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THESIS

Presented and publicly sustained by

ASSELI Brahim

For the fulfillment of the requirements for the degree of

DOCTORATE OF SCIENCES IN BIOLOGY

TOPIC

Screening of biological activities of *Hammada elegans* Botsch extracts

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List of publications and conference contributions

The publications

1) **Brahim Asseli**, Amar Djeridane, Reguia Mahfoudi, Mohamed Yousfi. High anti-inflammatory and antidiabetic activities of *Hammada elegans* (Bge.) Botsch (Chenopodiaceae) extracts: an *in vivo* assessment. *Journal of Diabetes & Metabolic Disorders.* 2021 19:53:57 Z.

2) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. Strong Antihemolytic and Antioxidant Properties of Aqueous Extract from Algerian *Hammada elegans* (Bge.) Botsch (Chenopodiaceae). *Current Enzyme Inhibition*. 2021, 17, 1-12.

3) Feriha Bensafiddine, **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. High Antioxidant Capacities and Anti-inflammatory Effects of *Hammada elegans* Botsch. Extracts: An *in vitro* Assessment. *Current Enzyme Inhibition*, 2019, 15, 55-68.

The conference contributions

1) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. High anti-inflammatory and antidiabetic activities of *Hammada elegans* (Bge.) Botsch (Chenopodiaceae) extracts: An *in vivo* assessment. Sixth MGIBR International Workshop of Aromatic, Medicinal and condiment plants Virtues and development prospects. That was held in 21-22 December 2020, Tlemcen University, Algeria.

2) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. Etude *in vitro* de l'effet antioxydant et anti-inflammatoire des extraits de trois plantes locales: *Atriplex halimus L., Cleome arabica L.* et *Hammada elegans* Botsch. Sixth MGIBR International Workshop of Aromatic, Medicinal and condiment plants Virtues and development prospects. That was held in 21-22 December 2020, Tlemcen University, Algeria.

List of publications and conference contributions

3) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. Etude des propriétés antioxydants et antihémolytiques des extraits d'une plante algérienne: *Hammada elegans* (Bge.) Botsch (Chenopodiaceae). Séminaire international sur les Sciences de la Matière (physique et chimie) en ligne (webinaire), organisé par Algerian Journal of Engineering, Architecture and Urbanism le 22 janvier 2021, Oran, Algerie.

4) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. High antihemolytic and antioxidant capacities of aqueous extract from Algerian *Hammada elegans* (Bge.) Botsch (Chenopodiaceae). Séminaire international sur les Sciences naturelles et de la vie en ligne (webinaire), organisé par Algerian Journal of Engineering, Architecture and Urbanism le 22 janvier 2021, Oran, Algerie.

5) **Brahim Asseli**, Reguia Mahfoudi, Amar Djeridane, Mohamed Yousfi. Antioxidant, anti-inflammatory and antidiabetic activities of *Hammada elegans* aqueous extract. Séminaire National « Ressources végétales, Produits Naturels et Santé » en ligne (webinaire) le 9 - 10 - 11 juin 2021, Université de Blida, Algerie.

Abstract

The present work was carried out to study the antioxidant capacities *in vitro* systems, and to evaluate for the first time the *in vivo* anti-inflammatory, anti-diabetic and acute toxicity properties of four extracts of aerial parts of *Hammada elegans* Botsch.

The evaluation of the total phenolic content reveals the presence of interesting amounts of polyphenols. The highest content is found in the aqueous extract (2.560 ± 0.008 mg GAE/g dw). The quantification of flavonoids shows that the aqueous extract has the best content (0.275 ± 0.001 mg QE/g dw). Also, the quantification of tannins led us to conclude that this plant is rich in tannins.

Evaluation of the antioxidant capacity by the four chemical assays showed that the aqueous extract exhibited the highest antioxidant capacities compared to the other extracts and standards. Its EC_{50} value for ABTS radical-scavenging activity was 0.265 ± 0.003 mg/L. Moreover, this extract showed high iron (II) chelating ability (EC_{50} = 0.958 ± 0.137 mg/L), and good antioxidant activity in the cupric ion reducing activity (CUPRAC) in a concentration dependent manner (EC_{50} were 0.709 ± 0.002 mg/L). Additionally, this extract had the best antihemolytic activity against AAPH-induced hemolysis (EC_{50} = 0.090 ± 0.004 mg/L). Our study revealed that the aqueous extract of *Hammada elegans* Botsch, is a potential source of antioxidants which possess a high protective effect of membrane against free radical.

The acute toxicity study shows no deaths in rats and no clinical signs of toxicity after administration of increasing doses of the extracts (50-1500 mg/kg bw).

The anti-inflammatory effects show that all extracts significantly inhibit rat paw edema (EC_{50} less than 345.51 ± 0.29 mg/kg bw). Therefore, the acetonic extract (EC_{50} = 157.45 ± 0.33 mg/kg bw) had the more active anti-inflammatory activity than that of the standard inhibitor "Ibuprofen".

The evaluation of the antidiabetic activities by three tests shows that: in the short-term test there is no significant decrease in blood sugar in normal rats, whereas in starch-induced hyperglycemia test the aqueous extract was significantly decreases hyperglycemia (57.21 ± 1.24 mg AEAC/kg bw) when compared to all tested extracts, while in the long-term test the acetonic extract was significantly decreases hyperglycemia (9.18 ± 0.72 mg GEAC/kg bw) when compared to all tested extracts.

In conclusion, this study shows that *Hammada elegans* Botsch extracts have good antioxidant capacities andseem to have potential therapeutic possibilities potential for the prevention and treatment of the inflammatory and diabetic effects. Its properties appear to be linked to its richness in bioactive molecules.

Keywords: Antidiabetic activity, Anti-inflammatory activity, Antioxidant capacity, Flavonoids, Polyphenols, Tannins, Rats Wista, *Hammada elegans* Botsch extracts.

ملخص

تم القيامُ بهذا العمل لدراسة القُدرات المضادة للأكسدة في الأنظمة المختبرية (*in vitro*)، ولتقييمُ ولأول مرة خصائص مُضادات الالتهاب، السكري والسمية الحادة في الجسم الحي (*in vivo*) لأربعة مستخلصات من الأجزاء الهوائية من نبات العجرم (*Hammada elegans* Botsch).

التقييم الاجمالي للمحتوى الفينولي الكلي كشف عن وجود كميات مثيرة للاهتمام من مادة البوليفينول. تم العثور على أعلى محتوى في المستخلص المائي (2,560 ± 0,008) ميليغرام مكافئ من حمض الغاليك بالنسبة لواحد غرام من المادة الجافة. يُوضح القياس الكمي للفلافونويد أن المستخلص المائي يحتوي على أفضل محتوى (0,275 ± 0,001) ميليغرام مكافئ من كيرسيتين بالنسبة لواحد غرام من المادة الجافة. كما قادنا أيضًا القياس الكمي للعفص إلى إستنتاج أن هذا النبات غني بالعفص.

تقييم قدرة مُضادات الأكسدة بأربع طرق كيميائية أظهر أن المستخلص المائي يمتلك أعلى قدرة مُضادة للأكسدة مقارنة بالمستخلصات والمعايير الأخرى. كانت قيمة EC_{50} لنشاط الكسح الجذري (ABTS) 0,265 ± 0,003 ميليغرام /لتر. علاوة على ذلك، أظهر هذا المستخلص قدرة عالية على كمش الحديد الثنائي (EC_{50} = 0,958 ± 0,137) ميليغرام /لتر، ونشاط جيد مُضاد للأكسدة في تقليل نشاط الأيون النحاسي (CUPRAC) بطريقة تعتمد على التركيز (EC_{50} كانت 0,709 ± 0,002 ميليغرام /لتر). بالإضافة إلى ذلك، كان لهذا المستخلص أفضل نشاط مضاد للتحلل ضد إنحلال الدم الناجم عن AAPH (EC_{50} = 0,090 ± 0,004) ميليغرام /لتر. كشفتْ دراستنا أن المستخلص المائي لنبات *Hammada elegans* Botsch هو مصدر محتمل لمُضادات الأكسدة التي تمتلك تأثيرًا وقائيًا عاليًا للغشاء ضد الجذور الحرة.

أظهرتْ دراسة السمية الحادة عدم حدوث وفيات في الجرذان ولا توجد علامات سمية إكلينيكية بعد إعطاء جرعات متزايدة من المستخلصات (50-1500) ميليغرام /كيلوغرام من وزن الجسم.

تُظهرالتأثيرات المضادة للالتهابات أن جميع المستخلصات تُثبط بشكل كبير وذمة مخلب الجرذ (EC_{50} أقل من 0,29 ± 345,51) ميليغرام /كيلوغرام من وزن الجسم. لذلك، كان للمستخلص الأسيتوني (EC_{50} = 157,45 ± 0,33) ميليغرام / كيلوغرام من وزن الجسم نشاطًا مضادًا للالتهابات أكثر من المثبط المرجعي "ايبيبروفان".

يُظهر تقييم الأنشطة المضادة لمرض السكر بثلاث اختبارات أنه: في الاختبار قصير المدى، لا يوجد انخفاض كبير في نسبة السكر في الدم في الجرذان العادية ، بينما في إختبار ارتفاع السكر في الدم الناجم عن النشا، كان المستخلص المائي يُقلل من ارتفاع السكر في الدم بشكل كبير (57,21 ± 1,24) ميليغرام قدرة مضادة لمرض السكر مكافئ من أكاربوز بالنسبة لواحد كيلغرام من وزن الجسم عند مقارنته بجميع المستخلصات المختبرة، بينما في الاختبار طويل المدى، كان المستخلص الأسيتوني يُقلل من إرتفاع السكر في الدم بشكل كبير (9,18 ± 0,72) ميليغرام قدرة مضادة لمرض السكر مكافئ من جليبوكلاميد بالنسبة لواحد كيلوغرام من وزن الجسم عند مقارنته بجميع المستخلصات المختبرة.

في الختام، تُظهر هذه الدراسة أن مستخلصات نبات *Hammada elegans* Botsch تتتمتع بقدرات جيدة كمُضادات للأكسدة ويبدو أن لديها إمكانيات علاجية محتملة للوقاية والعلاج من الآثار الالتهابية والسكري، ويبدو أن خصائصها مرتبطة بغناها بالجزيئات النشطة بيولوجيًا.

الكلمات المفتاحية: النشاطية المضادة اللأكسدة، النشاطية المُضادة للالتهاب، القدرة المُضادة للأكسدة، الفلافونويدات، البوليفينول، العفص، جردان ويستار، مستخلصات نبات *Hammada elegans* Botsch.

List of acronyms and abbreviations

AAEAS	**:**	Algerian Association of Experimental Animal Sciences.
AAPH	**:**	2,2'-Azobis (2-amidinopropane) dihydrochloride.
ABTS	**:**	2 2'-azino-bis (3-ethylbenzothiazoline-6- sulfonic acid).
AEAC	**:**	Acarbose Equivalent Antidiabetic Capacity.
AMADEOX	**:**	Modulating action of the activity of antioxidant enzymes.
BDE	**:**	Bond dissociation energy.
BHA	**:**	Butylhydroxyanisole.
BHT	**:**	Butylhydroxytoluene.
bw	**:**	Body Weight.
CAA	**:**	Cellular antioxidant activity.
CAT	**:**	Catalase.
CE	**:**	Catechin equivalents.
COX	**:**	Cyclooxygenase.
COX1	**:**	Cyclooxygenase1.
COX2	**:**	Cyclooxygenase2.
CP	**:**	Chelating power.
CUPRAC	**:**	Cupric ion Reducing Antioxidant Capacity.
DCFH-DA	**:**	Dichloro-dihydro-fluorescein diacetate assay.
DMSO	**:**	Dimethyl sulfoxide.
DNA	**:**	Deoxyribonucleic acid.
DPPH	**:**	1,1-Diphenyl-2-pycril-hydrazil.
EC_{50}	**:**	Half-Efficient Concentration.
EDTA	**:**	Ethylenediaminetetraacetic acid.
EMC-D	**:**	Encephalomyocarditis.

List of acronyms and abbreviations

EquiNox2	**:**	Equine phagocytic NADPH oxidase.
ESR	**:**	The erythrocyte sedimentation rate.
FBG	**:**	Fasting Blood Glucose.
FOX	**:**	The ferrous oxidation-xylenol orange assay.
FRAP	**:**	Ferric reducing-antioxidant power.
G	**:**	Glycaemia.
GAE	**:**	Gallic acid equivalents.
GEAC	**:**	Glibenclamide Equivalent Antidiabetic Capacity.
GPx	**:**	Glutathione peroxidase.
GSH	**:**	Glutathione.
GTG	**:**	Gold thioglucose.
H	**:**	Hour.
H_2O_2	**:**	Hydrogen peroxide.
HI	**:**	Haemolysis inhibition.
HOCl	**:**	Hypochlorous acid.
IC_{50}	**:**	50% Inhibition Concentration.
LD_{50}	**:**	Median lethal dose.
LOX	**:**	Lipooxygenases.
LPS	**:**	Lipopolysaccharide.
min	**:**	Minute.
MODY	**:**	Maturity Onset Diabetes of the Young.
nm	**:**	Nanometer.
$NO^{\bullet}$	**:**	Nitrogen monoxide.
$^1O_2$	**:**	Singlet oxygen.

List of acronyms and abbreviations

$O_2^{\bullet-}$	**:**	Superoxide radical.
OECD	**:**	Organization for Economic Cooperation and Development.
$OH^{\bullet}$	**:**	Hydroxyl radicals.
ONAB	**:**	National Office of Livestock Foods.
ONOO-	**:**	Peroxynitrite.
ORAC	**:**	Absorption capacity of oxygenated radicals.
P	**:**	Probability value.
PAF	**:**	Platelet activating factor.
PBS	**:**	Phosphate buffered saline.
PCA	**:**	Principal component analysis.
PD	**:**	Percentage decrease.
PG	**:**	Propyl gallate.
PGD2	**:**	Prostaglandin D2.
PGE2	**:**	Prostaglandin E2.
PI	**:**	Percentage of inhibition.
QE	**:**	Quercetin equivalents.
RAF	**:**	Relative activity factor.
RBC	**:**	Red blood cells.
$RO^{\bullet}$	**:**	Alkoxyl radical.
$ROO^{\bullet}$	**:**	Peroxyl radical.
ROOH	**:**	Hydroperoxides.
ROS	**:**	Reactive oxygen species.
RP	**:**	Reducing power.
SD	**:**	Standard deviation.

List of acronyms and abbreviations

SIEFED	**:**	Specific Immunological Extraction Followed by Enzymatic Detection.
SOD	**:**	Superoxide dismutase.
STZ	**:**	Streptozotocin.
TBHQ	**:**	Tertiary-butylhydroquinone.
TPTZ	**:**	2,4,6-Tris (2-pyridyl)-s-triazine.
TRAP	**:**	Telomerase repeated amplification protocol.
V	**:**	Volume of edema.
W	**:**	Weight.
Y	**:**	Yield.

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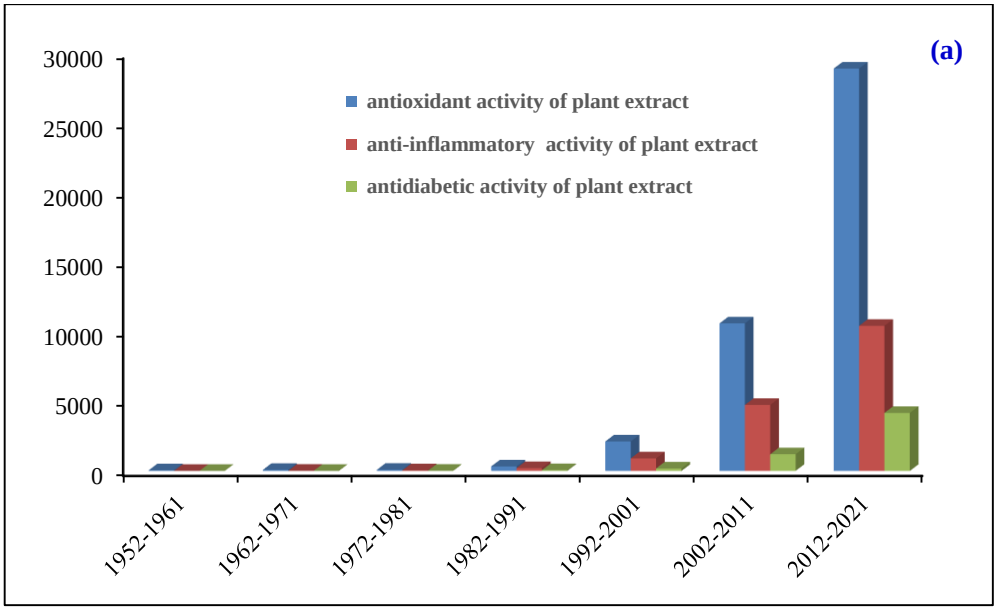
Introduction

The human body has evolved a highly antioxidant protection systems, that functions interactively and synergistically to neutralize free radicals. Thus, antioxidants defense may be based on several mechanisms namely: scavenging antiradical capacity; metal chelating capacity; reducing capacity; and inhibition of oxidative enzymes (**Jacob, 1995**). Therefore, overproduction of free radicals (FR) in general and reactive oxygen species (ROS) in particular can cause oxidative damage to biomolecules, eventually leading to many chronic illnesses such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, diabetes, and chronic inflammation (**Freidovich, 1999**). Then, plants rich in such phytochemicals could be used in the treatment of medical conditions associated with chronic hyperglycemia, oxidative stress and inflammation.

Inflammation is then, a homeostatic process whose purpose is to limit tissue destruction, destroy the causal agent and activate the tissue repair process by activating a whole cascade of chemical reactions. The inflammatory reaction occasionally exceeds its objectives and causes harmful effects (**Nathan, 2002; Barton, 2008**). The anti-inflammatory therapy is generally carried out by synthetic molecules of non-steroidal or steroidal anti-inflammatory type (corticosteroids), these drugs are widely used, but their side effects are sometimes serious, in particular the toxicity on the renal and the digestive systems (digestive irritations up to gastric ulceration) (**Das et *al*., 2010**).

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. It is characterized by hyperglycemia and accompanied by various chronic vascular complications (**Nickavar and Mosazadeh, 2009; Feng et *al*., 2011**). Diabetes is a major cause of morbidity and mortality worldwide and it contributes substantially to healthcare costs. In 2017 diabetes accounted for 425 million cases and its prevalence will rise to 629 million by 2040 (**Canto et *al*., 2019**). Among the various treatment strategies, diet therapy, pharmacotherapy, and insulin therapy are the main treatment options available to control diabetes, in addition to wide range of glucose lowering drugs which exert their hypoglycemic effects through various mechanisms (**Bhatt et *al*., 2019**). However, these treatment options have not gained much significance as these treatment strategies are commonly associated with disadvantages, such as drug resistance, side effects, and toxicity.

The therapeutic values of medicinal plants are similar in some chemical metabolites that produce a definite biological action on the human body (**Karaman et *al*., 2013**). Hence, regardless of the presence of the anti-inflammatory and hypoglycemic pharmacological drugs, supplementation of herbal based drugs to treat diabetes mellitusor inflammation is now a promising and novel treatment strategy due to its safe and non-toxic nature (**Palazzetti et *al*., 2003; Karaman et *al*., 2013**). It's for these reasons that researches on antioxidants, anti-inflammatory and antidiabetic activity of medicinal plants for understanding their role has grown exponentially. A search of 'Pubmed' and 'Science Direct' database using the term « antioxidant activity of plant extract », «anti-inflammatory activity of plant extract», and « antidiabetic activity of plant extract » revealed availability of plethora of literature. While prior to 1940 there was not even a single reference on the term antioxidant, anti-inflammatory and antidiabetic. But, using the terms « antioxidant activity of plant extract », « anti-inflammatory activity of plant extract», and « antidiabetic activity of plant extract » , approximately 116 , 10 and 4 citations respectively in the database 'Pubmed' and 432, 371, 19 citations respectively in the database 'Science Direct' were obtained for the period 1952 to 1971. However, the number of citations pf the terms for the terms « antioxidant activity of plant extract », « anti-inflammatory activity of plant extract», and « antidiabetic activity of plant extract ») was significantly increased to 39494 , 15125, 5351 citations respectively in the 'Pubmed' database and to 96213, 63423 and 5351 citations respectively in the 'Science Direct' database between 2002 and 2021 (**Figure 1**). These results clearly shows that in recent years, research on antioxidants, anti-inflammatory and antidiabetic agents in medicinal plants has recognized a huge popularity, indicating that a large number of plants worldwide have been analyzed for their antioxidant, anti-inflammatory and antidiabetic activity. So, it would be necessary to focus more on Algerian medicinal plants.



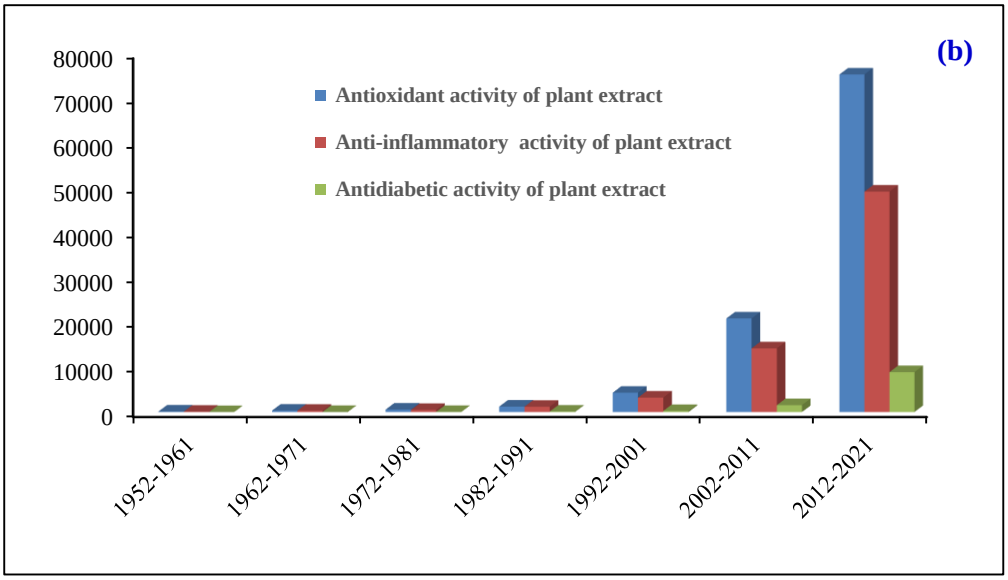


Figure 1. Results of PubMed (a) and Science Direct (b) search on terms « antioxidant activityof plant extract », « anti-inflammatory activityof plant extract», and « antidiabetic activity of plant extract » over the years.

Algeria is one of the richest Arab countries; its flora comprises 3,139 species consisting of 3,744 taxa including 464 endemic and 1,818 more or less rare species. This inventory is now evaluated at 4,449 taxa including 3,951 native taxa and 498 introduced to Algeria (**Dobignard and Chatelain, 2010**). However, few studies have been carried out in this country on the phytochemical plan as well on the pharmacological plan of local plant species. Moreover, a large

number of traditional herbal medicines have been used in the steep area of Algeria (Laghouat) since ancient times to treat different ailments.

In this scientific evaluation of medical plants potential, we have selected, for study purposes, a plant from Laghouat region in Algeria, from Chenopodiaceae family: *Hammada elegans* (Bge.) Botsch is a xerophytic plant widely distributed in rocky grounds and popularly known as (Ajram). The most important factor in choosing this plant that there is no previous study *in vivo* systemsof the anti-inflammatory and anti-diabetic activities, in addition to the acute toxicity of *Hammada elegans* Botsch extracts. Therefore, the research program is structured around five parts:

- ✓ The first part of this work was dedicated to the preparation of plants extracts, total phenolics, flavonoids and tannins quantification.

- ✓ The second part focused on the evaluation of antioxidant of extracts taken by*in vitro* measures, and their potential for trapping power for the $ABTS^{\bullet+}$ Radical (ABTS Test.), their potential to modulate the redox system (CUPRAC Test), their power of chelating ferrous iron, and their antihemolytic property (Hemolysis Test).

- ✓ The third part was destined for the acute toxicity test of these extracts on Albino Wistar rats.

- ✓ The fourth part was meant for the anti-inflammatory activity of these extracts by *in vivo* measuring of their power of inhibitory potency of rat paw edema induced by carrageenan.

- ✓ Finally part was meant for the examination of *in vivo* antidiabetic activity in normal rats and diabetic rats. We used three procedures: short term test, starch hyperglycemia test and long-term test.

Bibliographic review

I. Antioxidant activity

I.1. Definitions

I.1.1. Oxidative stress

Oxidative stress, sometimes called oxidative stress, in biological systems, oxidative stress is the consequence of an imbalance between the production of free radicals and the destruction of antioxidant defenses by antioxidant systems, so it is an imbalance in the antioxidant/oxidant balance in favor of pro-oxidants. It is also defined as a state in which the level of intermediate toxic reactive oxygen overcomes the host's endogenous antioxidant defenses; it develops when there is an overproduction of free radicals and a deficit of antioxidants, i.e. the oxidizing molecules are produced faster than they can be neutralized by the organism, therefore the origin of oxidative stress is mainly free radicals (**Koppenol, 2001; Atamer et *al*., 2008**).

Oxidative stress is the main initial cause of several diseases: cancer, cataract, amyotrophic lateral sclerosis, acute respiratory distress syndrome, pulmonary edema, accelerated ageing. It is also one of the factors that potentiate the appearance of multifactorial diseases such as diabetes, Alzheimer's disease, rheumatism, carcinogenesis, cardiovascular diseases, inflammation..… (**Surveswaran et *al*., 2007; Bekro et *al*., 2008**).

I.1.2. Free radicals and reactive oxygen species (ROS)

Free radicals are atoms, molecules or parts of molecules containing one or more unpaired electrons in their extreme orbit (**Goto et *al*., 2008**), this molecule is very unstable and reacts quickly with other components, trying to capture the electron needed to acquire stability, A chain reaction begins when a free radical attacks the nearest stable molecule by taking its electron out of it, and the attacked molecule itself becomes a free radical, thus initiating a chain reaction and we witness the destruction of long chains of molecules accompanied by cascading cellular degradation (**Dacosta, 2003; Goto et *al*., 2008**). Among all the radical species likely to be formed in cells, it is necessary to distinguish a restricted set of radical compounds which play a particular role in physiology and which we shall call primary free radicals, which derive directly from oxygen. The other free radicals, known as secondary radicals [Peroxyl radical (ROO•), Alkoxyl

radical ($RO^{\bullet}$)], are formed by the reaction of these primary radicals with biochemical compounds in the cell (**Atamer et *al*., 2008**).

Oxygen is the source of life for aerobic organisms. But oxygen can also be a source of aggression for these organisms (**Ekoumou, 2003**). All primary free radicals are often referred to as reactive oxygen species (ERO) or reactive oxygen species (ROS)(**Gueye, 2007**) which are various forms of active oxygen, they include oxygenated free radicals (chemical species possessing a single unpaired electron) such as : the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), nitrogen monoxide ($NO^{\bullet}$),..... and also certain reactive oxygen derivatives that are non-radical (not possessing a single electron) and whose toxicity is important, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$), …. (**Halliwell and Whiteman, 2004**).

