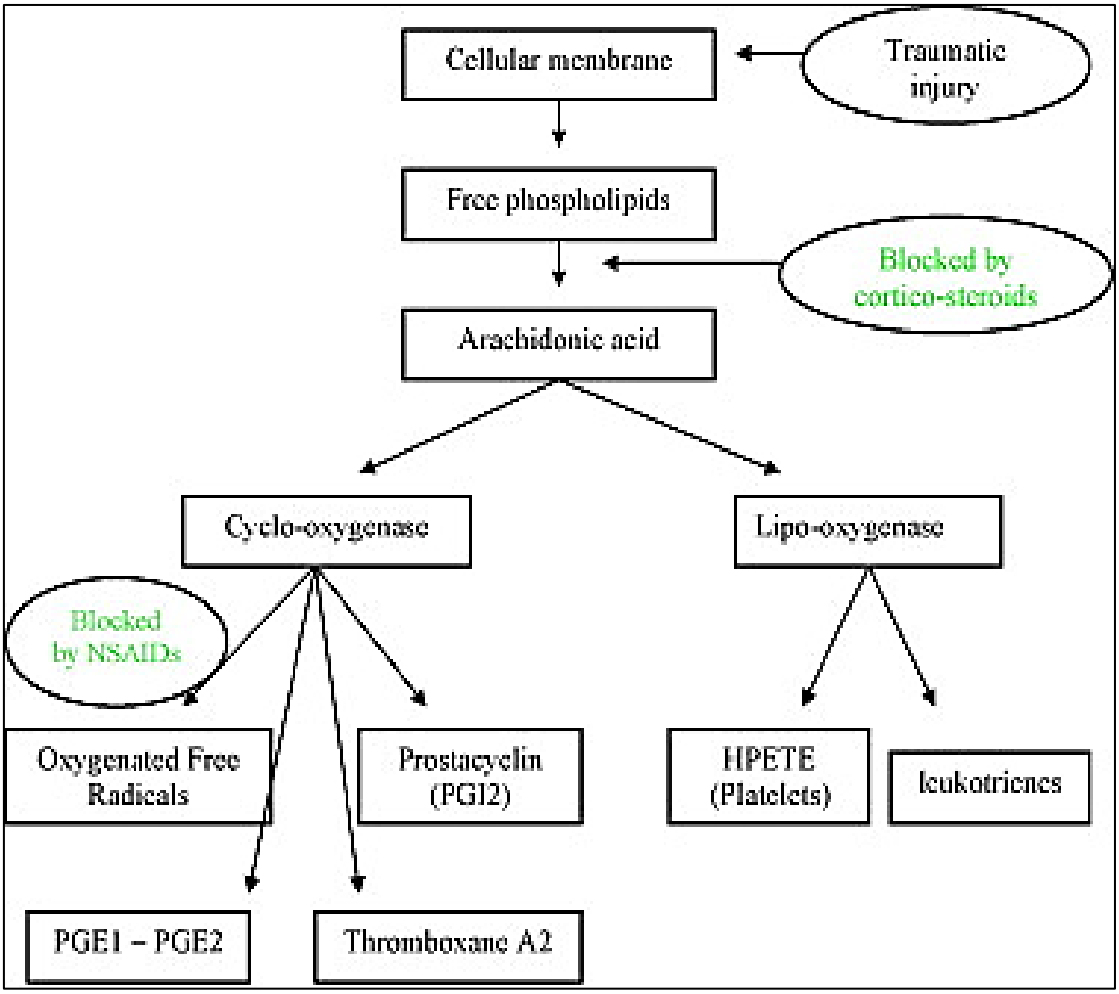


Figure 7. Arachidonic acid cascade and sites of anti-inflammatory action (Ziltener *et al*., 2010).

6.6.2. Steroidal anti-inflammatory drugs

Steroidal anti-inflammatory drugs (glucocorticoids) are a large family of drugs derived from cortisol. They are the most effective treatment used for chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, and autoimmune disease (Payne and Adcock, 2001). Their mechanism of action (**Figure 8**) is the same as that of

endogenous glucocorticoids. They induce an increase in the transcription of genes encoding anti-inflammatory proteins such as lipocortin-1 and interleukin 10, with an inhibition of the expression of several genes encoding pro-inflammatory proteins including cytokines, proteins, enzymes, receptors and adhesion molecules (Barnes, 1998).

As with NSAIDs, the use of glucocorticoids is associated with many side effects. The risk of developing these effects increases with the duration of treatment and the dosage. Various disorders can be observed, acute disorders such as arterial hypertension, deregulation of the natural synthesis of glucocorticoids, euphoria with insomnia up to acute psychosis and the appearance of peptic ulcers. Chronic disorders can also manifest themselves such as osteoporosis, cataracts and weight gain (Henzen, 2003).

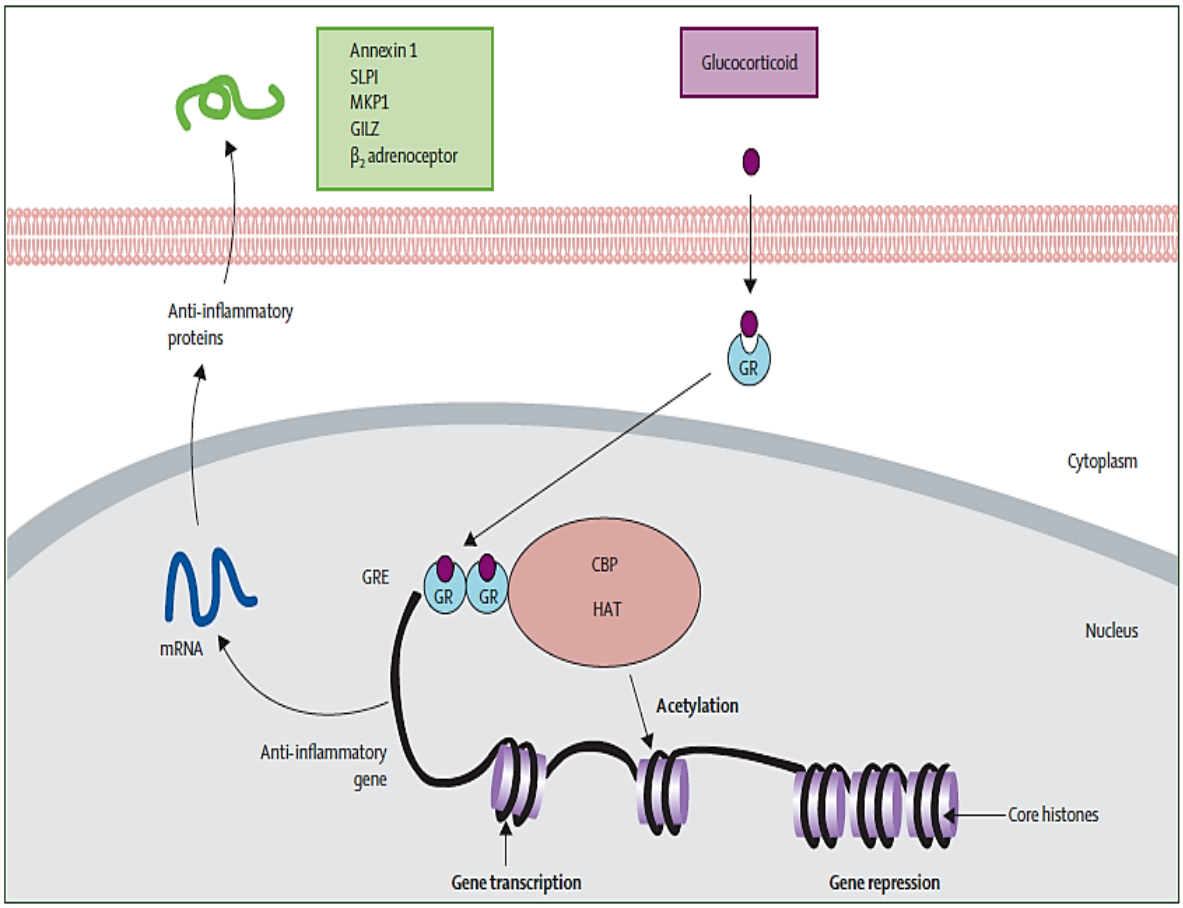


Figure 8. Mechanism of action of glucocorticoids (Barnes and Adcock, 2009).

GRE : Glucocorticoid Response Element; **CBP :** Cyclic AMP Response Element Binding Protein; **HAT:** Histone Acetyl transferase; **GR:** Glucocorticoid Receptor.

6.6.3. Anti-inflammatory drugs of plant origin

Plants are widely used in traditional medicine to relieve patients with certain inflammatory conditions such as rheumatoid arthritis, asthma, osteoarthritis, gout, allergic rhinitis, gastric and duodenal ulcers (Setty and Sigal, 2005; Wiart, 2006).

The anti-inflammatory activity of plants comes down to their content in bioactive secondary metabolites such as polyphenols, sterols, alkaloids, saponins, coumarins, terpenes, etc. These active substances can act at several stages of the inflammatory reaction by inhibiting the metabolism of arachidonic acid, the signal transduction mechanisms involved in the activation of inflammatory cells, the synthesis of pro-inflammatory cytokines, the expression of adhesion molecules, the activation of nuclear factor kappa-B and production of reactive oxygen species (Duwiejua and Zeitlin, 1993).Studies conducted *in vitro* and *in vivo* have shown the anti-inflammatory effect of a large number of plants as well as the mechanism of action.

7. TOXICITY

The study of toxicity covers a wide variety of areas. Indeed, from drugs to chemical weapons through plants, animals, industrial products, and many others. Humans are constantly exposed to toxicity, while other studies have shown that acute poisoning is the leading cause of hospitalization in developed countries and the second leading cause of death for individuals in developing countries (Viala and Botta, 2005).

Toxicity is a characteristic of any substance which, on contact or after presentation in an organism, has the property of causing dysfunction at the molecular, cellular or organic level (Bismuth *et al*., 1987), is an excellent criterion of orientation of the search for pharmacological activity.

The toxicity assessment is based on adequate qualitative (non-measurable) or quantitative (measurable) studies. There are several types of studies that allow us to assess the effects of a toxicant: epidemiological studies, which compare several groups of individuals or case studies, as well as in vitro studies performed on tissue cultures or cells (Gerin *et al*., 2003).

7.1. Toxic substances

Toxics are substances capable of disrupting the normal functioning of a living organism and can act at the point of contact (local effect) or enter the body. They are of natural source (eg dust, pollen), artificial (eg urea formaldehyde), chemical (eg acetone), or biological (eg aflatoxins) (Gilles, 2004). The main ways to absorb them are:

- Inhalation (respiratory tract)
- absorption through the skin (dermal route)
- ingestion (digestive tract)

The toxic action of a chemical substance for animal organisms is conditioned by its toxico kinetics and by the sensitivity of different animal species;

- Toxics not absorbed by the oral or percutaneous route will have a localized action.
- Toxics capable of entering the body will have a more diverse action.

This action depends on the biotransformations undergone which can give rise to more toxic metabolites and also on the location and accumulation of the substance and these metabolites in the body. The elimination of a toxic substance plays an essential role in its toxicity, when a toxic substance or its metabolites are rapidly excreted, they do not concentrate and do not damage cells (Gilles, 2004).

7.2. Toxic effect

The toxic effect is the result of an often complex process following an interaction between the toxicant and the organism, it is linked to the route of absorption, to the severity, to the time of appearance, and to the type of lesions, and it can cause a series of physiological and metabolic reaction. Some toxic effects are reversible, they go away more or less quickly after stopping exposure, while others are irreversible, they persist or even intensify after stopping exposure.

An acute or immediate effect is felt in a relatively short time (minutes, hours, days), while a chronic or delayed effect only manifests itself after a relatively long and permanent exposure time (weeks, months, or years) (Vaubaurdolle, 2007).

A morphological effect results in a change in the morphology of a tissue visible under light or electron microscopy, while a functional effect determines a change in the functions of an organ (liver, kidney) (Ben Youssef and Belguith, 2014).

7.3. Forms of intoxication

Toxicity is classified according to frequency of administration and duration of exposure in different forms; acute, subacute, subchronic, and chronic (Gilles, 2004).

7.3.1. Acute toxicity (short term)

Acute toxicity results from exposure to the toxicant over a short period of time relative to the body's life span. It is usually associated with a high dose but can also be associated with a lower concentration of a very toxic substance. It leads to serious physiological disorders or even death of organisms. The pathologies considered are necessarily rapidly developing because they are detected using short-term tests (Fleuretine *et al*., 1990).

Acute toxicity can be measured by the lethal concentration (LC50) for a percentage of the population exposed for a specified period of time. The effector concentration (EC) is that causing an observed effect. Thus, the LC50 (48 h) defines the dose which causes the death of 50% of organisms exposed to a toxicant for 48 hours. The lethal dose (LD50) is the dose transferred into the body by ingestion, inhalation, or contact, causing the death of 50% of individuals (Fleuretine *et al*., 1990).

- **Lethal dose LD_{50}**

The median lethal dose (LD_{50}) is the statistically deduced single dose, believed to cause the death of 50% of the animals to which the substance was administered (Gourlay-France *et al*., 2011). The product is generally administered to rats or mice divided into several groups at increasing doses sufficient to achieve a percentage mortality ranging between 0% and 100%.

The LD_{50} index is frequently used to express acute toxicity as well as to classify and compare toxicants. However, it has a very limited value, since it only concerns mortality and gives no information on the mechanisms involved and the nature of the lesions (Vaubaurdolle, 2007). It is expressed in mg of product per kg of body weight. The LD for a chemical

substance must always be indicated for a given animal species and for a determined route of administration (Lecoq *et al*., 2009).

➢ **Determination of LD_{50}**

The product tested is administered to a known species at different doses, the animals are observed for a period of 14 days following administration, any sign of toxicity will be noted, The construction of a curve giving the percentage of mortality as a function of the dose leads to determining the dose which would be the LD_{50} (Ben Youssef and Belguith, 2014).

➢ **Different methods for determining the LD_{50}**

The most common methods used to calculate LD50 are the graphical method of Litchflied and Wilcoxon (1949), the Gausso-logarithmic paper plot of Miller and Tainter (1944), and the moving average method of Thomson (1947) and weil (1952) (Ben Youssef and Belguith., 2014). However, to estimate the acute oral toxicity of substances with low toxicity or lacking toxicological data, the guidelines for testing chemicals described by the Organization for Economic Co-operation and Development OECD 425, (2008) are now widely used.

7.3.2. Subacute toxicity

It corresponds to the repeated administration of a product, over a period not exceeding 3 months. It makes it possible to identify the organ or the system on which the toxicant acts preferentially (Vaubaurdolle, 2007).

7.3.3. Long-term toxicity

Like the previous one, it is evaluated after repeated exposure to low concentrations of a given chemical, but on the other hand throughout the life of the animal (2 years in rodents such as rats or mice). It makes it possible to determine the long-term effects of a given product as a function of the total absorbed dose, which makes it possible to set threshold doses (or exposure limit values, in the case of the working environment) (Vaubaurdolle, 2007).

MATERIALS AND METHODS

The aim of this study was to evaluate the therapeutic effect of two Algerian endemic plants: *Genista Saharae (Coss. And Dur.) & Ononis Angustissima (Lam)* (Quezel and Santa, 1962-1963), by testing the different biological activities of its extracts obtained by subcritical water. An optimization of the extraction parameters was carried out firstly according to the yield of polyphenols, and then various biological tests were carried out. The experimental part was carried out at the level of biotechnology laboratory, University of *Novi Sad, Serbia*, CRBT of *Constantine*, and laboratory of applied microbiology of the University of *Ferhat Abbas-Sétif* -1-.

1. MATERIAL

1.1. Plant material

Genista saharae was harvested in Maiter Oued in the region of Bou Saada, South of M'sila (Algeria), and *Ononis angustissima* was harvested in the region of Hadjeb, west of Biskra. Both of plant samples were collected during flowering stage in April of 2019. The identification of collected plant samples was carried out by the Laboratory of development of natural biological resources (LVRBN, University Ferhat Abbas Setif). The aerial parts of each plant sample were dried, grounded in a blender, and stored in dark at ambient temperature until use.

1.2. Animals

Swiss albino female mice obtained from the Pasteur Institute in Algiers, weighing between 25 and 30 g were used in this study These mice are housed in polypropylene cages at room temperature, with free access to water and a standard diet provided by the Béjaïa National Office for Cattle Food (ONAB). After an adaptation period of 15 days, they were weighed and divided into groups of 5 mice each.

1.3. Chemical reagents and equipment

1.3.1. Chemical reagents

Several chemical reagents and solvents were used in this work. Among which: distilled water ΔH_2O, nitrogen (N_2) with a purity of 99 % (Messer, Germany), acetonitrile, formic acid, acetylsalicylic acid ASA, sulfuric acid H_2SO_4, hydrochloric acid (HCl), acetic acid (CH_3COOH), acetic anhydride, aluminum trichloride (AlCl3), gallic acid , methanol

CH_3OH, ethanol C_2H_5OH, 1-2,2-diphenyl-1-picryl hydrazyl (DPPH•), K_3Fe ferricyanide $(CN)_6$, trichloroacetic acid (TCA), butylated hydroxy toluene (BHT), Butylated hydroxyanisole (BHA), chloroform ($CHCl_3$), ferric chloride ($FeCl_3$), ammonia (NH_4OH), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), β-carotene, linoleic acid, Tween 40, monopotassium phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4) and fresh bovine blood, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), cis-diamminedichloroplatinum (Cis-DDP), Muller–Hinton broth (MHB). Almost all of the products came from the Sigma-Aldrich (Germany), Fluka (France), Riedel-de Haén and Prolabo laboratories. All chemicals and reagents were of analytical grade.

1.3.2. Equipment

House-made subcritical water extractor/reactor, UV-Vis spectrophotometer double beam (JENWAY 6305 UV / VIS), water bath (Memmert), pH meter (Hanna), magnetic stirrer (SCILOGEX), vortex (VELP), refrigerated centrifuge (ROTINA 420 R - HETTICH), Balance (OHAUS), freeze-dryer (CHRIST GAMMA 2-16 LSC plus).

2. METHODS

2.1. Phytochemical study

2.1.1. Subcritical water extraction

To obtain crude extracts of both plants, the fine powder obtained after cleaning, drying and grinding, was subjected to extraction with subcritical water. Subcritical water extraction (SWE) was performed in a house-made subcritical water extractor/reactor presented in **Figure 9**. Total capacity of high-pressure stainless steel vessel (6) was 1.7L. Pressurization of the vessel was performed with 99.999% nitrogen (Messer, Germany) through valve (3). Nitrogen was used in order to prevent oxidation at high temperatures. The operating pressure in the vessel was monitored by in-built manometer (2) (Inol, Slovenija, model IM 811A12). The process temperature was measured by a thermocouple Pt100 (4) and regulated by a temperature controller (8) (Nigos, Serbia, model 1011P). Vibrating platform (7) was used in order to enhance mass transfer and prevent local overheating in contact with heater (11) (Švarc-Gajić *et al*., 2017).

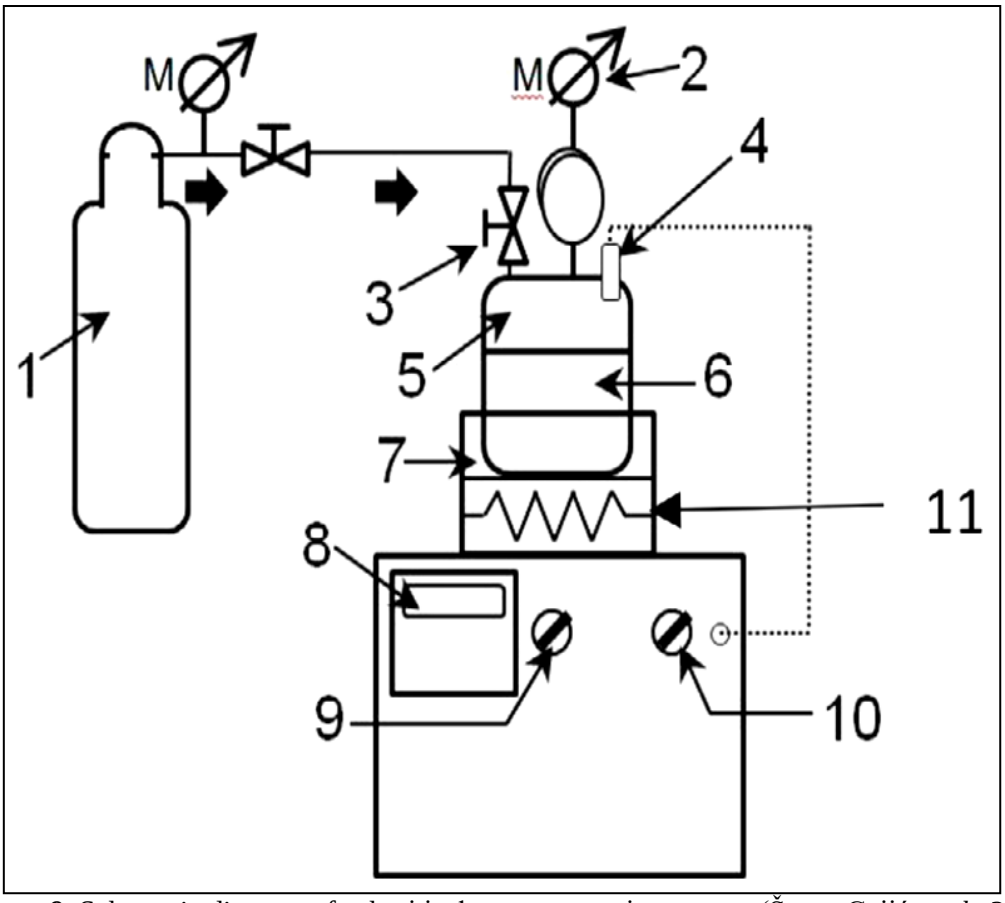


Figure 9. Schematic diagram of subcritical water extraction system (Švarc-Gajić *et al*., 2017); (1) nitrogen cylinder; (2) manometer; (3) input gas valve; (4) thermocouple for temperature measurement; (5) coverlid of extraction vassel; (6) extraction vassel; (7) vibrating platform; (8) digital temperature controller; (9) main swich; (10) swich for the vibrating platform; (11) heating plate**.**

In all the extractions performed, the sample/distilled water ratio was 1:20. The extraction temperature (110–185 ° C), the extraction pressure (10-90 bar), and the extraction time (15-60 min) were studied as independent variables in the first part of optimization, then the optimum values were retained for subsequent extraction, while the stirring speed (3 Hz) was kept constant. After extraction, the extraction vessel was immediately cooled in a continuous flow water bath at 20 ° C. Depressurization was performed by valve opening and purging nitrogen through a valve. Obtained extracts were filtered and stored in the refrigerator at 4 ° C until analysis.

