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THEME

Study of the antioxidant capacities and inhibitory effect of *Cleome arabica L* on α-amylase

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Abstract:

Cleome arabica L belongs to the family of Capparaceae. It has attracted a great deal of attention in recent years for its pharmaceutical potentials due to its antioxidant, antihypercholisterolemic, anti-cancer and chemoprotective activities for the treatment of a number of diseases.

The main aim of this study was to evaluate the α-amylase inhibitory and antioxidant activities of extracts from *Cleome arabica* collected on two seasons (January and November,2015, December 2016 and May 2017) in the town of Laghouat, steppe region of Algeria. The additional purpose of the study was to investigate a new natural inhibition of α-amylase using an *in vitro* model, as well as to find a natural anti-diabetic compound from the plant. The extracts were prepared with two solvent systems: hydroalcoholic (80/20) and acetonic solvent (70/30) systems. The crude hydromethanolic extracts were obtained by maceration in (M/W) (80/20) of eleven *Cleome arabica* leaves samples from November and January, the total phenolic content in the ethyl acetate fraction ranged between 0.341±0.009 and 0.751±0.018mg GAE/g dry matter and 0.170±0.0005 to 0.490±0.008mgGAE/g for the dichloromethane fraction, and between 0.060 ±0.004 to 0.453± 0.020mgGAE/g for the n-hexane fraction. The amount of flavonoids content in the ethyl acetate fraction ranged from 0.172±0.001 to 0.682±0.003 mg QE/g. The results of the condensed tannins in the ethyl acetate fraction varied between 0.172±0.008 to 0.332±0.007 mg CE/g, we conclude that the ethyl acetate is the best extractor of flavonoids and the condensed tannins belonging only to this fraction.

The total phenolics of ultrasound assisted extraction of hydro-Methanolic (80/20) and hydro-Acetonic (70/30) extracts present in different solvents of *C.arabica* parts (leaves, pods ,seeds, stems and roots) vary from **2.87 to 107.21** mgGAE/100g at the beginning of maturity stage (**December**) and rang between **3,981 to 99.6** mgGAE/100g at the end of maturity stage (**May**), the ethyl acetate fraction proved to be richer in total phenolics and flavonoids (**107.21**mgGAE/100g and **88.50** mgQE/100g) respectively in hydro-acetonic extract of leaves in the beginning of maturity stage , No remarkable differences were found in both extraction systems and methods, antioxidant activity was assessed using different tests: ferric reducing ability power (FRAP, DPPH,CUPRAC,ABTS and Phosphomolybdenum complex methods . The highest antioxidant capacities of *Cleome arabica* leaves extracts for November and January were detected by ABTS with EC50 values ranged from (0.009 to 0.013 mg/ml). PM-complex, ABTS,CUPRAC and FRAP were strongly positively correlated with each other , the EC50 values of hydro methanolic and acetonic extract in different fractions of *Cleome arabica parts* were approximately ranged between 0.0086 and 0.5 mg/ml for **December** and **May** months evaluated with DPPH ,antidiabetic activity was investigated by α-amylase inhibition tests , The hydro- acetonic extract showed the best activity against α-amylase compared with hydro-methanolic extract . The IC50 values were very low in **ethyl acetate** in both pods and seeds organ cited there IC50 are (IC50= 0.11mg/ml), (IC50 = 1.03mg/ml), respectively . Similar case with **n-hexane** fractions with (IC50 = 0.6 mg/ml, EC50 = 1.08 mg/ml) for stems and seeds respectively in the beginning of maturity stage .

This study is the first report on potential inhibition of these plant extracts on the digestive enzyme, α-amylase. The results indicate that *Cleome arabica* of Algeria is a powerful natural antioxidant and that it could also provide natural biologically active agents to be used in the management of diabetes.

Keywords: *Cleome arabica*, antioxidant activity, α-amylase inhibitory effect

Résumé

Cleome arabica L appartient à la famille des Capparaceae. Il a attiré beaucoup d'attention ces dernières années pour ses potentiels pharmaceutiques en raison de ses activités antioxydantes, antihypercholistérolémiques, anticancéreuses et chimioprotectrices pour le traitement d'un certain nombre de maladies.

L'objectif principale de ce travail consiste à évaluer les activités antioxydantes et inhibitrices de l'α-amylase d'extraits de *Cleome arabica L* collectés sur deux saisons (Janvier et Novembre 2015, Décembre 2016 et Mai 2017) dans la ville de Laghouat, région des steppes en Algérie. De plus, l'objectif de l'étude était d'étudier une nouvelle inhibition naturelle de l'α-amylase à l'aide d'un modèle in vitro, ainsi que de trouver un composé anti-diabétique naturel extrait de la plante. Les extraits ont été préparés avec deux systèmes de solvants: hydroalcoolique (80/20) et les systèmes de solvants acétoniques (70/30), Les extraits bruts hydrométhanoliques ont été obtenus par macération de onze échantillons de feuilles de *Cleome arabica* de mois de Novembre et Janvier et pour les différents organes de la plante pour le mois de Décembre ,tandis que, une ultrasonication a été faite pour les échantillons de mois de Mai , la teneur totale en composés phénoliques dans la fraction d'acétate d'éthyle de onze échantillons de feuilles de *Cleome arabica* de mois de Novembre et Janvier varie de 0,341 ± 0,009 à 0,751 ± 0,018 mg EAG /g de matière sèche et 0,170 ± 0,0005 à 0,490 ± 0,008 mg EAG/g pour la fraction de dichlorométhane, et entre 0,060 ± 0,004 à 0,453 ± 0,020 mg EAG /g pour la fraction de n-hexane. La teneur en flavonoïdes dans la fraction d'acétate d'éthyle varie de 0,172 ± 0,001 à 0,682 ± 0,003 mg EQ/g. Les résultats des tannins condensés dans la fraction d'acétate d'éthyle varie entre 0,172 ± 0,008 et 0,332 ± 0,007 mg EC /g. Nous concluons que l'acétate d'éthyle est le meilleur extracteur de flavonoïdes et de tanins condensés n'appartenant qu'à cette fraction.

Les composés phénoliques totaux des extraits hydrométhanoliques (80/20) et hydroacétoniques (70/30) présentent dans différents solvants de parties de *C.arabica L* (feuilles, gousses, graines, tige et racines) varie de 2,87 à 107,21 mgEAG / 100g au début de la maturité (Décembre) et varie entre 3,981 et 99,6 mgEAG / 100 g à la fin du stade de maturité (Mai), la fraction acétate d'éthyle s'est révélée plus riche en composés phénoliques et flavonoïdes totaux (107,21 mgEAG / 100 g et 88,50 mg EQ/ 100 g) respectivement dans l'extrait acétonique de feuilles au période de floraison , l'activité antioxydante a été évaluée à l'aide de différents tests: pouvoir réducteur de pouvoir ferrique (FRAP), DPPH, CUPRAC, ABTS et Phosphomolybdenum complex method, les capacités antioxydantes les plus puissants des extraits de feuilles de *Cleome arabica L* pour Novembre et Janvier ont été détectées par ABTS avec EC50 = 0.009mg/ml. Les complexes PM, ABTS, CUPRAC et FRAP étaient fortement corrélés positivement. Les valeurs de IC50 des extraits hydrométhanoliques et hydroacétoniques dans différentes fractions de parties de *Cleome arabica* comprisent entre 0,002 et 0,5 mg/ml, évaluées avec DPPH, l'activité anti α-amylase a été étudiée par des tests d'inhibition de l'α-amylase. L'extrait hydro-acétonique a montré la meilleure activité contre l'α-amylase par rapport à l'extrait hydro-méthanolique, les valeurs de la IC50 étaient très faibles dans l'acétate d'éthyle pour les gousses et les grains cités (IC50 = 0,11 mg/ml), (IC50 = 1,03mg/ml) respectivement, pour les fractions de n-hexane(IC50=0,6 mg/ml, IC50 = 1,08 mg/ml) respectivement pour les tiges et les graines aux floraisons.

Cette étude est le premier rapport sur l'inhibition potentielle de ces extraits de plantes sur l'enzyme digestive, l'α-amylase. Les résultats indiquent que *Cleome arabica* d'Algérie est un puissant antioxydant naturel et pourrait également fournir des agents biologiquement actifs naturels à utiliser dans la gestion du diabète.

Mots-clés : *Cleome arabica*, activité antioxydante, activité inhibitrice de α-amylase.

الملخص:

لقد جذبت *Cleome arabica L* اهتمامًا كبيرًا في السنوات الأخيرة بسبب أنشطتها المضادة للأكسدة ومضادات الكوليسترول في الدم ومضادات السرطان والوقاية الكيميائية لعلاج عدد من الأمراض, كانت الدراسة الرئيسية هي تقييم الأنشطة المثبطة لأنزيم الألفا أميلاز ومضادات الأكسدة لمستخلصات النبتة, لذا ركزنا كمرحلة أولية لهذا العمل الى إستخلاص المركبات الفينولية و تقدير كمياتها لإحدى عشر عينة من أوراق نبتة النتينة المحلية في الهيدروميثانول (80/20) لشهري يناير ونوفمبر2015 ,كما نقعت عينات كل من الأوراق,القرون,السيقان ,الجذور والبذور لشهر ديسمبر في كل من الهيدرو ميثانول (80/20) والهيدروأستون (70/30) بينما استخدمت الموجات فوق الصوتية في استخلاص المركبات الفينولية لشهر مايو 2017 لذات العينات للمذيبين.

أوضحت النتائج المتحصل عليها من تنقيع أوراق النبتة في محلول الهيدروميثانول (80/20) وجود كميات متفاوتة للفينولات في المستخلصات الثلاثة المتفاوتة القطبية خلال الشهرين يناير و نوفمبر حيث قدرت بـ : 0.0009 ± 0.34 الى 0.018 ± 0.751 mgEAG/g من المادة الجافة فيما يخص مستخلص الإثيل أسيتات , 0,0005 ± 0,170 إلى 0,008 ± 0,490 mg EAG/g لثنائي كلورالميثان و 0,060 ± 0,004 إلى 0,020 ± 0,453 mg EAG / g بالنسبة ل ن-هكسان .حيث قدر مستخلص الإثيل أسيتات الأكثر إستخراجية للفلافونيدات والعفصيات لإفتقار مستخلصات ثنائي كلور الميثان و ن . هكسان من هذه الأخيرة.كما أننا لم نلتمس الإختلاف في قدرة إستخلاص الفينولات لطريقتي الإستخلاص المعتمدة . ليظل مستخلص الإثيل أستات الأكثر إستخراجية للفينولات و الفلافونيدات (107.21 و 88.50 mg/ g 100 EAG) من المادة الجافة على الترتيب لمستخلصات الأستون في بداية نضج النبتة (ديسمبر) لتكتسب هذه الأخيرة قدرة معتبرة في مواجهة الجذور الحرة مقارنة بمضادات الأكسدة المرجعية التي قدرت ب 0.010 mg/ml بالنسبة ل DPPH و ABTS لشهري يناير و نوفمبر و 0.002 إلى 0.5 mg/ml بالنسبة ل DPPH لشهري ديسمبر و مايو لجميع أعضاء النبتة, كما سجلت علاقة إيجابية بين الطرق المستعملة لتقدير النشاط المضاد للأكسدة PM و FRAP, CUPRAC, ABTS فيما فسر ذلك أن للتقنيات المستعملة ذات نفس آليات التمخلب على عكس ما سجل بين هذه الأخيرة و كمية الفلافونيدات التي فسرت بعدم ارتباطها بصفة كبيرة بنشاط الأكسدة .

أوضحت النتائج المحصل عليها لمستخلص النتينة الهيدرو ميثانولي و الهيدروأسيتوني قدرة تثبيطية لإنزيم الألفا أميلاز جد فعالة خصت عينات بداية الإزهار(ديسمبر) و التي قدرت بـ: IC_{50} = 0.11 mg/ml و IC_{50} = 01.03 mg/ml لكل من القرون و البذور لمستخلص الإثيل أستيات على الترتيب, كما عرف مستخلص ن.هكسان النشاط ذاته بالنسبة للبذور والسيقان(IC50 =0,6 mg/ml, IC50 =1,08mg/ml)على الترتيب.

هذه الدراسة هي التقرير الأول عن التثبيط المحتمل لهذه المستخلصات النباتية على الإنزيم الهضمي ، α-amylase. تشير النتائج إلى أن *Cleome arabica* الجزائر من مضادات الأكسدة الطبيعية القوية ويمكن أن يوفر أيضًا عوامل نشطة بيولوجيا لإستخدامها في علاج مرض السكري

الكلمات المفتاحية :

النتينة *Cleome arabica* – نشاط مضاد للأكسدة – ضد الألفا أميلاز.

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EC	Enzyme commission
T2D	TypeII diabete
PM	Phosphomolybdenum complex
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
FRAP	Ferric ion reducing antioxidant power
CUPRAC	Cupric ion reducing antioxidant capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
BHT	Butylhydroxytoluène
TBHQ	Tert-butylhydroquinone
DM	Dry mattre
r	Correlation coefficient
GAE	Gallic Acid Equivallent
VCE	Vitamin C equivallent
QE	Quercetin equivallent
CE	Catechin equivalents
R^2	Adjusted relative error
LC-MS	Liquid chromatography-mass spectrometry
EC50	Effective Concentration of Half-Maximal Response
IC50	Inhibitory Concentration of Half-Maximal Response
Abs	Absorbance
I%	Inhibition rate
ND	Note determined
nm	Nanometer
h	Hour
UV-Vis	Ultra violet-Visible
pH	Potential of Hydrogen
µL	Microliter
TEAC	Trolox Equivalent Antioxidant Capacity
SSF	Solid-State Fermentation
SMF	Submerged Fermentation

Summary

Summary

PART II: EXPERIMENTAL SECTION AND RESULTS

Summary

GENERAL INTRODUCTION

According to conservative estimates, there are on earth about 400,000 secondary plant metabolites in1998, to more than 2.140.000 by 2014, and about 30,000 of them have been chemically isolated [1][2]. Scientific papers encourage researchers and clinicians to work hard in order to achieve three main goals: namely, to clarify the main active ingredients which can be extracted from medicinal plants; more purposefully, to clarify their role in the treatment of present diseases, and to find out to what extent they can be used to produce or synthesize more effective drugs.

Our laboratory has an objective of applying modern techniques of extraction and identification of bioactive elements from medicinal plants, holding the belief that the treatment of many diseases lay in nature. It has an active presence of more than twenty years in characterizing the physico-chemical properties of plants (mainly grown in the Algerian steppe region), and in trying to identify their potential in different life domains such as : medicine [3], biochemistry [4][5] and even in corrosion for the protection of mild and X71steel[6][7].

***C**leome arabica* belongs to the family of Capparaceae which has several species widely grown in tropical and subtropical regions of the world, more specifically in the steppe climate of North Africa. Also known as *Spider flower, it is an* adapted species in the desert areas of Algeria. The richness of this plant, locally called *"Netteina"*, lies in its various metabolites, and leads to a significant variation in phenolic compounds between plant organs. Leaves, pods, stems, roots and seeds. This variation is noticeable for phenolic acids flavonoids, terpenoids and alkaloids which are also known to have antioxidant and inhibitory proprieties involved in several diseases.

***C**leome arabica*, is among the plants that have received wide attention from our laboratory in the last decade. The first investigation was part of a screening study with 19 other plants. The second work resulted in the isolation of a new steroid compound [8]. Other works in different labs have been carried out later in order to study its effect in the treatment of abdominal and rheumatic pains [9], chemoprotective of its phenolic extract related research [10], which covers a number of glucosylated and rhamnosylated flavonols [11]. new dammarane triterpene were isolated by A .LADHARI, [12], for their antihypercholisterolemic effect [13] and finally, for the anti-cancer effects of *Cleome arabica* leaves extract by C .TIGRINE ,in 2018[14].

Life expectancy of the population increases along with individuals living unhealthy lifestyles. So too, does the prevalence of age-related diseases causing the most deaths worldwide such as Type II Diabetes (T2D)

and Alzheimer's disease (AD). Diabetes is a metabolic disorder that affects more than 425 million people worldwide in 2017[15]. This disease occurs due to absolute or relative deficiency of insulin secretion with or without varying degrees of insulin resistance. In many respects, Alzheimer's is a brain form of diabetes [16]. Even in the earliest stages of the disease, the brain's ability to metabolize sugar is reduced. Normally, insulin plays a significant role in helping the brain take up sugar from the blood. Consequently, the brain cells practically starve to death; patients are required to control their blood glucose with medications or by an exercise program of various enzymes in the digestive tract. Pancreas α-amylase (E.C.3.2.1.1) digests carbohydrates to glucose, Retardation of carbohydrate digestion by inhibition of enzymes such as α-amylase would lead to blood glucose level reduction and hence could be considered as a therapeutic strategy for the treatment of diabetes. α-amylase inhibitors therefore have a therapeutic role as one group of drugs introduced in the management of diabetes. α-amylase inhibitors are currently in clinical use such as acarbose and miglitol which prevent the digestion of carbohydrates and provide short-term glycemic control. The drawback of such inhibitors is their non-specificity in targeting different glycosidases. Also, these inhibitors are known to produce serious side effects that limit their use as a therapeutic drug. Therefore, natural extracts from available traditional medicinal plants are an important area of investigation with great potential for discovery of new antidiabetic drugs. Different plants have been reported to show α-amylase inhibitory effect and so may be relevant to the treatment of diabetes. More than 400 traditional plants are used for their hypoglycaemic properties by populations of diabetics throughout the world [17]. Harmless natural amylase and glucosidase inhibitors have been reported from plant sources. In several cases, the use of the plants and their respective therapeutic prescription in popular medicine are not easy to understand, and we can question if there is a relation between the therapeutic properties of these plants and their inhibitory effects on α-amylase. The phenolic compounds present in these plants may also serve as lead compounds for the synthesis of a series of inhibitors.

Coinciding with the emergence of these diseases, in the 20th century, the biological and medical scientists have been convinced by a new concept of **oxidative stress** that is a situation where the cell no longer controls the excessive presence of toxic oxygen radicals, a situation which researchers implicate in most human diseases [18].

To consider a natural compound or a drug as an antioxidant substance, it is necessary to investigate its antioxidant properties *in vitro* and then to evaluate its antioxidant functions in biological systems. The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but they may be also

influenced by other organic and inorganic compounds such as coumarins, phenolic acids and antioxidant micronutrients, e.g., Cu, Mn, Zn [19]. Phenolic compounds are a class of naturally occurring pigments with ubiquitous distribution in the plant Kingdom [20]. They display a remarkable biological property. Which may have a significant role in the decrease of oxidative stress, but the choice of plant organ and the optimal harvesting time is necessary for the production of plant active products with high medicinal value. **The L**aboratory of **F**undamental **S**ciences "LFS "is engaged in the study of more than 20 Traditional Algerian plants used in diabetes therapy as strong inhibitors of α-Amylase activity [21]. The interest of our group in various medicinal plants that grow in Algeria and their effects against α-amylase [22] oriented us to deepen the study on the *Cleome arabica* plant still further, by introducing other factors which may give better and reliable results.

This study has three parts: the first part is a bibliographical research consisting of three chapters. **(Chapter I.1)** treats the quantification of total phenolic, flavonoids and tannin contents of *Cleome arabica* leaves extract in different season (November and January,2015) as long as we can select the season where *Cleome arabica* is at its highest production of phenolic compounds. The antioxidants are known as compounds that inhibit or suspend the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [23]. Moreover, they have the capacity to keep the living cells from oxidative harm that happens due to the formation of free radicals and reactive oxygen species during most of the metabolic activity. Taking into account the complexity of the oxidation processes, there is no single method of reflecting the antioxidant profile of a sample. Therefore, the response obtained by means of different and complementary tests must be combined. For such end, our choice has been on the use of the five chemical tests (ABTS, FRAP: ferric reducing antioxidant power, DPPH , Phosphomolybdenum complex , and CUPric Reducing Antioxidant Capacity (**CUPRAC**) used for the first time to evaluate the antioxidant activity of *C.arabica L* extracts are summarized in **Chapter I.2**. We used the results to identify the correlation between them in different seasons and compared with those of butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and ascorbic acid (VC) which have been extensively used as standard antioxidants in the experiments. Different organs of *Cleome arabica* (leaves, pods, stems, roots and seeds) were studied on their inhibitory effect on α-amylase in two seasons (December,2016 and May,2017) in different extraction systems summarized in **Chapter I.3.** To the best of our knowledge, the inhibitory effect of *Cleome arabica* against alpha-amylase has never been investigated.

The second part concerns the experimental section, which has treated the three chapters previously cited, included all the used materials and chemicals products as well as the methodology of all assays throughout the study. Statistical analysis that was used for analysis of raw data is illustrated in this part. Results followed by a discussion of all methods used in this study are summarized in the third part.

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PART I.

BIBLIOGRAFIC SECTION

I.1: An Overview About Medicinal Plants

I.1.1. Medicinal Plants:

The term of medicinal plants include numerous types of plants used in herbalism and some of these plants have a medicinal activities [1]. They are considered as a rich source of ingredients which can be used in drug development and synthesis, used by 80% of the world population as the only available medicines especially in developing countries [2]. Commonly consider that herbal drugs are cheaper and safer as compared to synthetic drugs and may be used without or minimum side effects [2]3]. Some of these plants are considered as important source of nutrition and clinical microbiologists have great interest in screening of medicinal plants for new therapeutics. These plants comprise ginger, green tea, walnuts and some others plants[1]. The active principles of many drugs found in plants are secondary metabolites [4].

I.1.1.1. *Cleome arabica L:*

The flora of the Sahara has about 500 taxa of higher plants [5], part of which remains today as used by indigenous medicinal plants. It is an annual herb in the family of Capparidaceae. called "crucifères des pays chauds". This northern species grows in the Maghreb and the regions Saharan, common in the Hodna (M'sila) and in some parts of the Sahara Algerian: *C. arabica* [5] . The plant kingdom is under constant attack by herbivores, for this, and to protect against intermittent attacks of herbivores, various morphological, anatomical and physiological adaptations are recognized and was able to tolerate well osmotically and saline-stressful habitats of the arid ecosystems during germination stage. The ability possessed by plants to protect themselves against natural enemies has been reviewed in detail for centuries in order to be exploited for agronomic purposes [6].

a. Systematic Position:

Abbey .H et al, (1963) [7], Ozenda (1991)[5], and Chweya Menzava (1997) [8] and Judd et al (2002) [9] classify *Cleome arabica* as follows:

Table I.1: systematic classification of *cleome arabica*

Branch	Spermaphytes	Sub-order	Capparidineae
Sub- branch	Angiosperms	Family	Capparidaceae
Class	Dicotylédons	Subfamily	Capparidoideæ Tribe Cleomoideae
Sub-class	Dillenidae	Genre	Cleome
Ordre	Capparales	Species	Cleome arabica

b. Botanical Description:

The name Cleome comes from the Greek 'Kleio' which means circle. It is a perennial plant of 30 cm in height, with erect and branched stems, *C. arabica* has small hairy leaves, trifoliate to lanceolate leaflets. The flowers have petals that range in color from yellow to dark purple. The fruit is a hairy pod 2 to 5 cm in length (**Figure I.1**) at the base of the petiole. It is a plant odor fetid, toxic and has hallucinogenic effects[10].The stripes glands secrete a viscous substance [11] .

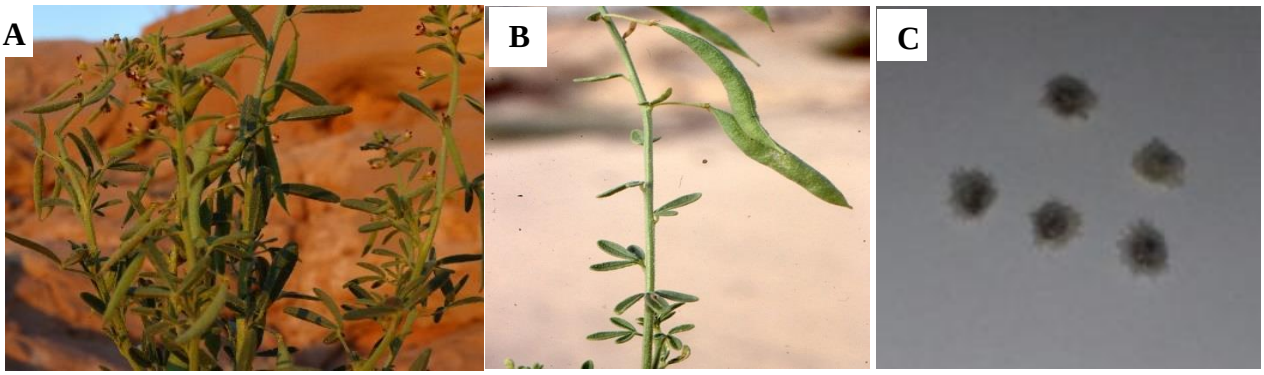


Figure I.1: *Cleome arabica* collected from Dayat-Elhamam, Sidi Makhlouf, Laghouat, Algeria (October 2015).

A: Aerial part, B: pods, C: seeds

c. Geographical Distribution:

This species is common in deserts and savannahs of tropical tamariçaies floor, up in the lower Mediterranean level on stony slopes and in the sandy gullies. This is a common species throughout the northern Sahara, Egypt and tropical Africa [5][12]. (**Figure I.2).**

In the Saharan region, *C. arabica* is found on rocks, sand and gravel. In Tripolitan it is very common in the Saharan part reaches to the coast, in Tunisia, it is common in the South, reached Ain Cherchira, Kerkennah islands to the north and Djerba islands. In Algeria, the plant is common in the basin of Hodna and fairly common in the Saharan Atlas. In Morocco, it is common in the Saharan Atlas and the northern Sahara.

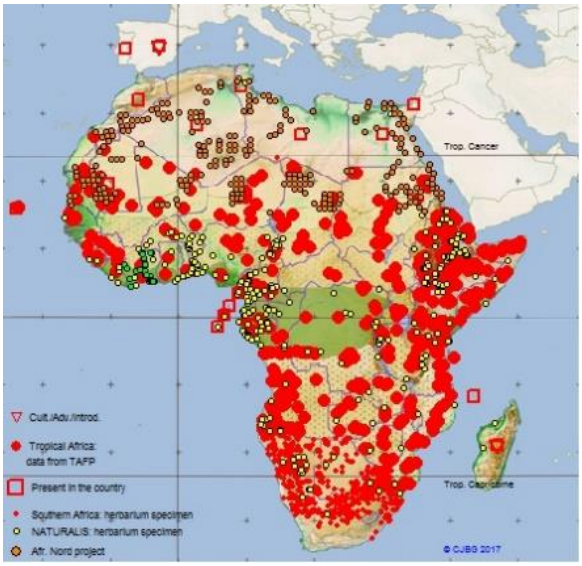


Figure I.2: Distribution of *Cleome arabica* in the Africa (2012) [13].

d. Ecology:

Germination is a critical stage in the life cycle of plants and tends to be highly changeable over space and time. Several environmental factors such as light, salinity, temperature and soil moisture simultaneously influence germination [14] and initial establishment of plants in arid and semi-arid regions[15]. Under such conditions, seed germination and seedling appearance are often limited by temperature when moisture conditions are favorable [16]. Temperature changes may affect a number of processes controlling seed germinability, including membrane permeability and the activity of membrane-bound and cytosolic enzymes[17]. Another crucial factor determining germination and seedling growth is soil moisture and, therefore, plays an important role in determining the distribution designs of species [18]. Although higher osmotically- and saline-stressful habitats of the arid environments decrease germination (Figure I.3).

Since it is known that plants can self-regulate their densities and distribution in nature via allelopathic interactions[19], in general, annual species need to control their germination closely to minimize the risk of local extinction caused by germinating under unfavorable conditions.

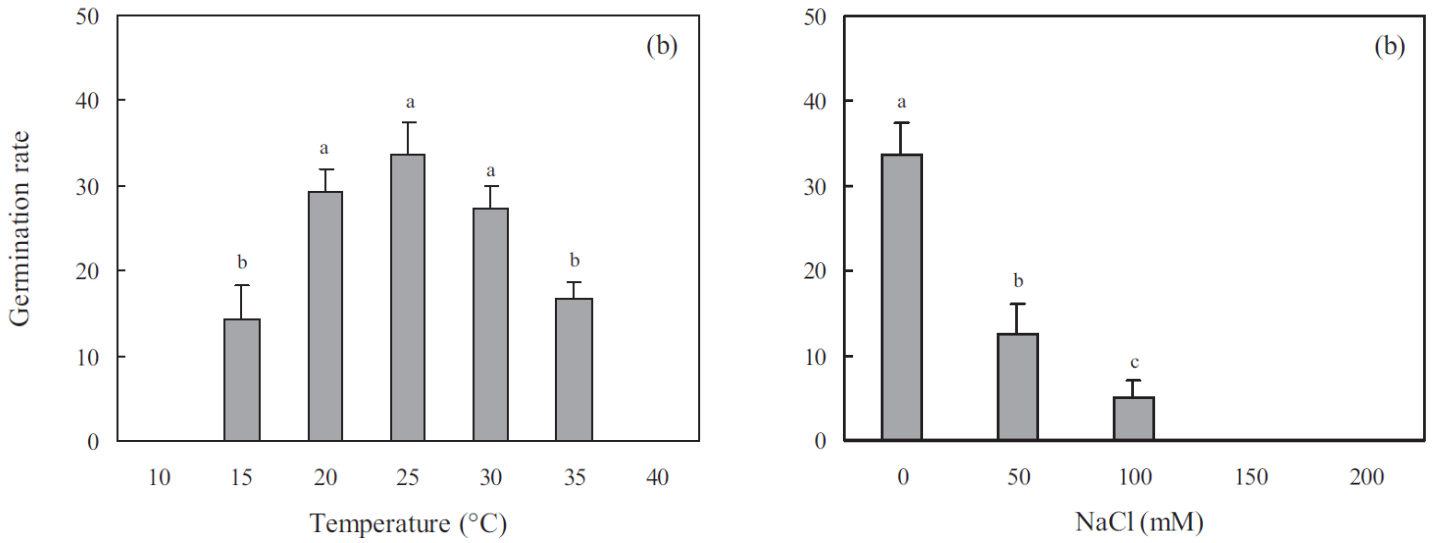
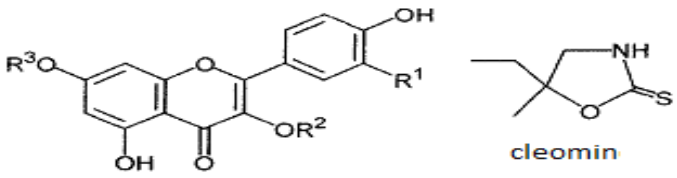


Figure I.3: Effect of salinity and temperature on seed germination of *Cleome arabica* [20].

e. Socio-Economic Position:

In Hoggar dried *Cleome arabica* leaves or powder are added to food as a diuretic for the treatment of rheumatism, in traditional medicine, *C.embylocarpa* is used as a sedative and for pain relief when associated with *Juniperus phoenicia*, with *Hammada scoparium* for headaches and with *Artimisia herba alba* as gastric, colic, and influenza and vomiting treatment [21]. Anti-inflammatory effect [22], or to cause perspiration [23]. The inhabitants of the region Boussaâda use the leaves of *Cleome arabica* in poultices for external application on the skin, to treat certain rheumatic [24] . They heat the dried leaves of the plant in a water bath and squeeze it to remove excess liquid, cover the painful part, previously lubricated with olive oil, with the still hot plant. Leave for 2-3 hours, the poultice is renewed several times. In Mauritania, women add the powder of the whole plant to milk and drinks, to get a fattening food. Similarly, they mix the leaves with grilled food to treat kidney and back disorders and give virility to men [23]. The leaves and roots of some species of the genus Cleome, such as *C. Rosea L.*, *C. viscosa L.*, *C. gynandra L.* and *C. L. africana*, are used in several regions of the world against the traditional pharmacopoeia diarrhea. They have anti-inflammatory, antimicrobial, anti-arthritic, anti-proliferative, anti-oxidant, anti-neoplastic effects. The aqueous extract of *C. viscosa L* is used as an analgesic, antipyretic and as hypoglycemic[34-37].Various side groups of compounds which triterpenes, the anthroquinones, flavonoids, saponins, steroids, resins, lectins, glycosides, tannins and other phenolic compounds, and alkaloids have been isolated from Capparidaceae including species of the genus Cleome[26]. In the Algerian Sahara, this plant does not present pastoral interest because it is not grazed by camels and little appreciated by goats and sheep [12].

The Cleome genus contains 601 species from the family of Cleomaceae [27] of which 7 are only used in traditional medicine[28], Nine known flavonol glycosides and cleomin have been isolated from the leaves and twigs of *C.arabica* [29] (**Figure.I.4).** *Cleome arabica* species is a well-known medicinal plant with traditional and pharmacological importance is studied in this work.



	R^1	R^2	R^3
1	OH	Glucosyl	Rhamnosyl
2	H	Glucosyl	Rhamnosyl
3	OCH_3	Glucosyl	Rhamnosyl
4	OH	Rhamnosyl	Rhamnosyl
5	H	Rhamnosyl	Rhamnosyl
6	OCH_3	Rhamnosyl	Rhamnosyl
7	OH	Glucosyl	H
8	H	Glucosyl	H
9	OCH_3	Glucosyl	H

Figure I.4: The Constituents of Leaves and Stems of *Cleome arabica* [29].

f. Biological Activities:

The biological activities of *Cleome arabica* are little studied. According to (Selloum and bourich ,2003) [22], a yellow precipitate obtained from the leaves of this plant, using the extraction system methanol/water followed by ethyl acetate, has had a remarkable anti-inflammatory effect in vivo, reducing paw edema in rats, and in vitro by modulating the activity of the lipoxygenase and leukotriene B4 generation and prostaglandin E2 polymorphonuclears' by neutrophils stimulated with calcium ionophore.Thus, by inhibiting their chemotaxis. Similarly, Selloum and colleagues (2004) [30] showed that this precipitate inhibits respiratory flamed stimulated neutrophils detected by chemiluminescence. The family of Capparidaceae is rich in flavonoids. They are present in several plants Cléome kind including *Cleome arabica*, *Cleome spinosa, Cléome amphicarpa, Cléome brachycarpa, Cleome chrysantha* and *Cléome droserifolia* [40,43].

I.1.2. Phenolic Compound:

More than 8000 molecules produced in plants are identified which three main classes: phenolic compounds, terpenoids, and alkaloids[44,45]. These molecules have different roles in the plant, noting the

climatic aggressions; biotic stress (pathogens, injuries, symbiosis) or abiotics (light, UV radiation, low temperature, deficiencies). They also contribute to the organoleptic quality of foods derived from plants (color, astringency, aroma, bitterness) [34].

A. Polyphenols in Plants: **Location and Interest:**

At the cell level, phenolic compounds are mainly distributed in two compartments: the vacuoles and the wall. In the vacuoles, the polyphenols are conjugated, with sugars or organic acids, which allows to increase their solubility and limit their toxicity to the cell. At the level of the wall, we find mainly lignin and related flavonoids to parietal structures. Phenolic compounds are synthesized in the cytosol. Part of the enzymes involved in the biosynthesis of phenylpropanoids is linked to the membranes of the endoplasmic reticulum, where they are organized into metabolons [35]. At the tissue level, the localization of polyphenols is related to their role in the plant and can be very characteristic. Within the sheets, the distribution of compounds is variable, for example anthocyanins and flavonoids are mostly present in the epidermis.

At the level of the whole plant, it should be noted that some compounds are not accumulated only in well-defined organs. In apple, for example, phenolic compounds are involved in the colouring of the skin via the anthocyanins, and in the organoleptic quality of the flesh, especially for bitterness or astringency [36].

Phenolic compounds play an important role in the metabolism of plant but also can react in plant interactions with their biological and physical environment (relationships with bacteria, fungi, insects, UV resistance). All categories of phenolic compounds are involved in resistance mechanisms ,They provide communication between cells, between plants, between plants and animals [37].

B. **Stability of Plant Phenolic Compounds:**

Phenolic compounds are secondary metabolites found in cereals, coffee beans, fruits, olives, vegetables, and tea leaves. A study on the stability of structurally different phenolic compounds in buffers in the pH range 3–11 revealed that caffeic, chlorogenic and gallic acid were not stable to high pH.

By contrast, catechin, epigallocatechin, ferulic acid, rutin, and cinnamic acid resisted pH-induced degradation. The results suggest that if a specific phenolic compound is found to be unstable under food-processing conditions, it may not be effective as an antioxidant, anticarcinogen, or antibiotic when present in foods subjected to heat or high pH.

I.1.2.1. Classification:

In the table **I.2**, Harborne and Simmonds (1964)[38] have classified these compounds in groups according to the number of carbon atoms in the molecule.

Table I.2: Classification of phenolic compounds

Structure	Class
C_6	Simple phenols
C_6-C_1	Phenolic acid and derived compounds
C_6-C_2	Acetophenone et phenylacetic acids
C_6-C_3	Cinnamic acid ,coumarin,isocoumarin ,chromones
C_{15}	Flavanols,flavonones,anthocyanines and anthocyanidin
C_{30}	biflavonyles
C_6-C_1-C_6,C_6-C_1-C_6	Benzophenone,xanthones and stelbene
C_6,C_{10},C_{14}	quinons
C_{18}	betacyanins
Lignin,neolignanes	Dimers or oligomers
lignin	polymers
Tanins	condensed and hydrolyzable

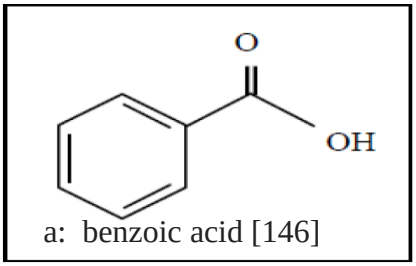
And on the other hand, on the basic skeleton structure, main classes are widespread [39] .

a. Monomeric Polyphenols:

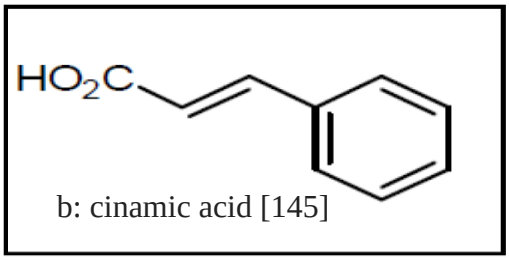
Phenolic acids:

Phenolic acids, have an acid and several phenol functions, They are colorless and rather rare in nature [40]. They are divided into two classes: benzoic acid derivatives (hydroxycinnamic acids) and derivatives of cinnamic acid (hydroxybenzoic acids) [41]. Phenolic acids are derived from benzoic acid are hydroxybenzoic and have a basic general structure of type (C6-C1) (Figure I.5a), these molecules often exist in the form of esters or glycosides [42] . The most common are salicylic acid and gallic acid [43].