The excessive production of free radicals produced from endogenous sources (are products of the organism's reactions) and exogenous sources (cigarette smoke, ultraviolet rays, radiation, transition metals, medicines.....)(**Pastre, 2005**) causes direct damage to biological molecules: oxidation of DNA (deoxyribonucleic acid), proteins, lipids and carbohydrates, but also secondary damage due to the cytotoxic and mutagenic nature of the metabolites released in particular during the oxidation of lipids (**Surveswaran et *al*., 2007**). In addition, the toxic effect of free radicals can lead to the alteration of cellular functions such as cell division and apoptosis and thus cell death (**Valko et *al*., 2007**).

I.1.3. Antioxidant

When the production of free radicals becomes too great, our antioxidant reserves may become insufficient to neutralize the harmful effect of free radical oxidation on our tissues and cells. This is why the body has developed very effective defense systems against the production of free radicals. The molecules controlling this production are called "antioxidants" (**Goto et *al*., 2008**). We then speak of "oxidative stress".

Antioxidants are all substances which, present in a low concentration compared to that of the oxidizable substrate, significantly delay or inhibit the oxidation of this substrate, and whose products of the reaction between the oxidant and the antioxidant must not be toxic and do not connect the radical reaction (**Parihar et *al*., 2008**). These antioxidants have two origins, one

provided by food, while the other is endogenous, represented by antioxidant enzymes directly synthesized by the body (**Droge, 2002**).

I.2. Defense system against free radicals

The organism is determined from a set of highly effective antioxidant defense systems in order to reduce the concentration of oxidant species in the body.Antioxidants are very diverse compounds that group together molecules with enzymatic and non-enzymatic activity (**Joël et *al*., 2002; Garrel et *al*., 2007**).

The three main enzymatic defense systems involve superoxydes dismutases (SOD), catalases (CAT) and glutathione peroxidases (GPX):

- Superoxide dismutases (SOD) SOD is one of the most important cellular enzymes that catalyzes the dismutation of the superoxide anion into oxygen and hydrogen peroxide (**Landis and Tower, 2005**).
- Catalase (CAT) is an enzyme located mainly in red blood cells and liver peroxisomes. It converts two molecules of H_2O_2 into H_2O and O_2 (**Valko et *al*., 2006**).
- Glutathione peroxidase and reductase (GSHPX) are capable of reducing on the one hand hydrogen peroxide into water molecules, and on the other hand organic hydroperoxides (ROOH) into alcohols. During this reaction, which requires the intervention of two molecules of glutathione (GSH), these are transformed into glutathione disulphide (GSSG) (**Valko et *al*, 2006**).

Non-enzymatic antioxidants, on the other hand, mainly comprise trace elements, reduced glutathione (GSH), uric acid, vitamins A, C and E and polyphenols.

- Trace elements such as copper, zinc, manganese, selenium and iron are essential metals in the defense against oxidative stress. These trace elements act as cofactors to maintain the catalytic activity of antioxidant enzymes (**Ahmet, 2003**).
- Glutathione plays a major role in protecting lipids, proteins and nucleic acids against oxidation (**Stamler and Slivka, 1996**). In situations of oxidative stress, its protective and detoxifying role results mainly from its function as a coenzyme of GSHPX. It also

interacts synergistically with other components of the antioxidant protective system such as vitamin C or vitamin E (**Gerard-Monnier and Chaudière, 1996**).

- Uric acid is a major terminal product of the purine metabolism in humans during intense physical exertion. Uric acid is a powerful free radical reducer; it reduces peroxyl and hydroxyl radicals and also neutralizes the superoxide anion (**Ames et *al*., 1981**).
- β-carotene (provitamin A) has the ability to capture singlet oxygen and is found in green vegetables, salad, carrots, spinach, apricots, papaya, melon, pumpkin and other yellow fruits (**Valko et *al*., 2006**).
- Vitamin C (ascorbic acid) is water-soluble. It plays a role in preventing oxidation in plasma and extracellular fluids, of which it is considered the most important antioxidant (**Fain, 2004**). Its action is both direct and indirect, acting directly on ROS (superoxides, hydroxyl, singlet oxygen, lipid radicals) and indirectly through its action of regeneration of vitamin E and GSH (**Duarte et *al*., 2007**).
- Vitamin E (tocopherol) is considered the main antioxidant. It intervenes directly at the level of biological membranes where it traps free radicals before they reach their targets. Vitamin E is liposoluble; it binds to membranes and can thus sequester free radicals preventing the propagation of lipid peroxidation reactions (**Pryor, 2000; Valko et *al*., 2006**).
- Polyphenols include a large number of natural substances of plant origin. Almost all of them exhibit marked antioxidant activity. Polyphenols possess the ideal chemical structure free radical scavenging activity, and have been shown to be more effective antioxidants in vitro than tocopherols, ascorbate, anthocyanins, flavonoids and tannins (**Boizot and Charpentier, 2006; Arora et *al*., 2000**).The antioxidant properties of polyphenols derive from their high reactivity as hydrogen or electron donors, the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (functional chain break), and their ability to chelate the metal ion transition (end of the Fenton reaction) (**Vertuani et *al*., 2004**). A very large body of experimental data now pleads in favor of their involvement in the prevention of degenerative diseases such as cancer, cardiovascular disease, osteoporosis, inflammatory diseases and diabetes.... (**Rock, 2003**).

In addition, there are other types of antioxidants such as:

- In the food industry, synthetic antioxidants such as butylated hydroxyanisole (BHA), butyl hydroxytoluene (BHT), propyl gallate (PG) and ter-butylhydroquinone (TBHQ) are widely used because they are effective and less expensive than natural antioxidants. However, their safety is much debated, as they are likely to have side effects and even toxicity (**Lisu et *al*., 2003**).
- Proteins circulating in the serum can take up free metal ions which are potentially toxic; these are transferrin and lactoferrin for iron and ceruloplasmin for copper. They act as chelating agents and keep the metal ions in an inactive form compared to the possible combination with hydrogen peroxide (**Jacques and André, 2004**).

II. 3. Methods for evaluating antioxidant activity

Many methods have been developed to evaluate the antioxidant activity of natural extracts. These methods differ from one another in terms of reaction mechanisms, substrate and antioxidant, reaction states and the form in which the results are expressed. A better understanding of these methods contributes to the correct interpretation of the results (**Magalhaes and Coll, 2008**).

Moreover, today, methods for assessing antioxidant activity are not standardized. Many factors come into play. Indeed, the method chosen is strongly linked to the reaction parameters: the solvent, the reaction time and the pH, etc. (**Magalhaes and Coll, 2008**).

There is a parallel increase in the interest in antioxidants and in the application of methods to estimate the effectiveness of these substances (**Bensafiddine, 2019**). Many methods are used to evaluate the antioxidant activity of a pure product or a mixture. Most of these methods are based on the staining or decolonization of a reagent in the reaction medium. However, these methods are not standardized and can vary greatly from one laboratory to another. However, the most commonly used methods for measuring antioxidant activity include:

II.3.1. Chemical methods:

Spectrophotometric, fluorimetric and chemiluminescence methods:

β-carotene, ORAC, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, FRAP, CUPRAC, DCFH-DA, FOX, TRAP, Iron chelation, others: $O_2^{\bullet}$, $OH^{\bullet}$, $ONOO^-$, HOCl.... (**Georgieva et *al*., 2010**).

II.3.2. Chemiluminescence methods:

Without amplifier, with amplifier, techniques for determining an antioxidant activity on lipoperoxidation, techniques for determining an antioxidant activity on lipoperoxidation, measurement of lipoperoxidation by the FOX method (**Celik et *al*., 2010**).

II.3.3. Methods based on the use of cells (cell-based assays):

CAA: cellular antioxidant activity, expression of antioxidant enzymes vs. inhibition of pro-oxidant enzymes, activation vs. repression of redox transcription factors (**Yonghui et *al*., 2016**).

II.3.4. Anticatalytic methods:

SIEFED method, EquiNox2 method (**Nyssen et *al*., 2018**).

II.3.5. Combined methods:

AMADEOX, lipoperoxidation: detection of lipid radicals by ESR, point on other methods (**Aranda-Merino et *al*., 2019**).

II. Anti-inflammatory activity

II.1. General informations

Inflammation is a coordinated adaptive process induced by microbial infection or tissue injury. It requires fine regulation, which is generally beneficial, and leads to the elimination of possible pathogens and the return to homeostasis of the injured tissue (**Barton, 2008**). The inflammatory reaction is responsible for local phenomena characterized by 4 cardinal signs which are redness,

heat, pain and œdema and by a series of cellular and biochemical processes: leukocyte, platelet and macrophage influx, release of arachidonic acid derivatives (prostaglandins including PGE2, thromboxanes and leukotrienes), platelet activating factor (PAF), haemoglobin, vasodilating amines such as histamine, serotonin and kinins, proteolytic enzymes and superoxide ions (**Binard and Saraux, 2006; Yam et *al*., 2009**). Inflammation is usually a beneficial process: Its purpose is to mobilize the immune system to eliminate the pathogen and repair tissue damage. Sometimes inflammation can be harmful because of the aggressiveness of the pathogen, its persistence, the site of the inflammation, or abnormal regulation of the inflammatory process **(Kirassian, 2015; Bounihi, 2016**). The main function of inflammation is to eliminate the aggressive agent and allow tissue repair. Short-term inflammation, known as acute inflammation, is a beneficial phenomenon for the organism which enables it to regain its physiological integrity. Whereas the negative aspect of the inflammation occurs when it becomes permanent and becomes a chronic inflammation (**Weill et *al*., 2003**).

II.2. Types of inflammation

Inflammation is classified into two categories according to the duration and kinetics of the inflammatory process (**Charles et *al*., 2010; Mansour, 2015**).

II.2.1. Acute inflammation

Acute inflammation is the body's immediate response to an aggressive agent. It is short-lived and can last from a few days to a few weeks, often abrupt in onset and characterized by intense vasculo-exudative phenomena. Acute inflammations heal spontaneously or with treatment, but can leave after-effects if there is significant tissue destruction. (**Weill et *al*., 2003; Serhan et *al*., 2010; Charles et *al*., 2010**). Acute inflammation is characterized by three main phases: an immediate vascular phase (of the order of minutes) characterized by changes in local microcirculation, a subsequent cellular phase characterized by the mobilization of numerous immune cells which will allow the elimination of pathogenic microorganisms and damaged tissue, and a healing phase which in a few days will lead to the restoration of the tissue (**Weill et *al*., 2003; Male et *al*., 2007**). Acute inflammation is a generally beneficial reaction for the organism since it helps it to defend itself against an aggression, and then to repair the damaged tissue (**Serhan et *al*., 2010**).

II.2.2. Chronic inflammation

Chronic inflammation is a prolonged inflammation, morphologically defined by the presence of lymphocytes, macrophages, and plasma cells in the tissue. In many cases, the chronic inflammatory response may persist for long periods (several months or years) (**Serhan et *al*., 2010; Charles et *al*., 2010**) When the pathogen persists due to failure of the acute inflammatory response or an inappropriate response, the balance between pro-inflammatory and anti-inflammatory molecules is disturbed and the inflammatory response evolves towards chronicity causing serious anatomical and functional sequelae. This imbalance is at the origin of many pathologies characterized by significant tissue destruction (**Mebirouk, 2017**). Chronic inflammation is also caused in the case of certain autoimmune diseases, and is thus characterized by a long duration (**Trabssa, 2015**).

II.3. Mechanism of the inflammatory reaction

The inflammatory reaction is a normal response of the body to immune or non-immune attacks. It is also a reaction of the connective tissue and vessels. The onset and course of the inflammation is governed by nerve reflexes and above all by endogenous chemical mediators (histamine, serotonin). Recent studies have brought to light an interaction between the pro-inflammatory enzymes Lipooxygenases (LOX) and diseases such as cancer, heart disease or asthma. Cyclooxygenase enzymes are also heavily involved in a number of diseases (**Bernard, 2003;Mena-Rejon et *al*., 2009**). In response to physical or chemical disturbance, activation of phospholipase A2 occurs, which hydrolyses the ester bonds of membrane phospholipids and releases arachidonic acid derivatives, which in turn are metabolized in two possible ways: (i) the lipooxygenase pathway which transforms it into leukotriene and (ii) the cyclooxygenase pathway which transforms it mainly into prostaglandin.Leukotrienes increase capillary permeability and exert chemoatractivity on the polynuclear cells. Prostaglandins produce local vasodilatation, promote œdema and leukocyte influx, in addition, they depress certain immune mechanisms and potentiate the algogenic effects of bradykinin (**Devillier, 2005; Venereol, 2005; Mena-Rejon et *al*., 2009**).

The inflammatory reaction involves a coordinated sequence of events:

- The first phase (the early phase or vascular phase), when a tissue undergoes aggression, specialized cells, the mast cells, release histamine and serotonin which stimulate vasodilatation in the affected part, causing erythema, redness and local heat.
  The (overloaded) capillaries release fluid that seeps into the tissues, causing swelling and a painful sensation.
- The second phase (the secondary phase or cell phase) is characterized by an extra-vascular migration of leukocytes and the release of cytokines, which are responsible for cell activation and the release of mediators. From then on, a succession of events within the inflammatory lesion leads to: the phagocytosis of external agents, the capture and presentation of antigens and the production of free radicals. In addition, cytokines act at the systemic level to increase the host's defense in the form of fever.
- The third phase (regeneration phase) consists of the formation of a granulation tissue. The leukocytes flow in from the macrophages and fibroblasts appear. The granulation tissue is a young connective tissue, rich in fibroblasts and capillaries, poor in connective fibers. This newly formed tissue can be individualized as a granuloma.
- The fourth phase (the terminal phase) is a phase of sclerosis of the newly formed tissue, which, when invaded by connective fibers, loses its elasticity and becomes sclerotic (**Salamatou, 2003; Timbo, 2003; Bertrand, 2005**).

II.4. Anti-inflammatory drugs and their pharmacological targets

II.4.1. Synthetic anti-inflammatory drugs

There are two main classes of drugs with anti-inflammatory effects:

II.4.1.1. Steroidal anti-inflammatory drugs (AIS) or glucocorticoids

They are synthetic derivatives of natural hormones, such as cortisol and cortisone, from which they are distinguished by their greater anti-inflammatory power (**Bannwarth et *al*., 2005**). Unlike non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids are capable of inhibiting all phases of the inflammatory reaction (**Muster, 2005**). They bind to intracellular receptors, the complex formed acting at the DNA level and modifying the transcription of many genes. In the

context of inflammation, glucocorticoids inhibit the transcription of COX2 genes (whereas they have no effect on COX1), phospholipase A2 and therefore inhibit both prostaglandin and leukotriene synthesis. This explains the great effectiveness of glucocorticoids on inflammatory phenomena. Glucocorticoids also reduce cell migration to the inflammatory site, modulate the cellular functions of lymphocytes and macrophages and modify the production of cytokines. (**Devillier, 2005; Venereol, 2005**).

II.4.1.2. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely used therapeutic classes in the world because of their anti-inflammatory, anti-pyretic and analgesic properties (**Trabsa, 2015**) (**Table 1**). The widely accepted mechanism of action of non-steroidal anti-inflammatory drugs is the inhibition of the conversion of arachidonic acid to cyclic endoperoxides by the enzyme cyclooxygenase (**Blain, 2002**). The inhibition of cyclooxygenases by NSAIDs therefore has an anti-inflammatory effect which is primarily related to COX2 inhibition, (**Turpin and Webber, 2012; Trabsa, 2015**), and reduces the physiological effects of prostaglandins **(Burmester, 2000)**. Noting as an example diclofenac, which has an equivalent effect on COX1 and COX2 (**Trabsa, 2015**). However, their long-term therapeutic use is often associated with adverse effects such as gastrointestinal ulcers and renal failure (**Mansour, 2015**).

Table 1. Examples of non-steroidal anti-inflammatory drugs (**Mansour, 2015; Trabsa, 2015**).

Structural class	Scientific name	Trade name
Salicylates	Salicylic acetyl	Aspirin®
	Diflusinal	Dolobid®
Derivatives of propenoic acid	Ibuprofen	Ibuprofen®
	Fenoprofen calcium	Nalfon®
	Flurbiprofen	Ansaid®
	Ketoprofen	Nalfon®
Acetic acid derivatives	Diclofenac	Voltarene®
Indoles	Indoles	Indocine®
	Indomethacin	Tolectine®
	Tolmetin	Clinoril®

II.4.2. Plant-based anti-inflammatory drugs

Medicinal plants are widely used in traditional medicine throughout the world for the relief of inflammatory diseases such as rheumatoid arthritis, asthma, bronchitis, eczema, osteoarthritis, gout, allergic rhinitis, gastric and duodenal ulcers...etc (**Setty and Sigal, 2005; Wiart, 2006**). The number of phytochemical compounds found in the plant kingdom is very vast, and their spectrum of activity is just as great. Some of these phytochemical compounds have anti-inflammatory properties. Many are believed to act by blocking the cyclooxygenase and lipoxygenase pathways and by other mechanisms. Several examples of plants can be cited (**Table 2**).

Table 2. Examples of medicinal plants with anti-inflammatory activities (**Grant et *al*., 2007; Marrassini et *al*., 2010; Kim et *al*., 2012; Mansour, 2015**).

Scientific name and family	Plant part used	Use
Zingiber officinale ; Zingiberaceae	Rhizome	Osteoarthritis, migraine, rheumatic pain
Helleborus orientalis L.; Ranunculaceae	Roots	Rheumatic pain
Urtica dioica ; Urticaceae	Leaves, Roots	Allergic rhinitis, eczema drop, rheumatic pain
Laurocerasus officinalis R.; Rosaceae	Leaves	Fever, pharyngitis, stomach pain, hemorrhoid
Curcuma longa ; Zingiberaceae	Rhizomes	Rheumatic pain, systemic lupus, psoriasis, kidney infections
Nerium oleander L.; Apocynaceae	Flowers	Pain, headaches
Harpagophytum procumbens ; Pédaliacées	tuber	Osteoarthritis, low back pain, headaches, fever
Rhododendron ponticum L.; Ericaceae	Leaves,	Colds, toothache
Juglans regia L.; Juglandaceae	Leaves, Fruits	Rheumatic pain, fever, eczema, rheumatic pain, malaria
Ocimum basilicum ; Lamiacees	Leaves	Low back pain, osteoarthritis
Oenothera biennis ; Onagraceae	Seeds	Rheumatic pain

II.5. Methods for assessing anti-inflammatory activity

There are many methods for studying anti-inflammatory drugs. After creating the inflammation in laboratory animals, the effects on the different phases of the inflammation are investigated. Among these methods we mention:

II.5.1. Inhibition of pro-inflammatory enzymes

These are generally "*In vitro*" methods; they are based on the inhibition of specific enzymes linked to inflammation. Examples are 12-lipoxygenase (12-LOX), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (**Wangensteen et *al*., 2006; Ziakas et *al*., 2006**).

II.5.2. Membrane stabilization

In general, there are several advantages to choosing erythrocytes as a model to study the mechanism of membrane stabilization. The surface area of the erythrocyte membrane can be accurately estimated and the erythrocyte is free of intracellular organelles. Thus, any effect of a substance on osmotic haemolysis could justifiably be interpreted as an effect on the membrane itself (**Shobana and vidhya, 2016**). Red blood cells are a good model for following the movement of water, placed in a hypotonic solution, their bursting (haemolysis) leaves only a membrane ghost and haemoglobin in solution transforming the opaque suspension of cells into a translucent red solution of haemoglobin (**Arbos et *al*., 2008**).

II.5.3. Protein denaturing

Protein denaturing is a process in which proteins lose their tertiary and secondary structure under the effect of certain substances, such as acid or strong base concentrated in organic salt, an organic solvent or heat. Most proteins lose their biological function when denatured. Denaturation of these molecules is a well-documented cause of inflammation (**Leelaprakash and Mohandass, 2011**). The denaturation mechanism probably involves the change in the electrostatic, hydrogen, hydrophobic and bisulphide bond (**Thanh et *al*., 2017**).

II.5.4. Ultraviolet rays erythema in guinea pigs

It is a question of assessing the intensity of the red colouring of the depilated skin on the back of the guinea pig subjected to ultraviolet rays, in the absence and then in the presence of anti-inflammatory drugs. This test can be carried out on mice (**Cohen, 1981**).

II.5.5. Arthritis with Freund's adjuvant

The primary and secondary inflammation caused by intra-articular injection of Freud's adjuvant (a suspension of killed tubercle bacilli) into the hind leg of the rat is reduced by anti-inflammatory substances, resulting in an oedematous reaction which develops immediately (primary inflammation) (**Cohen, 1981**).

II.5.6. Capillary permeability in rabbits

Turpentine essence or croton oil is applied to the depilated skin of the albino rabbit. Plasma exudation is revealed by the intravenous injection of Trypan blue or Evans bluewhich binds to plasma proteins. The extent of the skin blue stain is proportional to the capillary permeability. The extent of diffusion of the blue into the fundamental substance of the dermis is reduced in the presence of anti-inflammatory drugs (**Coyen, 1981**).

II.5.7. Local inflammation of the ear

This technique consists of creating an inflammation in the rat's ear, by local application of croton oil. This inflammation can be reduced by local application of anti-inflammatory substances (**Colot, 1972**).

II.5.8. Rat paw edema volume

Acute inflammation is characterized by classic symptoms such as heat, redness, swelling and pain. Measurement of rat paw edema is therefore an excellent tool for the quantification of skin inflammationinduced by phlogistic agents such as carrageenan, formalin, ovalbumin, kaolin (**Ozbek et *al*., 2005**). For our study we used this method with carrageenan as a pro-inflammatory substance (**Winter et *al*., 1962; Epa et *al*., 2015**) which is based on the inhibition of carrageenan-induced paw edema by injecting carrageenan solution into the subplantar space of the right hind

paw of the rat. The evolution of edema of the right hind rat paw was determined following carrageenan injection by using a digital caliper before and after induction of edema (**Sameh et *al*., 2016; Elion et *al*., 2014**).

III. Antidiabetic activity

III.1. General informations on diabetes

Blood glucose is the concentration of glucose in the blood. Glucose is an essential molecule for cellular functioning as it is the main source of energy. Supplied by food, it enters the body in the intestine and is distributed throughout the body via the blood. Part of the blood glucose is transformed into glycogen, a form of glucose reserve, stored mainly in the liver and can be mobilized at any time to compensate for low blood sugar levels (**Laxmi et *al*., 2010**).

Diabetes mellitus is a chronic metabolic disorder linked to a deficiency in either the secretion or action of insulin, or both, characterized by chronic hyperglycaemia, impaired metabolism of carbohydrates, proteins and fats, and an increased risk of vascular complications (**Punthakee et *al*., 2018**). This leads to subsequent acute and chronic complications affecting many organs including the eyes, kidneys, nerves, hearts and vessels (**Canivell and Gomis, 2014**). The term 'diabetes mellitus' has been attributed to the pathology because of the sweet taste of urine (**Sharma et *al*., 2008**).Diabetes is an incurable disease, yet it can be managed effectively (**Raccah, 2004**).

Diabetes is defined as a blood sugar level of more than 1.26 g/l (7 mmol/1) after an 8-hour fast and checked twice. It is also defined by the presence of symptoms of diabetes (polyuria, polydipsia, weight loss) associated with a blood glucose level (on venous plasma) greater than or equal to 2 g/1 (11.1 mmol/1) as well as by a blood glucose level greater than or equal to 2 g/1 (11.1 mmol/l) 2 hours after an oral load of 75 g of glucose (**Scheen and Luyckx, 2010**). It ispathology of glucose metabolism caused by a dysfunction of the cells β of the Langerhans islets, which secrete insulin, or by poor assimilation of the latter by peripheral tissues (insulin resistance) (**Wens et *al*., 2005**).

III.2. Classification of diabetes

The American Diabetes Association has classified diabetes into 4 major categories since 1997. These are: type 1 diabetes, type 2 diabetes, gestational diabetes and other specific types of diabetes (**Ndomou et *al*., 2014**).

III. 2.1. Type 1 diabetes

Type 1 diabetes, also known as insulin-dependent diabetes, is a condition of chronic hyperglycaemia, caused by the autoimmune destruction of the insulin-producing beta cells of the pancreatic islets by immune system antigens. As a result, the body is no longer able to produce the insulin the cells need, leading to hyperglycaemia and secondary complications (**Principi et *al*., 2017; Chih-Wei et *al*., 2018**).The initial molecular mechanisms that lead to the autoimmune reaction are still not fully identified (**Giudicelli and Cattan, 2001**). The destruction of ß cells by an autoimmune process is authenticated by the presence of islet cell anti-insulin antibodies (**Spinas and Lehmann. 2001**). This form of diabetes occurs mainly in children and adolescents, may also occur in young adults (< 40 years) and concerns 10% of diabetics (**Usher-Smith et *al*., 2012**).

III.2.2. Type 2 diabetes

Type 2 diabetes is a complex metabolic disorder resulting from chronic alterations associated with the corresponding insensitivity of tissues to the actions of insulin (**Brendan et *al*., 2017**). It is also called non-insulin diabetes, which is characterized by two major abnormalities: a disturbance in the effects of insulin on its target tissues (insulin resistance), and a disturbance in the secretion of pancreatic hormones (insulin deficiency), leading to a state of hyperglycaemia (**Wenzhen et *al*., 2018; Virally et *al*., 2007**), either of these two characteristics being able to dominate to a variable degree. It can evolve without symptoms for several years and generate complications without having been diagnosed (**Fagot, 2010**). Obesity is considered to be the main cause of type 2 diabetes in subjects who are genetically predisposed to the disease (**Mercaldo et *al*., 2017**). Generally occurs in mature subjects (over 40 years of age). It is the most common form (90% of cases) in all regions of the world (**Portha, 2003**).

III.2.3. Gestational diabetes

This type of diabetes occurs during pregnancy, especially during the 2nd or 3rd trimester when the insulin requirements are much higher than normal. In addition, certain factors such as growth hormones and placental hormones reduce the action of insulin. It develops in almost 4% to 7% of women. Gestational diabetes usually disappears after childbirth, but women who have had gestational diabetes are more likely to develop type 2 diabetes later in life (**Kim and Lee 2005; Pirson et *al*., 2016**).

III.2.4. Other types of diabetes: secondary (specific) diabetes

Diabetes mellitus can be secondary to pancreatopathy (chronic or acute pancreatitis, tumor, and haemochromatosis), various endocrinopathies (pheochromocytomas, acromegaly, Cushing's syndrome, hyperthyroidism, pancreatic and digestive endocrine tumors), and dysfunctions of genetic origin of the cells β (diabetes MODY [Maturity Onset Diabetes of the Young] and mitochondrial diabetes). It can also be the cause of drugs, chemical compounds or toxic compounds (**Maugendre et *al.*, 1995; OMS, 1999**).