In order to determine extraction yield (EY), 2 mL of liquid extracts was dried at 60°C until a constant mass. Further calculation of the total extraction yield was done according to the procedure described in Pharmacopoeia (Anekpankul *et al*., 2007).

2.1.2. Determination of total phenolic content

Total phenolic content was measured by the Folin-Ciocalteu method described by (Li *et al*., 2007). This method is based on the reduction in alkaline media of the phosphotungstic mixture of the Folin reagent by the oxidizable groups of the phenolic compounds, leading to the formation of reduction products of blue color. The latter exhibit an absorption maximum at 765 nm, the intensity being proportional to the amount of polyphenols present in the sample (Georgé *et al*., 2005).

The reaction mixture consisted of 400 µL of sample or standard solution and 2 mL of Folin-Ciocalteu reagent diluted 1:10. After 4 minutes 1.6 mL of 7.5%, (w/v) Na_2CO_3 was added. After 90 min of incubation at room temperature, the absorbance was measured at 765 nm. The blank was prepared by replacing the extract with distilled water. Gallic acid (0-200 mg/L) was used for the standard calibration curve. Results were expressed as mg gallic acid equivalent per g dry plant material (mg GAE / g), and calculated from the regression equation of the gallic acid calibration curve (**Figure 10**) in mean value ± SD (n = 3).

2.1.3. Determination of total flavonoid content

The determination of the flavonoids of the two extracts was carried out according to the aluminum trichloride ($AlCl_3$) method described by Bahorun *et al*., (1996). Flavonoids have a free hydroxyl group in a position capable of giving, in the presence of aluminum chloride, a yellowish complex by chelation of the Al^{+3} ion. The yellow coloration produced is proportional to the amount of flavonoids present in the extract (Basli *et al*., 2012). Briefly, 2 ml of 2% $AlCl_3$ was added to 2 mL of the extract or standard solution. After 10 minutes, the absorbance was measured at 430 nm. Rutin (0-125 mg/L) dissolved in distilled water was used as a standard (**Figure 10**). The results were expressed in mg of rutin equivalent per g of dry plant material (mg RE/g), and calculated as a mean value ± SD (n = 3)

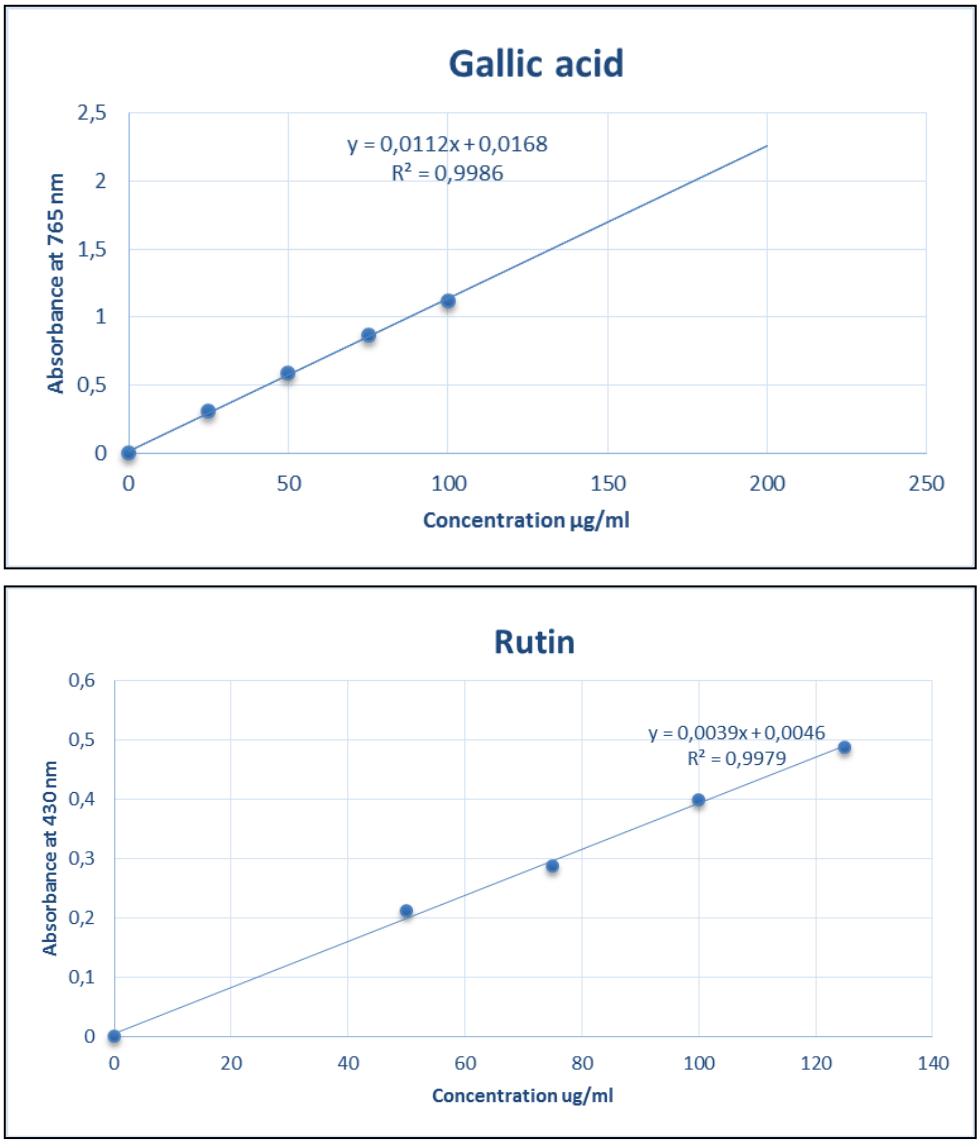


Figure10. Calibration curve of galic acid and rutin.

2.1.4. Identification and quantification of phenolic compounds by RP-HPLC-UV/Vis

Reversed-Phase High-Performance Liquid Chromatography with Ultraviolet/Visible detector (RP-HPLC-UV/Vis) is an important analytical technique with strong chromophores that absorb light in the wavelength region from 200 nm to 800 nm (Venkatesh *et al*., 2008). Numerous publications and research papers focus on separation methods to detect phenolic antioxidants using RP-HPLC-UV/Vis (Chawla and Mrig, 2009; Capitán-Vallvey *et al*., 2004; Lee *et al*., 2006).

Chromatograms were recorded using tree different wavelength (280 nm, 320 nm, and 360 nm) depending of the maximum absorption of the phenolic compounds. Separation was performed on a Luna C-18 RP column, 5 mm, 250 x 4.6 mm with a C18 guard column, 4 x 30 mm (both from Phenomenex, Torrance, CA, USA). The chromatographic conditions were the

following: flow rate 1.0 mL/min, sample injection volume of 20 µL and mobile phase A (acetonitrile) and B (1% formic acid). A gradient profile was used as follows: 0–10 min from 10 to 25% B; 10–20 min linear rise up to 60% B, and from 20 min to 30 min linear rise up to 70% B, followed by 10 minutes reverse to initial 10% B with additional 5 min of equilibration time, Sample injection volume of 20 µl (Šaponjac *et al*., 2015).

The quantification was conducted at 280 nm for monomeric flavan-3-ols (catechin and epicatechin), for hydroxybenzoic acids (gallic, vanillic, protocatechuic, syringic, p-hydroxybenzoic), at 320 nm for caffeic acid, chlorogenic acid, p-coumaric acid, gentisic acid, sinapic acid, and rosmarinic acid, and at 360 nm for rutin, luteolin, kaemferol, and quercetin. Lab Solutions software (Shimadzu Corporation, Kyoto, Japan) was used for control and data processing. Analytes in each extract were identified by comparing their retention times and UV-VIS spectra with those of standard compounds. Peak purity was checked to exclude any contribution from interfering peaks. The concentrations of individual phenolic compounds in extracts were determined using external standard calibration curves in the concentration range of 1 to 50 mg/L using a mixture of 17 standards. Results were means of triplicate injections and expressed as mg/g dry extract.

The results represented are means ± standard deviation. Statistical analyses were carried out using Origin 8.0 SRO software package and Microsoft Office Excel 2010 software. Significant differences were calculated by ANOVA ($p < 0.05$).

2.2. The *in vitro* evaluation of biological activities

2.2.1. The evaluation of antioxidant activity

2.2.1.1. Reducing power

The reducing power of the SW/extracts is measured by the direct reduction of the ferric form $Fe^{3+}(CN^{-})_6$ into a ferrous form $Fe^{2+}(CN^{-})_6$ which is determined by the spectrophotometric detection of the complex $(Fe^{3+})_4[Fe^{2+}(CN^{-})_6]_3$ having a strong absorption at 700 nm (Oyaizu, 1986). Different concentrations of extracts and BHT (Butylated Hydroxytoluen) are mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide $[K_3Fe(CN)_6]$ at 1%. After 20 minutes of incubation at 50°C, 2.5 mL of Trichloroacetic acid TCA (10%) are added to the reaction medium, and then the mixture is centrifuged at 3000 rpm for 10 minutes. Then 2.5 mL of supernatant is mixed with 2.5 mL of

distilled water and 0.5 mL of $Fecl_3$ (0.1%) and the absorbance is read at 700 nm against a blank without $Fecl_3$.

EC_{50}s are calculated from the graph of absorbance versus sample concentration.

2.2.1.2. DPPH radical-scavenging assay

For the DPPH radical-scavenging assay the procedure followed the method of Espin *et al,*. (2000) in which the samples express their antioxidant activity by the reduction of purple colored DPPH to the yellow colored diphenylpicrylhydrazine derivatives whose intensity is inversely proportional to the capacity of antioxidants (Cuendet *et al*., 1997; Burits and Bucar, 2000). It makes it possible to evaluate the rate of reduction of DPPH • and therefore provides a practical means of measuring the antioxidant power of studied extracts.

Briefly, 0.5 mL of the extract or standard (BHT) solutions at various concentrations was mixed with 0.5 mL (0.1 mM) of 2,2- diphenyl-1-picryl-hydrazyl (DPPH•) solution. The mixtures were incubated at room temperature for 30 min at room temperature in the dark, the absorbance was measured at λ= 517 nm by UV-Vis spectrophotometer. The control contains all reagents except the test sample, which is replaced with an equal volume of methanol. Methanol was used to set zero of transmittance. The percentage scavenging of DPPH• radical was calculated by the following equation:

% Inhibition = [(AC - AS) /AC] × 100

Where: **AC** is the absorbance of the control solution; **AS** is the absorbance of the sample (standard or extracts).

The inhibitory concentration of 50% of DPPH• activity (IC_{50}) of each extract was then calculated from the equation that determines the percentage of inhibition as a function of the concentration of the inhibitor. It was expressed in µg/mL and compared with that of BHT.

To better characterize the antiradical power, two other parameters are calculated: the effector concentration at 50% (EC_{50} = IC_{50}/mg of DPPH/mL) and the antiradical power (ARP) which is only the inverse of the EC_{50} (ARP = $1/EC_{50}$).

Note: The EC_{50} takes into account the concentration of DPPH• present in the reaction medium (Prakash *et al*., 2007).

2.2.1.3. *β*-Carotene Bleaching Assay

The β-carotene bleaching assay for evaluating antioxidant activity is one of the common methods used in the field of food chemistry. The principle of the method is based on the discoloration of yellowish color of a β-carotene solution due to the breaking of π-conjugation by addition reaction of lipid or lipid peroxyl radical (L· or LOO·) to a C=C double bond of β-carotene. The radical species is generated from the autoxidation of linoleic acid by heating under air atmosphere. When the appropriate antioxidant is added to the solution, the discoloration can be retarded by competing reaction between β-carotene and antioxidant with the subjected radicals (Al-Saikhan *et al*., 1995).

The technique developed by Taga *et al*., (1984) consisted of measuring the bleaching of β-carotene resulting from oxidation by degradation products of linoleic acid was adapted. Briefly, 1.0 mg β-carotene was dissolved in 10 mL chloroform. The absorbance was tested after adding 0.2 mL of the solution to 5 mL of chloroform, then reading the absorbance of this solution at 470 nm. A reading between 0.6 and 0.9 indicated a workable concentration of β-carotene. One mL of β-carotene chloroform solution was added with a pipette to a boiling flask that contained 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed using a rotary evaporator at 40°C, 50 mL of oxygenated distilled water was slowly added to the flask with vigorous agitation to form an emulsion. Five mL of the emulsion was added to 0.2 mL of the extracts solution, ethanol/water (negative control), or reference antioxidants BHT and BHA. A blank consisting of 20 mg linoleic acid, 200 mg Tween 40 and 50 mL oxygenated water was used to bring the spectrophotometer to zero. The tubes were placed in an agitating water bath at 50°C. Absorbance measurements were made at 15 min intervals for 120 min.

The antioxidant activity percentage was calculated by the following equation:

$$\mathbf{AA\ (\%) = [1- (A_{H0} - A_{Ht}) / (A_{C0} - A_{Ct})] \times 100}$$

Where: AA (%): Antioxidant activity percentage;

A_{H0}: absorbance value of β-carotene in presence of the extract measured at t = 0;

A_{C0}: absorbance value of β-carotene in presence of negative control measured at t = 0;

A_{Ht}: absorbance value of β-carotene in presence of the extract measured at t = 120 min;

A_{Ct}: absorbance value of β-carotene in presence of negative control measured at t = 120 min.

2.2.2. Anti-proliferative activity assay

The influence of *G.saharae* and *O.angustissima* SW/extracts on the growth of malignantly transformed cell lines, as well as on a mouse fibroblasts (L929) cell line, was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.

Cell lines were donated from the collection of cell lines of the Institute of Virology, Vaccines and Serums "Torlak", Belgrade. The following cell lines were used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma-HeLa derivative) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2×10^5 cell/mL; 100 μL/well) in 96-well cell culture plates in nutrient medium (Minimum Essential Medium (MEM) Eagle supplemented with 5% of Hep2c, RD, L2OB, and L929) and grown at 37 ◦C in humidified atmosphere for 24 h. Then, tested extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100 μL/well) and cells were incubated at 37 ◦C in humidified atmosphere for 48 h. Pure nutrient medium (100 μL) represented positive control for each cell line.

After incubation period, supernatants were discarded and MTT (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500 g/mL) was added to each well (100 μL/well). Immediately after, all wells were incubated at 37 ◦C in humidified atmosphere for 4 h. Reactions were halted by adding 100 μL of sodium dodecyl sulfate (SDS) (10% in 10 mM HCl). After overnight incubation at 37 ◦C, absorbance was measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA). The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against A580. Corresponding cells (grown in flasks), after cell count by haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/DMEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (ut supra).

The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated

extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the cis-diamminedichloroplatinum (Cis-DDP) determined in positive control wells as the 100% growth (Mosmann, 1983; Shiradkar *et al*., 2011; Baviskar *et al*., 2012; Švarc-Gajić *et al*., 2017). All experiments were done in triplicates.

2.2.3. Antimicrobial activity assay

Anti-microbial activity was determined by microdilution method in 96 multi-well microtiter plates (Sarker *et al*., 2007) testing five different concentrations of *G.saharae* and *O.angustissima* SW/extracts (19.5, 39.1, 78.1, 156.2 and 312.5 μg/mL) against six bacterial strains, two Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) and four Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153), as well as two fungal strains (*Candida albicans* ATCC 10231, *Aspergillus Niger* ATCC 16404).

The identification of the tested microorganisms was confirmed by the Laboratory of Mycology of the Department of Microbiology of the Torlak Institute in Belgrade, Serbia. Analysis was performed by using both bacterial and fungal strains. Standard antibiotic Amricin was used to control the sensitivity of the tested bacteria, whereas Nystatin was used as a control against tested yeast strains. All tests were performed in Muller–Hinton broth (MHB) except for those for yeast, in which case Sabouraud dextrose broth was used. A volume of 100 μL stock solutions of extracts (in methanol, 200 μL/mL) were pipetted into the first row of the plate. 50 μL of Mueller-Hinton or Sabouraud dextrose broth (supplemented with Tween 80 to a final concentration of 0.5% (v/v)) were added to other wells. A volume of 50 μL from the first test wells was pipetted into the second well of each microtiter line, and then 50 μL of scalar dilution was transferred from the second to the twelfth well. 10 μL of resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water) and 30 μL of nutrient broth were added to each well.

Finally, 10 μL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) were added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Plates were wrapped loosely with cling film to prevent

dehydration and prepared in triplicates. The plates were placed in an incubator at 37 ºC for 24 hours for the bacteria, and at 28 ºC for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the measured inhibitory concentration (MIC value). The average of three values was calculated, and the obtained value was taken as the MIC for the tested compound and standard drug.

2.2.4. Anti-diabetic Activity

- ✓ **Anti-hyperglycemic Activity (AHgA) by a-Glucosidase Bioassay**

α-Glucosidase inhibitory activity was performed by the previous method (Palanisamy *et al*., 2011) with some modifications. Sample solution (2 mg/mL; 50 µL) was mixed with glutathione (50 µL), *α*-glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20, Sigma) (50 µL) in phosphate buffer (pH 6.8) and PNPG (4-N-trophenyl--d-glucopyranoside) (50 µL) in a 96-well microplate and incubated for 15 min at 37 ◦C. Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (*α*-glucosidase) solution. The reaction was then stopped with the addition of sodium carbonate (50 µL, 0.2 M). The sample and blank absorbances were read at 400 nm with a micro plate reader (Tecan, Grodig, Austria). The absorbance of the blank was subtracted from that of the sample and the *α*-glucosidase inhibitory activity was expressed as acarbose equivalent (mg ACE/g extract).

- ✓ **Alpha amylase inhibition assay**

α-Amylase inhibitory activity was performed using Caraway Somogyi iodine/potassium iodide (IKI) method (Yang *et al*., 2012) with some modifications. Sample solution (25 µL) was mixed with *α* -amylase solution (ex-porcine pancreas, EC 3.2.1.1, Sigma) (50 µL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well microplate and incubated for 10 min at 37 ◦C. After pre incubation, the reaction was initiated with the addition of starch solution (50 µL, 0.05%). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (*α*-amylase) solution. The reaction mixture was incubated 10 min at 37 ◦C. The reaction was then stopped with the addition of HCl (25 µL, 1 M). This was followed by addition of the iodine-potassium iodide solution (100 µL). The sample and blank absorbances were read at 630 nm using a micro plate reader (Tecan, Grodig, Austria). In the presence of an α-amylase inhibitors less starch would be hydrolyzed into maltose or glucose, and the absorbance value would be decreased.

The absorbance of the blank was subtracted from that of the sample and the α-amylase inhibitory activity was expressed as acarbose equivalent (mg ACE/g extract).

2.2.5. Anti-inflammatory activity (AIA) by protein denaturation assay

In vitro assessment of anti-inflammatory activity of SW/extracts was determined by protein denaturation bioassay, using bovine serum albumin (BSA), according to the method adopted by Lavanya *et al*. (2010). This method consists of preparing four solutions:

1- The test solution (0.5 mL) composed of 0.45 mL of the aqueous solution of bovine serum albumin (BSA) 5% and 0.05 mL of each SW/extract with a concentration of 250 µg/mL.

2- The test control solution (0.5 mL) composed of 0.45 mL of the aqueous solution of 5% BSA and 0.05 ml of distilled water.

3- The product control solution (0.5 mL) composed of 0.45 mL of distilled water and 0.05 mL of each SW/extract with a concentration of 250 µg/mL.

4- The standard test solution (0.5 mL) consists of 0.45 mL of the aqueous solution of BSA 5% and 0.05 mL of the solution of the reference anti-inflammatory grug Diclofenac sodium with a concentration of 250 µg/mL.

The pHs of all the mentioned solutions above are adjusted to 6.3 with a solution of HCl (1N), then incubated at 37° C. for 20 min. Then, the temperature was increased to 57°C for 3 min followed by cooling of the tubes. Then, 2.5 mL of the phosphate buffered saline solution (pH 6.3) was added to all previously mentioned solutions.

Absorbance was measured by a UV–visible spectrophotometer at 416 nm, and percent inhibition of protein denaturation was calculated according to the following equation:

% of inhibition = [100 - (A of test solution – A of product control solution) × 100]/ A of test control solution

The control represents 100% of the denatured proteins and the results are compared with sodium Diclofenac (250 µg/mL) (Williams *et al*., 2008; Sangita *et al*., 2012).

2.3. Toxicity evaluation

2.3.1. Evaluation of the acute oral toxicity

In order to assess any possible risk of toxicity during biological tests, it was necessary to carry out toxicity tests on SW/extracts from the studied plants *G.saharae* (SWG) and *O.angustissima* (SWO). This can be determined by several methods, of which we cite as examples: the method of Dragstedt and Lang, the method of Karberet Behrens (Stowtchiva, 1988), the method of Litchfield and Wilcoxon (Dupont et al., 1970) ... etc. However, these methods require a large number of animals.

In our approach, we adopted the guidelines for the testing of chemicals OECD 425. (2008) which is based on the *Bruce* method of studying acute oral toxicity and dose adjustment methods. This procedure, described by the Organization for Economic Co-operation and Development (OECD), helps to reduce the number of animals necessary to estimate acute oral toxicity to a minimum. On the other hand, the limit test offers an effective means of identifying substances which may be of low toxicity or whose toxicological data are unavailable as in our case.

In addition to the observation of physiological and/or behavioral signs of toxicity, the test makes it possible to estimate the lethal dose 50 (LD_{50}) which represents the dose of a substance which, administered to laboratory animals, causes the death of half of them.

The LD_{50} makes it possible to measure the toxicity of a substance, and to establish toxicity classes (Stowtchiva, 1988; Oduola *et al*., 2007). In general, the lower the LD_{50}, the more toxic the substance. The opposite is also true: the higher the LD_{50}, the lower the toxicity, as shown in **Table 3**.

Table 3. Toxicity Classes in Laboratory Mice according to Diezi scale (Diezi, 1989).

Oral LD_{50}	**Toxicity index**
LD_{50} <5 mg / kg	very toxic
5 mg / kg <LD_{50} <500 mg / kg	toxic

500 mg / kg <LD_{50} <5000 mg / kg	low toxicity
LD_{50}> 5000 mg / kg	not toxic (harmless)

Practically, two doses of each extract of our plants (2 and 5 g / kg) were tested on groups of five mice at most, of homogeneous weight. First of all, the mice are divided into 5 lots as follows: (Lot1: Placebo, Lot2: 2g / kg of SWG, Lot3: 5g / kg of SWG, Lot4: 2g / kg of SWO, Lot5: 5g / kg SWO) (OECD 425, 2008). Mice in the placebo group were given the same volume of distilled water in the same way. Doses are administered orally (single dose) in a sequential fashion. If at least three animals die, the LD_{50} is assumed to be less than the administered dose, and the principal test is performed to determine the LD_{50}. Otherwise, the toxicity test is completed, and all that is needed is to note that the LD_{50} is greater than the doses administered.