Phenolic acid derived from cinnamic acid (Figure I.5b) are often esterified. The most common are cinnamic acid, caffeic acid, ferulic acid, p-coumaric acid and synaptic acid [40].



a: benzoic acid [146]



b: cinamic acid [145]

Figure I.5a: phenolic acid derived from benzoic acid

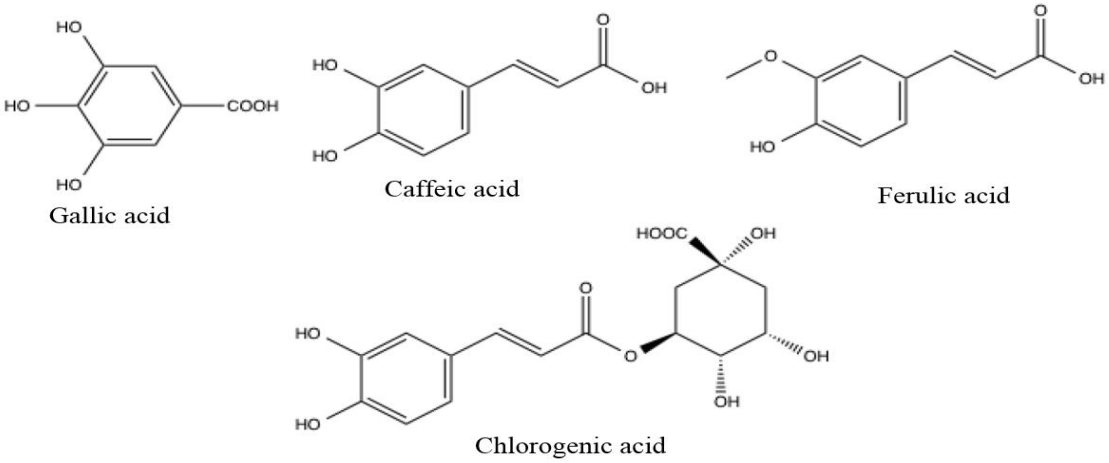


Figure I.5b: phenolic acid derived from cinnamic acid

Flavonoids:

With over 9000 natural compounds identified in nature, the flavonoid family is the largest group of polyphenolic compounds [44] that have been recorded which represent the third largest group of natural products following the alkaloids (12,000) and terpenoids (30,000)[45].

Chemical Structure and Classification of Flavonoids:

Flavonoids are characterized by a phenylbenzopyran chemical structure. The general structure includes a C15 (C6-C3-C6) skeleton joined to a chroman ring (benzopyran moiety). The heterocyclic benzopyran ring is known as the **C** ring, the fused aromatic ring as the **A** ring, and the phenyl constituent as the **B** ring (**FigureI.6**).

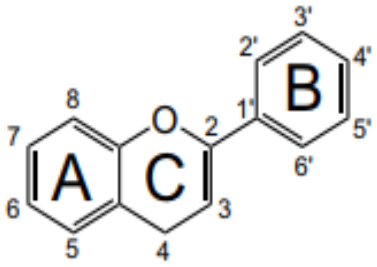


Figure I.6: Basic flavonoid skeleton structure with conventional numbering of carbons and naming of rings.

According to the position of the aromatic ring (A&B) to the benzopyrane, flavonoids can be grouped in four classes: major **flavonoids** (2-phenylbenzopyrans), **isoflavonoids** (3-benzopyrans), **neoflavonoids** (4-benzopyranes) and **minor flavonoids**. More than 5,000 different flavonoids have been identified [13] and they are divided into the following six main subclasses of flavonoids: flavonols, isoflavones, flavanols, flavanones, flavones, and anthocyanidins [58- 59]. These subclasses show distinct saturation and oxygenation of the C-ring, as well as positioning of the B-ring. Most flavonoid subclasses are diversely distributed throughout the plant kingdom and flavonoids often occur in several different food sources.

Distribution and Location:

Flavonoids are essential constituents of plants, they are distributed in all parts of the plant such as leaves, roots, stems, flowers, seeds and bark. They predominate, especially at the youth level, aerial organs as young leaves and flower buds [48]. Flavonoids are widely distributed among bryophytes, pteridophytes, chlorophytes and angiosperms [49].The forms heteroside water soluble flavonoids accumulate in vacuoles and depending on the species, are concentrated in the epidermis of the leaves or fall between the epidermis and the mesophilic. In the case of flowers, they are concentrated in the epidermal cells[50]. Therefore, vegetables and fruits consumed daily are rich in flavonoids. Similarly, they are found in large quantities in many medicinal and aromatic plant [51].

Bioavailability and Metabolism of Flavonoids:

The beneficial effects of flavonoids on the health of living beings depend not only their consumption levels but also their bioavailability, for better knowledge of it,it is essential to explain their protective effects against many diseases such as cardiovascular, neurodegenerative diseases, cancers [52]. Indeed, studies of Vanessa et al. (2003) [53] have shown that the bioavailability of flavonoids depends on the transmission capacity through the enterocytes, the intensity of intestinal secretion flavonoids conjugated to the intestinal lumen and into the blood and then of the biliary secretion. Most flavonoids enter the feed in the form of glycosides, most of which are consumed rutin and quercetin [54], Brown (1980) [55]showed that glycosides of quercetin does not absorb at the small intestine, because of their hydrophilic nature and relatively high molecular weight. However, [68-69] showed a rapid absorption glycosides of quercetin compared with flavonoid aglycones in humans. According to these authors, this is the glycosidic part which facilitated the passage of the flavonoid through the intestinal wall via the route of absorption of sugars. Day & Williamson (1999) [58] reported that the speed in the absorption of glycosides depends on the chemical nature of their sugars. They mentioned that the glycosides onion (quercetin-4'-glucoside and quercetin 3, 4'-diglucoside) are better absorbed glycosides apples, tea and even supplements rutin. They also showed that after the absorption of these glycosides and their passage through the enterocytes, they undergo deglycosylation via β-glucosidase cytosolic and via phlorizin hydrolase lactase. Then they are converted into glucuronides or sulfates. This phenomenon has been shown to phloridzin [59], the luteolin 7-O-glucoside [60], the glycoside quercetin [61] and kaempferol 3-O-glucoside[62]. Similarly, it has been proved the existence of quercetin 3-O-rhamnoglucoside.

In addition, a study on the pharmacokinetics of flavonoids in humans, using diosmin, 7- rhamnoglucoside of diosmetin, allowing the detection of the single diosmetin in plasma with a complete absence in the urine [63]. The remaining flavonoids that are not absorbed from the small intestine, pass into the colon where they undergo deglycosylation via β-glucosidase secreted by the intestinal flora [64]. Similarly, this bacterial flora can cut cycle pyrone ring C, producing phenylacetic acid and phenylpropionic acid, and other derivatives[55] ,The metabolites may be obtained for many. They can be returned to the intestinal lumen, as they can pass into the liver to undergo other changes. Finally, they secrete in the gallbladder or in the blood to be eliminated in the urine [58].

Biological and Pharmacological Properties of Flavonoids:

Phenolic compounds particularly flavonoids that are metabolites synthesized in large amounts by plants represent a defense system against pathogens microorganisms [50]. Similarly, flavonoids protect plants against UV radiation by absorbing both these radiations and neutralizing reactive oxygen species formed [65]. In humans, Flavonoids are powerful bioactive molecules and they are obstructive leads for cancer treatment due to their ability to induce apoptosis. Cytochrome P450, family 3, subfamilyA **(**CYP3A4), which is the most plentiful enzyme in the liver and beneficial in metabolizing a significant number of carcinogens and medications. Several studies revealed that Kaempferol, Quercetin , Apigenin, Naringin Quercetin and naringin can inhibit CYP3A [66].Flavonoids possess remarkable biological activities pharmacological which are summarized in (Table I.3).

Table. I. 3: Biological activities and pharmacological flavonoids in humans

Biological activities of phenolic compound		**References**
Antioxidant activity		[79-80]
Anti-cancer activity		[81-85]
Anti-ulcerative activity		[69]
Anti-inflammatory activity, antihyperglycaemic and hepatoprotective act antimicrobial activity		[22],[70][71]
Analgesic activity , antipyretic and antidiabetic activities		[72]
Anti-parasitic activity	Leishmania	[73]
	trypanosoma	[74],[75]
	Plasmodium folciparum	[76]
Antivirals activity		[77]
Antibacterial activity		[99-100]

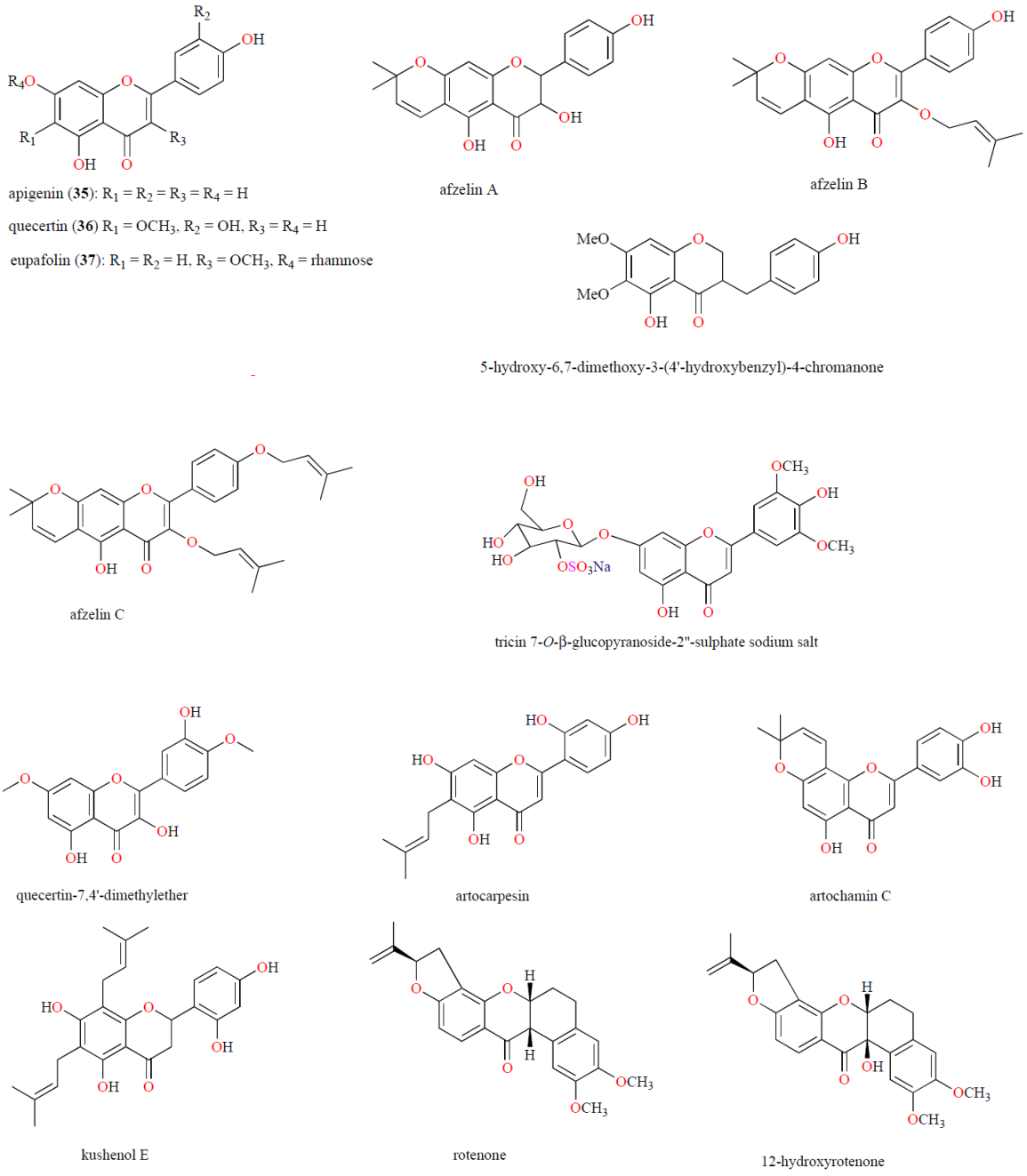


Figure.I.7: Flavonoids with cytotoxic activity, derived from plants used in Africa traditional medicine [80]

b. Polyphenols in The Form of Polymers:

Tannins:

Tannins are polyphenolic secondary metabolites of higher plants and are either galloyl esters and their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol-, catechin- and triterpenoid cores (gallotannins, ellagitannins and complex tannins), or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (condensed tannins)[81].

Classification:

1. Hydrolysable tannins:

These are based on esters of phenol carboxylic acids (gallic acid) with a central carbohydrate core for example: - gallotannins (gallic acid, quinic acid, tannic acid) - ellagitannins (ellagic acid, castalagin, vescalagin, etc.) - hydrolysable tannin oligomers (agrimoniin, rugosin D) - caffeic acid derivatives (chlorogenic acid, caffeetannin, dicaffeoylquinic acid, rosmarinic acid).

2. Condensed tannins:

Structurally related to flavonoids, these tannins are distributed widely in nature and constitute a heterogeneous group, Condensed tannins are chemically oligomers group of polyhydroxyflavan-3-o1 and polyhydroxyflavan 3,4-diols (leucoanthocyanidin) or oligomers of a combination of those two compounds. The basic flavonoid structure in condensed tannins [82].

3. Complex tannins:

The complex tannins are a series of compounds found to occur widely in plants containing both hydrolysable and non-hydrolysable or condensed tannins. Complex tannins are shown to contain a hydrolysable tannin moiety in their molecules connected through a carbon-carbon linkage to flavan-3-ol (flavano-ellagitannin), procyanidin (procyanidinoellagitannin) and flavonoid glucoside (flavono-ellagitannin) moieties for example : (stenophyllanin A, acutissimin B, mongolicain A, stenophynin A, etc.). It enhances structure, aids color stabilization and provides antioxidant protection. It is less reactive and more polymerized than some other tannins.

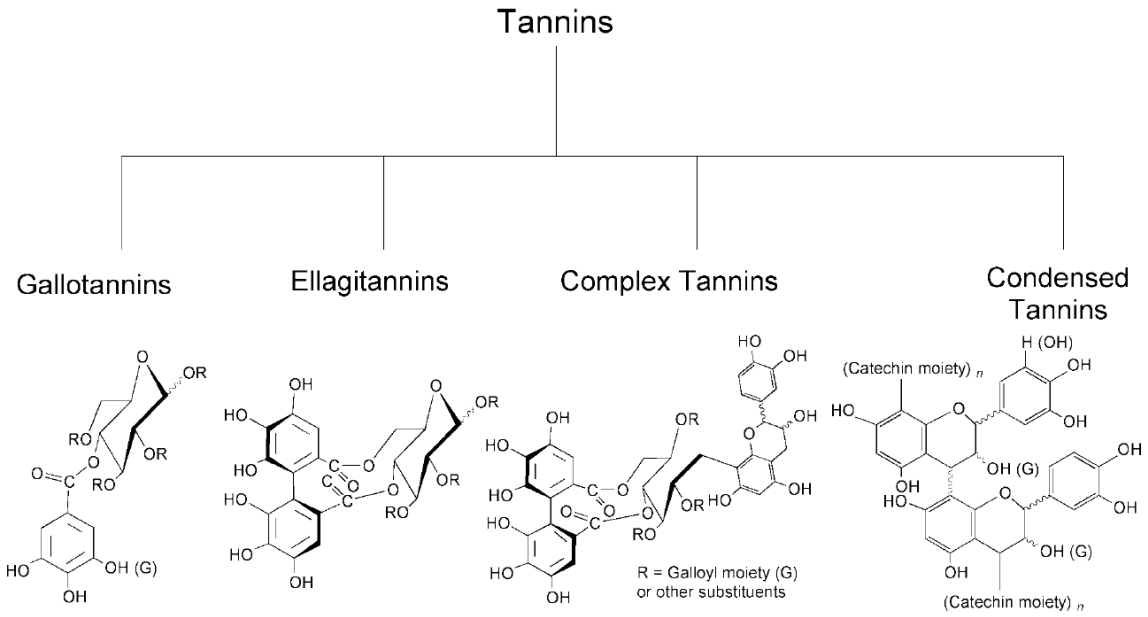


Figure I.8. Classification of the tannins

Biological and Pharmacological Properties of Tannins:

Recently the tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as AIDS and various cancers[83]. The search for new compounds lead for the development of novel pharmaceuticals has become increasingly important, especially as the biological action of tannin-containing plant extracts has been well documented. During the last twenty years many representatives of this class of compounds have been isolated and characterized, currently known tannins with unambiguously determined structures already number far more than 1000 natural products. In extensive biological tests many representatives of this class were found to have antiviral, antibacterial, and especially, antitumor activity. For example, certain tannins can selectively inhibit HIV replication [81].

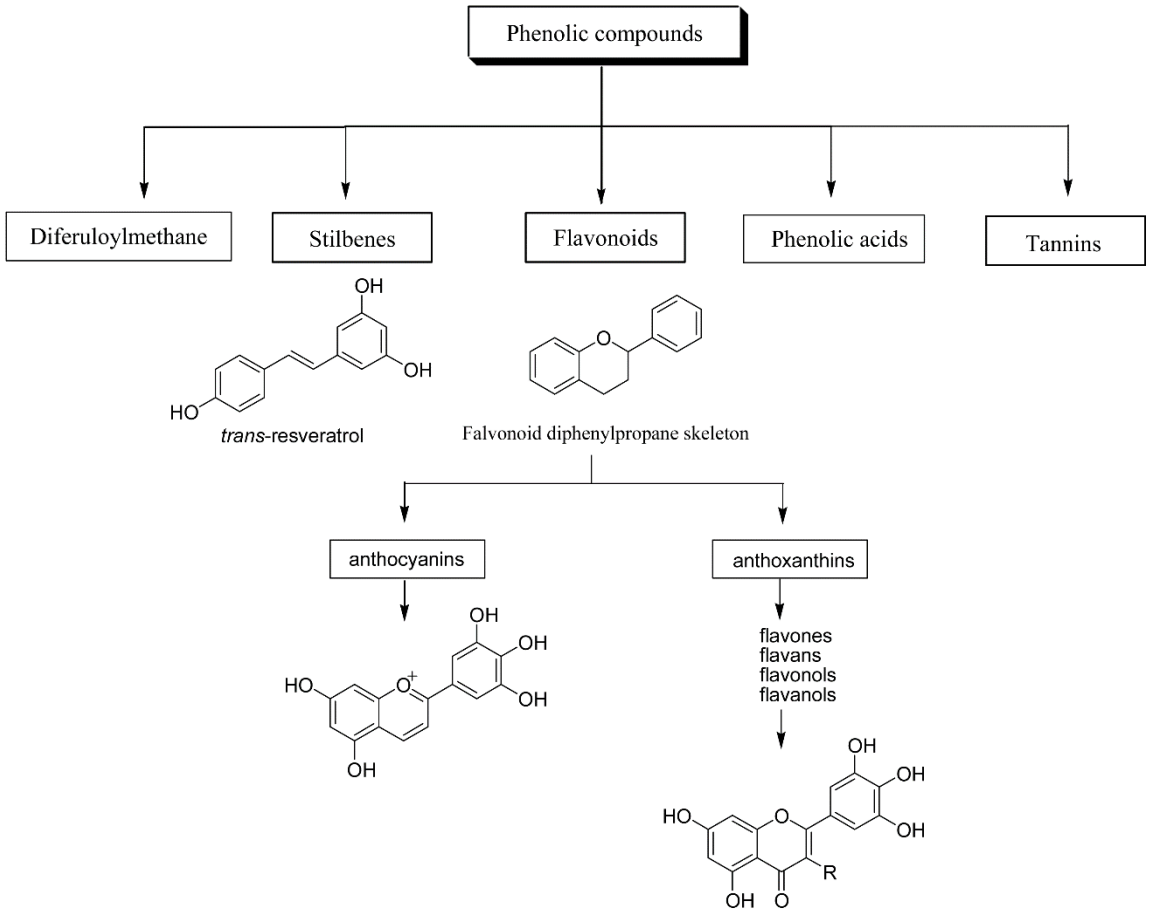


Figure I.9. Classification of dietary polyphenols

In general, phenolic compounds are poorly absorbed in the small intestine, it is estimated that 90-95% of dietary phenolics accumulate in the colon [84].

A detailed study in this chapter is performed on the statistical quantification of the total phenolic, flavonoid and tannins content of hydro-methanolic extract of eleven samples of *Cleome arabica* leaves collected in distance of five meter between one to other of *Cleome arabica* ssp. in different seasons (November, January,2015) in order to find or select the season where the plant is at peak production of phenolic compound and their relation with antioxidant activity in various seasons and enzyme inhibition study in current chapter .

I.1.2.2. Phenolic Compounds Analysis Methods:

The separation, the detection, the identification and the quantification of the molecules are carried out by several analytical and especially chromatographic methods.

Actually, chromatographic methods coupled to various detectors occupy the most important place in the molecular screening because they make it possible to simultaneously characterize a very wide range of molecules with a better specificity and a greater sensitivity compared to the old methods.

They are applicable to all biological media, whatever their nature, after an extraction step made necessary because of the complexity of these matrices.

The physico-chemical properties of the analytes condition the detection by chromatography, which explains why there is no chromatographic method which allows the detection of all the substances and that a good strategy imposes the disposition of several colorimetric, spectrophotometric methods, and complementary chromatography.

Table I.4. presents the different spectrometric methods and chromatographs, mentioning the advantages and limitations of each method (MADI, A.2018).

Table I.4.Methods and Chromatographies, The Advantages and Disadvantages

Methods	**Advantages**	**Disadvantages**
Colorimetric	Simple, fast, available, direct.	Lack of specificity and sensitivity, subjectivity of interpretation. need a white and a positive to each test.
Spectrophotometric	Quantification and / or spectral identification simple and fast for certain compounds.	Lack of specificity and sensitivity, separation necessary preliminary
TLC (Thin layer chromatography)	Simple material, available, inexpensive, separative method non-destructive robust, allows the identification of hundreds of molecules and their metabolites; facilitates specific tests identification and allows the collection of fractions after separation, the new methods are semi-quantitative, Automatable and give a better identification	Relatively long to achieve; prior extraction of necessary analytes. lack of sensitivity and resolution, influence of local conditions on results, difficult interpretation and delicate in the presence of many metabolites.
LC-MS	Rapid identification and quantification of a very large number of even polar and thermolabile compounds, great sensitivity and specificity, low sample volume	High cost, lack of standardization of spectrum libraries mass.

I.2: Evaluation of the Antioxidant Potency of Phenolic Extracts:

I.2.1. Generalities:

Antioxidants are thought to show a very important role in the body defense system against reactive oxygen species (ROS) [85]. They are substances that may protect cells against the effects of free radicals. Free radicals are molecules produced when the body breaks down food, or by environmental exposures like tobacco smoke and radiation. Antioxidants are a chemical species, molecule, piece of molecule or simple atom, [86] capable of having an independent existence ("free") containing one or more single electrons (unpaired electron on an orbital) [87]. This gives them a high reactivity and hence a very short life time. Indeed, the free radical will always tend to fill its orbit by capturing an electron to become more stable [88]. Hence, radicals are generally less stable than non-radicals, although their reactivity varies. Free radicals are capable of reacting indiscriminately with any molecules with which they come in contact [89]. Free radicals can damage cells and may play a role in heart disease, cancer, Alzheimer, DNA damage and other diseases[112-115]. Studies submitted that a diet high in antioxidants from fruits and vegetables is related to a lower risk of cancer [116-117] and Alzheimer disease [93], cardiovascular and Parkinson's disease,[119-120]. Dietary plants cover mutable biochemical families and totals of antioxidants. It has been imagined that plant antioxidants may contribute to the beneficial health effects [96][97].

Numerous in vitro procedures have been developed to measure antioxidant capacities of biological samples. The most popularly used organic radical producers 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) assay, 2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS) assay, cupric ion reducing capability (CUPRAC) assay and one method works with metal ions for oxidation ferric ion reducing antioxidant power (FRAP) assay [98]and oxygen radical absorbance capacity (ORAC) assay. These methods differ from each other comes under free radical scavenging activity. all of these assays be combined to provide a full profile of antioxidant capacity ,The aim of this research was to combine and compare the efficiency of ABTS, DPPH, FRAP, CUPRAC and PM complexe assays to estimate antioxidant activities and their correlations with total phenolics contents in aqueous methanolic *Cleome arabica* leaves extracts in two month (November and January).

I.2.2. Oxidative Stress and Type 2 Diabetes:

In general, diabetes involves metabolic disorders. However, most of the accessory metabolic pathways activated in the pathology of diabetes are accompanied by an overproduction of highly reactive oxidative species, implicated in the onset of various complications (Figure I.10). The causes of the complications and pathologies associated with diabetes are on the one hand directly oxidative mechanisms due to the state of chronic oxidative stress altering the biological molecules, but are also the deleterious pathways for the cells activated by the ROS [99].

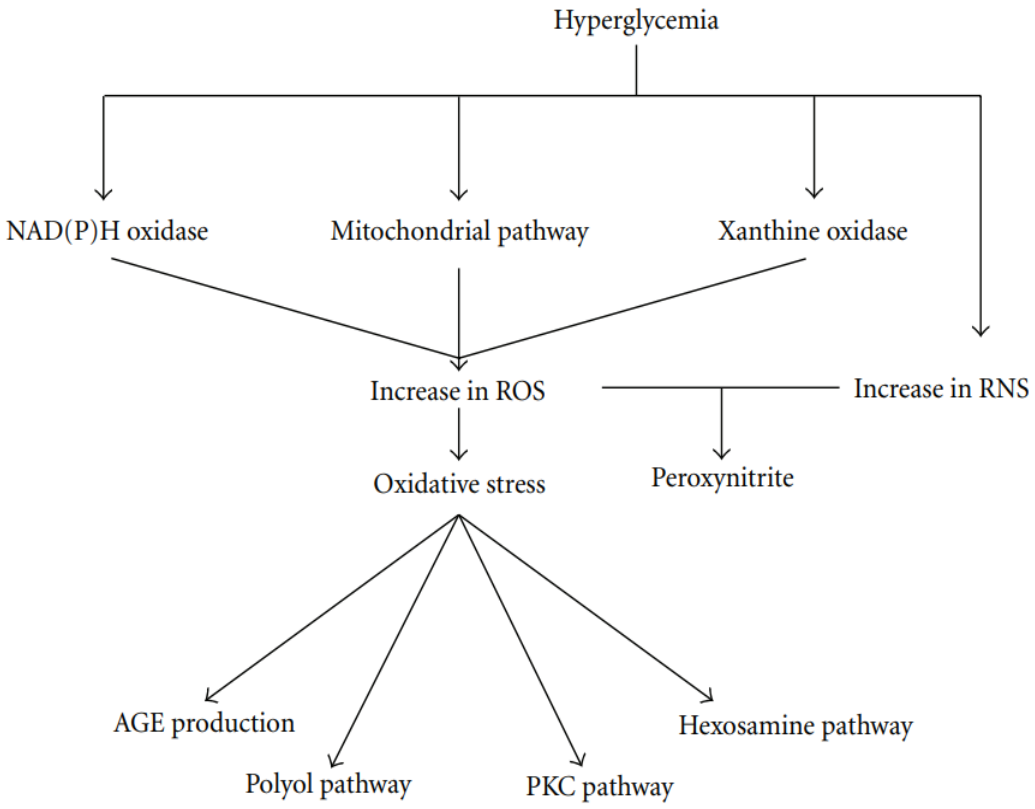


Figure I.10.The main pathways generating diabetic complications, reactive nitrogen species (RNS) (According to Evans et al., 2002 [19]**)**

I.2.2.1. Oxygen, Reactive Oxygen Species (Ros):

Our body needs energy to function properly. Cells transform the nutrients brought by the energy and water supply. This transformation generates about 2% of oxygen molecules [100]. Our cells convert a small portion of O_2 into potentially toxic metabolites: reactive oxygen species (ROS). There are several types of ROS, radicals or non-radical. EROs (essential for reactive oxygen species) are likely to participate in the degradation of biomolecules (lipids, proteins, DNA, glucose). The main EROs are reduced forms of O_2 [101], the superoxide anion (O2 • -, reduction to 1 electron), the hydroxyl radical (OH •, reduction to 3electrons), but also the radicals oxyl (RO •), peroxyl (ROO •) and nitric oxide (NO •).ORS also includes non-radical species, including hydrogen peroxide (H2O2, reduction to 2 electrons), singlet dioxygen (1O2), hypochlorous acid (HOCl), ozone (O3) and peroxynitrite (ONOO-) (Figure I.11).

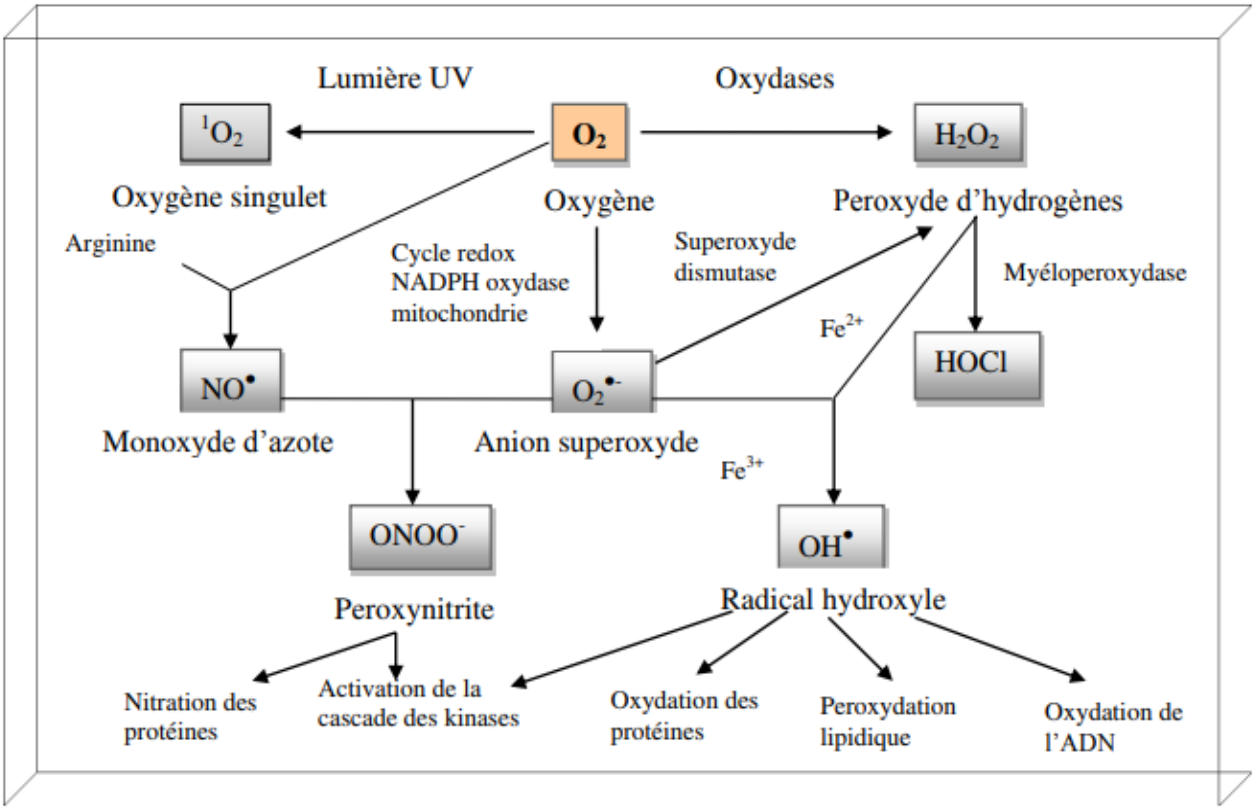
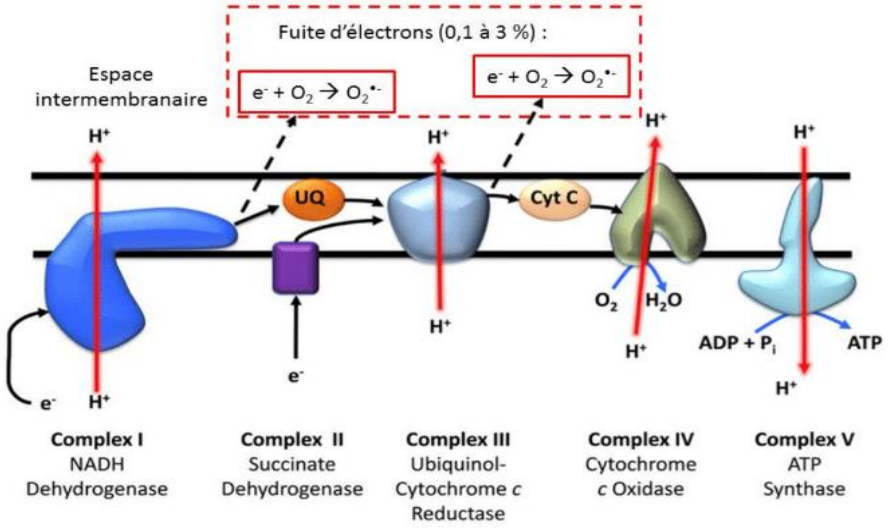


Figure I.11: Main pathways of formation of reactive oxygen species [21-22].

I.2.2.2. ROS Production by The Hyperglycemia-Induced Respiratory Chain:

At the level of the respiratory chain, the metabolites resulting from the Krebs cycle provide the electrons necessary for the operation of the proton pumps allowing the establishment of a potential membrane used for the synthesis of ATP. The transfer of these electrons is provided by the different complexes within the inner mitochondrial membrane. In case of **hyperglycemia**, the oxidation of glucose is increased, causing the growth of the membrane potential, which, when it reaches a critical threshold, blocks the transfer of e- at the level of the complex III. The unmatched e-escapes then escape ubiquinone to produce the superoxide anion by reacting with the final acceptor, O2 (Figure I.12)[102]



FigureI.12. leakage of electrons source of ROS in the chain of transport of electrons

From(Ghouleh et al,2011)[24] *UQ;ubiquinone ,Cys C:cytochrome C*

I.2.3. Source of Antioxidants:

Vitamin C, Vitamin E, α-carotene, Licopein, Selenium, Polyphenol, Glutathione, Peroxidase, Cystine are the main sources of antioxidants. Fruit juices, beverages and hot drinks contain high amounts of antioxidants, like polyphenols, vitamin C, vitamin E, Maillard reaction products, β-carotene, and lycopene[103]. The consumption of fruit juices, beverages and hot drinks was found to reduce the morbidity and mortality caused by degenerative diseases [104].

I.2.3.1. Fruits and Vegetables and Type 2 Diabetes:

Different epidemiological studies may be contradictory about the beneficial effect of a diet rich in fruits and vegetables with regard to diabetes or not very affirmative as to the preventive potential of fruits and vegetables, but suggest, however, a positive trend on health in general. The administration of specific fruits or vegetables in humans has sometimes helped to better account for their protective potential. For example, administration of a daily dose of lyophilisate of strawberry or strawberry drink for several weeks, respectively in women with metabolic syndrome and in hyperlipidemic men and women, has reduced adverse oxidative phenomena [106-107].

The recommendations based on epidemiological studies are such that fruits and vegetables ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract [108-109].

I.2.4. Function of Some Antioxidants:

The Food and Drug Administration (FDA) defines antioxidants only as dietary supplements to be taken in addition to normal food consumption in an effort to prevent these diseases [109]. Antioxidants are known to play a key role in the protective influence exerted by plant foods[110] .

-Function of Vitamin C: Vitamin C intake is inversely related to cancer, with protective effects shown for cancer of the lung, breast, pancreas, stomach, cervix, rectum and oral cavity [111]. In stressful situations adrenal glands react by releasing hormones that trigger the “fight or flight” reaction. It has been indicated that 200mg of vitamin C a day may reduce the levels of stress hormones. Stress affects the immune system. Mega doses of vitamin C increase the levels of antibody that fights against germs and viruses in both stressed and unstressed rats, with greater antibody increase in the unstressed rats [112].

-Vitamin E: Vitamin E is one of the most important lipid-soluble primary defense antioxidants [113]. It is a generic term used for several naturally occurring tocopherols and tocotrienols. In its function as a chain-breaking antioxidant, vitamin E rapidly transfers its phenolic H-atom to a lipid peroxyl radical, converting it into a lipid hydroperoxide and a vitamin E radical. Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation and responsible for protecting Poly Unsaturated Fatty Acid (PUFA) present in cell membrane and density lipoprotein (LDL) against lipid peroxidation[114] and many other functions.

-β-Carotene: Beta-carotene has antioxidant properties that can help neutralize free radicals – reactive oxygen molecules potentially damaging lipids in cell membranes and genetic material, which may lead to the development of cardiovascular disease and cancer. At present, it is unclear whether some beneficial effects of β-carotene and other carotenoids in humans are result of their antioxidant activity or other non-antioxidant mechanisms. In vitro studies indicate that carotenoids can also inhibit the oxidation of fats under certain conditions. They may have anti-atherosclerotic potential, but their effects on humans appear to be more complex [115]. Indeed, it is a required oligo element for the synthesis and function of about 20-40 enzymes, that help prevent cellular damage from natural by-products of oxygen metabolism, called reactive oxygen species (ROS) or free radicals [116] [147].

-Glutathione: Dolas and Gotmare (2015)[118], reported that Glutathione protects cells from toxins such as free radicals. The human body produces glutathione from the synthesis of three key amino acids-cysteine, glycine and glutamic acid. Food sources with the highest amounts of naturally occurring glutathione include; asparagus, avocado, grapefruit, squash, potato, cantaloupe, peach, zucchini, spinach, broccoli, watermelon, and strawberries. Fish, meat, and foods which yield sulfur containing amino acids (e.g. eggs) are the preferred sources for maintaining and increasing bodily glutathione levels.

