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Etude analytique de la composition bioactive de l'espèce *Calotropis procera* appartenant à la flore saharienne et Activity insecticide.

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Le : 25/01/2024

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THESIS

For obtaining the LMD Doctoral Degree in Chemistry

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Analytical study of the bioactive composition of *Calotropis procera* species belonging to the Saharan flora and Insecticidal study.

Publicly presented and supported by:

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DEDICATION

A special dedication of my grateful feelings,

To my father: Abdelkader

To my brother:Brahim

“May Allah have mercy upon them”
&
To my dear mother: Alalia

“May Allah protect her”

Who encouraged me enormously.

To my brothers Smail, Moussa, Bouamama
&
My sistesr Asma, Kaltoum, Zineb, Naziha,
&
My nieces Souria, Wfaa

Who were always here for me.

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List of abbreviations

C. procera	*C. procera*
CC	Column Chromatography
$CHCl_3$	Chloroform
DCM	Dichloromethane
DEPT	Distortionless-Enhancement by-Polarization-Transfer
EtAc	Ethyl Acetate
EtOH	Ethanol
Etp	Petroleum Ether
FA	Formic Acid
GC-MS	Gas Chromatography/ Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
LC-MS	Liquid Chromatography–Mass Spectrometry
LD_{50}	The medium Lethal Dose
MeOD	Deuterated Methanol
MeOH	Methanol
n-BuOH	n-Butanol
NMR	Nuclear Magnetic Resonance Spectrometry
P. blanchardi	*Parlatoria blanchardi*
PTLC	Preparative Thin Layer Chromatography
RP	Repellency Percentage
RT	Retention time
T.castaneum	*Tribolium castaneum*
TFA	Total Fixed Alkaloids
TLC	Thin Layer Chromatography
VAH	Volatile Alkaloids from *C. procera* dried aerial parts hydrolats

VDA	Volatile Dried Aerial parts
VDF	Volatile Dried Flowers
VLC	Vacuum Liquid Chromatography
Y	Extraction Yield
KI	Potassium iodide

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ABSTRACT

The present scientific contribution focuses on the determination of the volatile and non-volatile chemical composition of *C.procera*, which is a toxic Saharan plant collected from Djanet (Tasili N'Ajjer) region; as well as on the evaluation of the in vitro insecticidal activity of its latex and the three extracts (Etp, DCM, EtOH), in addition to the isolated compounds from the ethyl acetate (EtAc) extract of the aerial part of the species.

First of all, the ethnopharmacological study results have showed numerous uses of this plant in different preparation modes, such as maceration, for treatment of many diseases as dermatoses. On this basis, preliminary phytochemical tests were carried out on the latex and three crude extracts (Etp, DCM, and EtOH) prepared from the aerial part of *C. procera*, they revealed the presence of polyphenols, flavonoids, tannins, sterols, terpenes as well as alkaloids and glycosides.

Gas chromatography coupled with mass spectrometry (GC/MS) analysis of *C. procera* volatile fractions allowed the diagnosis of a rich chemical composition, with more or less significant presence percentages; among other there are: Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-, *p*-vinylguaiacol, Palmitic acid, Dotriacontane.

The purification and identification using different chromatographic methods and spectroscopic analysis such as NMR, IR, led to identify Rutin and Gallic Acid in EtAc extract while; Epicatechin, Rutin, and Quercetin were identified in n- Butanol extract.

To determine the total fixed alkaloids and the volatile ones from *C. procera* aerial parts different steps of separation and physico-chemical analysis were used. On one side, HPLC analysis allowed the identification of Atropine in two sub-fractions; on the other side, GC/MS analysis led to identify 32 compounds in VAH1 where Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- was the most abundant constituent; while, 12 compounds were identified in VAH2, where 13-Docosenamide, (Z)- was the major.

The evaluation of insecticidal and repellent activity in vitro of *C. procera* extracts against *P.blanchardi targ* and *T. castaneum* showed the ethanolic extract significant insecticidal activity on all *P. blanchardi* stages, reflected by an LD_{50} equal to 0.65 ± 0.01 mg/ml, which was assessed after 24 hours. Likewise, a significant repellent rate on *T. castaneum* adults is found, it is estimated at 96.66 ± 5.77%, compared to the other extracts, after 5 hours.

On another hand, the evaluation of the insecticidal activity of isolated compounds from the ethyl acetate extract showed that Rutin has a significant insecticidal activity on all

(*P.blanchardi*) stages estimated at 71.73%, compared to Gallic Acid, which gave 59.44% after 24 hours at 0.5 mg/ml.

Keywords: *Calotropis procera*, Tassili N'Ajjer, HPLC-DAD, GC-MS, NMR, Bio-insecticide activity, *Tribolium castaneum*, *Parlatoria blanchardi*.

RESUME

La présente contribution scientifique basé sur la détermination de la composition chimique volatile et non volatile de *C. procera*, qui est une plante saharienne toxique récoltée dans la région de Djanet (Tasili N'Ajjer) ; ainsi que sur l'évaluation de l'activité insecticide in vitro de son latex et des trois extraits (Etp, DCM, EtOH), en plus des composés isolés de l'extrait dacétate d'éthyle (EtAc) de la partie aérienne de l'espèce.

Tout d'abord, les résultats de l'étude ethnopharmacologique ont montré de nombreuses utilisations de cette plante dans différents modes de préparation, comme la macération, pour le traitement de nombreuses maladies comme les dermatoses. Sur cette base, des tests phytochimiques préliminaires ont été réalisés sur le latex et trois extraits bruts (Etp, DCM, EtOH) préparés à partir de la partie aérienne de *C. procera*, ils ont révélé la présence de polyphénols, flavonoïdes, tanins, stérols, terpènes ainsi que alcaloïdes et glycosides.

L'analyse par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC/MS) des fractions volatiles de *C. procera* a permis de diagnostiquer une composition chimique riche, avec des pourcentages de présence plus ou moins importants ; entre autres, il y a le phénol, le 2,2'-méthylènebis[6-(1,1-diméthyléthyl)-4-méthyl-, le p-vinylgaïacol, l'acide palmitique, le dotriacontane.

La purification et l'identification à l'aide de différentes méthodes chromatographiques et d'analyses spectroscopiques telles que la RMN, l'IR, a conduit à identifier la Rutine et l'Acide Gallique dans l'extrait d'EtAc tandis que ; L'Epicatéchine, la Rutine et la Quercétine ont été identifiées dans l'extrait de n-butanol.

Pour déterminer les alcaloïdes fixes totaux et volatils des parties aériennes de *C. procera*, différentes étapes de séparation et d'analyse physico-chimique ont été utilisées. D'un côté, l'analyse HPLC a permis l'identification de l'Atropine en deux sous-fractions ; d'autre part, l'analyse GC/MS a permis d'identifier 32 composés dans VAH1 où le Phénol, 2,2'-méthylènebis[6-(1,1-diméthyléthyl)-4-méthyl- était le constituant le plus abondant ; tandis que 12 composés ont été identifiés dans VAH2, où le 13-docosénamide, (Z) - était le le majeur.

L'évaluation de l'activité insecticide et répulsive in vitro des extraits de *C. procera* contre *P.blanchardi* et *T. castaneum* a montré que l'extrait éthanolique avait une activité insecticide significative sur tous les stades de *P. blanchardi*, reflétée par une DL50 égale à 0,65 ± 0,01 mg/ml, qui a été évaluée après 24 heures. De même, on retrouve un taux répulsif

significatif sur *T. castaneum* adultes, il est estimé à 96,66 ± 5,77%, par rapport aux autres extraits, après 5 heures.

D'autre part, l'évaluation de l'activité insecticide des composés isolés de l'extrait d'acétate d'éthyle a montré que la Rutine a une activité insecticide significative sur tous les stades (*P. blanchardi*) estimée à 71,73%, par rapport à l'Acide Gallique qui a donné 59,44% après 24 heures à 0,5 mg/ml.