The five groups were observed individually (different behavioral disturbances of the animals, compared to that of the untreated group, such as agitation, respiration, asthenia, and death) at least once during the first 30 minutes following the administration of the product, and regularly during the first 24 hours (with particular attention during the first 4 hours), then daily thereafter, in order to record late effects including the number of deaths.

Therefore, the assessment of toxic effects is made based on the examination of behavior and mortality rate. The total observation period is 14 days. The animals are kept at room temperature with free access to water and food

2.4. Statistical analysis

The results of tests performed in vitro are expressed as mean value ± SD. EC_{50} and IC_{50} are calculated by linear regression from the [% inhibition = f (concentrations)] curve.

The significance of the differences between the control and the different tests is determined by the univariate ANOVA test followed by the Dunnett / Tukey test for multiple comparisons. The differences are considered statistically significant at the threshold of 0.05 ($p \leq 0.05$).

RESULTS AND DISCUSSION

1. Optimisation of the extraction parameters

The aim of this step was to define optimal conditions for SWE of phenolic compounds from *O. angustissima* and *G. saharae*. Afterward, various biological tests of SW/extracts obtained in optimal parameters were carried out. According to available literature, subcritical water extraction hasn`t been previously applied in the extraction of these plant matrices.

1.1.The influence of the extraction temperature

Temperature is the most important factor in the SWE process, influencing extraction efficiency and selectivity (Hawthorne *et al*., 2000; Švarc-Gajić, 2012; Švarc-Gajić, and Cvetanović., 2014; Švarc-Gajić *et al*,. 2017). The influence of the extraction temperature on the extraction efficiency was investigated at six different temperatures (110-185ºC), applying the extraction time of 30 min, agitation frequency of 3 Hz, and pressure of 20 bar. The temperature influence was observed by measuring yields of total phenols (expressed as mg of gallic acid equivalent per g of dry plant material (mg GAE/g)) and flavonoids (expressed as mg of rutin equivalent per g of dry weight of plant (mg RE/g)) (**Table 4**).

Table 4. The influence of the extraction temperature on the yields of total phenols and flavonoids.

Temperature (°C)	*O. angustissima*		*G. saharae*	
	TPC[a] (mg GAE/g)	TFC[b] (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)
110	11.30 ±0.34 [c]	9.13 ±0.14	18.03 ±0.24	8.68 ±0.18
125	11.55 ±0.3	7.81 ±0.2	19.93 ±0.19	7.59 ±0.09
140	11.67 ±0.32	8.29 ±0.2	20.90 ±0.29	7.73 ±0.15
155	16.19 ±0.12	6.99 ±0.13	24.80 ±0.43	7.04 ±0.15
170	15.34 ±0.36	6.19 ±0.13	21.82 ±0.19	6.33 ±0.07
185	13.88 ±0.4	5.11 ±0.16	18.58 ±0.31	4.79 ±0.11

[a] mean total phenols content (GAE: gallic acid equivalents).
[b] mean total flavonoids content (RE: rutin equivalents).
[c] mean± SD

Total phenols content increased with the extraction temperature from 110 to 155ºC for both studied plants, *O. angustissima,* and *G. saharae*. With further temperature increase, a decrease in phenolic content was observed probably due to their degradation. The highest concentration of total phenols for *O. angustissima* (16.19 mg GAE/g) and *G. saharae* (24.80 mg GAE/g) was achieved at the temperature of 155°C (**Table 4**).

The highest concentration of total flavonoids was observed at the lowest tested temprature (110°C) for both plants, *O. angustissima* (9.13 mg RE/g), and *G. saharae* (8.68 mg RE/g). With further temperature increase there was a slight decrease in flavonoids content for both plant sampels (**Table 4**).

The increase in phenolic content with the temperature could be explained by enhancement of the diffusion coefficients, and solubility of the target compounds with temperature increase. Decreased viscosity and surface tension of the solvent are allowing better contact with porous solid samples (Mustafa and Turner, 2011; Švarc-Gajić, 2012; Tomšik *et al*., 2017). The most pronounced temperature influence however, is on the polarity, by varying the extraction temperature, the dielectric constant of water can be modulated affecting it`s selectivity. Consequently, the polarity of water at elevated temperatures becomes equivalent to that of common organic solvents, targeting specific chemical class. This implies that more polar solutes soluble in ambient water are extracted efficiently at lower temperatures, whereas moderately polar and non-polar compounds require less polar solvent and higher temperatures (Smith, 2006; Švarc-Gajić *et al*,. 2017). It was therefore shown that major phenolics classes in analyzed plant samples are such that they are the best solubilized with water at 155°C for both studied plants (*O. angustissima* and *G. saharae*).

The temperature affects physicochemical properties of water, but also causes degradation of the thermally labile analytes (Kronholm *et al*., 2007). Thus at higher temperatures, it can be assumed that a part of phenols and flavonoids was degraded due to high water reactivity and strong hydrolytic potential of superheated water (Švarc-Gajić, and Cvetanović., 2014), causing the drop in the extraction yields of phenols above 155°C for the stidied plants, and also the steadily decreasing the flavonoids content above 110°C.

Several previous studies of SWE conducted with other plant samples (Švarc-Gajić, and Cvetanović., 2014; Cvetanović *et al*., 2017; Tomšik *et al*., 2017; Švarc-Gajić *et al*., 2018; Nastic and Švarc-Gajić., 2018) have reported similar fashion of the decrease in phenols content with the temperature increase. The reported optimal temperatures for total phenols in

SWE of *Teucrium montanum, Chamomilla matricaria, Matricaria recutita,* (*Prunus avium, Prunus cerasus*), and *Allium ursinum L.* were 160 °C, 130°C, 160°C, 150°C, and 179°C respectivelly.

1.2.The influence of the extraction pressure

The principal operational parameter in SWE is the temperature owing to effects described earlier. In SWE applied pressure mostly serves to maintain water in its liquid state, even though slight efects on water polarity with pressure increase have been reported. Namely, water polarity negligibly increases with pressure, not favoring the process of the extraction of less polar solutes, and making the process operationally less convenient (Švarc-Gajić, 2012). Elevated pressures, however, allow better solvent penetration into the pores of extracted medium making the solvent/solute contact more intimate and accelerating the process. Unlike temperature, the pressure has no significant effect on the extraction efficiency by subcritical water (Cvetanović *et al*., 2017; Švarc-Gajić *et al*., 2018). Previous studies have also shown that elevated pressures didn't improve the recovery of compounds from natural sources by SWE (Deng *et al*., 2004; Deng *et al*., 2005; Kim *et al*., 2009). However high pressures applied during the extraction help to control problems related to the formation of air bubbles within the matrix, which hinder solvent contact with the matrix (Mustafa and Turner 2011; Švarc-Gajić, 2012). In order to determine the influence of pressure on the recovery of phenols from studied plant materials, investigation was carried out at previously defined optimal temperature of 155°C, applying agitation rate of 3 Hz, and extraction time of 30 min. The influence of this operational parameter was observed at four different pressures (10, 30, 50, and 70 bar). **Table 5** shows extraction yields of phenols and flavonoids obtained by SWE at different pressures.

Investigation of the pressure influence revealed complex and diverse effects for both plant matrices and both chemical classes. In all cases studied here, the pressure did slightly affect flavonoid content. The calculated differences between minimal and maximal calculated contents for total phenols and flavonoids at different investigated pressures is shown in **Tables 6 and 7**.

Results and discussion

Table 5. The influence of the extraction pressure on the yields of phenols and flavonoids.

Pressure (bar)	*O. angustissima*		*G. saharae*	
	TPC (mg GAE/g)	TFC (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)
10	17.21 ±0.1 [a]	7.63 ±0.06	19.47 ±0.07	6.61 ±0.12
30	17.70 ±0.06	6.98 ±0.12	21.24 ±0.1	8.15 ±0.18
50	18.61 ±0.11	6.15 ±0.05	23.59 ±0.25	8.30 ±0.04
70	18.29 ±0.14	5.48 ±0.05	23.44 ±0.13	7.37 ±0.11

[a] ±SD

Table 6. Minimum and maximum extraction efficiencies of phenols at different pressures.

	Pressure (bar)	*O. angustissima*	*G. saharae*
Min TPC[a] (mg GAE/g)	10	17.21	19.47
Max TPC[b] (mg GAE/g)	50	18.61	23.59
Relative TPC change (%)		8.13	21.16

Min TPC[a]: Minimum TPC.
Max TPC[b]: Maximum TPC.

Table 7. Minimum and maximum extraction efficiencies of flavonoids at different pressures.

	Min TFC[a] (mg RE/g)	Max TFC[b] (mg RE/g)	Relative TFC change (%)
O. angustissima	5.48 (70 bar)	7.63 (10 bar)	39.23
G. saharae	6.61 (10 bar)	8.30 (50 bar)	25.57

Min TFC[a]: Minimum TFC.
Max TFC[b]: Maximum TFC.

The pressure of 50 bars showed to be the optimal for extracting phenols from both studied plant samples, and flavonoids from *G. saharae* (Table 2). This was in slight collision with other previously reported studies, in which, for most of plant samples optimal pressure in SWE was 20 bar (Švarc-Gajić *et al*., 2018) and 30 bar (Švarc-Gajić and Cvetanović., 2014; Cvetanović *et al*., 2017). Švarc-Gajić *et al*. (2018) extracted phenols from *Prunus avium* and *Prunus cerasus* stems reaching highest values at 20 bar, whereas Cvetanović *et al*. (2017) and Švarc-Gajić *et al*. (2014) reported the optimal pressure of 30 bar for phenols extraction from *Chamomilla matricaria* and *Matricaria recutita*.

Previous studies reported that the optimal pressures for flavonoids extraction from *Matricaria recutita* (Švarc-Gajić and Cvetanović., 2014) and *Chamomilla matricaria* (Cvetanović *et al*., 2017) were 30 and 45 bar, respectively.

For *O. angustissima* the lowest pressure of 10 bar provided the highest flavonoids content (7,63 mg RE/g) making elucidation of the pressure influence complex. It is obvious from our results that plant matrix has pronounced effect on the interaction of subcritical water at different pressure, stressing the necessity to carefully optimize this operational parameter for every plant matrix. Defined optimal pressure for SWE of phenols from *O. angustissima*, and *G. saharae*, was 50 bars, and kept constant in the following experiments.

1.3.The influence of the extraction time

Defining the optimal extraction time is important since prolonged extraction can induce degradation of the target compounds (Švarc-Gajić *et al*., 2018). In order to determine the influence of time on the recovery of phenols from studied plant materials, investigation was carried out at previously defined optimal temperature of 155°C, and optimal pressure of 50 bars, applying agitation rate of 3 Hz (**Figures 11 and 12)**.

The highest concentration of total phenols and flavonoids was observed at the lowest extraction time (15 min) for both plants. Further prolongation of the extraction led to a slight decrease in phenols and flavonoids contents (**Figures 11** and **12).**

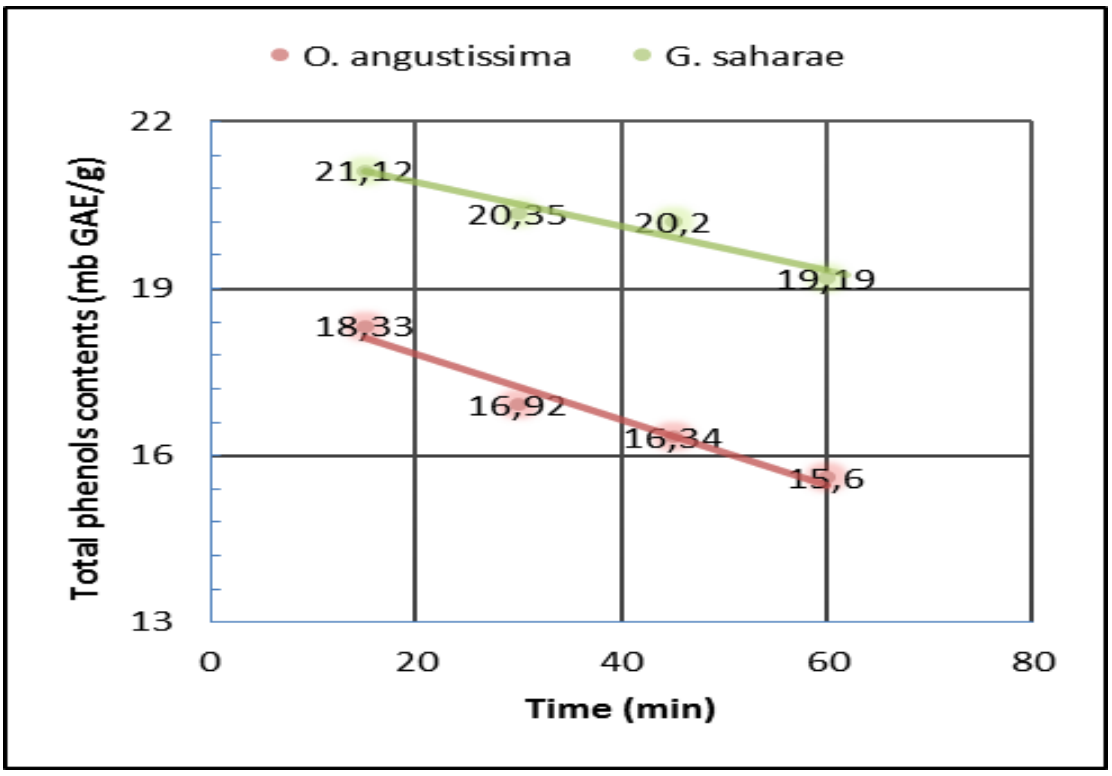


Figure 11. The influence of the extraction time on the yields of phenols.

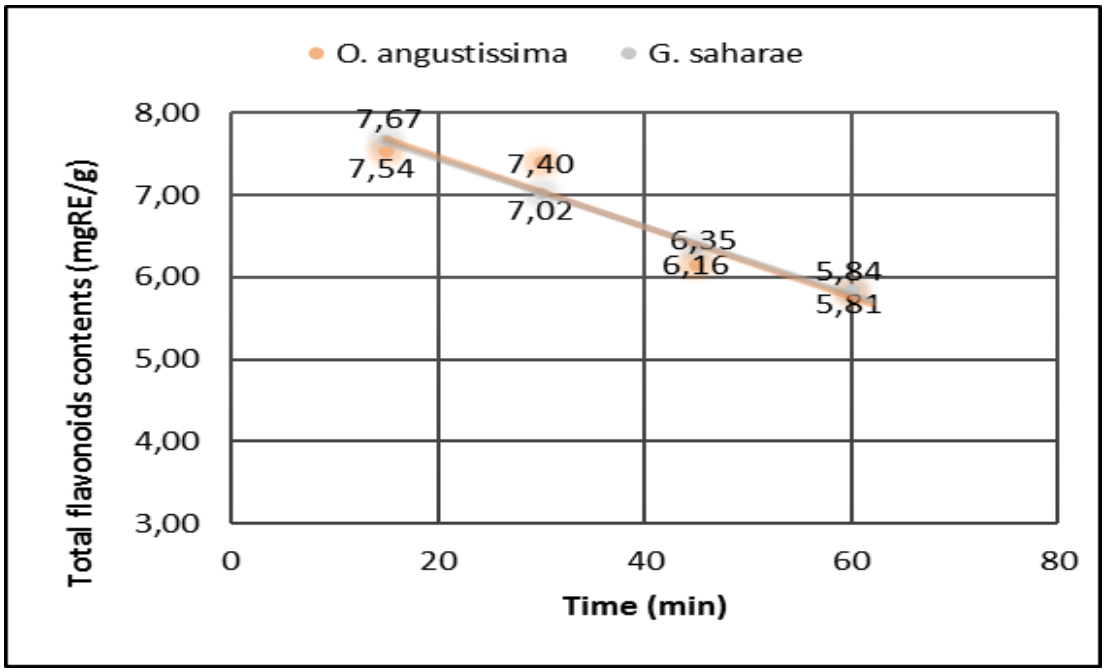


Figure 12. The influence of the extraction time on the yields of flavonoids.

The decrease in phenolic and flavonoids content with time could be explained by analyte degradation with longer extraction times. In fact, many previous studies confirm that longer extraction causes analyte degradation (Tomšik *et al*., 2017; Cvetanović *et al*., 2017; Švarc-Gajić *et al*., 2018; Nastić and Švarc-Gajić, 2018). The reported optimal times for phenols in SWE of *Prunus avium*, *Prunus cerasus, Chamomilla matricaria*, *Teucrium montanum,* and *Allium ursinum L* were 30 min for *Prunus avium*, *Prunus cerasus, Chamomilla matricaria*, *Teucrium montanum* and 10 min for *Allium ursinum L*. According to these results, extraction time of 15 min was sufficient for the recovery of phenols and flavonoids by SWE and was adopted as optimal. Relatively short extraction times with good yields of the target compounds represent one more advantage of subcritical water extraction (Švarc-Gajić *et al*., 2018), making this technology efficient, time and energy saving.

This idea may be implemented in the exploitation of medicinal plants at semi- and industrial level bringing the idea of flow-through technology since short extraction times are required.**Table 8** summerises maximal observed contents of total phenols and flavonoids for both plant samples, and operational parameters at which those were achieved.

Table 8. The maximum total phenols and flavonoids contents of *O. angustissima,* and *G. saharae* extracts.

	O. angustissima	*G. saharae*
TPC (mg GAE/g dry weight)	18.33 ±0.32	21.12 ±0.48
TPC (mg GAE/g extract)	109.02 ±1.88	111.88 ±2.52
TFC (mg RE/g)	7.54 ±0.06	7.67 ±0.15
Optimal temperature (°C)	155	
Optimal pressure (bar)	50	
Optimal time (min)	15	

For *O. angustissima,* the total phenols obtained in our study for subcritical water extracts (109.02 mg GAE/g extract) were comparable to those obtained in aqueous extracts (118.55 mg GAE/g extract) (Guettaf *et al*., 2016), but higher (18.33 mg GAE/g dry weight) in comparison to methanolic extracts (12.03 mg GAE/g dry weight) (Djeridane *et al*., 2010) obtained after 48 hour of maceration in 80% methanol.

The content of total phenols in methanolic extracts of *G. saharae* growing in Oued Souf region (Algerian desert) (1.33 mg GAE/g extract) was significantly lower in comparison to contents determined in this study (111.88 mg GAE/g extract) (Chouikh *et al*., 2018). The

authors applied 72 hour of maceration in methanol. The contents determined in this work were also higher than that found by Meriane *et al*. (2014) in the MeOH extract of different parts of *G. saharae* from the region of Oued El-Maadher, Boussaâda, Wilaya of M'Sila, Algeria. In roots the authors determined 93.3 mg pyrogallol equivalent/g extract, whereas in flowers they calculated the content of 90.67 pyrogallol equivalent/g extract. The contents of total phenols determined by Guettaf *et al*. (2016) in aqueous (130.44 mg GAE/g extract) and ethyl acetate (459.28 mg/g extract) extracts of the same plant, and that grown in Ghardaya-Algeria, respectively, were higher of those found in this study (Bouchouka *et al*., 2012). The content of plants secondary metabolites in addition to being dependent on the applied extraction technique depends also on other factors, such as plant variety, geographical region, climate, soil composition, etc.

2. Identification and quantification of phenolic compounds by RP-HPLC-UV/Vis

High performance liquid chromatography combined with various detectors is the universal method for qualitative and quantitative analysis of phenolic compounds. Optimal extracts of *O. angustissima,* and *G. saharae* obtained at temperature of 155 °C, pressure of 50 bar, extraction time of 15 min, and agitation rate of 3 Hz were analyzed by HPLC with UV/VS detector. The content of phenolic compounds in the subcritical water extracts was estimated from calibration curves (**Table 9**).

Table 9. Polyphenolic compounds content (mg/g dry extract) in *G. saharae* and *O. angustissima* SW/extracts identified and quantified by HPLC.

Polyphenolic compounds	***G. saharae* (mg/g)**	***O. angustissima* (mg/g)**
Gallic acid	Nd	3.01±0.13b
Protocatechuic acid	51.43±2.04a	17.42±0.45c
Epicatechin	Nd	4.47±0.19b
Catechin	Nd	7.21±0.19b
Caffeic acid	0.33±0.002a	0.39±0.01c
Syringic acid	16.83±0.72a	10.66±0.51c
p-Coumaric acid	1.16±0.04a	0.63±0.01b
Vanillic acid	Nd	Nd
Sinapic acid	1.55±0.04a	0.76±0.01c
Chlorogenic acid	Nd	1.35±0.02b

p-Hydroxybenzoic	161.64±6.98a	95.14±4.28c
Gentisic acid	Nd	1.668±0.037a
Rosmarinic acid	Nd	Nd
Rutin	0.39±0.01a	Nd
Quercetin	0.57±0.02a	0.28±0.01b
Luteolin	Nd	0.01±0.001b
Kaemferol	Nd	0.08±0.003a
Total	233.888	141.391

Nd: not determined, below limit of detection. Values are the mean ± SD (n = 3). Different letters in raw indicate significantly different values ($p < 0.05$) among the results.

The sum of the individual phenolic compounds identified by HPLC for both plant extracts was higher than total phenolic content calculated previously by spectrophotometric assay. According to HPLC-UV/VS analysis, three hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic) were found to be the dominant phenolic compounds among the analysed acids in both plant extracts, with deferent concentrations. Where, p-Hydroxybenzoic acid was found to be the main phenolic compound in *G. saharae* (161.64 mg/g), and *O. angustissima* (95.14 mg/g) extracts. Protocatechuic acid was relatively high in both extracts (51.43 and 17.42 mg/g for *G. saharae* and *O. angustissima* extracts, respectively), as well as syringic acid (16.83 and 10.66 mg/g for *G. saharae* and *O. angustissima* extracts, respectively). The amount of all these three dominant phenolic acids was significantly higher in *G. saharae* extract, which align with the deference in total phenolic compounds detected by HPLC in this study.

Two monomeric flavan-3-ols, namely catechin and epicatechin, were detected in *O. angustissima* extract with relatively high amount, but not in the extract of *G. saharae.* Their content in tested extract was 7.21 and 4.47 mg/g, respectively.