III.3. Treatment of diabetes

III.3.1. Treatment of diabetes mellitus with insulin and synthetic drugs

The treatment of diabetes mellitus depends on the type of diabetes and the degree of insulin deficiency:

- The treatment of type 1 diabetes is based on a vital prescription of insulin combined with diet, physical activity, self-monitoring, education and regular medical monitoring (**Bailey, 1999; Wang et *al*., 2012**).
- The initial treatment of type 2 diabetes is primarily based on diet and physical exercise. Muscle and joint functions reduce fat deposits, particularly abdominal fat, linked to insulin resistance and its consequences, often resulting in weight loss (**Tossou et *al*., 1995; Koski, 2006**). Three classes of oral antidiabetic drugs with hypoglycaemic effects through different mechanisms of action are available to clinicians treating type 2 diabetics who are insufficiently controlled by diet alone: Hypoglycaemic sulfonamides

(glibenclamide, gliburide, glimepiride and tolbutamide); Biguanides (metformin); Intestinal α-glucosidase inhibitors represented by acarbose and miglitol (**Harrigan et *al*., 2001**). Hypoglycaemic sulfonamides stimulate insulin secretion without influencing its synthesis (**Melander, 1996**). They bind to a specific receptor on the membrane of β-pancreatic cells. The binding of sulfonylureas to their specific receptors leads to the closure of ATP-dependent potassium channels, which causes membrane depolarization and the secondary opening of calcium channels. The influx of calcium into the cytoplasm of β-pancreatic cells induces exocytosis of insulin-containing vesicles in a similar way to that observed after glucose stimulation (**Ilarde et *al*., 1994**). Hypoglycaemia is the most serious side effect associated with the use of hypoglycaemic sulfonamides (**Defronzo, 1999**). Biguanides decrease hepatic glucose production (inhibition of neoglucogenesis and glycogenolysis), promote the uptake of glucose by the muscle and stimulate glycolysis. Biguanides also inhibit intestinal glucose absorption without any effect on insulin secretion. Side effects include anorexia, which can lead to moderate weight loss, metallic taste, nausea, vomiting, abdominal pain and diarrhoea. The most serious side effect of biguanides is lactic acidosis (**Gan et *al*., 1992; Orban et *al*., 2006**). Inhibitors of intestinal carbohydrate absorption, these are pseudo-tetrasaccharides of bacterial origin, these structural analogues of dietary oligosaccharides competitively and reversibly inhibit the α-glucosidases of the brush border of the small intestine (glucoamylase, maltase, isomaltase and sucrase). Inhibitors of intestinal α-glucosidases slow down the enzymatic cleavage of food sugars into mono and disaccharides, which are then, absorbed into the ileum, at this level the absorption of glucose after a meal is thus delayed in time. Inhibitors of α-glucosidases are essentially active on postprandial hyperglycaemia (**Reuser et *al*., 1994**).The side effects of this family are digestive and sometimes very disabling (flatulence, diarrhea, abdominal pain (**Baron, 1998**).

III.3.2. Treatment of diabetes mellitus with medicinal plants

In recent decades, special attention has been paid to the use of medicinal plants. One of the most dynamic areas of current research is the evaluation of traditional herbal medicines in the treatment and control of diabetes (**Grover et *al*., 2002**). Several plants played an important role in the treatment of diabetes. The existence of several compounds of plant origin seemed to give this beneficial effect (**Marles and Farnsworth, 1994**). Plants represent the major form of traditional treatment worldwide. It is characterized by its positive effects with fewer serious side effects.

The use of traditional medicine is widespread and is of increasing health and economic importance. In developing countries, the routine use of traditional medicine is accessible and affordable, especially for the world's poorest patients (**Oubré et *al*., 1997; OMS, 2002**). Several examples of plants can be cited (**Table 3**).

Table 3. Examples of medicinal plants with antidiabetic activities

Plant and family	Plant partused	Mechanism of action	References
Allium cepa (Onion) ; Alliaceae	Onion bulbs	• Stimulate insulin secretion. • Compete with insulin forinactivating sites inthe liver.	(**Eidi et *al*., 2006**)
Allium sativum (Garlic); Alliaceae.	Garlic gloves	• Stimulate insulin secretion. • Inhibit glucose production by the liver.	(**Augusti and Sheela, 1996**)
Aloe vera (Aloebarbedensis); Aspholedeceae	Leaf, pulp and gel	• Stimulate synthesis and/or release of insulin. • Alter the activity of carbohydrate metabolizingenzymes.	(**Tanaka et *al*., 2006**)
Catharanthus roseus; Apocynaceae	Fresh leaf juice	• Increase hepatic utilization of glucose. • Suppress activities of gluconeogenic enzymes.	(**Singh et *al*., 2001**)
Cinnamomum cassie (Chinese cinnamon); Lauraceae	Bark	• Enhance insulin action. • Increase glucose uptake and glycogen synthesis.	(**Jarvill-Taylor et *al*., 2001**)
Coccinia indica; Cucurbitaceae	Leaves	• Suppress glucose-6-phosphatase. • Stimulate glycogen synthase activity and reduction of phosphorylase activity.	(**Kumar et *al*., 1993**)
Fiscus bengalensis; Moraceae	Leaves and bark	• Increase insulin secretion. • Inhibit insulinase activity.	(**Augusti et *al*., 1994**)
Gymmema slyvestre (Gurnar); Asclepiadaceae	Leaves	• Stimulate insulin secretion from rat Islets. • Decrease the activity of gluconeogenic enzymes. • Induce β-cell regeneration.	(**Shanmugasundaram et *al*., 1990**)
Momordica cymbalaria (Bitter Melon); Cucurbiteae	Fruit pulp, seeds, leaves and wholeplant.	• Stimulate insulin secretion. • Suppress the activity of gluconeogenic enzymes.	(**Rao et *al*., 1999**)

		• Increase β-cells in diabetic rats.	
Polygala senega; Polygalaceae	Rhizomes	• Decrease hepatic glucose output. • Increase insulin sensitivity.	**(Bnouham et *al*., 2006)**
Trigonella foemum-Graecum (Fenugreek); Falcaceae	Seeds	• Slow digestion and absorption of carbohydrates. • Increase glucose-induced insulin secretion. • Enhancement of peripheral insulin action.	**(Hannan et *al*., 2007)**
Syzigium cumini (Eugeniajanbolaria); Mytaceae	Seeds, leaves and fruit pulp	•Stimulate kinases involved in peripheral utilizationof glucose.	**(Kumar et *al*., 2008)**

III.4. Methods for assessing antidiabetic activity

Experimental studies of diabetes in animal models and advanced *in vitro* techniques are essential for the improvement of knowledge and clear understanding of the pathology and pathogenesis, and to find new therapy. Animal models of diabetes are therefore, greatly useful in biomedical studies because they offer the promise of new insights into human diabetes. Most of the available models are based on rodents because of their small size, shorter generation intervals and economic considerations. Experimental diabetes mellitus studied by several methods that include: chemical, surgical and genetic manipulations (**Kumar et *al*., 2012**). It is also very important to select appropriate animal model for the screening of new chemical entities (NCEs) and other therapeutic modalities for the treatment of diabetes (**Chattopadhyay et *al*., 1997**). The main aim of the present review is to being together all various *in vivo* animal models and *in vitro* techniques for carrying diabetes research.There are many methodsto evaluate the antidiabetic activity of natural extracts. Among these methods we mention:

III. 4.1. *In vitro* techniques

III. 4.1.1. Assay of amylase inhibition

In vitro amylase inhibition can be studied by adding the test sample was allowed to react with α-amylase enzyme and Incubated, add starch solution. After incubation dinitrosalicylic acid reagent was added to both control and test. Keep this mixture in boiling water bath for few minutes.

The absorbance was taken at 540 nm using spectrophotometer and the percentage inhibition and IC_{50} value was calculatedv (**Hansotia and Drucker, 2005**).

III.4.1.2. Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibition activity was performed by incubating α-glucosidase enzyme solution with phosphate buffer which contains test samples of different connections at 37°C for 1 hr in maltose solution. The reaction mixture was kept in boiling water few min and cooled. Glucose reagent was added and its absorbance was measured at 540 nm to estimate the amount of liberated glucose from maltose by the action of α-glucosidase enzyme. The percentage of inhibition and IC_{50} was calculated (**Lelliott and Vidal-Puig, 2004**).

III. 4.2. *In vivo* techniques

III. 4.2.1. Alloxan induced diabetes

Alloxan is most widely used in experimental diabetic research. Alloxan produces selective necrosis of the beta cells of pancreas. The alloxan is administered by various routes like intravenous, intraperitoneal and subcutaneous. Alloxan is used for induction of diabetes in experimental animals such as mice, rats, rabbits and dogs. The routes and dose of alloxan required may vary depending upon the animal species (**Federiuk et *al*., 2004; Iranloye et *al*., 2011**).

III. 4.2.2. Streptozotocin induced diabetes

Streptozotocin (STZ) is a naturally occurring chemical it particularly produces toxic to the beta cells of the pancreas. It is used in medical research as an animal model for hyperglycemia (**Brentjens and Saltz, 2001**). STZ alters the blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is due to the decreased in blood insulin levels. At last hyperglycemia develops and blood insulin levels drops. STZ impairs glucose oxidation and decreases insulin synthesis and release (**Bedoya et *al*., 1996**).

III. 4.2.3. Dithizone induced diabetes

Dithizone is an organosulfur compound, it have chelating property. Dithizone is used in induction of diabetes in experimental animals. In dithizonised diabetic animals, the levels of zinc, iron, and

potassium in the blood were found to be higher than normal, dithizone has permeates membranes and complex zinc inside liposomes, then release of protons, this enhances diabetogenicity (**Nakajima et *al*., 1985; Graham et *al*., 2000**).

III. 4.2.4. Insulin antibodies induced diabetes

The insulin antibodies have the affinity and capacity to bind insulin. Insulin deficiency mechanism may cause greater postprandial hyperglycemia because antibody-bound insulin is unavailable to tissues, but the prolongation of postprandial hyperinsulinemia may leads to hyperglycemia (**Van Haeften et *al*., 1986**).

III. 4.2.5. Ferric nitrilotriacetate induction of diabetes

In experimental animals parenteral administration on of large daily dose of ferric nitrilotriacetate for 60 days manifest diabetic symptoms such as hyperglycemia, glycosuria, ketonemia andketonuria (**Marchand-Brustel, 1999**).

III. 4.2.6. Goldthioglucose obese diabetic mouse model

Gold thioglucose (GTG) is a diabetogenic compound, which manifest obesity induced Type -2 diabetes. The intraperitoneal administration GTG in experimental animal gradually develops obesity, hyperinsulinemia, hyperglycemia, insulin resistance for a period of 16- 20 weeks. The GTG is transported in particular to the cells and causes necrotic lesions, which is responsible for the development of hyperphagia and obesity. It also increases body lipid, hepatic lipogenesis and triglyceride secretion, increased adipose tissue lipogenesis and decreases glucose metabolism (**Jaidane and Hober, 2008**).

III. 4.2.7. Virus Induced Diabetes

Viruses produce diabetes mellitus by destroying and infecting pancreatic beta cells. Various human viruses used for inducing diabetes include RNA picornoviruses, Coxackie B4, encephalomylocarditis (EMC-D and M variants), Mengo-2T, reovirus, and lymphocytic choriomeningitis (**Utsugi et *al*., 1992**).

III. 4.2.8. D-Variant Encephalomyocarditis (EMC-D)

EMC- D virus can infect and destroy pancreatic beta cells in mice and produce insulin dependent hyperglycemia (**Lansdown and Brown, 1976**), EMC-D virus known as NDK25. Intraperitoneal injection of NDK25 develops non-insulin dependent diabetes mellitus (**Horwitz et *al*., 1998**).

III. 4.2.9. Coxsackie viruses

Coxsackie viruses also cause diabetes in mice; it can infect and destroy pancreatic acinar cells. Coxsackie B4 virus is strongly associated with the development of insulin-dependent diabetes mellitus in humans. Diabetes induced by Coxsackie virus infection release of sequestered islet antigen resulting in the re- stimulation of auto reactive T cells **(Heather et *al*., 2012**).

III. 4.2.10. Growth hormone induced diabetes

Repeated administration of growth hormone in higher experimental animals induces diabetes with ketonuria and ketonemia. Prolonged administration of growth hormone produced permanent diabetes; there was loss of pancreatic islets tissues and of beta cells (**Friedman, 2000**).

III. 4.2.11. Corticosteroid induced diabetes

Corticosteroid induces diabetes, which is called steroid diabetes. The prednisolone and dexamethasone cause steroid diabetes. Glucocorticoids stimulate gluconeogenesis, in the liver, resulting in increase in hepatic glucose and induce insulin resistance and hyperglycemia (**Shafrir, 2003**).

IV. *Hammada elegans* (Bge.) Botsch

IV.1. Systematic

The systematics of *Hammada elegans* Botsch is presented in the **Table 4**.

Table 4. Systematics of *Hammada elegans* Botsch (**Guy, 1979**).

Reign	Plantes
Sub-reign	Plantesvasculaire
Deviation	Tracheophytes
Sub -deviation	Angiospermes
Class	Dicotyledones
Sub-class	Caryophyllidees
Order	Caryophyllales
families	Chenopodiacees
Genre	*Hammada*
Species	*Hammada elegans*
sub-species	*Hammada elgans* Botsch
Vernacular name	El Ajram (العجرم)

IV.2. Botanical description

Hammada elegans Botsch (**Figure 2**) is a shrub of 20-40 cm height, with erect branches, green in youth, becoming whitish with age, jointed, cylindrical, with thin and fragile articles of 10-12 Ø 2mm. Opposite leaves fused into a very short cup with ciliated margins, reduced to 2 cuspidate tips, with briefly woolly hairy axils. Solitary leaves ♀♂ with upper flower axils with 2 short bracts 1-1.2 mm ≤ flower, scarce, drowned in tomerntum. Perianth globose with 5 membranous sepals 1.5-2 mm, stamens 5+5 woolly obtuse staminodes on back 0.5 mm; ovary strawy, style with 2 stigmas. Perianth fruiting Ø 6-8 mm with 5 subegalegalpapyracae wings. Fruits with thick pericardium separable from vertical seed (**Baba Aissa, 2000**).

Figure 2. Sketch of aerial parts of *Hammada elegans* Botsch (Sidi Makhlouf-Laghouat).

IV.3. Chemical composition and pharmaceutical properties

Recently, it has been reported by our research group that the phytochemical analysis of the aerial parts of *Hammada elegans* (Bge.) Botsch showed the presence of alkaloids, *C*-heterosides, reduced *O*-Heterosides, anthocyanins, saponins, tannins, reducing components, flavonoids, sterols and terpenes (**Bensafieddine et *al*., 2019**). According to **Labiodh** and collaborators (**Labiodh et *al*., 2019**), leaf extracts of *Hammada elegans* Botsch is rich in alkaloids, saponins, reducing components, tannins, *C*-heterosides and flavonoids. Qualitative analyses of the extracts by CCM confirmed the presence of these components in the aerial parts of *Hammada elegans* Botsch.

Studies of *Hammada elegans* Botsch biological activities are scarce. However, according to our research group; **Bensafieddine** and collaborators indicated that acetonic, hydroacetonic, methanolic and hydromethanolic extracts from *Hammada elegans* Botsch are endowed with remarkable antioxidant activity *in vitro*. These extracts displayed chelating effect on ferrous ions (Chelation Test), reducing of copper activity (CUPRAC Test), antiradical activity ($NO^{\bullet}$ Test) and antilipoperoxidative activity (Lipoperoxidation Test), the acetonic extract exhibited strong antioxidant activities compared to all extracts. Likewise, the acetone extract of *Hammada elegans* Botsch still exhibited the best anti-inflammatory activity *in vitro* (anti-lipooxygenase and anti-cyclooxygenase) compared to all the other extracts and reference inhibitors (aspirin and ibuprofen) (**Bensafieddine et *al*., 2019**). On the other hand, **Labiodh** and collaborators showed that

extracts of *Hammada elegans* Botsch revealed high radical scavenging activities (DPPH assay), good reducing of copper activity (CUPRAC Test), low chelating powers (Chelation Test), and important xanthine oxidase inhibition. The diethyl ethers and acetonic fractions of *Hammada elegans* Botsch exhibited strong antioxidant activities compared to all extracts. These fractions also demonstrated a better chelating capacity of iron, a strong reducing power of copper and a great inhibiting effect of xanthine oxidase compared to all referenced antioxidants used (**Labiodh et *al*., 2019**). According to **Djokhdem** and collaborators, that the acetonic and methanolic extracts of *Hammada elegans* Botsch had potent antilipoperoxidation effect, defined as inhibition percentage of linoleic acid and sunflower peroxidation assay (**Djokhdem et *al*., 2016**).

Material and methods

I. Materials

I.1. Biological materials

I.1.1. Plant material

Hammada elegans Botsch was collected in May 2018 from the region of Sidi Makhlouf (at 40 km at the north of Laghouat in the steppe region of Algeria). Information on the plants (local name, medicinal uses, parts of the plant used, methods of preparation and administration) were collected from local inhabitants knowledgeable about the plants' curative properties. The aerial plant parts (leaves + stems + flowers) were thoroughly washed with tap water, dried at room temperature, and ground to a fine powder for using in extraction later.

I.1.2. Animal material

Male Adult Albino Wistar rats (2-3 months old) weighing between 180 and 340 g were brought back from the Pasteur Institute of Algeria, The animals divided into groups are housed in polypropylene cages at conditions of temperature (25 ± 5 ^{0}C), subjected to a light / dark cycle of 12 / 12h and relative humidity 40–70%with free access to water and food. The food is supplied by the National Office of Livestock Foods (ONAB) of El-Kssar-Bejaia of Algeria. The rats were given a 7 day adaptation period before use (**Figure 3**). All the experimental procedures were conducted in accordance with the ethical guidelines for the care and use of laboratory animals provided by Algerian Association of Experimental Animal Sciences (AAEAS).

Figure 3. Strain of Wistar rats and their food

I.2. Chemical products, materials and instruments

The list of chemicals and reagents, glassware, apparatus and other equipment are listed in **Table 5**. All products used in this work are of a high analytical grade.

Table 5. List of chemical products, materials and instruments.

Products	Mark
Potassium phosphate monobasic, potassium phosphate dibasic, starch, 2 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) (ABTS), Alloxan, Neocuproine (2,9-dimethyl-1,10-phenanthroline) (Nc), Methanol, Ethylene diamine tetra-acetic (EDTA), Iron (II) sulphate heptahydrated ($FeSO_4.7H_2O$), butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ), Dimethyl sulfoxide (DMSO), vanillin, Gallic acid, catechin, quercetin, Folin-ciocalteu reagent, aluminum chloride ($AlCl_3$), Sodium phosphate monobasic, Sodium phosphate dibasic, Sodium chloride, α-tocopherol, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), Carrageenan, 2,4,6-tripyridyl-s-triazine (TPTZ).	Sigma-Aldrich
Copper (II) chloride dihydrate ($CuCl_2$. $2H_2O$), HCl (hydrochloric acid 37%)	Riedel de Haën
sodium carbonate (Na_2CO_3).	Pearce
Acetone, Hexane, ascorbic acid.	Prolabo
Hydrogen peroxide (H_2O_2).	Sialchim
0.9% NaCl solution, Diclofenac , Ibuprofen, Acarbose, Glibenclamide.	
Instruments and equipments	
Qualitative filter paper Ø15cm, Analytical balance (KERN, ABS 220-4), Micropipette 100-1000µl (Isolab), Micropipette 10-200µl (Accumax), Quartz cuvette, Bain marie (Memmert), Rotavapor, Ultrasounddevice (Shimadzu), Centrifuged (Model 2650), spectrophotometer UV/Visible (Shimadzu 1800), Etuve (Memmert), digital caliper, gastric tube, glucometer at test strips (DIAGNO-CHECK).	

II. Methods

II.1. Preparation of extracts

In our work we chose the hot extraction "Soxhlet" which is a simple and suitable method allowing to repeat the extraction cycle with crude solvent infinitely until the complete exhaustion of the solute in the raw material. After having finely ground the plant material , a mass of approximately 62g each time has been extracted by four increasing polarity solvent systems: Hexane (0.0); Acetone (5.4); Methanol (6.6); Water (9.0) “The values between parentheses indicate the polarity indices with respect to water”. After each extraction, the solvent used is evaporated under vacuum using a rotary evaporator. Then, the recovered residue is weighed and then dissolved in different volumes of dimethyl sulfoxide (DMSO) solvent. Then, we obtained four different extracts: hexanic extract, acetonic extract, methanolic extracts and aqueous extract. The extracts were stored at 4 ° C and protected from light until use.

The yield extraction which is expressed as a percentage was calculated by the following formula:

$$\mathbf{Y}\ (\%) = \frac{\mathbf{W_1 - W_2}}{\mathbf{W_3}} \times \mathbf{100}$$

Where: Y(%): Yield; **W_1:** Weight of the tube after evaporation;**W_2 :** Weight of the tube before evaporation (empty tube);**W_3:** Weight of the starting vegetable powder.

II.2. Determination of total phenolics compound

The amount of total phenolics in the samples was determined with the Folin-Ciocalteu reagent using the method of **Singleton and Ross (1965)** with slightmodifications. This yellow-coloured reagent consists of a mixture of phosphotungstic acid and phosphomolybdic acid. When the polyphenols are oxidized, they reduce the Folin-Ciocalteu reagent into a blue-coloured complex consisting of tungsten oxide and molybdenum. The colour intensity is proportional to the levels of oxidized phenolic compounds (**Boizot and Charpentier, 2006**). This method was employed to evaluate the phenolic content of the samples. A standard curve was obtained using gallic acid as a standard. Different concentrations of gallic acid were prepared in distilled water, and their absorbances were recorded at 760 nm. The procedure is as follows: as follows: 100 µL of each

sample was added to 500 µL of the aqueous solution of Folin-Ciocalteu reagent at 10%. The solutions are mixed and incubated at room temperature for 2 min and then 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 30 min in the dark at room temperature. The absorbance of all samples was measured at 760 nm using a spectrophotometer (Shimadzu -UV-1800) and the results were expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g dw). All the assays were carried out at least in triplicate.

II.3. Estimation of flavonoids content

The flavonoids content in extracts was determined spectrophotometrically according to **Quettier-Deleu** and collaborators (**Quettier-Deleu et *al*., 2000**), using a method based on the formation of a complex flavonoid–aluminum, having the absorptivity maximum at 430 nm. Quercetin was used to make the calibration curve. 0.5 mL of diluted sample was separately mixed with 0.5 mL of 2% aluminum chloride methanolic solution. After incubation at room temperature for 20 min, the absorbance of the reaction mixture was measured at 430 nm using spectrophotometer (Shimadzu -UV-1800) and the flavonoids content was expressed as mgquercetin equivalents per gram dry weight (mg QE/g dw).

II.4. Determination of condensed tannin content

Condensed tannin content was estimated using the vanillin assay method (**Julkunen-Titto, 1985**) with slight modifications. This method is based on the ability of reacting with the vanillin units of condensed tannins in the presence of acid to produce a colored complex measured at 500 nm. The reactivity of vanillin with tannins involved only the first unit of polymer. 200 µL of each extract was added to 1.5 mL of vanillin reagent (4% w/v in methanol) and vortexed.Then, 0.75 mL concentrated (37 %) HC1 was added and allowed to react at room temperature for 20 min. The absorbance of all samples was measured at 500 nm using a spectrophotometer (Shimadzu -UV-1800). Catechin was used to prepare the standard curve. The results were expressed as mg catechin equivalents per gram dry weight (mg CE/g dw).

II.5. The *in vitro* of antioxidant capacity evaluation

Many methods are used to evaluate the antioxidant activity of pure compounds or extracts. Most of these methods are based on the colouring or discolouration of a reagent in the reaction medium. In our study, we used four different *in vitro* chemical tests: $ABTS^{\bullet+}$ test, copper reduction test (CUPRAC), the iron chelating test and antihemolytic test.

Measurements of optical densities in the presence of each extract solution or standards at different dilutions allowed us to express the antioxidant power by the parameter EC_{50} (median effective concentration) which represents the concentration of the antioxidant needed to:

- Scavenge 50% of total free radicals ($ABTS^{\bullet+}$ assay);
- Reduce 50% of total copper ions (CUPRAC assay);
- Chelate 50% of ferrous iron ions initially introduced (Chelating assay);
- Inhibit 50% of red blood cells initially introduced (Antihemolytic assay).

The EC_{50} values are calculated graphically by the linear regressions of the graphs representing the variation of the percentages of antioxidant activity according to different concentrations of the tested extracts. Vitamin C, Vitamin E, BHA (butylhydroxyanisole), BHT (butylhydroxytoluene), TBHQ (tertiary-butylhydroquinone) and EDTA (Ethylene diamine tetra acetic acid) were employed as positive controls and the antioxidant activity of the extracts was expressed as EC_{50} values. The values are presented as the means of triplicate analysis. The absorbance of all samples in all antioxidant assays was measured using a spectrophotometer (Shimadzu -UV-1800).

II.5.1. Determination of ABTS radical cation scavenging activity

The total antioxidant capacity of the samples was estimated using the ($ABTS/H_2O_2$/HRP) system for $ABTS^{\bullet+}$ radical formation by using a slightly modified method of **Chen** and collaborators (**Chen et *al.*, 2004**). In this test, the relative capacity of antioxidants to scavenge the $ABTS^{\bullet+}$ radical was measured.The working solution contained 20 mM ABTS and 0.2 g/L horseradish peroxidase prepared in phosphate buffer, pH = 6.9. The reaction was initiated with 1 mM hydrogen peroxide. 100 µL of diluted sample was mixed with 1 mL of the working solution

(green $ABTS^{\bullet+}$ radical cation); the reading was taking after 5 min at 734 nm. The percentage of inhibition (PI%) was calculated according to the formula:

$$\mathbf{PI}\% = \frac{\mathbf{A_{control} - A_{sample}}}{\mathbf{A_{control}}} \times \mathbf{100}$$

Where;

$\mathbf{A_{control}}$: absorbance without extract or standard; $\mathbf{A_{sample}}$: absorbance with extract or standard.

II.5.2. Determination of copper reducing activity (CUPRAC)

The evaluation of the reductive capacity of our extracts was carried out using the CUPRAC test. The CUPRAC (Cupric ion Reducing Antioxidant Capacity) method is based on the monitoring of the decrease in increased absorbance of the Neocuproine (Nc)-copper ions complex [Nc-Cu^{2+}]. In the presence of an antioxidant, the copper-neocuproine complex is reduced and this reaction is quantified spectrophotometrically at a wavelength of 465 nm. In our work, the reducing power of the extracts studied is determined by using a slightly modified method of **Apak** and collaborators (**Apak et *al*., 2007**). 0.1 ml sample was taken and added to 0.5 mL of 0.01 M Cu (II) chloride, 0.5 ml of ethanol solution neocuproine (Nc) $7.5.10^{-3}$ M and 1 ml of 1 M ammonium acetate buffer solutions (pH 7). After 30 min, the absorbance at 459 nm was recorded against blank. The antioxidant effect of the various extracts tested was firstly expressed in terms of the reducing power (RP %) estimated according to the following formula:

$$\mathbf{RP}(\%) = \frac{\mathbf{A_{sample}}}{\mathbf{A_{control}}} \times \mathbf{100}$$

Where;

$\mathbf{A_{control}}$: absorbance without extract or standard; $\mathbf{A_{sample}}$: absorbance with extract or standard.