Mots clés : *Calotropis procera*, Tassili N'Ajjer, HPLC-DAD, GC-MS, RMN, Activité bio-insecticide, *Tribolium castaneum*, *Parlatoria blanchardi.*

ملخص

هذه المساهمة العلمية مبنية على تحديد المركبات الكيميائية الطيارة و الغير طيارة لـ *C. procera* ، وهو نبات صحراوي سام يتم حصاده في منطقة جانت (Tasili N'Ajjer) ؛ بالإضافة إلى تقييم نشاط المبيدات الحشرية في المختبر لمادة اللاتكس الخاصة به والمستخلصات الثلاثة (Etp، DCM، EtOH) ، بالإضافة إلى المركبات المعزولة من مستخلص أسيتات الإيثيل (EtAc) للجزء الهوائي لهذه النبتة .

كبداية، أظهرت نتائج دراسة علم الأدوية التقليدي استخدامات عديدة لهذا النبات بطرق تحضير مختلفة، مثل التنقيع، لعلاج العديد من الأمراض كالأمراض الجلدية. على هذا الأساس، أجريت اختبارات كيميائية نباتية أولية على مادة اللاتكس وثلاثة مستخلصات خام (Etp، DCM، EtOH) محضرة من الجزء الهوائي ل *C. procera*، حيث كشفت عن وجود مادة البوليفينول والفلافونويد والعفص والستيرولات والتربينات وكذلك قلويدات وجليكوسيدات.

أتاح التحليل باستخدام كروماتوغرافيا الغاز المقترن بمقياس الطيف الكتلي (GC / MS) للأجزاء المتطايرة من *C. procera* إمكانية تشخيص تركيبة كيميائية غنية، مع نسب وجود أكثر أو أقل؛ من بينها:

Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-, *p*-vinylguaiacol, Palmitic acid, .Dotriacontane

قادتنا التنقية باستخدام طرق كروماتوجرافية مختلفة والتحليلات الطيفية مثل الرنين المغناطيسي النووي NMR والأشعة تحت الحمراء IR للتعرف على Rutin و Gallic Acid في مستخلص EtAc بينما؛ تم التعرف على Epicatechin و Rutin و Quercetin في مستخلص n -BuOH.

لتحديد مجموع القلويدات الثابتة الكلية والمتطايرة للأجزاء الهوائية من *C. procera*، تم استخدام مراحل مختلفة من الفصل والتحليل الفيزيائي الكيميائي. من ناحية، سمح تحليل HPLC بتحديد ال Atropine في كسرين فرعيين؛ من ناحية أخرى أدى تحليل GC / MS إلى تحديد 32 مركبًا في VAH1 حيث كان Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-] هو الأكثر وفرة ؛ بينما ، تم تحديد 12 مركبًا في VAH2 حيث كان 13-Docosenamide, (Z)- هو المركب الرئيسي.

أظهر تقييم النشاط المبيد للحشرات وطارد الحشرات في المختبر لمستخلصات *C. procera* ضد *P.blanchardi* و *T. castaneum* أن المستخلص الإيثانولي كان له نشاط مبيد حشري بارز على جميع مراحل *P. blanchardi* ينعكس بمقدار LD_{50} 0.01 ± 0.65 مغ/مل والذي تم تقييمه بعد 24 ساعة.

كما لوحظ وجود نسبة كبيرة من المواد الطاردة للحشرات على *T. castaneum* البالغة، حيث تقدر بنحو96 ,66±5.77% ، مقارنة بالمستخلصات الأخرى ، بعد 5 ساعات.

من ناحية أخرى، أظهر تقييم فعالية المبيدات الحشرية للمركبات المعزولة من مستخلص أسيتات الإيثيل أن Rutin له نشاط مبيد حشري بارز على جميع مراحل *(P. blanchardi)* يقدر بنحو 71.73٪ ، مقارنة بـGallic acid الذي أعطى 59.44٪ بعد 24 ساعة عند 0.5 مغ / مل.

الكلمات المفتاحية: *Calotropis procera* , طاسيلي ناجر , RMN, GC-Ms, HPLC-DAD , *نشاط مبيدات الحشرات الحيوية*, *Tribolium castaneum, Parlatoria blanchardi* .

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General introduction

Recently, the search for biologically active natural materials has become of great interest, especially if they are obtained from bioresources in their native state. In fact, their potential bio-effects are supposed to be closely similar in enhancing health conditions; these bioproducts are hunted to substitute the industrial ones whose side effects are discutable [1].

Natural substances are those produced by living organism which commonly used as food and as a treatment for both humans and animals, because of its medicinal properties as antioxidants and antibacterial agents. Plants are considered one of the natural stores of natural materials, even the toxic ones that contain milky substance (latex) [2].

Most of these latexe's species as Apocynaceae and Asclepidiaceae remain to be scrutinized for their biological usefulness. Bioactive potential of latex is reported in the scientific literature against various pathogenic organisms [1,2].

Indeed, the green parts of *C.* plant produce and accumulate the latex as a defense strategy against organisms such as virus, fungi, and insects; this explains its use by the local population to combat some skin fungi infections [3,4].

On the light of the bibliographic research, our choice was oriented towards *C. procera* as a potential natural resource of bioactive chemicals. Actually, the species appears very interesting to carry out our study; in addition, a limited number of scientific reports on it as a toxic species commonly used in traditional medicine in the Algerian Sahara were found. *C. procera* belongs to the family Asclepiadaceae, it is an Algerian medicinal plant locally known as "Kranka"; it was reported that it contained many secondary metabolites such as, cardenolides, steroids, tannins, and saponins [16].

Regarding the uses of *C. procera;* some researchers have described that in folk medicine the whole plant is used to treat common diseases; however, others have stated that the most important parts of this plant used for medicinal purposes are leaves and latex [5, 6, 7].

The present contribution focuses on the analysis of the chemical composition of *C. procera* by purifying, characterizing and quantifying the alkaloid and phenolic compounds, as well as the volatile fraction, using different physico-chemical analytical methods after an investigation on the uses of this plant in traditional medicine. Furthermore, the study aims to evaluate the effectiveness of these samples against several discomforts encountered in the agro-food and toxicological sector. Overall, our study is divided into two parts:

1. **Bibliographic study; contains two chapters:**

- Generalities on secondary metabolites.
- The chemical composition of *C. procera*

2. Experimental parts; contains four chapters:

- Ethnopharmacological and Phytochemical study.
- Extraction and characterization of volatile compounds by GC-MS.
- Purification, identification and characterization of phenolic compound in EtAc and n-Butanol fractions by TLC, PTLC, HPLC, LC-MS, FT-IR, and RMN.
- Purification, identification and characterization of fixed Alkaloids (TFA) and volatile Alkaloids (VAH) by TLC, HPLC, GC-MS.
- Evaluation of the Bio-insecticidal activity of *C. procera* extracts, *C. procera* latex, isolated Rutin and Gallic acid against *T. castaneum* adults and *P. blanchardi* adults and larvae.

Our study will end with a general conclusion gathering all the results obtained with some perspectives.

Part one:
Literature review

Chapter I:

Generalities on secondary metabolites

I.1. Introduction

A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs [8] . Since ancient times, people have searched for natural sources to treat their diseases; the beginnings of the medicinal plants' use were instinctive, as is the case with animals [9]. Studies have been carried out globally to verify the efficacy of medicinal plant and some of the findings have led to the production of plant based medicines. Currently, the global market value of medicinal plant products exceeds $100 billion per annum [8].

Medicinal plants have a large group of secondary metabolites (SMs) based on their chemical composition, these have biological activities that can improve human health.but they also represent important value in perfume, agrochemical, cosmetic industries [10].

Many SMs such as alkaloids, terpenoids, and phenylpropanoids are being considered for drug development [11].