Gallic acid, one of hydroxybenzoic acids, has been detected with good amount in *O. angustissima* extract (3.01 g/mg), but not in the extract of *G. saharae.*

Four hydroxycinnamic acid derivatives, namely caffeic, p-Coumaric, sinapic, and chlorogenic acids, were present in *O. angustissima* extract (0.39, 0.63, 0.76, and 1.35 mg/g, respectively), while just three of them with the exception of chlorogenic acid, were detected in the extract of *G. saharae* (0.33, 1.16, and 1.55 mg/g) for (caffeic, p-Coumaric, and sinapic acid, respectively).

Low content of both luteolin and kaemferol, was detected in *O. angustissima* extract (0.01 and 0.08 mg/g), while they were totally absent in in the extract of *G. saharae*.

Rutin and quercetin were detected in the extract of *G. saharae*, but just quercetin in *O. angustissima* extract. While vanillic and rosmarinic acids, were not detected in both tested samples.

The difference between the sum of individual phenolic compounds identified by HPLC, and total phenolic content calculated previously by spectrophotometric assay can be explained by the fact that Folin-Ciocalteu method is not selective.

Scientific data on the phenolic compounds of *G. saharae* is difficult to compare because of the lack of studies.

In CHOUIKH *et al*. (2018) study, the analysis of methanolic extracts of *G. saharae* growing in Oued Souf region (Algerian desert) by HPLC has confirmed the presence of some phenolic compounds such as Gallic acid, Caffeic acid, Vanillin, ρ-Coumaric acid and Rutin. These compositions were found in different concentrations between various stages of plant, where ρ-Coumaric acid (65.76 µg/mg Extract) was the dominant phenolic acid among limited number of phenolic compounds used as standards in this study. Vast and deferent dominant compounds were detected in SW/extract of *G. saharae*, with higher amount, such as p-Hydroxybenzoic acid, the main phenolic compound (161.64 mg/g extract).

In the ethanol extract of *Genista tinctoria*, a specie belongs to the Fabaceae family, deferent phenolic acids were identified by HPLC (Hanganu *et al*., 2016).

Considering the 19 standard compounds used in this study, Luteolin (1.12 mg/g plant material) was found in the largest quantities. Thus, this comparative study showed large qualitative and quantitative differences between the analyzed extracts.

Such difference between separated and dominant phenolic compounds reported in available studies and the present study, could have been related to the number of factors, such as genetic, environmental factors, variety, etc. (Melicháčová *et al*., 2010), as well as to applied extraction technique and solvent. In the present study, three hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic) were found to be the dominant phenolic compounds, with high amounts, as well as other phenolic compounds, demonstrating the potential of SWE technique for the recovery of phenolic compounds.

As for *O. angustissima,* there is no scientific data on the phenolic compounds of its SW/extract, nor extracts obtained by organic solvents, to compare with. However, in this study, SW/extract of *O. angustissima* showed a similar HPLC profile of principal phenolic compounds to that of SW/extract of *G. saharae,* where the main phenolic compounds were hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic) with less content. On the other hand, the SW/extract of *O. angustissima* has shown to be more diverse regarding phenolic compounds, with the presence of different compounds such as catechin, epicatechin with a considered amount, and luteolin and kaemferol with low concentrations. These compounds were not detected in the SW/extract of *G. saharae.*

In general, the phenolic compounds profile of both plant extracts analyzed by HPLC seems to be richer in the favor of *G. saharae,* and more diverse in the favor of *O. angustissima*.

Furthermore, the biological activity of extracts of different plants has been associated with phenolic compounds. If we keep in mind that each of the identified components possesses high biological activity, it can be concluded that together they represent very powerful mixture. Based on this, it can be assumed that the high potency of the obtained extracts is due to synergistic effect of all compounds, which are present in it.

3. The *in vitro* evaluation of biological activities

3.1. The evaluation of antioxidant activity

The role of free radical reactions in disease pathology is well established and is known to be involved in many acute and chronic disorders (Wakamatsu *et al*., 2008). Studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants, such as phenolics, flavonoids, tannins, and proanthocyanidins. Data from *in vitro* studies suggest that flavonoids have the ability to influence important cellular and molecular mechanisms related to carcinogenesis. Earlier research papers demonstrated correlation between the phenolic content in plants and their antioxidant power (Song *et al*., 2014). This activity of natural phenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers and as potential metal ion chelators (Rice-Evans *et al*., 1995).

In order to assess the antioxidant potential of the optimal extracts of *O. angustissima,* and *G. saharae* obtained by SWE, it is important to combine several antioxidant tests. Indeed, the antioxidant activity can be attributed to different mechanisms such as the scavenging of

free radicals, the prevention of the initiation of a chain of reactions producing ROS and the decomposition of peroxides (Ozen, 2009). By applying three different assays for radical scavenging activity, the antioxidant capacity of *O. angustissima,* and *G. saharae* extracts was determined.

3.1.1. Reducing power

The antioxidant activity of natural compounds may have a reciprocal correlation with their reducing power (Duh and Yen, 1997; Yen and Duh, 1995). A reducing power is only the ability to donate electrons. The presence of reducing compounds in the extracts, reduces the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be assessed by monitoring the increase in the intensity of the color that turns from yellow to green at 700 nm (Chang *et al*., 2008). This intensity is relative to the reducing power of the sample studied (Yadav *et al*., 2012). Thus, the reducing power of these compounds can serve as a significant indicator of their potential antioxidant activity (Liu *et al*., 2013). The high reducing power of flavonoids suggested their remarkable power to donate electrons to reactive free radicals, thus converting them into more stable non-reactive species and finally ending the chain reaction of free radicals (Zha *et al*., 2009).

The results show that the SW/extracts of *G. saharae* and *O. angustissima* have an average reducing power but dependent concentration (**Figure 13**). Indeed, at a concentration of 600 µg/mL, *O. angustissima* showed almost equal potency to that of *G. saharae* with a minimal difference in favor of *O. angustissima*. On the other hand, BHT shows maximum reducing power only at 50 µg/mL. In short, the differences between the extracts and the BHT are statistically significant (***; p <0.001), conversely, the difference between the extracts themselves is statistically insignificant (ns; p> 0.05). The EC50s are shown in **Table 10**.

Table 10. The EC_{50}s of *G. Saharae*, *O. angustissima* extracts, and BHT.

Parameter / **Sample**	**EC50** (µg/ml)
BHT	15,15 ± 1,47
G. saharae	578,16 ± 4,35 *******
O.angustissima	547,06 ± 40,54 *******

Values represent the mean of three trials ± SD. The comparison is made against the BHT. ***: p <0.001.

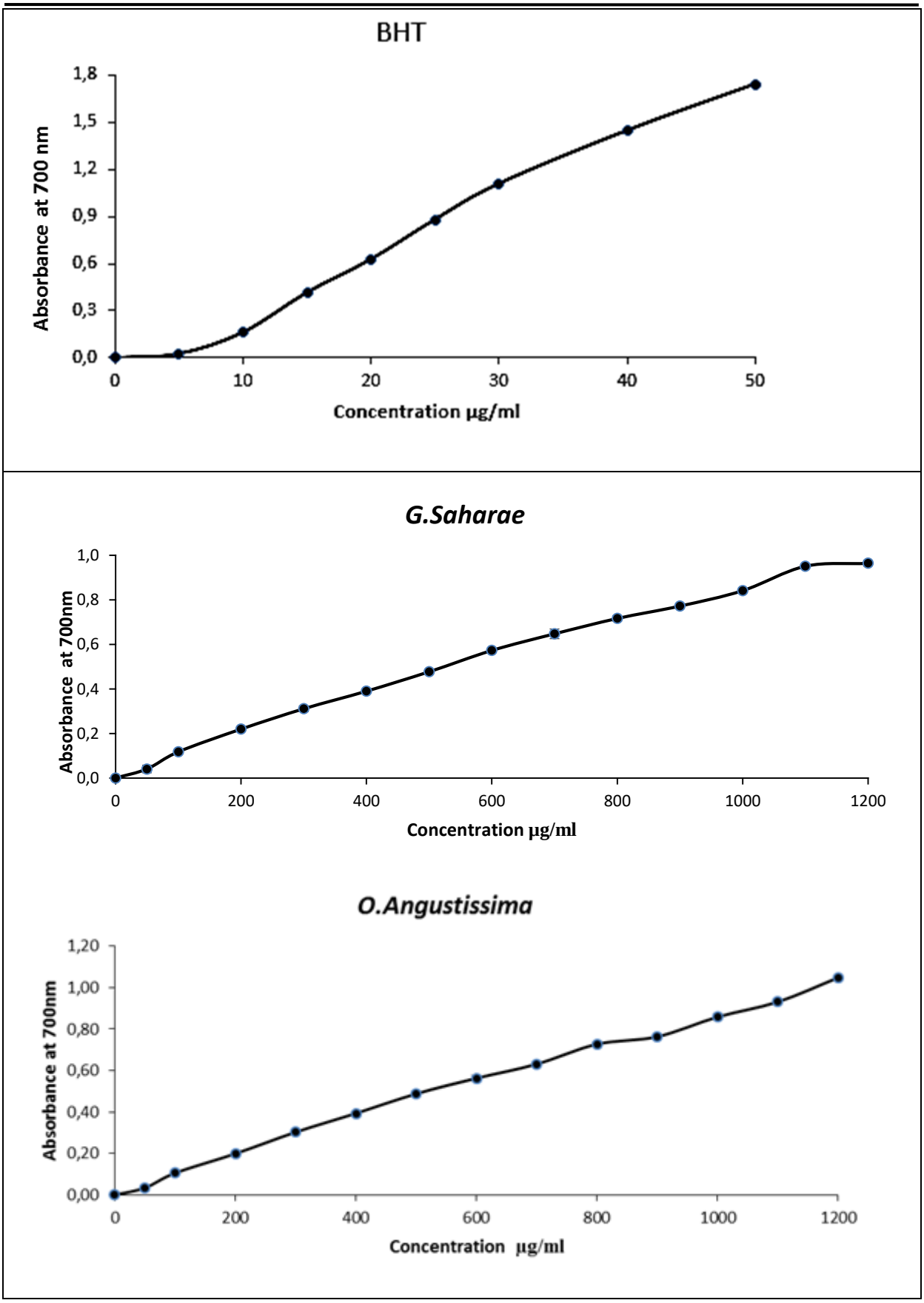


Figure 13. Reducing power of SW/extracts of *G saharae* and *O angustissima* and BHT at different concentrations.

Values represent the mean of three trials ± SD.

Obtained EC50 values of the reducing power show that both SW/extracts have an average or even low reducing capacity. Indeed, the two values are much higher than that of BHT. This demonstrates their inability to donate electrons and therefore their inability to neutralize free radicals. The study by Ghribi *et al*. (2015) shows a low reducing power of the butanolic extract of *O. angustissima* (IC 50 = 112.91 µg/mL). On the other hand, the same study reveals a good reducing power of the ethanolic extract supposed to be rich in flavonoids (IC 50 = 63.42 µg / mL).

According to Le *et al*. (2007), the reducing power could be due to the presence of flavonoids which are major electron donors. On the other hand, the reducing effect of the studied extracts as well as their flavonoid content are significantly identical. This could indicate that there are correlations between their flavonoids content and their reducing activities. In fact, both SW/extracts are poor in terms of compounds responsible for the reducing power (flavonoids). In addition, interference due to the presence of other compounds in the extract is often a limiting factor in the reducing power test.

These results agree with previous tests regarding the amount of flavonoids and free radical scavenging activity (Ksouri *et al*., 2009).

3.1.2. DPPH radical-scavenging activity

Obtained results show that the extracts of the aerial part of both plants have a concentration-dependent anti-free radical activity (**Figure 14**).

G. Saharae exerted an anti-radical activity of 70% at the concentration of 300 µg/mL. Its effect is better than that of *O. Angustissima*, which achieves the same activity level at a higher concentration estimated at 400 µg/mL. However, both SW/extracts have a lower anti-free radical activity than that of BHT, used as a standard antioxidant, which shows a relatively greater effect (same anti-free radical activity at 70 µg / mL concentration).

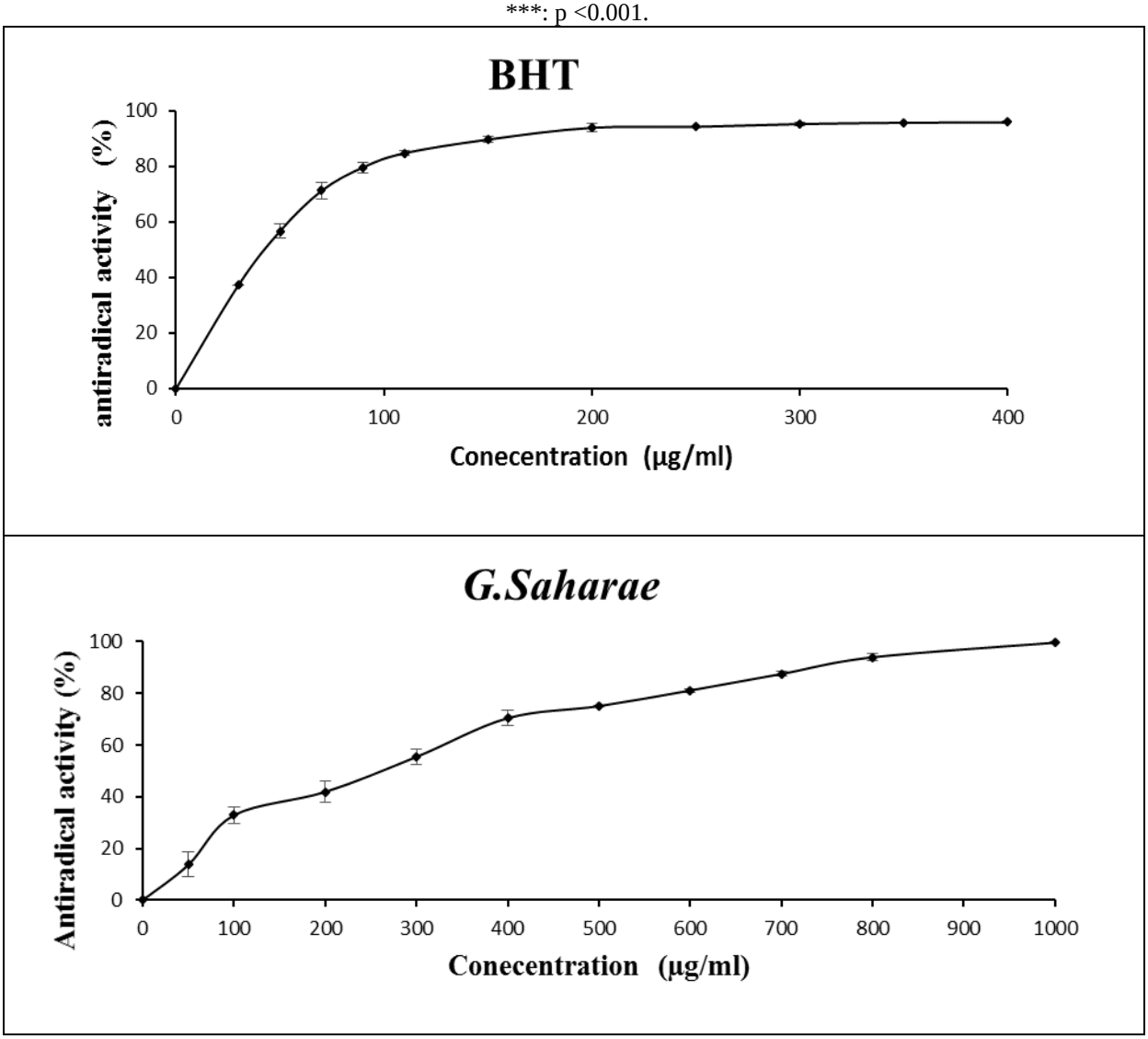
The differences in the so-called scavenger effect between the extracts and BHT on the one hand, and between the extracts themselves on the other hand, are statistically significant (***; $p < 0.001$). **Table 11** and **Figure 14** show the 50% inhibitory concentrations (IC_{50}) of the two SW/extracts and BHT, as well as the 50% effective concentration (EC_{50}) and the antiradical power (ARP).

Results and discussion

Table 11. IC_{50}, EC_{50} and ARP values of SW/extracts of *Genista Saharae*, *Ononis Angustissima* and BHT.

Parameter / Sample	**IC_{50}** (µg/ml)	**EC_{50}** (µg/µg DPPH)	**ARP**
BHT	44,35 ± 3,10	0,56 ± 0,03	1,76 ± 0,12
O.Angustissima	267,26 ± 8,43 ***	3,42 ± 0,10	0,29 ± 0,01
G.Saharae	191,35 ±14,80 ***	2,45 ± 0,18	0,40 ± 0,03

Values represent the mean of three tests ± SD. The comparison of the IC50 is made against the BHT.
***: p <0.001.



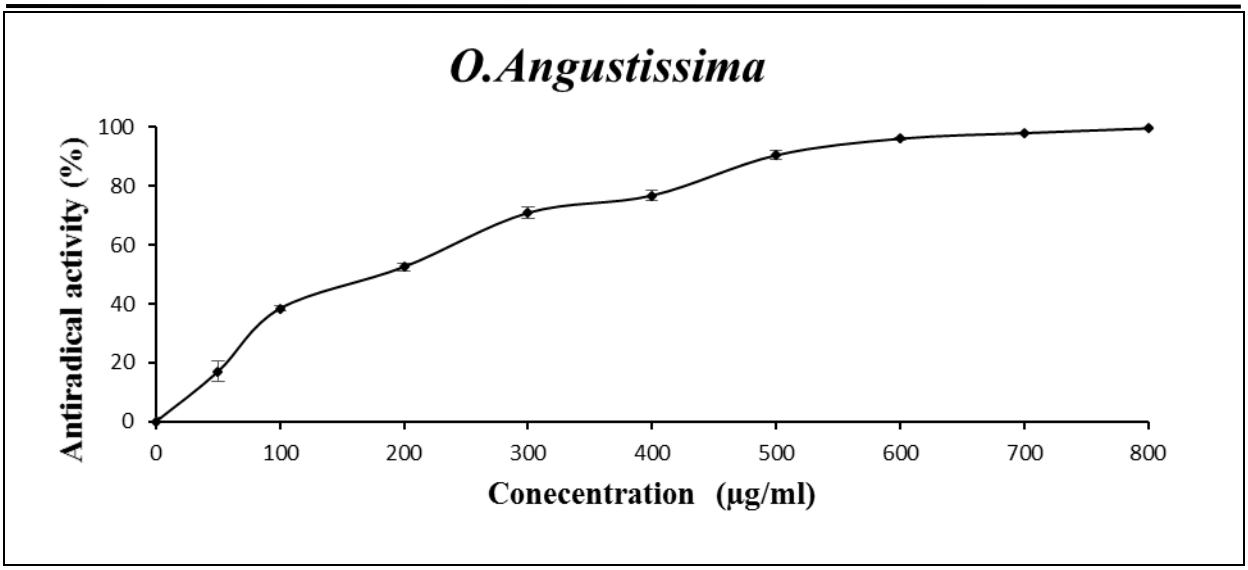


Figure 14. Anti-free radical activity of the SW/extracts of *G. Saharae* and *O. Angustissima* and the reference against the DPPH radical.

Each value represents the mean value of 3 tests ± SD.

The anti-free radical activity is very important because of the deleterious role of free radicals in the food sector and in biological systems (Gulçin *et al*., 2010). The DPPH radical method, used in this study, is a common procedure in which the antioxidant activity of the test sample is estimated by the degree of discoloration of the DPPH solution. This purple chromogen is easy to use, has high sensitivity, and allows rapid analysis of the antioxidant activity of large number of samples while providing reproducible results (Gulçin *et al*., 2010).

The results of the present study show that the SW/extracts of *G. Saharae* and *O. Angustissima* have moderate scavenging effects with respect to the DPPH radical. Indeed, the IC_{50} values obtained with these extracts are respectively of the order of 191 and 276 µg / mL, respectively. The BHT used as a reference antioxidant is by far more active than the two extracts (IC_{50} = 44 µg / mL). Ghribi *et al*. (2015) demonstrated that the butanolic extract of *Ononis angustissima* harvested from Tunisia has a DPPH scavenging capacity similar to that obtained in our study with an IC_{50} = 189.86 µg / mL. This similarity of results between the butanol extract and the SW/extract suggests that the two solvents have similar extracting power of the compounds responsible for the scavenging of DPPH (flavonoids). Indeed, the same study reports a good scavenging effect with an IC_{50} = 24.48 µg / mL obtained by the ethanolic extract of *O. Angustissima*. This result suggests that ethanol might be a better flavonoid extractor than the previous two solvents. Likewise, Tawaha *et al*. (2007) found that the methanolic extract of *O. Natrix* (the closest species to that of *Angustissima*) has good total antioxidant activity (76 µmol ET / g of extract). According to Ghribi *et al*. (2015), isoflavones were found to be major contributors to the antioxidant activity of *O. Angustissima*.

On the other hand, Meriane *et al*. (2014) found that the methanolic extract of *G. Saharae* has a greater DPPH radical scavenging effect than that of our SW/extract (respectively, IC50 = 8.27 Vs 276 µg / mL). Likewise, the study by Bouchouka *et al*. (2011) on the ethyl acetate fraction of *G. Saharae* reveals a strong scavenging effect of DPPH (26.52 mg EVC / g of extract).

According to Meriane *et al*. (2014), polar and nonpolar isoflavones appear to be the main contributors to the antioxidant activity of *G. Saharae*. Whereas, other phenolic compounds do not react with DPPH, the low activity of the SW/extracts is probably due to the low content of flavonoids which are known to be phenolic compounds with the greatest capacity to trap radical species, and reactive forms of oxygen (Hennebelle *et al*., 2004). This correlation has been reported by other authors. Selles *et al*. (2012) have shown that there is a very significant correlation between the content of total polyphenols and the scavenger activity of the DPPH radical.

The difference in activity noted between organic and SW/extracts is therefore attributed to the fact that extracts based on organic solvents are richer in polar secondary metabolites, of medium and low polarity (Kintzios *et al*., 2010).

Since *G. Saharae* is relatively richer in flavonoids than *O. Angustissima* (respectively, 7.67 and 7.54 mg RE/g), its ability to scavenge DPPH radical is relatively higher. It also shows that there is a clear correlation between total polyphenol content and antioxidant activity. And could indicate that polyphenols are responsible for this activity. According to Nijveldt *et al*. (2001), flavonoids in particular, are active in the process of scavenging free radicals. The scavenging effect of flavonoids on free radicals depends on the presence of free OH groups, in particular 3-OH, with a 3 ', 4'-catechol (dihydroxy) configuration (Heim *et al*., 2002). The number and / or the position of the hydroxyl groups on the nuclei of these molecules as well as the substitutions on the B and A rings with the presence of the C2-C3 double bond in conjugation with the 4-oxo function on the C ring reinforce the antioxidant activity of flavonoids.