II.5.3. Determination of ferrous ion chelating activity

The Fe^{2+} ion chelation test highlights the ability of a molecule to fix Fe^{2+} ions. Fe^{2+} ions play an important role in the production of free radicals, especially during the Fenton reaction, which occurs each time a molecule of H_2O_2 is in contact with Fe^{2+} ions and which is at the origin of the production of hydroxyl radicals ($OH^{\bullet}$), one of the most reactive radicals. Iron also plays a role in

the propagation phase of lipoperoxidation as well as in the formation of the O_2^- radical (**Huang et *al.*, 2005**). Ferrous ion chelating activity was measured by inhibition of the formation of iron (II)-2, 4, 6-tripyridyl-s-triazine (TPTZ) complex. The assay was conducted according to a slightly modified method described by **Dinis** and collaborators (**Dinis et *al.*, 1994**) except that ferrozine was substituted by 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ). The reaction mixture contained 100µL of a sample or EDTA, 100 µl of $FeCl_2$ (2 mM in water); 100µl of TPTZ (5 mM in methanol) and 2900µL of methanol. The mixture was shaken well and allowed to react at room temperature for 10 min. The absorbance of the Fe^{2+}-TPTZ complex was measured at 595 nm against a blank. The chelating activity is firstly expressed in chelating power (CP %) using the following formula:

$$\mathbf{CP}\ (\%) = \frac{\mathbf{A_{control} - A_{sample}}}{\mathbf{A_{control}}} \times \mathbf{100}$$

Where;

$\mathbf{A_{control}}$: absorbance without extract or standard; $\mathbf{A_{sample}}$: absorbance with extract or standard.

II.5.4. Antihemolytic activity against AAPH induced erythrocyte hemolysis

Hemolysis is an indicator of free radical damage affecting the membrane of red blood cells (RBC) which might be counteracted by antioxidants. In this assay, AAPH ((2,2'-Azobis(2-amidinopropane) dihydrochloride)) was used to generate free radicals, which could attack the RBC membrane and eventually causehemolysis (**Yang et *al.*, 1987**). Blood was obtained from healthy male volunteer (42 years old) who was non-smoker and not receiving any pharmacological treatment. Blood was centrifuged at 2000x g for 10 min and the plasma and buffy coat were removed. Erythrocytes were washed three-times with 3 volumes of PBS, pH 7.4. During the last wash, the erythrocytes were centrifuged at 2000 xg for 10 min to obtain a constantly packed cell preparation. In the test system, 10% hematocrit of RBC suspension was prepared in PBS solution, pH 7.4. 3 mL of the cell suspension was pre-incubated with 150 µL of various concentrations of extracts at 37°C for 15 minutes. Then, 300 µL of 50 mM AAPH solution were added and the reaction mixture was shaken gently while being incubated at 37°C for 3 h. Subsequently, the samples were centrifuged at 2000 × g for 10 min and the absorbance of supernatant was measured spectrophotometrically at 540 nm. The antihemolytic activity was

firstly expressed as a percentage of haemolysis inhibition (HI%) according to the following formula:

$$\mathbf{HI}(\%) = \frac{\mathbf{A_{control} - A_{sample}}}{\mathbf{A_{control}}} \times \mathbf{100}$$

Where;

$\mathbf{A_{control}}$: absorbance without extract or standard; $\mathbf{A_{sample}}$: absorbance with extract or standard.

II.6. Acute toxicity study in rats

In order to avoid any possible risk of toxicity during biological tests, it was necessary to carry out toxicity tests. The acute toxicity test of the extract was carried out according to the method described by the Organization for Economic Cooperation and Development **OECD** (**2008**), **Essai n°425** (**OECD, 2008**). This test is carried out on a population of 110 male rats, divided into 22 groups of 5 rats and kept on an empty stomach 16 hours before the start of each experiment. Each group receives orally 1mL (**Figure 4**) of extract of *Hammada elegans* Botsch as follows:

- **Group1 (witness):** mineral water.
- **Groups 2-8:** Acetonic extract at dosages of 50, 100, 200, 300, 500, 1000 and 1500 mg/kg bw, respectively.
- **Groups 9-15:** Aqueous extract at dosages of 50, 100, 200, 300, 500, 1000 and 1500 mg/kg bw, respectively**.**
- **Groups 16-22:** Methanolic extract at dosages of 50, 100, 200, 300, 500, 1000 and 1500 mg/kg bw, respectively.

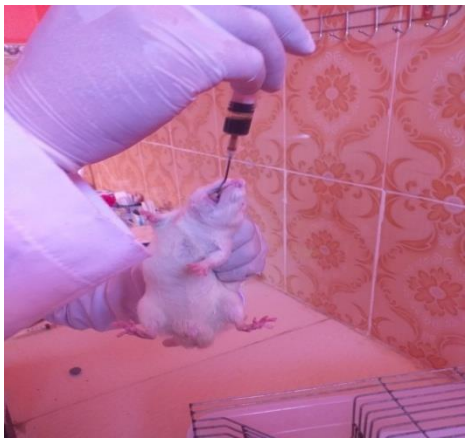


Figure 4. Oral gavage administration of doses to rats

Rats are deprived of food but no water for 2 hours after administration. They are observed individually from the first 30 minutes after administration and regularly during the first 24 hours, then daily thereafter, the observation period for 14 days, for any abnormal manifestation; coma, reduced activity, diarrhea, vomiting, shivering, restlessness, convulsion and mortality (**Carlos et *al*., 2015**).

II.7. The *in vivo* evaluation of anti-inflammatory capacity

The anti-inflammatory activity of the extracts was carried out using the method described by Winter and collaborators (**Winter et *al*., 1962**) which is based on the inhibition of carrageenan-induced paw edema by injecting carrageenan solution into the subplantar space of the right hind paw of the rat. For this study, 112 rats were used and deprived of food for 16 hours before we start the experimentation. Then, they were divided into 22 groups (n = 5-7). One hour before injecting carrageenan solution, each group received orally with help of gastric tube different treatments as follow:

- **Group 1 (witness):** 1 mL of mineral water.
- **Groups 2-5 (standard):**Diclofenac with dosages of 10, 25, 50 and 100 mg/kg bw, respectively.
- **Groups6-9(standard):**Ibuprofen with dosages of 100, 150 and 300 mg/kg bw, respectively.
- **Groups10-13:**Acetonic extract with dosages of 50, 100, 150 and 200 mg/kg bw, respectively
- **Groups14-17:**Aqueous extract with dosages of 50, 100, 200 and 350 mg/kg bw, respectively.
- **Groups 18-22:**Methanolic extract with dosages of 50, 100, 150, 200 and 300 mg/kg bw, respectively.

One hour after the administration of treatment, each rat received through injection in the right hind paw 100 µl of 1% Carrageenan suspension dissolved in 0.9% NaCl solution to induce inflammation (**Winter et *al*., 1962; Epa et *al*., 2015**) (**Figure 5**).

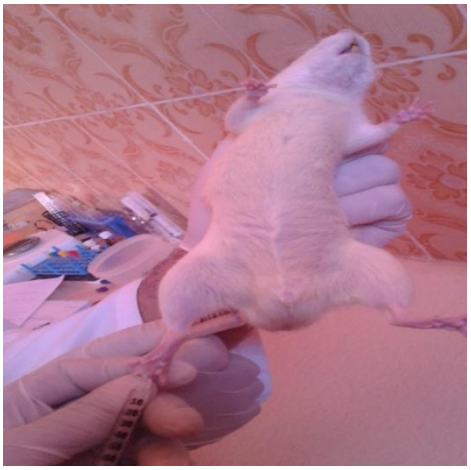
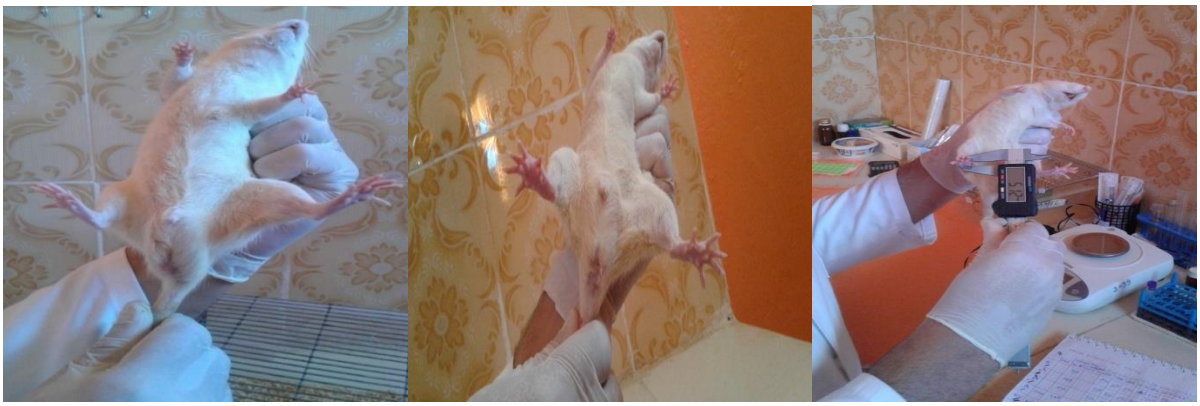


Figure 5. Sub-plantar injection of carrageenan.

The evolution of edema of the right hind paw was determined every 30 minutes up to 360 minutes following carrageenan injection by using a digital caliper before and after induction of edema (**Elion et *al*., 2014; Sameh et *al*., 2016**) (**Figure 6**).



(a) Paw before the injection. (b) Paw after the injection. (c) Measuring the diameter of the Paw.

Figure 6. Measuring the swelling diameter of the paw using a digital caliper.

The Anti-inflammatory activity was evaluated firstly as a percentage of inhibition of rat paw edema (PI %) according to the formula shown below:

$$\mathbf{PI}(\%) = \frac{\mathbf{V_c - V_s}}{\mathbf{V_c}} \times \mathbf{100}$$

Where;

V_c: rat paw edema volume of witness group (control group).**V_s:** rat paw edema volume of treated groups by extracts or standards.

The percentage of anti-inflammatory inhibition of each extract was plotted against different concentrations and the concentration which caused 50% inhibition was taken as the efficient concentration EC_{50}. The anti-inflammatory activities of standards (ibuprofen and diclofenac) were also determined using the same procedure.

II.8. The *in vivo* antidiabetic activity

In order to study the *in vivo* anti-diabetic activity of the extracts of *Hammada elegans*, we used 119 Albino Wistar rats distributed between normal rats (non-diabetic) and rats made diabetic by alloxan by subcutaneously with a dose of 150 mg /kg bw (experimental diabetes),and we used three procedures: short- term test, starch-induced hyperglycemia test and long-term test.

II.8.1. Short-term study

20 normal rats were divided into 4 groups of 5 animals and each group was orally received 1 mL of a single dose of the extract as shown below:

- **Group1:** Normal witnesses rats receiving mineral water.
- **Group2:** Normal treated rats with 100 mg/kg bw of acetonic extract.
- **Group3:** Normal treated rats with 100 mg/kg bw of aqueous extract.
- **Group4:** Normal treated rats with 100 mg/kg bw of methanolic extract.

The blood sugar level (Glycaemia) of each rat in the different groups was determined by a glucometer at test strips (DIAGNO-CHECK, Algeria) from 2 µL collected from the tip of the tail vein at time 0 minute, 60, 90, 120, 180, 240 and 360 minutes (**Figure 7**). The results were expressed in terms of milligrams of glucose per deciliter of blood (**Birru et *al*., 2015**).

Figure 7. Measuring blood sugar using a glucometer at test strips

II.8.2. Starch-induced hyperglycemia test

This assay examines the plant extracts effect on the hyperglycemia caused in normal rats by a starch solution (3g/kg bw) administered intra-gastric gavage simultaneously with a solution of different tested extracts. Acarbose was used as positive control. Therefore, 45 normal rats (non-diabetic) were divided into 9 groups of 5 animals and each group was treated as follows:

- **Group 1 (normal witnesses):** Normal rats receiving mineral water.
- **Group 2 (negative witnesses):** Normal rats treated with starch at 3g/kg bw
- **Groups 3-6 (standard):** Normal rats treated with starch (3g/kg bw) + acarbose at doses of 10, 40, 70 and 90 mg/kg bw, respectively.
- **Group7:** Normal rats treated with starch (3g/kg bw) + 100 mg/kg bw of acetonic extract.
- **Group 8:** Normal rats treated with starch (3g/kg bw) + 100 mg/kg bw of aqueous extract.
- **Group 9:** Normal rats treated with starch (3g/kg bw) +100 mg/kg bw of methanolic extract.

The glycaemia was determined by a glucometer at test strips (DIAGNO-CHECK, Algeria) from 2 µL collected from the tip of the tail vein at time 0, 30, 60, 90, 120, 150, 180 and 240 minutes. The results have been expressed in terms of milligrams of glucose per deciliter of blood (**Aslan et *al*., 2006**).

The antidiabetic activity was firstly expressed as a percentage decrease in blood sugar (PD%) using the following formula:

$$\mathbf{PD}(\%) = \frac{\mathbf{G_c - G_s}}{\mathbf{G_c}} \times \mathbf{100}$$

Where ;

$\mathbf{G_c}$**:** Glycaemia level of rats in the negative control group.$\mathbf{G_s}$**:** Glycaemia level of rats in treated groups with extracts or acarbose.

The antidiabetic activity of each extract was calculated then from the calibration curve of acarbose and expressed in milligrams of acarbose equivalent antidiabetic capacity (AEAC) per kilogram of body weight (AEAC mg/kg bw).

II.8.3. Long-term test

Diabetes was induced in fasted overnight animals by intraperitoneal injection of alloxan monohydrate dissolved in sterile saline water at a dose of 150 mg/kg body (**Celiktas et *al*., 2007; Matteucci and Giampietro, 2008; Rohilla and Ali, 2012**). Diabetes was detected after 3 days and the rats with blood glucose level greater than or equal to 300 mg/dL (**Warda et *al*., 2009; Eidi and Maryam, 2009**) were considered diabetic and they are included in our study. Glibenclamide has been used as a positive control. The selected diabetic animals were divided in to 8 groups (n = 6) and one more group of normal non-alloxanized animals was also added in the study, each group was orally received a single extract dose and four standard doses as indicated follows:

- **Group1 (normal control):** Receives 1 mL of mineral water.
- **Group 2 (negative control, alloxan-induced):** Receives 1 mL of mineral water.
- **Groups 3-6 (diabetic-induced):** Receives 1 mL of glibenclamide at doses of 2, 5, 7 and 10 mg/kg bw, respectively.
- **Group 7 (diabetic-induced):** Receives 1 mL of acetonic extract at 100 mg/kg bw dose.
- **Group 8 (diabetic-induced):** Receives 1 mL of aqueous extract at 100 mg/kg bw dose.
- **Group 9 (diabetic-induced):** Receives 1 mL of methanolic extract at 100 mg/kg bw dose.

The preparations (drug, extracts and water) are given orally to the rats of 9 groups twice a day for 13 days.

Two tracking points were specified on the treated rats.

A) Blood was taken from the tail of the rat before administration of the extracts for the determination of blood glucose level. The blood sugar level is measured every two days using a commercial glucometer at test strips (DIAGNO-CHECK, Algeria). The results have been expressed in terms of milligram of glucose per deciliter of blood (**Aslan et *al*., 2006**). The Antidiabetic activity was evaluated firstly as a percentage decrease in blood sugar (PD %) using the following formula:

$$\mathbf{PD}(\%) = \frac{\mathbf{G_c - G_s}}{\mathbf{G_c}} \times \mathbf{100}$$

Where;

$\mathbf{G_c}$: Glycaemia level of rats in the negative control group. $\mathbf{G_s}$: Glycaemia level of rats in treated groups with extracts or glibenclamide.

The antidiabetic activity of each extract was calculated then from the calibration curve of glibenclamide and expressed in milligrams of glibenclamide equivalent antidiabetic capacity (GEAC) per kilogram of body weight (GEAC mg/kg bw).

B) During the period of the experience, the rats were weighed every three days thanks to a balance in grams (g).

II.9. Statistical Analysis

Each *in vitro* experiment was replicated three or five times (n = 3 or 5), and results were expressed as means ± SD. Results for the *in vivo* assessment are presented as mean ± standard deviation (SD) for five rats in each group. The EC_{50} values were calculated from the equations of the curves plotted by Excel. One way analysis of variance (ANOVA) followed by Tukey's and Dunnett tests was used for statistical analysis to determine the difference between the groups. The significance difference if any among the groups at p value < 0.05 was considered statistically significant. Pearson's correlation coefficient was determined to determine the variations between each plant extract in terms of the antioxidant, and anti-inflammatory and antidiabetic acticities using XLSTAT 2020.5.03 with a tolerance of 0.0001 and a 95% confidence interval.

Results and discussion

I. Extraction yield and phenolics quantifications

The extraction was done after drying the plant in the shade and making it powder. In fact, the use of dry material is recommended as long as certain compounds (especially glycosides) may be subjected to enzymatic degradation when the plant material is fresh or not dried. In addition, moisture-induced microbial fermentations can be the cause of this degradation. Drying the plant in the dark prevents chemical transformations such as isomerization and degradation caused by ultraviolet radiation from sunlight. The use of powder in place of the whole plant is intended to improve extraction by making the sample more homogeneous, increase the surface area of contact with the solvent and facilitate its penetration into cells that are not destroyed after grinding.

Due to the use of numerous plant species as a source of phytotherapeutic products, the study of their biological activities have boomed in recent years (**Choirunnisa et *al*., 2016; Boro et *al*., 2019; Chaves et *al*., 2020**). In plants, the main compounds withbiological activities are phenols, as they have an aromatic ring that allows the stabilization and relocation of the unpaired electrons of their structure, thus facilitating the donation of hydrogen atoms and electrons from their hydroxyl groups (**Aparadh et *al*., 2012; Banothua et *al*., 2017**). The total phenol content varies depending on the plant species, plant tissue, developmental stage, and environmental factors, such as temperature, water stress, and light conditions (**Hameed et *al*., 2017; Chanthasri et *al*., 2018**).

The total phenolic content, the flavonoid content and the condensed tannin amount were analyzed using Folin-Ciocalteu, aluminum chloride and vanillin assays, respectively. The total phenolic content, the content of flavonoids andthe content of tannins of each extract have been determined from the standard gallic acid calibration curve (**Figure 8**) the standard quercetin calibration curve (**Figure 9**) and the standard catechin calibration curve (**Figure 10**), respectively. The contents of total phenolics, flavonoids and condensed tannins of each extract wereas mg gallic acid equivalents per gram dry weight (mg GAE/g dw), mg quercetin equivalents per gram dry weight (mg QE/g dw), and catechin equivalents per gram dry weight (mg CE/g dw), respectively.

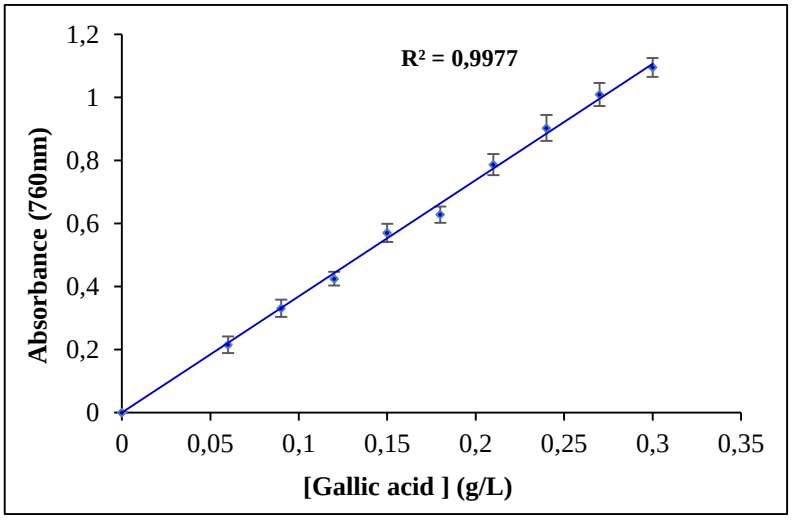


Figure 8. Standard curve of gallic acid for the determination of total polyphenols.

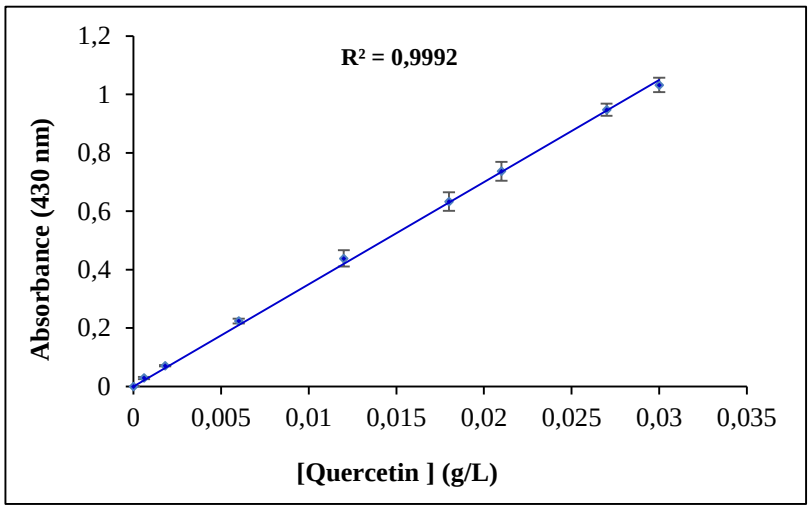


Figure 9. Standard curve of quercetin for the determination of total flavonoids

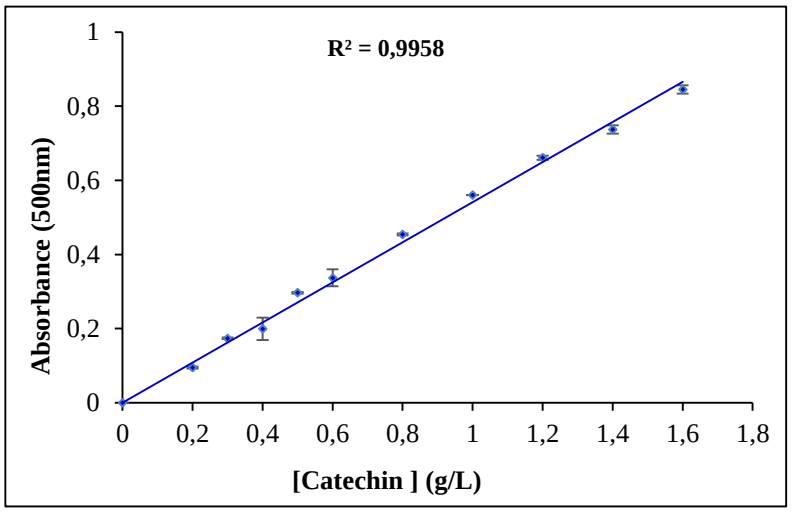


Figure 10. Standard curve of catechin for the determination of tannins.

The results ofextractionyield, total phenolics content, flavonoids contentand condensed tannins content determined in the extracts of *Hammada elegans* Botsch are shown in **Table 6**. The results representing the means of three parallel tests ± standard deviation (SD).

Table 6. Extraction yield, total phenolics content, flavonoids content and condensed tannins Condensed of *Hammada elegans* Botsch extracts.

Extracts	Extraction yield (%)	Total phenolic content (mg GAE/g dw)[a]	Total flavonoid content (mg QE/g dw)[b]	Total tannin content (mg CE/g dw)[c]
Hexanic	02.25	0.060 ±0.000[a]	0.072 ±0.001[a]	5.220 ±0.182[a]
Acetonic	01.64	0.302 ±0.008[b]	0.112 ±0.002[b]	8.122 ±0.056[b]
Methanolic	14.18	1.025 ±0.005[c]	0.150 ±0.001[c]	6.563 ±0.086[a]
Aqueous	20.36	2.560 ±0.008[d]	0.275 ±0.001[d]	8.347 ±0.123[c]

[a] milligrams of gallic acid equivalent per gram of dry weight of plant. [b] milligrams of quercetin equivalent per gram of dry weight of plant. [c] milligrams of catechin equivalent per gram of dry weight of plant. The mean with the different upper case letters are significantly different at $p < 0.0001$.

The different crude extracts obtained were slightly viscous with greenish color. Extract residue yields were calculated as the dry weight percentage of dry plant material (**Table 6**). The crude extract yields of *Hammada elegans* Botsch varied significantly depending on the solvent extraction system (1.64 to 20.36 %). The highest value was observed in the aqueous extract, while the lowest one was detected in acetonic extract. The acetonic extract, which marked the lowest amount of dry residues compared to the other extracts, suggests that the plant is rich in polar compounds. Furthermore, extract yield has also been reported to depend on the method and conditions in which the extraction is performed, on the plant species, its geographic origin, harvest time, and conditions and duration of storage (**Wael, et *al*., 2006**). Moreover, variations in the concentration of secondary metabolites are the result of both biotic and abiotic factors. Quantitative and qualitative analysis of the dynamics and distribution of secondary metabolites, the impact of environmental factors on their structure as well as biological activity provide answers to many current issues in several disciplines.

The results of the of total phenolics content varied widely in the investigated *Hammada elegans* Botsch extracts,and ranged from 0.060 ± 0.000 to 2.560 ± 0.008 mg GAE/g of dry material (**Table 6**). The maximum total phenolic levels were observed in the aqueous extract; whereas, the lowest contents were marked in hexanic extract. Wile, the amount of flavonoids varied from

0.072 ± 0.001 to 0.275 ± 0.001 mg QE/g of dry material. The greatest amount of flavonoids was recorded in the aqueous extract; whereas, the lowest flavonoids contents were marked in hexanic extract. Whereas, the content of the condensed tannins in the *Hammada elegans* Botsch extracts varied between 5.220 ± 0.182 and 8.347 ± 0.123 mg CE/g of dry material. The maximum total tannins levels were observed in the aqueous extract; whereas, the lowest contents were marked in hexanic extract. On other hand, the total phenolic content in the tested sample in this study was lower than reported for other herbs (**Djeridane et *al*., 2006; Djeridane et *al*., 2007; Skowyra, 2014**), however, the obtained phenolic content in the aqueous plant extract was lower than those reported for the other medicinal and common dietary plants (**Deng et *al*., 2013; Djeridane et *al*., 2015; Bensafieddine et *al*., 2019**).

In this study, we conclude that the aqueous extract exhibited higher content of phenolics compound, flavonoids and tannins condensed than other extracts. The use of different solvent for extraction certainly affected extraction productivity of phenolic compounds. According to **Sulaiman** and collaborators (**Sulaiman et *al*., 2011**), the difference in polarities of extracting solvents might influence the solubility of chemical constituents in a sample and its extraction yield. Therefore, the selection of an appropriate solvent system is one of the most relevant steps in optimizing the recovery of total phenolics, total flavonoids, total condensed tannins and other bioactive compounds from extracts. It is evident from the present investigation that *Hammada elegans* Botsch are a good source of phenolics as compared to other species and some of its biological effects could be attributed to the presence of these valuable constituents (**Shahidi et *al*., 1992; Tanaka et *al*., 1998**). It can also be deduced that period of collect of the plant and methods of extraction were important factors in optimization, which can affect variations of yield. Phenolic compounds are known as powerful chain breaking antioxidants (**Shahidi et *al*., 1992; Hatanoet *al*., 1989**), and are very important plant constituents because of their scavenging ability due to their hydroxyl groups (**Duh et *al*., 1999**). It has been suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested at up to 1 g daily from a diet rich in fruits and vegetables (**Tanaka et *al*., 1998**). Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties (**Djeridane et *al*., 2010**).