I.2.5. Antioxidant Activity:

Free radicals, or reactive oxygen species (ROS) and reactive nitrogen species (RNS), are known to cause damage to lipids, proteins, enzymes and nucleic acids leading to cell or tissue injury implicated in the process of ageing [119]. Wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson, and coronary heart pathologies are due to these free radicals and oxidative stress. Aerobic organism developed antioxidant defense mechanisms that reduce the damage caused by ROS and RNS entities. The defense mechanism can be both enzymatic and non-enzymatic. In the enzymatic mechanisms, several enzymes such as superoxide dismutase, catalase, glutathione reductase, peroxidase and nitric oxide synthase are involved. Whereas non enzymatic mechanisms are restricted to antioxidants and trapping agents such as ascorbic acid, α-tocopherol, β-carotene, glutathione, flavonoids, uric acid, cysteine, vitamin-K, serum albumin, bilirubin, and trace elements such as zinc and selenium [120]. In the past few years, natural antioxidants have generated considerable interest in preventive medicine [151-152]. Plants produce a huge amount of antioxidants and they can represent a potential source of new compounds having antioxidant properties[120] .

I.2.6. Methods for Studying the Anti-Radical Potential of Natural Compounds:

The study of the anti-radical power of a sample can be approached by many different techniques, which can be separated into 2 categories: those based on a proton transfer mechanism (Hydrogen Atom Transfer: HAT) and those that implement evidence of an electron transfer (Electron Transfer: ET). These two reaction mechanisms lead to the same result, that is to say the neutralization of the radical species involved in the reaction, but depending on the antioxidant considered, the kinetics of ET and HAT will be different[123]. Indeed, some molecules will exert their radical scavenging capacity preferentially by direct exchange of protons, while others will pass through a transfer of e- which can be coupled in a second time to a proton transfer in a strongly accepting solvent bonds hydrogen. The structure of antioxidants is not the only factor influencing their reactivity in one direction or other. The reaction medium, that is to say the nature of the solvent (polarity, pH etc.) would also be critical. So, the HAT mechanism is generally favored in a polar environments[124]. Each analytical method therefore focuses on one or the other mechanism by detecting the ability of a sample to reduce a particular type of radical species: peroxyl radicals, superoxide anion, singlet oxygen, hydroxyl radical or other synthetic radicals used to facilitate their detection by the presence of a chromophore.

In the case of HAT tests, these are mainly competitive reactions between the antioxidant and a substrate detectable against peroxyl radicals generated from an initiator of azo-type radicals, by thermal dissociation, the substrate will serve as a probe to follow the reaction. Among these methods, mention may be made of ORAC (Oxygen Radical Absorbance Capacity), TRAP (Total Radical Trapping Antioxidant Parameter), The advantage of these methods is the notion of kinetics, allowing to follow and to include in the interpretation of the results of the effects of both latency of the substrate consumption by the radicals, but also the variations of the speed of their oxidation[123].

In the case of ET tests, these are generally so-called final reading methods, which involve easily detectable radicals by the presence of a chromophore or a fluorophore, which is destroyed by the reduction induced by an anti-oxidant as follow**:**

Probe (radical) + e- (an antioxidant) = reduced probe + oxidized antioxidant

These methods include Total Polyphenols by Folin Ciocalteu, TEAC (Trolox Equivalent Antioxidant Capacity), FRAP (Ferric ion Reducing Antioxidant Power), ABTS, DPPH (Radical Scavenging Capacity

Assay), CUPRAC. Depending on the radical used, the reaction takes place with more or less rapid kinetics. In the case of DPPH, a long time is necessary to lead to the depletion of antioxidants by their oxidation, thus testifying to a very slow kinetics. In the example of the radical cation ABTS • +, involved in the TEAC method, in a few minutes, the majority of anti-radical molecules reacted, making this technique very appreciable because of its speed. In contrast, ET-based tests actually reflect the reducing capacity of molecules, describing only a part of the possible antioxidant mechanism. Indeed, the chelating effects for example are not taken into account.

Finally, other methods still make it possible to specifically measure the trapping of certain reactive species such as the hydroxyl radical (Hydroxyl Radical Scavenging Assay), singlet oxygen (Singlet Oxygen Scavenging Capacity Assay). However, all these methods based on the demonstration of a chemical reactivity are not enough to confirm a bio-activity. Indeed, it is essential to confirm the biological activity by other adapted methods using biological models in vitro and / or in vivo. Each method gives us different information about the antioxidant capacity of the tested extract. This includes the mechanism of antioxidant activity (H-transfer like ORAC or single electron transfer like FRAP and CUPRAC, ABTS and DPPH are somewhere considered to have a mixed mechanism or rather H-transfer), the ability to quench ROS or metal ion reduction. Nevertheless, influence on the activity has the temperature, pH, which could be different in each tested method. Thus, we are able to determine the full antioxidant capacity of our extract. This is our objective in this chapter consiste in the development, or the optimization of analytical methods allowing to carry out a screening of eleven samples of *C.arabica* in different season on the evaluation of their antioxidant potential. In this perspective, the first objective was to study the total anti-radical power of this plant extracts through conventional methods.

I.2.7. Statistical Analysis:

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Analysis of variance was used to test any difference in antioxidant activities resulting from these methods. Correlations among data obtained were calculated using Pearson's correlation coefficient (r).

I.3. Effect of Polyphenols on Alpha-Amylase:

I.3.1. Effect of Some Plants Extract on Alpha Amylase:

Thirteen plant species which are claimed to have anti-diabetic activity (based on folk medicine and/or scientific reports) were tested for alpha amylase inhibitory activity. Two of the screened plants exhibited significant (more than 80%) alpha amylase inhibitory activity. IC50 of these plants was estimated based on the dried crude extract and found to be 0.08, and 0.2 mg/ml for Aloe Vera and *Paronychia argentea* respectively**.,** In *A. Vera* the activity was most likely due to cinnamic acid derivatives [125]. *Bryophyllum pinnatum* {(Lam) Oken} leaves are employed as food and as traditional medicines. *B. pinnatum* aqueous extract also had considerably α-amylase and α-glucosidase inhibitory activities with IC50 values 149.20 ± 14.44 µg/mL and 126.15 ± 9.76 µg/mL respectively. their findings indicated that ethyl acetate fraction contained a considerably higher ($p < 0.05$) amount of total phenolic, flavonoids, total antioxidant, FRAP, metal ion, ABTS and DPPH radical scavenging activity than other solvent fractions. Furthermore, the ethyl acetate fraction elicited a significantly higher ($p < 0.05$) inhibitory effects on α-glucosidase (IC50 = 70.90 ± 1.23 µg/ml), α-amylase (IC50 = 62.45 ± 1.22 µg/ml) activities than other fractions[126] , Twenty-one naturally occurring flavonoids were tested for inhibitory activities against alpha-glucosidase (EC 3.2.1.20) and alpha-amylase (EC 3.2.1.1). Luteolin, amentoflavone, luteolin 7-O-glucoside, and daidzein were the strongest inhibitors among the compounds tested. Luteolin inhibited alpha-glucosidase by 36% at the concentration of 0.5 mg/ml and was stronger than acarbose[127].

I.3.1.1. Why *Cleome arabica*?

Many plants produce secondary metabolites (PSM) that inhibit digestive enzymes of herbivores, thus limiting nutrient availability. In response, some special herbivores have evolved digestive enzymes that are resistant to inhibition. Monoterpenes are a class of chemical compounds that have not been investigated as digestive inhibitors despite their potency at inhibiting other enzymes, such as acetylcholinesterase [128], We investigated the potential for interactions between monoterpenes and flavonoids in the *Cleome arabica* that can be extracted with methanol-water and then with solvent of different polarities, ethyl acetate, dichloromethane and n-hexane and the digestive enzyme, which we believe that is an monoterpene-rich Cleome species [129]. We also evaluated chromatographically, and showed to have excellent enzymatic action. Thus, the exploration for new, and novel enzyme inhibitors with progressive therapeutic potential and fewer side effects are required to treat diseases in mankind as well as in animals.

I.3.2. Alpha-Amylase:

I.3.2.1. Definition:

α-amylase is endoglucanase enzyme widely present in plants, animals, bacteria, and fungi. The alpha-amylases are the calcium metalloenzymes which can't function in the absence of **calcium** [160-161]. There are many digestive enzymes in humans and among them the most important one is pancreatic alpha-amylase **(EC 3.2.1.1).** This later are convened as family 13 of the glycosyl hydrolases**.** It is made up of amylose, a straight-chain α-1, 4 linked polymer of about 105 units and amylopectin, a branched chain polymer with α-1,4 linked glucose with branch points made up of α-1,6 linkages that contains about 106 units of glucose that act as a catalysis in the reaction which involves the hydrolysis of the alpha-1,4 glycosidic linkages of the starch, amylopectin, amylose, glycogen, and numerous maltodextrins and is responsible for starch digestion. The other important enzyme is alpha-glucosidase or maltase (EC 3.2.1.20) which catalyzes the final step of the digestive process of carbohydrates mainly starch by acting upon 1,4-alpha bonds and producing glucose as the final product [132]. If there will be excess conversion of starch to sugars, it will increase the sugar level in blood, then the role of insulin will come into action by ordering cells to metabolize the excess sugar moieties and store as energy sources i. e. glycogen. This cycle is endlessly happening in a healthy person. But in some cases, due to excess activity of amylase enzyme and insulin deficiency or resistance to insulin, level of blood glucose arises which might result in hyper glycaemia. To control hyper glycaemia several studies on inhibition of amylase enzyme activity is being studied. A typical α-amylase has a three dimensional (3D) structure with three characteristic domains A, B and C creating a barrel form (FigureI.13) [163-164]. The A domain are highly conserved residues of amino acid which par-take in catalysis and binding of substrates [134]. The B domain is involved in binding of Ca2+ and participates in binding of substrate. The C-domain's role, a second protrusion in front of or behind the A domain, however, is not fully known but a study by Holm et al[135].

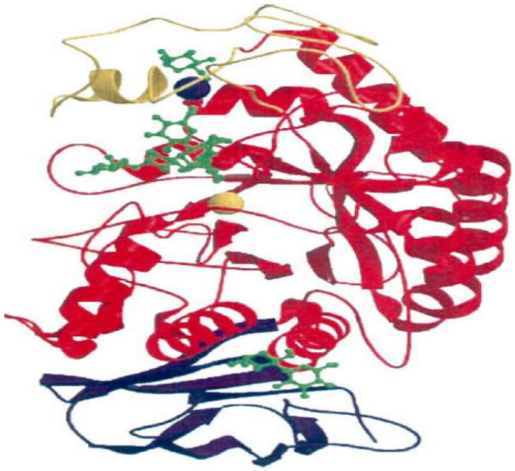


Figure I.13. Structure of α-amylase. Domain A is shown in red, domain B in yellow and domain C in purple. In the catalytic center, the calcium ion is shown in the blue sphere and the chloride ion in the yellow sphere.

I.3.2.2. Amylase Localization, Regulation and Deficiency:

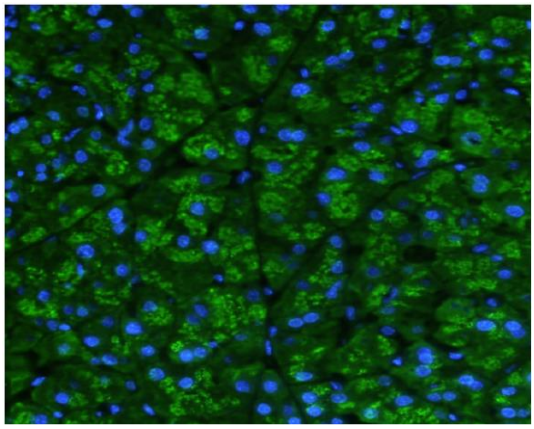
In animals α-amylase occurs in pancreas, parotid, liver, serum, urine and occasionally in smaller amounts in other tissues or tumors; the major salivary and pancreatic amylase proteins are very similar. Salivary amylase initiates carbohydrate digestion in the mouth and pancreatic amylase is the main enzyme for luminal digestion of carbohydrate in the small intestine. Human pancreatic α-amylase is synthesized as a protein of 57 KDa for which the cDNA predicts a protein of 512 amino acids. This includes a signal sequence, amylase isolated from human pancreatic juice has 496 amino acids. In various species the reported molecular weight for amylase is 50-57 kDa and consists of a single chain protein with one carbohydrate (some species have an isoform with none) and an isoelectric point of 7.1. Human pancreatic juice amylase has no sugar groups and exists as two isoforms of pI 7.2 and 6.6 termed HPA I and HPA II. Five disulfide bridges have been described in porcine pancreatic amylase and most species have one or two free sulphydryl groups.

Pancreatic amylase is similar to other secretory proteins and is synthesized in the rough endoplasmic reticulum. It then moves through the Golgi apparatus and is stored in secretory granules until the time it undergoes regulated secretion. Immunostaining reveals its concentration in the granules (FigureI.14). As with other pancreatic enzymes, the amount of amylase in the pancreas is regulated by the amount of dietary substrate [166-168]. Rodents, which eat a carbohydrate rich diet, synthesize more amylase, which is the most abundant pancreatic digestive enzyme in these species. This effect occurs at the transcriptional level and the

effect is mediated in large part by insulin. With diabetes, the pancreatic amylase falls but can recover following insulin treatment [169-170] . This is especially prominent in rats where in experimental diabetes pancreatic amylase falls to only a few percent of normal. A 30 base pair insulin responsive element was identified in the mouse Amy2-2 gene (22). The fact that mice express more than one functional Amy2 gene explains why total pancreatic amylase does not fall in diabetes as much as it does in rats.

Ca^{2+} binding is an important biochemical phenomenon with many widespread consequences. One of the reasons why Ca^{2+} has been so extensively utilized by nature may be the variability of its coordination. In spite of its preference toward octahedral coordination, up to nine ligands can be found in crystalline complexes involving calcium-carboxylate and calcium-water interactions [139] .

A complete structural description of these mechanisms is necessary to understand the ways in which proteins' activities can be modulated by Ca2+. It should also-be noted that purely structural Ca^{2+} sites contribute substantially to the stability of the proteins. We can find two calcium ions in the catalytic site of alpha amylase structure, one of them is the secondary calcium-binding site in *A. Oryzae* α-amylase in binding with three important amino acid (glu230,Asp206,Asp297),(Figure I.15) have all been implicated in the hydrolysis of the substrate, [139] the reduction of catalytic activity is consistent with the binding of Ca^{2+} at the low-affinity site.



FigureI.14. Immunoflourescent localization of amylase in mouse pancreas (green). Nuclei are stained blue with DAPI

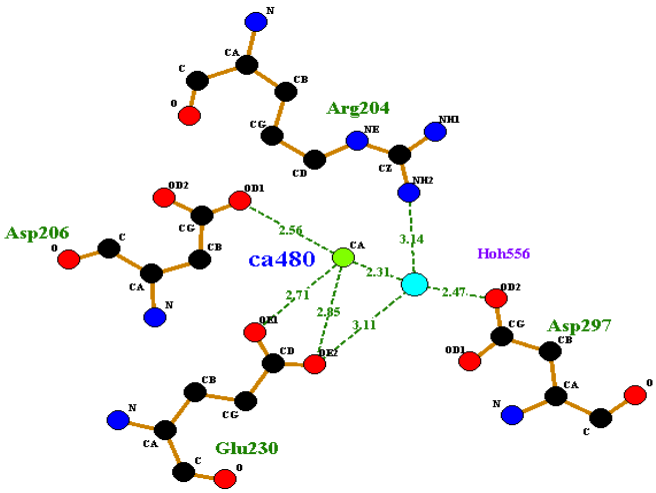


Figure I.15: The active site of alpha-amylase contains a trio of acidic groups that do most of the work, Ca480 represented with green sphere, Figure taken with ligplot.

I.3.2.3. Most Sources and Application of α-Amylases:

α-amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases are widely distributed and are one of the most studied enzymes. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries, can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. The production of α-amylase is essential for conversion of starch into oligosaccharides. Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato [133]. Various physical and chemical factors have been known to affect the production of α-amylase such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture and agitation with regards to SSF and SmF, respectively. Interactions of these parameters are reported to have a significant influence on the production of the enzyme.

Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization. Among bacteria, *Bacillus sp*. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase. Fungi belonging to the genus Aspergillus have been most commonly employed for the production of α-amylase. Production of enzymes by solid-state fermentation (SSF) using these moulds turned a cost-effective production technique. Detailed literature is available on various microbial sources for the production of amylases [140]. Among the fungi,

most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37 °C.

pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium, Fungi of Aspergillus sp. such as *A. oryzae, A. ficuum and A.* niger were found to give significant yields of α-amylase at pH=5.0–6.0 in SmF [140].

The Aspergillus species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α-amylase. *Aspergillus oryzae* has been largely used in the production of food such as soy sauce, organic acid such as citric and acetic acids and commercial enzymes including α-amylase[133].

An unusual feature of α-amylases isolated from different sources is the substantial divergence observed in their primary sequences. Only minimal homology is observed in sequence alignments and this is restricted for the most part to four short segments (4-9 residues in length) of polypeptide chain. In contrast, structural studies on a limited number of these enzymes have shown that greater homology exists between them with respect to polypeptide chain folding. α-amylase structures com pleted thus far include those of barley, pig and the fungal enzymes from *Aspergillus oryzae* and *Aspergillus niger*. These results also show that these α-amylases have similar active site regions that are centred about three highly conserved carboxylate groups. However, although some progress has been made in understanding the roles of these residues in the catalytic event mediated by α-amylase ,much of this process remains poorly understood[141].

I.3.2.4. Amylase Inhibitors in Clinical Medicine:

Amylase inhibitors particularly Acarbose, a pseudo-tetrasacharide, have been used in the treatment of type 2 diabetes where they have moderate effects to reduce peak postprandial glucose and Hemoglobin A1c. Normally, they are used as a second line medication often in conjunction with metformin [142]. Evidence also exists that acarbose can reduce dumping syndrome after bariatric surgery [143]. A host of natural plant products with amylase inhibitor activities have been proposed or studied as a treatment for diabetes but little conclusive evidence exists for their effectiveness.

I.4. Diabetes Mellitus:

Diabetes mellitus can be described as a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism due to defects in insulin secretion, insulin action, or both (Adolfo et al., 2008). According to International Diabetes Federation, around 194 million people live with diabetes and the epidemiological estimates that by 2025 there will be 333 million diabetes sufferers worldwide.

I.4.1. Types of Diabetes: The three main types of diabetes are: Type 1 diabetes, Type 2 diabetes, Gestational diabetes

Type I Diabetes: Type I diabetes is an autoimmune disease. An autoimmune disease results when the body's system for fighting infection (the immune system) turns against a part of the body. In diabetes, the immune system attacks and destroys the insulin-producing beta cells in the pancreas. The pancreas then produces little or no insulin. At present, scientists do not know exactly what causes the body's immune system to attack the beta cells, but they believe that autoimmune, genetic, and environmental factors, possibly viruses, are involved. It develops most often in children and young adults but can appear at any age. Symptoms of type I diabetes usually develop over a short period, although beta cell destruction can begin years earlier. Symptoms may include increased thirst and urination, constant hunger, weight loss, blurred vision, and extreme fatigue. If not diagnosed and treated with insulin, a person with type I diabetes can lapse into a life-threatening diabetic coma, also known as diabetic ketoacidosis.

Type II Diabetes: Type II diabetes mellitus is an increasingly common disorder of carbohydrate and lipid metabolism two important characteristics of this disease are insulin resistance, the failure of peripheral tissues; including liver, muscle, and adipose tissue, to respond to physiologic doses of insulin, and failure of pancreatic β-cells to properly secrete insulin in response to elevated blood glucose levels. Obesity is a significant risk factor for the development of type II diabetes mellitus. An extremely lean and lipoatrophic models have revealed a similar predisposition to developing diabetes. Although it may seem paradoxical that both increased adiposity and severely reduced fat mass cause diabetes, a common pathophysiologic process in fat may be responsible for the predisposition to develop hyperglycemia in both conditions (Nadler and Attie, 2001)[144]. Broadhurst proposed the major causative factors for Non-Insulin Dependent-Diabetes Mellitus (NIDDM) involving obesity and over fatness; carbohydrate and fat over nutrition; lack of polyunsaturated fatty acids (PUFA) in plasma membranes and unbalanced triglyceride intake; chromium

deficiency; and lack of soluble fiber and relevant beneficial phytochemicals .NIDDM is a complex disease that is currently thought to be influenced by more than a single gene or environmental factor. Although the relative contribution of genetic and environmental factors to the development of NIDDM differs among individuals, patients generally have two common metabolic abnormalities: insulin resistance and defects in glucose-stimulated insulin secretion, which lead to disease state.

Gestational Diabetes: Some women develop gestational diabetes late in pregnancy. Although this form of diabetes usually disappears after the birth of the baby, women who have had gestational diabetes have a 20 to 50% chance of developing type II diabetes within 5 to 10 years. Maintaining a reasonable body weight and being physically active may help prevent development of type 2 diabetes. As with type 2 diabetes, gestational diabetes occurs more often in some ethnic groups and among women with a family history of diabetes. Gestational diabetes is caused by the hormones of pregnancy or a shortage of insulin. Women with gestational diabetes may not experience any symptoms. [NIH, 2006, http://diabetes.niddk.nih.gov/dm/pubs/overview/index.aspx]

Treatment for type II diabetes, present oral therapeutics available for the treatment of type II diabetes can be broadly classified into following categories based on their mode of action Sulfonylureas- improve insulin production. Metformin (Biguanide class)-regulates inappropriate release of glucose by liver and corrects the insulin resistance. Thiazolidinediones(glitazones)- attenuates insulin resistance. α-amylase inhibitor inhibits starch digestion in intestine. α-glucosidase inhibitor inhibits digestion of maltose and related oligosaccharides.

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PART II.

EXPERIMENTAL SECTION

In this section, we will present the experimental process in the quantification of total phenolic, including the chemicals used and the plant preparation. We will investigate, also, the flavonoids and tannins content of hydro-methanolic *Cleome arabica* leaves extract in two months, November and January 2015 for eleven samples, and the results for their antiradical scavenging activity, using five antioxidant activity assays. We will present at the end of the study the inhibitory effect of *Cleome arabica* parts on α-amylase in two months, December 2016 and May 2017 in different extraction systems.

II.1. Materials and Chemical Reagents:

All reagents used in this experiment were obtained from Biochem, Sigma–Aldrich, Fluka, Precision balance type OHAUS, Vernier caliper, Rotary evaporator type BUCHI, pH meter type INOLAB, Magnetic stirrer-hotplate type HEIDOLPH, UV / visible spectrophotometer type SHIMADZU UV-1601, Bath Marie or water bath type MEMMERT, Ultrasonic Cleaner, type Joident at 40 kHz**.**

II.2. Plant Collection:

The plant (*Cleome arabica*) was collected in two different seasons (two major phonological stages), the beginning of maturity stage (November 2015 and January 2015, December 2016) and the end of maturity stage (May 2017). All samples were harvested at Laghouat municipality in the steppe region of Algeria.

The plant was identified by the members of the Laboratory of Fundamental Sciences (LSF), University of Laghouat, and an herbarium exemplar was deposited into the herbarium of the laboratory. The plant was separated into leaves, pods, stems, seeds and roots. The different parts were dried, grinded and kept until analysis.

II.3. Extraction of Phenolic Compound:

Extraction is one of the most important steps in sample pre-treatment. Generally, it is a separation process where the distribution of the analyte (in this case, a phenolic compound) between two immiscible phases is made in order to arrive at the appropriate distribution coefficient. The extraction procedure is sequential and systematically carried out using an aqueous organic solvent to extract phenolic compounds in fruit and vegetable samples. This traditional method is called liquid-liquid extraction (LLE) and different extraction solvents have been mentioned in the literature such as ethanol, acetone or methanol, or a mixture with water [1]. Soxhlet system is used to extract the lipidic fraction from food and other solid samples like in our case, using suitable solvents. Although it is not specific for phenolic compounds extraction, usually the

extraction yields are compared to those obtained with another type of polyphenol extraction systems, the extraction must be performed with the most adequate solvent and under ideally predetermined analytical conditions of temperature and pH. Moreover, it is essential to take into account the polyphenolic structure because these compounds may have multiple hydroxyl groups that can be conjugated to sugars, acids or alkyl groups. Thus, the polarities of phenolic compounds vary significantly, and it is difficult to develop a single method for optimum extraction of all phenolic compounds. Hence, the optimization of the extraction procedure is essential for an accurate assay of phenolic compounds from different food matrices [2].

For this reason, modern extraction and isolation techniques will be described as alternative techniques to considerably reduce solvent consumption, solvent release into the environment, and human exposure, and accelerate the extraction process. These modern techniques include: supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).The principal steps in the extraction of polyphenols and flavonoids from *Cleome arabica* parts are summarized in (Figure II.1) included:

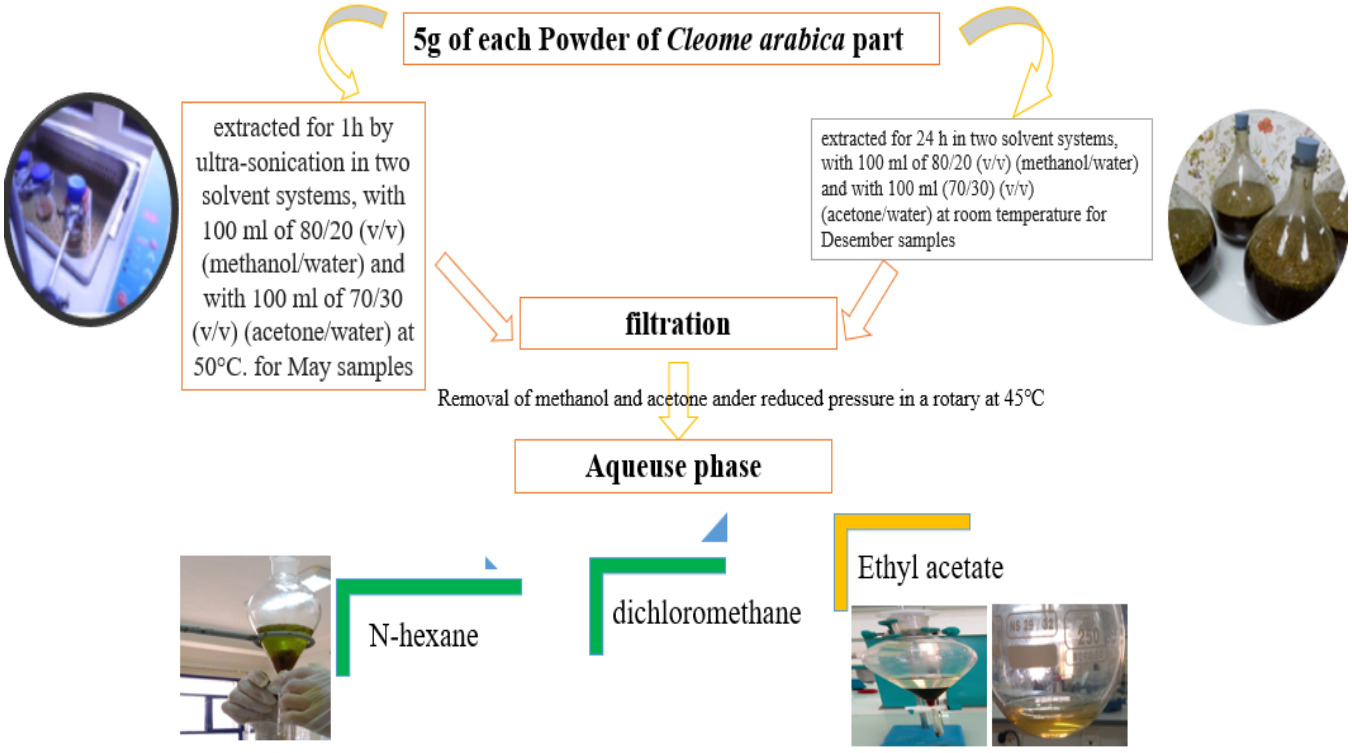


Figure II.1: principle steps in the extraction of polyphenols and flavonoids from *Cleome arabica* parts

II.3.1. Solid–Liquid Extraction (Maceration):

Solid-liquid extraction or leaching can be defined as a mass transport phenomenon in which,, solid contained in a solid matrix, migrates into a solvent brought into contact with the matrix. Mass transport phenomena can be enhanced by changes in concentration gradients, diffusion coefficients or boundary layer [2]. It is a unit operation extensively used to recover many important food components: sucrose in cane or beets, lipids from oil seeds, proteins in oil seed meals, phytochemicals from plants, functional hydrocolloids from algae and **polyphenol compounds from plants**, fruits, vegetables, etc… [3].

Extraction efficiency is known to be a function of process conditions. Several factors affect the concentration of the desired components in the extract: temperature, liquid-solid ratio, flow rate and particle size, and the time of extraction. The most common solvents extraction methods are those using acidified methanol or ethanol. From these methods, extraction with methanol is often the most efficient; in fact, it has been found that in anthocyanin extractions from grape pulp, the extraction with methanol is 20% more effective than that with ethanol, and 73% more effective than water extraction. Nevertheless, in food industry ethanol is preferred, due to the toxicity of methanol [3].

For our samples, a Maceration was carried out for *Cleome arabica* samples collected in different seasons (two major phonological stages) in an arid-type climate with dry and hot summers, and with cold winters. During the coldest month (November, December & January 2016), the collect was done at 70km northern Laghouat city. This plant was identified and studied earlier by our group Khacheba, Djeridane, & Yousfi in 2014 at LSF [4]. The different data (local name, medicinal uses, parts of the plants used, and method of preparation) were obtained from inhabitants having knowledge of the healing properties of these plants. *Cleome arabica* was separated into leaves, pods, stems, seeds and roots, and then dried and grinded. For leaves the lipid was removed by soxhlet extraction with petroleum ether for 24h. Five grams of each powder were extracted for 24 h in two solvent systems, with 100 ml of 80/20 (v/v) (methanol/water) and with 100 ml (70/30) (v/v) (acetone/water) at room temperature.

We did study only one system of extraction 80/20 (v/v) (methanol/water) for eleven samples of *C.arabica* leaves for **November** and **January 2015**.

II.3.2. Ultrasound-Assisted Extraction Method (UAE):

It is a technique that started in the 1950s with laboratory scale experiments. The extraction of bioactive compounds under ultrasound irradiation about 20 to 100 KHz offers high reproducibility in shorter times, simplified manipulation, reduced solvent consumption, and at temperature and lower energy input [5].

Usually, UAE performed in experimental work has indirect sonication by using a small cleaning bath. Additional agitation or shaking is attached to avoid standing waves or the formation of solid free regions for the preferential passage of the ultrasonic waves. Very few plant materials require more than two hours of sonication since vegetal tissue consists of cells surrounded by walls. During sonication, cavitation process causes swelling of cells or breakdown of cell walls, which allows high diffusion rate across cell walls [6]. Ultrasonic cavitation is the momentary creation of "bubbles" in the fluid which immediately and violently implode to produce millions of microscopic jets of liquid which gently scrubs the parts which are submerged in the tank. In our case, This extraction was carried out for ***Cleome arabica*** at fruiting stage (May, 2017) collected at 30 Km south of Laghouat. This extraction was carried out for May samples. Five grams of each powder were extracted for 1h by ultra-sound in two hydro-alcoholic solvent systems, with 100ml of 80/20 (v/v) (methanol/water) and with 100ml of 70/30 (v/v) (acetone/water) at 50°C. After filtration and removal of methanol and acetone under reduced pressure in a rotary evaporator at 45°C.

II.3.3. Liquid-Liquid Extraction (LLE) :

Liquid-liquid extraction is a mass transfer operation in which a liquid solution (the feed) initially containing one or more solutes is thoroughly mixed with an immiscible or nearly immiscible liquid (solvent). The solvent exhibits preferential affinity or selectivity towards one or more of the components in the feed and has a different density. Two streams result from this contact: the first one is the extract which is the solvent rich solution containing the desired extracted solute, the second one is the raffinate, the residual feed solution containing little solute [3].

Solubility of phenolics is governed by their chemical nature in the plant, which may vary from simple to very highly polymerized [2]. Plant materials may contain varying quantities of phenolic acids, phenylpropanoids, anthocyanins, and tannins, among others. There is a possibility of interaction between phenolics and other plant components such as carbohydrates and proteins that may lead to the formation of complexes that may be quite insoluble. Likewise, the solubility of phenolics is affected by the polarity of solvent(s) used. Therefore, it is very difficult **to develop an extraction procedure suitable for the extraction of all plant**

phenolics. Additional steps may be required to remove the unwanted phenolics and non-phenolic substances such as waxes, terpenes, fats, and chlorophylls. The current official analytical method for extracting phenolic compounds is liquid-liquid extraction (LLE) for liquid samples. The process of degradation can be triggered both by external and internal factors. Light, together with air and temperature are the most important factors that facilitate degradation reactions. The extraction temperature usually needs to be high in order to minimize the duration of the process. For these reasons, these traditional extraction sample methods have been replaced by other methodologies which are more sensitive, selective, fast, and environmentally friendly [8]. In our case the liquid-liquid extraction is preformed after Ultrasound-Assisted Extraction and solid-liquid extraction (maceration). After filtration and removal of organic phase under reduced pressure in a rotary evaporator 45°C, the remaining aqueous solutions were sequentially extracted. The sequential extraction was carried out with organic solvents having increasing polarity: n-hexane, dichloromethane and ethyl acetate three times, the filtrate was extracted in the respective organic solvents until total exhaustion. Organic extracts were evaporated after that to dryness under reduced pressure at 45 °C. Dry fractions have powder aspect with white color for ethyl acetate, and green for n-hexane and dark green for dichloromethane, samples were stored in the methanol at 4 °C until use.

II.4. Analytical Method:

In order to analyze the percentage plant yield extract, the yield of evaporated dried extracts based on dry weight basis was calculated by using gravimetric analysis as the following equation:

$$\boldsymbol{yield}\left(\frac{\boldsymbol{g}}{\boldsymbol{100g\ of\ dry\ plant\ material}}\right) = \frac{\boldsymbol{w1}}{\boldsymbol{w2}} \times \mathbf{100}\%$$

Where W1 is the weight of the extract after the solvent evaporates and W2 is the weight of the dry plant material.

II.5. Quantification of Phenolic Compound:

II.5.1. Spectroscopic Determination of Total Phenols:

Although quantitative determination of polyphenols is hampered by their structural complexity and diversity, several methods have been used to determine polyphenols in plant extracts. Assuming that quantification of individual polyphenols does not adequately reveal the proportion of polymeric procyanidins, then, spectrophotometry in the ultraviolet region may be a useful tool to help solve this problem. Colorimetric

reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and at a low-cost [9]. However, it is important that colorimetric assay need to use a reference substance, then, this method measures the total concentration of phenolic hydroxyl groups in the plant extract. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex that can be quantified by visible-light spectrophotometry. The Folin-Ciocalteu method is described in several pharmacopoeias.

The reaction forms a blue chromophore constituted by a phosphotungstic phosphomolybdenum complex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. However, this reagent rapidly decomposes in alkaline solutions, which makes it necessary to use an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. To solve this problem, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity. This reagent is based on the early work of Singleton & Ross method [10] which is a colorimetric oxidation/reduction method for phenolic compounds.

The intensity of light absorption at 760 nm wavelength is proportional to the concentration of phenols. Briefly, a 100µl of the diluted leaf extracts samples was added to 500µl of Folin–Ciocalteu reagent. After 3min, 2ml of saturated sodium carbonate solution (2%) was added. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 764 nm. The calibration curve was prepared with Gallic acid solutions, and the results are given as Gallic acid equivalents (GAE).

Calibration Curve: a standard calibration curve was obtained from the gallic acid solutions of mass concentration ranging from 0.05 to 0.3 mg/ml. Volumes of 100 µl of each solution were introduced into test tubes, followed by the addition of 500 µl of the Folin-Ciocalteu reagent (10 times diluted). After incubation for 2 minutes, 2 ml of carbonates 2% sodium was added, then the solutions were shaken immediately and kept in the dark for 30 minutes at room temperature. The absorbance of each solution was determined at 760 nm against a blank on SHIMADZU UV-1601. The readings of the optical density at 760 nm, solutions thus prepared to make it possible to draw the calibration curve of gallic acid (Figure 1.annex).

II.5.2. Spectroscopic Determination of Total Flavonoids Content:

The total flavonoids content of *C. arabica* leaves extracts was determined using a spectrophotometric method of Laimaison and Carnat [11] with slight modification using a method based on the formation of a

complex flavonoid–aluminium, having the absorptivity maximum at 430 nm. Briefly, 1 ml of sample or standard (quercitin) was mixed with 1ml (w/v) aluminum chloride ($AlCl_3$) 2%. Absorbance of the colored flavonoid–aluminum complex was measured immediately at 430 nm with SHIMADZU UV-1601 UV–Vis spectrophotometer versus a blank. The blank consisted of 1 mL of 50% (v/ v) distillated water instead of samples. The Total flavonoid content of the *C.arabica* extract was expressed as mg quercetin equivalent (CE)/ 100 g of dry weight material. All samples were analyzed in triplicate.

Calibration Curve:

Quercetin was used as a standard to plot the calibration curve. A standard solution of Quercetin prepared in methanol, was diluted to obtain dilute solutions of mass concentration which vary between 0.005 and 0.02 mg / ml. In a 10 ml volumetric flask. We introduced 1ml of each solution thus prepared, 1ml of AlCl3.6H2O 2% was added. The solution is well homogenized and the absorbance was measured immediately at 430 nm after 20mn incubation time against a blank using the same spectrophotometer for the previous assay. In using the absorbance values, thus obtained, the calibration curve of the Quercetin is plotted (Figure.2/annex).

II.5.3. Tannin Condensed Content:

The condensed tannins are determined by the vanillin method with slight modification, the Broadhurst et al, (1978) [12]. This method is based on the ability of vanillin to react with condensed tannin units in the presence of acid to produce a colored complex measured at 500 nm. The reactivity of vanillin with tannins implies only the first unit of the polymer. 5ml of reagent comprises 0.5% vanillin and 4% HCL prepared in the methanol added to 1ml of diluted extract. The reagent and diluted extract pre-incubated in a water bath at 20ºC for 10 minutes before the reaction starts and then the mixture is incubated for 15 minutes. The reading of the absorbance is carried out in a Visible/UV spectrophotometer of SHIMADZU type 1601, at a wavelength of 500 nm against a white.

A calibration curve is performed in parallel under the same operating conditions using catechin as a positive control.

Calibration Curve: Catechin was used as a standard to plot the calibration curve. A standard solution of catechin prepared in methanol, was diluted to obtain dilute solutions of mass concentration which vary between 0.1 and 1 mg/ml. In a 10 ml volumetric flask, 5ml of reagent comprises 0.5% vanillin and 4% HCL prepared in the methanol added to 1ml of diluted extract. The solution is well homogenized and the

absorbance was measured immediately at 500 nm after 15mn incubation time against a blank using the same spectrophotometer for the previous assay. In using the absorbance values thus obtained, the calibration curve of the catechin is plotted (Figure3/annex).