All in all, our present study was consistent with previous reports. The moderate activity of our SW/extracts is due either to the lack of the SW/extracts in terms of flavonoids, or to the presence of other molecules, which affect the scavenging capacity in terms of the capacity of hydrogen donations. Additionally, both SW/extracts are believed to contain polar compounds such as flavonoids and glycosides, which may be responsible for their moderate antioxidant activity (Benbrinis, 2012).

3.1.3. *β*-Carotene Bleaching

Lipids oxidation in food poses serious problems for the food industry because it leads to qualitative (rancidity) and nutritional (loss of vitamins) alterations. It is at the origin of bad tastes, odors and toxic products (peroxides, aldehydes) which affect both the nutritional values of foods and the health of consumers (Gulcin *et al*., 2010). Lipid oxidation can also take place *in vivo* and is associated with several pathologies, namely cardiovascular disease, aging and cancer (Ramarathnam *et al*., 1995). Thus, it is very important to look for inhibitors of lipid peroxidation both in foodstuffs and in the body (Bougatef *et al*., 2009). In the present study, the β-carotene bleaching test was performed to assess the lipid oxidation inhibitory activity of SW/extracts of *G. saharae* and *O. angustissima*.

Figure 15 represents the bleaching kinetics of β-carotene in the presence and absence of SW/extracts of *Genista Saharae* and *Ononis Angustissima* as well as of BHT and BHA which represent reference antioxidants. The initial absorbances of the aqueous and methanolic negative controls are 0.989 and 1.015 reaching, after 120 min, minimum values of 0.223 and 0.299 respectively, indicating total peroxidation of β-carotene, while the absorbances of the SW/extracts of *Genista Saharae* and *Ononis Angustissima* vary from 1.010 and 1.034 at time 0 to final values at time 120 min of 0.846 and 0.678 respectively, indicating a strong antioxidant activity compared to the negative control. However, the absorbances of BHT and BHA remain stable throughout the 120 minutes. The results show that *G. Saharae* possesses remarkable antioxidant activity reaching the rate of 77%, which is much higher than that of *O. Angustissima,* which does not exceed the rate of 54% **(Figure 16**).

Based on this fact, the inhibitory activity of β carotene peroxidation exerted by *G. Saharae* is almost equal to those of BHA and BHT considered as reference, the statistical analysis of which reveals a non-significant value (ns; $p > 0.05$), namely that the inhibitory activity of BHA and BHT are of the order of 80% and 84% respectively.

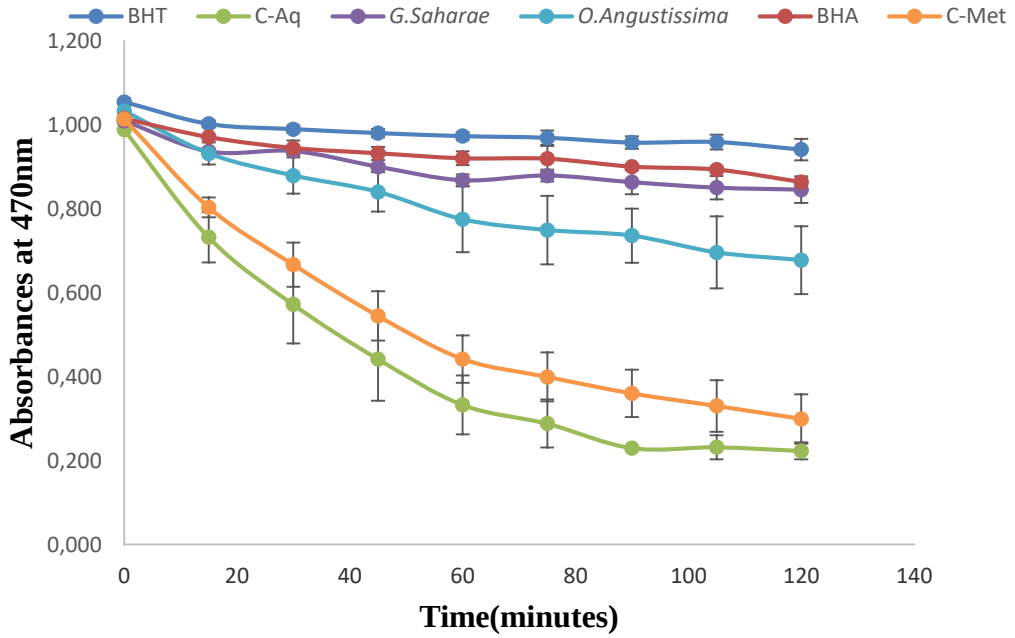


Figure 15. Bleaching kinetics of β-carotene in the presence and absence of SW/extracts of *G.saharae*, *O.angustissima*, BHT and BHA .

Values represent the mean of three measurements ± SD. Control 1: aqueous; Control 2: methanolic.

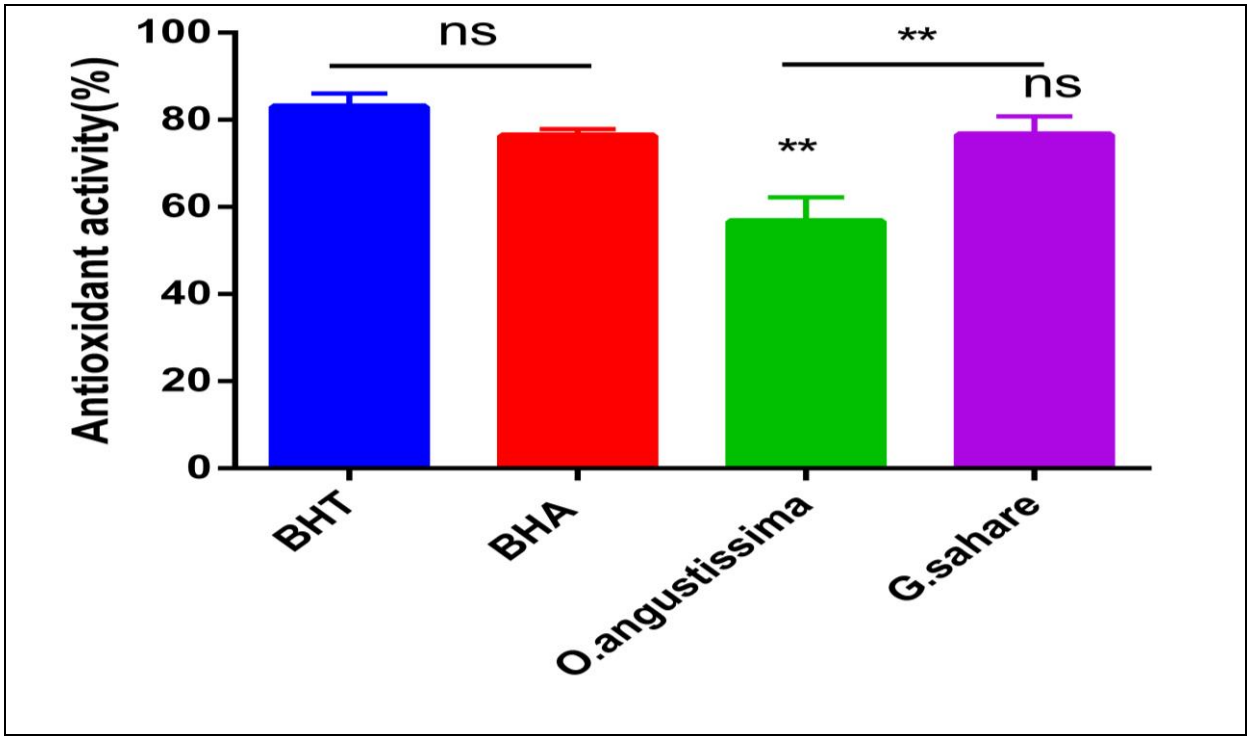


Figure 16. Antioxidant activity of SW/extracts of *G.saharae*, *O.angustissima*, BHT and BHA in the β-carotene/linoleic acid system.

Values are the mean of three tests ± SD. The comparison is made with the BHA; **: P≤0.01, ns: not significant.

The β-carotene test is widely used to measure the antioxidant activity of bioactive compounds, as β-carotene is extremely susceptible to free radicals resulting from the oxidation of linoleic acid (Kumazawa *et al*., 2002). This is due to the ability of carotenoids to

neutralize free radicals while losing their coloration, which results in a decrease in absorbance at 470 nm (Barros *et al*., 2007). The importance of this test lies in the use of β-carotene as a coloring agent in drinks, so its discoloration will clearly be the cause of the reduction in the quality of these products (Bougatef *et al*., 2009).

During lipid oxidation, antioxidants work in different ways including scavenging free radicals, breaking down peroxides and chelating metal ions. Often more than one mechanism is involved to have a synergistic effect (Moure *et al.*, 2001).

The obtained results show that at a concentration of 2 mg/mL, the SW/extract of *O. angustissima* exerts a moderate antioxidant activity, while that of *G. saharae*, exerts a very high effect on lipid peroxidation (80%). Indeed, the bleaching of β-carotene was significantly slowed down in the presence of *G. saharae*. A previous study on the *Genista cephalantha* species confirms our results by showing an inhibition rate of β carotene equal to 70.75% (Kaoutar *et al*., 2014). These results suggest that extracts of the *Genista* genus have a considerable capacity to react with free radicals to convert them into non-reactive species and to interrupt the chain of radical reactions. It also reflects the solubility of the antioxidant compounds of the two extracts in water. Compounds that possess this characteristic can be used in food systems (Moure *et al*., 2001). According to Maggi *et al* (2009), the antioxidant activity of a compound is very often related to the presence of easily oxidizable portions such as a hydroxyl group on a hydrocarbon. Flavonoids inhibit lipid peroxidation at an early stage through the scavenger activity of peroxide radicals as they can interrupt a chain of radical reactions through the hydrogen donation property (Sandhar *et al*., 2011). Several reports have, aligning with the present study, also indicated that *Genista* species contain a variety of secondary metabolites of various types, which have been shown to be biologically active (Dixon *et al*., 1995; Boumaza *et al*., 2006). As long as *G. saharae* contains more total polyphenols than *O. angustissim*a, there is a strong correlation between the total polyphenol content of the two extracts and their antioxidant activities. Indeed, the antioxidant activity of the SW/extracts of *G. saharae* and *O. angustissima* is due to the presence of phenolic acids and flavonoids.

Furthermore, the effect of *G. saharae* is very close to that of BHT and BHA, which were chosen in this work for comparative purposes, as they are most often used in food preservation. The ability of *G. saharae* to modify lipid peroxidation induced by free radicals is related not only to structural characteristics of antioxidants, but also to their ability to interact with and penetrate lipid bilayers (Antonella *et al*., 1995). The structure and

lipophilicity of polyphenols have been shown to be determining factors in the antioxidative property, possibly affecting the depth of incorporation of these compounds into the lipid phase of the membrane (Djeridane *et al*., 2010).

The obtained results indicated moderate antioxidant potential of the examined extract. This can be affected by its composition. The relationship between structure and activity can be proposed from series of the phenolics. Generally, phenolic compounds with *o*-dihydroxyl group in aromatic ring possess stronger antioxidant activity than monophenolics (Rice-Evans, Miller, & Paganga, 1996). Furthermore, high amounts of rosmarinic acid, chlorogenic acid, quercetin as well as other phenols can be directly linked with the antioxidant activity of the tested extract.

3.2. Anti-proliferative activity (Cytotoxic activity)

According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products (Richardson, 2001). Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers (Birt *et al*, 2001, Greenberg *et al*, 1994, Michaud *et al*, 2000, Yuvaraj *et al*, 2010). More than 60% of currently used anticancer agents are derived in one way or another from natural sources (Balatas, Cragg and Newman, 2003). However, many of their potential effects, especially anti-cancer effect, have not been well studied. Numerous published research reports cytotoxic effects of phytochemicals thus modern medicine is looking for new models for anti-cancer drugs with improved characteristics (Svarc-Gaji, 2013).

In current study, the cytotoxic activity of optimal extracts of *O. angustissima*, and *G. saharae* obtained by SWE in wide range of mass concentrations from one to 100 µg/mL was evaluated and compared with cis-diamminedichloroplatinum (cis-DDP) or Cisplatin, a standard chemotherapeutic agent effective in the treatment of great number of cancers and tumors.

The MTT assay to test for cytotoxicity is a quantitative and reliable colorimetric test that is based on the enzymatic reduction of yellow water-soluble MTT dye by mitochondrial succinate dehydrogenase to form a purple formazan product (insoluble in water) which measures viability, proliferation and the activation of cells. The MTT assay measures cell membrane integrity by determining mitochondrial activity through an enzymatic reaction on the reduction of MTT to formazan (Azurah *et al*., 2011). The amount of formazan produced is

assumed to be directly proportional to the cell number in a range of cells lines (Gerlier and Thomasset, 1986; Mosmann, 1983).

MTT assay was performed with three different cell lines: cell line derived from human rhabdomyosarcoma (RD), cell line derived from human cervix carcinoma Hep2c (HeLa) and fibroblast cell line from murine (L2OB), as well as on a mouse fibroblasts (L929) cell line. Calculated cytotoxic activity of the tested extracts against tested cell lines is shown in **Table 12.**

Table 12. *In vitro* cytotoxic activity of *O. angustissima,* and *G. saharae* extracts, obtained by SWE.

Samples	**IC_{50} (µg/mL)[a]**			
	Hep2c cells [b]	**RD cells [c]**	**L2OB cells [d]**	**L929 cells [e]**
O. angustissima	39,79±0,83	32,45±0,55	28,27±0,51	84,49 ± 0,42
G. saharae	31,25±0,79	29,45±0,43	18,34±0,73	78,01 ± 0,59
(*Cis*-DDP)[f]	0.94±0.55	1.40±0.97	0.72±0.64	52.23±0,92
American National Cancer Institute (NCI), the criterion of cytotoxic activity for plant extracts is IC_{50}< 30 µg/mL				

[a] Mean value of three replicate ±SD.
[b] Cell line derived from human cervix carcinoma.
[c] Cell line derived from human rhabdomyosarcoma.
[d] Cell line derived from murine fibroblast.
[e] mouse fibroblasts cell line.
[f] Cis-diamminedichloroplatinum.

Cellular viability and proliferation are considered to be important functional characteristics of healthy and actively growing cells. Therefore, an increase in cell viability indicates cell proliferation in a non-toxic environment while a decrease in cell proliferation indicates cell death due to toxicity.

In cells treated with both optimal *G. saharae* and *O. angustissima* SW/extracts, a considerable dose-dependent inhibition of cell growth was observed.

The most effective cell growth inhibition activity and extremely low IC_{50} value of 18.34 µg/mL was observed in fibroblast cell line from murine (L2OB) treated with *G. saharae* SW/extract. The lowest IC_{50} value (28.27 µg/mL) was calculated in the same cell line treated with *O. angustissima* SW/extract. Slight higher IC_{50} values were calculated in the case

of the cell line derived from human rhabdomyosarcoma (RD) (29.45 and 32.45 µg/mL) for *G. saharae* and *O. angustissima* SW/extract, respectively, while in the case of Hep2c cells, the highest values of (31.25, 39.79 µg/mL) were seen for *G. saharae* and *O. angustissima* SW/extracts, respectively. Inhibitory concentrations for cis- diamminedichloroplatinum (Cis-DDP) were in the range from 0.72 to 1.4 µg/ml. In comparison to standard cytotoxic agents, the extracts had significantly lower activity.

Comparing the two plants anti-proliferative activity, we notice that *G. saharae* has lower IC_{50} values than *O. angustissima,* which could be explained by its higher and diverse content of phenolic compounds shown previously in the HPLC analysis.

Mašković *et al*. (2015) used the same cell lines during the evaluation of the anti-proliferative effects of ethanolic extracts of *Onosma aucheriana*. For three cell lines, Hep2c, RD cells, and L2OB cells (IC_{50} of 40.34, 50.57, 25.54 µg/mL, respectively), subcritical water extracts of *G. saharae* and *O. angustissima* had much higher activities.

To the best knowledge, there are no previous literature reports on cellular-protective, cancer-preventive and cytotoxic effects of *G. saharae* and *O. angustissima* extracts obtained by conventional extraction techniques, or by subcritical water. Thus, the Anti –proliferative activity of both extracts seems to be significant, especially for *G. saharae* SW/extract.

The obtained extracts were rich in biologically active molecules. However, due to the synergistic effects of natural compounds, further investigation is necessary to clearly understand the mechanisms of action of *G. saharae* and *O. angustissima* constituents as modulators of cytotoxic activity.

3.3. Antimicrobial activity

Due to general tendency of modern medicine to search for novel natural bioactive compounds and increased resistance of pathogens to synthetic antibiotics, many investigations are focused on natural products with promising antimicrobial activity. The antimicrobial activity of subcritical water extracts of *G.saharae* and *O.angustissima* was studied for five different concentrations (19.5, 39.1, 78.1, 156.2 and 312.5µg/mL) against six bacterial strains, two Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) and four Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Proteus vulgaris* ATCC 13315 and *Proteus mirabilis* ATCC 14153), as well as two fungal strains (*Candida albicans* ATCC 10231 and *Aspergillus Niger* ATCC 16404). The measured

inhibitory concentrations (MIC values) for investigated extracts were compared with those determined for Amricin and Nystatine, standard anti-bacterial and anti-fungal compounds, and presented in **table 13**. Data show that extracts exhibited antibacterial activity against all tested strains but with different degrees of effectiveness.

Table 13. Antimicrobial activity of subcritical water extracts of *G.saharae* and *O.angustissima.*

	MIC value (μg/mL)			
Microbial strains	*O.angustissima*	*G.saharae*	Amricin	Nystatin
Staphylococcus aureus ATCC 25923	78.13 ± 0.7	78.13 ± 0.3	0.97 ± 0.03	/
Klebsiella pneumoniae ATCC 13883	78.13 ± 0.8	78.13 ± 0.7	0.49 ± 0.02	/
Escherichia coli ATCC 25922	156.25 ± 0.5	78.13 ± 0.3	0.97 ± 0.03	/
Proteus vulgaris ATCC 13315	312.5 ± 0.8	156.25 ± 0.7	0.49 ± 0.02	/
Proteus mirabilis ATCC 14153	156.25 ± 0.8	156.25 ± 0.7	0.49 ± 0.02	/
Bacillus subtilis ATCC 6633	78.13 ± 0.1	156.25 ± 0.3	0.24 ± 0.02	/
Candida albicans ATCC 10231	78.13 ± 0.5	312.5 ± 0.3	/	1.95 ± 0.10
Aspergillus niger ATCC 16404	78.13 ± 0.3	78.125 ± 0.2	/	0.97 ± 0.03

Both *G.saharae* and *O.angustissima* SW/extracts demonstrated antimicrobial activity within the MIC range from 78.13 μg /mL to 312.5 μg /mL.

For *O.angustissima* SW/extract, the strongest antibacterial activity was recorded against *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Bacillus subtilis* (MIC = 78.13 μg /mL), while the antifungal activity was the same for both fungi, *Candida albicans* and *Aspergillus niger* used in this study with (MIC = 78.13 μg /mL). A moderate antibacterial activity was registered against *Escherichia coli* and *Proteus mirabilis* (MIC = 156.25 μg /mL), and relatively weak against *Proteus vulgaris* (MIC = 312.5 μg /mL).

As for *G.saharae* SW/extract, the strongest antibacterial activity was recorded against *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Escherichia coli* (MIC = 78.13 μg /mL), while in the case of fungi the strongest activity, was against *Aspergillus niger* (MIC = 78.13 μg /mL), which was much better comparing to that against *Candida albicans* (MIC = 312.5

μg /mL). A moderate antibacterial activity was registered against *Proteus vulgaris, Proteus mirabilis,* and *Bacillus subtilis* (MIC = 156.25 μg /mL).

Both plant extracts were equally strong against the Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*) (MIC = 78.13 μg /mL), however, *G.saharae* SW/extract showed better activity against Gram-negative bacterial strains. Whereas, in the case of fungi, *O.angustissima* SW/extract had stronger activity against *Candida albicans* (MIC = 78.13 μg /mL).

In the study of Bouchouka *et al*., (2012), the antibacterial activity of water, hexane, and ethyl acetate extracts of *G.saharae,* against three bacterial strains, *S. aureus, E. coli* and *P. aeruginosa*, was carried out by the disc diffusion method. Among extract fractions tested, only the ethyl acetate fraction of *G. sahara* exhibited an antibacterial activity against *S. aureus*. None of the plant extract fractions showed activity against the gram (-) organisms; *E. coli* and *P. aeruginosa*. This was the only antimicrobial study realized for *G.saharae*, to which *G.saharae* extract obtained with subcritical water expressed much better activity for wider bacterial strains. This leads to assumption that subcritical water extracts contained wider array of compounds with antimicrobial properties or to higher concentrations of compounds with antimicrobial compounds.

As for *O.angustissima*, there were no data related to the antimicrobial activity of any of its ewtracts.

In comparison to antimicrobial activity of supercritical extracts of oregano (Stamenic, M *et al*., 2014), known as one of the richest sources of natural antimicrobial compounds, antimicrobial activity of subcritical water extracts of *G.saharae* and *O.angustissima* demonstrated better activity. MIC values for supercritical extracts of oregano against *Staphylococcus aureus* were in the range from 320 to 2560 μg/mL depending on the extraction conditions. Close MIC values for oregano extract were calculated also for *Klebsiella pneumonia,* while in the case of *Escherichia coli*, these values were even higher than 2560 μg/mL. In comparison to subcritical water extracts of other plants, the results obtained in this study were comparable to data reached for subcritical water extracts of chamomile (Cvetanović *et al*., 2015), and ginger (Švarc-Gajić *et al*., 2017). To our knowledge, there are no data published on antibacterial and antifungal properties of *G.saharae* and *O.angustissima* extracts achieved by SCW. The obtained results imply that *G.saharae* and *O.angustissima* extracts obtained with subcritical water are good source of the antimicrobial agents.