II. The *in vitro* antioxidant capacity evaluation

Free radicals are involved in the normal physiology of living organisms. Under certain conditions, the excess of free radicals and reactive oxygen species has been reported to induce cellular damage and to be involved in several human diseases (**Thong et *al*., 2008**).There are several methods for the determination of antioxidant activities. The chemical complexity of extracts, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour could lead to scattered results, depending on the test employed (**Ozturk et *al*., 2007**). Therefore, Basic classification of *in vitro* of reactions is based on type SET-based assay and HAT-based assay (**Karadag et *al*., 2009**). The SET-assay is based on the ability of antioxidant to transfer one electron by reducing oxidant, and the degree of color change is correlated to the concentration of antioxidant in the sample (**Prior et *al*., 2005**). Meanwhile, HAT-based assay measures the ability of antioxidant to quench radical by hydrogen donation (**Prior et *al*., 2005**). SET and HAT mechanism almost always occur together, and the mechanism that appear predominantly is influenced by ionization potential (DIP), bond dissociation energy (BDE), redox potential, pH, and solvent (**Prior et *al*., 2005; Karadag et *al*., 2009**). Therefore, the antioxidant capacities of our extracts were evaluated by 4 different *in vitro* analytical methods: the scavenging activity of 2,20- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS assay), cupric ion reducing capacity (CUPRAC assay), the evaluation of reducing power and the ferrous chelating power (chelating assay) and for the evaluation antihemolytic activity against AAPH induced erythrocyte hemolysis (hemolysis assay).

Thus, the measurements of optical densities in the presence of each extract solution at different dilutions allowed us to express the antioxidant power by the effective concentration parameter EC_{50}. However, low EC_{50} values indicated higher antioxidant capacities. Therefore, the simplest approach in interpreting the data is to plot the percentage of antioxidant capacity as a function of the concentration of the antioxidant agent (extracts and standards), thus obtaining a concentration range that give antioxidant rates within the range $\leq 50\% \leq$. EC_{50} values are determined graphically using regression on the plot representing the percentage of antioxidant capacity as a function of different concentrations of the tested extracts or standards (**Figures 11-14**). Vitamin C, Vitamin E, TBHQ, BHT, BHA and EDTA were used as positive controls and their antioxidant capacities were also determined using the same procedure. The results representing the means of three parallel tests ± standard deviation (SD).

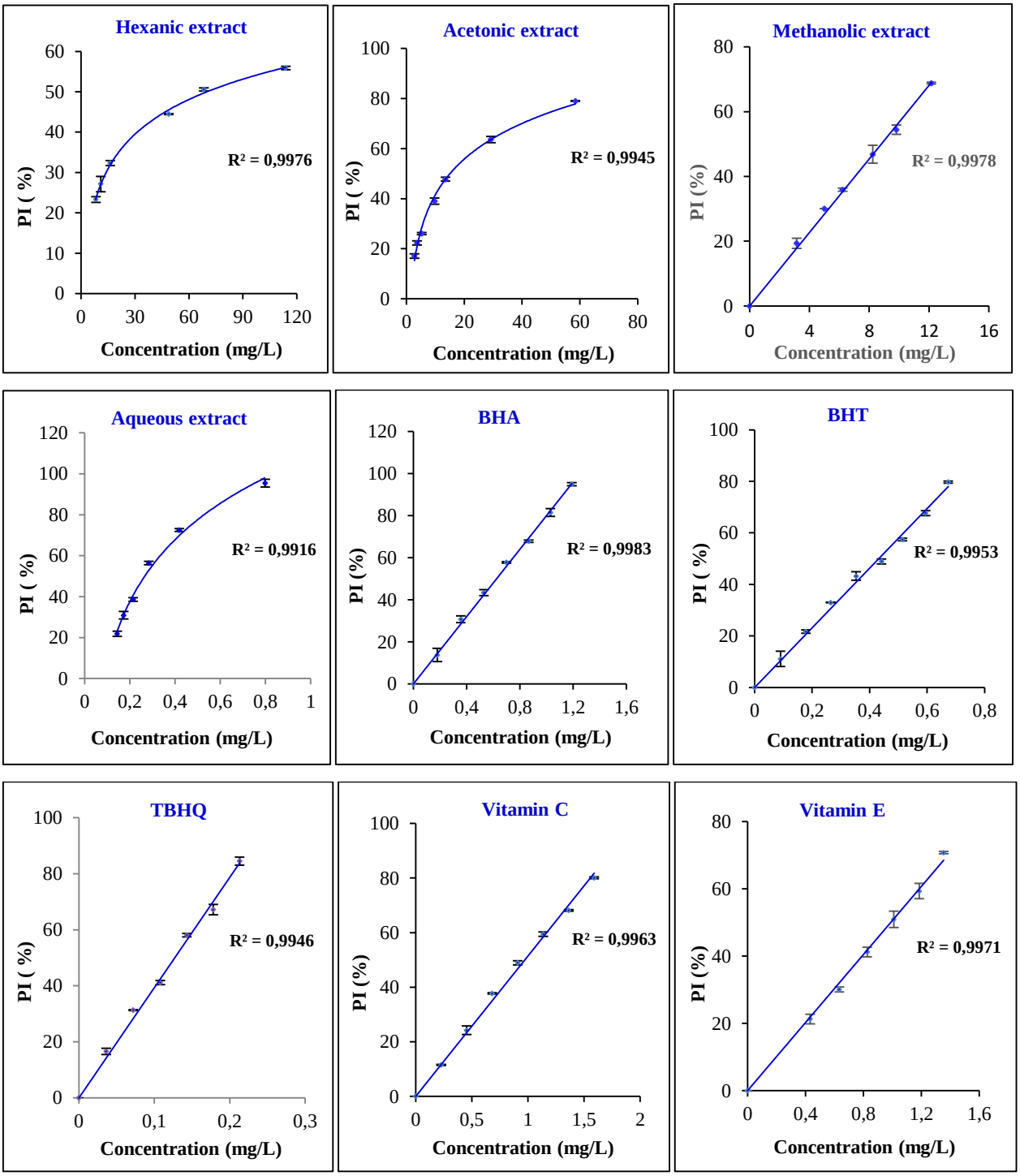


Figure 11. Antiradicalar Power Curves (ABTS Test).

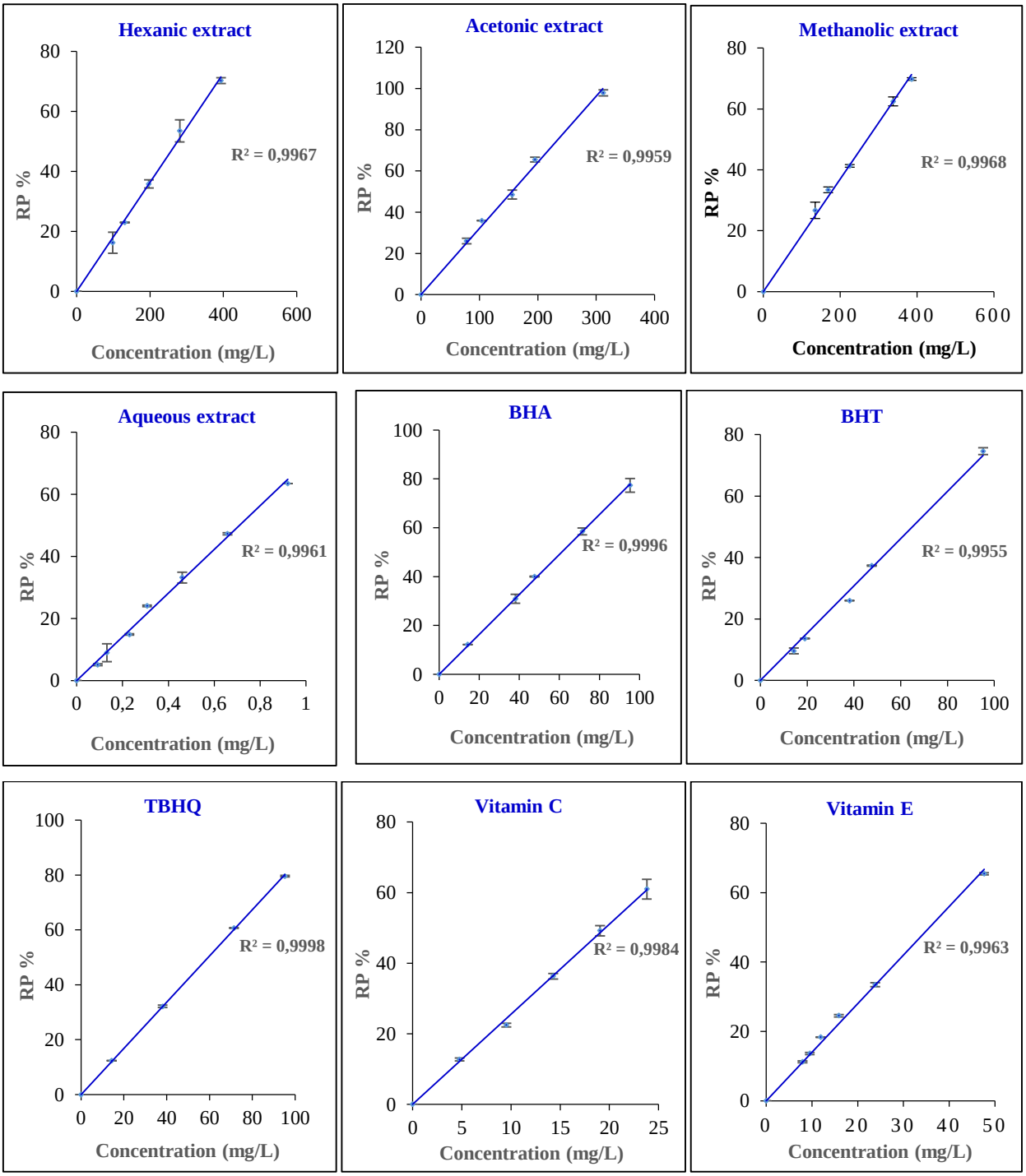


Figure 12. Reducing power curves (CUPRAC Test).

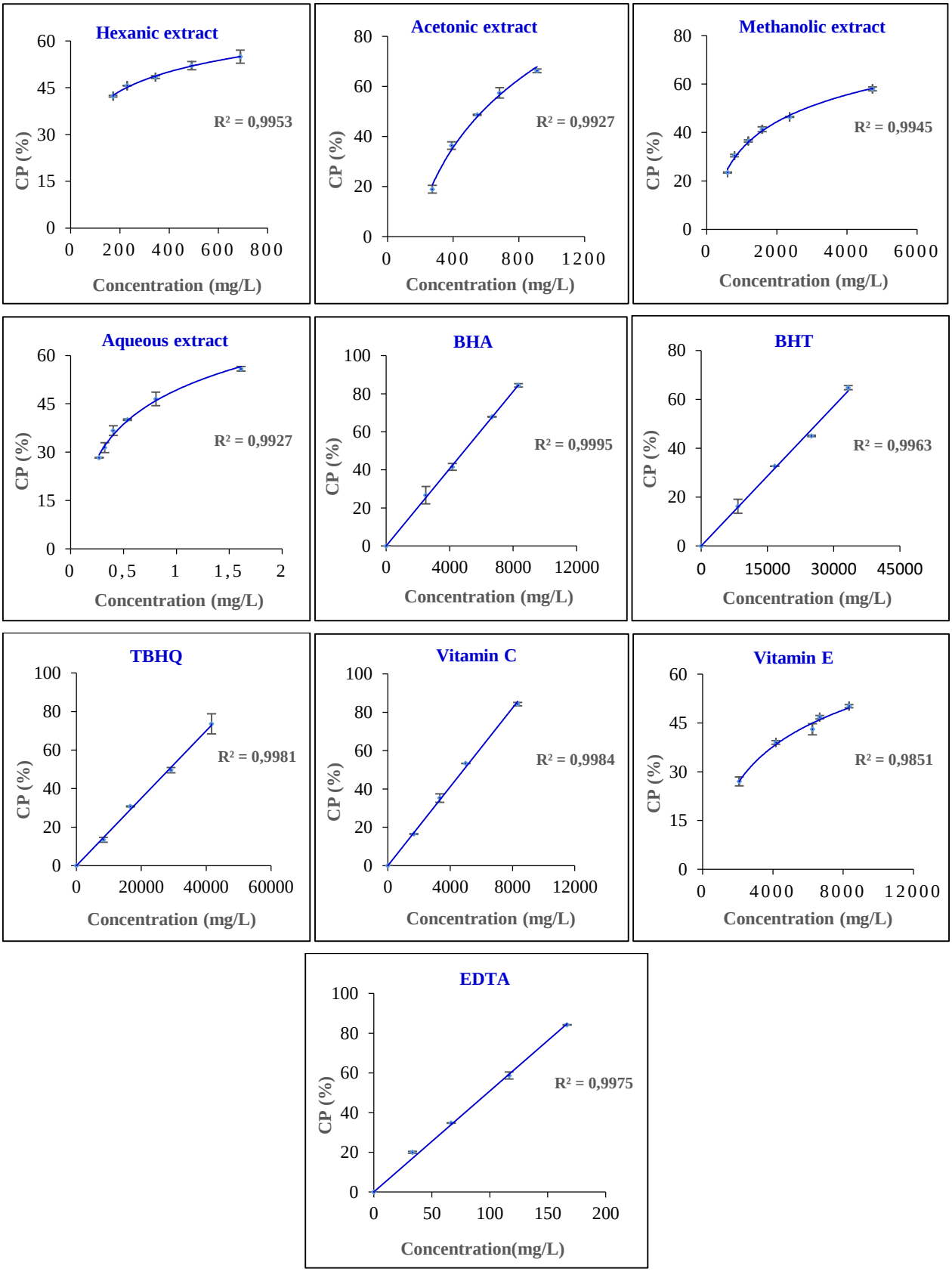


Figure 13. Chelating power curves (Chelating Test).

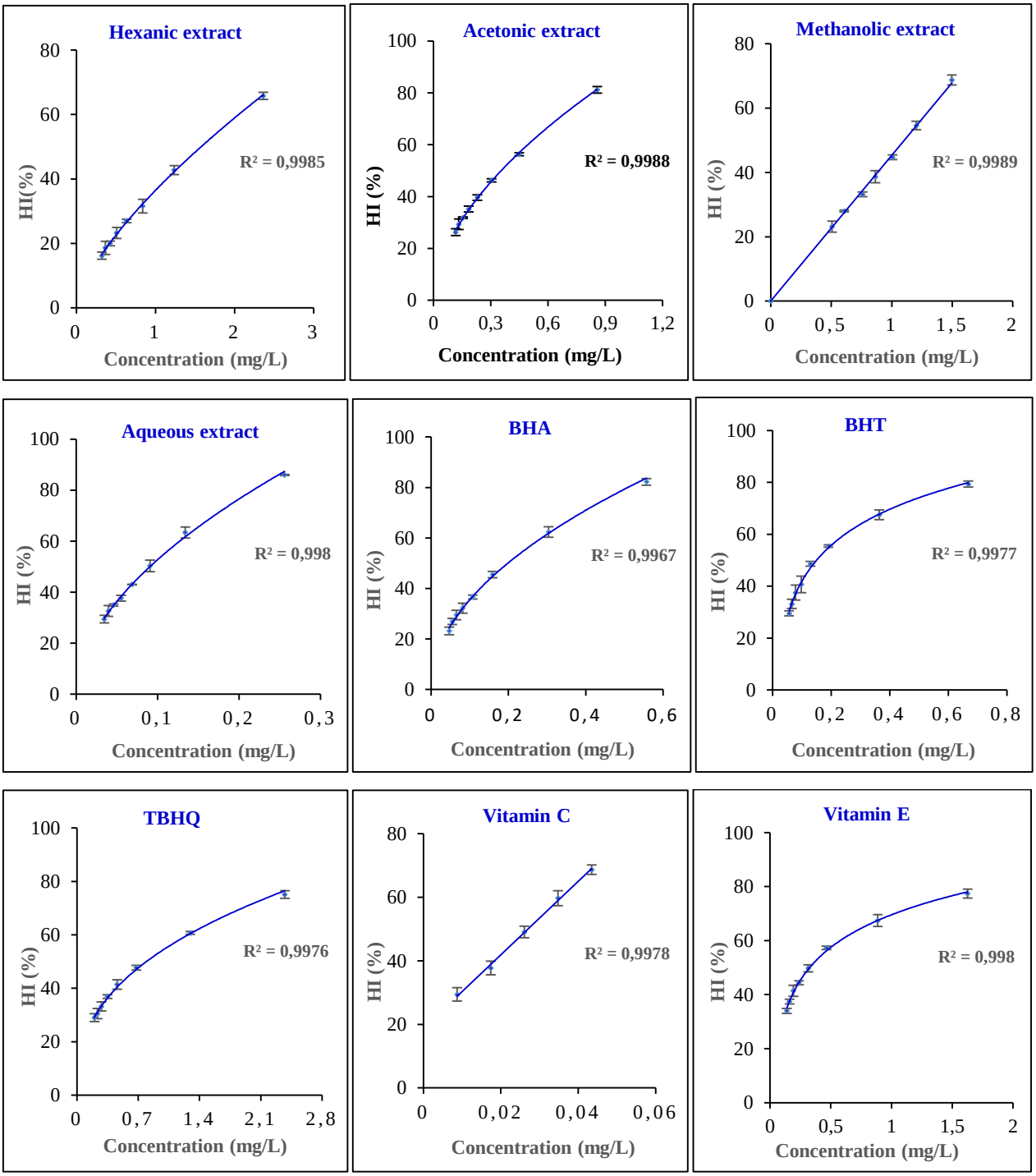


Figure14. Hemolysis inhibition power curves (Hemolysis Test).

II.1. ABTS radical scavenging activity

The improved $ABTS^{\bullet+}$ method was used to determine the antioxidant activity of *Hammada elegans* Botsch extracts examined in this work. This method is widely used to assess the total amount of radicals that can be scavenged by an antioxidant, *i.e*. the antioxidant capacity. The advantage of this assay is that $ABTS^{\bullet+}$ radical has high reactivity and, therefore reacts with a higher number of antioxidants, especially hydrophilic ones such as phenolic compounds (**Boussaada et *al*., 2008**). The green radicalcation $ABTS^{\bullet+}$ has absorbance peaks at 630, 734 and 812 nm. On interaction with antioxidants, the radical is reduced suppressing the absorbance of the green radical cation in a dose-dependent way. Moreover, the advantage of this assay, that $ABTS^{\bullet+}$ radical has high reactivity and therefore reacts with a higher number of antioxidants, especially hydrophilic ones such as phenolic compounds. The EC_{50} values obtained through this antiradicalar screening are shown in **Table 7**.

Table 7. EC_{50} values of the different extracts and standards measured by the ABTS test.

	EC_{50} (mg/L)	**Standard**	**EC_{50} (mg/L)**
Hexanic extract	69.951 ± 1.757^{a}	**BHA**	0.624 ± 0.000^{d}
Acetonic extract	15.072 ± 0.166^{b}	**BHT**	0.432 ± 0.000^{d}
Methanolic extract	8.800 ± 0.103^{c}	**TBHQ**	0.127 ± 0.001^{d}
Aqueous extract	0.265 ± 0.003^{d}	**Vitamin C**	0.972 ± 0.000^{d}
		Vitamin E	0.988 ± 0.016^{d}

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

The obtained results (**Table 7**) indicate that all plant extracts showed a beneficial effect against free radical cation damage (fast and effective scavengers of the $ABTS^{\bullet+}$ radical). Their EC_{50} values ranged between 0.265 ± 0.003 and 69.951 ± 1.757 mg/L. But, comparatively to the standards (BHA, BHT, vitamin C and vitamin E), only the aqueous extract ($EC_{50} = 0.265 \pm 0.003$mg/L) was significantly ($p < 0.0001$) the best inhibitor. Its effect was three times as BHA (0.624 ± 0.000 mg/L), two times as BHT (0.432 ± 0.000 mg/L) and four and a half times as vitamin C, vitamin E (0.972 ± 0.000, 0.988 ± 0.016 mg/L), respectively. As positive controls, TBHQ ($EC_{50} = 0.127 \pm 0.001$ mg/L) showed a higher effect against free radical cation damage when compared to all extracts. In fact, secondary metabolites, particularly polyphenols, are known in bibliography for their antiradical effect by neutralizing free radicals. However, the results of phytochemical tests

reveal that the important antiradical effect of the aqueous extract of *Hammada elegans* Botsch is probably related to its richness in flavonoids and tannins. These compounds are endowed with antioxidant activity by releasing a hydrogen atom from their hydroxyl groups. So, *Hammada elegans* Botsch extracts remain very advantageous by their abilities to trap free radicals despite their lower antioxidant power than synthetic antioxidants. This antioxidant potential offers this plant a great interest in the prevention against cardiovascular diseases, cancers, diabetes, neurodegenerative diseases, inflammation and aging. Some antioxidants are capable of retarding the formation of the $ABTS^{\bullet+}$ radical, and others removing it to give rise to new radicals. For example, some phenolic acids whose structure-activity relationship contributes to interaction with various hydrophobic (such as cell membrane) and hydrophilic (such as cytoplasm) biological structures (**Hernández-Rodríguez et *al*., 2019**).The phenolic contents of *Hammada elegans* Botsch including two principal families: flavonoids and condensed tannins, could have potent effects on reducing the $ABTS^{\bullet+}$ radical. These findings of this study are in good accordance with previous studies that reported the higher potent ability of flavonoids and condensed tannins to remove $ABTS^{\bullet+}$ radicals (**Piluzza and Bullitta, 2011; Rebaya et *al*., 2015; Bisognin et *al*., 2019**). However, the confirmation of these results needs to be done through additional *in vivo* studies. In the case of extracts, these compounds may interact with each other through synergism or antagonism, which may interfere with the ability to eliminate free radicals (**Lü et *al*., 2010; Huyut et *al*., 2017**). Here we hypothesize that the flavonoids ant condensed tannins contributed to the $ABTS^{\bullet+}$ radical scavenging presented by the extracts. Moreover, the best antioxidant activity of the aqueous extract of *Hammada elegans* Botsch tested in this study was higher than reported for most of the other medicinal plants (**Monika and Olszewsk, 2011; Saeed et *al*., 2012; Tamuly et *al*., 2014**).

II.2. Determination of copper reducing activity (CUPRAC)

The ability to donate electrons in an oxidation reduction reaction can also be used in the assessment of the antioxidant activity of a compound. This capacity is called reducing power. Thus, the reductive activity of *Hammada elegans* Botsch extracts was evaluated using the CUPRAC method. The latter is a simple, fast and reproducible test. It is universal can be applied in plants as well as plasmas and in organic and aqueous extracts (**Apak et *al*., 2007; Badarinath et *al*., 2010**).

The presence of reducers in the plant extracts causes the reduction of Cu^{2+} in the copper form. Therefore, formed Cu^{+} can be evaluated by measuring and monitoring the decrease in green color density in the reaction medium at 456 nm. In other words, the $CuCl_2$/neocuproine system gives the method sensitivity for the "semi quantitative" determination of the concentrations of the extracts, which participate in the redox reaction. The results of this test are summarized in**Table 8**.

Table 8. EC_{50} values of the different extracts and standards measured by the CUPRAC test.

Extract	EC_{50} (mg/L)	Standard	EC_{50} (mg/L)
Hexanic extract	264.212 ± 5.675[a]	**BHA**	61.130 ± 1.257[c]
Acetonic extract	155.951 ± 0.608[a]	**BHT**	65.021 ± 0.550[c]
Methanolic extract	270.687 ± 0.072[b]	**TBHQ**	59.438 ± 0.039[c]
Aqueous extract	0.709 ± 0.002[f]	**Vitamin C**	19.609 ± 0.644[e]
		Vitamin E	35.409 ± 0.129[d]

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

First of all, we found that the reducing power of plant extracts is dose-dependent (concentration-dependent). Thus, all the extracts studied reveal moderately interesting reducing properties, which are manifested by low EC_{50} values. The obtained values were in the range of 0.709 ± 0.002 mg/L for the aqueous extract of the *Hammada elegans* Botsch sample to 270.687 ± 0.072 mg/L for the methanolic extract. Aqueous extract showed a high significant ($p < 0.0001$) reducing ability when compared to all extracts. It is 381.7 times more active than methanolic extract, 372.6 times more active than hexane extract and 219.9 greater than acetonic extract. Also, the reducing effect of BHT, BHA, TBHQ, vitamin C and vitamin E were less than the aqueous extract of *Hammada elegans* Botsch, as positive controls, but more active than other extracts: (hexanic extract: 264.212 ± 5.675 mg/L, acetonic extract: 155.951 ± 0.608 mg /L, methanolic extract: 270.687 ± 0.072 mg/L).Thus, the aqueous extract must be capable of donating electrons to the free radicals to stabilize the oxidation in the actual biological and food system. Furthermore, according to the content of total phenolics, flavonoids and condensed tannins of the aqueous extracts of *Hammada elegans* Botsch, their good reducing effect is probably attributed to their richness in tannins and flavonoids. Therefore, this finding is in accordance with the findings of previous studies (**Abu-Lafi et *al.*, 2020; Chandel et *al.*, 2020; Pande and Chanda, 2020**), possibly reflecting a higher reducing power of plant extracts.

II.3. Determination of ferrous ion chelating activity

The current treatment of heavy metal toxicity (such as iron) is based primarily on metal chelation, in addition to a few enzyme and hormonal correctors, but the latter has other complications and drawbacks (**Cardani and Farina, 1970**). In case of serious poisoning chelation agents such as EDTA by infusion are used. These treatments will accelerate the urinary elimination of the metal but also eliminate iron (**Burgat and Pinault, 1995**). All drug treatments have side effects such as EDTA (nephrotoxicity, gastrointestinal toxicity, increased occasional disorders). Similarly, recent research has focused on herbal medicine, which is considered to be a more effective and less dangerous treatment. Thus, the use of plants and plant-based chelating products is increased very rapidly (**Hans, 2007**).

Antioxidants may not necessarily show their activity to react directly with free radicals, but slow down the rate of oxidation by several mechanisms like chelation of prooxidant transition metals (copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel). Fe^{2+} is an essential catalyst for lipid peroxidation due to its higher activity and pro-oxidant action (**Ahn and Kim, 1998**). Iron chelation is based on the inhibition of the formation of the Fe (II)-TPTZ complex (2,4,6-Tripyridyl-s-Triazine). TPTZ reacts with divalent ions to form a highly soluble violet complex in organic solvents. In the presence of chelating agents, the formation of this complex is disturbed leading to a decrease in the color that is followed by the spectrophotometer. We used EDTA as a composite of references.

From the reduction profiles obtained for our extracts, it seems that the variation of the reducing power versus the concentration of the extracts gives very important linearity plots. To better characterize the effectiveness of extracts, their reducing activities were expressed in terms of EC_{50}. The results of the determination of the antioxidant activity of different extracts using the iron chelating power method are presented in **Table 9**.

Table 9. EC_{50} values of the different extracts and standards measured by the iron chelating test.