II.6. Evaluation of the Antioxidant Potency of Phenolic Extracts:

II.6.1. Methods of Total Antioxidant Capacity Assessment:

The various analytical methods of evaluation of the antioxidant capacity fall into distinct categories (Table II.1) [13].

TableII.1: Methods of Total Antioxidant Capacity Assessment

Antioxidant	capacity assay Principle of the method	End-product determination
Spectrometry		
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
phosphomolybdenum method	Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex.	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry

II.6.1.1. Spectrometric Technique:

Spectrometrics techniques [13-14] rely on the reaction of a radical, radical cation or complex with an antioxidant molecule capable to donate a hydrogen atom.

II.6.1.1.1. DPPH Free Radical Scavenging Activity Assay:

No antioxidant assay is simpler or less expensive to run than the DPPH assay, which accounts for its popularity and extensive use [15]. Needed only the reagent, some cuvettes and a UV–vis spectro-photometer, In the present study, the 2,2–Diphenyl–picrylhydrazyl (DPPH•) radical scavenging method is used to study

the antioxidant activity of ethyl acetate, chloroform and n-hexane soluble fractions obtained from the different parts of *Cleome arabica*. The (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not dimerize, as happens with mostly free radicals. The delocalization on the DPPH• molecule determines the occurrence of a purple color, with an absorption band with a maximum around 520nm. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet color. The antioxidant activity of *C.arabica* L extract, with respect to the radical DPPH, was evaluated spectrophotometrically by following the reduction of this radical which is accompanied by its passage from the violet color to the yellow color measurable at 517 nm. The antioxidant compounds neutralize the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH, thereby changing the color from purple to the yellow colored stable diamagnetic molecule diphenylpicrylhydrazine. The degree of discoloration indicates the scavenging potential of the extract or antioxidant in terms of hydrogen donating ability. According to the method reported by (Cotelle et al, 1996) [16] with minor modifications to the method of [17-18]. Briefly, ethanolic solution extract and standard solutions were prepared , the 250µM DPPH ethanolic solution was prepared and kept in the dark until use; the mixtures between the DPPH and the extract were shaken vigorously and let to react in the dark for 30 min at room temperature. After that, the absorbance was read at 517 nm. Radical scavenging capacity was expressed as percentage effect (E %) and calculated using the following equation:

$$Percentage\ effect\ (E\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100$$

Consequently, the absorbance diminution depends linearly on the antioxidant concentration. Ascorbic acid, BHT and TBHQ are used as a standard antioxidant (**Figure4/annex**). Different concentration samples were used in order to obtain antiradical curves for calculating the EC50 values. Antiradical curves were plotted referring to concentration on the x axis and their relative scavenging capacity on the y axis. The EC50 values were processed using +6 points . ET−based assay can be summarized as follows:

DPPH: DPPH. + ArOH → DPPH + ArO. + H+

The percentages of inhibition, thus determined in previous plots, allowed us to determine the 50% inhibition concentration (EC50) of the antioxidants present in the phenol extracts expressed in mg/ml, which represent the concentration of the necessary inhibitor to decrease 50% free radicals in the reaction medium.

Figure 5/annex represents the EC50 values of some samples chosen in November and January. Similarly, we calculated the EC50s of vitamin C, TBHQ and BHA for comparison with those of phenolic extracts. EC50 values obtained are summarized in **Table II.2**.

II.6.1.1.2. ABTS Radical Cation Decolorization Assay:

The ABTS cation radical (ABTS•+) which absorbs at 743 nm (giving a bluish-green color) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)) [76]. ABTS assay was described by (Re et al., 1999, Serpen et al.2008) [19-20] with minor modification. The radical cation ABTS + occurs immediately after the addition of 100 µl The ABTS•+ radical cation was generated by chemical oxidation with potassium persulfate $K_2S_2O_8$ solution (70mM); the mixture was brought to room temperature and put in the dark for 24 hours before use. 1 ml of this solution was diluted by a buffered solution at pH 6.8 to 7.0 phosphate prepared by dissolving 0.4477g of sodium hydrogen phosphate hydrate twelve times (Na_2HPO_4-$12H_2O$), 0.195 g of hydrated sodium dihydrogenphosphate (NaH_2PO_4-$2H_2O$) and 2.25 g of chloride sodium (NaCl) in 250ml of distilled water. The optical density of the pre solution incubated at 37°C for 15 min is adjusted to (0.70 ± 0.002) at 734 nm. In the presence of hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization.

100 µl of each diluted extract in distilled water and admixed with 1ml of the ABTS + solution. The percentage inhibition (PI) of ABTS + for each sample was calculated according to the formula bellow:

$$Percentage\ inhibition\ (PI\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100$$

Although other wavelengths such as 415 and 645 nm have been used in the ABTS assay, the 734 nm peak wavelength has been predominantly preferred due to less interference from plant pigments.

In the presence of hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. We also tested vitamin C, TBHQ and BHT as standard (**Figure6/annex)**. The %AI of the extracted phenols was expressed as IC50, with the IC50 being defined as the concentration of test

sample required to inhibit the formation of active radicals by 50%. ET−based assay can be summarized as follows:

$$\mathbf{ABTS + K2S2O8 \rightarrow ABTS^{\cdot +},\ \lambda_{max}=734\ nm\ ABTS^{\cdot +} + ArOH \rightarrow ABTS + ArO^{\cdot} + H^{+},}$$

Examples of the EC50 values of some samples chosen from November and January are summarized in (**Figure 7/annex)**

II.6.1.1.3. Evaluation of Total Antioxidant Capacity by Phosphomolybdenum Method:

The antioxidant activity of the *C.arabica* L extract was evaluated by the phosphomolybdenum method according to the method of Prieto (1999) [21]. The assay is based on the reduction of molybdates Mo (VI) to molybdenum Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid p^H. The followed scheme is to combine 0.1ml extract with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank, 0.1ml of distillate water was used in place of the test sample. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 70°C for 90 min. We subjected the samples to a fast cooling before measuring the absorbance of the solution at 695 nm using a spectrophotometer [21]. The antioxidant capacity of each sample was expressed as an ascorbic acid (A.A) equivalent. The values are presented as the means of triplicate analysis. The reaction equation of ET−based assay can be summarized as follows:

$$\mathbf{Mo\ (VI)\ (yellow) + e^{-}\ (from\ AH) \rightarrow Mo\ (V)\ (blue)\ at\ \lambda_{max} = 695\ nm,}$$

(Figure 8&9) in the annex represented the antiradical curves of standard anti-oxidant evaluated with molybdate method, and the inhibition rate of some January and November samples evaluated by molybdate method, respectively.

II.6.1.1.4. Ferric Ion Reducing Antioxidant Power (Frap) Assay:

The ferric ion reducing antioxidant power (FRAP) method was used to measure the decreasing capacity of *C. arabica L* extracts. This method was carried out with slight modifications of [65-66] . The FRAP test solution was mixed in this order at the volume ratio of (10:1:1). Acid buffer pH = 3.5; 2,4,6-solution tripyridyl-s-triazine (TPTZ) and the iron III chloride solution, respectively. The following protocol is used for the buffer acid (pH = 3.5) must be dissolved 0.310 g CH_3COONa, 3H2O in a 100 ml flask, then add 1.6 ml of pure acetic acid, and complete with distilled water up to gauge. The TPTZ solution is prepared by

dissolving 157 mg of this compound in 50 ml distillate water and 0.165ml of HCl 40 mM. For the solution of ferric chloride (20 mM), we have dissolved 0.2723g of $FeCl_3$, 6H2O in a flask 50 ml and added it to distilled water.

For samples to be tested, these are mixed in the same proportions as to plot the standard curve, the reagent (1000 µl), and the solution of the test compound (100µ L). The optical density is read after 1h at 593nm. The ability "electron donor" of the sample is measured by the change in color due to the formation of the complex (Fe^{2+} TPTZ), and the equivalent in Fe^{2+} is determined. The reaction equation of ET−based assay can be summarized as follows: FRAP:

$$\mathbf{Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)2^{2+} + ArO^{\cdot} + H^{+},\ \lambda_{max}=593nm}$$

where TPTZ: 2, 4, 6-tripyridyl-s-triazine ligand.

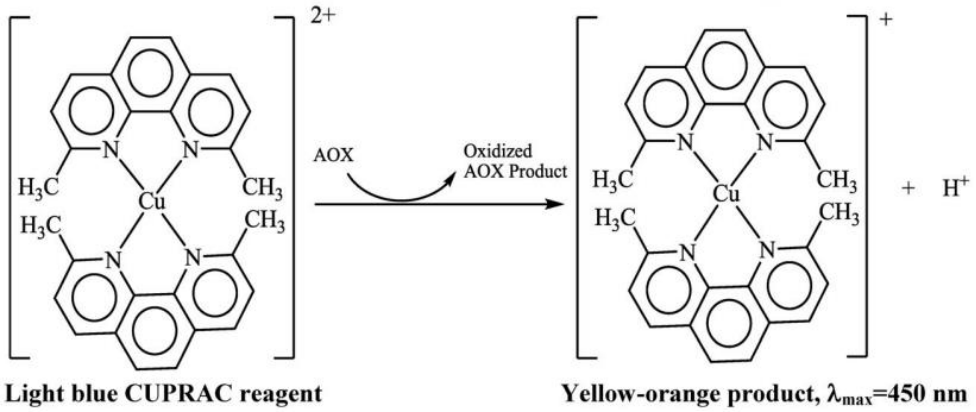
All measurements were repeated three times. Total antioxidant capacity in the measuring systems, expressed as VC equivalents, was calculated. Correlation coefficient (R^2) for the test curve ranged between 0.99 to 1.00.

Figures 10&11 in the annex represent the antiradical curves of standard anti-oxidant evaluated with the FRAP method and the inhibition rate of some January and November samples evaluated by the FRAP method, respectively.

II.6.1.1.5. Copper Reduction (CUPRAC):

The evaluation of the reducing capacity of our samples was carried out using the test CUPRAC. Based on the reduction of increased absorbance of Neocuproine complex (Nc)-ions cupric [Nc2-Cu2 +]. Indeed, in the presence of an antioxidant, the copper complex neocuproine is reduced, and this reaction was quantified spectrophotometrically at a wavelength of 456 nm[24]. The preparation scheme for the measurement of the antioxidant properties was the following:1 ml of ammonium acetate buffer (1M, pH = 7> m = 19.66 g in 250 ml water) was mixed with 100 µl of diluted extract to be tested, 500 µl of Neocuproine (7, 4 mM => m = 0.039g in 25ml ethanol) and 500 µl of $CuCl_2.2H_2O$ 10.1mM(0.01 m => m = 0.1722g in 100ml water). The mixture is incubated for 1h, then the reduced complex [-Nc Cu2 +] was quantitated spectrophotometrically at a wavelength of 456 nm.

In case of blank = solvent instead of extract.



The reaction equation of ET−based assay can be summarized as follow:

CUPRAC: n Cu(Nc)$_2$ $^{2+}$ + Ar(OH)n → n Cu(Nc)$_2$ $^{+}$ + Ar(=O)n + n H^{+}, where the polyphenol is oxidized to the corresponding quinone, and the reduction product, i.e., bis(neocuproine)copper(I) chelate shows an absorption maximum at 456 nm**.** The redox reaction with reducing poly - phenols and vitamins C, TBHQ and BHT is measured at 450nm, the orange–yellow color is due to the Cu(I)-Nc charge-transfer complex formed. Liberated protons are buffered in ammonium acetate medium. **Figures 12&13,** in the annex, represent the antiradical curves of standard anti-oxidant evaluated with the CUPRAC method, and the inhibition rate of some January and November samples evaluated by the CUPRAC method, respectively.

II.7. Effect of Polyphenols on Alpha-Amylase:

II.7.1. Aspergillus Orzsae A-Amylase Inhibitory Activity Assay:

α-Amylases are starch degrading enzymes that catalyze the hydrolysis of α-1,4-O-glycosidic bonds in polysaccharides with the preservation of α-anomeric configuration in the products, [28]. The inhibitory activity of *C. arabica* fractions of α – amylase from Aspergillus oryzae was assayed using a method adopted by khacheba et al.2014 [4] on its substrate starch using 3,5 – dinitrosalliculic acid (DNS) as a reagent [29]. Figure II.2 represents the different steps of α-amylase inhibitory effect of *Cleome arabica* extracts.

In brief, 200 µL of sodium phosphate buffer saline (pH = 6,9) was mixed with 100 µL of soluble starch (1 %) and incubated with 100 µL of suitable aliquots of the test sample at 37 °C for 5 min, then the reaction was started by the addition of 100 µL of *Aspergillus oryzae* α – amylase (13 U/ml; one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per minute under assay conditions) (Khacheba et al., 2014) [30]. After incubation at 37 °C for 2 min, the

reaction was stopped by adding 3,5 – dinitrosalliculic acid (DNS) and boiled for 5 min at 100°C. The mixture was cooled and diluted with distilled water. The absorbance of the mixture was measured at 530 nm and α – amylase inhibition was expressed as percentage of inhibition.

$$\textbf{\textit{Inhibitory activity}}\ (\%) = [(\boldsymbol{A0} - \boldsymbol{A\,S})\,/\boldsymbol{A0}]\,\boldsymbol{x}\,\mathbf{100}$$

A_0 : Absorbance of control without inhibitor. A_S : Absorbance of test Sample with inhibitor.

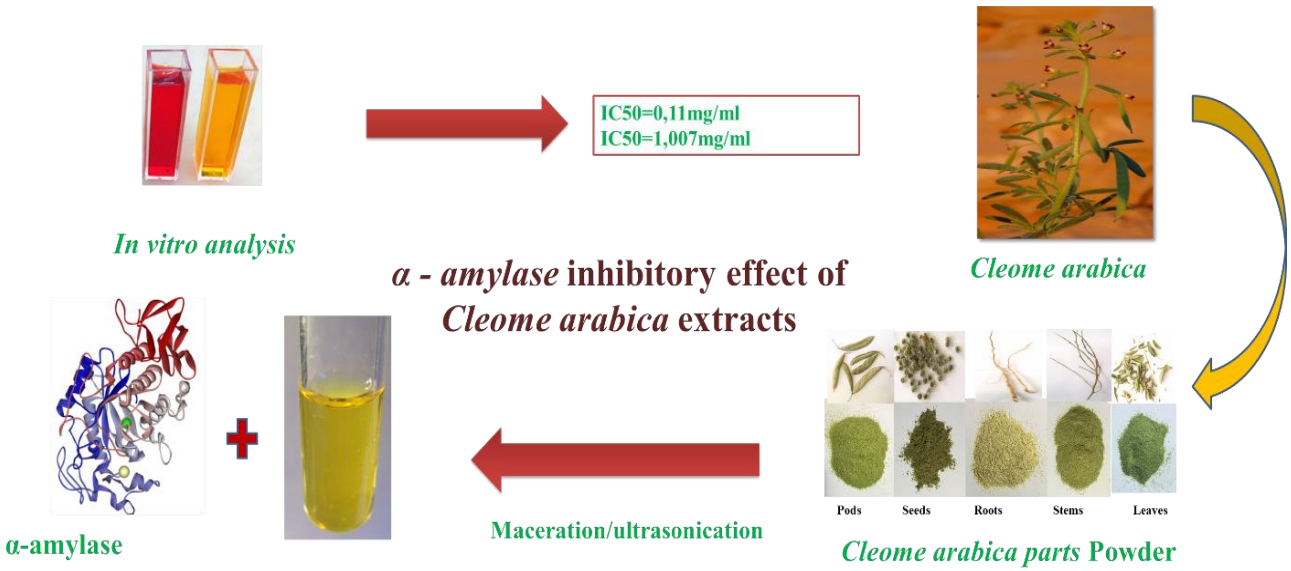


Figure II.2: Different steps of α-amylase inhibitory effect of *Cleome arabica* extracts

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PART III:

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

In this section, we will present results of the total measured phenolic, flavonoids and tannins content of hydro-methanolic of *Cleome arabica* leaves extract in two months for eleven samples. We will evaluate their antiradical scavenging activity with five antioxidant activity assays, including discussion of the mechanisms underlying each assay. We will study and discuss the previous results together with their inhibitory effect of *Cleome arabica* parts on α-amylase.

III.1. Content of Total Phenolic, Flavonoids and Condensed Tannins:

For the purpose of extraction experiment using plant/ herb, is that all of extraction solvents / chemicals should not have toxic effect(s) or strong interfering efforts to living cells, animals, and human beings. For the organic solvents applied for extraction, some of them have high toxicity to living cells, such as methanol, Acetone, Chloroform, Dichloromethane, etc.. Some solvents in low boiling point have high risk in laboratory and manufactory safety issues, it is better to optout these chemicals/solvents. Whereas on extraction using the solvents of Water, Ethanol, Ethyl Acetate, and Hexane, which have no toxic effect(s) or minor interfering efforts to the living cells, animals, and human beings, you only need to do one blank test on water base, water extraction product mainly contains (metals, ions, high hydrophilic compounds, and water soluble proteins/enzymes, glycoproteins, peptides, amino acids, nucleotides, sugars, and polysaccharides).

Ethanol, methanol extraction product mainly contains high hydrophilic compounds (including very polar neutral, basic and acidic compounds, amino acids, nucleotides, sugars, and polysaccharides).

Ethyl Acetate extraction product mainly contains medium hydrophobic compounds (including medium and low polar neutral, basic and acidic compounds), of which, steroids, wax, fatty acids, alkaloids, and polar-chain carbonated polymers are expected.

Hexane extraction product mainly contains low or non-polar hydrophobic compounds with extremely high lipophilicity of which low polar neutral compounds, steroids and high carbon fatty acids are expected). the extraction solvent selected depends upon solubility of our compound of interest. Methanol /water, dichloromethane, ethyl-acetate are better extraction solvent of secondary metabolites. The active compounds explored in this study are hydrophobic means better extracts with organic solvents. Most of flavonoids shows medium polarity, so methanol is expected to be better than water. So methanol 80% was selected because generally flavonoids in plants are in the glycoside form [1].

The extraction solvents carry off non-phenolic substances such as sugars, dyes and proteins which may interfere during any phenolic evaluation [2], Aqueous methanolic extraction proved to be the most effective method for extracting phenolic materials and other antioxidative compounds [3]. The extracts obtained were stored in the refrigerator before being analyzed. The phenolic extracts thus obtained generally have a viscous appearance, and sometimes in the form of powder for ethyl acetate extract, of green, yellow or brown color.

The results of the total phenolic content in the ethyl acetate fraction ranged between 0.341±0.00 and 0.751 ± 0.02mg GAE/g dry matter and 0.170±0.00 to 0.490±0.01mgGAE/g for the dichloromethane fraction, and between 0.060 ±0.00 to 0.453± 0.02mgGAE/g for the n-hexane fraction.

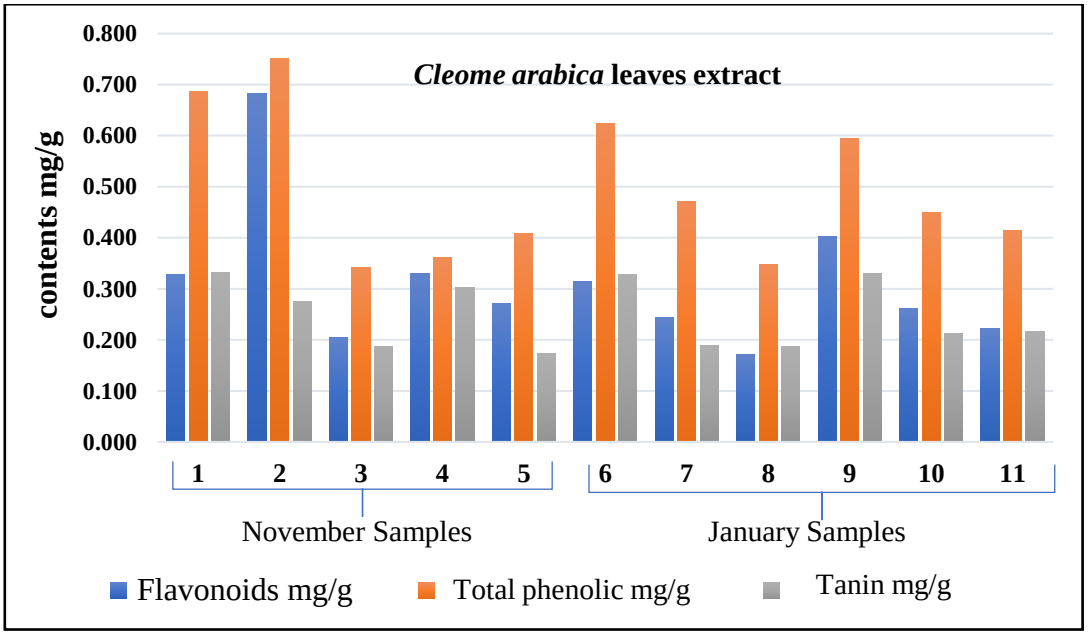
The total phenolic content in many samples tested in this study was higher than reported for the other medicinal plants [4]. But we marked that the total phenolic content in the tested plant was lower than those reported for the most of other Asian medicinal and common dietary plants [5-6]. (Tigrine, 2014) [7] Showed that *Cleome arabica* Leaves extract is relatively rich in polyphenols, with a rate of 322.1 ± 3.44 mg gallic acid equivalent / g DM. This may be related to the distribution of secondary metabolites, may change during plant development, Also with harsh climatic conditions (very low temperature in winter, solar exposure, drought, salinity), which stimulate the biosynthesis of secondary metabolites such as polyphenols [8]. In addition the organ analyzed, the region and date of harvest, the extraction and analytical method and the solvents that are used [9]. Indeed, the phenolic content of a plant depends on a number of intrinsic factors (genetics) and extrinsic (cultural practices, maturity at harvest and storage conditions). In addition the quantification method may also influence the estimation of the total phenol content [10].

In our study, we chose a hydro-alcoholic mixture (methanol-water) with a ratio (80/20) by volume and then purification with ethyl acetate. This choice of this methodology is based on a literature indicating the use of this type of solvents to extract phenolic compounds with important activities [11-12]. However, adding water to methanol in the ratio of M:W 80:20 or 70:30 further increase the polarity of the solvent mixture, which is suitable for recovery of polar compounds from a polar-non polar mixture.

In our work, the dosage of flavonoid content was determined spectrometrically by the aluminum chloride method which is used specifically for the quantification of flavones and flavonols. Therefore, the results obtained do not reveal the exact total flavonoid amounts in the plant investigated, or the method not be successful for all type of plant specially the plant contains a height contain of

chlorophyll. For this raison to obtain a close estimate of these substances in these plants, use the technique of 2,4-dinitrophenylhydrazine as a complementary method for the determination of flavanones and dihydroflavonol [13].

The amount of flavonoids content in the ethyl acetate fraction ranged from 0.172±0.001 to 0.682±0.003 mg EQ/g. for the tannins content, we observe the same results as flavonoids exists only in the ethyl acetate fraction, The results of the condensed tannins in the ethyl acetate fraction varied between 0.172±0.008 to 0.332±0.007 mgEC/g. we conclude that the ethyl acetate is the best extractors of flavonoids and the condensed tannins. The results of total phenolic content, flavonoids and tannins condensed content are shown in graph columns (**FigureIII.1&III.2**). The greatest amount of flavonoids was recorded in the **ethyl acetate** fraction but no quantity was registered in the dichloromethane and n-hexane fraction. We can confirm that the most amount of flavonoids in this plant are in glyosidic form [14].



FigureIII.1: comparison between the levels of total phenols, flavonoids and tannins for the ethyl acetate fraction of *Cleome arabica* leaves extract.

It can be deduced then that almost all these samples are in possession of a material rich in flavonoids in the rate greater than 50% (**figure III.2**). Thus, it can be deduced that the analysis of polyphenols by spectroscopic methods gives us a good idea about the quantitative and qualitative identification of these compounds.

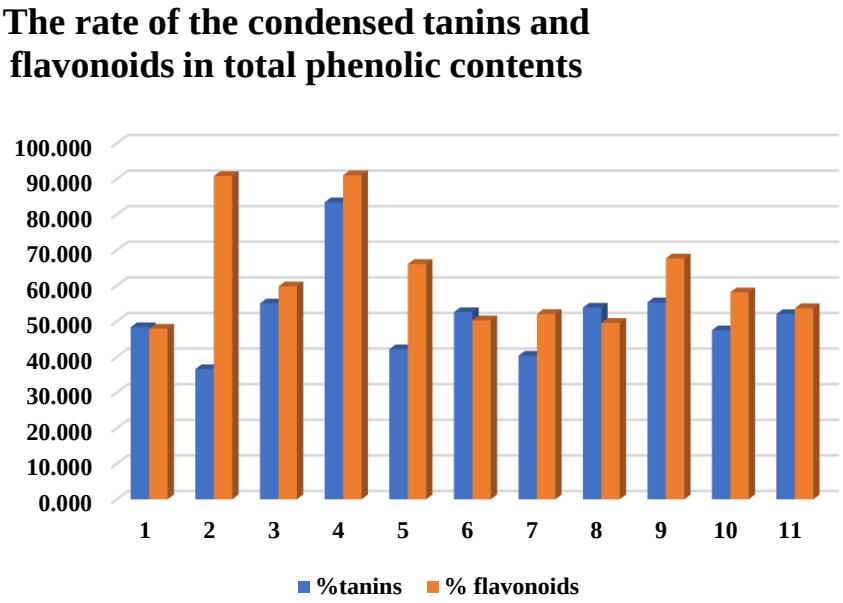


Figure III.2: The rate of condensed tannins and flavonoids in total phenolic content.

From the graph shown in (**Figure III.3**), it is clear that there is a good positive correlation between the total phenol content and the level of flavonoids. This result can be translated by the fact that the amount of flavonoids varies proportionally with all the polyphenol content might be because flavonoids are one of the major phenolic compounds of *Cleome arabica.*

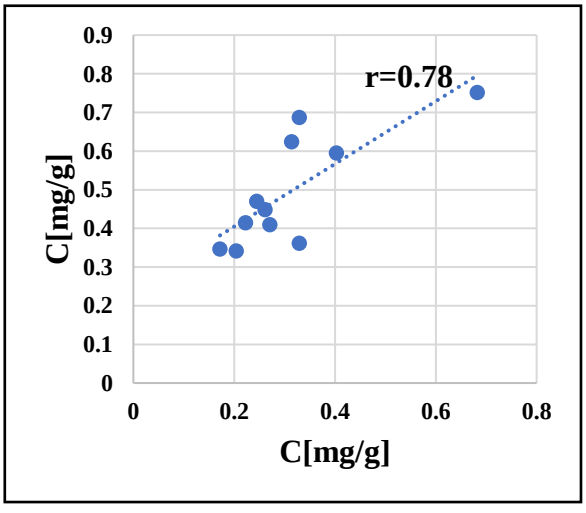


Figure III.3: Correlation curve between the total phenolic content and total flavonoid content

The high content of polyphenols in the hydro-methanoic extract is related to the high solubility of phenols in polar solvents. The type and structure of the concentration of flavonoids, condensed tannins

in plant extracts depends on the polarity of the solvents used in the preparation of the extracts [5][10],sequential extraction stay better . We observed that the flavonoid and tannins content decreases with the season until its lack in May (see paraghIII.5) so it`s effect with the harvesting time.

III.2. Antioxidant Activity of Test Samples:

The total antioxidant capacities of *C.arabica* leaves extract are summarized in **Table III.1**. Each method gave us different information about the antioxidant capacity of the tested extract. The highest antioxidant capacities were detected by ABTS with extract series concentration from 0.1 to 0.8 mgGAE/g and EC50 values ranged from (0.009±0.000 to 0.013±0.000 mg/ml) which is better than that of BHT (0.021mg/ml), TBHQ (0.023mg/ml) and ascorbic acid (0.057mg/ml). However, the lowest anti-oxidant activity was registered between (0.16±0.02 and 0.446 ±0.027mg/ml), (0.166 ± 0.002 to 0.323 ±0.004mg/ml) and (0.097±0.003 to 0.232 ±0.0.0025mg/ml) for PMC assay, CUPRAC, FRAP assay, respectively (see tableIII.1) [44]. This difference in the results is due to a different reaction chemistry involved in different in-vitro assays. The antioxidant activity is usually measured by quantifying the ability of antioxidant compounds (electron donors/reducing agents) to quench the free radicals. The free radicals are very labile species which are often generated at uncontrolled rate, in reaction medium, due to fluctuation in reaction conditions. Therefore, we must study our samples from different sides. To highlight the major ones, the antioxidant compound activity (say for polyphenols) is a function of pH, light and presence of pro-oxidants in reaction medium which often results in variations in the results obtained. One should always test the different antioxidant activity assays and try to find correlation among them (make down).An example all the more reason could be that the CUPRAC method can determine the antioxidant substances containing sulfhydryl group (SH), for example the proteins. The CUPRAC method is a simple and inexpensive antioxidant capacity assay for dietary polyphenols (both natural and synthetic antioxidant phenols), vitamins C and E, and human serum antioxidants, utilizing the copper(II)-neocuproine reagent as the chromogenic oxidizing agent. This method has distinct advantages over other ET−based assays, namely simplicity, availability and stability of reagents, reproducibility over a wide concentration range, selection of working pH at physiological pH (as opposed to Folin and FRAP methods, which work at alkaline and acidic pH, respectively), applicability to both hydrophilic and lipophilic antioxidants (unlike Folin and DPPH), completion of the redox reactions for most common flavonoids within reasonable time (unlike FRAP)[15]. Not all methods measure protein-thiols, or smaller molecule -SH compounds of different origins (such as GSH, with FRAP. Also, The CUPRAC method is advantageous over FRAP since the redox chemistry of copper(II) -as opposed to that of chemically inert high-spin ferric ion having half-

filled d-orbitals in its electronic configuration- should involve faster kinetics. The bis (neocuproine) copper (I) cation chromophore is soluble both in water and in organic media. The CUPRAC method describes the development of a simple and widely applicable antioxidant capacity assay for flavonoids, phenolic acids, hydroxycinnamic acids, thiols, synthetic antioxidants, and vitamins C and E[16]. The CUPRAC reagent is much more stable and easily accessible than the chromogenic radical reagents (e.g., ABTS, DPPH, etc.)[16]. The redox reaction giving rise to a colored chelate of Cu (I)-Nc is relatively insensitive to a number of parameters, adversely affecting radicals reagents such as DPPH, e.g., air, sunlight, humidity, and pH, to a certain extent. In this experiment, the ABTS method was used to confirm the results from the DPPH method assay since both methods are based on a similar antioxidant mechanism and the extracts used in both tests were ethanol soluble. All samples extracts are comparable without significant differences.

For the case of DPPH and ABTS radicals, the mechanism (particularly in ethyl acetate) is very often by electron transfer, furthermore, the antioxidant activity of *C.arabica* doesn't change with the different seasons of the year. All these are in- vitro models and they do not point to a particular disease model. Some scientists claim that all plants have an antioxidant activity; so, unless this activity plays a beneficial role in a diseased model of oxidative stress, it has no relevance. Consequently, it will be important to measure the antioxidant activity in- vivo, most especially, in a diseased model where excessive free radicals are produced.

III.3. Antioxidant Capacity of The Important Standards:

There are some relevant organic compounds used as antioxidant standards, comprising Ascorbic acid, BHT and TBHQ which were moderately evaluated for their standing of antioxidant power by DPPH, ABTS, FRAP, PM- complex and CUPRAC assays. A lower IC_{50} and EC_{50} values correspond to a larger scavenging activity. As shown in Table III.2, the DPPH radical scavenging activities of these reference compounds were comparatively evaluated: ascorbic acid possessed the highest radical scavenging activity, 0.001±0.00g/l as compared with TBHQ 0.04±0.00g/l, BHT 0.168±0.004g/l, the ranks of the scavenging activity were found in similar trends with DPPH and PM-complex assays; ascorbic acid >TBHQ > BHT. On the contrary, the ranks of the scavenging activity were found in similar trends with CUPRAC and ABTS, BHT>TBHQ>ascorbic acid. The linearity of calibration curves allowed quantification of antioxidant activity using any of the standards listed above. DPPH, ABTS, PM-complex, CUPRAC and FRAP values (Table III.1) for the antioxidant activity of these standards were used for investigating the correlation coefficients. The antioxidant

activity of many samples of *C.arabica* L extract tested in this study was higher than reported for most of other medicinal plants. These results of the study are in good accordance with previous studies (Table III.2).

Table III.1: Antioxidant activity of examined *Cleome arabica* L ethyl acetate extracts and standard antioxidants determined by DPPH, ABTS, FRAP, CUPRAC and PM assays.

Samples code	CUPRAC g/l	PM g/l	FRAP g/l	DPPH g/l	ABTS g/l
N1	0.323 ± 0.00	0.446±0.02	0.158 ±0.00	0.015 ± 0.00	0.013 ± 0.00
N2	0.22 ± 0.00	0.262±0.00	0.232 ±0.00	0.01 ± 0.00	0.009 ± 0.00
N3	0.24 ± 0.00	0.248±0.00	0.099 ±0.00	0.013 ± 0.00	0.01 ± 0.00
N4	0.166 ± 0.00	0.162±0.02	0.053 ±0.00	0.010 ±0.00	0.009 ± 0.00
N5	0.261 ± 0.00	0.243±0.00	0.094 ±0.00	0.017 ±0.00	0.01 ± 0.00
J1	0.296± 0.01	0.29±0.00	0.117 ±0.00	0.017 ±0.00	0.01 ± 0.00
J2	0.265± 0.00	0.273±0.01	0.104±0.00	0.023 ±0.00	0.01 ± 0.00
J3	0.255±0.00	0.314±0.00	0.12 ±0.00	0.018 ±0.00	0.01 ± 0.00
J4	0.24±0.00	0.238±0.01	0.097 ±0.00	0.02 ±0.00	0.009 ± 0.00
J5	0.27±0.01	0.287±0.00	0.119 ±0.00	0.018 ±0.00	0.01 ± 0.00
J6	0.25±0.00	0.303±0.01	0.11 ±0.00	0.025 ±0.00	0.009 ±0.00
BHT	0.093 ±0.00	2.495±0.05	0.145±0.00	0.168±0.00	0.021±0.00
TBHQ	0.2845 ±0.00	0.593±0.00	0.056±0.00	0.04±0.00	0.023±0.00
VC	0.366±0.00	0.443±0.00	0.109±0.00	0.001±0.00	0.057±0.00

Each value is a mean ± SD of triplicate analysis, N: November, J: January.

Table III.2: EC50 of *C. arabica L* extract compared with the previous studies.

Plant	DPPH	ABTS	references
Methanol extract of S. thymbra	0.391 ± 0.45 mg/ml	0.178 ± 0.80 mg/ml	[17]
Lemon Essential oils	6.68 ± 0.33 mg/ml	2.97 ± 0.18 mg/ml	[18]
Avocado	41.2±6.4 mgVCE/100g	70.0±10.1mgVCE/100g	[19]
Apple	143.7±47.3 mgVCE/100g	158.7±35.6mgVCE/100g	[19]
Lemon	101.2±2.0 mgVCE/100g	145.8±13.7mgVCE/100g	[19]
Cleome Arabica	13.15 ± 0.01 mg/l	/	[5]
Cleome Arabica	0.00488 mg/ml	/	[6]
Ethyl acetate extract of R.vesicarius	0.140 ± 0.007 mg/ml	/	[20]
Nepeta nepetella	1.45 ± 0.07 mg/ml	/	[21]
Lemon balm	16.35 ± 0.82mg cm-3	10.37 ± 0.62mg cm-3	[18]

In order to get a visual presentation of the antioxidant activity, we plotted in figure III.4 a 3D a bar chart of the antioxidant activity as function of the used method and test samples. We have observed rapid and strong inhibition of both, DPPH radical or ABTS radical cation, after the addition of *C.arabica L* extract. Compared to other methods, from the methodological point of view, the DPPH method is suggested as easy and perfect with regard to measuring the antioxidant activity of fruits and vegetable juices or extracts. The results are highly reproducible and comparable to other free radical scavenging methods such as ABTS [22].

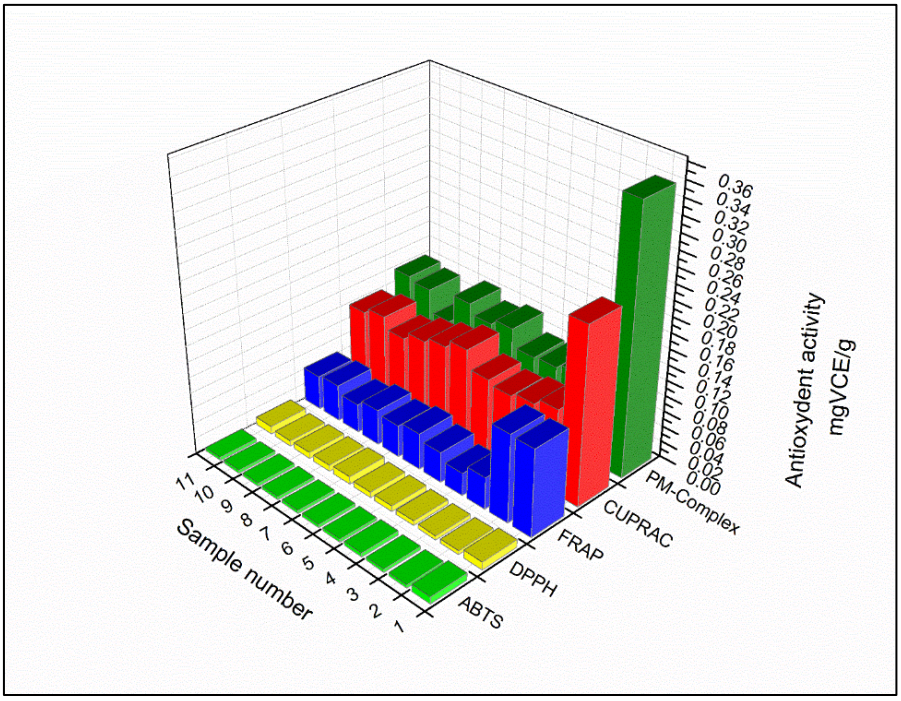


Figure III.4: This figure is a graphical presentation of table III. 2, which show the antioxidant activity levels, from the strongest evaluation method (ABTS) to the weakest (Phosphomolybdate complex).