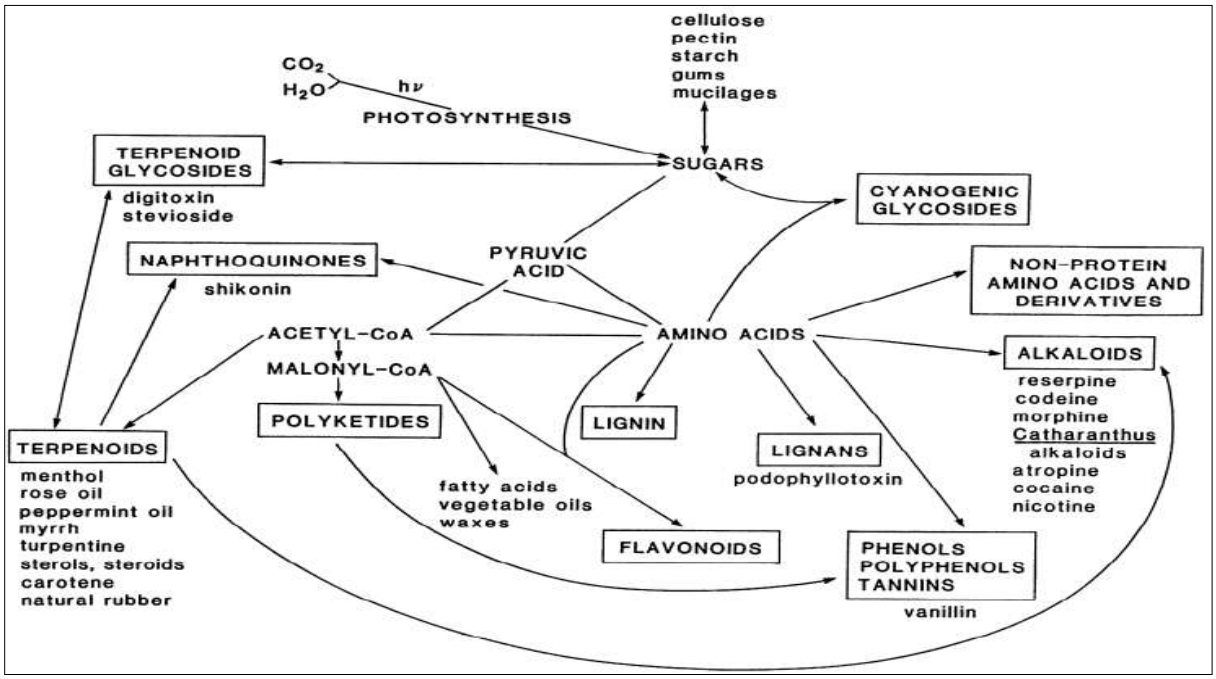


Figure I.1. Biosynthetic origin of some commercially important plant derived compounds (major groups of secondary metabolites) [12].

I.2. Alkaloids

I.2.1. Presentation

In the plant kingdom, alkaloids, cyanogenic glucosides/glucosinolates and nonprotein amino acids represent the major classes of nitrogen containing ‘‘secondary metabolites’’ [13].

An alkaloid is an organic basic compound (most often of plant origin), heterocyclic with nitrogen as the heteroatom, with a complex molecular structure, the concept of being derived from amino acids was added, together with the idea that the nitrogen should be in a heterocyclic ring [13,14]

According to their shapes and origins, alkaloids have several classifications; they are divided into three categories: true and proto alkaloids are derived from amino acids, whereas pseudo alkaloids are not derived from these compounds [15].

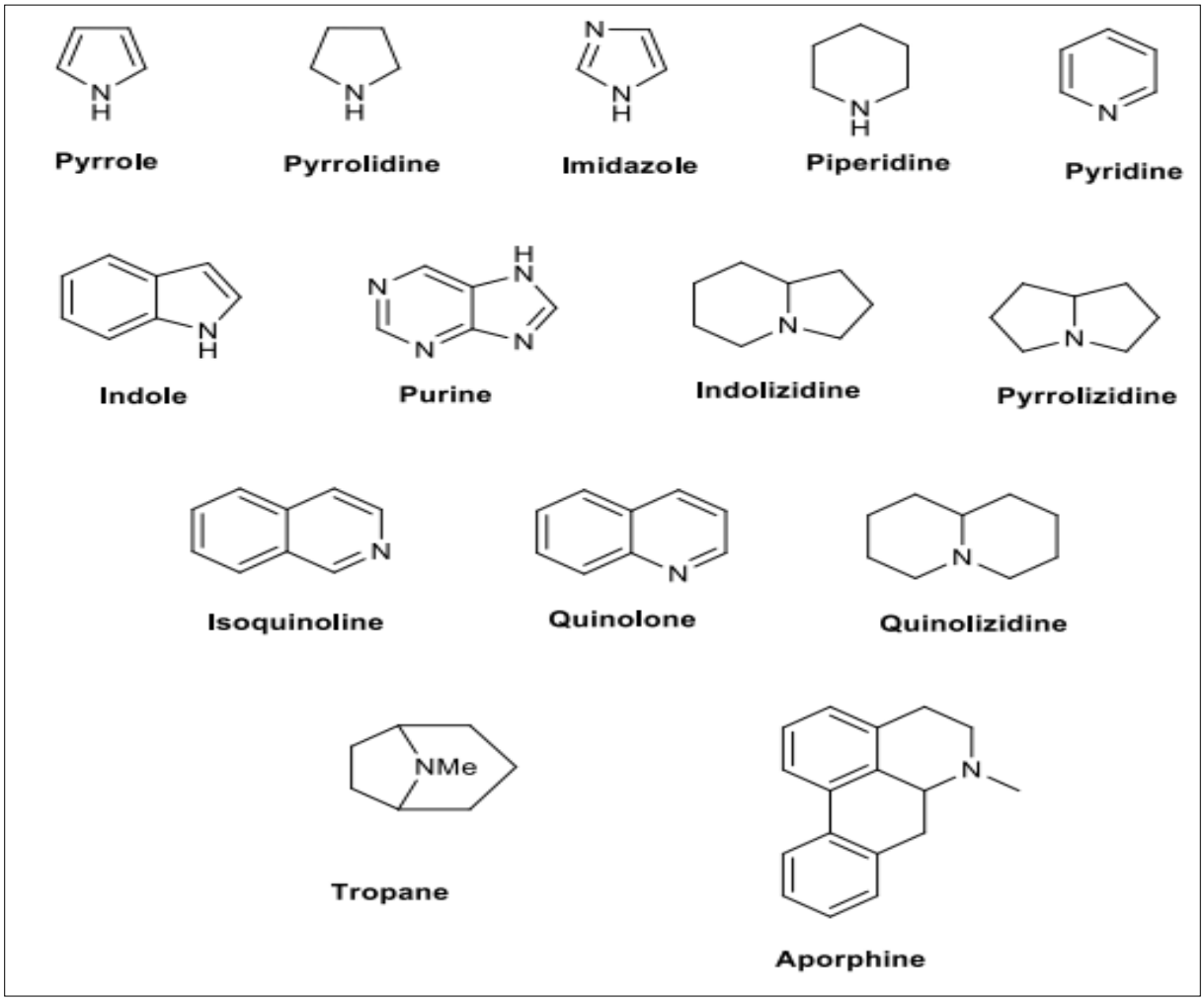


Figure I.2. The 14 sub-groups of alkaloids.

I.2. 2.Classification

- **True-alkaloids (Heterocyclic alkaloids):** are the compounds which derive from amino acid and a heterocyclic ring with nitrogen. Alkaloids discovered in these groups have been the most prevalent genuine alkaloids found in nature e.g. Atropine, Nicotine, Cocaine, Quinine, Dopamine, Morphine, Geissospermine, Piperine, Berberine, and Gasoline [16].

- **Proto-alkaloids (Non-heterocyclics):** are the compounds which contain nitrogen atom derived from an amino acid which is not a part of the heterocyclic ring e.g. Adrenaline, Mescaline, Colchicine, Cathinone, and other proto alkaloids Hordenine,Pcilocin; they are uncommon in nature. Mescaline is a phenyl ethylamine alkaloid derived from the Lophophora williamsii plant [16,17,18].