3.4. Anti-inflammatory activity (*in vitro*)

Numerous studies have evaluated the inhibitory effect of protein denaturation from different plant extracts on inflammation *in vitro*. Proteins denaturation is one of the inflammation causes (Bagdad *et al*., 2011; Mizushima *et al*., 1968). The production of auto antigens in inflammatory diseases may be due to the denaturation of proteins *in vivo*. The possible mechanism of denaturation is the alteration of electrostatic, hydrogen, hydrophobic, and disulfide bonds that maintain the three-dimensional structure of proteins (Bagdad *et al*. 2011; Sangeetha *et al*. 2011). Non-steroidal anti-inflammatory drugs like phenylbutazone and indomethacin have been shown not only to inhibit the synthesis of pro-inflammatory prostaglandins, but also to inhibit protein denaturation (Sangeetha *et al,* 2011, Adarshvm *et al*. 2001). They thereby prevent the denaturation of heat-treated albumin at physiological pH (pH: 6.2 to 6.5).

The results of the anti-inflammatory activity *in vitro* of SW/extracts of *G.saharae*, *O.angustissima,* and a reference anti-inflammatory drug (Diclofenac sodium) are shown in **table 14.** This test consists of evaluating the percentages of inhibition of denaturation of bovine serum albumin (BSA).

Table 14: Percentage inhibition of BSA denaturation at the concentration of 250 µg/mL.

Sample	% inhibition of protein denaturation
Diclofenac sodium	90,77 ± 0,20
G.saharae	63,92 ± 1,58 **
O.angustissima	44,29 ± 3,96 ***

Values are the mean of three replicates ± SD. The comparison is made with Diclofenac sodium. ***: P≤0.001, **: P≤0.01.

According to the obtained results (**table 14**), both studied SW/extracts inhibited the denaturation of BSA at the concentration of 250 µg/mL, but at different rates. Indeed, the percentage inhibition of BSA denaturation was 63.92 % and 44.29 %, for *G.saharae*, and *O.angustissima*, respectively.

We can notice that the *G.saharae* SW/extract is more efficient than the *O.angustissima* SW/extract with a difference of 19.63%. The result obtained for *G.saharae* SW/extract is close to that obtained for diclofenac sodium, an anti-inflammatory drug used as a standard with a percentage inhibition of around 90.77 % at the same concentration. The

differences between the extracts and diclofenac sodium are statistically significant (**; P≤0.01; ***: P≤0.001) compared to *G.saharae* and *O.angustissima* SW/extracts, respectively.

These results show that both samples are able to control protein denaturation. However, *G.saharae* has a stronger inhibitory effect than that of *O.angustissima* (64% and 44%, respectively).

Our results are in agreement with those of Bremner *et al*. (2009) who reported a potent anti-inflammatory effect *in vitro* of *Genista Ramosissima* and *Ononis Ramosissima* species by inhibiting the activation of cytokines (TNFα and PGE2). Likewise, Bello *et al*. (1995) reported an *in vitro* anti-inflammatory effect of the *Genista Patens* species. On the one hand, the evaluation of the anti-inflammatory activity of *Genista Tenera* by an *in vitro* test involving the inhibition of cyclo-oxygenase shows an inhibition of the activation of COX-1 equal to 47.5% (Batista *et al*. 2015). On the other hand, genistein, a phytoestrogen found in the *Genista* genus, inhibits pro-inflammatory cytokines *in vitro*, such as IL-1β and IL-6, (Kim *et al*., 2014).

According to Kar *et al*. (2012), antioxidant activity may be responsible for anti-inflammatory activity *in vitro*. This hypothesis is consistent with that obtained in our study, which shows a correlation between the relative potent antioxidant activity and the anti-inflammatory activity of *G.saharae* SW/extract. Thus, the denaturing inhibitory activity of BSA can be attributed to the presence of different bioactive phenolic compounds, having the ability to bind cations and other biomolecules, thus attributing the ability to protect denaturing proteins (Oyedapo, 2001).

It can be concluded from this, that *G.saharae* SW/extract has a considerable anti-inflammatory effect *in vitro* against protein denaturation and that further definitive studies are needed to determine the mechanisms and constituents behind its anti-inflammatory actions.

3.5. Anti-diabetic activity

The prevalence of metabolic and life-style diseases have reached alarming rate during past decades. Diabetes is a global health problem. The prevalence of *diabetes mellitus* (DM) is increasing dramatically in recent years and it is estimated that about 430 million of people were affected by diabetes in 2014 (WHO, 2016).

The recent surge of antidiabetic drugs on the market exploits a wide range of therapeutic targets due to the variety of pathogenic abnormalities associated with diabetes and

its related complications. Different mechanistic classes of oral drugs used in the first line treatment of diabetes include inhibitors of carbohydrate digestion, which delay postprandial absorption of monosaccharides (α-glucosidase and α-amylase inhibitors) (Gonçalves and Romano, 2017).

Key enzyme inhibitory theory is one of the most accepted theories for DM. *α*-amylase and *α* -glucosidase are key enzymes in the catabolism of carbohydrates. The enzymes catalyze the hydrolyzing of *α*-(1,4) bond in the carbohydrate and, therefore, the blood glucose level is increasing. At this point, the inhibition of these enzymes could be considered as important tool for the management of blood glucose level in DM patients (Wang *et al*., 2012).

Several enzyme inhibitors (acarbose and voglibose for amylase/glucosidase) have been chemically produced, however, the use of synthetic inhibitors may be accompanied with side effects like gastrointestinal disturbances and hepatotoxicity (Buchholz and Melzig, 2016; Mocan *et al*., 2016; Annamalai *et al*., 2017; Sang *et al*., 2017). Plant secondary metabolites have been reported to exhibit great potential to act as enzyme inhibitors with less pronounced side effects (Pereira *et al*., 2017).

The antidiabetic potentials of *G.saharae* and *O.angustissima* extracts achieved by SCW were tested against, α-glucosidase and α- amylase, using a micro-plate reader. The results were expressed as acarbose equivalents (mmol ACAE/g extract) (**Table 15)**.

Table 15. Enzyme inhibitory effects of *G.saharae* and *O.angustissima* SW/extracts.

	G.saharae	***O.angustissima***
α-Glucosidase inhibition (mmol ACE/g extract)	0.14 ± 0.01a	1.13 ± 0.18
α-Amylase inhibition (mmol ACE/g extract)	3.75 ± 0.34	4.34 ± 0.57

[a] Values expressed are means ± SD of three parallel measurements. **ACE**: Acarbose equivalent.

Tested SW/extracts demonstrated inhibitory effects against both enzymes α-amylase and α-glucosidase. In both applied assays, *O.angustissima* exhibited stronger enzyme-inhibitory effects compared to *G.saharae*. These finding were in contrast to total phenolic content and antioxidant activity where *G.saharae* extracts showed higher activity, owing to the fact that tested bioactivities are probably mostly related to other non-phenolic inhibitors. Similar to our results, Katanić *et al*. (2017) reported no correlation between phenolic content, antioxidant properties and enzyme inhibitory activities (α-amylase, tyrosinase and cholinesterase) for methanolic extract of *Digitalis lamarckii*.

Nevertheless, gallic, protocatechuic and chlorogenic acids, which were dominant components in *O.angustissima* and *G.saharae* SW/extracts, probably contributed to enzyme inhibitory effects. The compounds were reported as effective antidiabetic agents *in vivo* and *in vitro* studies (Adefegha *et al*., 2015; Oboh *et al*., 2015; Oboh *et al*., 2016; Cen *et al*., 2016).

Apparently, both SW/extracts were more active toward the α-amylase than α-glucosidase.

Ozer *et al*. (2018) reported strong correlation between protocatechuic, syringic, p-coumaric, p- hydroxybenzoic acids as well luteolin, and enzyme inhibitory activities against α-amylase and α-glucosidase.

Tested *O.angustissima* and *G.saharae* SW/extracts exhibited weaker inhibitory activity against α-amylase and α-glucosidase in comparison with ethylacetate extract of *Stachys annua* (1.78 mmol ACE/g and 6.18 mmol ACE/g extract, respectively) (Kocak *et al*., 2017). In comparison to subcritical water extracts of *Sambucus ebulus* root (Cvetanović *et al*., 2017), SW/extracts prepared from *O.angustissima* and *G.saharae* exhibited weaker α-amylase inhibitory effect, but stronger α-glucosidase inhibitory effect.

According to our knowledge, enzyme inhibitory activity of *O.angustissima* and *G.saharae* SW/extracts has not previously been reported. Therefore, our research may be treated as a first report of enzyme inhibitory activities of subcritical water extracts obtained from *O.angustissima* and *G.saharae*.

Taking into account that extracts were made by using water as a solvent, the step of their further purification can be avoided. Due to the safety of subcritical water such extracts are convenient for oral functional formulations.

4. Toxicity evaluation

4.1. Evaluation of the acute oral toxicity

The main objective of acute toxicity studies is to identify a single dose causing major adverse effects or life-threatening toxicity, which often involves an estimate of the minimum dose that can cause lethality.

After oral administration of subcritical water extracts obtained from *O.angustissima* and *G.saharae* to mice, the findings were almost identical for both extracts. Indeed, we note that there are no signs of intoxication and mortality in connection with the first injected dose

(2000 mg/kg). During the first six hours of observation, no mortality was observed in mice treated with this dose, but rather comforting signs compared to controls such as hyperactivity and increased heart rate were observed.

The signs observed previously disappeared around the 2nd day and more or less serious signs took over; isolation, loss of appetite, poor activity, slight drowsiness. The signs observed on the 2nd day disappeared around the 3rd day and they were not reconstituted for the remaining 12 days. These results suggest that the LD_{50} is located well above 2g/kg.

Given the absence on one side of serious signs of toxicity (cut-off breathing, loss of balance, muscle contractions, partial or total paralysis, etc.) and on the other side no mortality has been observed with the 2000 mg/kg dose, we switched to the 5000 mg/kg dose recommended by the OECD 425. (2008).

The observations were almost the same as those for the 2000 mg/kg dose during the 14 days of the experiment (1st day: hyperactivity and increased heart rate, 2nd day: isolation, loss of appetite, low activity, and slight drowsiness. The signs observed on the 2nd day disappeared around the 3rd day and they were not reconstituted during the 12 remaining days). Regarding mortality, only one death was recorded during the 7th day (**Table 16**). This is a mouse fed with *O.angustissima* SW/ extract at a dose of 5000 mg/kg. At the limit dose of 5000 mg/kg of *O.angustissima* and *G.saharae* extracts, the number of animals that died in total is less than three so the test is terminated (at the end of an observation period of 14 days during of which no additional dose was given) and the LD_{50} of *O.angustissima* and *G.saharae* extracts is determined to be greater than 5000 mg/kg (OECD 425, 2008).

According to the classification of Diezi (1989), the SWE extracts of *O.angustissima* and *G.saharae* are not toxic; because the LD_{50} of both plants is greater than 5000 mg/kg. Thus *O.angustissima* and *G.saharae* SW/extracts can be considered safe when administered orally unless subsequent studies prove otherwise by evaluating other parameters namely; weight growth, blood count (hematological and biochemical), and functional tests particularly those relating to external organs as well as the basis of necrotic reports, accompanied by the associated histological examinations (Diallo, 2005).

Table 16. Mortality rate of treated mice expressed as a number of individuals per 5 treated animals.

Administered dose	*G.saharae*	*O.angustissima*
2000mg/kg	0	0
5000mg/kg	0	1

Results and discussion

The clinical maps of female mice treated with SWE of *G. Saharae* and *O. Angustissima* under acute toxicity conditions are almost identical. They are characterized by an increased heart rate and mild drowsiness, mild signs that disappear after the second day. These signs are probably due to a blockade of muscarinic M2 receptors, leading to suppression of vagal tone (Kenneth *et al*., 2001). Serious signs such as respiratory distress, paralysis of the legs, and convulsions were not observed, thus indicating that there was no damage to the central nervous system by blocking the production of acetylcholine in the synapses of the central nervous system (Goulle *et al*., 2004). The majority of surviving animals recovered a normal appearance until the 14th day, with the exception of a single animal treated with 5000 mg/Kg of *O.angustissima* SW/ extract which died on the 7th by respiratory arrest associated with convulsions.

All in all, no obvious signs of acute toxicity could be observed in animals treated with 5000 mg/kg of SWE of *G. Saharae* and *O. Angustissima*. In addition, the mortality rate was less than 3/5 animals for both extracts. This suggests that the LD_{50s} of *G. Saharae* and *O. Angustissima* are greater than 5000 mg/kg.

Few toxicological studies on the genus *Genista* have been carried out. The study by Bello *et al*. (1995) on the methanolic extract of the *Genista Patens* species confirms our results on the low toxicity or harmlessness of the *Genista* genus with an LD_{50}> 3000 mg/Kg. According to Gauthier-Pilters, (1969), the *Genista saharae* from Erg-Mauritania-, is very grazed and appreciated in spring and summer, it must be absolutely avoided in winter by males, since high consumption causes them retention of urine resulting in a few days in death. However, our harvest was made during the month of April when the toxicity of the plant is minimal or zero. The toxicity of this plant may be due to the high alkaloid content during winter.

The genus *Ononis* in turn is very little studied from the toxicological point of view. Montero *et al*. (1988) reported that no toxicity was observed following intraperitoneal administration of *Ononis Pubescens* extract. This observation is consistent with the results obtained by our study.

According to the classification of Diezi (1989), both SWE extracts of *G. Saharae* and *O. Angustissima* are non-toxic; because their LD_{50} is greater than 5000 mg/kg. Both extracts can be considered safe and can be administered orally.

Results and discussion

Comprehensive studies of sub-acute and chronic toxicity focusing on behavioral and physiological changes (weight change, relative organ mass, biochemical and histological data) are possible to better determine the long-term effects.

CONCLUSION

Conclusion

In this study, the therapeutic effect of two Algerian endemic plants (*O. angustissima and G. saharae*) with medicinal properties was evaluated. The efficiency of subcritical water extraction of phenols and flavonoids from these plants was determined. Optimal parameters for SWE were (155°C, 15min, and 50 bars) for both studied plant samples. At defined optimal extraction parameters, maximum contents of phenols in the extracts of *O. angustissima and G. saharae* were18.33, and 21.12 mg GAE/g dry weight, respectively. The chemical profile of tested extracts defined by RP-HPLC-UV/Vis, showed the dominance of three hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic). Two monomeric flavan-3-ols, namely catechin and epicatechin, were detected in *O. angustissima* extract with relatively high amount, but not in the extract of *G. saharae*. Both chemical profiles, and bioactivities were comparable for *O. angustissima*, and *G. saharae*. However, *G. saharae* exhibited slightly stronger antioxidant capacity and higher content of phenolic compounds. Tested extracts demonstrated inhibitory effects against both enzymes α-amylase and α-glucosidase. In both applied assays, *O.angustissima* exhibited stronger enzyme-inhibitory effects compared to *G.saharae*.

The study of the anti-inflammatory activity in vitro carried out by the method of inhibiting denaturation of proteins of extracts reveals a remarkable capacity of inhibition.

An anti-proliferative activity has been confirmed for both extracts, where, the most effective cell growth inhibition activity IC_{50} value of 18.34 µg/mL was observed in L2OB cells treated with *G. saharae*. While the lowest IC_{50} value (28.27 µg/mL) was calculated in the same cell line treated with *O. angustissima*. Slight higher IC_{50} values were calculated in the case of line derived from human rhabdomyosarcoma (RD) (29.45 and 32.45 µg/mL) for *G. saharae* and *O. angustissima* SW/extract, respectively, while in the case of Hep2c cells, the highest values of (31.25, 39.79 µg/mL) were seen for *G. saharae* and *O. angustissima* extracts, respectively. *G. saharae* seems to have better anti-proliferative activity, which could be explained by its higher and diverse content of phenolic compounds shown in the HPLC analysis.

The extracts also have shown an antimicrobial activity. Both plant extracts were equally strong against the Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*) (MIC = 78.13 µg /mL), however, *G.saharae* SW/extract showed better

activity against Gram-negative bacterial strains. Whereas, in the case of fungi, *O.angustissima* extract had stronger activity against *Candida albicans* (MIC = 78.13 µg /mL), and equal against *Aspergillus niger* (MIC = 78.13 µg /mL).

The acute toxicity study classified both extracts in the category of harmless plants with an LD_{50} greater than 5000 mg / Kg.

Owing to the safety of the used solvent, excellent yields of bioactive compounds from medicinal plants, the correlation between the content of total polyphenols and the good pharmacological power exhibited by the two plants, which supports their traditional use in the management of various pathologies. The extracts may have good potential to be used in pharmaceutical industry.

All obtained results are of great interest but only constitute a first step in the search for biologically active natural substances and sources. Complementary, precise and in-depth studies remain necessary to be able to confirm the results highlighted. Many perspectives can be envisaged which can be summarized in the following points:

- Use of advanced techniques for the isolation and identification of the active ingredients responsible for the therapeutic effects.

- Use cell cultures and isolated cells *in vitro* to estimate antioxidant and anti-inflammatory activity to confirm previous results.

- To be able to demonstrate a possible synergy between various bioactive compounds, *in vivo* investigations would be necessary to determine the biological targets, and to understand the tissue and molecular mechanism involved.

- In-depth pharmaco-toxicological studies on the two plants, in order to determine the long-term effects.

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ANNEX

APTEFF, 51, 1-206 (2020) UDC: 633.88:66.061:547.56(65)
DOI: https://doi.org/10.2298/APT2051191B BIBLID: 1450-7188 (2020) 51, 191-206

Original scientific paper

SUBCRITICAL WATER EXTRACTION OF POLYPHENOLS FROM ENDEMIC ALGERIAN PLANTS WITH MEDICINAL PROPERTIES

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Ephedra alata, Ononis angustissima, and Genista saharae are endemic Algerian plants with pharmacological potential, used for centuries in traditional medicine. Herein the efficiency of subcritical water extraction (SWE) of phenols and flavonoids from these plants was evaluated by spectrophotometric assays. The most important operational parameters of the technique (temperature, pressure, time) were optimized for each plant based on phenols yield. At defined optimal extraction conditions (140 °C for E. alata, 155 °C for O. angustissima and G. saharae, 50 bars, 15min), maximum contents of phenols in the extracts were 16.13, 18.33, and 21.12 mg GAE/g dry weight, for E. alata, O. angustissima, and G. saharae, respectively. Considering the safety of the used solvent, excellent yields of phenols and short extraction time, subcritical water extraction can efficiently be used in exploitation of pharmacological potentials of the studied plants.

Keywords: *Ephedra alata, Ononis angustissima, Genista saharae,* subcritical water extraction, phenols, flavonoids.

INTRODUCTION

Medicinal plants have been used in Algeria for centuries for their valuable bioactive compounds to treat different ailments (1). Besides its desertic and semi-desertic areas, Algeria is characterized by large pool of plants with high pharmacological potential (1, 2). Many of plant sources still haven`t been sufficiently explored. *Ephedra alata*, *Ononis angustissima* and *Genista saharae* are promising Saharan endemic medicinal plants used to treat many diseases by the local population.

Ephedra is a genus of non-flowering seed plants belonging to the Ephedraceae family (3), which includes approximately 67 species, growing mainly in the desert areas of Asia, America, Europe and North Africa (4). Among these species, *Ephedra alata* Decne (Alanda, Arabic) is particularly interesting for its ephedrine alkaloids (5), which acts on the sympathetic nervous system as a sympathomimetics (6). Ephedrine, the major *E.alata* alkaloid is widely used pharmaceutical for prevention of arterial hypotension during spinal anesthesia. It is commonly used as nasal decongestant and appetite-suppressant (7).

Besides alkaloids, the plant represents good source of polyphenolic compounds (8). The decoction of *E. alata* stems have been used in folk medicine as a stimulant, deob-

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APTEFF, 51, 1-206 (2020) UDC: 633.88:66.061:547.56(65)
DOI: https://doi.org/10.2298/APT2051191B BIBLID: 1450-7188 (2020) 51, 191-206

Original scientific paper

struent, to treat different disorders (kidney, bronchi, circular system, digestive system), to relieve asthma attack and as antifungal agent (9). Traditionally, the plant stems are chewed to treat bacterial and fungal infections (10). Previous *in vitro* studies have shown that its aqueous extracts exhibit anticancer activities, inducing apoptosis, inhibiting proliferation, inducing cell cycle arrest, and supressing tumorangiogenesis (11, 12). Furthermore, extracts show strong antimicrobial effects (13, 14).

The genus Ononis, belongs to the Fabaceae family and comprises 75 species that grow in Europe, Asia and the Mediterranean region (15). In Algeria more than 34 species have been identified (16). Members of the genus have been shown to exhibit antipyretic, antibiotic, analgesic, anti-inflammatory, cytotoxic, and antidiabetic activities (17, 18, 19). In traditional Turkish medicine, Ononis species were used for many centuries for their antiseptic, antimicrobial and diuretic properties (17, 18, 19). Ononis decoctions are reported to be useful in the treatment of rheumatic and skin diseases (18). *Ononis angustissi*ma Lam. is endemic plant of the North of Algerian Sahara (Guardaia, Bechar, Biskra, Boussaada and Beni Abbas) (20). The decoction of its aerial parts is used in traditional medicine for its hemostatic properties (21). Literature search indicate that *O. angustissima* hasn't been sufficiently studied and data on its chemical profile are scarce. However, it has been reported that its aerial parts are a good source of antioxidant polyphenols and flavonoids (22, 24, 20).

The Genista genus, from the family of Fabaceae, consists of 87 species, mainly represented in the Mediterranean area. Among them, 11 species are endemic in Algeria (25). Genista species have various uses throughout Mediterranean area. Different plant parts are used as dietary sources, animal feed, or in medicinal applications. Antioxidant properties of crude extracts of *G. tenera, G. sessifolia* and *G. tinctoria, G. cadasonenesis, G. Sandrasica* and *G. vuralii* have been reported in the literature (26, 27, 28, 29). *Genista saharae Coss. & Dur. Section Spartidium Spach.* (formerly *Spartidium saharae Coss. & Dur*) is a Saharian endemic shrub that grows in North Africa (Algeria, Libya, Morocco, Tunisia, and Egypt) (25, 30). According to ethnobotanical reports, aerial parts of *G. Saharae* were traditionally used for treating respiratory diseases and for its diuretic properties. Published studies also indicate that *G. sahara* aerial parts have strong antibacterial (31, 32) and antioxidant (23) properties. Some previous studies have focused on the chemical composition of this species revealing that the plant is a good source of antioxidant phenolic compounds such as O- and C-glycosylflavonoids and isoflavonoids (33, 34, 35).

Phenolic compounds are synthesyzed by plants as secondary metabolites necessary for their growth and physiology (36). These chemicals are continuously in the focus of scientific research because of their potent effects and because they represent the most abundant phytochemicals in human diets. These phytochemicals exhibit array of biological effects important for health protection, disease prevention and overall wellbeing (37). Phenolic compounds have protective role in diseases caused by oxidative damage (coronary and heart disease, stroke and cancers) (38, 39, 40). These molecules act against free radicals through antioxidant, redox and metal chelation capacity, acting as reducing agents, hydrogen donors or singlet oxygen quenchers (38).