III.4. Correlation Between Tests:

The (Table III.3) summarized the results of correlation coefficient between antioxidant activity assays, it is clear from this study that the different methods used for the determination of antioxidant capacity are highly correlated. Correlation analysis for phytochemical contents with IC50 values of radical scavenging and/or antioxidant ability of extract. EC50 of ABTS, CUPRAC and FRAP showed a correlation value (r: 0.713, 0.948, 0.985) with PM-complex respectively. it could be suggested that PM-complexe, ABTS, CUPRAC and FRAP were strongly positively correlated with each other (Figure III.5B). This could be explicated by their same reaction mechanisms which belong,for all of them, to the electron transfer reaction method [23]. While non-significant correlation was observed with DPPH, due to the fact that they cannot reflect the same stage of the oxidation process. Therefore, the antioxidant activity should be determined by more than one method and should measure different oxidation products, and this means that the evaluation of the antioxidant activity requires the use of a multiple-method approach, with different mechanisms of inhibition. A positive correlation was also observed between flavonoids and total polyphenolic (r: 0.781), confirming that flavonoids are the most important group of phenols in plants [24]. Furthermore, we can say that the phenolics comprise flavonoids, phenolic acids, simple phenols, stilbenes, coumarins, isoflavonoids, condensed tannins (known as proanthocyanidins), lignans, etc . The levels of these constituents widely fluctuate in plants

and plant parts during different seasons. The type of phenolics and amount of individual phenolic compound present in the sample is one important factor in the antioxidant effect. The potential and presumptive antioxidant capacity of each of these components depend on the kind of test system employed to measure oxidant (including free radical) generation .The total phenolic and flavonoids showed significant correlation (r: 0.646, 0.782) with FRAP, respectively (Figure III.5A), but no significant correlation was observed between flavonoids and (CUPRAC, DPPH, PM-complex, ABTS). This observation may suggest that the reducing power exhibited by the different fractions is not only a function of flavonoids, but also of the existence of other constituents with an antioxidant potential [25]. In order to get clear picture, it is recommended that the compound of interest should be isolated/purified and correlated with different assays. This will give a better estimation and correlation of the antioxidant activity with compound of interest under investigation. In addition, correlations among the antioxidant activity based on DPPH, ABTS, CUPRAC, PM-complex and FRAP assays were positively high and ranged between 0.413 and 0.985; there was a positive and greatly significant relationship for PM-complex & CUPRAC (r = 0.985). Significant correlations were also noted between PM-complex & ABTS value (r = 0.966) and ABTS vs CUPRAC value (r = 0.948). Furthermore, we registered a positive correlation between PM-complex and FRAP (r= 0.713). REC vs FRAP (r= 0.692), This strong correlation is due to the fact that these four methods are based on the generation of a free radical by different mechanisms followed by the detection and quantification of an end point in the reaction. The addition of an antioxidant inhibits the development of the end point, an inhibition that is an expression of the antioxidant capacity of the studied sample [26]. Moderately positively correlated with total flavonoids content vs FRAP: r = 0.646. These results indicate that the antioxidant activities proved by these tests are ensured. Probably by the same active molecules not flavonoids recapitulate the Spearmans–Rho correlation coefficients were reported in TableIII.4. A high flavonoid content evaluated with aluminum chloride colorimetry does not certainly mean a high antioxidant capacity, the low correlation observed between either ABTS/DPPH , CUPRAC, PM-complex results and total flavonoids content was due to the nature of measurement techniques.[27], A weaker correlation was observed between the antioxidant activities and the flavonoid contents, which shows that the presence of a considerable amount of these compounds in a plant does not always imply a corresponding antioxidant potential[28].

Table III.3: Spearmen's–Rho coefficients for the correlation between antioxidant capacities measured by ABTS, DPPH, PM, FRAP and CUPRAC assays, total phenolic and flavonoids content of *Cleome arabica* L extract.

	Phenolics	Flavonoids	ABTS	FRAP	CUPRAC	DPPH	Molybdate
Phenolics	1	0.781	0.377	0.782	0.516	0.086	0.459
Flavonoids	0.781	1	0.0496	0.64	0.057	0.399	0.048
ABTS	0.377	0.0496	1	0.629	0.9477	0.413	0.96
FRAP	0.782	0.646	0.629	1	0.692	0.075	0.713
CUPRAC	0.516	0.057	0.948	0.691	1	0.516	0.985
DPPH	0.0858	-0.399	0.413	0.075	0.516	1	0.521
phosphomolybdenum complex	0.459	0.0478	0.966	0.713	0.985	0.521	1

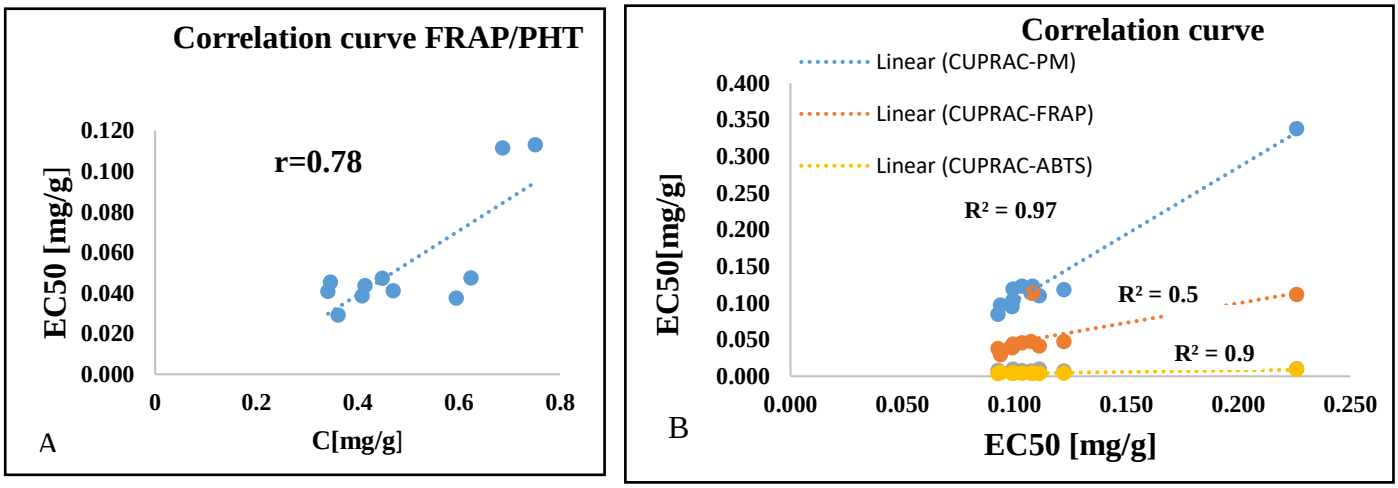
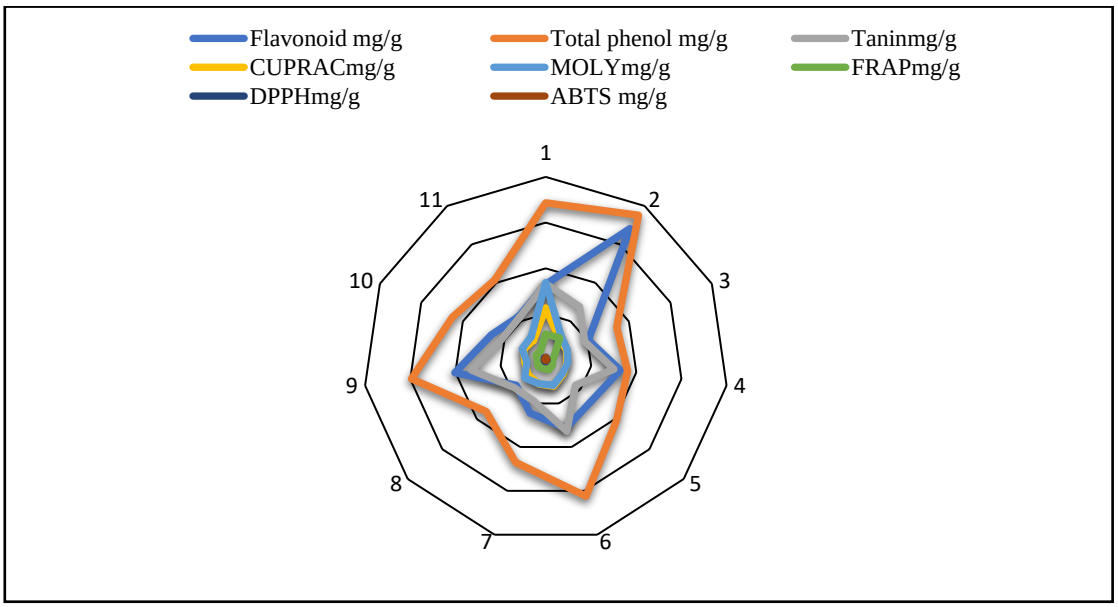


Figure III.5: Correlation graphs for FRAP IC50mg/g values and (A) total phenolic contents, (B). Correlation curve between CUPRAC/PM-complexe,CUPRAC/FRAP,CUPRAC/ABTS.

Figure III.6 represents a radal chart of the tested antioxidant activities, total phenolic, flavonoids and tannins content for eleven samples. The results show that plant samples with high polyphenol contents,

flavonoids and tannins contents have the weakest antioxidant powers. This implies that there is no influence of the flavonoid concentration on the activity, but it is the type of the molecules which act on free radicals. A simple explanation for this variation is that the activity of flavonoids to trap radicals depends essentially on their structure. The position of the double bond in the C ring as well as the number and / or position of the hydroxyl groups (OH) are the most important elements to explain the increase or decrease of the antiradical activity of our phenolic extracts.



FigureIII.6: Quantification and Antioxidant Activities of all samples Radal Chart

In this chapter, we performed a detailed study of the antioxidant activity of *Cleome arabica* leaves extract, in different seasons evaluated with five assays. It was concluded that the hydromethanolic extract of *C.arabica* leaves possesses significant antioxidant activity, meanwhile phenolic compounds were a major support in antioxidant capacity of three organic solvents that were used. A previous study reported that antioxidant capacity depends on the solvent used (A. Floegel et al.2011)[19]. We confirm this remark due to the fact that antioxidant activity is higher in the ethyl acetate fraction than dichloromethane and n-hexane due to the absence of flavonoids in these two fractions. This leads us to investigate ethyl acetate fraction furthermore, the comparison of antioxidant capacities measured by ABTS and DPPH, CUPRAC, FRAP & PM-complex assay does not depend on the growing seasons. Our results showed that these plants can be used as alternative artificial antioxidants used in pharmaceutical industry. The result was better than obtained by certain dietary antioxidants rich in polyphenols and flavonoids extracted from *Cleome arabica* (Djeridane et al., 2010).

III.5. The Effect of *Cleome arabica* Parts in α-Amylase:

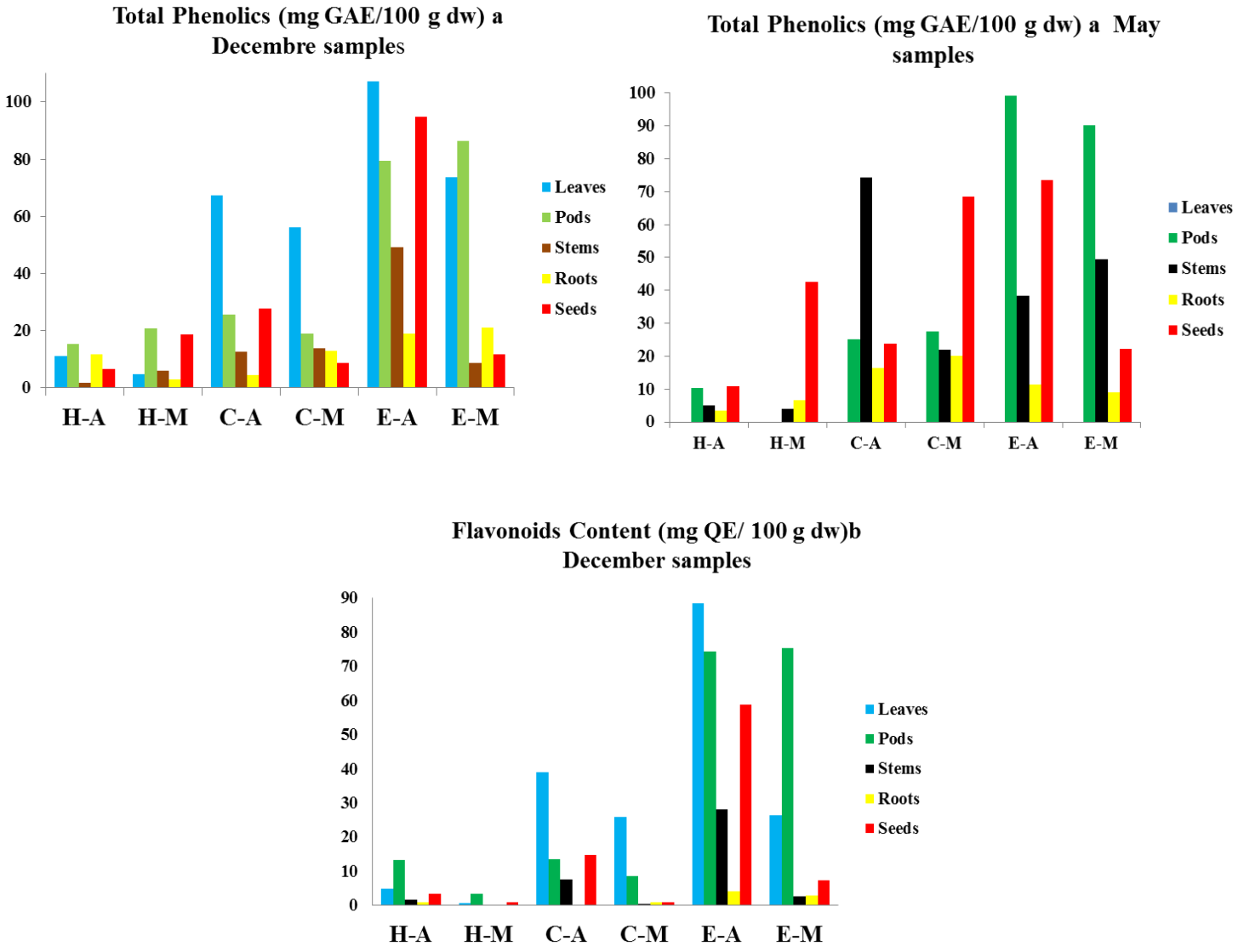
III.5.1. Total phenolic content:

Compared with classical extraction methods like maceration and hot extraction, the ultrasound assisted extraction proved to be simpler but more effective procedure to obtain active compounds from medicinal plants[29-30]. The use of ultrasound decreased significantly the total time of treatment and in addition, it was our target. This extraction method was more effective for the steroids and most of the triterpenes.[31]. It is also one of the preferable alternative methods due to the cavitation effect caused that allows to reduce the solvent needed increasing the release of the desired compounds with low energy requirements [32]. **No** remarkable **differences** were found in *Cleome arabica* extracted by both methods this is similar that found with literatures [33][34].

The quantification of *Cleome arabica* parts polyphenols content was analysed in order to investigate any correlation between the later one and their antioxidant / α-amylase inhibition activity, and also the number of hydroxyl groups related in the nature of polyphenol and their effect in α-amylase. The amounts of total phenolic in the extracts were determined spectrophotometrically conferring to the Folin Ciocalteau reagent (FCR), taking as gallic acid equivalent. The values of total phenols content found in *C.arabica* parts extracts are shown in Figure III.3. The total phenolics of methanolic and acetonic extracts in different solvent of *C.arabica* parts (leaves, pods ,seeds, stem and roots) vary from **2.87** to **107.21** mg/100g at the beginning of maturity stage **(December),** and rang between **3,981** to **99.6** mg/100g at the end of maturity stage **(May),** The different content of phenols and flavonoids in diverse season is very clear, and indicate that the beginning of maturity stage extract has a higher total phenol than the end of maturity stage. The opposite was observed only at ethyl acetate fraction for May samples (end of maturity stage). This can be explained by a possible manipulation error, or that the ethyl acetate fraction could not extract all the bioactive polar compounds in the beginning of maturity stage. These results consolidate our argument that more than one polar solvent must be used to confirm a trend. Moreover, the ethyl acetate fraction was found to be richer in total phenolics and flavonoids (107.21mg/100g and 88.50 mg/100g) respectively in hydro-acetonic extract of the leaves in the **beginning** of maturity stage was found. We can conclude that the **ethyl acetate** is more extractor of polyphenol than dichloromethane and n- hexane [35-36], which may be correlated to their antioxidant and enzyme inhibition properties. Overall, leaves cover a high total phenolic content compared to other organ plant values (see **FigureIII.7**). Generally, acetone solvent is more extractor of phenols compared to methanol [37] ,but the phenolic content of methanolic extract in different fractions of seeds was higher in May compared to December, this can be related to the fact that seeds appear later that other organs. The diversity in flowering period assures continuous

production and maturation of seeds, most seeds can germinate under favorable environmental conditions (high temperature and oxygen content following tillage operations) [38]. We observed that the harvesting time and extraction solvent influence in the quantity of the phenol extracted, which fluctuate in different organ-plant in divers stages of its life. The total phenolic content of *Cleome arabica* was higher than reported for the most of other medicinal and mutual dietary plants [39]. Our results show the best agreement between the total phenolic content and their total flavonoid content of leaves, pods, stems, roots and seeds extract in the beginning maturity stage with correlation coefficient r= (**0.91, 0.98, 0.96, 0.89, 0.98)** respectively (Figure III.10). For the end of maturity stage, we cannot estimate the presence of flavonoids in our plant parts extract. Similarly that found by [40], so the current study in several month is very important for select the best month or season where the plant is at peak production of secondary metabolite [40].

Consequently, the phenolic plants create one of the major groups of compounds that act as enzyme inhibitor, it was mandatory to determine their total amount in the certain plant extracts [41].



A: Acetonic system, **M:** Methanolic system.**H**: Hexanic fraction, **C:** Cloroform fraction,

E: Ethyl acetate fraction.

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

Figure III.7: Graphical presentation shown the total phenolic content and flavonoids content of *C,arabica* parts in December and may month.

III.5.2. Antioxidant Capacity:

We use **DPPH** reagent as a stable free radical in ethanol that easily accepts an electron or hybrid radical and is converted to a stable diamagnetic molecule by reacting with suitable reducing agents. DPPH radicals formed into equivalent hydrazine in 30min (the detail work in chapter II). The EC50 values of ascorbic acid, hydromethanolic and acetonic extract in different fractions of *Cleome arabica* were approximately ranged between 0.0086 and 0.5 mg/ml, the EC50 values of the free radical scavenging activity are summarised in **TableIII.4** with regression coefficients (R^2) of 0.99, these regression coefficients indicate a strong antioxidant potential of this plant similar to that of ascorbic acid, BHT and TBHQ as standards . The antioxidant activity might be attributed to phenolic content, triterpenoids and flavonoid content of the tested plant. Comparable finding were reported by other groups in their *in vitro* studies [43- 44]**.** The results of Table III.4 show the scavenging effect of *C. arabica* fractions on DPPH radical are in the following order: *ethylacetate* > *chloroform* > *n-hexane* fractions in December in all plant organs for acetonic extract/fractions. Moreover, for May samples the order of DPPH values along different fractions was as follow: *chloroform* > *ethyl acetate* > *n-hexane* in the same extract. It was clear that the ethyl acetate fraction contains a higher content of the compounds that have an antioxidant propriety compared with other fractions. On the other hand, the DPPH assay was linearly not correlated to their total phenolic compounds with growth season [44]. In order to get sure of the existence of a correlation between the antioxidant activity and its growth, we have must study its activity in several months. We observe that the plant extract of *Cleome arabica* has a significant antioxidant potential comparable to other works [17-19]. The trend was not comparable in the methanol and acetone extract, it can be perceived that is no important correlation between the phenolic content of the extracts and their antiradical activity as evaluated by the DPPH assay ($r < 0.1$) for the pods, stem and seeds. The results differ from the findings from some medicinal species [45]. There are numerous reasons to explain the ambiguous relationship between the antioxidant activity and the total phenolics. As the total phenolic content did not include all the antioxidants as well as the synergism among the antioxidants in the mixture made for antioxidant activity [5] (see chapter II).

Table III.4. Free radical (DPPH) scavenging activity of the Maceration and the Ultrasonication extracts.

	December						May					
	Maceration Extracts						Ultrasonication extracts					
	Methanol/water (80/20)			Acetone/water (70/30)			Methanol/water (80/20)			Acetone/water (70/30)		
	Hexane	CF	ethyl acetate	Hex-ane	CF	ethyl acetate	Hexane	*CF*	ethyl acetate	Hexane	CF	ethyl acetate
Parts of plant	IC50 (mg/ml)											
Leaves	ND	0.950±0.019	0.696±0.173	ND	0.08±0.032	0.158±0.037	ND	ND	ND	ND	ND	ND
Pods	ND	0.355±0.034	0.386±0.005	ND	0.145±0.025	0.0094±0.003	ND	0.811±0.033	0.093	ND	0.621±0.024	0.195±0.01
Stem	ND	0.436±0.008	1.934±0.00	ND	0.161±0.002	0.048±0.0008	2,642±0,593	0,257±0,024	0,141±0,008	1,803±0,100	0,272±0,003	0,126±0,004
Roots	ND	0.0029±0.002	0.282±0.002	ND	0.404±0.027	0.221±0.0045	0,078±0,011	0,315±0,006	0,254±0,002	0,273±0,019	0,190±0,001	0,254±0,005
Seeds	ND	0.49±0.095	0.560±0.020	ND	1.21±0.07	0.087±0.000	0.171±0.01	0.055±0.00	0.193±0.006	0.511±0.011	0.353±0.016	0.481±0.001
	Antioxydant Standards											
BHT	0.168±0.004											
TBHQ	0.04±0.001											
VC	0.001±0.000											

ND: Not determined

Is there a relation between the antioxidant activity, total phenolic content and alpha amylase inhibitory effect?

To get an answer, we focus and profound our understanding of the beneficial potential of *C. arabica* on diabetic mellitus treatment, which were not investigated before for in vitro α-amylase inhibitory effect.

III.5.3. α-amylase Inhibitory Effect:

The plant extract indicated a highest α-amylase inhibition an increasing of its concentration, The acetonic extract showed the best activity against α-amylase compared with methanolic extract, the IC50 values was very low in ethyl acetate in both pods and seeds cited there IC50 are (IC50 = 0.11mg/ml), (IC50 = 1.03mg/ml) respectively, reflect on the stronger inhibitory effect against alpha amylase, similar to n-hexane fractions with (IC50 = 0.6 mg/ml, EC50 = 1.08 mg/ml) for stems and seeds respectively in the **beginning of maturity** stage in hydro-acetonic extract. IC50 values of 1,007 and 1,525 mg/ml for the n-hexane fraction of acetone and methanol system respectively of roots in the end of the maturity stage (May), the height inhibition activity against α-amylase was found in the n-hexane fraction for all organ plant in hydro-acetonic extract for the beginning of maturity stage (see TableIII.2). The inhibition rate against α-amylase inhibitory activity of the five-best extract/fraction in the beginning and end of the maturity stage summarized in Figure III.8&9.

Considerable correlation is estimated between the total phenolic content in different fraction extract and their α-amylase inhibitory activity in the beginning of maturity stage of **leaves** with r=0.8. This result is better than found by other literatures [46]. Medium correlation recorded with the stems r=0.5 and a weak correlation was found with roots r=0.10 with no significant correlation for the **seeds** and **pods** r= (-0.1, -0.25) respectively in the beginning maturity stage. **At the end** of maturity stage, we recorded a significant correlation between the total phenolic content and their IC50 values against α-amylase for both **roots** and **pods** r= (0.87, 0.7) respectively. Mediocre correlation was detected with seeds with r= 0.65, and a weak correlation was found with stems with r=0.36. The antioxidant activity was not related with α-amylase inhibition activity of *Cleome arabica* parts extract.

The comparison between the inhibition activity of *Cleome arabica* in two seasons gives us the period when the *Cleome arabica* is at its peak of activity against α-amylase that is during the beginning of maturity stage. Moreover, we have observed that the extractor system is very important to screen. The polar phenolic compounds extracted with **ethyl acetate** exhibit a best antioxidant activity in the majority of the plant organ. On the other hand, the amount compounds extracted with **n-hexane** and ethyl acetate cover a higher α-amylase inhibitory activity in the beginning of maturity stage. Consequently, the phenolic compounds exhibit the antioxidant activity is in structure and polarity different that react against the enzyme or in some

cases with no phenolic nature. We also detected that the chlorophyll concentration gets higher with age. Thus, the period of the collect is of great importance. It should also be noted that rain precipitation play an important role in the activity of the plant, and may affect the results. Thus, further studies are needed for this important plant.

The study of possible correlations/no-correlation between the total phenolic content and their anti-oxidant/enzyme inhibition escort us to find the responsible compound group for these activities, but the study of inhibitory effect of this plant against α-amylase rests insufficient, high performance liquid chromatography constitutes a crucial, reliable technique for the characterization of phenolic compounds due to its adaptability. Therefor isolation compounds are necessary to get an idea regarding the type of phytochemicals responsible in this activity. Afterward a correlation between these compounds and their free radical scavenging activity and inhibitory effect study again.

Table III.5. IC_{50} values of the extracts of the Maceration and the Ultrasonication extracts.

	December						May					
	Maceration Extracts						Ultrasonication extracts					
	Methanol/water (80/20)			Acetone/water (70/30)			Methanol/water (80/20)			Acetone/water (70/30)		
	Hexane	CF	ethyl acetate	Hexane	CF	ethyl acetate	Hexane	*CF*	ethyl acetate	Hexane	CF	ethyl acetate
Parts of plant	**IC50 (mg/ml)**											
Leaves	**1.19±0.12**	**7.95±1.95**	**18.45±0.64**	**3.15±1.24**	**6.86±1.6**	**12.85±2.93**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
Pods	**7.09±1.19**	**2.76±0.46**	**5.16±0.12**	**1.9±0.11**	**13.93±1.08**	**0.11±0.01**	**15,57±0,17**	**17,371±1,41**	**no activity**	**4,98 ±0,19**	**6,72±0,40**	**no activity**
Stem	**8.01±1.31**	**1.64±0.07**	**4.76±0.19**	**0.6±0.09**	**0.79±0.79**	**5.35±0.16**	**2.832±0.13**	**6.134±0.85**	**9,379±0,16**	**1.63±0.35**	**3.064±0.48**	**7.112±0.01**
Roots	**1.67±0.19**	**3.73±0.66**	**2.16±0.16**	**1.31±0.14**	**3.14±0.35**	**2.85±0.40**	**1,525±0,23**	**11,088±0,69**	**2,42±0,25**	**1,007±0,04**	**3,811±0,24**	**4,097±0,56**
Seeds	**08.71±1.01**	**2.59±0.15**	**1.77±1.77**	**1.08±0.06**	**2.35±0.17**	**1.03±0.07**	**14,419±0,32**	**2,797±0,16**	**2,543±0,05**	**2,316±0,03**	**3,643±0,08**	**35,248±0,52**

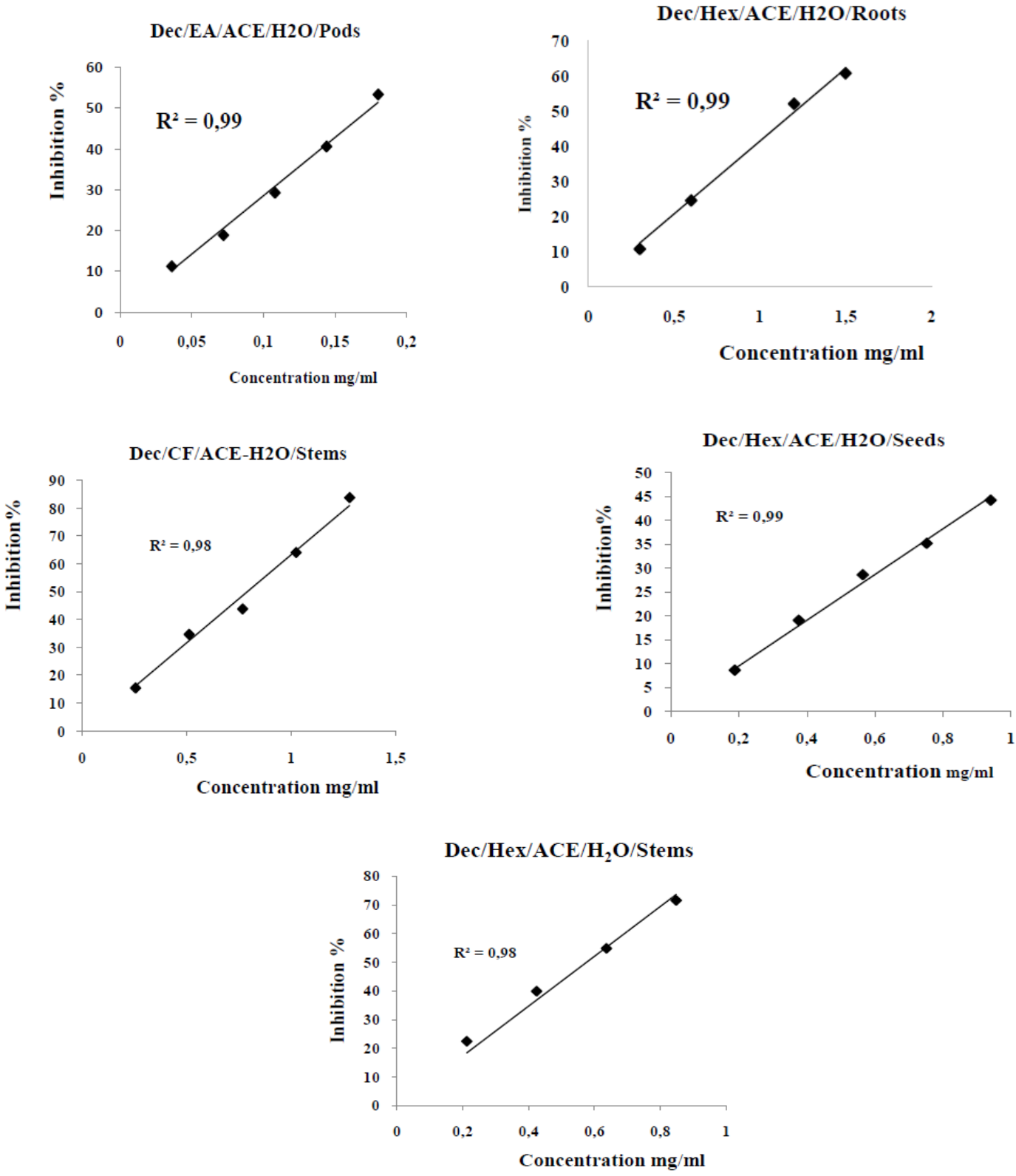
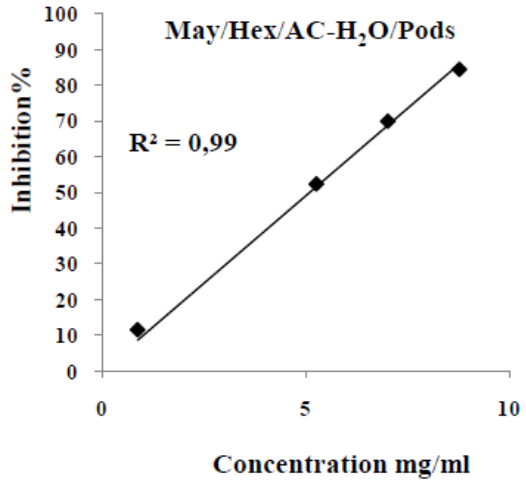
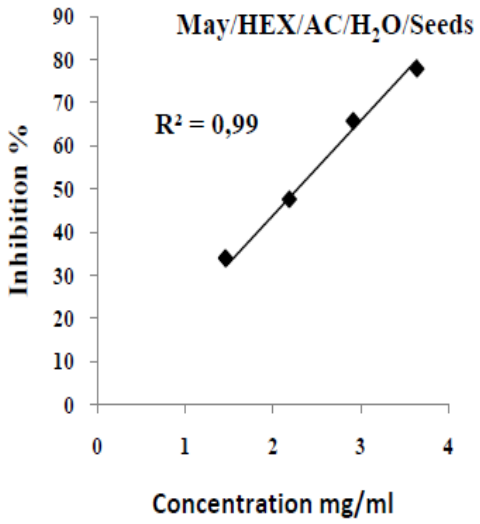
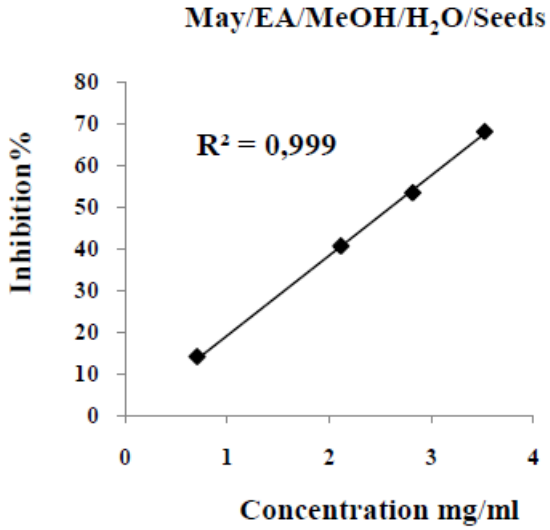


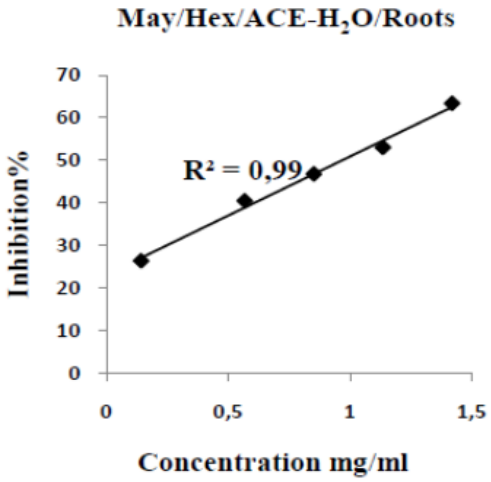
Figure III.8. The inhibition rate against α-amylase inhibition activity of the five best extract in the begnning of maturity stage.

Dec : December; Hex: Hexane; ACE : Acetone; EA : Ethyacetate; CF : Cloroform.









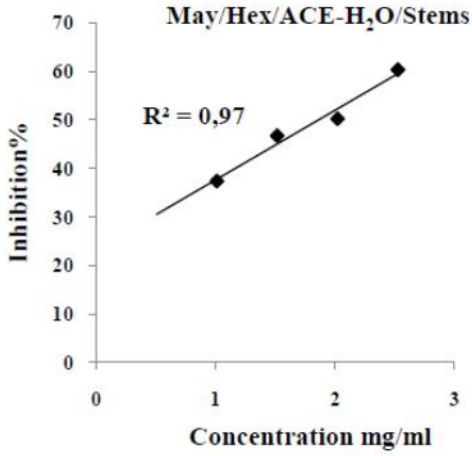
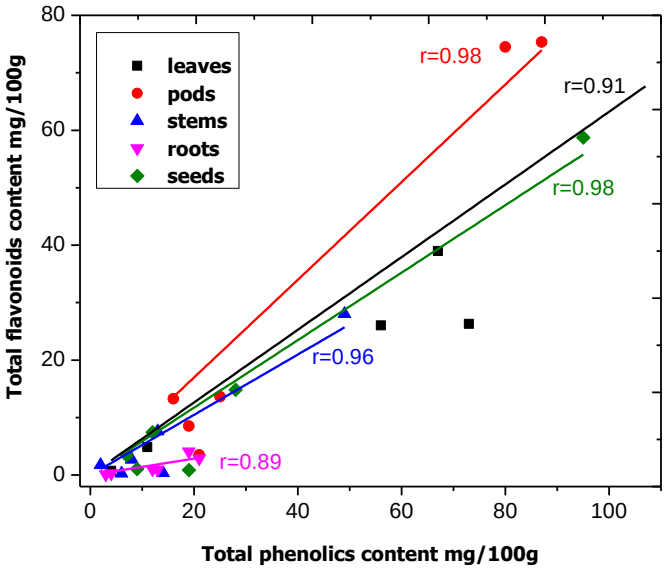


Figure III.9. The inhibition rate against α-amylase activity of the five best extract in the end of maturity stage.



FigureIII.10. Linear correlation between the total phenolic content and their total flavonoids content of each organ plant.

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GENERAL CONCLUSION

GENERAL CONCLUSSION

This thesis contributes to the valorization of natural resources of our region Laghouat, in central Algeria, that have antioxidant and hypoglycemic activity. For this reason, we have conducted a profound in vitro study of the antioxidant and α-amylase inhibitory effect of the *Cleome arabica*, studied for the first time its parts extract in two months, using different extraction systems. The effect of *Cleome arabica* harvesting time on total phenolic content, total flavonoid and tannin contents and their antioxidant capacity of eleven samples, was investigated in this study. Harvesting times (from November,2015 to May 2017) affect the content of phenolic, flavonoids and tannin content, then its effect goes decreasing by the end of maturity stage until its shortage in May Month. We found that the harvesting time and extraction solvent influence in the quantity of the phenol extracted, which fluctuates between 34.1and 75.1 mg GAE/100g dry matter in the ethyl acetate fraction and 17.0 to 49.0mg GAE/100g for the dichloromethane fraction and between 6.0 to 45.3 mg GAE/100g for the n-hexane fraction. The amount of flavonoids content in the ethyl acetate fraction found between 17.2 & 68.2 mg QE/100g and varying between 17.2 to 33.2 mg CE/100g for the tannins content, means that ethyl acetate is more an extractor to the total phenolic than dichloromethane and n-Hexane. The total phenolics of hydro-methanolic and hydro-acetonic extracts in different solvents of *C.arabica* parts (leaves, pods ,seeds, stem and roots) vary from 2.87 to 107.21 mg/100g at the **beginning of maturity stage** (December), and range between 3,981 to 99.6 mg/100g **at the end of maturity stage** (May). The results indicate that the beginning of maturity stage extract has a much higher total phenol than the end of maturity stage .

The disturbance of phenolic compounds varies more in different organs of the plant than the content increase does in areal parts. Flavonoids represent a high ratio in phenolic compounds focused only in ethyl acetate fraction in the rate greater than 50% of total phenolic content, meaning that most of the flavonoids of *Cleome arabica* are flavonoids glycosides which can be easily and perfectly extracted using the hydromethanolic and hydroacetonic systems.