Extract	EC_{50} (mg/mL)	Standard	EC_{50} (mg/mL)
Hexanic extract	3.951 ± 0.799 ^{de}	**BHA**	4.926 ± 0.034^{d}
Acetonic extract	0.577 ± 0.003^{e}	**BHT**	26.315 ± 0.002^{b}
Methanolic extract	2.808 ± 0.076^{f}	**TBHQ**	28.594 ± 0.001^{a}
Aqueous extract	0.958 ± 0.001^{f} **(mg/L)**	**Vitamin C**	4.854 ± 0.001^{d}
		Vitamin E	8.783 ± 0.003^{c}
		EDTA	0.098 ± 0.001^{f}

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

Results from this test showed that the iron chelating ability of our extracts: (hexanic, acetonic, and methanolic) revealed a good activity compared with references: (BHA: 4.926 ± 0.034 mg/mL, BHT: 26.315 ± 0.002 mg/mL, TBHQ: 28.594 ± 0.001 mg/mL, vitamin C: 4.854 ± 0.001 mg/mL, vitamin E: 8.783 ± 0.003 mg/mL) (**Table 9**). However, EDTA (0.098 ± 0.001mg/mL) displayed higher chelating power activity than the extracts: (hexanic, acetonic, and methanolic).The aqueous extract of *Hammada elegans* Botsch (EC_{50} = 0.958 ± 0.001 mg/L) have the highest significant ($p < 0.0001$) chelating power against Fe^{2+}-TPTZ complex compared to all extracts and positive controls. It is 102.7 times more active than EDTA, 602.4 times more active than acetonic extract. The EC_{50} of the chelating power of the other extracts is present in **Table 9**. Indeed, according to the phytochemical screening of the extracts of *Hammada elegans* Botsch (**Bensafieddine et *al*., 2019; Labioth et *al*., 2019**), the good effect of this extracts is probably attributed to its richness in alkaloids, saponosides and *C*-glycosides. The content of total phenolics, flavonoids and condensed tannins of extracts of *Hammada elegans* Botsch exhibited the existence of rich total phenolics, flavonoids and condensed tannins in the aqueous extract. Also, the aqueous extract exhibits strong chelating properties in comparison to other samples. Thereby, his ability to chelate transition metal ions may be due to the presence of tannins (**Ghasemzadeh et *al*., 2010; Tiong et *al*., 2013**).Also, this may be due to higher extractability of iron chelators antioxidant via aqueous solvent systems (**Tiong et *al*., 2013**). However, these findings are in line with documented chelating ability of different extracts of other plants that water extracts were stronger chelating agent than organic extracts (**Moukette et *al*., 2015; González-Palma et *al*., 2016; Ekin et *al*., 2017**). On the other hand, the best chelating potential of the aqueous extract could be due to the presence of compounds other than phenolics, flavonoids and tannins.

Previous studies showed that polysaccharides from some medicinal plants (**Be Miller, 2019**) possess their abilities to chelate metal ion.

The results obtained in this study clearly showed that the aqueous extract of *Hammada elegans* Botsch have strong antioxidant capacity. Because synthetic antioxidants such as BHA and BHT have some adverse effects, the extract of this plant species may be useful for treating oxidative damage in the food and pharmaceutical industries. However, further studies to isolate and identify active compounds, especially phenolics, and *in vivo* studies are needed to better understand their action mechanisms as pharmacological agents.

II.4. Antihemolytic activity against AAPH induced erythrocyte hemolysis

The protective effect of the extract on cellular oxidative damage was *in vitro* evaluated by hemolysis, TBARS formation and hemoglobin oxidation induced by AAPH in human erythrocytes. Erythrocyte membrane is mostly susceptible to free radicals attack due to its high content of poly-unsaturated fatty acids, as well as molecular oxy-gen transport by hemoglobin (**García-Becerra et *al*., 2006**). Lipid peroxidation in human erythrocyte membrane mediated by AAPH induces membrane damage and subsequent hemolysis (**Aman et *al*., 2013**). In our study, we used a test *in vitro* based on submit erythrocyte to free radical aggression. All of antioxidant present in the extract is mobilized to the fight of oxidant induced by AAPH attack and protect the integrity of erythrocytes resulting in the Hemolysis inhibition. This assay is useful for screening studies on various molecules and their metabolites, especially molecules, which are having an oxidizing or antioxidizing activity and, on the other hand, molecules having a long-term action (**Girodon et *al*., 1997; Lesgards et *al*., 2002; Stocker et *al*., 2003; Djeridane et *al*., 2010**).

In order to evaluate the action of the extracts on the inhibition Lipid peroxidation in human erythrocyte membrane, the hemolysis method was chosen using AAPH as the initiator of lipid substrate oxidation and membrane damage and subsequent hemolysis.This method is based on the absorbance of supernatant was measured spectrophotometrically at 540 nm. Then, the antihemolytic activity expressed in terms of EC_{50} was then calculated from the plots representing the variation of the Hemolysis inhibition percentage according to the mass concentrations of the extracts. The results of this test are presented in **Table 10**.

Table 10. EC_{50} values of the different extracts and standards measured by the hemolysis assays.

Extract	EC_{50} (mg/L)	Standard	EC_{50} (mg/L)
Hexanic extract	1.575 ± 0.061^{a}	**BHA**	0.198 ± 0.013^{e}
Acetonic extract	0.355 ± 0.008^{b}	**BHT**	0.150 ± 0.012^{ef}
Methanolic extract	1.104 ± 0.005^{d}	**TBHQ**	0.792 ± 0.033^{c}
Aqueous extract	0.090 ± 0.004^{fg}	**Vitamin C**	0.027 ± 0.001^{g}
		Vitamin E	0.325 ± 0.008^{d}

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

As shown in **Table 10** it is clear that all extracts inhibit the hemolysis activity, their EC_{50} values ranged between 0.090 ± 0.004 to 1.575 ± 0.061 mg/L. The maximum hemolysis inhibition capacity was detected in the aqueous extract of *Hammada elegans* Botsch ($EC_{50} = 0.090 \pm 0.004$ mg/L), while the lowest activity was shown in the hexanic extract ($EC_{50} = 1.575 \pm 0.061$mg/L). Otherwise, these results plainly show that ascorbic acid exhibited significantly ($p < 0.0001$) antihemolytic activity greater than that of all extracts with EC_{50} of 0.027 ± 0.001 mg/L. However, in comparison with the other standards (BHT, BHA, vitamin E and TBHQ), only the aqueous extract of *Hammada elegans* Botsch shows the most important efficiency. It is 8.8 times more active than TBHQ, 3.61 greater than vitamin E, 2.2 greater than BHA and 1.66 greater than BHT while, for the other extracts, the acetonic extract of *Hammada elegans* Botsch showed a high antihemolytic activity of 0.355 ± 0.008 mg/L. It is more active than TBHQ ($EC_{50} = 0.792 \pm 0.033$ mg/L). Moreover, the reducing of hemolysis by acetonic extract was lower than presented for vitamin C, vitamin E , BHA and BHT ($EC_{50} = 0.027 \pm 0.001$, 0.325 ± 0.008, 0.198 ± 0.013 and 0.150 ± 0.012 mg/L) respectively. Moreover, the most potent antihemolytic activity of the aqueous extract was significantly higher than that of other studied plant extracts (**Khalili et *al*., 2014; Meziti et *al*., 2019; Karim et *al*., 2020**).

The incubation of red blood cells with the tested extracts did not induce hemolysis, indicating that the extracts are nontoxic and harmless for the cells. The pre-incubation of erythrocytes with different concentrations of the extract for 180 min followed by AAPH challenge resulted in a potent protective effect against AAPH-induced erythrocyte hemolysis. This capacity may be due to the existence of phenolic compounds like flavonoids and anthocyanins. The phytochemical screening the hydromethanolic extract of *Hammada elegans* Botsch showed the presence of

tannins (**Bensafieddine et *al*., 2019; Labioth et *al*., 2019**). The content of total phenolics, flavonoids and condensed tannins of extracts of *Hammada elegans* Botsch exhibited the existence of rich condensed tannins in the aqueous extract. Recent studies have shown that many compounds like tannins contribute significantly to the antiperoxidative activity (**Abdille et *al*., 2014**). Our results show that this plant has a protective and stabilizing effect of cell membrane, and therefore we will consider studying their antiatherogenic effect.

III. Acute toxicity assessment

Toxicity assessment of plant extracts or molecules with proven therapeutic use is of utmost important factor to prevent any risk to human health (**Ifeoma and Oluwakanyinsola, 2013**). Moreover, identification of the toxic agent will permit either its elimination to obtain non-toxic active extracts, or modification of its dose or chemical structure if it carries the biological activity (**Ifeoma and Oluwakanyinsola, 2013**). Therefore, depending on the tenure of drug administration imparted to animals, pharmacological medicine estimation is especially of 3 types like acute, sub-acute and chronic pharmacological studies. The acute toxicity measure is that one dose is employed in every animal on one occasion so as to estimate the gross behavior and additionally LD_{50} or median dose. So as to judge the toxic nature of pharmaceutically bioactive compound gift within the plant extract or its formulation, acute oral toxicity is that the initiative to be performed. Acute toxicity measurement deals with that dose that kills five hundred of the tested group of animals (**Lapornik et *al*., 2015**).

Hammada elegans Botsch extracts was first tested for acute toxicity in Wistar rats. The extracts were administered orally at increasing single doses (50-1500 mg/kg bw). The results representing the means of five parallel tests ± standard deviation (SD). During the 14 days of observation, no deaths were observed, but some signs of toxicity (reduced activity and restlessness) were seen in the rats treated with high doses of the methanolic extract (1000-1500 mg/kg bw) compared to the negative control group. However, these disorders did not last more than two hours after the gavage of the methanolic extract. This difference in acute toxicity between the different extracts may be explained by a qualitative or quantitative difference in the secondary metabolites content since alcohols are known to bring out metabolites, which is not the case of water due to the destruction of plant cell walls (**Lapornik et *al*., 2015**).

Rats treated with the various doses of the extracts had no significant change in body weight. Therefore, *Hammada elegans* Bosch extracts did not induce any toxic effect on rat organs going by this indicator, since the relative weights of the rats were not significantly different from control values.In conclusion and according to the acute toxicity test results of *Hammada elegans* Botsch extracts the rats survived after 14 days of observation, which implies that the LD_{50} is greater than 1500 mg/kg bw (LD_{50}: the median lethal dose that kill 50% of the animals). Consequently, the toxicity test results encouraged us to undertake in anti-inflammatory and anti-diabetic studies of *Hammada elegans* Botsch extracts *in vivo.*

Finally, the safe and efficacious nature of three different extracts prepared from *Hammada elegans* Bosch was confirmed and established by acute oral toxicity check conducted as per the regulations of OECD. The uniform and stable behavior and response of animals throughout the experimentation of two weeks offered the safe and harmless behavior of extracts in animals up to dose of even up to 1500 mg/kg bw. Any studies further are guaranteed together with sub-acute and chronic pharmacological evaluations to ensure the fruitful use of these extracts for numerous medical specialty functions and other pharmacological applications. Hence, *Hammada elegans* Bosch have a broad safety margin in experimental animals might be due to interaction of ingredients.

IV. The *in vivo* anti-inflammatory capacity evaluation

Acute inflammation is characterized by classic symptoms such as heat, redness, swelling and pain. Measurement of rat paw edema is therefore an excellent tool for the quantification of skin inflammation induced by phlogistic agents such as carrageenan, formalin, ovalbumin, kaolin (**Winter et *al.*, 1962**). Therefore**, c**arrageenan-induced paw edema is a widely used method to study the inflammatory process of the skin, to identify potential anti-inflammatory agents for the treatment of skin disorders, and in the search for extracts and anti-inflammatory compounds that act at various levels (**Subhan et *al.*, 2007**). Edema or swelling, one of the key signs of acute inflammation, is an important parameter to consider when evaluating agents with potential anti-inflammatory activity. Carrageenan-induced rat paw edema provokes the release of several inflammatory mediators such as histamine, serotonin, leukotrienes, bradykinin and prostaglandins (**Akindele and Adeyemi, 2007; Buapool et *al.*, 2013**). The inflammatory response is dual phase: the first phase (1-2 hours after carrageenan injection) results primarily from the concomitant release

of inflammation mediators such as serotonin, histamine and kinin. The second phase (3-6 hours after carrageenan injection) is marked by the release of bradykinins, leukotrienes and prostaglandins into the tissues, mediated by pro-inflammatory lipoxygenase (LOX) and cyclooxygenases (COX) enzymes (**Yam et *al*., 2009**). However, this phase may extend over more than 6 hours (**Perez-Gurrero et *al*., 2001**). Inhibiting the biosynthesis of inflammatory mediators and blocking the activity of these enzymes can thus be a key to effective treatment of many recent-onset inflammatory diseases (**Koolman and Roehm, 2005**).

The *in vivo* anti-inflammatory effect of *Hammada elegans* Botsch extracts is investigated here for the first time using the test of the rat paw edema induced by injection of a carrageenan solution (**Winter et *al*., 1962**). The assessment was based on the percentage of edema inhibition calculated in relation to the control group (treated with mineral water). The subplantar injection of carrageenan in the control group caused edema with a maximum volume after 4 to 6 h of injection. The oral pretreatment of rats by extracts of *Hammada elegans* Botsch or standards significantly prevented the formation of edema. Indeed, the treatment of rats with different doses of extractspresented the highest edema inhibition after 300 minutes (between 20 and 60%) (**Figure15**). However, for ibuprofen and diclofenac, which were used as standard anti-inflammatory agents, the degree of inhibition of edema started with weak percentageinitially and the values decreased by time until 360 minutes (**Figure 15**).

The time course curves of the studied extracts with were similar to the anti-inflammatory standards, showed that the mechanism of anti-inflammatory activity of these extracts may involve inhibition of a combination of the pathways involved in the carrageenan induced inflammation i.e. serotonergic, histaminergic, kinins or prostaglandins pathways. Lycorine has been shown to block lipopolysaccharide (LPS) induced production of pro-inflammatory mediators *via* mechanisms including up-regulation of inducible nitric oxide synthase and cyclooxygenase-2 protein levels in RAW264. 7 cells as well as reducing the release of nitric oxide, PGE 2, TNF-a and IL-6 and also inhibit P38 MAPK and JAK-STAT signaling pathways (**Minkah and Danquah, 2021; Shafiq et *al*., 2021**). These mechanisms may partly explain the anti-inflammatory activities observed. This study may demonstrate the relevance of the extracts *Hammada elegans* Botsch in managing inflammation, however, further studies are required to isolate the phytochemicals responsible for the anti-inflammatory, anti-infective and antipyretic activities as well as elucidate the exact mechanism for the biological effects observed.

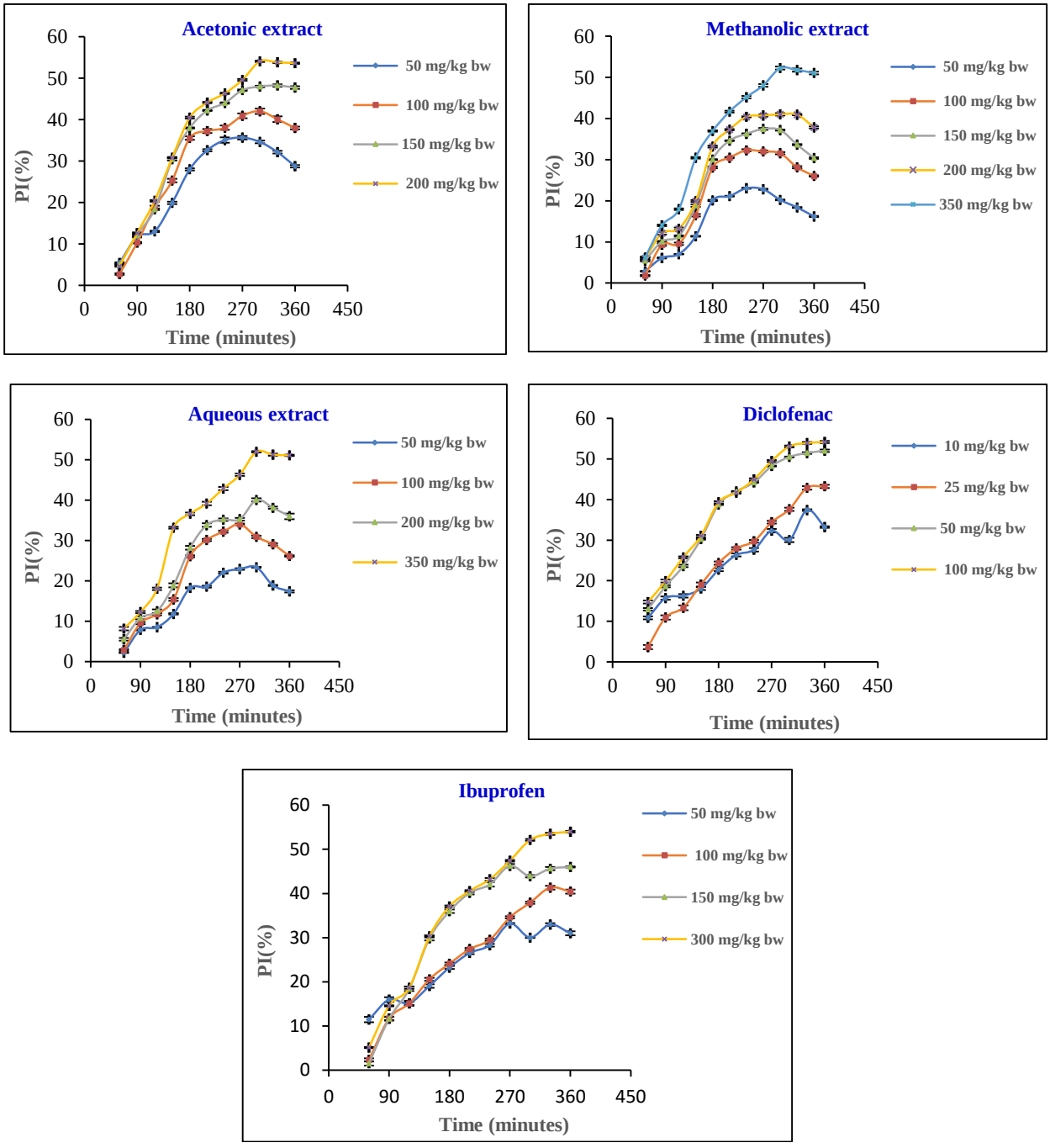
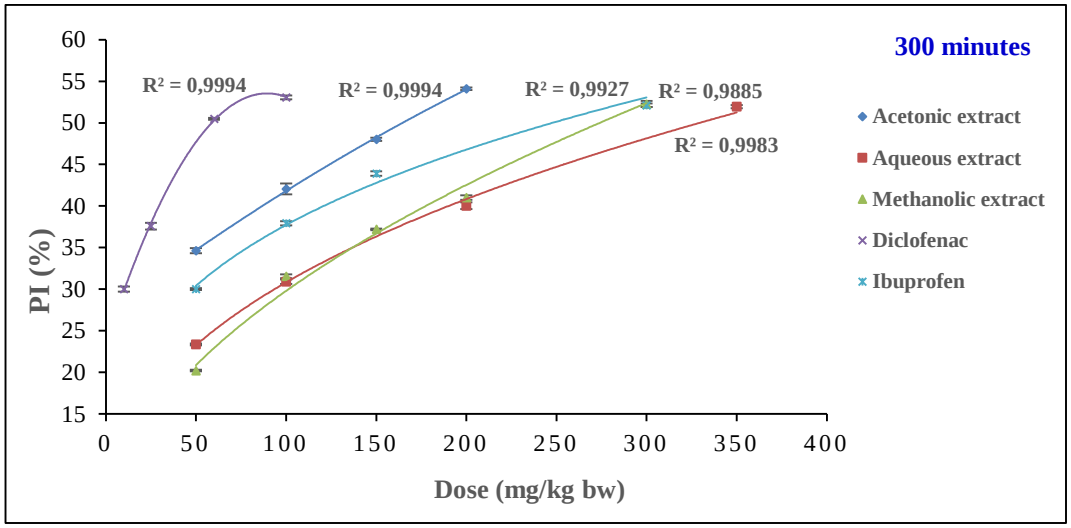
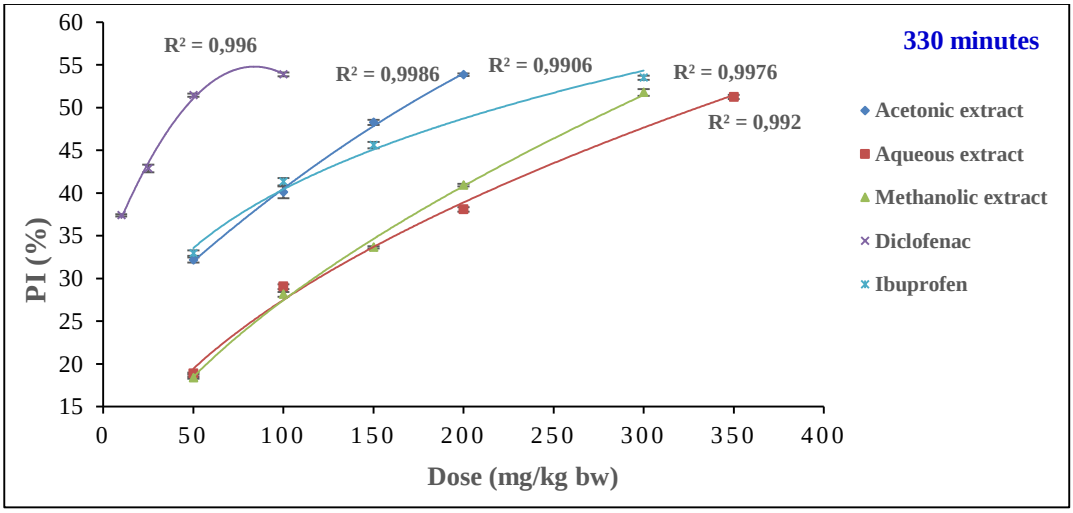


Figure 15. Effect of *Hammada elegans* Botsch extracts and standardson carrageenan-induced paw edema in rat.

Therefore, EC_{50} values were determined graphically using regression on the plot representing percentage of edema inhibition as a function of extract dose at 300, 330 and 360 minutes (**Figure 16)**. Diclofenac and ibuprofen were used as positive controls.





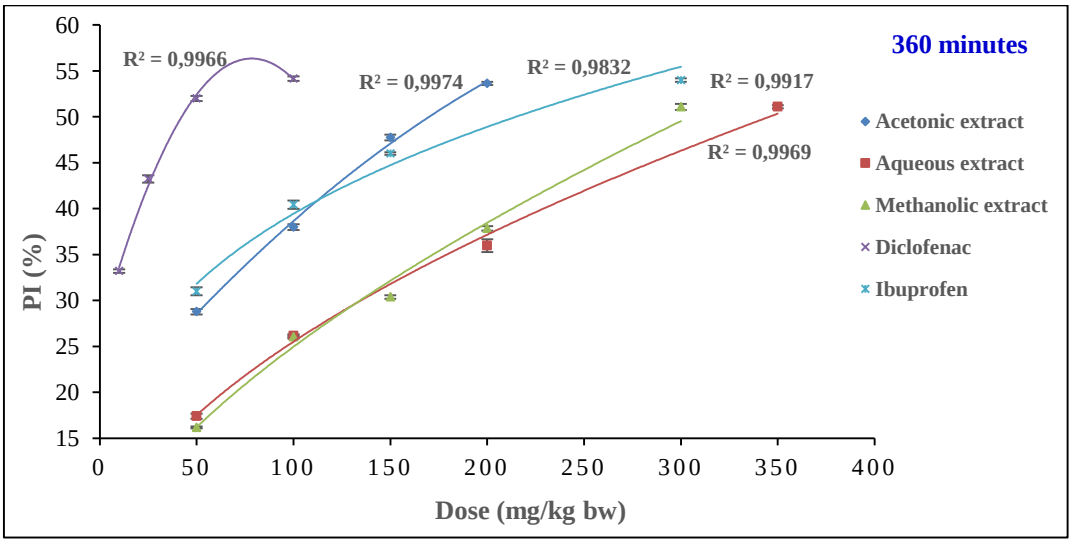


Figure 16. Concentration-response curves for anti-inflammatory inhibition capacity of *Hammada elegans* Botsch extracts and standards measured at 300, 330 and 360 minutes. Each value represents the mean SD of results in five rats ($p < 0.001$).

The results of the anti-inflammatory activity of extracts and standards representing the means of five parallel tests ± standard deviation (SD) are reported in **Table 11**.

Table 11. Anti-inflammatory activity measured at 300, 330 and 360 minutes and expressed as EC_{50} (mg/kg bw) values of examined extracts and standards determined by the rat leg edema inhibition test.

	EC_{50} (mg/kg bw)		
	300 minutes	**330 minutes**	**360 minutes**
Acetonic extract	157.45 ± 0.33^{a}	159.52 ± 0.36^{a}	164.08 ± 0.27^{a}
Aqueous extract	329.59 ± 0.24^{b}	330.08 ± 0.22^{b}	345.51 ± 0.29^{b}
Methanolic extract	273.97 ± 0.21^{c}	285.09 ± 0.21^{c}	304.53 ± 0.19^{c}
Diclofenac	59.33 ± 0.26^{d}	45.83 ± 0.24^{d}	42.49 ± 0.27^{d}
Ibuprofen	247.62 ± 0.20^{e}	220.14 ± 0.34^{e}	214.96 ± 0.30^{e}

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

As shown in **Table 11,** it is clear that extracts significantly ($p < 0.0001$) inhibit the inflammation activity in a dose-dependent manner. EC_{50} values range from 157.45 ± 0.33 to 345.51 ± 0.29 mg/kg bw at 300 minutes and persist with the same significance up to 360 minutes. However, compared to the anti-inflammatory drugs reference (diclofenac and ibuprofen), all tested extracts did not show significant inhibitory effect on the rat paw edema except for the acetonic extract which showed EC_{50} = 157.45 ± 0.33, 159.52 ± 0.36 and 164.08 ± 0.27 mg/kg bw at 300, 330 and 360 minutes, respectively. This extract exhibited a more active anti-inflammatory activity than the reference ibuprofen (EC_{50} = 247.62 ± 0.20, 220.14 ± 0.34 and 214.96 ± 0.30 mg/kg bw at 300, 330 and 360 minutes, respectively) following carrageenan injection. On the other hand, the aqueous extract of *Hammada elegans* Botsch showed the lowest anti-inflammatory potency of all tested extracts, with an EC_{50} of 329.59 ± 0.24, 330.08 ± 0.22 and 345.51 ± 0.29 mg/kg bw at 300, 330 and 360 minutes, respectively following the edema induction.

These results are in agreement with those of **Bensafieddine** and collaborators (**Bensafieddine et *al*., 2019**) who evaluated the anti-inflammatory effect *in vitro*, using acetonic and methanolic extracts of *Hammada elegans* Botsch *via* COX and LOX inhibition tests, and confirmed the higher inhibition potency of theacetonic extract (EC_{50} = 0.38 ± 0.00 mg/mL) towards LOX compared to the methanolic extract, ibuprofen and aspirin with EC_{50} values equivalent to 3.08 ± 0.00 mg/mL, 0.68 ± 0.01and 0.70 ± 0.00 mg/mL respectively. Furthermore, the acetonic extract of

Hammada elegans Botsch (EC_{50} = 0.25 ± 0.00 mg/mL) exhibited a higher inhibition power towards COX in comparison to the methanolic extract (EC_{50} = 1.11 ± 0.09 mg/mL) and anti-inflammatory drugs: ibuprofen (EC_{50} = 0.35 ± 0.00 mg/mL) and aspirin (EC_{50} = 0.64 ± 0.00 mg/mL).

Indeed, the best anti-inflammatory effect of diclofenac is due to its rapid pharmacokinetic properties and a bioavailability of 65% with oral administration. The lowest action of the extracts could be explained both by the pharmacokinetic and pharmacodynamics behaviour of the active secondary metabolites. Indeed, the digestion and absorption of phytochemicals are slow processes especially for glycosylated forms because of the resistance of the osidic bonds resulting in decrease of bioavailability. On the pharmacodynamic level, the inflammatory process is complex and involves several cell signaling pathways in addition to the free radicals production which is responsible for tissue degeneration.