Phenolic compounds have been extracted from plants sources by using various conventional extraction techniques such as maceration or Soxhlet extraction (41, 42, 43). However, these methods have a number of obvious disadvantages, such as long duration,

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consumption of organic solvents and limited efficiency towards different classes of phenolics (44). A variety of innovative extraction techniques have been developed for the extraction of bioactive compounds from natural sources, including subcritical water extraction (SWE) (45, 46, 47, 48, 49), supercritical fluid extraction (SFE) (50), ultrasound-assisted extraction (UAE) (51), microwave-assisted extraction (MAE) (52), ultrahigh pressure-assisted extraction (UPE) (43) and pulsed electric field extraction (PEF) (53). In the recovery of bioactive compounds from plant sources SWE captures more and more attention due to its safety, superior efficiency, selectivity and environment-friendly nature. As a solvent, subcritical water has the advantages of high diffusivity, low viscosity, and low surface tension, making the extraction process more efficient due to more intimate contact with sample matrix, improved solubility, and enhanced desorption kinetics (47, 61). Literature search reports subcritical water extraction of different bioactive ingredients such as polysaccharides, proteins, antioxidants, and polyphenols from plant sources (54, 55, 56). As an environmentally-friendly and efficient extraction technique, SWE shows great potential for application in different fields considering the safety of obtained extracts, superior chemical composition and compatibility of the extracts with food, pharmaceutical and cosmetic products (44).

According to available literature, there are no reports on the use of subcritical water for the recovery of polyphenols from *Ephedra alata*, *Ononis angustissima,* and *Genista saharae*. Thus, the aim of this study was to evaluate the efficiency of SWE for obtaining *E. alata*, *O. angustissima,* and *G. saharae* extracts with high content of polyphenols. The influence of the extraction temperature, pressure and time on the extraction yield has been investigated. Total content of phenolic compounds (TPC) was determined by Folin-Ciocalteau method. For every plant species, the most important operational parameters were optimized for the highest content of polyphenols.

MATERIALS AND METHODS

Chemicals and reagents

Folin Ciocalteau's phenol reagent and rutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Aluminium chloride hexahydrate and sodium carbonate were purchased from Merck (Darmstadt, Germany). Gallic acid monohydrate (GA; *purum*), was acquired from Sigma-Aldrich (Steinheim, Germany). Nitrogen was of 99.999% purity (Messer, Germany). All other chemical and reagents were of analytical reagent grade**.**

Plant material

Genista saharae was harvested in Maiter Oued in the region of Bou Saada, South of M'sila, and *Ononis angustissima* was harvested in the region of Hadjeb, west of Biskra.

Both of plant samples were collected during flowering stage in April of 2019. However *Ephedra alata* was harvested in the region of El Hadjira, North of Ouargla. The identification of collected plant samples was carried out by the Laboratory of development of natural biological resources (LVRBN) at the University of Setif. The aerial parts

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of each plant sample were dried, grounded in a blender, and stored in dark at ambient temperature until use.

Subcritical water extraction

SWE was performed in a house-made subcritical water extractor. Extraction procedure and apparatus were described previously (48). Total capacity of high-pressure stainless-steel vessel was 1.7 L. Pressurization of the extraction vessel was performed with 99.999% nitrogen (Messer, Germany). In all experimental runs, sample to distilled water ratio was 1:20 w/w. Extraction temperature (110–185 °C), extraction pressure (10-90 bar), and extraction time (15-60 min) were investigated as independent variables, while agitation rate (3 Hz) was held constant. After the extraction, the process vessel was immediately cooled in flow-through water-bath at 20 °C. Depressurization was done by valve opening and purging nitrogen through a valve. Obtained extracts were filtrated and stored in the refrigerator at 4 °C until analysis.

Determination of extraction yield

In order to determine extraction yield (EY), 2 ml of the total extracts volume obtained after the extraction was dried at 60 °C until a constant mass, weigted and used to calculate the total extraction yield. Further calculation was done according to the procedure described in Pharmacopoeia (57).

Determination of total phenolic content

Total phenolic content was measured by the Folin– Ciocalteu method (58). The reaction mixture consisted of 400 µl of the sample or standard solution and 2 ml of 1:10 v/v diluted Folin–Ciocalteu reagent. After 4 minutes, 1.6 ml of Na_2CO_3 7.5% (w/v) was added. After 90 min of incubation at room temperature, the absorbance was measured at 765 nm. The blank was prepared by replacing the extract with distilled water. Gallic acid (0–200 mg/l) was used for the standard calibration curve. The results were expressed as mg of gallic acid equivalent per g of dry plant material (mg GAE/g), and calculated as mean value ± SD (n = 3).

Determination of total flavonoid content

Flavonoids content in the extracts was determined by colorimetric assay with $AlCl_3$ according to the method described by Bahorun et al. (59). Briefly, 2 ml of 2 % $AlCl_3$, was added to 2 ml of the extract or standard solution. After 10 minutes, the absorbance was measured at 430 nm. Rutin (0–125 mg/l) dissolved in distilled water, was used as a standard. Results were expressed as mg of rutin equivalent per g of dry weight of plant (mg RE/g), and calculated as mean value ± SD (n = 3).

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Optimisation of the extraction parameters

The influence of the extraction temperature

The aim of the present study was to define optimal conditions for SWE of phenolic compounds from *E. alata*, *O. angustissima* and *G. saharae*. According to available literature, subcritical water extraction hasn`t been previously applied in the extraction of these plant matrices.

Temperature is the most important factor in the SWE process, influencing extraction efficiency and selectivity (47, 49, 48, 60). As a solvent, subcritical water has the advantages of high diffusivity, low viscosity and low surface tension, making the extraction process more efficient due to more intimate contact with sample matrix, improved solubility and enhanced desorption kinetics (47, 61).

The influence of the extraction temperature on the extraction efficiency was investigated at six different temperatures (110-185 °C), applying the extraction time of 30 min, agitation frequency of 3 Hz, and pressure of 20 bar. The temperature influence was observed by measuring yields of total phenols (expressed as mg of gallic acid equivalent per g of dry plant material (mg GAE/g)) and flavonoids (expressed as mg of rutin equivalent per g of dry weight of plant (mg RE/g)) (Table1).

Table 1. The influence of the extraction temperature on the yields of total phenols and flavonoids

Temperature (°C)	*E. alata*		*O. angustissima*		*G. saharae*	
	TPC[a] (mg GAE/g)	TFC[b] (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)
110	11.75 ±0.12[c]	5.62 ±0.19	11.30 ±0.34	9.13 ±0.14	18.03 ±0.24	8.68 ±0.18
125	15.12 ±0.18	5.28 ±0.06	11.55 ±0.3	7.81 ±0.2	19.93 ±0.19	7.59 ±0.09
140	15.58 ±0.17	4.22 ±0.12	11.67 ±0.32	8.29 ±0.2	20.90 ±0.29	7.73 ±0.15
155	14.53 ±0.09	3.74 ±0.06	16.19 ±0.12	6.99 ±0.13	24.80 ±0.43	7.04 ±0.15
170	13.90 ±0.99	3.33 ±0.05	15.34 ±0.36	6.19 ±0.13	21.82 ±0.19	6.33 ±0.07
185	14.94 ±0.19	2.76 ±0.05	13.88 ±0.4	5.11 ±0.16	18.58 ±0.31	4.79 ±0.11

[a] mean total phenols content (GAE: gallic acid equivalents).
[b] mean total flavonoids content (RE: rutin equivalents).
[c] mean± SD

Total phenols content increased with the extraction temperature from 110 to 155 °C for *O. angustissima* and *G. saharae*, and from 110 to 140 °C for *E. alata*, With further temperature increase, a drop in phenolic content was observed probably due to their degradation. The highest concentration of total phenols for *O. angustissima* (16.19 mg GAE/g) and *G. saharae* (24.80 mg GAE/g) was achieved at the temperature of 155 °C whereas the temperature of 140 °C was the optimal for *E. alata* (15.58 mg GAE/g) (Table 1).

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The highest concentration of total flavonoids was observed at the lowest tested temperature (110 °C) for all three plants, *O. angustissima* (9.13 mg RE/g), *G. saharae* (8.68 mg RE/g) and *E. alata* (5.62 mg RE/g). With further temperature increase there was a slight decrease in flavonoids content for all three plant sampels (Table 1).

The increase in phenolic content with the temperature could be explained by enhancement of the diffusion coefficients, and solubility of the target compounds with temperature increase. Decreased viscosity and surface tension of the solvent are allowing better contact with porous solid samples (62, 47, 63). The most pronounced temperature influence however, is on the polarity, by varying the extraction temperature, the dielectric constant of water can be modulated affecting it`s selectivity. Consequently, the polarity of water at elevated temperatures becomes equivalent to that of common organic solvents, targeting specific chemical class. This implies that more polar solutes soluble in ambient water are extracted efficiently at lower temperatures, whereas moderately polar and non-polar compounds require less polar solvent and higher temperatures (64, 48). It was therefore shown that major phenolics classes in analyzed plant samples are such that they are the best solubilized with water at 155 °C (*O. angustissima* and *G. saharae*) and 140 °C (*E. alata*).

The temperature affects physicochemical properties of water, but also causes degradation of the thermally labile analytes (65). Thus at higher temperatures, it can be assumed that a part of phenols and flavonoids was degraded due to high water reactivity and strong hydrolytic potential of superheated water (49), causing the drop in the extraction yields of phenols above 155 °C for *O. angustissima* and *G. saharae*, and 140 °C for *E. alata*, and also the steadily decreasing the flavonoids content above 110 °C.

Several previous studies of SWE conducted with other plant samples (66, 67, 49, 63, 68) have reported similar fashion of the decrease in phenols content with the temperature increase. The reported optimal temperatures for total phenols in SWE of *Teucrium montanum*, *Chamomilla matricaria*, *Matricaria recutita,* (*Prunus avium, Prunus cerasus*), and *Allium ursinum L.* were 160 °C, 130 °C, 160 °C, 150 °C, and 179 °C respectivelly.

The influence of the extraction pressure

The principal operational parameter in SWE is the temperature owing to effects described earlier. In SWE applied pressure mostly serves to maintain water in its liquid state, even though slight efects on water polarity with pressure increase have been reported. Namely, water polarity negligibly increases with pressure, not favoring the process of the extraction of less polar solutes, and making the process operationally less convenient (47). Elevated pressures, however, allow better solvent penetration into the pores of extracted medium making the solvent/solute contact more intimate and accelerating the process. Unlike temperature, the pressure has no significant effect on the extraction efficiency by subcritical water (67, 68). Previous studies have also shown that elevated pressures didn't improve the recovery of compounds from natural sources by SWE (71, 72, 73). However high pressures applied during the extraction help to control problems related to the formation of air bubbles within the matrix, which hinder solvent contact with the matrix (47, 62). In order to determine the influence of pressure on the recovery of phenols from studied plant materials, investigation was carried out at previously defined optimal temperatures of 155 °C for *O. angustissima* and *G. saharae*, and 140 °C for

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E. alata, applying agitation rate of 3 Hz, and extraction time of 30 min. The influence of this operational parameter was observed at four different pressures (10, 30, 50, and 70 bar). Table 2 shows extraction yields of phenols and flavonoids obtained by SWE at different pressures.

Table 2. The influence of the extraction pressure on the yields of phenols and flavonoids.

Pressure (bar)	*E. alata*		*O. angustissima*		*G. saharae*	
	TPC (mg GAE/g)	TFC (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)	TPC (mg GAE/g)	TFC (mg RE/g)
10	14.13 ±0.6 [a]	4.05 ±0.09	17.21 ±0.1	7.63 ±0.06	19.47 ±0.07	6.61 ±0.12
30	14.78 ±0.82	4.06 ±0.05	17.70 ±0.06	6.98 ±0.12	21.24 ±0.1	8.15 ±0.18
50	17.15 ±0.52	4.36 ±0.04	18.61 ±0.11	6.15 ±0.05	23.59 ±0.25	8.30 ±0.04
70	14.99 ±0.05	4.63 ±0.02	18.29 ±0.14	5.48 ±0.05	23.44 ±0.13	7.37 ±0.11

[a] ±SD

Investigation of the pressure influence revealed complex and diverse effects for different plant matrices and different chemical classes. In all cases studied here, the pressure did slightly affect flavonoid content. The calculated differences between minimal and maximal calculated contents for total phenols and flavonoids at different investigated pressures is shown in Tables 3 and 4.

The pressure of 50 bars showed to be the optimal for extracting phenols from all studied plant samples, and flavonoids from *G. saharae* (Table 2). This was in slight collision with other previously reported studies, in which, for most of plant samples optimal pressure in SWE was 20 bar (68) and 30 bar (67, 49). Švarc-Gajić, J et al. (68) extracted phenols from *Prunus avium* and *Prunus cerasus* stems reaching highest values at 20 bar, whereas Cvetanović, A et al. (67) and Švarc-Gajić et al. (49) reported the optimal pressure of 30 bar for phenols extraction from *Chamomilla matricaria* and *Matricaria recutita*.

Suprisingly, the highest flavonoids content for *E. alat*a (4.63 mg RE/g) was achieved at 70 bars, which was quite high pressure in comparisson to other studied plant matrices. Previous studies reported that the optimal pressures for flavonoids extraction from *Matricaria recutita* (49) and *Chamomilla matricaria* (67) were 30 and 45 bar, respectively.

Table 3. Minimum and maximuma extraction efficiencies of phenols at different pressures.

	Pression (bar)	E. alata	*O. angustissima*	*G. saharae*
Min TPC[a] (mg GAE/g)	10	14.13	17.21	19.47
Max TPC[b] (mg GAE/g)	50	17.15	18.61	23.59
Relative TPC change (%)		21.37	8.13	21.16

Min TPC[a]: Minimum TPC.
Max TPC[b]: Maximum TPC.

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Table 4. Minimum and maximuma extraction efficiencies of flavonoids at different pressures

	Min TFC[a] (mg RE/g)	Max TFC[b] (mg RE/g)	Relative TFC change (%)
E. alata	4.05 (10 bar)	4.63 (70 bar)	14.32
O. angustissima	5.48 (70 bar)	7.63 (10 bar)	39.23
G. saharae	6.61 (10 bar)	8.30 (50 bar)	25.57

Min TFC[a]: Minimum TFC.
Max TFC[b]: Maximum TFC.

For *O. angustissima* the lowest pressure of 10 bar provided the highest flavonoids content (7,63 mg RE/g) making elucidation of the pressure influence complex. It is obvious from our results that plant matrix has pronounced effect on the interaction of subcritical water at different pressure, stressing the necessity to carefully optimize this operational parameter for every plant matrix.

Defined optimal pressure for SWE of phenols from *O. angustissima*, *G. saharae*, *E. alata* was 50 bars, and kept constant in the following experiments.

The influence of the extraction time

Defining the optimal extraction time is important since prolonged extraction can induce degradation of the target compounds (68). In order to determine the influence of time on the recovery of phenols from studied plant materials, investigation was carried out at previously defined optimal temperatures of 155 °C for *O. angustissima* and *G. Saharae*, and 140 °C for *E. alata*, and optimal pressure of 50 bars, applying agitation rate of 3 Hz. Figures 1 and 2 summarize extraction yields of phenols and flavonoids obtained by SWE at different times.

The highest concentration of total phenols and flavonoids was observed at the lowest extraction time (15 min) for all three plants. Further prolongation of the extraction led to a slight decrease in phenols and flavonoids contents (Figures 1, 2).

The decrease in phenolic and flavonoids content with time could be explained by analyte degradation with longer extraction times. In fact, many previous studies confirm that longer extraction causes analyte degradation (63, 66, 67, 68). The reported optimal times for phenols in SWE of *Prunus avium*, *Prunus cerasus, Chamomilla matricaria*, *Teucrium montanum,* and *Allium ursinum L* were 30 min for *Prunus avium*, *Prunus cerasus, Chamomilla matricaria*, *Teucrium montanum* and 10 min for *Allium ursinum L*. According to these results, extraction time of 15 min was sufficient for the recovery of phenols and flavonoids by SWE and was adopted as optimal. Relatively short extraction times with good yields of the target compounds represent one more advantage of subcritical water extraction (68), making this technology efficient, time and energy saving.

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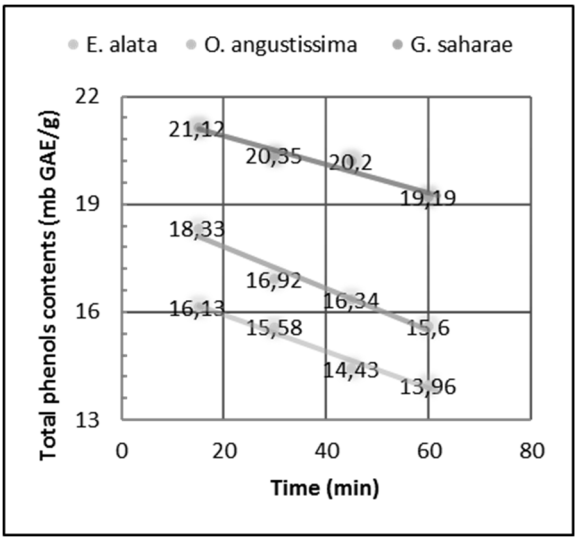

Figure 1. The influence of the extraction time on the yields of phenols

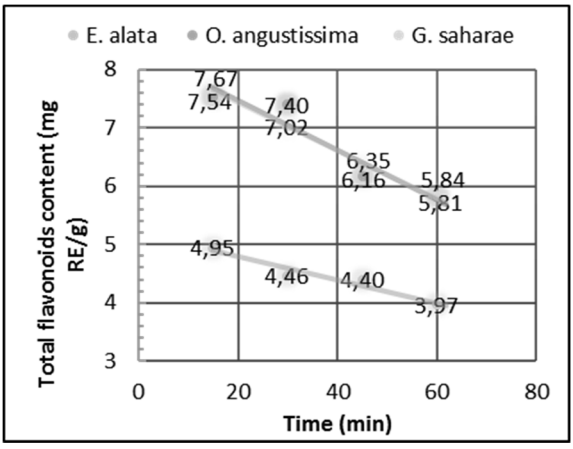

Figure 2. The influence of the extraction time on the yields of flavonoids

This idea may be implemented in the exploitation of medicinal plants at semi- and industrial level bringing the idea of flow-through technology since short extraction times are required.

Table 5 summerises maximal observed contents of total phenols and flavonoids for all three plant samples, and operational parameters at which those were achieved.

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Table 5. The maximum total phenols and flavonoids contents of *E. alata*, *O. angustissima* and *G. saharae* extracts.

	E. alata	*O. angustissima*	*G. saharae*
TPC (mg GAE/g dry weight)	16.13 ±0.3	18.33 ±0.32	21.12 ±0.48
TPC (mg GAE/g extract)	95.46 ±1.75	109.02 ±1.88	111.88 ±2.52
TFC (mg RE/g)	4.95 ±0.06	7.54 ±0.06	7.67 ±0.15
Optimal temperature (°C)	140	155	
Optimal pressure (bar)	50		
Optimal time (min)	15		

In ethanolic extract of Palestinian *E.alata,* determined total phenolic content was 9.18 mg GAE/g of extract whereas in methanolic extract the content was significantly higher (47.62 mg GAE/g of extract) (10). The contents determined in this study applying SWE were much higher, being more than two fold greater in comparison to methanolic extract. Total phenols in infusions, decoctions, and EtOH/H_2O extracts of Algerian *E. alata* harvested in the region of Tebessa determined by LC-DAD-ESI/MS were 294 mg/g extract, 380 mg/g, and 240 mg/g, respectively (27). Danciu et al. (74) determined total phenols content (156.23 mg GAE/g extract) in hydroalcoholic extracts of Tunisian *E. alata*, which was higher than the total phenols extracted by SCW in our study. The content of plants secondary metabolites in addition to being dependent on the applied extraction technique depends also on other factors, such as plant variety, geographical region, climate, soil composition, etc.

For *O. angustissima*, the total phenols obtained in our study for subcritical water extracts (109.02 mg GAE/g extract) were comparable to those obtained in aqueous extracts (118.55 mg GAE/g extract) (22), but higher (18.33 mg GAE/g dry weight) in comparison to methanolic extracts (12.03 mg GAE/g dry weight) (24) obtained after 48 hour of maceration in 80% methanol.

The content of total phenols in methanolic extracts of *G. saharae* growing in Oued Souf region (Algerian desert) (1.33 GAE/g extract) was significantly lower in comparison to contents determined in this study (111.88 mg GAE/g extract) (75). The authors applied 72 hour of maceration in methanol. The contents determined in this work were also higher than that found by Meriane et al. (33) in the MeOH extract of different parts of *G. saharae* from the region of Oued El-Maadher, Boussaâda, Wilaya of M'Sila, Algeria. In roots the authors determined 93.3 mg pyrogallol equivalent/g extract, whereas in flowers they calculated the content of 90.67 pyrogallol equivalent/g extract. The contents of total phenols determined by Guettaf et al. (23) in aqueous (130.44 mg GAE/g extract) and

ethyl acetate (459.28 mg/g extract) extracts of the same plant, and that grown in Ghardaya-Algeria, respectively, were higher of those found in this study (31).

CONCLUSION

In this study, the efficiency of subcritical water extraction of phenols and flavonoids from three endemic Algerian plants (*E. alata, O. angustissima, and G. saharae*) with medicinal properties was evaluated. The most important extraction parameters of the technique (temperature, pressure, time) were optimized for each plant. Optimal temperature for SWE of *E. alata* was 140 °C, whereas maximum yield of phenols and flavonoids in extracts of *O. angustissima* and *G. saharae* were achieved applying extraction temperature of 155 °C. For all studied plant samples, maximum yields of phenols were achieved at relatively short extraction time of 15 min at the pressing of 50 bars. At defined optimal extraction parameters, maximum contents of phenols in the extracts of *E. alata, O. angustissima, and G. saharae* were 16.13, 18.33, and 21.12 mg GAE/g dry weight, respectively.

Owing to the safety of the used solvent and excellent yields of bioactive compounds from medicinal plants, the extracts may have good potential to be used in pharmaceutical industry. Presented study, thus, represents the first step to more throughout chemical and biological study of these extracts.

Acknowledgements

The authors are grateful to the Serbian Ministry of Education, Science and Technological Development (451-03-68/2020-14/200134), and the Algerian Ministry of Higher Education and Scientific Research (DGRSDT).