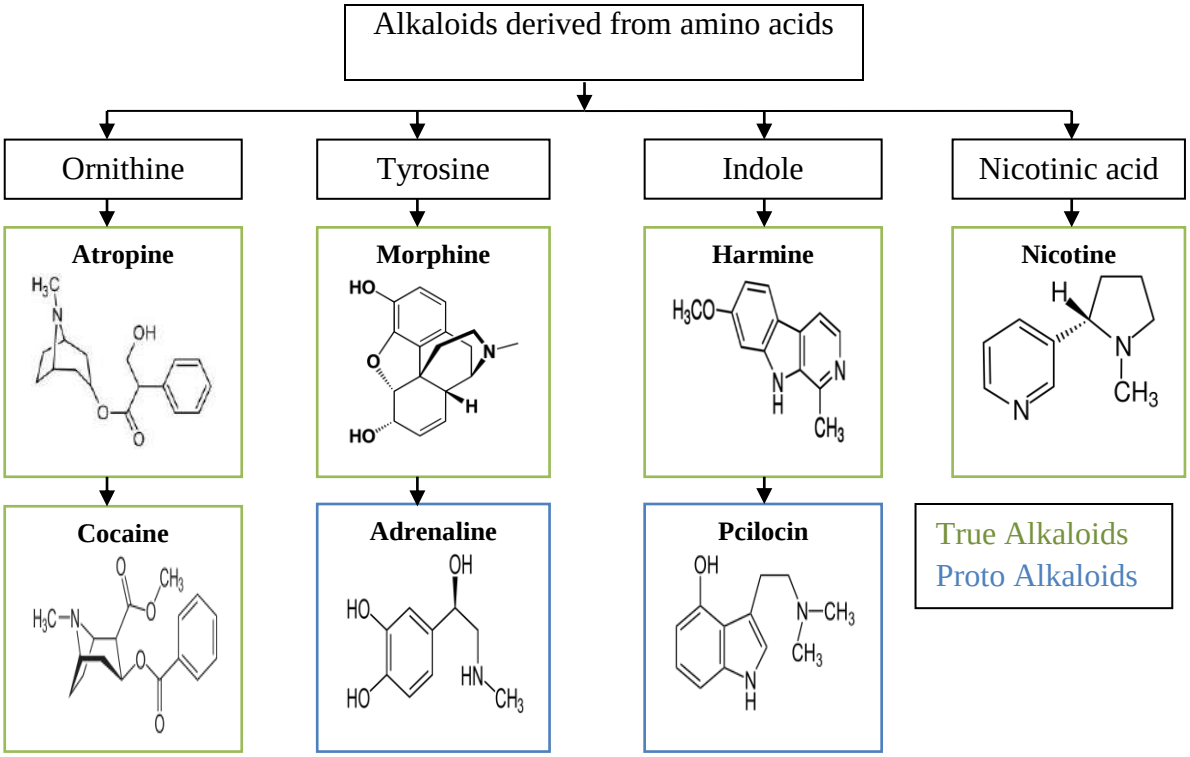


Figure I.3. Schematic representation of true and proto-alkaloids.

- **Pseudo-alkaloids:** are the compounds that do not originate from amino acids, they produced by amination or transamination of amino acid precursors or post cursors. Pseudo alkaloids could be acetate and phenylalanine-derived or terpenoid, as well as steroidal alkaloids e.g Coniine, Capsaicin, Ephedrine, Solanidine, Caffeine, the Obromine, and Pinidine [16].

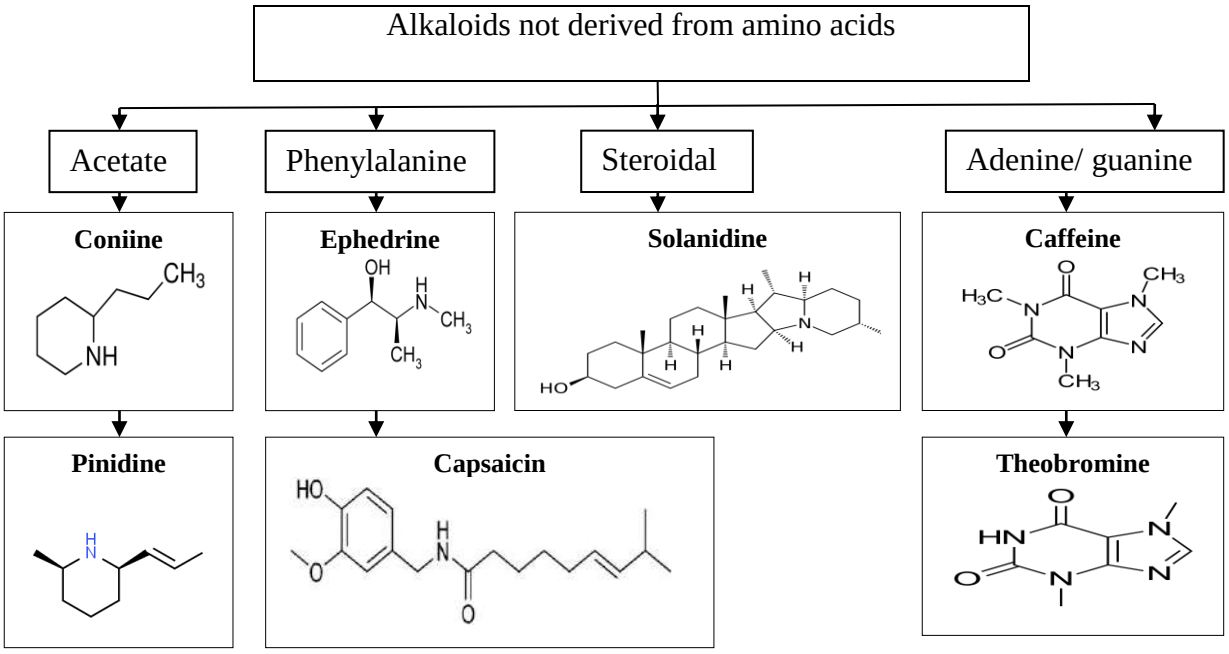


Figure I.4. Schematic representation of pseudo-alkaloids.

I.2.3.Properties of alkaloids

- Alkaloids are compounds of a basic character; they form salts with mineral acids (hydrochloride, sulfate, and nitrate) or organic acids (tartrates, sulfamates, maleates) [19].
- Alkaloid salts have different solubility (in different solvents such as water and dilute alcohols) with different pH except in very rare cases they are insoluble in organic solvents [19].
- Alkaloids are precipitated with some specific reagents (alkaline reagents), these take place in a slightly acidic aqueous medium [19].
- Generally, alkaloids are extremely toxic, they are bitter in taste but often optically active substances, colorless, crystalline or liquid at room temperature [20].

I.2.4. Biological activities of alkaloids

For a long time, many alkaloids extracted from plants have been used for treating a large number of ailments including: snakebite, fever and insanity and even today it still prominent drug [21]. Due to their involvement in defense, the pharmacological actions of alkaloids remain the subject of interest by many researchers and scientists through many studies, as for Quinolone alkaloids like quinine and quinidine that are used as antimalarial drugs [22]; indole alkaloids as antitumor drugs [23]; the berberine showed anti-diabetic effect [24] , anti-hypertensive, anti-inflammatory, antioxidant, antidepressant, hepatoprotective

activity, and anti-cancer activities [25,26]. Many other alkaloids (piperidine alkaloid) exhibit insecticidal and fungicidal activity [27,28,29].

I.3. Phenolics

I.3.1. Presentation

Phenolic compounds are secondary metabolites (SMs) widely spread throughout the plant kingdom with around 8000 different phenolic structures [30].

These phenols are composed of an aromatic ring with one or more hydroxyl groups. Although, phenolic compounds can be present in their free form in plants; they are generally present bounded to sugars or proteins [31].

I.3.2. Classification

Usually, phenolic compounds are classified in different ways. According to their carbon chain, they can be divided into 16 classes [32]; but in relation with the most important structures found in the human diet, polyphenols and simple phenols are classified as presented in **Figure I.5**.