Furthermore, by comparing the five antioxidant activities assays using (ABTS, DPPH, FRAP, CUPRAC and Phosphomolybdenum complex method) with their ability of using different stable radicals to react with antioxidants, ABTS and DPPH assays have been proved a good factor for determining the antioxidant quality of *C. arabica* with EC50 values varying between (0.009 to 0.013 mg/ml) detected by ABTS in the ethyl acetate fraction which remains better than that of the standard. Dichloromethane and n-hexane may be due to the absence of flavonoids in these two fractions. This led us to investigate ethyl acetate fraction still further. Thus, EC50 of ABTS, CUPRAC and FRAP showed a positive correlation (r: 0.713, 0.948, 0.985) with PM-complex, respectively. It could be suggested that PM-complex, ABTS, CUPRAC and FRAP were strongly positively correlated with each other , This could be explicated by their same reaction mechanisms which all belong to the electron transfer reaction method [2]. The results

of Radal chart show that plant samples with high polyphenol contents, flavonoids and tannins contents have the weakest antioxidant powers. This implies that there is no influence of the flavonoid content on the activity, but it is the type and structure of the molecules which act on free radicals. Considerable correlation is estimated between the total phenolic content and their α-amylase inhibitory activity in the beginning of maturity stage of **leaves** (r=0.8), when the weaker correlation has been found with other organs of the plant . This could be explicate that the phenolic compounds that react against the α-amylase are in nature and structure different in plant parts.

The combination of antioxidant tests in the assessment of the bioactivity of natural products in *Cleome arabica* leaves showed a highest antioxidant capacity which does not depend on the growing season. Our results are not in accordance with those noted with other plants [1]. This research highlights the need for further investigation for several months. The results obtained are hardly comparable due to the different mechanisms, redox potentials, pH and solvent dependencies, etc, of various assays. The n-hexane extract provided a significant inhibition of the enzyme compared to chloroform and ethyl acetate extract, suggesting that the plant contained potential apolar inhibitor compounds which may contribute to its inhibition effect on α-amylase in a hydro-acetonic system. Aqueous acetone extract of *Cleome arabica* parts displayed most effective inhibition against the α-amylase enzyme in the beginning of maturity stage in the ethyl acetate fraction for the pods and seeds with IC50 are (IC50 = 0.11mg/ml), (IC50 = 1.03mg/ml), respectively; similar to n-hexane fractions with (IC50 = 0.6 mg/ml, EC50 = 1.08 mg/ml) for stems and seeds, respectively in the **beginning of maturity**. Similarly, aqueous acetone extract of *Cleome arabica* parts exhibited most effective inhibition **(**IC50=1.007mg/ml, IC50=1.63mg/ml) for roots and stems, respectively in the n-hexane extract for the end of maturity stage. We can conclude from the results of correlation between the total phenolic content and their effect against the enzyme, that the phenolic compounds exhibit the antioxidant activity which is in nature and structure different from that which reacts against the enzyme.

In order to get a clear depiction of the content of this plant, it is recommended that the compounds of interest should be isolated/purified and correlated with different antioxidant activity assays and that their effect on α-amylase be studied. Likewise, in-depth studies in vivo, using diabetic mice are desired to result in the application of molecules isolated from this plant as an alternative to chemical molecules with undesirable effects. In the silico approach, analysis of the selected compounds identified from HPLC-MS could lead to further development to find the potent α-amylase, α-glucosidase inhibitors for the treatment of diabetes Mellitus.

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ANNEX 1

This annex regroup all calibration curves of total phenolic flavonoid and tannin content, also Anti-radical curves of standard anti-oxidant and Inhibition rate of January and November samples evaluated by five antioxidant activity assays.

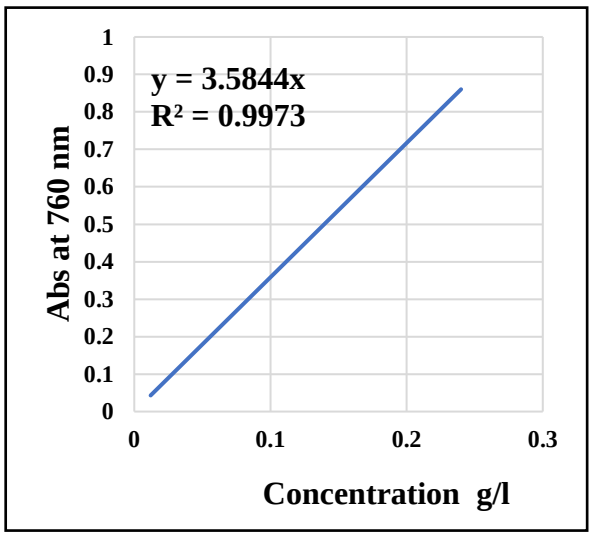


Figure 1: Gallic acid Calibration curve.

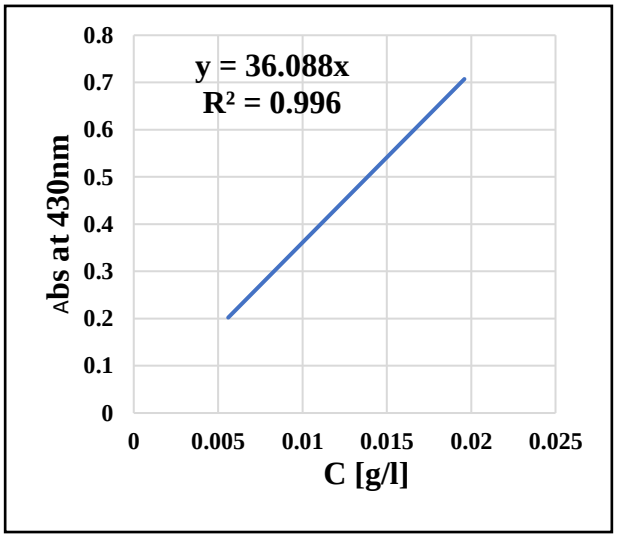


Figure 2: Quercetin Calibration curve

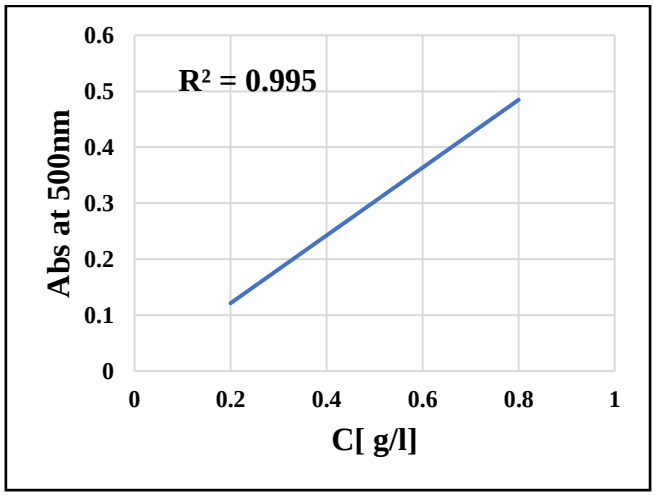


Figure 3: Catechin Calibration curve

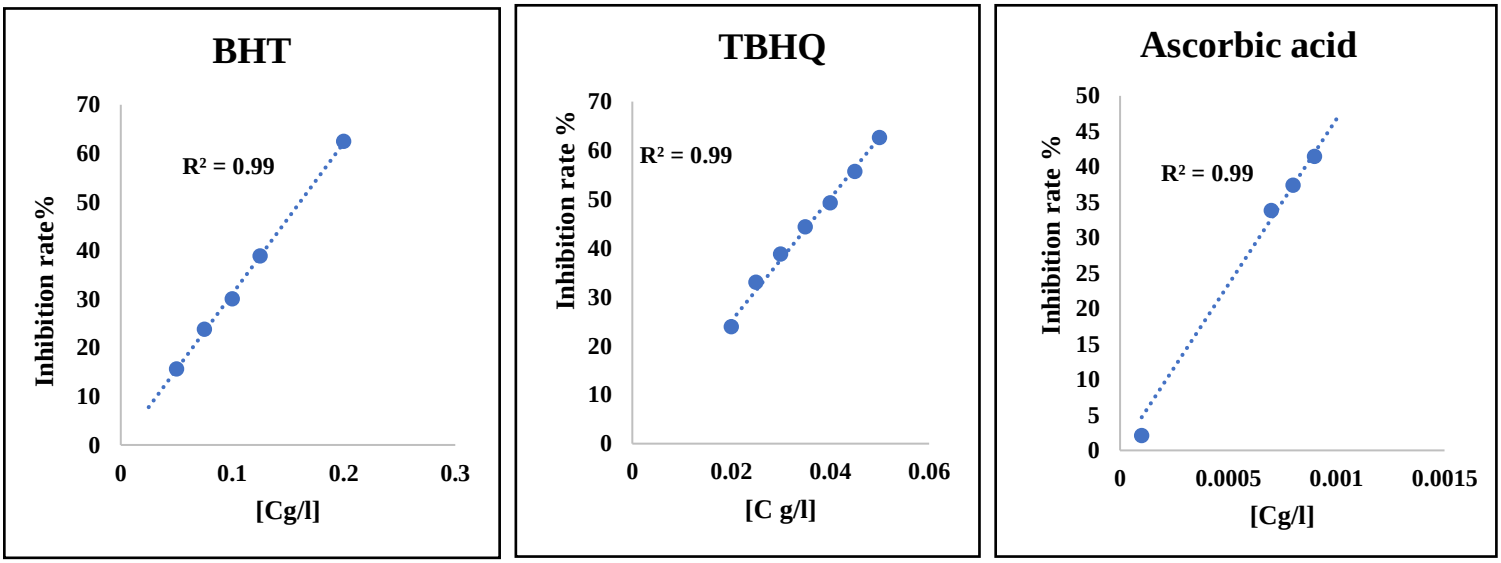


Figure 4. Antiradical curves of standard anti-oxidant evaluated with DPPH method

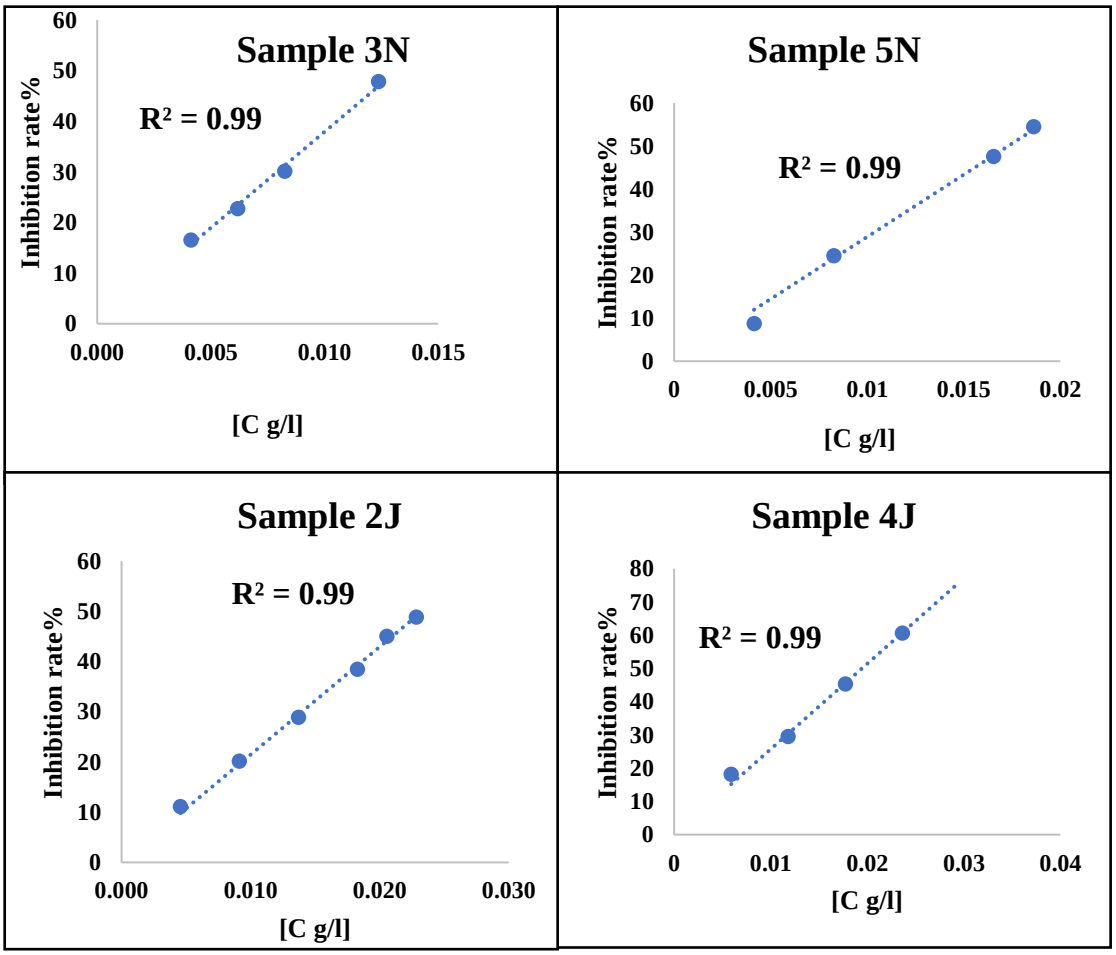


Figure5: Inhibition rate of January and November samples evaluated by DPPH method

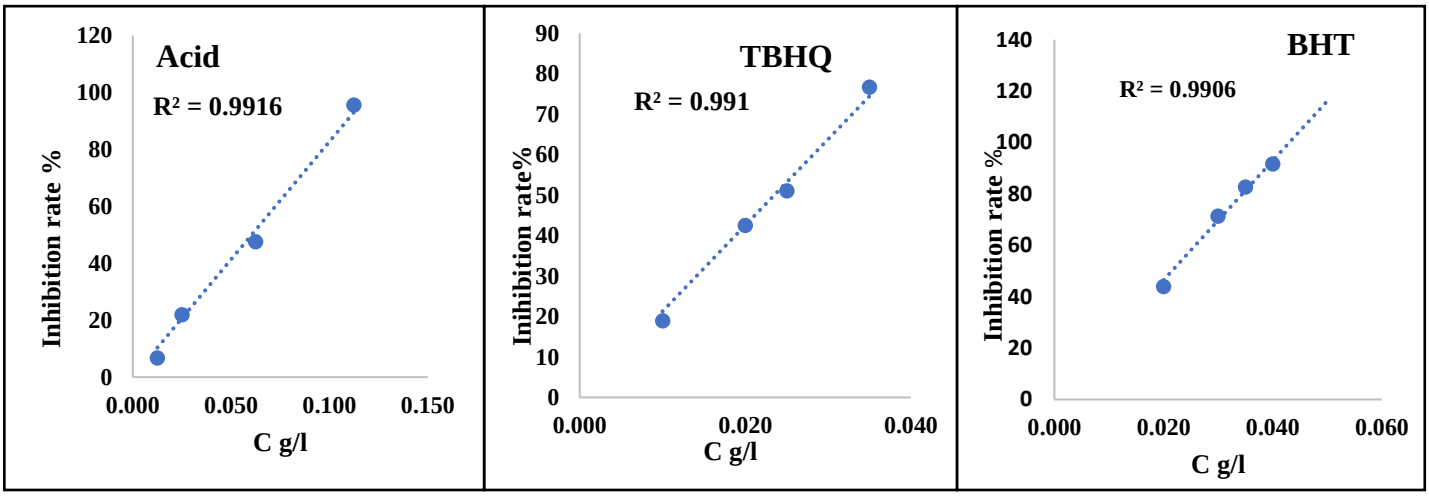


Figure 6. Antiradical curves of standard anti-oxidant evaluated with ABTS method

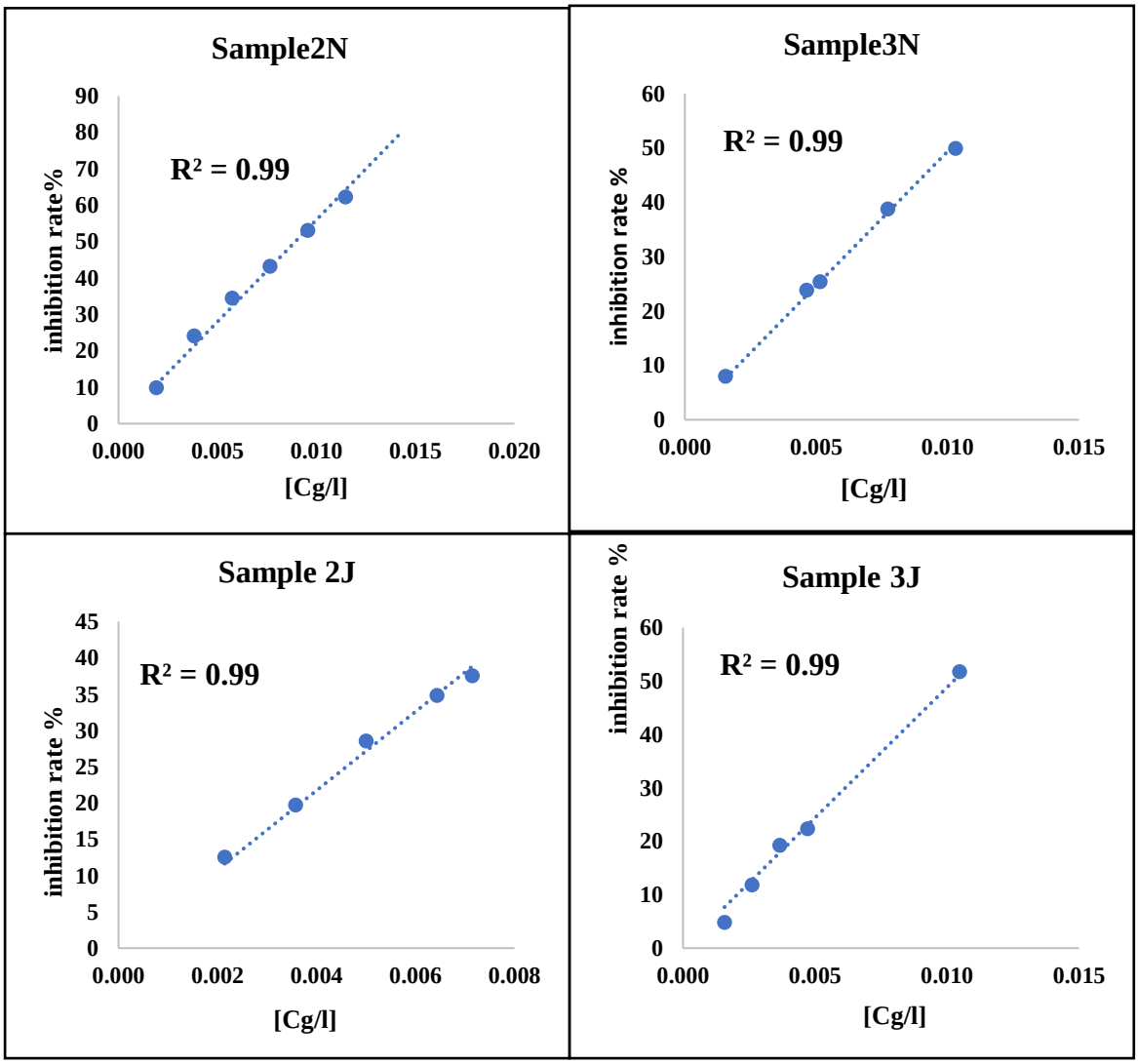
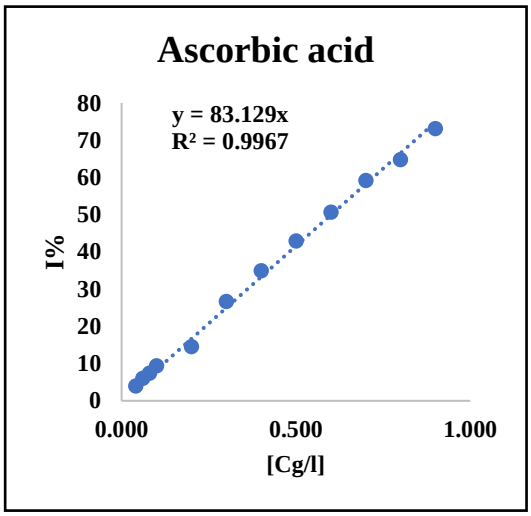
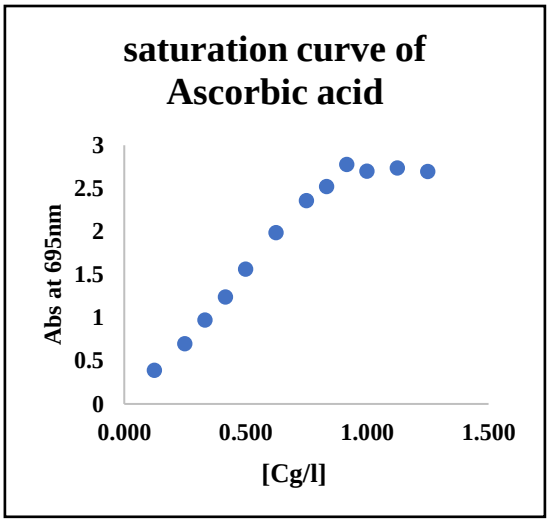
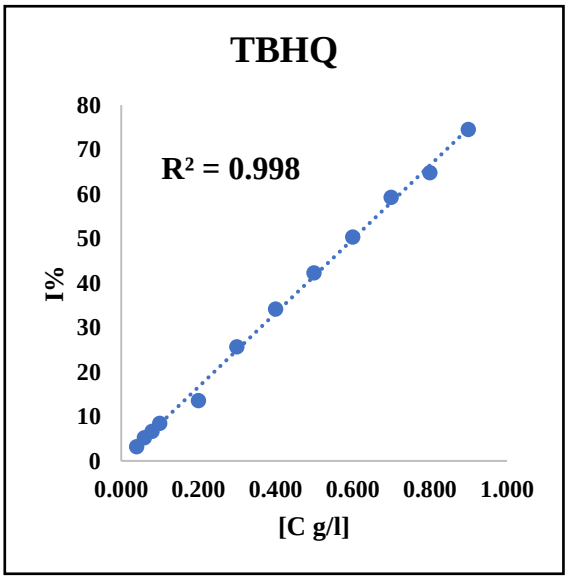


Figure 7: Inhibition rate of January and November samples evaluated by ABTS method.







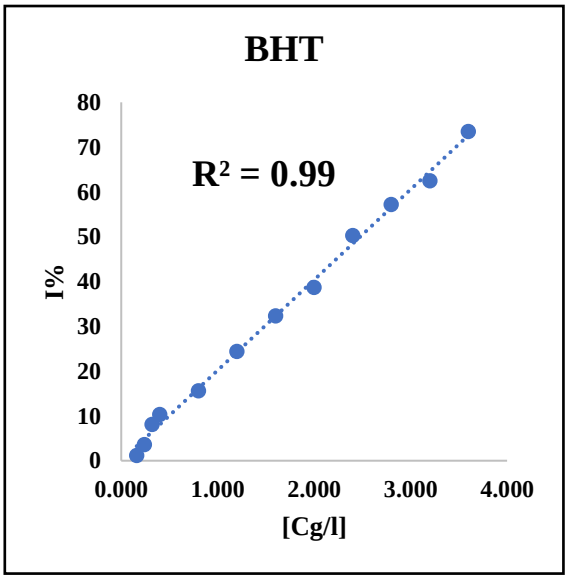


Figure 8: Antiradical curves of standard anti-oxydant evaluated with molybdate method

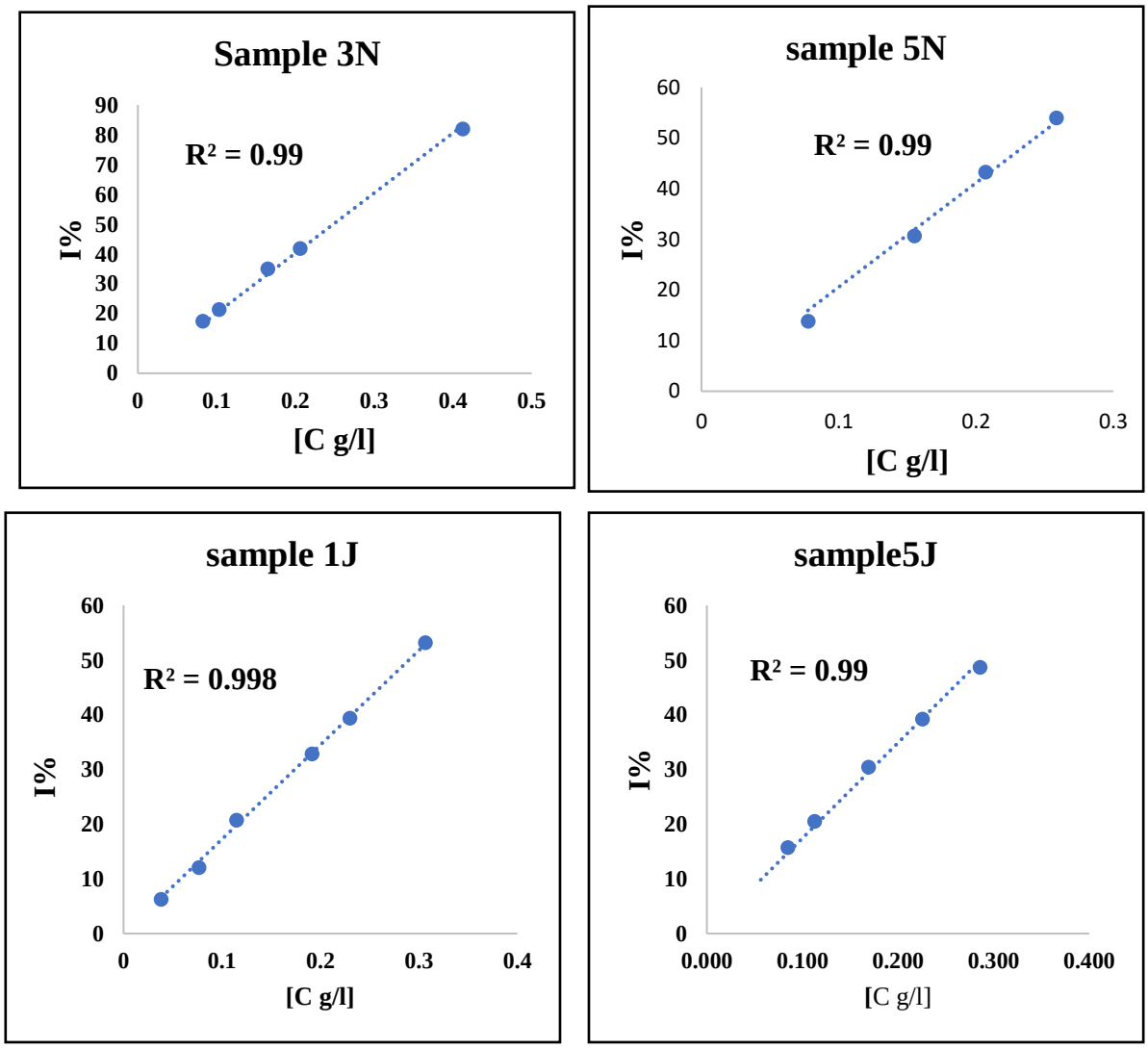
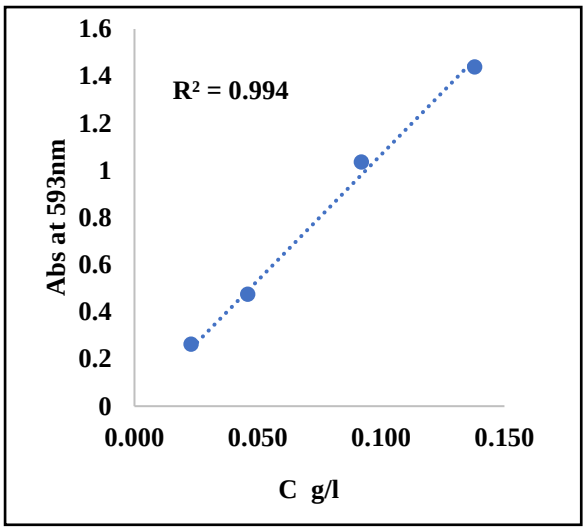
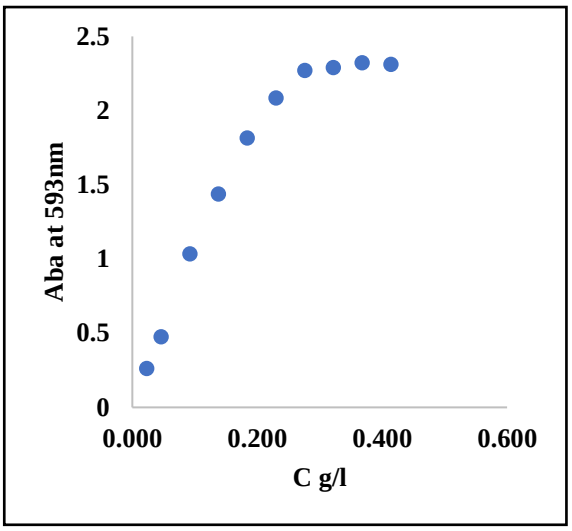


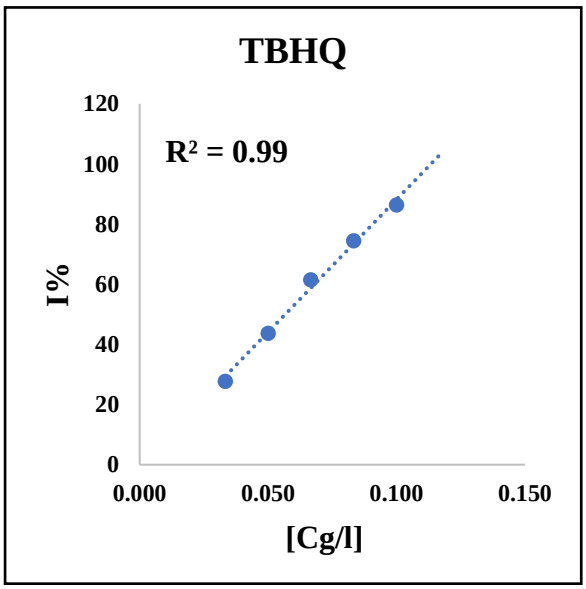
Figure 9: Inhibition rate of January and November samples evaluated by molybdate method.



Calibration curve of Ascorbic acid



Saturation curve of Ascorbic acid



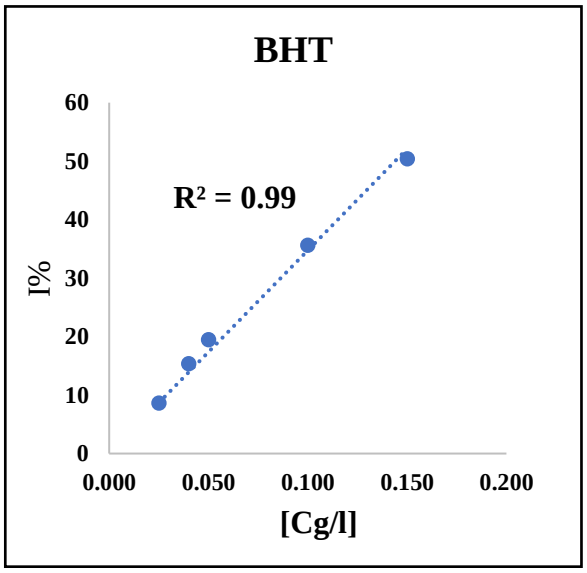


Figure 10. Antiradical curves of standard anti-oxydant evaluated with FRAP method

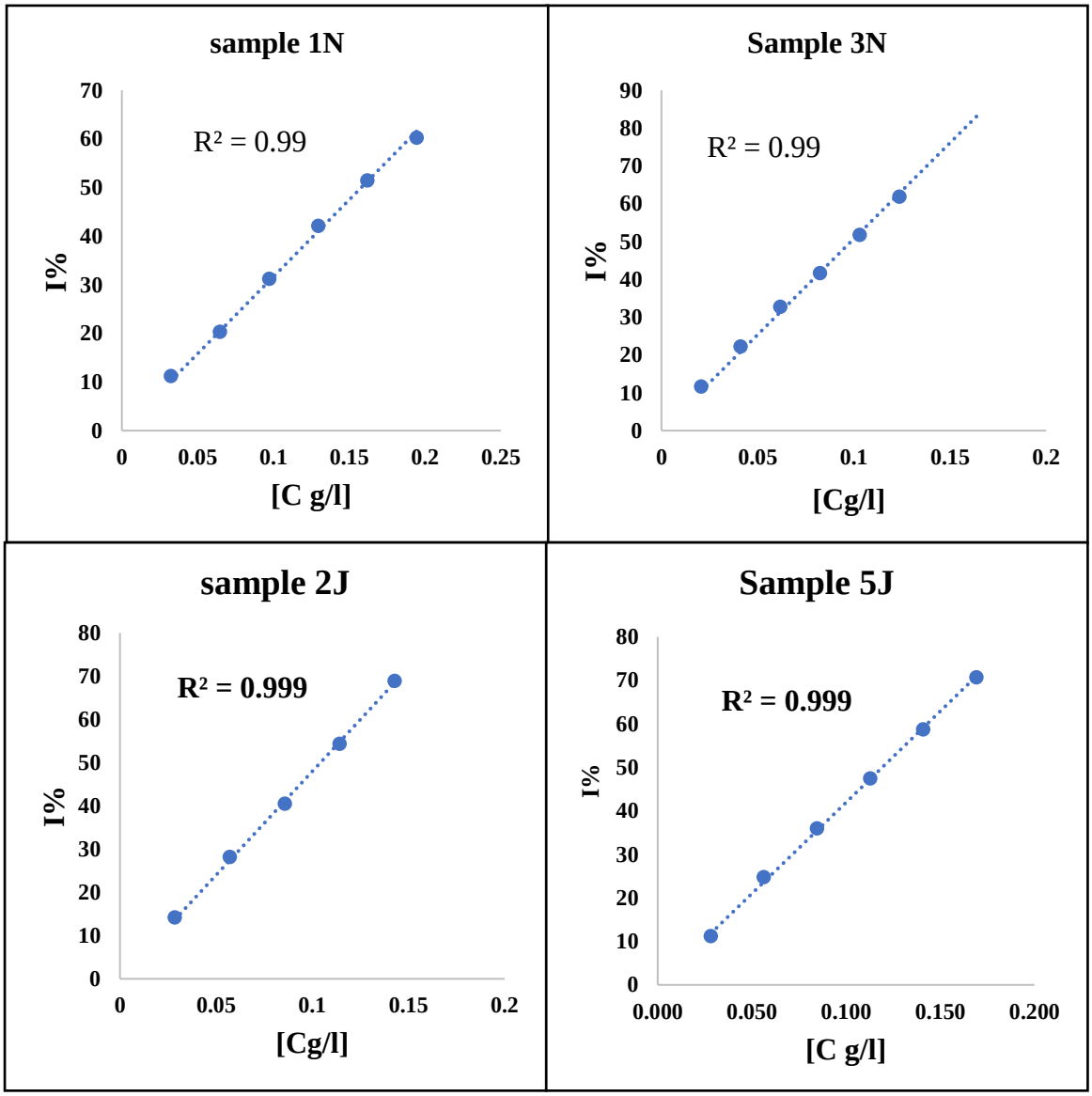
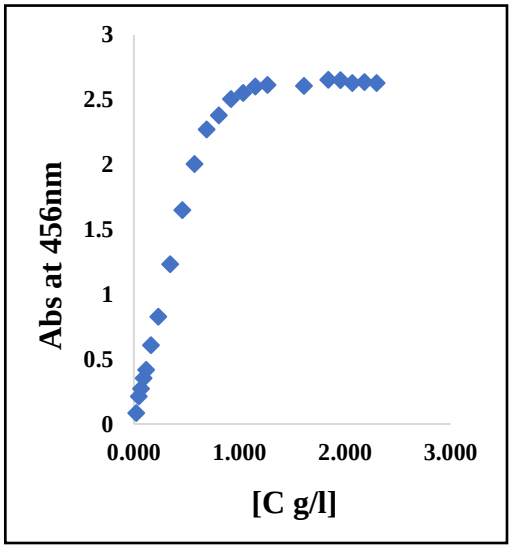
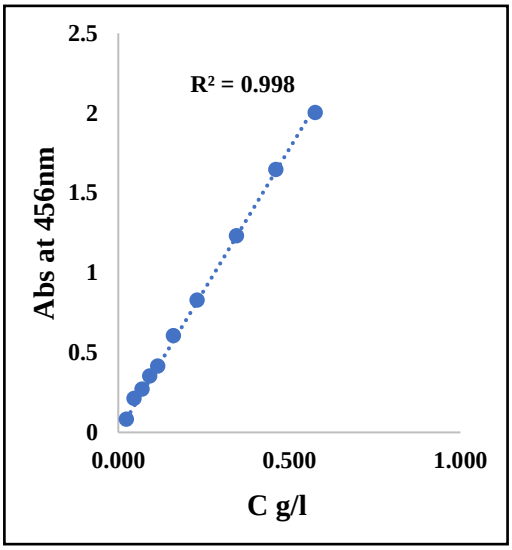


Figure 11: Inhibition rate of January and November samples evaluated by FRAP method