The world is richly endowed with diverse medicinal plants with anti-inflammatory activities that have been shown to be effective in the treatment of inflammatory conditions in traditional medicine. Interestingly, scientists have examined some of these medicinal plants and documented their biological and therapeutic activities. However, as far as we know, no work has been devoted to express the anti-inflammatory properties of medicinal plants in term of EC_{50} values but their anti-inflammatory effects have been expressed by the percentage inhibition of the inflammatory reaction produced by carrageenan at a single dose of extract. Therefore, it is unsuitable to compare our results to those reported by other studies. But, if we concentrated the comparison in single doses, our results are comparable with other research carry out on some plant extracts. **Ratnasooriya** and collaborator smarked reduction in paw edema (28-59%) of an aqueous leaf extract of *Ixora coccinea* (Rubiaceae) in rats after oral administration (500, 1000 and 1500 mg/kg bw) (**Ratnasooriya et *al*., 2005**) Moreover, **Manjit** and collaborators (**Manjit et *al*., 2006**). have proved that the aqueous extract of *Mirabilis jalapa* Linn. has shown significant ($P < 0.05$) inhibition of paw oedema, 37.5% and 54.0% on at the doses of 200 and 400 mg/kg, respectively. Also, **Girish** and collaborators (**Girish et *al*., 2016**) reported that the aqueous and ethanolic extracts of *A. lebbeck* leaves using carrageenan-induced paw edema at doses of 50, 100, and 200 mg/kg bw (oral administration) showed a dose-dependent and significant ($p < 0.05$) inhibition of carrageenan-induced hind paw edema with maximum percentage inhibition (PI) values of 22.34, 30.85, 39.36 respectively. Furthermore, **Abdulmalik** and collaborators

(**Abdulmalik et *al*., 2017**) have demonstrated that the methanolic, aqueous, and hexanic extracts of *Calendula arvensis* had significant anti-inflammatory activity at the doses of 300 and 500 mg/kg bw ($p < 0.001$) with a significant reduction and inhibition of edema with 51.08, 71.33 and 63.38induced by carrageenan and on experimental trauma induced rat paw edema, respectively. In addition, **Achinta** and collaborators (**Achinta et *al*., 2007**) have confirmed that the methanolic extract of *Phyllanthus reticulatus* Poir. (Euphorbiaceae) at the 300 mg/kg dose level showed 40.03% inhibition of edema. On other and, the anti-inflammatory properties of oral administration of essential oil at the doses of 200, 400 mg/kg bw, respectively, showed significant reduction and inhibition of edema with 61.76% and 70.58%, respectively, induced by carrageenan at 6 h when compared with control and standard drug (indomethacin) (**Amina et *al*., 2013**) Another study of the anti-inflammatory activity of *Brassica juncea* methanolic extract against carrageenan-induced paw edema test at the dose of 1000 mg/kg bw, showed a significant anti-inflammatory activity with 65.98% reduction in the paw volume (**Yogendra et *al*., 2014**). Furthermore, it was observed that the ethanolic extract of *Ficus virens* (400 mg/kg bw) exhibits maximum anti-inflammatory activity against carrageenan induced hind paw edema with an inhibition of 66.46 % (**Abdul et *al*., 2013**). Moreover, **Ana** and collaborators showed that the hydroalcoholic extract of aerial parts of *Herissanti tiubae* at 50, 100 or 200 mg/kg bw did not reduce carrageenan-induced paw edema (**Ana et *al*., 2016**).

V. The *in vivo* evaluation of antidiabetic activity

Diabetes is a disorder of glucose metabolism that disrupts the body's storage and use of glucose. These metabolic disorders include alterations in the metabolism of carbohydrates, fats and proteins associated with absolute or relative deficiencies in insulin secretion and/or insulin action (**Daisy et *al*., 2013**). Moreover, chronic hyperglycemia is associated with specific organ complications affecting particularly the eyes, kidneys, nerves, heart and blood vessels (**Drouin et *al*., 1999**).

In our study, the anti-hyperglycemic activity of our extracts was evaluated *in vivo* using three tests: short-term test (in non-diabetic rats), starch-induced hyperglycemia test (in non-diabetic rats) and long-term alloxan test (experimental diabetes). Moreover, there have been no previous reports, at least to the best of our knowledge, on the *in vivo* antidiabetic activity of this plant

extracts. This was the first study to evaluate the *in vivo* antidiabetic inhibitory effect of *Hammada elegans* Botsch extracts in normal and alloxan-induced diabetic rats.

V.1. Short-term study

In the short-test assay, the effect of the plant on fasting blood sugar levels in normal rats was been assessed. After fasting, the animal is in a post-absorptive state, and any extract or molecule likely to lower blood sugar levels under these conditions must act by inhibiting hepatic and renal glucose production either directly or indirectly through the release of insulin (**Shrayy and Gerich, 2010**).

Assessment of the effect of the acetonic, methanolic and aqueous extracts of *Hammada elegans* Botsch on fasting blood glucose levels in normal rats was conducted by administering orally 100 mg/kg bw of the extracts. The mean values of five replicates ± standard deviation (SD) are calculated and presented in the **Figure 17**.

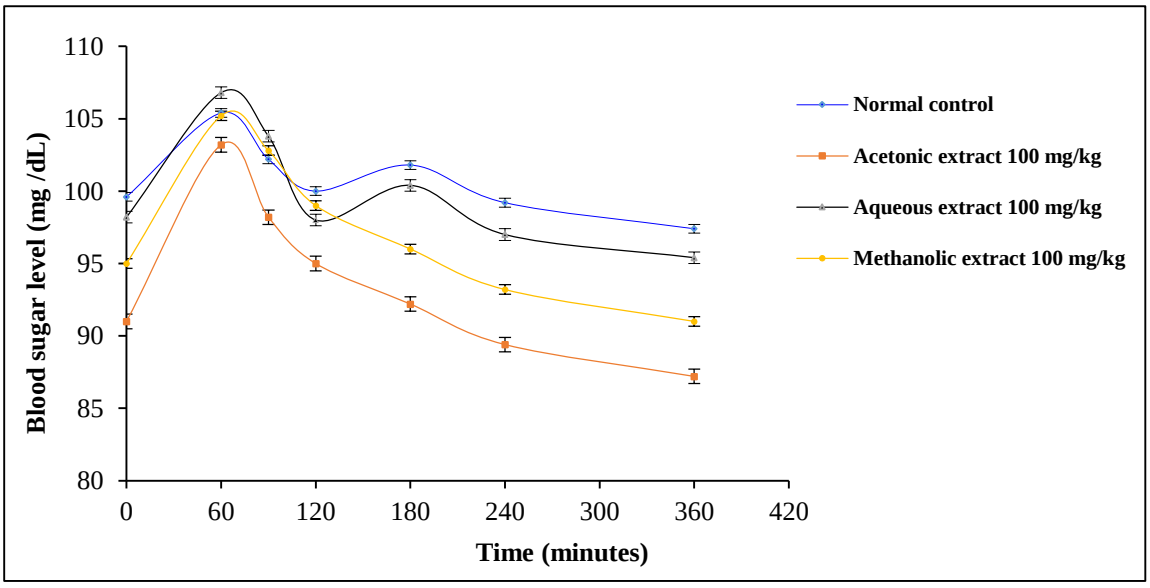


Figure 17. Changes in blood glucose level in normal rats treated with extracts of *Hammada elegans* Botsch (100 mg/kg bw) measured by the short-term test. n = 5 ± SD for each group. $p < 0.0001$.

As shown in the above Figure, fasting blood glucose (FBG) levels were within the range of 99.60 ± 0.89 and 91.00 ± 1.41 mg/dL in all the groups at 0 h. Single dose administration of all extracts (100 mg/kg bw) did not significantly decrease the blood glucose level at various time intervals compared to the control group treated with mineral water (**Figure 17**). Additionally, the

comparison between extracts and the control group showed a significant reduction ($p < 0.0001$) in glycaemia in rats treated with the acetonic extract; its maximum percentage decrease in fasting glycaemia (10.30%) was detected after 6 h. Moreover, the marqued reduction of glycaemia levels of the acetonic extract at 100 mg/kg bw dose, indicated that this extract can produce significantly hypoglycemia with high-doses administration.

V.2. Starch-induced hyperglycemia test

In the starch-induced hyperglycemia assay, the effect of the plant extracts induced in normal rats following an oral administration of a starch solution (3g/kg bw) has been assessed. Hence, any extract or molecule capable of reducing hyperglycemia can be delayed by reducing the rate of digestion of starch *via* the inhibition of α-amylase and α-glucosides enzymes, thereby retarding glucose absorption (**Ali et *al*., 2006; Lee and Lee, 2010**) and therefore promoting the use of glucose by muscles or by stimulating the secretion of insulin by the pancreas (**Shrayyef and Gerich, 2010**).

To determine the potential action of the tested *Hammada elegans* Botsch extracts, particularly their inhibitory action on α-amylase and α-glucosides in the digestive tract, acarbose was employed as positive control. (**Figure 18**).

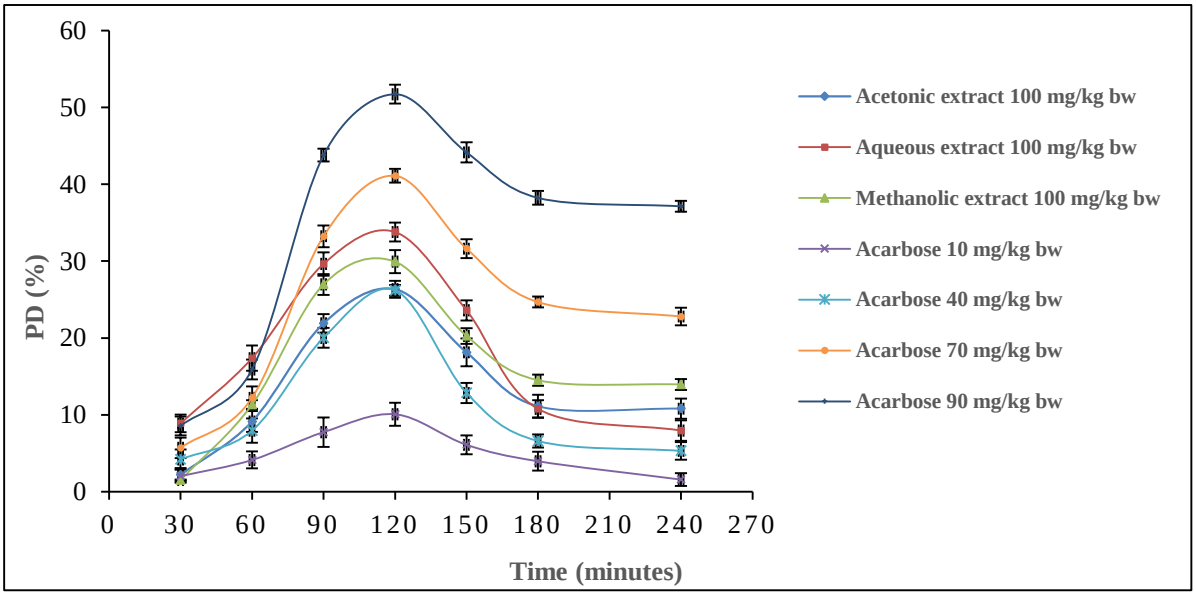


Figure 18. Kinetics of extracts and acarbose in the starch-induced hyperglycemia test. n = *5* ± SD for each group. $p < 0.0001$.

The glucose lowering effect (inhibition percentage) produced by plant extracts and acarbose increases significantly ($p < 0.0001$) with time until 120 minutes. The antihyperglycemic power was then, raised in all treated rats compared to normal control group. High total area under the curve reflects high efficacy of the extract. The results from the starch tolerance reveals that oral administration of all extracts of *Hammada elegans* Botsch to rats fed with both starch significantly suppressed the rise in postprandial hyperglycaemia in all the diabetic treated rats, whereas administration of distilled water to diabetic control rats did not affect the rise in postprandial hyperglycaemia. Therefore, the reduction in the blood glucose level by the *Hammada elegans* Botsch aqueous extract treated rats compared to the antidiabetic standard suggest that this extract slowed down the digestion and absorption of starch in the animals more likely to the other extracts. Therefore, to compare the antihyperglycemic activity of extracts to that of acarbose, their antidiabetic activity was calculated from the calibration curve of acarbose (**Figure 19**) and expressed in milligrams of acarbose equivalent antidiabetic capacity (AEAC) per kilogram body weight at the optimum effective time (120 minutes).

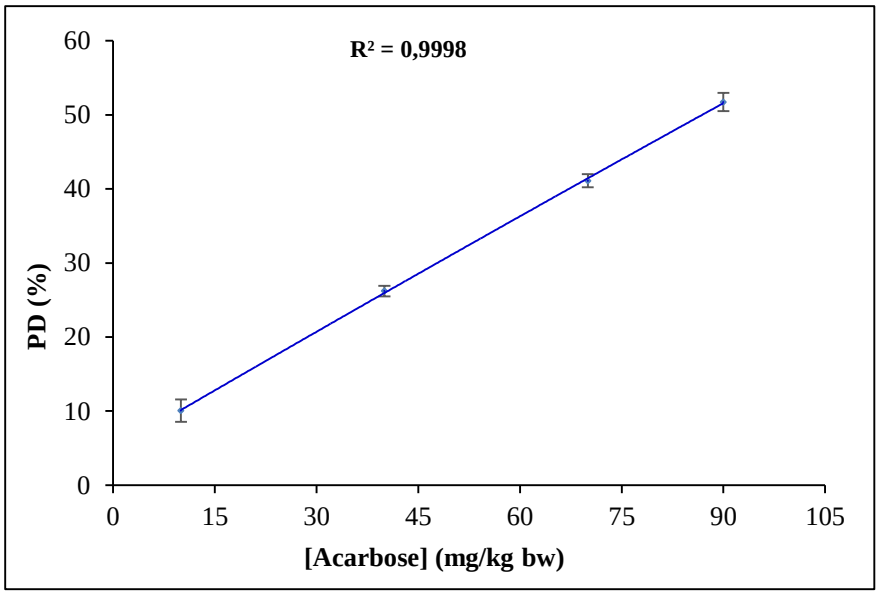


Figure 19. Calibration curve of acarbose (Starch-induced hyperglycemia test) n = 5 ± SD for each group. $p < 0.0001$.

The antidiabetic activity corresponding to each plant extract was then calculated. The AEAC parameter is defined as the dose of acarbose giving the same antidiabetic activity equivalent to 100 mg/kg bw of extract and higher AEAC values means higher hypoglycemic capacity. These

values which representing the mean values of five replicates ± standard deviation (SD) were recorded in the **Table 12**.

Table 12. Antidiabetic activity of *Hammada elegans* Botsch extracts (at dose of 100 mg/kg bw) measured by starch-induced hyperglycemia and expressed as AEAC (mg/kg bw).

	AEAC (mg/kg bw)(120 minutes)	**Relative activity factor (RAF)**
Acetonic extract	44.60 ±1.09[a]	2.24
Methanolic extract	50.68 ±0.83[b]	1.97
Aqueous extract	57.22 ±1.24[c]	1.74

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.0001$.

The obtained results show that all extracts reduce significantly ($p < 0.0001$) postprandial hyperglycemia induced in normal rats following oral administration of a starch solution. The calculated AEAC values were in the range of 44.60 ± 1.09 - 57.21 ± 1.24 mg /kg bw. The aqueous extract of *Hammada elegans* Botsch significantly ($p < 0.0001$) reduced starch-induced postprandial hyperglycemia compared to all the tested extracts (AEAC = 57.21 ± 1.24 mg/kg bw). Conversely, the poor antihyperglycemic activity has been observed for the acetonic extract (AEAC = 44.60 ± 1.09 mg/kg bw). Furthermore, the starch-induced glycemic capacity of all extracts was significantly ($p < 0.0001$) less in rats pre-treated with standard intestinal α-glucosidase enzyme inhibitory drug acarbose, i.e. acarbose is 2.24, 1.97 and 1.74 times more active than acetonic, methanolic and aqueous extracts respectively (**Table 12**).

Otherwise, starch-induced hyperglycemia activity of *Hammada elegans* Botsch extracts tested in this study showed a significant antidiabetic capacity when compared with other medicinal plants. For example, **Ashok** and collaborators have founded that postprandial glycemic load in rats pre-treated with chickpea raw seeds and their sprouts were 2-6% lesser than the control group of rats. However,the pre-treatment of rats with *Cicer arietinum* Linn. seeds and sprouts methanolic extract could not affect starch-induced postprandial glycaemia (**Ashok et *al*., 2013**). In addition, the researches of **Rammohan** and **Asmawi** demonstrate that 500 mg and 1000 mg/kg bw ethanolic extract of *Andrographis paniculata* reduces moderately the blood glucose concentration after starch and sucrose loading in normal and diabetic rats (**Rammohan and Asmawi, 2006**). Although, **Violet** and collaborators have found that *Eryngium creticum* Lam. extract treatment rats did not demonstrate any decrease in overall glycemic excursion versus the control and drug-

treated animals (**Violet et *al.*, 2011**). Moreover, using normal rats, **Elsnoussi** and collaborators have found that treated rats receiving 250 mg/kg and 500 mg/kg bw of ethanolic extract of *Orthosiphon stamineus* did not show any significant decrease in blood glucose levels compared to control rats. Only a dose of 1000 mg/kg bw of the extract reduced the blood glucose levels significantly after starch loading (**Elsnoussi et *al.*, 2015**).

In conclusion and according to above comparison, our study finds that all studied *Hammada elegans* Botsch extracts could significantly mitigate starch-induced postprandial glycemic excursion and reduce postprandial glycemic burden. This plant therefore, may become dietary supplement of choice before consumption of starch-rich meals. Phytochemicals have been linked to positive health benefits (**Marcello and Franco, 2009; Jun et *al.*, 2009**). and results from this study show the effectiveness in lowering the blood glucose levels this is probably due to the inhibition of starch degradation by α-amylase and α-glucosidases retarding glucose absorption relevant to type 2 diabetes management. This action might be attributed to the bioactive compounds largely present in the tested extracts of *Hammada elegans* Botsch. In this study, tested extracts from *Hammada elegans* Botsch offers a prospective therapeutic approach for the management of type 2 diabetes mellitus and may be beneficial for borderline diabetic patients.

V.3. Long-term test

In long term-treatment of alloxan induced diabetic rats the degree of protection of *Hammada elegans* Botsch extracts was determined by measuring blood glucose after a daily administration of the extracts for 13 days. Hence, the pancreas is the primary organ involved in the detection of the body's dietary and energy states via regulation of blood glucose, and release of insulin in response to high glucose levels. Alloxan is one of the common substances used to induce diabetes mellitus in a wide variety of species by damaging the insulin-secreting cells of the pancreas. This inflicts damage to a large number of β cells, resulting in a decrease in the release of endogenous insulin, leading thereby to decreased glucose utilization by tissues (**Sharma et *al.*, 2010**). Induction of experimental diabetes in animal models is essential to advance knowledge and understanding of the various aspects of pathogenesis, with the ultimate goal of developing new therapies (**Pinheiro et *al.*, 2011**).

Firstly, it was observed that the standard drug glibenclamide and all extracts lowered the blood glucose level significantly ($p < 0.0001$) in the diabetic rats on 7th, 9th, 11th and 13th days as compared to initial (0 hour) blood serum glucose levels (**Figure 20**).

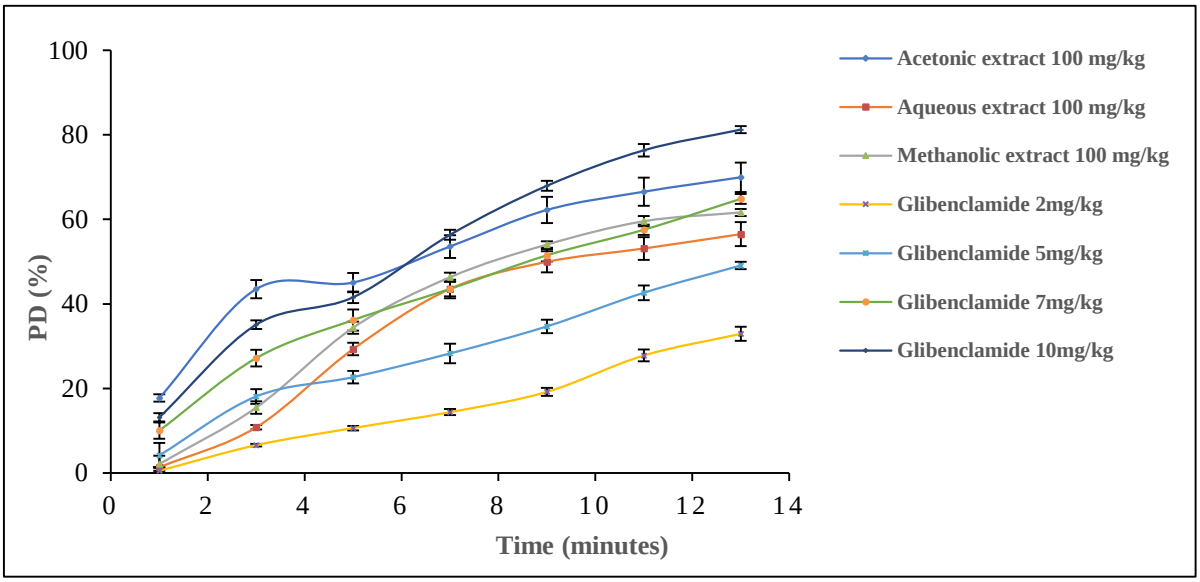


Figure 20. Kinetics of extracts and glibenclamide in alloxan-induced diabetic rats test. n = 6 ± SD for each group. $p < 0.0001$.

A marked increase in fasting blood glucose level was recorded in alloxan-induced diabetic rats when compare with the normal control rats. Glibenclamide induces a significant reduction ($P < 0.001$) in blood glucose levels when used in treating alloxan induced-diabetic rats. It was noticed also, that all extracts exhibited a similar significant hypoglycemic activity (100 mg/kg bw) throughout the entire period of the treatment. But, researchers reported that petroleum ether and water extracts did not have a significant effect on blood glucose levels in diabetic rats (**Ali et *al.*, 2012**). However, aqueous extracts of *Hammada elegans* Botsch showed lower blood glucose level activity compared to diabetic rats. Most probably, the responsible compounds that give the antidiabetic effect were not extracted due to the polarity of the solvent.It could be due then, to extraction, solvent system, improper experimental design, or other factors.Moreover, our extracts are more active when compared to previous studies used a much higher dose daily. For example, different extracts (methanol, chloroform, ethyl acetate, n-butanol, and petroleum ether) of *Phytelephas macrocarpa* at 1000 mg/kg bw, in *in vivo* animal study (12-day), did not show blood glucose-lowering activity (**Azad and Sulaiman, 2020**). Also, **Khouri** and **Daradka** indicated that only treatment with high dose of *Orchis anatolica* roots ethanolic extract (800 mg/kg/body

weight) reduces blood sugar values to significant levels ($P < 0.001$) on alloxan-induced diabetic rats (**Khouri and Daradka, 2013**). Furthermore, **Mohammed** and collaborators demonstrated that the methanolic whole plant extract of *Vinca rosea* at high dose (500 mg/kg bw) exhibited only significant antihyperglycemic activity than at low dose (300 mg/kg bw) in diabetic rats (**Mohammed et *al.*, 2010**). Similarly, **Nasab** and collaborators proved that the administration of *Trigonella foenum graecum* seed aqueous extract and *Cordia myxa* fruit aqueous extract to diabetic rats did not caused reduction in the elevated levels of serum glucoseonly at doses higher than 870 mg/kg bw and 500 mg/kg bw, respectively (**Nasab et *al.*, 2017**).

Hence, to compare the antidiabetic activity of each extract with that of standard in diabetic rats treated for 13 days with daily doses (100 mg/kg bw), their antihyperglycemic activity has been calculated then from the calibration curve of glibenclamide at 7th days (**Figure 21**).

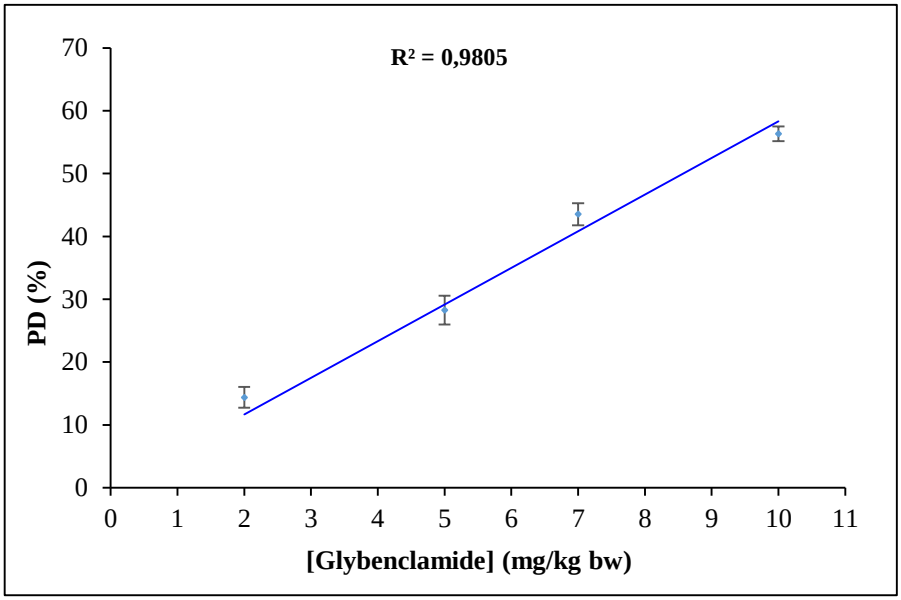


Figure 21. Calibration curves of glibenclamide (long-term test) established at 7th days. n = 6 ± SD for each group. $p < 0.0001$.

The antidiabetic activity of extracts was expressed, in milligrams Glibenclamide Equivalent Antidiabetic Capacity (GEAC) per kilogram of body weight of rat. The GEAC parameter is defined as the dose of glibenclamide giving the similar antidiabetic activity equivalent to 100 mg/kg bw of extract and higher GEAC values means higher anti-hyperglycemic activities. These values represent the mean values of 6 replicates ± standard deviation (SD). The results obtained are presented in **Table 13**.

Table 13. Antidiabetic activity of *Hammada elegans* Botsch extracts (at dose of 100 mg/kg bw) measured by long-term testand expressed as GEAC (mg/kg bw).

	GEAC (mg/kg bw) (7 days)	Relative activity factor (RAF)
Acetonic extract	9.18 ±0.72[a]	10.86
Methanolic extract	7.93 ±1.08[b]	12.61
Aqueous extract	7.43±1.21[b]	13.45

The data are expressed as mean ± standard deviation (n = 5). The mean with the different upper case letters are significantly different at $p < 0.001$.

The calculated values range from 7.43 ± 1.21 to 9.18 ± 0.75 mg GEAC/kg bw. Treatment of diabetic rats with the acetonic extract was quite effective in reducing glycaemia compared to the other extracts (GEAC = 9.18 ± 0.75 mg/kg bw). While the lowest antidiabetic activity was noted for the aqueous extract (GEAC = 7.43 ± 1.21 mg/kg bw). Furthermore, at different days of administration, all extracts showed significant antihyperglycemic potential ($p < 0.0001$) compared to glibenclamide; i.e. these results revealed that all studied extracts exhibited an antidiabetic effect less than this of glibenclamide; its effect is 10.86, 12.61 and 13.45 times more active than acetonic, methanolic and aqueous extracts respectively.