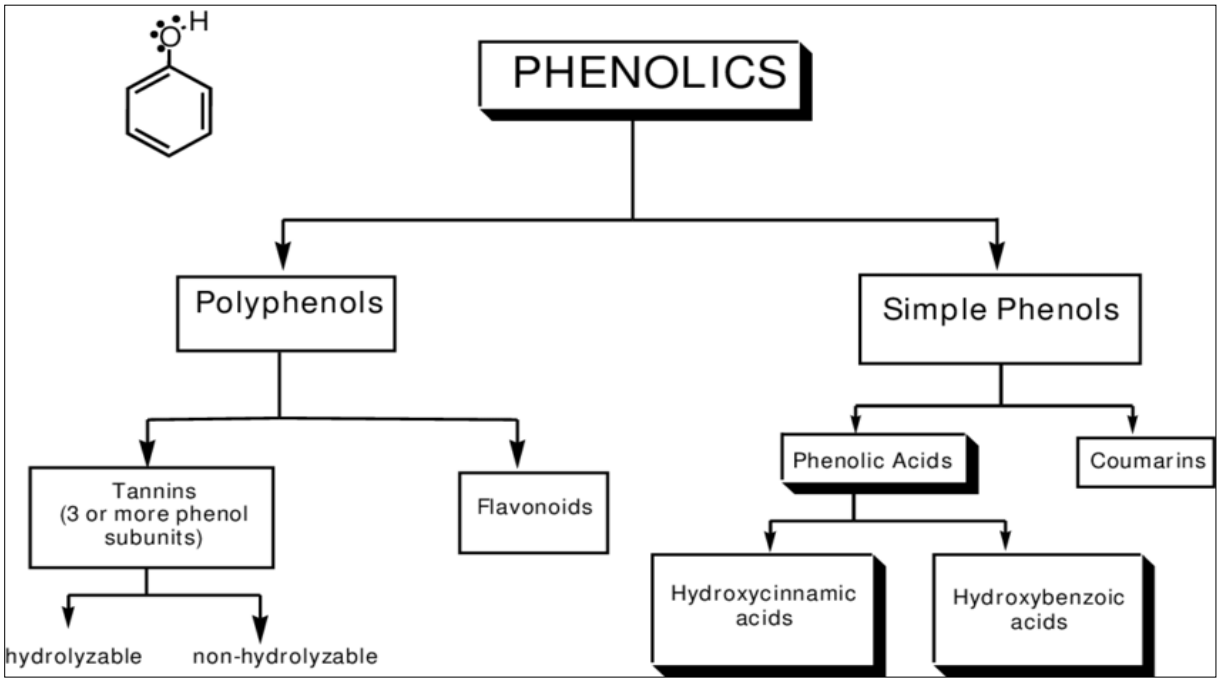


Figure I.5. Schematic classification of polyphenols [33].

- **Phenolic acids**

Phenolic acids described as compounds having two distinguishing constitutive carbon frameworks; the hydroxycinnamic and hydroxybenzoic structures and derivatives **Figure I.6.**

The benzoic acids have seven carbon atoms (C6 -C1) and are the simplest phenolic acids found in nature, Cinnamic acids have nine carbon atoms (C6 -C3), but the most commonly found in vegetables are with seven. The structural differences between various phenolic acids are small, originating from the number and positions of the hydroxyl and methoxy groups on the aromatic ring [33,34].

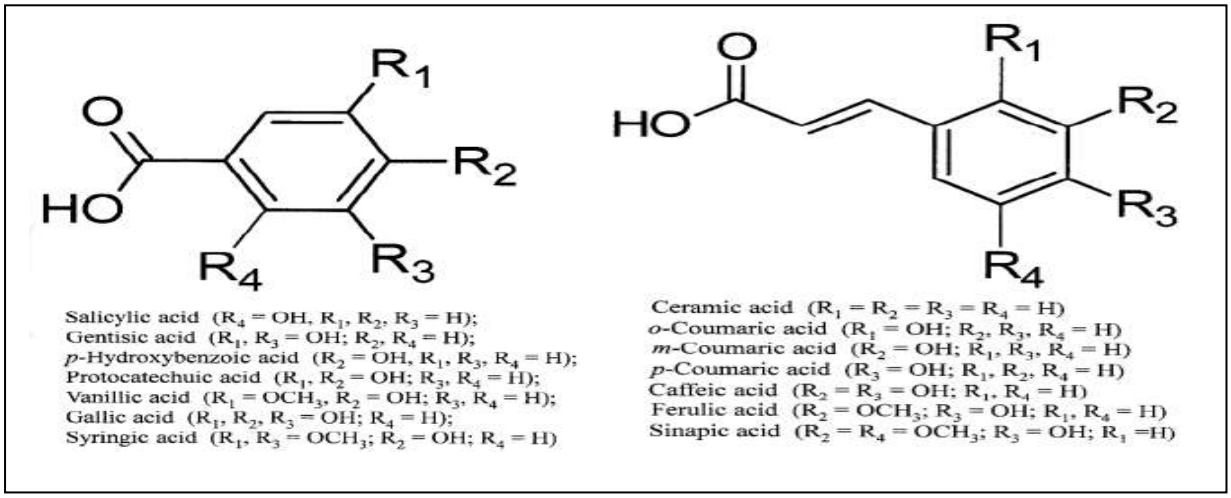


Figure I.6. Structures and substitution patterns for prominent phenolic acids [32].

- **Flavonoids**

Flavonoids are important group widely distributed among the plant, they constitute the major group of phenolic compounds and they are responsible for the blue, purple, yellow, orange and red colors in plants [35]. Structurally, they are formed of more than one benzene ring in their structure (a range of C15 Aromatic compounds), and due to the variation in the number and positions of hydroxyl groups as well as in their range of alkylation and glycosylation, flavonoids can be divided into six subclasses: flavones, isoflavones, flavonols, anthocyanins, flavanols and flavanones (**Figure I.7**) [36].

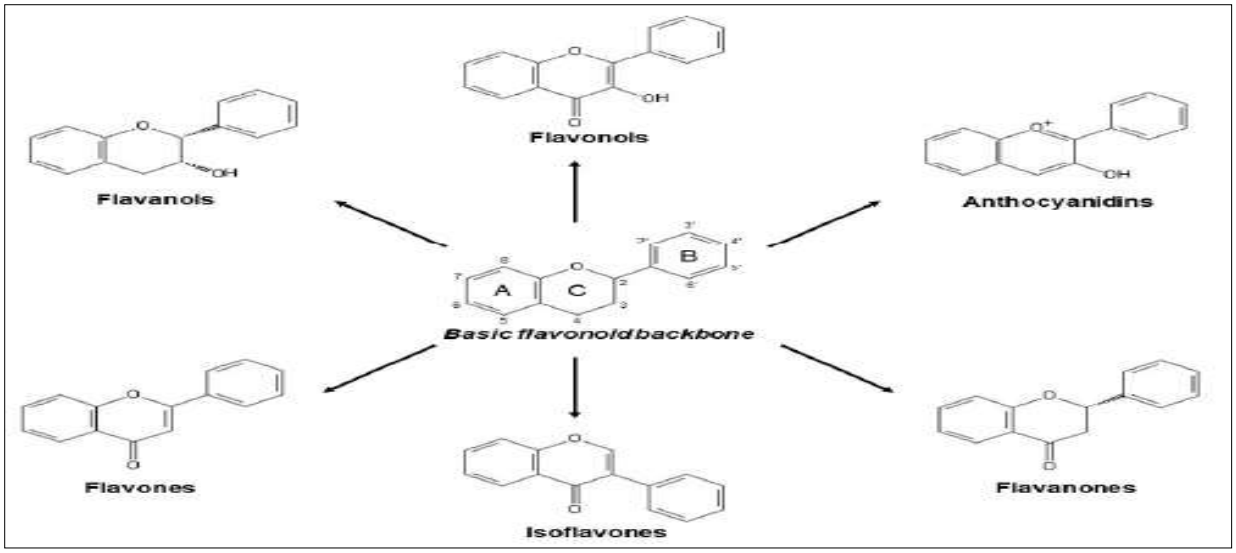


Figure I.7. Basic flavonoid structure and main types of flavonoids [37].