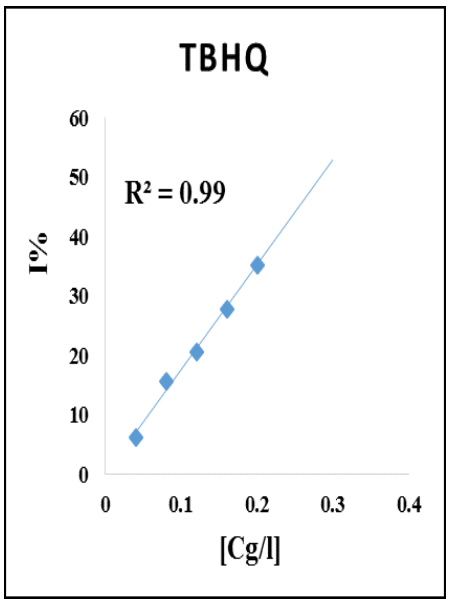
ANNEX 1

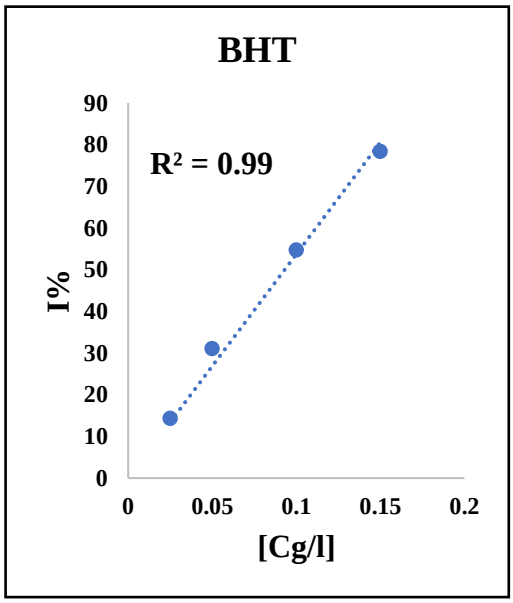


Saturation curve of Vitamin C



Calibration curve of Vitamin C





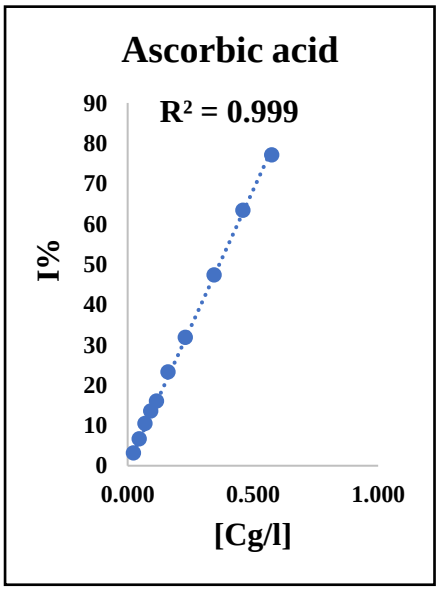


Figure 12: Antiradical curves of standard anti-oxydant evaluated with CUPRAC method

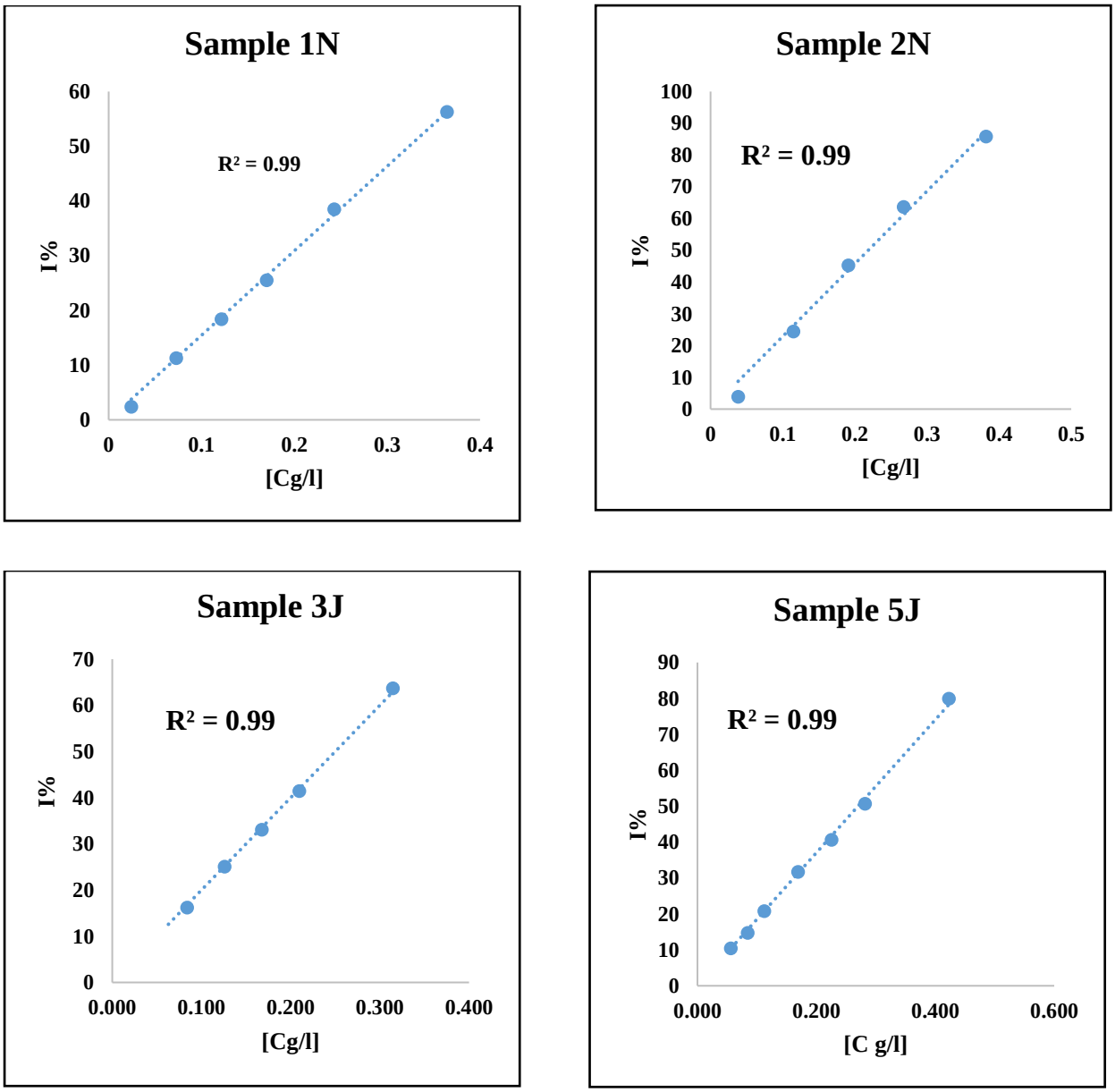


Figure 13: Inhibition rate of January and November samples evaluated by CUPRAC method

List of Scientific Contributions :

1)- Original Paper: α-amylase Inhibitory Effect and Antioxidant Activity of Cleome arabica Depending on Seasonal Variations, *Current Enzyme Inhibition* 14.3 (2018): 217-225: Bentham Science Publishers

Seglab Fatiha, Khacheba Ihcen*, Boussoussa Hadjer, Chaoua Hosseyn, Djeridane Amar and Mohamed yousfi

2)-Original Paper: High in vitro Antioxidant Capacities of Algerian Cleome arabica Leaves' Extracts

Hautes capacités antioxydantes in vitro des extraits de feuilles de Cleome arabica d'Algérie

F. Seglab · C. Hamia · I. Khacheba · A. Djeridane · M. Yousfi

3) - Communication: Antidiabetic and Antioxydant Activities of Various Extracts From Different parts of Cleome arabica Grown in Algeria. In Qatar Foundation Annual Research Conference Proceedings (Vol. 2018, No. 2, p. HBPD795). Qatar: HBKU Press.

Seglab, F.atiha, Mohamed, Y., & Khcheba, I. (2018, March)

4)- Communication: Comparison Of Five Evaluation Antioxidant Activity Methods On Cleome arabica Leaves Extracts And Their In Vitro Antidiabetic Activity In Different Season.Relizane : 1er Séminaire National sur l'Environnement et le Développement Durable

Seglab Fatiha, Khacheba Ihcen, Chaoua Hosseyn,and Mohamed yousfi

5)- Communication: Six Algerian medicinal plants used in diabetes therapy as strong inhibitor of α-glucosidase activity, Eloud:Seminaire International Sur Les Plant Medicinal(SIPM2018)

Abir bakhawa, Khacheba Ihcen, boussoussa hadjer,**Seglab fatiha** ,Noussaiba bensayah and Mohamed yousfi

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Current Enzyme Inhibition, 2018, *14*, 1-9

RESEARCH ARTICLE

α-amylase Inhibitory Effect and Antioxidant Activity of *Cleome arabica* Depending on Seasonal Variations

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ARTICLE HISTORY

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Abstract: ***Background*:** *Cleome arabica* belongs to the family of Capparaceae. It has attracted a great deal of attention in recent years for its pharmaceutical potentials due to its antioxidant, antihypercholisterolemic, anti-cancer and chemoprotective activities for the treatment of a number of diseases.

***Objective*:** The main study was to evaluate the α-amylase inhibitory and antioxidant activities of extracts from *Cleome arabica* collected on two seasons (December 2016 and May 2017) in the town of Laghouat steppe region of Algeria. In addition, the purpose of the study was to investigate a new natural inhibition of α-amylase using an *in vitro* model, as well as to find a natural anti-diabetic compound from the plant.

***Method*:** The extracts were prepared with two solvent systems: hydroalcoholic and acetonic solvent systems. The polyphenolic contents of methanol and acetone extracted samples were evaluated *in vitro* in two different ways: total phenolic contents and total flavonoid content. The antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl radical, test enzyme inhibitory effect against α-amylase of n-hexane, chloroform and ethyl acetate were investigated with spectrophotometric methods.

***Results*:** The phenolics and flavonoid contents varied from (2.87 to 107.21mg Gallic acid equivalent/100g dry matter) and from (0.12 to 88.50 mg Quercetin equivalent /100g) respectively for the beginning of maturity stage (December) in all plant organs. The radical scavenging activity values of the 1, 1-diphenyl-2-picrylhydrazyl radical test ranged between 0.009 to 0.481 mg/ml in different plant organ extracts. The inhibitory effect of the *Cleome arabica* extracts on α-amylase was also investigated and concentrations that gave 50% inhibition of maximal activity were found in acetonic extract with finest values (0.11 and 1.01 mg/ml) in the beginning and the end of maturity stage respectively at ethyl acetate and n- hexane fraction for the pods and roots, respectively.

***Conclusion*:** This study is the first report on potential inhibition of these plant extracts on the digestive enzyme, α-amylase. The results indicate that *Cleome arabica* of Algeria is a powerful natural antioxidant and also could provide natural biologically active agents to be used in the management of diabetes.

Keywords: Antioxidant activity, *Cleome arabica,* phenolic compounds, α-amylase.

1. INTRODUCTION

Cleome arabica belongs to the family of *Capparaceae* which has several species widely grown in tropical and subtropical regions of the world [1], more common in the steppe climate of North Africa. Also known as *Spider flower*, this species is widely distributed in the desert areas of Algeria (*"Netteina"* locally called). The entire plant has a disagreeable odour; especially in its maturity that disappears after dryness. The Herders of The steppe region know its potential medicinal effect, by observing its consummation by certain animals in the herd.

Cleome arabica covers an aggregate of rhamnosylated and glucosylated flavonols, kaempferol, isorhamnetin and the 11-α-acetylbrachy-carbone-22(23)-ene, which are the best inhibitory compounds isolated recently [1-4] and thus possible to use for the protection of liver, heart and kidney functions, and in the treatment of abdominal and rheumatic pains [5]. It is a plant that has received much research attention and has been extensively investigated for its pharmaceutical potential due to its antioxidant, antihypercholisterole-

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mic, anti-cancer and chemoprotective activities for the treatment of a number of diseases and pathological conditions [4-6], it also possesses antimicrobial and antifungal activities [4-7].

The richness of *Cleome arabica* with various secondary metabolite leads to a significant variation in phenolic compounds between plant organs (Leaves, pods stems, roots and seeds). This variation is observed for phenolic acids, flavonoids, terpenoids and alkaloids [8].

Diabetes mellitus is one of the important chronic diseases which corresponds to a rise in blood glucose related to insulin resistance and / or insulinopenia [9]. One therapeutic approach to decrease the hyperglycemia is to retard and reduce the digestion and absorption of ingested carbohydrate in the digestive organs by the inhibition of enzymes involved in their digestion such as α-amylase [9, 10].

Various prescribed antidiabetic drugs, are currently available to reduce, control and manage diabetes mellitus, such as acarbose, miglitol, voglibose, biguanides, sulfonylureas, meglitinides and thiazolidinediones. But most classes of these pharmaceutical drugs have serious side/adverse effects such as flatulence, diarrhea and abdominal pain. Thus, finding new α-amylase inhibitors with minor side effects are of great importance. Research interest in this field has lately been focused on finding new, naturally occurring and safe specific α-amylase inhibitors from medicinal as well as non-medical plants and herbs. Therefore, safer natural amylase inhibitors have been reported from plant sources [11-21]. However, the choice of organ and the optimal harvesting time is necessary for the production of plant active products with high medicinal value. Therefore, the aim of this study is to evaluate phenolic compounds, and *in vitro* antidiabetic (by inhibition of α-amylase) and antioxidant effects of *Cleome arabica* collected in the different season in the town of Laghouat in the steppe region of Algeria. With the purpose of finding the factors that may give better and reliable results about the antioxidant and α-amylase inhibitory activities of *Cleome arabica* parts. To the best of our knowledge, the inhibitory effect of Algerian *Cleome arabica* against α-amylase has never been investigated before. Also, this work is a continuity of studies on *Cleome arabica* [2] and other antidiabetic studies on Algerian plants realized in our laboratory [18, 22-24].

2. MATERIALS AND METHODS

2.1. Plant Material

The plant (*Cleome arabica*) was collected in two different seasons (two major phonological stages) : December 2016 and May 2017. The two samples were harvested at Laghouat city in the steppe region of Algeria.

The plant was identified by Pr Djeridane Amar a member on the laboratory of Fundamental Sciences, University of Laghouat, and a voucher specimen was deposited into the herbarium of the laboratory of Fundamental Sciences under numbers : 02/2016/CA and 04/2017/CA for December 2016 and May 2017 samples respectively. The plant was separated into leaves, pods, stem, seeds and roots. The different parts were dried, ground and kept until analysis.

2.2. Reagents

All chemicals reagents were from Sigma (USA), Aldrich (Milwaukee, USA), Fluka Chemie (Buchs, Switzerland), and Merck (Germany). The solvents used were of analytical grade.

2.3. Extraction

2.3.1. Maceration Extracts

Extraction was carried out for December sample. Five grams of each powder was extracted for 24 h in two solvent systems, with 100ml of 80/20 (v/v) (methanol/water) and with 100ml (70/30) (v/v) (acetone/water) at room temperature. After filtration and removal of methanol and acetone under reduced pressure in a rotary evaporator at 45°C, the remaining aqueous solutions were sequentially extracted until total exhaustion with organic solvents having an increasing polarity such as: n-hexane, chloroform and ethyl acetate. The organic fraction was dried with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 10 mL of methanol and kept at 4°C.

2.3.2. Ultrasonication Extracts

This extraction was carried out for May sample. Five grams of each powder was extracted for 1h by ultrasonication in two solvent systems, with 100ml of 80/20 (v/v) (methanol/water) and with 100ml of 70/20 (v/v) (acetone/water) at 50°C. After filtration and removal of methanol and acetone under reduced pressure in a rotary evaporator 45°C, the remaining aqueous solutions were sequentially extracted until total exhaustion with organic solvents having increasing polarity such as: n-hexane, dichloromethane and ethyl acetate. The organic fraction was dried with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 10 mL of methanol and kept at 4°C.

2.4. Determination of Total Phenolics Compound

The amount of total phenolics in the samples was determined spectrophotometrically with the Folin-Ciocalteu reagent using the method of Singleton and Rossi [25] (Singleton and Rossi 1965) modified by [26, 27]. The procedure is as follows: 100 µL of each sample was added to 500µL of the aqueous solution of Folin-Ciocalteu reagent at 10%. After 3min of incubation at room temperature, 2mL of 2% (w/v) sodium carbonate in water was added. Blanks were prepared by replacing the reagent with water to correct for the interfering compound. After 30 min of incubation in the dark at room temperature, the absorbance of all samples was measured at 760 nm using the Shimadzu UV-1601 UV-Visible spectrophotometer [28]. The Gallic acid was used as a standard and all the assays were carried out at least in triplicate.

2.5. Quantification of Flavonoids Content

The flavonoids content in the extracts was determined spectrophotometrically according to the method of Laimaison and Carnat [29] (Laimaison and Carnat 1991),

modified by [26, 30], using a method based on the formation of the complex flavonoids-aluminium, having an absorption maximum at 430 nm. Quercetin was used for the calibration curve.1mL of diluted sample was mixed with 1mL 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 with a Shimadzu UV-1601 UV-visible spectrophotometer. All samples were analyzed in triplicate and the flavonoids content is expressed in mg Quercetin equivalent (RQ) per 100 g of dry weight material [31].

2.6. Evaluation of Antioxidant Activity (DPPH Assay)

The DPPH radical scavenging assay was performed according to the method reported by [32]. With minor modifications [33, 34].

The solution of DPPH· in ethanol (100μM) was prepared daily before measurements. Various concentrations of 500 µl of sample solution diluted in ethanol were added to500 µl of the DPPH· radical solution. The mixture was then shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorption was measured at 517 nm using the same spectrophotometer.

The percentage scavenging of the DPPH· free radical was measured using the following equation:

$$\text{Percentage effect (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

The antioxidant activity of the extract was expressed as an IC_{50} value defined as the concentration (in mg/ml) of the extract that inhibited 50% of DPPH radicals. The DPPH radical scavenging activity obtained for each plant extract was compared with that of Vitamin C and Vitamin E as a reference compound.

2.8. Assay for Aspergillus Oryzsae α-amylase Inhibitory Activity

α-amylases are starch degrading enzymes that catalyze the hydrolysis of α-1,4-O-glycosidic bonds in polysaccharides with the preservation of α-anomeric configuration in the products, [35]. The inhibitory activity of *C. arabica* fractions on α-amylase from *Aspergillus oryzae* was assayed, using a method adopted from [36], on its substrate starch using 3,5-dinitrosalliculic acid (DNS) as a reagent [37].

In brief, 200 µL of sodium phosphate buffer saline (pH = 6,9) was mixed with 100 µL of soluble starch (1 %) and incubated with 100 µL of suitable aliquots of the test sample at 37 °C for 5 min, then the reaction was started by the addition of 100 µL of *Aspergillus oryzae* α-amylase (13 U/ml ; one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per minute under assay conditions) (Khacheba *et al.* 2014) after incubation at 37°C for 2 min, the reaction was stopped by adding 3,5-dinitrosalliculic acid (DNS) and boiled for 5 min at 100°C.

The mixture was cooled and diluted with distilled water. The absorbance of the mixture was measured at 530 nm and α-amylase inhibition was expressed as a percentage of inhibition.

$$\text{Inhibitory activity (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

$A_{control}$: Absorbance without inhibitor.

A_{sample} : Absorbance with inhibitor.

2.9. Statistical Analysis

Each data point in the results is the mean of three replicates. The statistical significance of the treatment effect was expressed as mean ± SD.

3. RESULTS AND DISCUSSION

3.1. Total Phenolic Content

The phenolic compounds constitute one of the major groups of compounds that act as antioxidant activity. It was mandatory to determine their total amount in the selected plant extracts [23, 27, 33, 38, 39]. The quantification of *Cleome arabica* parts polyphenols content was analysed in order to investigate the presence of any correlation between the latter one and their antioxidant and α-amylase inhibition activities.

The content of phenolic compounds was expressed in mg equivalent Gallic acid/100g of dry matter in the December extracts (beginning of maturity stage), varied between 2.87 and 107.21 mg/100 g, and varied between 3.98 and 99.6 mg/100 g in the May extracts (end of maturity stage). The highest amounts were found in the ethyl acetate fractions while the lowest amounts were found in n-hexane fractions. The leaves and pods showed the highest values in total phenols (107.21and 99.6mg/100g) respectively in acetonic fractions against roots which showed the lowest value2.87 mg/100g in methanlic fraction.

The phenolic content of methanolic extract in different fractions of seeds was higher in May as compared to December.

We can conclude that ethyl acetate is a better extractor of the phenolic compounds than chloroform and n- hexane. As the phenolic compounds have OH hydroxyl groups in their structures, they will be extractable by polar solvents such as acetate ethyl which justifies the importance of the amount of phenols in the latter.

We note that variation of seasons and the extraction solvents influence the quantity of the phenolic compounds extracted, so the total phenolic content changes in different organ and extraction solvent in the diverse stage of its life. The total phenolic content of *Cleome arabica* was higher than reported for the most of other medicinal and mutual dietary plants [38-40].

The highest amounts of flavonoids were found in ethyl acetate fractions. All the December fractions showed the presence of flavonoid while those of May do not show the presence of flavonoids in all the extracts which can be explained by the fact that they are synthesized when plants are in adverse conditions, including attack by herbivores, microorganisms and the presence of different species that compete for light, water and nutrients [41]. The plant increases the concentrations of phenolic compounds in the foliage due to

Table 1. Total Amount of *Cleome arabica* Phenolics Compounds and flavonoids.

	December						May					
	Maceration Extracts						Ultrasonication extracts					
	Methanol/water (80/20)			Actetone/water (70/30)			Methanol/water (80/20)			Actetone/water (70/30)		
	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate
Parts of plant	**Total Phenolics (mg GAE/100 g dw)a**											
Leaves	4.48 ± 0.2	56.32 ± 4.62	73.67 ± 0.83	11.21 ± 5.10	67.25 ± 2.38	107.21 ± 0.83	ND	ND	ND	ND	ND	ND
Pods	20.90 ± 2.15	18.87 ± 1.25	86.50 ± 2.92	15.34 ± 4.07	25.66 ± 1.72	79.59 ± 1.71	ND	27,53 ± 0.01	90.10 ± 0.00	10,32 ± 0.00	25,11 ± 0.00	99.60 ± 0.00
Stem	6.00 ± 1.13	13.95 ± 0.14	8.66 ± 0.08	1.87 ± 0.8	12.78 ± 2.35	49.32 ± 3.60	3,98 ±0.00	21,88 ± 0.02	49,54 ± 0.02	5,06 ± 0.01	74,37 ± 0.04	38,48 ± 0.03
Roots	2.87 ± 0,23	12.87 ± 0.,37	21.00 ± 0.51	11.86 ± 1.21	4.56 ± 0.43	19.04 ± 0.84	6,56 ±0.01	20,18 ± 0.04	8,97 ± 0.01	3,35 ± 0,01	16,46 ± 0.04	11,47 ± 0.02
Seeds	18.73 ± 1.92	8.76 ± 0.19	11.62 ± 0.51	6.49 ± 0.86	27.90 ± 1.48	95.00 ± 0.16	42,69 ±0.00	68,46 ± 0.00	22,23 ± 0.00	10,88 ± 0.00	23,71 ± 0.00	73,56 ± 0.00
Flavonoids Content (mg QE/ 100 g dw)b												
Leaves	0.72 ± 0.02	26.04 ± 3.04	26.31 ± 2.32	4.86 ± 1.71	38.92 ± 3.04	88.50 ± 0.12	ND	ND	ND	ND	ND	ND
Pods	3.53 ± 0.40	8.53 ± 1.31	75.34 ± 2.00	13.25 ± 3.29	13.61 ± 3.36	74.48 ± 2.97	ND	ND	ND	ND	ND	ND
Stem	0.27 ± 0.08	2.38 ± 0.18	2.71 ± 0.90	1.75 ± 0.46	7.61 ± 1.87	28.05 ± 1.23	ND	ND	ND	ND	ND	ND
Roots	0.12 ± 0.07	0.93 ± 0.15	2.87 ± 0.32	1.02 ± 0.42	0.30 ± 0.21	4.07 ± 1.43	ND	ND	ND	ND	ND	ND
Seeds	0.86 ± 0.15	1.03 ± 0.07	7.46 ± 0.52	3.43 ± 0.64	14.84 ± 0.88	58.71 ± 5.29	ND	ND	ND	ND	ND	ND

ND : Note determined
a. milligrams of gallic acid equivalent per gram of dry matter of plant.
b. milligrams of quercetin equivalent per gram of dry matter of plant

its potential toxic effects against herbivores and insects [42]. Our results presented the best agreement between the total phenolic content and their total flavonoid content of leaves, pods, stems, roots and seeds extracted in the beginning of maturity stage with Correlation coefficient r=(0.91, 0.98, 0.96, 0.89, 0.98), respectively.

3.2. DPPH Scavenging Activity

We have evaluated the antioxidant power of the different extracts from *Cleome arabica* in the hope of finding the existence of a relationship between the phenolic content, antioxidant activity and the inhibitory capacity against the α-amylase view that antioxidant therapy may be a useful strategy in the treatment of diabetes.

The evaluation of the antioxidant activity of our phenolic extracts was conducted by an *in vitro* chemical test. In this test, we were interested in measuring the activity of the free radical scavenging by the antioxidant fractions of our phenolic extracts, using the stable radical 1,1-diphenyl-2- picrylhydrazyl (DPPH). We have used DPPH as a stable free radical reagent that easily accepts an electron or hydrogen radical and converts into a stable diamagnetic molecule in 30 min. The ethanolic solution of DPPH radical shows a strong absorption band at 517 nm, which decreases in the presence of phenolic extracts.

In the current study, the scavenging activities of DPPH exerted by our phenolic extracts as well as that of Vitamin C and vitamin E, used as a synthetic antioxidant and taken as references were calculated from the linear % inhibition-concentration curves. The concentration of inhibitors was expressed in mg/ml.

The antioxidant efficacy of our extracts was determined by calculating the IC_{50} parameter which represents the inhibitor concentration (antioxidant) needed to achieve 50% reduction of free radicals. The results are summarized in Table **1**.

The IC_{50} values of the phenolic extracts of our plant generally vary from 0.002to 1.93 mg / ml. the lowest value was recorded for the chloroform extract of December in the Roots, whereas the highest value was recorded for n-hexane extract of May in the stems.

All the plant extracts showed a beneficial effect against free-radical damage similarly to the standard antioxidant: Vitamin C (IC_{50} : 0.0049 mg/ml) and Vitamin E (IC_{50} : 0.0029 mg/ml). We also observed that the plant extract has a significant antioxidant potential comparable to other works [43-45].

The results show a scavenging effect of *Cleome arabica* fractions on DPPH radical in the following order: ethyl acetate > chloroform > n-hexane fractions in December and May in all organ plant. The root extracts were a powerful antioxidant whereas the stem extracts were a weak antioxidant. We noted that the n-hexane extracts were less active against the DPPH ˙compared to the ethyl acetate and chloroform extracts. Two hypotheses can be put forward to explain

Table 2. Free radical (DPPH) scavenging activity of the different extracts of *Cleome arabica*.

	December						May					
	Maceration Extracts						Ultrasonication extracts					
	Methanol/water (80/20)			Actetone/water (70/30)			Methanol/water (80/20)			Actetone/water (70/30)		
	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate
Parts of plant	IC_{50} (mg/ml)											
Leaves	ND	0.95 ± 0.02	0.70 ± 0.17	ND	0.8 ± 0.032	0.16 ± 0.04	ND	ND	ND	ND	ND	ND
Pods	ND	0.32 ± 0,03	0,40 ± 0.0	ND	0,15 ± 0,03	0,01 ± 0.00	ND	0.81 ± 0.03	0.10 ± 0.01	ND	0,62 ± 0.02	0,20 ± 0,01
Stem	ND	0.44 ± 0.01	1.93 ± 0.00	ND	0.16 ± 0.00	0.05 ± 0.00	2,64 ± 0,59	0,26 ± 0,02	0,14 ± 0,01	1,80 ± 0,10	0,27 ± 0,00	0,13 ± 0,00
Roots	ND	0.003 ± 0.00	0.28 ± 0.00	ND	0.004 ± 0.03	0.22 ± 0.00	0,08 ± 0,01	0,31 ± 0,01	0,25 ± 0,00	0,27 ± 0,02	0,19 ± 0,00	0,25 ± 0,00
Seeds	ND	0.49 ± 0.10	0.56 ± 0.02	ND	1.21 ± 0.07	0.09 ± 0.00	0.17 ± 0.01	0.06 ± 0.00	0.19 ± 0.01	0.51 ± 0.01	0.35 ± 0.02	0.48 ± 0.00

ND: Note determined

their lesser effect: either that these extracts do not contain enough polyphenolic compounds due to the little polar character of the n-hexane, or that the polyphenols do not correspond to antioxidants with interesting properties. We can say also that the majority of phenolic compounds content in ethyl acetate and chloroform have a number of hydroxyl groups, where their reducing capacity is improved when two hydroxyl groups are oriented ortho or para [34]. The antioxidant activity might be attributed to phenolic content, triterpenoids and flavonoid content of the tested plant. The comparable finding was reported by another group in their *in vitro* studies [46, 47]. We also noted that all the extracts have a comparable activity with almost the same IC_{50}values. From this, we can conclude that the antioxidant capacity of these extracts could mainly be due to the presence of certain molecules potentially active of flavonoids in the phenolic extracts.

It was clear that the DPPH assay was linearly not correlated to their total phenolic compounds with growth season. In order to confirm that correlation exists between the antioxidant activity and its growth, we have to study its activity in several months. There is no important correlation between the phenolic content of the extracts and their antiradical activity as evaluated by the DPPH assay ($r< 0.1$) for the pods, stem and seeds. There are numerous reasons to explain the ambiguous relationship between the antioxidant activity and total phenolics as the total phenolics composition did not include all the antioxidants compounds or a set of synergistic compounds for antioxidant activity [2]. A detailed examination of the phenolic composition of the plant extracts is required for a comprehensive assessment of the individual compound exhibiting antioxidant activity.

4. INHIBITION OF FUNGAL α-AMYLASE

In order to find a natural inhibitor against the fungal α -amylase, we studied *in vitro* the effects of our phenolic extracts on the activities of the enzyme, at varying concentrations of extracts and substrates, to identify the organic extracts with the better inhibitory on the enzyme.

The enzymatic activity of α-amylase was titrated using starch as a substrate which releases maltose with a spectrophotometer detection after reaction with complexing agents.

The α-amylase inhibition activities were assayed in the presence of different concentrations of extracts (0.1 to 25 mg/ml). The IC_{50} values fluctuated from 0.11 -13.93 mg/ml for December and from 1.00-35.25 mg/ml for other extracts, which were obtained from the variation of the percentages of inhibition as a function of different concentrations of inhibitors. Figs. (**1** and **2**) show the curves of the five best extracts.

The best inhibitors were in the acetonic extracts compared with methanolic extracts in the majority of organs. The best value was recorded for the ethyl acetate fraction of December Pods with an IC_{50} value of 0. 11 mg/mL and ethyl acetate fraction of seeds were least potent with an IC_{50} value of 35.25 mg/mL (Table **2**). The higher activity of the acetonic extracts as compared to the methanolic extracts can be attributed to the presence of more active compounds with different structures extracted by acetone as compared to methanol.

The hexanic extracts showed the best IC_{50}values in both acetonic and methanolic systems with values close to each other. This can be explained by the fact that the two solvents systems used for the extraction : methanol/H_2O and acetone /H_2O may extract the same apolar molecules with near chemical structure and were able to react similarly against the enzyme and the extraction solvents in all the organs and showed different values of $IC_{50}$with the majority far from each other as the inhibition phenomenon is the result of a synergy between several molecules, these values could be lower if the molecules responsible for inhibition in the two solvents of extraction were together in the same reaction medium.

The comparison between the inhibition activity of *Cleome arabica* in two seasons gives us the period when the *Cleome arabica* is at its peak the activity against α-amylase that is during the beginning of the maturity stage. We can say that the plant source (the nature of the plant material, its origin, growth stage, climatic conditions for growth, degree

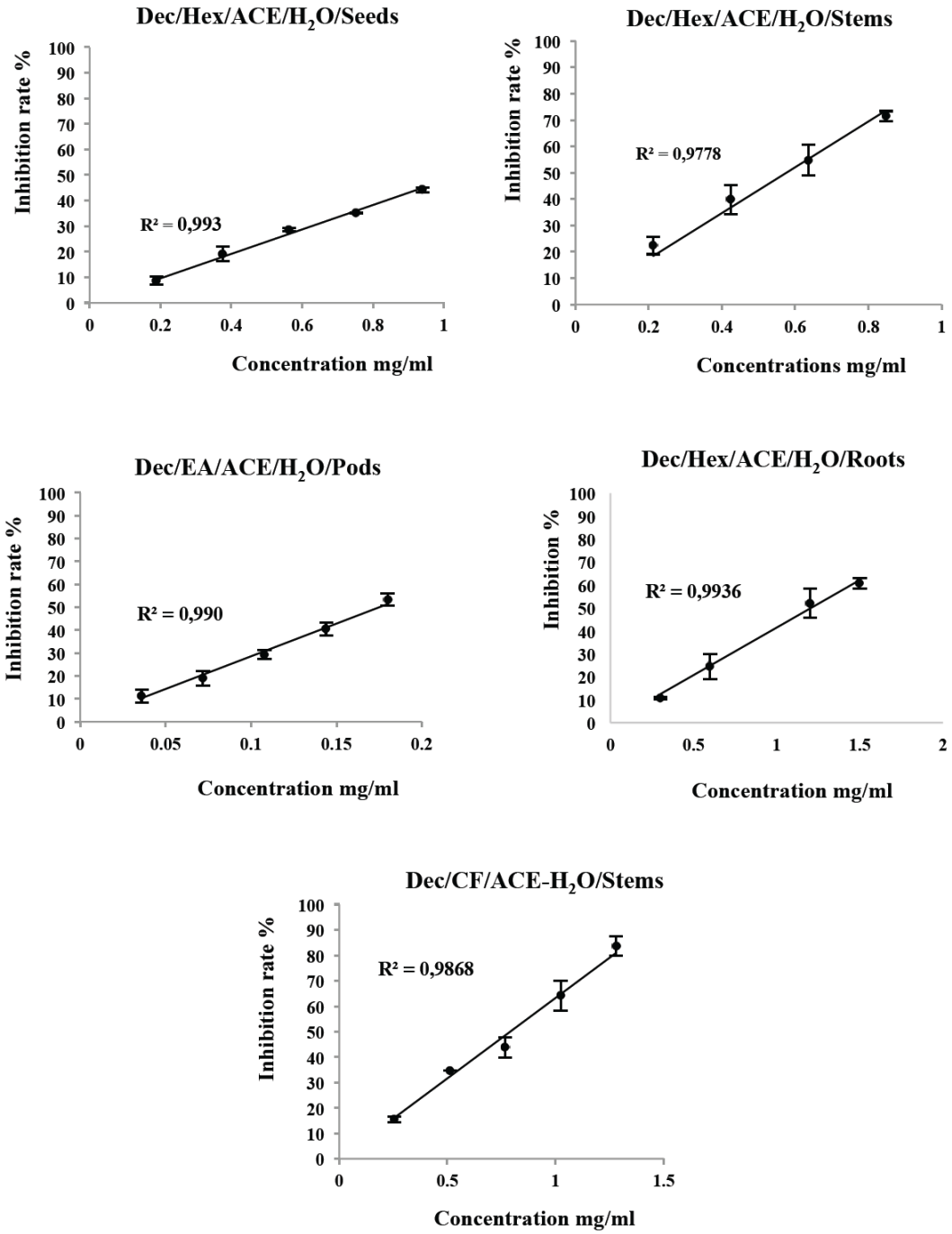

Fig. (1). The inhibition rate against α-amylase inhibition activity of the five best extract in the begnning of maturity stage.
Dec : December; Hex: Hexane; ACE : Acetone; EA : Ethyacetate; CF : Cloroform.

of processing, moisture content, and particle size) [48], remains the first point influencing the synthesis of inhibitory molecules and their action which are influenced by the atmospheric and climatic conditions under which the plant is grown. The synthesis of phenolic compounds and other secondary metabolites (responsible for the pharmacological effects of the plant) is often affected by environmental factors [49]. Moreover, we have observed that the extractor system is very important to screen. The polar phenolic compounds extracted with ethyl acetate exhibited the best antioxidant activity in the majority of the organ plant. On the other hand, the amount of compounds extracted with n-hexane cover a higher α-amylase inhibition activity. Consecontely, the phenolic compounds that exhibit the antioxidant activity and those react against the enzyme have different structure and polarity. We can conclude that the successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Also, the type of extraction, the variations in different extraction methods affect the quantity of secondary metabolite. The composition of an extract depends upon the type of extraction, time of extraction, temperature, nature of

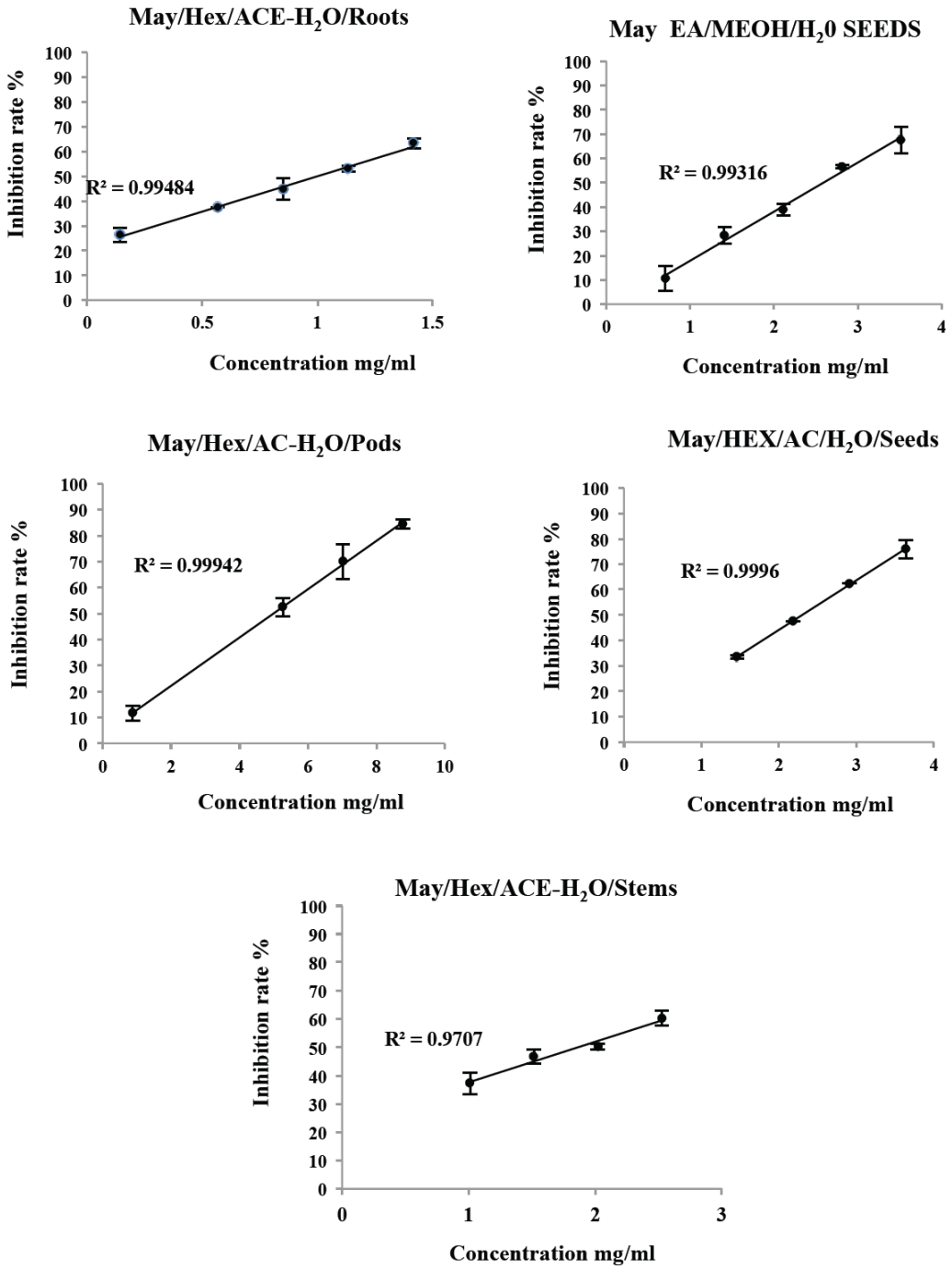

Fig. (2). The inhibition rate against α-amylase activity of the five best extract in the end of maturity stage.
Hex: Hexane; ACE : Acetone; EA : Ethyacetate; MeOH : Methanol.

solvent, solvent concentration, and the polarity of solvents, the length of the extraction period must also be taken into consideration, which depends on solvent used, pH of the solvent, temperature, particle size of the plant tissues, and the solvent to sample ratio [48]. On the other hand, by comparing the values of the IC_{50} to the contents of phenols and flavonoids, we found that the inhibitory activity was proportional to the concentration of phenolic compounds in only two cases, the leaves extract of the beginning maturity stage r = 0.98 and roots and pods extracts (r = 0.87 and r = 0.7 respectively). It may be thought that the inhibitory potency of the plant extracts is not limited to the phenolic content, but it may be due to the presence of other individual active compounds and was dependent not only on the concentration of individual inhibitors but also on the structure and interaction among them.