This study showed that *Hammada elegans* Botsch extracts produced a moderate decrease in blood glucose at 100 mg/kg bw in diabetic rats after 13th days of treatment compared to glibenclamide. However, the use of greater administration doses of extracts can produce a best hypoglycemia effects. Therefore, the antidiabetic effect of *Hammada elegans* Botsch extracts may be due to increased release of insulin from the existing β-cells of pancreas. Our findings are in agreement with those reported by several authors using alloxan-induced diabetic rats (**Pareek et *al*., 2009; Jiaa et *al*., 2009; Gireesh et *al*., 2009**). Further, the observed antidiabetic capacities of *Hammada elegans* Botsch extracts may also be due to enhanced glucose utilization by peripheral tissues. In this context, our study finds that all studied *Hammada elegans* Botsch extracts could significantly treatalloxan-induced diabetic mellitus and reduce postprandial glycemic burden comparatively to a number of other plants which have also been reported to exert hypoglycemic activity (**Onakpa and Asuzu, 2013; Haytham et *al*., 2014; Asra et *al*., 2020**).

One possible reason for the antidiabetic effect of extracts could be the presence of antioxidants in the plant that have been reported in a previous study (**Bensafieddine et *al*., 2019**). Antioxidants are protective agents that inactivate the ROS which cause cell damage (**Joseph et *al*., 2013**).

Interestingly, plants which have flavonoids, alkaloids, and glycosides have antioxidant activity and are claimed to possess anti-hyperglycemic effect (**Petchi et *al.*, 2013**).Therefore, the hypoglycemic effect of this plant could be associated with the presence total phenolics, saponins, tannins, flavonoids and alkaloids which have been associated with hypoglycemic activity (**Bensafieddine et *al.*, 2019**). Alkaloids promote the regeneration of pancreatic islets following destruction of the beta cells (**Middleton et *al.*, 2000**). Some tannins exhibits antidiabetic activity as demonstrated by **Broadhurst** and collaborators (**Broadhurst et *al.*, 2000**). Flavonoids are good amelioration blood glucose level and lipid parameters in alloxan-induced diabetic mice (**Li et *al.*, 2009**). Flavonoid have has been also shown to cause pancreatic beta cell degranulation. Indeed, previous researchers have demonstrated the hypoglycemic activity of triterpenoid glycosides and saponins (**Reher et *al.*, 1991; Kako et *al.*, 1997**). Thus on the basis of these findings, it is proposed that multi-components of *Hammada elegans* Botsch having insulin-secreting and β-cells protective attributes are proposed to be responsible for observed hypoglycemic effects.

On other hand, to determine the influence of the extracts on the body weight and growth of alloxan-induced diabetic rats, because the body weight loss is related to the diabetes complications .the rats' body weight was monitored periodically throughout the experiment. The results of the monitoring are shown in **Figure 22**.

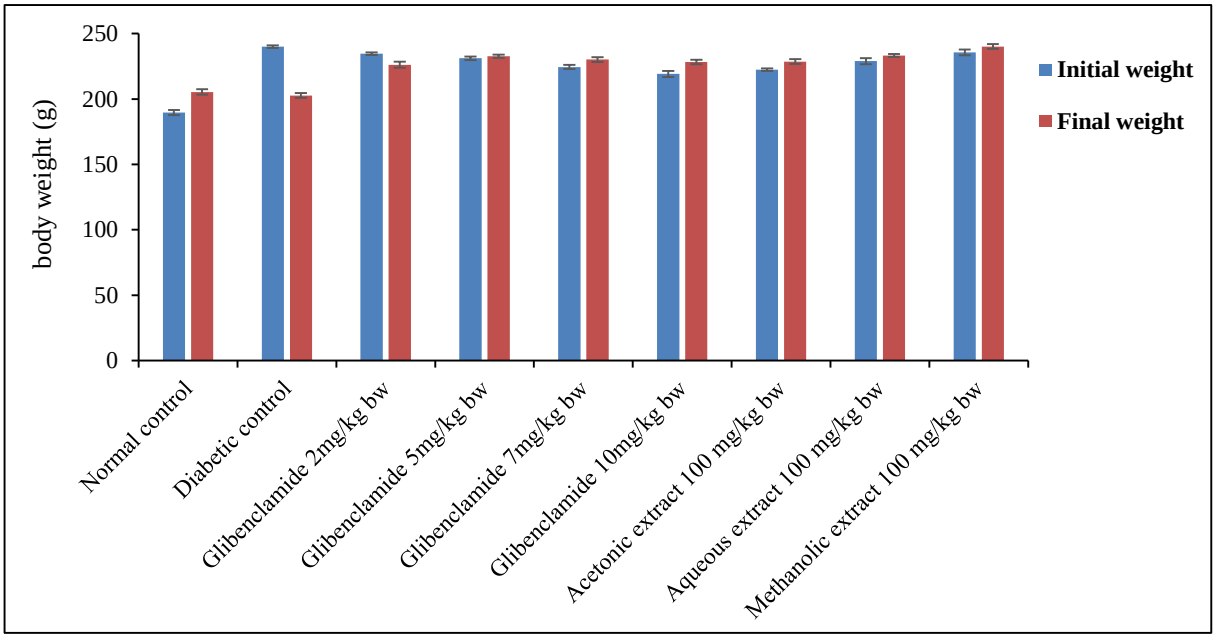


Figure 22. Effect of *Hammada elegans* Botsch extracts and glibenclamide on body weight (long-term test). n = 6 ± SD for each group. Values are presented as mean ± SD of 6 animals and significance was calculated as comparison to normal group. $p < 0.0001$.

The obtained results showed that the body weight gain was associated to the following groups: normal control, glibenclamide and *Hammada elegans* Botsch extracts. The first body weights were similar in normal and diabetic rats, while diabetic rats showed a significant ($p < 0.0001$) decrease in body weight on 13 th day comparatively to the normal rats. However, *Hammada elegans* Botsch extracts (100 mg/kg bw) caused significant ($p < 0.0001$) increase in body weight in comparison with diabetic rats.this decrease in weight is due to the lack of insulin, which leads to the degradation of lipids and proteins known to contribute to body weight (**Prabhakar and Doble, 2008; Rajagopal and Sasikala, 2008**). Therefore, the groups treated with the tested extracts of *Hammada elegans* Botsch and especially the acetonic extract showed a significant prevention of body weight loss, which could be the result of an ability to reduce hyperglycemia. On the contrary, the diabetic untreated group showed a progressive fall in body weight throughout the experimental period.

VI. Correlation between results of antioxidant, anti-inflammatory and antidiabetic activities and phenolic contents

The major part of the informed studies concluded that polyphenols are the principal composites responsible for the antioxidant, anti-inflammatory and antidiabetic activities of the tested plant extracts (**Aryaeian et *al*., 2017; Tungmunnithum et *al*., 2018; Mechchate et *al*., 2021**). In order to evaluate the presence of relationships between phenolic contents and their antioxidant, anti-inflammatory and antidiabetic activities, we have used Pearson's correlation coefficient. Pearson's (r) indicate the relationship can range from -1 to 1. An r of -1 indicates a perfect negative linear an r of 0 indicates no linear relationship between variables, and an r of 1 indicates a perfect positive linear relationship between variables. The Pearson's correlation between the different activities and phenolic content were regrouped in **Table 14**.

Table 14. Spearmans–Rho coefficients for the correlation between antioxidant, anti-inflammatory and antidiabetic activities and phenolic contents of *Hammada elegans* Botsch extracts.

	Total phenolics	Flavonoids	Tannins	ABTS	CUPRAC	Chelation	Hemolysis	Anti-inflammatory	AEAC	GEAC
Total phenolics	**1**									
Flavonoids	0.995	**1**								
Tannins	0.604	0.683	**1**							
ABTS	-0.698	-0.744	-0.846	**1**						
CUPRAC	-0.797	-0.842	-0.823	0.581	**1**					
Chelation	-0.627	-0.705	-0.989	0.776	0.889	**1**				
Hemolysis	-0.659	-0.734	-0.991	0.803	0.892	0,999	**1**			
Anti-inflammatory	0.497	0,413	-0,667	-0,595	0.234	0.607	0.557	**1**		
AEAC	0.294	0.202	0.815	-0.403	0.442	0.767	0.727	0.975	**1**	
GEAC	-0.233	-0.450	0.636	0.627	-0.193	-0.574	-0.523	-0.999	-0.965	**1**

According to the above **Table 14**, the results revealed excellent correlations between the antioxidant activities of the different extracts and their contents of tannins in ABTS scavenging assay (r = -0.846), CUPRAC assay (r = -0.823), ferrous chelation assay (r = -0.989), and hemolysis assay (r = - 0.991), meaning that the concentration of condensed tannin compounds may be a good indicator of the antioxidant capacity in the extracts. The negative correlation coefficient indicates that the lower EC_{50} value of the extract, is the higher in the tannins content, as they are inversely proportional, which is in accordance with the experimental data. However, among the four antioxidant activity methods, only the chelation and hemolysis assays provided a significant correlation with the tannins contents, with a p-value of 0.011 and 0.009, respectively. Instead, good negative and not significant correlations were found between the phenolic and flavonoid contents and the antioxidant activities of the extracts. The correlation coefficients determined for the four assays were ranged from r = -0.627 to r = -0.842, with p-values more

than 0.158. These results could indicate that the antioxidant activities possessed by the extracts may be caused by other compounds, such as vitamin, unquantified phenolics, could be contributing to the antioxidant capacity of these extracts.

Significant correlations between the content of phenolics, flavonoids and tannins in plants and their antioxidant activity have been observed in other studies. **Chaudhari** and **Mahajan** (**2015**) observed that the correlation coefficient was 0.911 between the content of phenolic compounds and the reducing power. Furthermore, **Phung et *al.,*** (**2017**) demonstrated a significantly high and positive correlation between the phenolic contents of *Castanea crenata* and antioxidant activities of extracts in in ABTS (r = 0.91) and reducing power (r = 0.99). Similarly, the tannin contents had a highly positive correlation to antioxidant activities (r = 0.47–0.64). Moreover, **Kasipandi et *al.,*** (**2019**) proved that phenolic, flavonoid and tannin contents of the *Rubus* fruit's extracts were positively associated with the antioxidant activity measured by the ABTS assay (Phenolics : r = 0.806, p = 0.002; Flavonoids: r = 0.753, p = 0.005; Tannins: r = 0.768, p = 0.004). The same situation was observed by **Bizuayehu et *al.,*** (**2016**) when the correlation was investigated between the level of total polyphenols, flavonoids and tannins contents in the tea crude extracts and the antioxidant activities. The result showed that a positive linear correlation between the antioxidant capacities and total polyphenols (r = 0.91), flavonoids (r = 0.80) and tannins (r = 0.73) content respectively. These results are in accordance found by **Shan et *al.,*** (**2011**), who while studying hydroethanolic extracts of *Castanea mollissima* Blume, also observed that total tannins content was more significantly negatively correlated to EC_{50} values of ABTS radicals scavenging (r = 0.817, $p < 0.05$) than those of polyphenols. Furthermore, high correlation coefficient was found between total phenolics in hydromethanolic extracts of *Dolichos lablab* L. and chelating capacity (r = 0.93) and antihemolytic activity (r = 0.839). Moreover, the content of tannins gave the well correlation r = 0.64 and r = 0.99 with chelating and antihemolytic assays, respectively (**Vellingiri et *al.,* 2013**). The same ascertainment was observed by **Tarik et *al.,*** (**2014**) which have shown high correlations between the levels of total polyphenols, flavonoids, and condensed tannins in three Algerian medicinal plants extracts and iron chelating assay with values of $r \geq 0.82$. In conclusion, the high significant correlations between total phenolics, flavonoids and condensed tannins with the four studied antioxidant assays suggest that polyphenols are important contributors in antioxidant activities. Moreover, **Hagerman et *al.,*** (**1998**) reported that the tannins have potent scavenging activity toward the free radicals and that the activity depends on the molecular

weight, the number of aromatic rings, and nature of hydroxyl groups. Therefore, antioxidant activities of these extracts cannot be predicted on the basis of their spectrophotometric quantifications alone, but will also require proper characterization of individual phenolic components.

The Pearson's correlation between the anti-inflammatory activity of extracts and phenolic contents were also determined (**Table 14**). Itis clear that the anti-inflammatory activity was poorly correlated with total phenolics (r = 0.497) and flavonoids (r = 0.413), respectively. These results indicate that the total phenolics and flavonoids levels in each extract werenotdirectlyrelated to the anti-inflammatory activities, but there is anothercompounds may be *more* active *than* the polyphenols like alkaloids. However, Pearson's correlation between anti-inflammatory effect and tannin contents showed a good negative correlation (r = -0.667). This negative correlation result suggests that 66.7% of anti-inflammatory activity of extracts is related to their contents in tannins. This finding was also in agreement with another reports in the literature (**Mota et *al*., 1985; Jeffers, 2006; Lima et *al*., 2007; Park et *al*., 2013; Fraga-Corral et *al*., 2021**).

On other hand, the results (**Table 14**) revealedalso poor correlations between the antidiabetic activities of the different extracts and their contents of total phenolics (r = 0.294 and r = -0.233) and flavonoids (r = 0.202 and r = -0.450) for starch-tolerance test and alloxan-induced diabetic assay, respectively.These weak correlation coefficients indicate that the antidiabetic activities possessed by the extracts may be principally caused by non-phenolic and non-flavonoid compounds present in the samples but also of the presence of other constituents with an antidiabetic potential. Therefore, good correlationswere observed between the antidiabetic activity of extracts and their contents in condensed tannins (r = 0.815 and r = 0.636), for starch-tolerance test and alloxan-induced diabetic assay, respectively; confirming that tannins are the most important group in extracts representing the antidiabetic agents. This result was also in agreement with previous studies (**Kunyanga et *al*., 2011; Kunyanga et *al*., 2011; Morada et *al*., 2016; Esmaie et *al*., 2019; Sanvee et *al*., 2021**).

Otherwise, using Pearson's correlation coefficient, the relationships between the different antioxidant, anti-inflammatory and antidiabetic assays has been studied. The obtained results (**Table 14**) indicated that EC_{50} of CUPRAC, chelation and hemolysis assays showed goodpositive

relationships (r = 0.581, 776 and 0.803) with ABTS, respectively. Moreover, omportant correlations were also noted between CUPRAC vs chelation (r = 0.888) and CUPRAC vs hemolysis(r = 0.892). Furthermore, we registered a high significant relationship between chelation and hemolysis assays (r = 0.999; p <0.05). This strong significant correlation suggests that 99.9% of antihemolytic agents of these extracts are also good chelator substances. Furthermore, other studies confirmed that the antihemolytic activities were generally increased with an increase in chelator concentration (**Janakiraman et *al*., 2015; Aaseth et *al*., 2016; Fibach and rachmilewitz, 2017; Blouin et *al*., 2020**).

Moreover, the relationships between anti-inflammatory and antidiabetic activities have been studied. The obtained results (**Table 14**) indicate the existence of high significant relationships (r = 0.975, -0.999; p < 0.05) between anti-inflammatory with starch-induced antidiabetic and alloxan-induced diabetic assays, respectively assays. This result suggests that 97.5% of anti-inflammatory agents of these extracts are also good antidiabetic agents in starch-induced normal rats, But. The negative correlation coefficient indicates that the anti-inflammatory activity of extracts is inversely proportional to the starch-induced antidiabetic activity, i.e. the extracts exhibiting significant anti-inflammatory effects showed the lowest hypoglycemic activities in oral starch-tolerance assay. The Pearson's correlation (**Table 14**) showed also excellent negative significant correlations (r = -0.965, p = 0.033) for all studied extracts between oral starch tolerance tests on both normal and diabetic rats measured at 300 minutes. This negative correlation coefficient indicates that the starch-induced antidiabetic activity of extracts is inversely proportional to the alloxan-induced antidiabetic capacity, i.e. the extracts exhibiting significant oral starch-tolerance activities showed the lowest hypoglycemic activities in diabetic rats.

In order to determine if exists the similarities and/or the differences within the antioxidant, anti-inflammatory and antidiabetic activities of the studied extracts within their phenolic contents, principal component analysis (PCA) was performed for the combined phenolic contents and the seven antioxidant, anti-inflammatory and antidiabetic activities parameters (**Figure 23**).

PCA was used to demonstrate how the ten parameters namely phenolic, flavonoids, tannins, ABTS, CUPRAC, iron chelating, hemolysis, anti-inflammatory, AEAC and GEAC contribute to the overall antioxidant, anti-inflammatory and antidiabetic activities of *Hammada elegans* Botsch

extracts. Firstly, PCA explained 100.00% of the total variance in the studied profile of the *Hammada elegans* Botsch extracts. As shown in **Figure 23**, loading factors for principal axes F1 and F2 represented 55.66 % and 44.34 % of the total information, respectively. Moreover, the loadings and percentage of variance for the first two principal components for extracts are shown in **Table 15**.

Table 15. Discriminate variables factors of phytochemicals content, antioxidant, anti-inflammatory and antidiabetic activities of *Hammada elegans* Botsch extracts.

	F1	F2
Phenolics	34.67%	-93.80%
Flavonoids	43.39%	-90.10%
Tannins	99.94%	3.37%
ABTS	-23.54%	97.19%
CUPRAC	-89.59%	44.43%
Chelation	-99.90%	4.42%
Hemolysis	-99.45%	10.46%
Anti-inflammatory	-64.12%	-76.73%
AEAC	-79.48%	-60.69%
GEAC	60.92%	79.30%

The first axis F1is highly positive correlated with tannins (99.94%), and also highly negative correlated with CUPRAC (-89.59%), chelation (-99.90%) and hemolysis (-99.45%) assays. While, axis F2 which represents 1.25 times as much as F1, ishighly positively correlated with ABTS (97.19%), and strongly positive correlated withtotal phenolics (-93.80 %) and flavonoids (-90.10%). In another hand, parameters namely anti-inflammatory and AEAC were moderately negative loaded with the two axis, their contribution were -64.12% and -79.48% for the first PC1, and -76.73% and -60.69% for the PC2, respectively. Whereas, the two axis F1 and F2 werealso positive associated with the anti-inflammatory parameter, by a contribution of 60.92% and 79.30%, respectively.

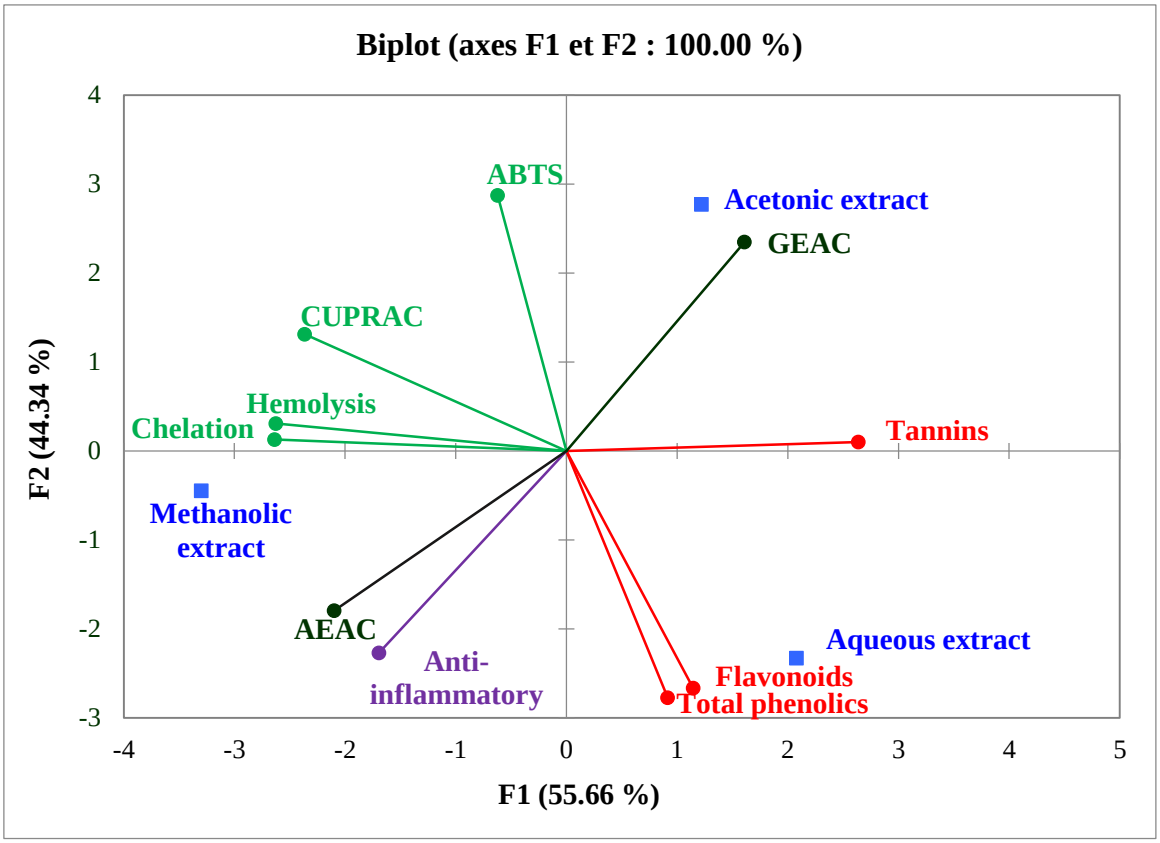


Figure 23. Loading plot obtained from PCA of the phenolic contents, the four antioxidant assays, the anti-inflammatory assay and the two antidiabetic tests of eleven *Hammada elegans* Botsch extracts.

Chelation, CUPRAC and hemolysis (**Figure 23**) are strongly negative correlated and grouped on the left side of the plot (F1). This relationship can be explained by the mechanism of three assays which are based on the measurement of the scavenging ability of the antioxidant towards transition metals.Whereas, ABTS is found along the positive side of PC2 which confirmed no significant correlation with other antioxidant assays. Similarly, anti-inflammatory and AEAC are strongly negative correlated with GEAC (**Figure 23**), this distribution confirmed the existence ofhigh significant correlation between anti-inflammatory and antidiabetic activities responses of extracts. The acetonic extract is on the positive side of the plot, it was in the same region with GEAC and the opposite side of anti-inflammatory which justified by the similarity of response on the two activities.So, the therapeutic potential of the acetonic extracts for diabetes may beexpected by minimizing the bioavailability ofglucose along with protecting the β-cells ofpancreas by scavenging NO and other stablefree radicals. This extractwhich is negatively related may necessarily be translated into antioxidant and antidiabetic activity. For aqueous extract, total phenolics, flavonoids and ABTS are highly loaded which indicate that the three

properties are closely related while PC2 implies a moderate relationship with CUPRAC, chelation and hemolysis tests. Evidently, PC1 is generally more correlated with the variable then PC2. This is to be expected because PCs are extracted successively, each one accounting for as much of the remaining variance as possible. The interrelationship of phenolics and antioxidant activities are important to group the variables based on the correlation and further understand how these variables contribute to the antioxidant activity of the plant extracts.Due to the nature of the functional groups being extracted the correlation of antioxidant assays for extracts showed similar patterns. They have the ability to react with the free radical either by scavenging or reducing. Thus, isolation and identification the compounds that are responsible for the antioxidant activity should be aimed for future study.

Loading of all the plant extracts and individual PC scores indicates that extracts with similar values for the variables explained by the PC appeared closely related, and those with large positive or negative scores are extremes in some variables. PCA score plot provided an overview of the similarities and differences between samples. F1 axis is highly negative correlated with methanolic extract (-3.29) and moderately positive correlated with aqueous (2.08) and acetonic (1.22) extracts. But, the acetonic extract which poorly correlated with F1 axis (1.22), it has the highest positive loading on PC2 (2.78). It is expected because this extract was proved to have the highest antioxidant activity and therefore stands out from other extracts. This result suggests that the aqueous extract was closely associated tannins and this extract which was in the opposite region with the four antioxidant activities, justify its best antioxidant power compared to the other extracts. Moreover, the methanolic extract is the same region with CUPRAC, chelation and hemolysis indicated its similarity of response on the three activities. Indeed, chelation and hemolysis assays which are in the opposite direction of tannin content, indicated a significant negative correlation ($r > 0.99$; $p < 0.001$) between the results obtained by ferrous chelation assay and hemolysis assay versus the amount of tannins.

Conclusion and perspectives

Despite the heterogeneous nature of the immense biodiversity of the African continent in general and of Algeria in particular, there has been little effort devoted to the development of therapeutic agents of medicinal plants. This is why we are interested in the study of *Hammada elegans* Botsch, a plant that is less frequently used in our country, because of ignorance of their nutritional and medical values. However, the main objective of this work was to evaluate the *in vitro* antioxidant activities and the *in vivo* anti-inflammatory and antidiabetic activities of this plant extracts. In the first part of this work, we are interested in the determination of total phenolics, flavonoids and tannins in the different extracts. The second part examined the antioxidant properties of extracts using four complementary chemical techniques. While, the third part has been focused on the anti-inflammatory and antidiabetic properties evaluationthese plant extracts on Albino Wistar rats. Indeed, the results obtained from this thesis lead us to conclude the following findings:

- The results obtained showed that the yields of crude extracts are variable depending on the extraction solvent system. The dry extracts obtained represent a viscous appearance of different colors with yields varying from 1.64% to 20.36%, the aqueous extract provided the most important yield (20.36%).
- The highest total phenolic content is found in the aqueous extract (2.560 ± 0.008 mg GAE/g dw). While, the quantification of flavonoids showed that the aqueous extract had the best content (0.275 ± 0.001 mg QE/g dw). Similarly, the tannins quantification, led us to conclude that this plant is rich in tannins.
- The evaluation of antioxidant activities revealed that all extracts possessing significant antioxidant activity. The aqueous extract had a very interesting activity compared to the other extracts.In addition, the aqueous extract still exhibited the best antihemolytic activity compared to other extracts and standards.
- Results of the acute toxicity of plant extracts did not show a consistent effect on animals survived after 14 days of observation, with a value of LD_{50} greater than 1500 mg/kg bw.
- The current study highlights for the first time the anti-inflammatory activityof the extracts *in vivo.* The acetonic extract represents displayed the most active extract, an activity which is also significantly superior to that of ibuprofen.

- The current study highlights also for the first time, the *in vivo* antidiabetic potential of *Hammada elegans* Botsch extracts by three assays. The short-term test indicated that there is no significant decrease in blood sugar in our rats treated with the extracts compared to the control group. The results of the hyperglycemia test caused by starch showed that the aqueous extract significantly reduces hyperglycemia compared to all the extracts and acarbose as potent antidiabetic drug, while the long-term test revealed that the acetonic extract significantly reduced hyperglycemia compared to other extracts and glibenclamide as potent antidiabetic drugduring 13 days of treatment of the alloxan diabetic rats.This extract also prevents the sever reduction in body weight.

Collectively, these results illustrated promising antioxidant capacities *in vitro* and anti-inflammatory and antidiabetic activities *in vivo* of *Hammada elegans* Bosch,especially the aqueous extract that has proven to be the most active one.Therefore, these results remain preliminary, requiring further in-depth studies to better focus on the effects revealed. Molecular-scale studies will be required to determine which active compounds of this species may be responsible for such effects and to investigate *in vivo* antioxidant activities of the active ingredients in order to determine their doses preventive and therapeutic. Further investigation is necessary to determine the exact phytoconstituents (s) responsible for antidiabetic and anti-inflammatory effects.

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