I.3.3. Biological activities of Phenolics

Interest in phenolic compounds has dramatically increased during the last decade despite the antioxidant activity and their possible benefits to human health, Phenolic acids are naturally found in fruits and vegetables, and they were proven active as: antidepressant [38], antihypertensive [39], anti-inflammatory, neuroprotective [40], anticancer and antidiarrheal agents [41].

In addition to being anti-oxidant agents, in vitro many studies also showed that flavonoids have anti-inflammatory, anti-allergic, hepato-protective, anti-thrombotic, antiviral, and anti-carcinogenic activities [4,42].

Regarding, tannins, they are used in the veterinary field as anthelmintic and antimicrobial agents [43,44].

I.4. Terpenoids

I.4.1. Presentation

Terpenes, also termed terpenoids or isoprenoids, are natural compounds found in several organisms belonging to the animal and plant kingdoms, they constitute the largest class of plant secondary metabolite (SMs) with more than 55,000 known compounds diversified in their structure, functions, and properties [45].

I.4.2. Classification

Based on the number of isoprene units present in their structure, Terpenes, are classified into hemiterpenes (C5H8), monoterpenes 2 (C5H8), sesquiterpenes 3 (C5H8), diterpenes 4 (C5H8), sesterterpenes 5 (C5H8), triterpenes 6 (C5H8), etc. **Figure I.8** [45].

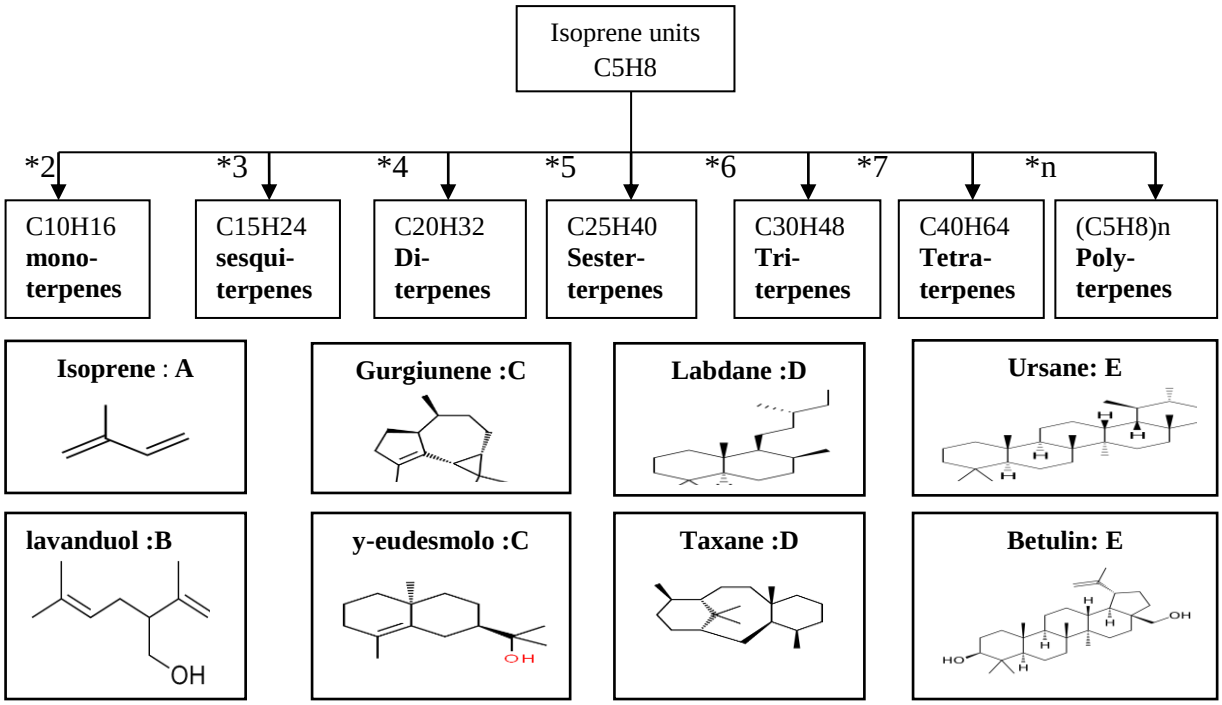


Figure I.8. Examples of terpenes structures and classification

A)Himiterpene , B)monoterpenes, C)sesquiterpenes, D)diterpenes, E)triterpenes [46].

I.4.3. Biological activities of Terpenoids

Several studies have attributed to this big family of compounds a range of pharmacological properties, such as anticancer, antimicrobial, antifungal [45]; various monoterpenes are toxic to insects, fungi, and bacteria [47,48,49].Terpenes also have various applications in industrial sectors, such as pharmaceutical, food, cosmetic, perfumery, agriculture, biopesticides [45].

I.5. Methods for analyzing plant metabolites

The analytical techniques that can be used to identify the constituents of a complex mixture are numerous and varied. However, constituents' identification and quantification of a natural mixture always remain delicate operations and often require the use of several complementary techniques [50,51]. Generally, the constituents of a natural complex mixture are identified in three ways (**Figure I.9)**.

- **Path A:** is especially well suited to routine analysis such as quality controls of samples for example essential oils or plant extracts, whose constituents have already been described in the literature. It involves the coupling of a chromatographic technique (CPG, HPLC), which allows the individualization and quantification of the constituents, with a spectroscopic technique (SM, IRTF, etc.), which allows their identification by comparison of their spectral data with those of known products [52].
- **Path B:** is recommended when the constituents of a mixture present difficulties in identification (structures of a new compound and/or very similar structures). Two steps are necessary: after purification/isolation of the compounds by different chromatographic techniques such as thin layer chromatography (TLC), column chromatography (CC), HPLC and preparative GC (CPGP) preceded either by fractional distillation or crystallization, then a structural analysis is carried out based on 1H and 13H NMR [52].
- **Path C:** intermediate between the two previous ones, implements 13C Nuclear Magnetic Resonance without prior separation of the compounds or proceeded by a fractionation step reduced to a minimum. This path, initiated by **Formácek** and **Kubeczka (1982a/1982b)** [53,54] has been developed, optimized and computerized by the "Chemistry and Biomass" team (UMR CNRS nº 6134-SPE-University of Corsica) for about twenty years to become a real analytical tool for identifying the constituents of a natural mixture.

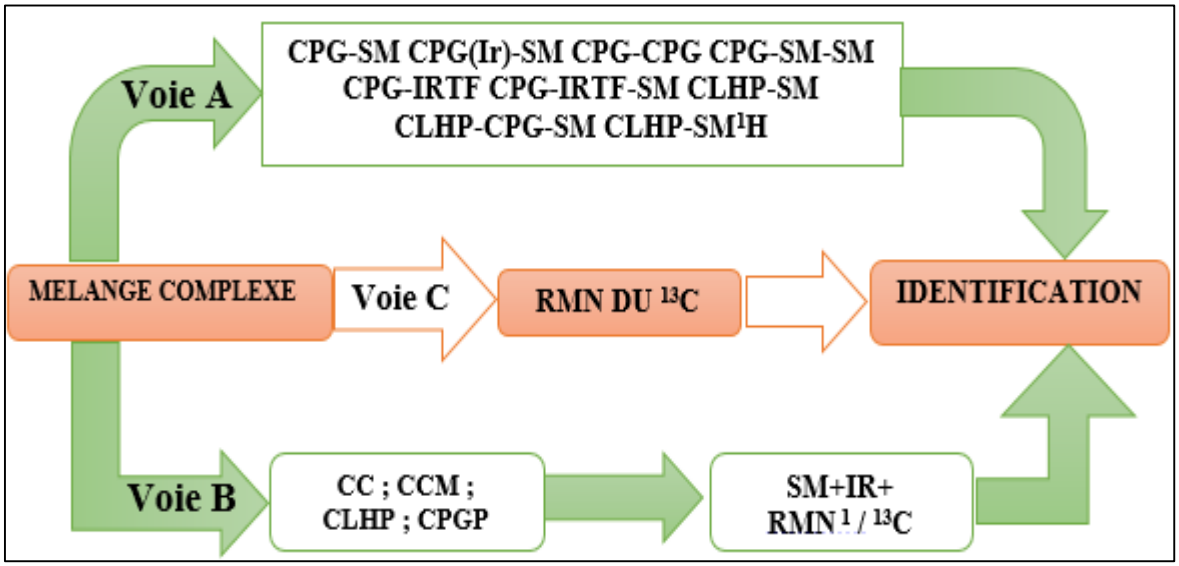


Figure I.9. The principal ways of analyzing a natural complex mixture [52].