The antioxidant activity was not related to α-amylase inhibition activity. The study of possible correlations/no-correlation between the total phenolic content and their antioxidant/enzyme inhibition activities escorts us to find the responsible compound group for these activities.

Table 3. IC_{50} values of the anti-α-amylase Activity of the different extracts of *Cleome arabica*.

	December						May					
	Maceration Extracts						Ultrasonication extracts					
	Methanol/water (80/20)			Actetone/water (70/30)			Methanol/water (80/20)			Actetone/water (70/30)		
	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate	Hexane	Chloroform	Ethyl Acetate
Parts of plant	IC_{50} (mg/ml)											
Leaves	1.19 ± 0.12	7.95 ± 1.95	18.45 ± 0.64	3.15 ± 1.24	6.86 ± 1.6	12.85 ± 2.93	ND	ND	ND	ND	ND	ND
Pods	7.09 ± 1.19	2.76 ± 0.46	5.1 6 ± 0.12	1.90 ± 0.11	13.93 ± 1.08	0.11 ± 0.01	15,57 ± 0,17	17,37 ± 1,41	ND	4,98 ± 0,19	6,72 ± 0,40	ND
Stem	8.01 ± 1.31	1.64 ± 0.07	4.76 ± 0.19	0.60 ± 0.09	0.79 ± 0.79	5.35 ± 0.16	2.83 ± 0.13	6.13 ± 0.85	9,38 ± 0,16	1.63 ± 0.35	3.06 ± 0.48	7.11 ± 0.01
Roots	1.67 ± 0.19	3.73 ± 0.66	2.16 ± 0.16	1.31 ± 0.14	3.14 ± 0.35	2.85 ± 0.40	1,52 ± 0,23	11,09 ± 0,69	2,42 ± 0,25	1,01 ± 0,04	3,81 ± 0,24	4,10 ± 0,56
Seeds	8.71 ± 1.01	2.59 ± 0.15	1.77 ± 1.77	1.08 ± 0.06	2.35 ± 0.17	1.03 ± 0.07	14,42 ± 0,32	2,80 ± 0,16	2,54 ± 0,05	2,32 ± 0,03	3,64 ± 0,08	35,24 ± 0,52

ND: Note determined

CONCLUSION

This study investigated the potential *in vitro* antidiabetic and antioxidant activities of different extracts from organs of *Cleome arabica* growing in Algeria and harvested in two seasons. All the extracts showed a significant antioxidant potential similar to the standard antioxidants such as Vitamin C and Vitamin E. All the sample extracts gave an inhibition activity against α -amylase which was found to be better in the sample of the beginning of maturity stage (December). The hexane extract provided a significant inhibition of the enzyme, suggesting that the plant contained potential apolar inhibitor compounds which may contribute to its inhibition effect on α-amylase. However, it seems that the extraction method has no influence on the inhibitory and antioxidant activities. Further studies need to isolate and identify the structures of the compounds responsible for the inhibition of the α-amylase enzyme. This is the first report of the α-amylase inhibitory activity of the different part of Algerian *Cleome arabica* species. This study shows the possibility of using *Cleome arabica* extract to decrease postprandial hyperglycaemia.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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PHARMACOGNOSIE

High in vitro Antioxidant Capacities of Algerian *Cleome arabica* Leaves' Extracts

Hautes capacités antioxydantes in vitro des extraits de feuilles de *Cleome arabica* d'Algérie

F. Seglab · C. Hamia · I. Khacheba · A. Djeridane · M. Yousfi

Abstract The aim of this study was to evaluate the antioxidant capacities of *Cleome arabica* leaves' extract collected on two seasons in the town of Laghouat steppe region of Algeria. Five common tests for measuring antioxidant capacities were used to compare with three standard antioxidants: measurement of free radical scavenging activity with 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) and 2,2′-azinobis(3-ethylbenzo-thiazo-line-6-sulfonate) ($ABTS^{\bullet+}$) radical cation, measurement of total antioxidant capacities with phosphomolybdenum, ferric reducing, and cupric reducing methods. The amount of the phenolic compounds was carried out by the quantification of total phenolic, total flavonoid, and condensed tannin contents in three organic solvents with varying polarities. The results show that the ethyl acetate is the best extractor solvent of flavonoids, while petroleum ether has the ability to extract more of terpenes. The values of quantification ranged from 0.341 to 0.751 mg of gallic acid equivalent/g of dry matter, from 0.172 to 0.682 mg of quercetin equivalent/g of dry matter and from 0.172 to 0.332 mg of catechin equivalent/g of dry matter for the total phenolic, flavonoids, and the condensed tannins, respectively. All the extract shows strong antioxidant activity, whose best are found in the ABTS and DPPH assay with IC_{50} values of 0.01 and 0.017 mg/ml, respectively, in a different season. These results suggest that the level of antioxidant activity in this plant varies to a great extent. They also suggest that phenolics in this plant provide substantial antioxidant activity. Upon achievement of this survey, an extra benefit of this medicinal plant may be found.

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Keywords *Cleome arabica* · Ethyl acetate extracts · Phenolic compounds · Antioxidant capacity · PCA

Résumé Le but de cette étude était d'évaluer les capacités antioxydantes des extraits de feuilles de *Cleome arabica* récoltées à deux saisons de la région de la steppique algérienne de Laghouat. Cinq tests des capacités antioxydantes ont été utilisés, comparés à trois antioxydants standards : mesure de l'activité de piégeage des radicaux libres avec le radical 1,1-diphényle 2-picrylhydrazyle (DPPH•) et le radical cation 2,2′-azinobis (3-éthylbenzo-thiazo-6-sulfonate) ($ABTS^{\bullet+}$), la mesure des capacités antioxydantes totales avec les méthodes du phosphomolybdate, la réduction du fer ferrique et la réduction du cuivre cuivrique. La quantité de composés phénoliques a été déterminée par la quantification de la teneur totale en composés phénoliques, de la teneur totale en flavonoïdes et de la teneur en tanins condensés dans trois solvants organiques de polarités variables. Les résultats montrent que l'acétate d'éthyle est le meilleur solvant d'extraction des flavonoïdes, tandis que l'éther de pétrole a la capacité d'extraire davantage de terpènes. Les valeurs de quantification variaient de 0,341 à 0,751 mg équivalent en acide gallique/g de la matière sèche, de 0,172 à 0,682 mg équivalent en quercétine/g de la matière sèche et de 0,172 à 0,332 mg équivalent en catéchine/g de la matière sèche pour les phénols totaux, les flavonoïdes et les tanins condensés respectivement. Tous les extraits montrent une forte activité antioxydante, les meilleurs étant trouvés dans les tests ABTS et DPPH avec des valeurs d'IC_{50} de 0,01 et 0,017 mg/ml respectivement au cours d'une saison différente. Ces résultats suggèrent que le niveau d'activité antioxydante de cette plante varie dans une large mesure. Ils suggèrent également que les composés phénoliques de cette plante fournissent une activité antioxydante substantielle. Après avoir réalisé cette étude, un avantage supplémentaire de cette plante médicinale pourrait être trouvé.

Mots clés *Cleome arabica* · Extraits d'acétate d'éthyle · Composés phénoliques · Capacité antioxydante · ACP

Introduction

In recent years, the world of biological and medical science has been convinced by a new concept of oxidative stress, that is, a situation where the cell no longer controls the excessive presence of toxic oxygen radicals, a situation which researchers implicate in most human diseases [1]. Plant materials have various beneficial medicinal effects which are typically due to the combinations of phytochemical compounds present in the plant, and their action is exceptional to specific plant species or groups [2,3]. Phenolic compounds are a class of naturally occurring pigments with ubiquitous distribution in plant kingdom [4]. They display a remarkable biological property, which may have an important role in the reduction of oxidative stress. Many antioxidants are proven to undergo an electron transfer mechanism upon exerting their antioxidant functions [5]. Some workers have attempted to establish quantitative correlations between the antioxidant activities and redox potentials for reductant antioxidants. *Cleome arabica* belongs to Capparaceae family that includes 150 species widely grown in tropical and subtropical regions of the world, which is also widespread in North Africa. This plant metabolizes a large number of secondary metabolites, mainly flavonoids, alkaloids, glycosides, organic acids, and glucosinolates [6,7]. It has been used in traditional medicine in the treatment of scabies and inflammation [6,8-10] as well as its leaves, which contain a number of glucosylated and rhamnosylated flavonols and possess anti-inflammatory activity which are used for the treatment of abdominal and rheumatic pains [11]. It also possesses antimicrobial, antifungal, and cytotoxic activity against P388 cells [12,13].

Despite the abundant research that has been carried out on the potential health benefits of *Cleome arabica*, little research was done about their biological activities, especially the antioxidant activity [4,14].

Taking into account the complexity of the oxidation processes, there is no single method of reflecting the antioxidant profile of a sample; therefore, the response obtained by means of different and complementary tests must be combined. The aim of this study is to evaluate phenolic compounds, in vitro antioxidant effects of *Cleome arabica* extracts collected in different seasons in the town of Laghouat in the steppe region of Algeria. In this work, we aim to assess the antioxidant potential of *Cleome arabica samples* using five chemical tests, (ABTS; ferric reducing antioxidant power, FRAP, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), phosphomolybdenum complex, and cupric reducing antioxidant [CUPRAC]). To find the correlation between them in different seasons and compare them with those of butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and ascorbic acid (VC) have been extensively used as standard antioxidants in the experiments, which can offer a wider picture of the antioxidants present in biological samples as it rather than considers the effect of single compounds, it considers the additive and synergistic effects of all antioxidants, and may, therefore, be useful to study the potential health benefits of antioxidants on oxidative stress-mediated diseases [15,16]. To the best of our knowledge, the antioxidant activities with various methods of Algerian *Cleome arabica* was never been investigated. Also, this work is a continuity of studies on *Cleome arabica* [17] and other antioxidant studies on Algerian plants realized in our laboratory [18–22].

Materials and Methods

Plant Material

The plant (*Cleome arabica*) was collected in two different seasons: January and November 2015. The two samples were harvested at Laghouat city in the steppe region of Algeria. A voucher specimen was deposited into the laboratory of Fundamental Sciences, University of Laghouat. The leaves were dried, grinded, and kept until analysis.

Reagents

All chemicals reagents were from Sigma (USA), Aldrich (Milwaukee, USA), Fluka Chemie (Buchs, Switzerland), and Merck (Germany). The used solvents were of analytical grade.

Preparation of Extracts

The fresh *Cleome arabica* material leaves were oven-dried and grinded. The lipid was removed by Soxhlet extraction with petroleum ether for 24 h. After that, the remaining powder was macerated in hydroalcoholic solvent system with 100 ml of 80/20 (v/v) (methanol/water) for 24 h at room temperature. After evaporation of water under reduced pressure, the organic fraction was extracted with a series of solvents of increasing polarity as hexane and dichloromethane to remove the pigments and lipid compounds. Then, the extraction was done with ethyl acetate until exhaustion. Organic extract was evaporated to dryness under reduced pressure at 45 °C. Dry fractions were stored in ethanol at 4 °C until use.

Determination of Total Phenolic Content

The total phenolic content was spectrophotometrically determined using the Folin–Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphotungstic and

phosphomolybdic acids (FC reagent). This reagent, based on the early work of Singleton & Ross method [23], is a colorimetric oxidation/reduction method for phenolic compounds. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. Briefly, a 100 µl of the diluted sample was added to 500 µl of Folin–Ciocalteu reagent. After 3 min, 2 ml of saturated sodium carbonate solution (2%) was added. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 764 nm. The calibration curve was prepared with Gallic acid solutions, and the results are given as Gallic acid equivalents (GAE).

Determination of Total Flavonoid Content

The total flavonoid content was determined using a spectrophotometric method of Lamaison and Carnat [24] with minor modification by Floegel et al. [25]. Briefly, 1 ml of sample or standard (quercetin) was mixed with 1 ml (w/v) of aluminum chloride ($AlCl_3$) 2%. Absorbance of the colored flavonoid–aluminum complex was measured immediately at 430 nm versus a blank. The total flavonoid content of the *Cleome arabica* L. extract was expressed as mg quercetin equivalent (QE)/100 g of dry weight material. All samples were analyzed in triplicate.

Determination of Tannin Condensed Content

The condensed tannins are determined by the vanillin method with slight modification [26,27]. This method is based on the ability of vanillin to react with condensed tannin units in the presence of acid to produce a colored complex measured at 500 nm. The reactivity of vanillin with tannins implies only the first unit of the polymer. 5 ml of reagent comprises 0.5% vanillin and 4% HCL prepared in the methanol added to 1 ml of diluted extract. The reagent and diluted extract preincubated in a water bath at 20 °C for 10 min before the reaction starts, and then the mixture is incubated for 15 min. The reading of the absorbance is carried out in a Visible/UV spectrophotometer of Shimadzu type 1601, at a wavelength of 500 nm against a white. A calibration curve is performed in parallel under the same operating conditions using catechin as a positive control.

Evaluation of Antioxidant Capacities

Phosphomolybdenum Assay

The antioxidant activity of the *Cleome arabica* L. extract was evaluated by the phosphomolybdenum method. The assay is based on the reduction of molybdates Mo (VI) to molybdenum Mo (V) Mo by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The followed scheme is to combine 0.1 ml extract with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In case of blank, 0.1 ml of distilled water was used in place of the test sample. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 70 ° C for 90 min. We subject the samples to a fast cooling before measuring the absorbance of the solution at 695 nm using a spectrophotometer [28]. The antioxidant capacity of each sample was expressed as ascorbic acid (A.A) equivalent. The values are presented as the means of triplicate analysis.

Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The ferric ion reducing antioxidant power (FRAP) method was used to measure the decreasing capacity of *Cleome arabica* L. extracts; this method was carried out with slight modifications [29,30]. FRAP reagent was prepared by mixing acid buffer, TPTZ solution and ferric chloride solution at the volume ratio of (10:1:1) respectively.

The following protocol is used for the buffer acid (pH = 3.5): first dissolve 0.310 g of CH_3COONa, $3H_2O$ in a 100-ml flask, then add 1.6 ml of pure acetic acid, and complete with distilled water up to gauge. The TPTZ solution is prepared by dissolving 157 mg of this compound in 50 ml distilled water and 0.165 ml of HCl 40 mmol/l. For the solution of ferric chloride (20 mmol/l), we have dissolved 0.2723 g of $FeCl_3$, $6H_2O$ in a 50-ml flask and added with distilled water.

For samples to be tested, they were mixed in the same proportions as to plot the standard curve, the reagent (1 000 µl), and the solution of the test compound (100 µl). The optical density is read after 1 h at 593 nm. The ability "electron donor" of the sample is measured by the change in color due to the formation of the complex (Fe^{2+} TPTZ) and the equivalent in Fe^{2+} is determined.

All measurements were repeated three times. Total antioxidant capacity in the measuring systems, expressed as VC equivalents, was calculated. Correlation coefficient (R^2) for the test curve ranged between 0.99 and 1.00.

Copper Reduction Capacity Assay (CUPRAC)

The evaluation of the reducing capacity of our samples was carried out using the test CUPRAC. It is based on the following by the reduction of increased absorbance of neocuproine complex (Nc)-ions cupric [Nc2-$Cu2^+$]. Indeed, in the presence of an antioxidant, the copper complex neocuproine is reduced and this reaction was quantified spectrophotometrically at a wavelength of 456 nm [31]. The preparation scheme for the measurement of the antioxidant properties

was the following: 1 ml of ammonium acetate buffer (1 M, pH = 7 > *m* = 19.66 g in 250 ml water) was mixed with 100 µl of diluted extract to be tested, 500 µl of neocuproine (7, 4 mM ≥ *m* = 0.039 g in 25 ml ethanol), and 500 µl of $CuCl_2$-$2H_2O$ 10.1 mM (0.01 m ≥ *m* = 0.1722 g in 100 ml water). The mixture is incubated for 1 h, and then the reduced complex [−Nc Cu2 +] was quantitated spectrophotometrically at a wavelength of 456 nm against a blank.

DPPH Free Radical Scavenging Activity Assay

Antioxidant activity of *Cleome arabica* L. extract with respect to the radical DPPH was evaluated spectrophotometrically by following the reduction of this radical which is accompanied by its passage from the violet color to the yellow color measurable at 517 nm. According to the method reported by Cotelle et al. [32] with minor modifications [33,34]. Briefly, ethanolic solution extract and standard solutions (control) were prepared and added to a 250 µM of DPPH ethanolic solution; the mixtures were shaken vigorously and left to stand in the dark for 30 min at room temperature, and then absorbance was read at 517 nm. Radical scavenging capacity was expressed as percentage effect (E%) and calculated using the following equation:

$$Percentage\ effect\ (E\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100$$

Different sample concentrations were used in order to obtain antiradical curves for calculating the EC_{50} values. Antiradical curves were plotted referring to concentration on the *x*-axis and their relative scavenging capacity on the *y*-axis. The EC_{50} values were processed using six or plus of points.

ABTS Radical Cation Decolorization Assay

ABTS assay was described by Re et al. and Serpen et al. [35,36] with minor modification. The radical cation $ABTS^{+}$.occurs immediately after the addition of 100 µl. The $ABTS^{\bullet+}$ radical cation was generated by chemical oxidation with potassium persulfate of a potassium persulphate $K_2S_2O_8$ solution (70 mM). The mixture is brought to room temperature and the dark for 24 h before use. 1 ml of this solution was diluted of a buffered solution at pH 6.8 to 7.0 phosphate prepared by dissolving 0.4477 g of sodium hydrogen phosphate hydrate 12 times (Na_2HPO_4-$12H_2O$), 0.195 g of hydrated sodium dihydrogen phosphate (NaH_2PO_4-$2H_2O$), and 2.25 g of chloride sodium (NaCl) in 250 ml of distilled water. The optical density of the presolution incubated at 37 °C for 15 min is adjusted to (0.70 ± 0.002) at 734 nm.

100 µl of each diluted extract in distilled water is admixed with 1 ml of the ABTS + solution. The percentage inhibition (PI) of ABTS + for each sample was calculated according to the formula below:

$$Percentage\ inhibition\ (PI\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100$$

Also, we tested vitamin C, TBHQ, and BHT as standard. The %AI of the extracted phenols was expressed as IC_{50}, with the IC_{50} being defined as the concentration of test sample required to inhibit the formation of active radicals by 50%.

Results and Discussion

Content of Total Phenolics, Flavonoids, and Condensed Tannins

The extraction solvents carry off non-phenolic substances such as sugars, dyes, and proteins which may interfere during any phenolic evaluation [37]. Aqueous ethanolic extraction proved to be the most effective method for extracting phenolic materials and other antioxidative compounds [38].

The results of the total phenolic content in the ethyl acetate fraction ranged between 0.341 and 0.751 mg EAG/g for the dry matter, 0.170 and 0.490 mg EAG/g for the dichloromethane fraction, and between 0.060 and 0.453 mg EAG/g for the petroleum ether fraction. The total phenolic content in many samples tested in this study was higher than reported for the other medicinal [39]. But we marked that the total phenolic content in the tested plant was lower than those reported for most of the other Asian medicinal and common dietary plants [14,15].

The amount of flavonoid content in the ethyl acetate fraction ranged from 0.172 to 0.682 mg EQ/g. The results of total phenolic, flavonoid, and tannin condensed contents are shown in table 1. The greatest amount of flavonoids was recorded in the ethyl acetate fraction but no quantity was registered in the dichloromethane and petroleum ether fraction.

Calycopterine; Quercetin glucoside; Quercetin 3,7-diglucoside; Kaempferol 3-G-7-Rhamnoside; Quercetin 7-Rhamnoside; Apigenin 6,8-di-CGlycoside; Kaempferol 3,7-diRhamnoside; Quercetin 3-G-7-rhamnoside; Kaempferol 3′methoxy-3,7 diRhamnoside; Kaempferol 7-Rhamnoside; Isorhamnetin.

For the tannins content, we observe the same result as flavonoids exist only in the ethyl acetate fraction. The tannins assay method used is that of vanillin described by Hagerman [27]. The results of the condensed tannins in the ethyl acetate fraction varied between 0.172 and 0.332 mgEC/g. We conclude that the ethyl acetate is the best extractor of flavonoids and the condensed tannins.

Measurement of Antioxidant Capacity Expressed as Vitamin C Equivalents

The antioxidant capacities of *Cleome arabica* L. extract are summarized in table 2. The highest antioxidant capacities were detected by ABTS with extract series concentration from 0.1 to 0.8 mgGAE/g, and EC_{50} values ranged from 0.371 to 0.909 mg VCE/100 g is better than that of BHT (0.021 mg/ml), TBHQ (0.023 mg/ml), and ascorbic acid (0.057 mg/ml). However, the lowest antioxidant activity was registered between (9.265 and 12.215 VCE mg/100g), (2.923 and 11.305 mg VCE/100 g), and (8.459 and 33.759 mg VCE/100 g) for CUPRAC assay, FRAP, and phosphomolybdenum complex (PM-complex) assay, respectively. There are different reasons that lead to these differences. One of these reasons could be that CUPRAC method can determine the antioxidant substances containing sulfhydryl group (SH), for example, the proteins. Not all methods measure protein thiols or smaller molecule -SH compounds of different origins such as GSH, with FRAP. Also, the

Table 1 Total amount of phenolic compound, flavonoids, and tannins condensed of *Cleome arabica* leaves' extract

Samples N°	Total phenolic content[a] mg/g	Total flavonoid content[b] mg/g	Total tannin content[c] mg/g
N1	0.686 ± 0.01	0.329 ± 0.002	0.332 ± 0.007
N2	0.751 ± 0.02	0.682 ± 0.003	0.274 ± 0.013
N3	0.341 ± 0.01	0.204 ± 0.005	0.188 ± 0.014
N4	0.362 ± 0.00	0.329 ± 0.003	0.302 ± 0.002
N5	0.409 ± 0.01	0.271 ± 0.004	0.172 ± 0.008
J1	0.624 ± 0.02	0.314 ± 0.002	0.328 ± 0.014
J2	0.470 ± 0.01	0.245 ± 0.001	0.190 ± 0.007
J3	0.347 ± 0.01	0.172 ± 0.002	0.187 ± 0.01
J4	0.595 ± 0.03	0.403 ± 0.001	0.329 ± 0.006
J5	0.449 ± 0.01	0.261 ± 0.002	0.213 ± 0.017
J6	0.415 ± 0.01	0.223 ± 0.003	0.216 ± 0.017

[a] mg of gallic acid/g of sample (mgGAE/g(
[b] mg of quercetin/g of sample (mgQE/g(
[c] mg of catechin/g of sample (CE/g(

Table 2 Antioxidant activity of examined *Cleome arabica* L. ethyl acetate extracts and standards determined by DPPH, ABTS, FRAP, CUPRAC, and PM assays

Samples' code	CUPRAC (g/l)	PM (g/l)	FRAP (g/l)	DPPH (g/l)	ABTS (g/l)
N1	0.323 ± 0.004	0.446 ± 0.027	0.158 ± 0.002	0.015 ± 0.000	0.013 ± 0.000
N2	0.22 ± 0.009	0.262 ± 0.006	0.232 ± 0.0025	0.01 ± 0.000	0.009 ± 0.000
N3	0.24 ± 0.0004	0.248 ± 0.006	0.099 ± 0.001	0.013 ± 0.000	0.01 ± 0.000
N4	0.166 ± 0.002	0.162 ± 0.020	0.053 ± 0.001	0.010 ± 0.000	0.009 ± 0.000
N5	0.261 ± 0.009	0.243 ± 0.003	0.094 ± 0.001	0.017 ± 0.000	0.01 ± 0.000
J1	0.296 ± 0.010	0.29 ± 0.002	0.117 ± 0.002	0.017 ± 0.000	0.01 ± 0.000
J2	0.265 ± 0.006	0.273 ± 0.011	0.104 ± 0.000	0.023 ± 0.000	0.01 ± 0.000
J3	0.255 ± 0.008	0.314 ± 0.001	0.12 ± 0.005	0.018 ± 0.000	0.01 ± 0.000
J4	0.24 ± 0.002	0.238 ± 0.017	0.097 ± 0.003	0.02 ± 0.001	0.009 ± 0.000
J5	0.27 ± 0.010	0.287 ± 0.003	0.119 ± 0.001	0.018 ± 0.001	0.01 ± 0.001
J6	0.25 ± 0.003	0.303 ± 0.010	0.11 ± 0.003	0.025 ± 0.002	0.009 ± 0.001
BHT	0.093 ± 0.002	2.495 ± 0.053	0.145 ± 0.001	0.168 ± 0.004	0.021 ± 0.000
TBHQ	0.2845 ± 0.004	0.593 ± 0.006	0.056 ± 0.000	0.04 ± 0.001	0.023 ± 0.000
VC	0.366 ± 0.002	0.443 ± 0.009	0.109 ± 0.000	0.001 ± 0.000	0.057 ± 0.005

N: November, J: January

CUPRAC method is advantageous over FRAP since the redox chemistry of copper(II) — as opposed to that of chemically inert high-spin ferric ion having half-filled d-orbitals in its electronic configuration — should involve faster kinetics. The bis-(neocuproine) copper (I) cation chromophore is soluble both in water and organic media; therefore, the CUPRAC method is capable to assay both hydrophilic and lipophilic antioxidants. In this experiment, the ABTS method was used to confirm the results from the DPPH method assay since both methods are based on a similar antioxidant mechanism and the extracts used in both tests were ethanol soluble. All sample extracts are comparable without significant differences.

For the case of DPPH and ABTS radicals, the mechanism (particularly in ethyl acetate) is very often electron transfer than the other methods; furthermore, the antioxidant activity of *Cleome arabica* does not change with the different seasons of the year.

Antioxidant Capacity of the Relevant Standards

Some relevant organic compounds are used as antioxidant standards, including ascorbic acid, and BHT and TBHQ were comparatively evaluated for their ranking of antioxidant power by DPPH, ABTS, FRAP, PM-complex, and CUPRAC assays. A lower IC_{50} and EC_{50} values correspond to a larger scavenging activity. As shown in table 2, the DPPH radical scavenging activities of these reference compounds were comparatively evaluated and ascorbic acid possessed the highest radical scavenging activity, 0.001 g/l as compared with TBHQ 0.04 g/l and BHT 0.168 g/l; and the ranks of the scavenging activity were found in similar trends with DPPH and PM-complex assays, ascorbic acid > TBHQ > BHT. On the contrary, the ranks of the scavenging activity were found in similar trends with CUPRAC and ABTS, BHT > TBHQ > ascorbic acid. The linearity of calibration curves allowed quantification of antioxidant activity using any of the standards listed above. DPPH, ABTS, PM-complex, CUPRAC, and FRAP values (Table 2) for the antioxidant activity of these standards were used for investigating the correlation coefficients. Antioxidant activity of many samples of *Cleome arabica* L. extract tested in this study was higher than reported for most of the other medicinal plants [40–44]. These results of the study are in good accordance with previous studies (Table 3).

We have observed rapid and strong inhibition of both DPPH radical and ABTS radical cations, after the addition of *Cleome arabica* L. extract. Compared to other methods, from the methodological point of view, the DPPH method is suggested as easy and perfect with regard to measuring the antioxidant activity of fruit and vegetable juices or extracts. The results are highly reproducible and comparable to other free radical scavenging methods such as ABTS [45].

Correlation Between Results of Antioxidant Activities and Phytochemical Constituents

It is clear from this study that the different methods used for the determination of antioxidant capacity are highly correlated. Correlation analysis is used for phytochemical contents with IC_{50} values of radical scavenging and/or antioxidant ability of extract. IC_{50} of ABTS, CUPRAC, and FRAP showed a significant correlation (r: 0.713, 0.948, 0.985) with PM-complex. While nonsignificant correlation was observed with DPPH, a positive correlation was also observed between flavonoids and total polyphenolic (r: 0.781), confirming that flavonoids are the most important group of phenols in plants [46]. The total phenolic and flavonoids showed significant correlation (r: 0.646, 0.782) with FRAP, respectively, but no significant correlation observed between flavonoids

Table 3 EC_{50} of *Cleome arabica* L extracts compared with previous studies

Plant	DPPH	ABTS	References
Satureja thymbra	0.391 ± 0.45 mg/ml	0.178 ± 0.80 mg/ml	[40,37]
Lemon essential oils	6.68 ± 0.33 mg/ml	2.97 ± 0.18 mg/ml	[27]
Avocado	41.2 ± 6.4 mg VCE/100 g	70.0 ± 10.1 mg VCE/100 g	[22]
Apple	143.7 ± 47.3 mg VCE/100 g	158.7 ± 35.6 mg VCE/100 g	[22]
Lemon	101.2 ± 2.0 mg VCE/100 g	145.8 ± 13.7 mg VCE/100 g	[22]
Cleome arabica	13.15 ± 0.01 mg/l	/	[11]
Anvillia radiata	≤ 0.380 mg/ml	0.486 ± 0.038 mg/ml	[38,41]
Cleome arabica	0.00488 mg/ml	/	[22]
Rumex vesicarius	0.140 ± 0.007 mg/ml	/	[38]
Nepeta nepetella	1.45 ± 0.07 mg/ml	/	[39,42]
Lemon balm	16.35 ± 0.82 mg/ml	10.37 ± 0.62 mg/ml	[27]
Dark tea	0.013 ± 0.001 mg/ml	/	[43]
Brick tea	0.043 ± 0.002 mg/ml	0.027 ± 0.001 mg/ml	[44]

(CUPRAC, DPPH, PM-complex, ABTS). This observation may suggest that the reducing power exhibited by the different fractions is not only a function of flavonoids but also of the presence of other constituents with an antioxidant potential [47]. In addition, correlations among antioxidant activity based on DPPH, ABTS, CUPRAC, PM-complex, and FRAP assays were positively high and ranged between 0.413 and 0.985. The results show that when all standard materials were relatively analyzed by statistics, there was a positive and greatly significant relationship for PM-complex and CUPRAC (r = 0.985). Statistically significant correlations were also noted between PM-complex and ABTS values (r = 0.966) and ABTS and CUPRAC values (r = 0.948). Furthermore, we registered a positive correlation between PM-complex and FRAP (r = 0.713) and REC and FRAP values (r = 0.692). This strong correlation is due to the fact that these four methods are based on the generation of a free radical by different mechanisms followed by the detection and quantification of an end point in the reaction. The addition of an antioxidant inhibits the development of the end point, an inhibition that is an expression of the antioxidant capacity of the sample studied [48]. Moderately positive correlation has been found between total flavonoids content vs FRAP: r = 0.646. These results indicate that the antioxidant activities proved by these tests are ensured, probably by the same active molecules not flavonoids (Table 4).

In this present study, PCA was used to identify the variation in the antioxidant capacity of the plant extracts and to demonstrate how the eight parameters, namely, DPPH, ABTS, CUPRAC, FRAP, PPM, total phenolic content, total flavonoid content, and total tannins content contribute to the overall antioxidant capacity of *Cleome arabica*.

In this manuscript, PCA was applied to the data set of 11 different extracts to separate the samples according to similarity of the antioxidant activity. This technique is very useful because it reduces original variables based on principal component and gives the relationship between analyzed extracts and applied methods. The results obtained for each method were adopted as columns and the extracts as rows.

First, principal component analysis (PCA) applied to the data explained 75.25% of the total variance in the antioxidant profile of the *Cleome arabica* extracts. Moreover, the use of this unsupervised classification method often permits a simple representation of different sample data and their correlations. Two principal components were extracted because they have eigenvalues higher than 1.0, as suggested by the Kaiser criterion [49]. Eigenvalues give the measure of the variance accounted by the corresponding eigenvectors (components) [50]. Principal component 1 (PC1) explained up to 41.83% of total variance and PC2 explained 33.41% (Figs 1, 2).

ABTS, FRAP, CUPRAC, FRAP, and PPM are strongly positive correlated and grouped on the right side of the plot (Fig. 2), whereas DPPH is found in opposition side which confirmed no significant correlation with other assays. The extract N4 is on the negative side of the plot from all others because it has the highest negative loadings on PC1 (−1.45).

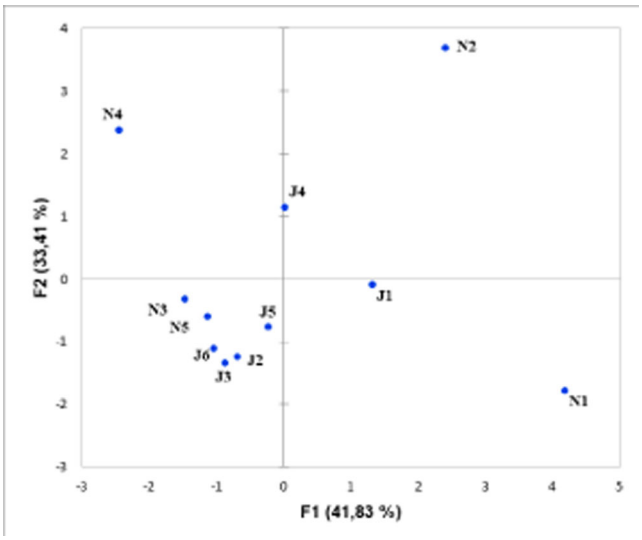

Fig. 1 Score plot obtained from PCA of the antioxidant capacities and the phenolic, flavonoid, and tannin contents of 11 *Cleome arabica* extracts

Table 4 Coefficients for the correlation between antioxidant capacities measured by ABTS, DPPH, PM, FRAP, and CUPRAC assays, total phenolic and flavonoid contents of *Cleome arabica* L. extracts

	Phenolics	Flavonoids	ABTS	FRAP	CUPRAC	DPPH	Molybdate
Phenolics	1	0.781	0.377	0.782	0.516	0.086	0.459
Flavonoids	0.781	1	0.0496	0.64	0.057	−0.399	0.048
ABTS	0.377	0.0496	1	0.629	0.9477	0.413	0.96
FRAP	0.782	0.646	0.629	1	0.692	0.075	0.713
CUPRAC	0.516	0.057	0.948	0.691	1	0.516	0.985
DPPH	0.0858	−0.399	0.413	0.075	0.516	1	0.521
Phosphomolybdenum	0.459	0.0478	0.966	0.713	0.985	0.521	1

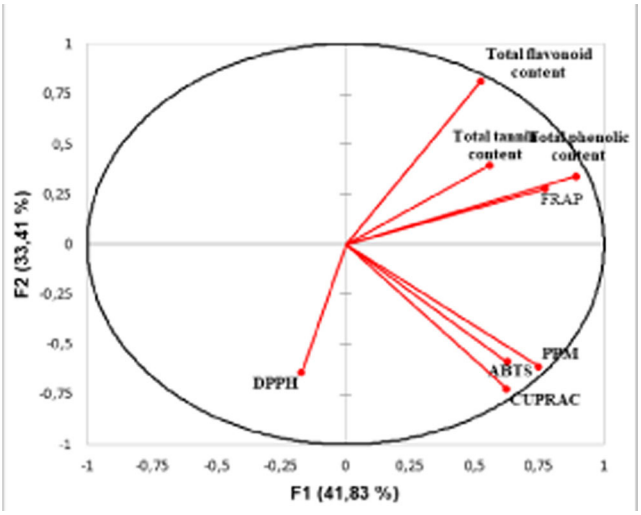

Fig. 2 Loading plot obtained from PCA of five antioxidant tests of 11 *Cleome arabica* extracts

It is expected because this extract was proved to have the highest antioxidant activity and therefore stands out from other extracts. In contrast, extract N1 has the highest negative loadings on PC2 (−1.78), and it is anticipated because this extract was proved to have the lowest antioxidant activity compared to other extracts. In contrast, a greater cluster of total phenolic, flavonoid, and tannins content with FRAP assay activity may account for their comparable activity due to their positional grouping at the same region of the plot (Fig. 2).

Conclusion

Up to date, the scientific documentation regarding antioxidant activity of plants extracts has been reported. Subsequently, we have explored in our study the antioxidant capacities of 11 extracts of local *Cleome arabica* leaves by utilizing five different in vitro assays. The present investigation provides useful information on antioxidant effects of these plant extracts, and the ethyl acetate fraction extract was showed maximum antioxidant power of all in vitro assays in comparison to the other extracts and standards.

Consequently, the present study provides scientific proof for traditional claim of Cleome arabica leaves as antioxidants. So, further in vivo studies are required to support the ethnomedicinal claim. Therefore, the present investigation will be supportive to the scientific documentation-related in vitro studies. Correlation between in vitro and in vivo studies may be helpful to understand the molecular mechanism of antioxidant process and to reveal phytochemicals of the extract responsible for this activity. Further studies need isolation and purification of active phytoconstituents with potent antioxidant activity.

Conflicts of interests the authors have no conflicts of interests to declare.

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