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UDC: 633.88:66.061:547.56(65)
BIBLID: 1450-7188 (2020) 51, 191-206

Original scientific paper

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Received: 30 August 2020
Accepted: 08 October 2020

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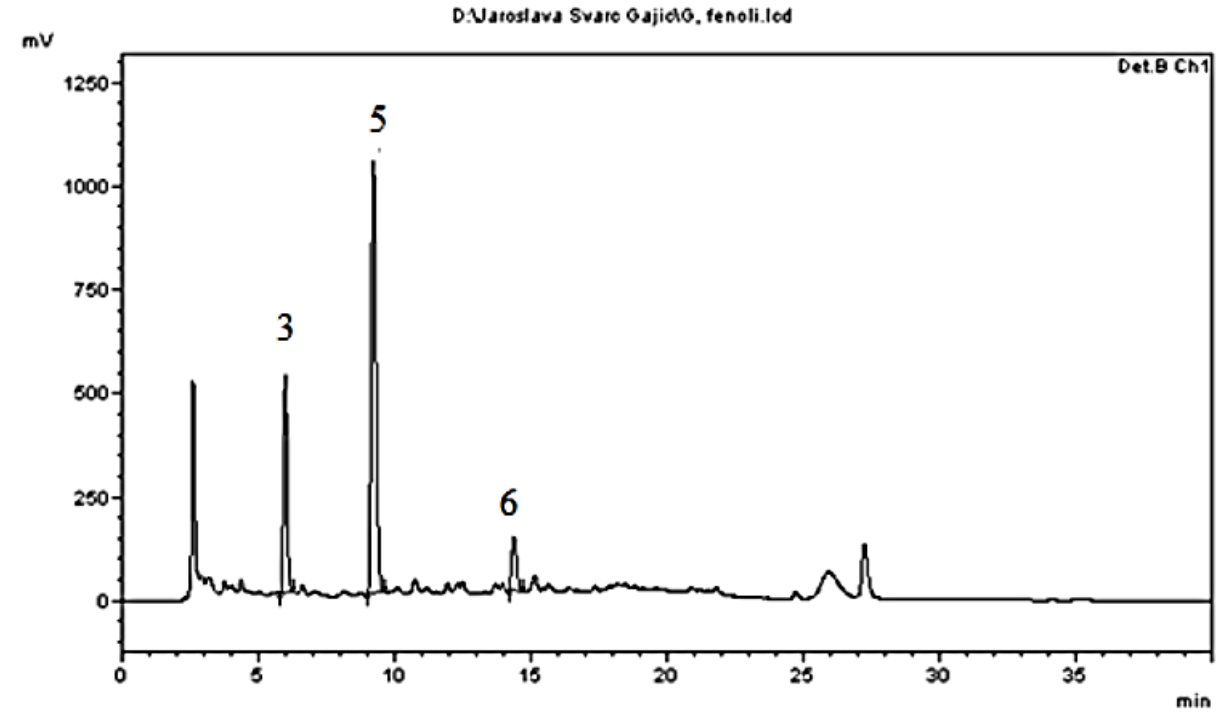
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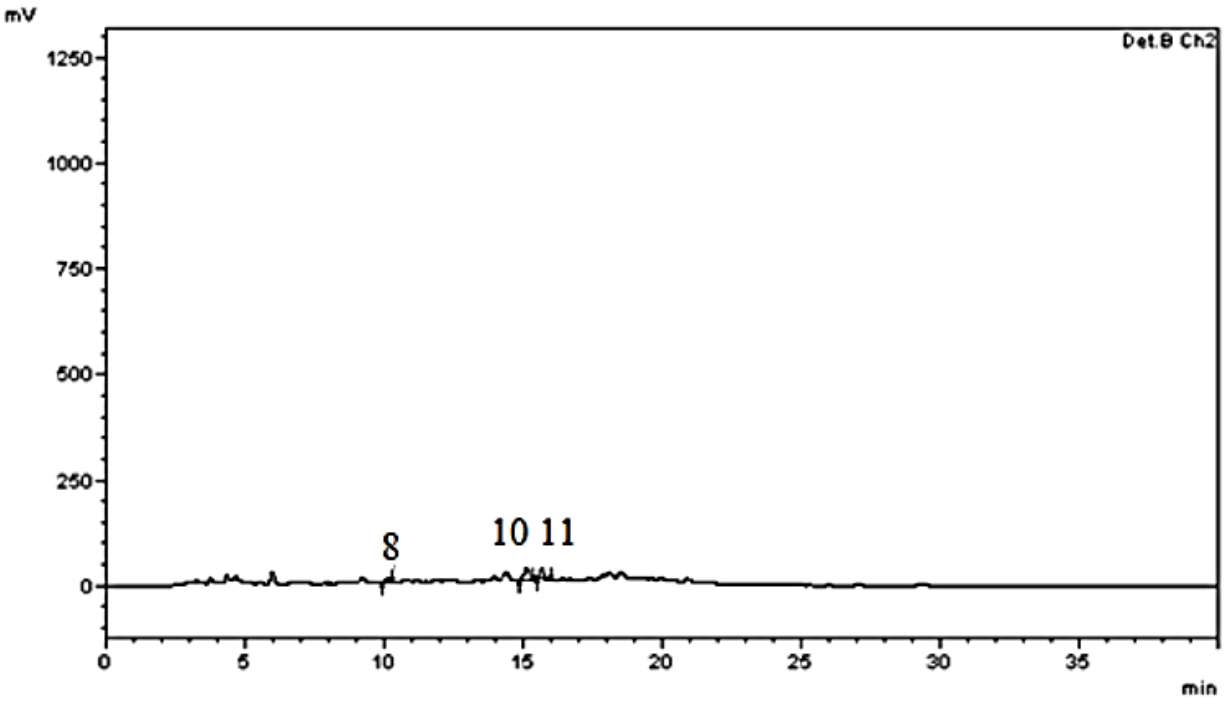
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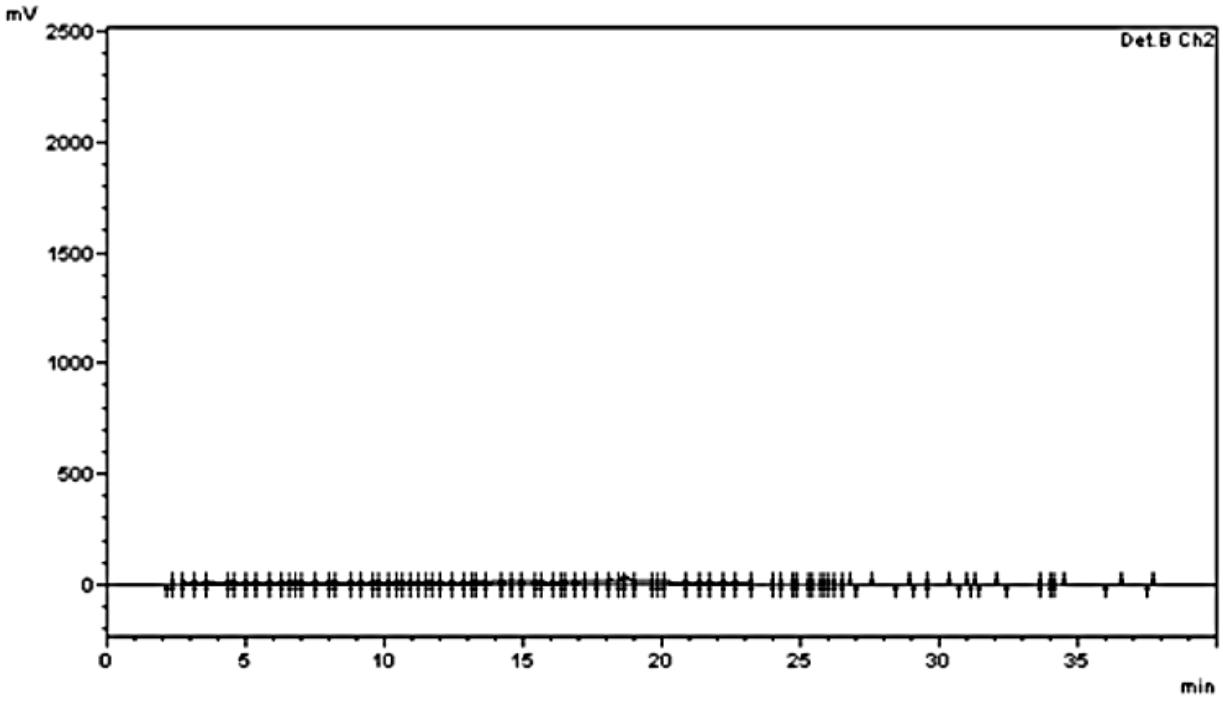
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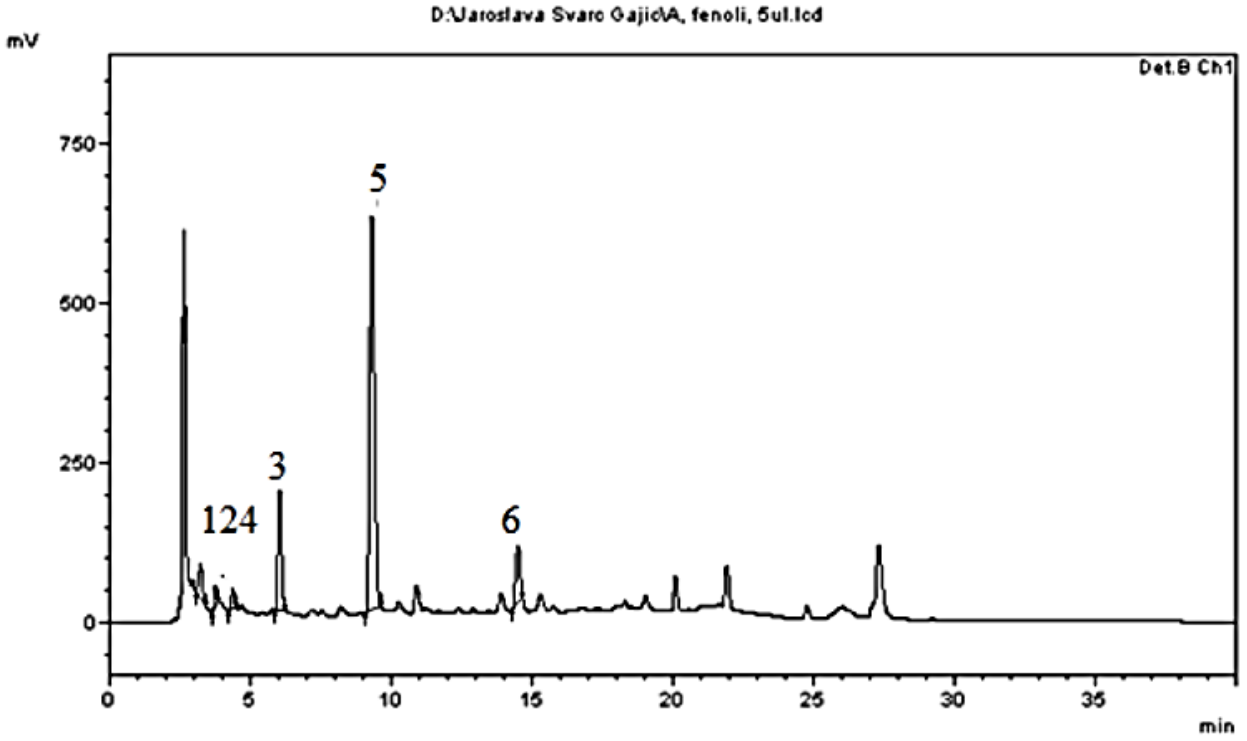
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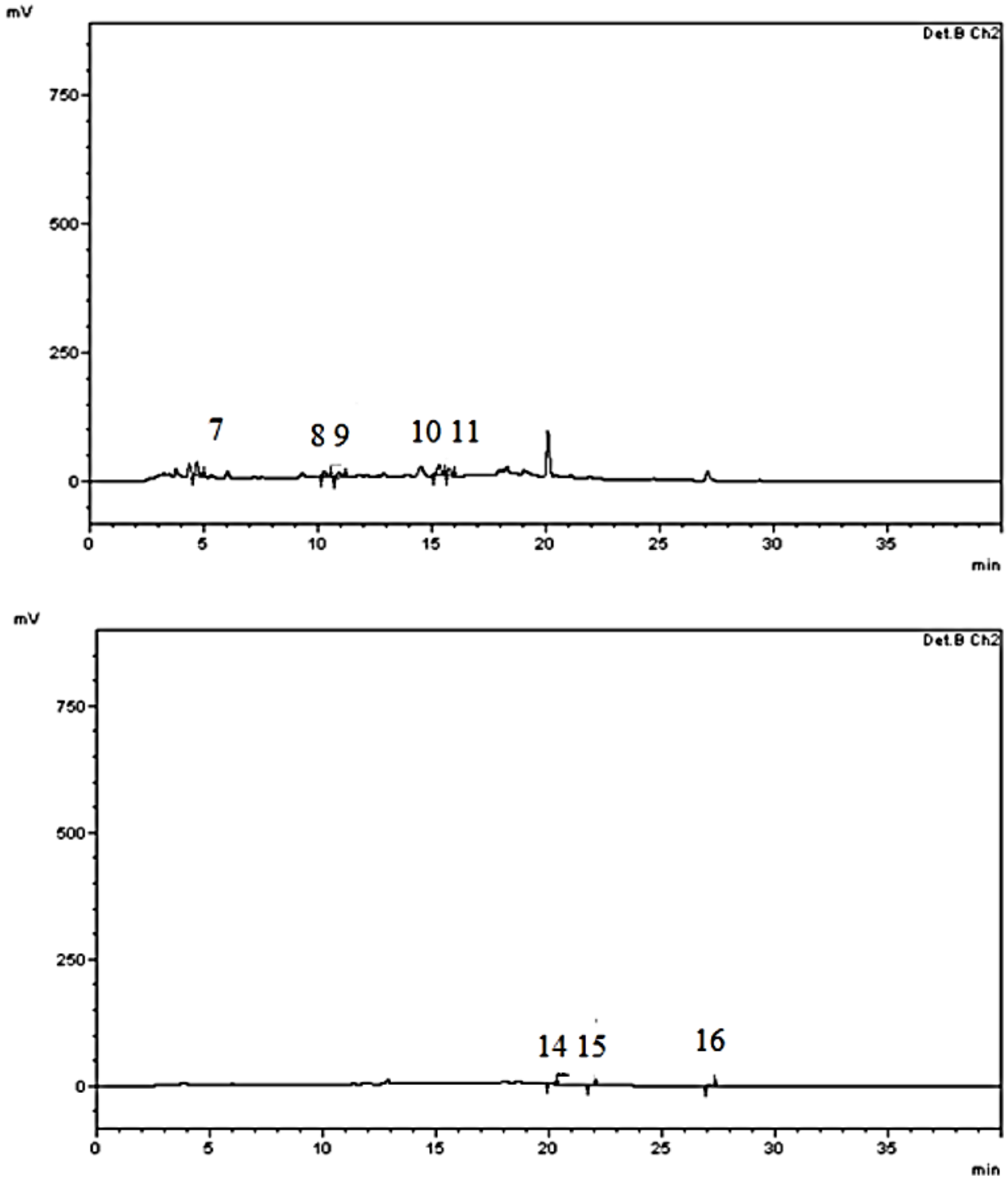

Figure captions

HPLC chromatograms of phenolics in G. *saharae* and *O. angustissima* on: A) 280 nm: 1-gallic acid; 2-catechin; 3-protocatechuic; 4-epicatechin; 5-phydroxybenzoic acid; 6-syringic acid; B) 320 nm: 7-chlorogenic acid; 8-caffeic acid; 9-gentisic acid; 10-p-coumaric acid; 11-sinapic acid; 12-rosmarinic acid; C) 360 nm: 13-rutin; 14-quercetin; 15-luteolin; 16-kaemferol.

ملخص

تعتبر *Ononis angustissima* و *Genista Saharae* من النباتات الجزائرية المتوطنة ذات القيمة الدوائية، وتستخدم منذ قرون في الطب التقليدي. تم في هذه الدراسة تم تقييم كفاءة استخراج الفينولات والفلافونويد من هذه النباتات بواسطة الماء دون الحرج (SWE)، عن طريق قياسات طيفية. تم بعد ذلك، اختبار؛ الأنشطة البيولوجية (في المختبر)، والسمية (في الجسم الحي) للمستخلصات المثلى. تم تحديد اهم العوامل التشغيلية لهذه التقنية (درجة الحرارة، الضغط، والوقت) لكل مستخلص بناءً على مردود الفينولات. في ظروف الاستخلاص المثلى المحددة (155 درجة مئوية، 50 بار، 15 دقيقة)، كان الحد الأقصى لمحتوى الفينولات في المستخلصات 18.33، و21.1 مغ GAE / غ وزن جاف، لـ *O. angustissima*، و *G. Saharae*، على التوالي. تم تقييم النشاط المضاد للأكسدة للمستخلصات من خلال فحوصات مختلفة بما في ذلك، DPPH و β-Carotene Bleaching. تم دراسة تأثير المستخلصات المضاد لمرض السكر في المختبر ضد α-amylase و α-glucosidase. تم تحديد التركيب الكيميائي للمستخلصات المختبرة بواسطة RP-HPLC-UV / Vis. كانت كل من التركيبة الكيميائية والنشاطات الحيوية قابلة للمقارنة بين *O. angustissima* و *G. saharae*. ومع ذلك، أظهرت *G. Saharae* قدرة مضادة للأكسدة أقوى قليلاً ومحتوى أعلى من المركبات الفينولية. ثلاث أحماض هيدروكسي بنزويك (p-Hydroxybenzoic، protocatechuic ، syringic) كانت هي المركبات الفينولية الرئيسية في المستخلصات المختبرة. أظهرت المستخلصات التي تم اختبارها آثارًا مثبطة ضد كل من إنزيم ألفا الأميليز و ألفا جلوكوزيداز. في كلا الفحصين التطبيقيين، أظهرت *O.angustissima* تأثيرات تثبيط إنزيمية أقوى مقارنة بـ *G.saharae*. تم إجراء اختبار MTT لاختبار النشاط المضاد للتكاثر للمستخلصات بثلاثة نسائل خلوية مختلفة. لوحظ أن نشاط تثبيط نمو الخلايا الأكثر فعالية بقيمة IC50 بقيمة 18.34 ميكروغرام / مل في خلايا الأرومة الليفية من الفئران (L2OB) المعالج بـ *G. Saharae*. تم حساب أدنى قيمة 28.27) IC50 ميكروغرام / مل) في نفس الخلية المعالج بـ *O. angustissima*. تمت دراسة النشاط المضاد للميكروبات لمستخلصاتنا ضد ست سلالات بكتيرية، بالإضافة إلى سلالتين من الفطريات. كان كلا المستخلصين النباتيين قويين بشكل متساوٍ ضد السلالات البكتيرية إيجابية الجرام (*Staphylococcus aureus*و Bacillus subtilis) (ميكروغرام / مل MIC = 78.13) ، ومع ذلك ، أظهرت *G.saharae* نشاطًا أفضل ضد السلالات البكتيرية سالبة الجرام. بينما، في حالة الفطريات، كان لمستخلص *O.angustissima* نشاط أقوى ضد *Candida albicans* (MIC = 78.13 ميكروغرام / مل)، ومتساوي ضد *Aspergillus niger* (MIC = 78.13 ميكروغرام / مل). تم فحص السمية الحادة للمستخلصات باستخدام الفئران. وجد أن الجرعة المميتة 50 أعلى من 5000 مغ / كغ. تشير النتائج التي تم الحصول عليها إلى أن مستخلصات الماء دون الحرج من *G. Saharae* و *O. angustissima* يمكن أن توفر؛ مضادات الأكسدة، مضادات ميكروبات، وعوامل مضادة للتكاثر، طبيعية وقوية لاستخدامها في علاج أمراض مختلفة.

الكلمات المفتاحية: *Ononis angustissima*، *Genista Saharae* ، استخلاص بواسطة الماء دون الحرج، الفينولات، الفلافونويد، السمية، التأثيرات البيولوجية.

Abstract

Ononis angustissima and *Genista saharae* are endemic Algerian plants with pharmacological potential, used for centuries in traditional medicine. Herein the efficiency of subcritical water extraction (SWE) of phenols and flavonoids from these plants was evaluated by spectrophotometric assays. Afterward, the optimal extracts were tested for its; biological activities (*in vitro)*, and toxicity (*in vivo*). The most important operational parameters of the technique (temperature, pressure, time) were optimized for each plant based on phenols yield. At defined optimal extraction conditions (155°C, 50 bars, 15min), maximum contents of phenols in the extracts were 18.33, and 21.12 mg GAE/g dry weight, for *O. angustissima* and *G. saharae*, respectively. The antioxidant activity of extracts was evaluated by different assays including, DPPH, and β-Carotene Bleaching Assays. The anti-diabetic effect of the extracts was investigated *in vitro* against α-amylase and α-glucosidase. Chemical profile of the tested extracts was defined by RP-HPLC-UV/Vis. Both chemical profiles and demonstrated bioactivities were comparable for *O. angustissima* and *G. saharae*. However, *G. saharae* exhibited slightly stronger antioxidant capacity and higher content of phenolic compounds. Three hydroxybenzoic acids (p-Hydroxybenzoic, protocatechuic, syringic) were the major phenolic compounds in the tested extracts. Tested extracts demonstrated inhibitory effects against both enzymes α-amylase and α-glucosidase. In both applied assays, *O.angustissima* exhibited stronger enzyme-inhibitory effects compared to *G.saharae*. The MTT assay to test the anti-proliferative activity of extracts was performed with three different cell lines. The most effective cell growth inhibition activity IC_{50} value of 18.34 µg/mL was observed in fibroblast cell line from murine (L2OB) treated with *G. saharae*. The lowest IC_{50} value (28.27 µg/mL) was calculated in the same cell line treated with *O. angustissima*. The antimicrobial activity of our extracts was studied against six bacterial strains, as well as two fungal strains. Both plant extracts were equally strong against the Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*) (MIC = 78.13 µg /mL). However, *G.saharae* SW/extract showed better activity against Gram-negative bacterial strains. Whereas, in the case of fungi *O.angustissima* extract had stronger activity against *Candida albicans* (MIC = 78.13 µg /mL), and equal against *Aspergillus niger* (MIC = 78.13 µg /mL). The acute toxicity of extracts was carried out using mice. The $LD_{50}$was found to be superior to 5000 mg/kg. Obtained results suggested that subcritical water extracts of *G. saharae* and *O. angustissima* could provide powerful natural; antioxidant, antimicrobial, and anti-proliferative agents, to be used in the management of different diseases.

Key words: *Ononis angustissima*, *Genista saharae*, subcritical water extraction, phenols, flavonoids, biological activities.