I.6. Conclusion

From the earliest times, Nature and traditional medicine have remained as source of medicinal agents used for the treatment of various ailments. The valorization of natural resources could constitute a solution to socio-economic progress, agro-alimentary, food and pharmaceuticals. For this reason, more investigations should be based on phytochemical studies, pharmacological and therapeutic potential of herbal medicines.

Chapter II:

Generalities on Calotropis procera (Ait.) R. Br.

II.1. Introduction

Since ancient times, humans have highly relied on the use of plants for their food, clothes, and fragrances and especially in the treatment of diseases [55]. The search for new pharmacological active agents from natural resources such as plants, led to the discovery of many clinical useful drugs [56].

II.2. Generalities on *Calotropis procera*

Calotropis genus is a perennial wild herb widely distributed in tropical and subtropical regions of Asia and Africa. *Calotropis procera* belongs to the family Asclepiadaceae and it is a xerophytic erect shrub, soft wooded, evergreen perennial shrub, commonly known as "milk weed" because it produces large quantity of latex [57].

The plant and its latex represent an important source for the preparation of folk medicine [58]; it is well known for the pharmacological properties of its latex which is used as a defense strategy against organisms such as virus, fungi, and insects [3,4].

In traditional medicine, C. species are used to treat skin infections like (leucoderma, eczema and leprosy) [59], antidote for scorpion and rabies [60,61]. Moreover, it offers various benefits for the environment where it grows, among others the settlement in sandy soils, prevention of soil erosion, natural production in weavering and medical industries [62].

II.3. Taxonomic classification [57]

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Gentianales
Family: Asclepiadaceae
Genus: C.
Species: C. procera

Figure II.1. Photo of *C. procera* (Djanet 10/10/2019).

II.4. Morphology

C. procera is a shrub, 2.5-6m meters tall, with large, almost sessile opposite leaves, entire, glaucous green, oval and leathery, have a waxy appearance, they are 15-30 (cm)long and 2.5-10 (cm) broad [63,58].

The flower petals are arranged in pentamerous form, small, cream or greenish-white at the base and purple violet at the extremity of the lobes also it has a deep root system [58], the flowering period ranged from March to October [64].

The fruit, exceeds 10 cm in diameter, is a large follicle, greenish, ovoid, stuffed with silky filaments containing flattened Seeds, All this plant parts contain viscous, abundant and very irritating latex [63].

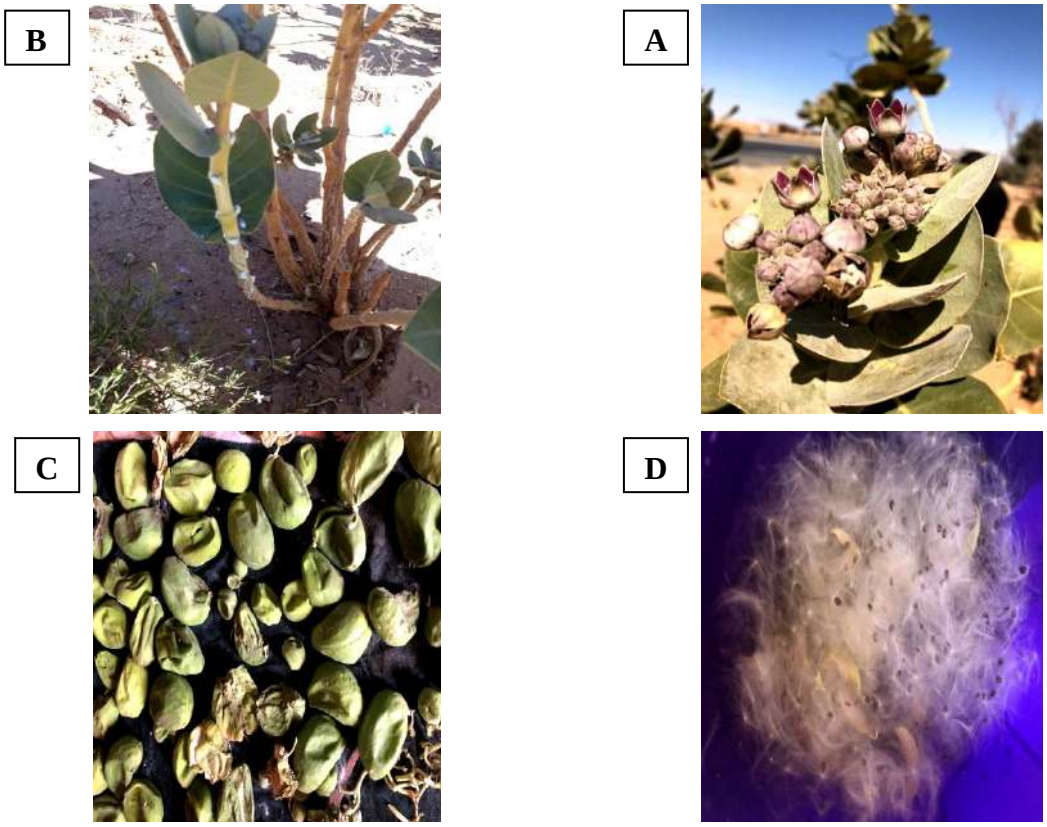


Figure II.2. Photos of *C. procera* organs: inflorescence **(A)**; latex oozing out of the stem and leaves **(B)**; follicle fruits **(C)**; seeds and silky filaments (fiber) **(D)**.
(Djanet 10/10/2019).

The plant is known in Arab-Greek medicine as Ushar or Madar [65], in Algeria it is called Kranka, Ushaar, Torha and Tourdja [63].

II.5. Geographical distribution

C. procera is native to Africa, Arabian Peninsula, Western Asia, the Indian Subcontinent, and Indo-China, However, the introduction of the plant outside its native boundaries has led to its naturalization in parts of Africa, Australia, and America [66].

The geographical board of *C. procera* is presented in **Figure II.3**. It is found in most parts of the world with a warm climate in dry, sandy and alkaline soils [67].

C. procera grows and spreads rapidly in high degree of abiotic stresses; tolerates high temperatures, salinity, drought resistant, to a relatively high degree and is quickly established as a weed along roadsides in rural and urban region [68].

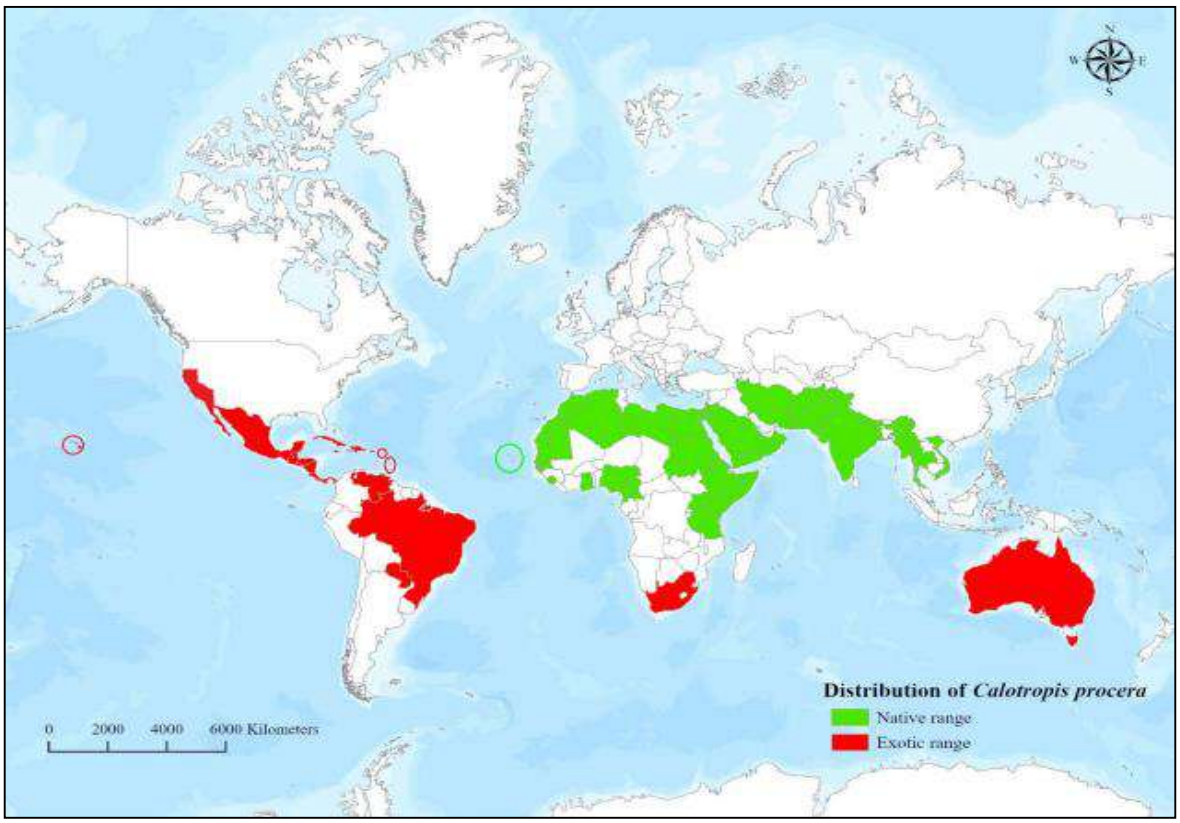


Figure II.3. Worldwide distribution of *C. procera* [69].

II.6.Traditional uses and Phytochemistry

C. procera organs are used, in traditionally medicine, to treat different diseases in different ways as summarized in the table **Table II.1**.

Table II.1. Traditional uses of *C. procera.*

Plant parts	Mode of preparation	Diseases and Illness	References
Stem		-skin diseases -enlargements of abdominal viscera -intestinal worms -leprosy and cure leucoderma	[68,70]
Root		Eczema, leprosy, elephantiasis, asthma, cough, rheumatism, diarrhoea and dysentery	
		Warts	[63]
		Snakebite	[71]
	Root decoction	Used to the treatment of breast cancer and gonorrhea	[72]
		Colds, cough and asthma	[63]
	Root bark powder	Calms dysentery, also it is a diaphoretic and an expectorant, treat camel mange	
Latex	Latex raw /diluted	-Comfort toothache -Purgative -Remove hair from hides	[73,74,75]
		- Applied directly to the warts	[63]
Flower		Digestive, stomachic, cough, asthma loss of appetite	[73,74,75]
	Flowers decoctions/ Ashes	Used for simple or infected wounds, eczema, scabies	[63]
Bark	Powder/ infusion	Treatment of leprosy and elephantiasis,	[71]
Leaves	Leaves Exudates	Scorpion bite treatment of infectious diseases	[76]
	Boiling leaves	Used to treat paralysis	[73-74-75]
	Leaves maceration	Taken to treat colds, cough and asthma	[63]
	Fresh leaves	Applied to painful joints and swelling	[77,78,79]
	Leaves Oil	Applied to paralyzed part	
	Leaves powder	Wound healing	[73-74-75]

II.7. Chemical constituents and Toxicity

C. procera is considered one of the few plants not to be fed to grazing animals because of its poisonous latex to humans and animals. On one hand, this latex, in contact with human eye, could be toxic and it can cause blindness and photophobia; other symptoms such as skin inflammation, vomiting, diarrhea, low blood pressure, and even death were recorded; on the other hand this material was used by tribes to poison arrows intended for hunting [80,81].

Table II.2 Shows the list of chemical constituents were isolated from *C. procera*

Table II.2. Chemical constituents isolated from *C. procera* different organs.

Part of plant	Chemical class	Chemical constituent	**Ref.**
Latex	Cardenolides	Calactoprocin/ Afrogenin/ Afroside/ 15β-Hydroxy uscharin/ 15β-Hydroxy calactin/ 12β-Hydroxycoroglaucigenin/ Procegenin A/ Procegenin B	[82]
		Uscharin / Uzarigenin / Syriogenin / Proceroside /Calotropagenin	[83]
	Steroids	3β,27-Dihydroxy-urs-18-en-13,28-olide/ β-Sitosterol	[84]
	Flavonoid	Quercetin-3- rutinoside	[85]
	Terpenes	β-amyrin/ Lupeol	[86]
	Proteins /Enzymes	Procerain/Procerain B	[87]
	Alkaloids	Uscharin / uscharidin / voruscharidin	[88]
Leaves	Flavonoids	5-Hydroxy-3,7-dimethoxyflavone-4'-O-β-Glucopyranoside/ Isorhamnetin/ Kaempferol/ Rutin	[89]
		Quercitin/ Kampferol	[90]
	Esters	Tridecyl ester	[91]
	Volatiles	1-Hexacosene/ Pentatriacontane/ R-Limonene/ Tridecane	[91]
		Myristicin/ Myristic acid /E-phytol/ Oxygenated sesquiterpenes/ Oxygenated diterpenes/ E,E-farnesyl acetone	[92]
		Mannosamine/ Pentatriacontane/ R-Limonene/ Tridecane	[91]
	Cardenolides	Uscharin /Uscharidin /Calotropin /Calotoxin /Uzarigénine/ Acide -19 –calotropine	[88]
	Terpenes	α-Amyrin/ α-Amyrin acetate / Urosolic acid	[93,94,95]
	Steroids	β-sitosterol / Procesterol	[96,97,98]
	Alkaloids	Mudarine	[99]
Root/Root bark	Cardenolides	Digitoxigenin /Digitoxin/Digoxigenin/ Calotoxin/ Proceragenin **(R)**	[100]
	Steroids	Cyclosadol/ Procesterol /Multiflorenol **(R)** /Calotroposides H–N/ Stigmasterol **(RB)**	[101-100]
	Terpenes	Pseudo-taraxasterol acetate/ Taraxasterol/ Calotroprocerol A/ Calotroproceryl acetate B **(RB)**	[101]
		Dihydrophytoyl tetraglucoside/ Phytyl iso-octyl ether/ Procerasesterterpenoyl triglucoside **(R)**	[102]
		Calotropenol/ Calotropenyl acetate/ β-Sitostenone **(R)**	[100]

	Esters	Calotropterpenyl ester **(RB)**	[104]
Flowers	Flavonoid	Quercetin3-O-rutinoside/ D-arabinose/ glucose/ glucosamine/ L-rhamnose	[104,105]
	Enzymes	3-Proteinase/ Calotropain (protease)	[104,105]
	Cardenolides	Uzarigenin	[104,105]
	Alcaloids	Uscharidin/ Voruscharin	[104,105]
	Volatiles	Myristicin/ Myristic acid /E-phytol/ Oxygenated sesquiterpenes/ Oxygenated diterpenes/ E,E-farnesyl acetone	[92]
	Lignans	7'-Methoxy-3'-O-demethyl-tanegool-9- O-βD glucopyranoside	[106]
Stem	Cardenolides	Calotropin/ Proceroside/ Calactin / Calotoxin/ Ascléposide/ Uzarigénin /Calotropagénine/ Coroglaucigénin	[84,88]
	Alcaloids	Uscharidin	[84]
	Volatiles	Myristicin/ Myristic acid /E-phytol/ Oxygenated sesquiterpenes/ Oxygenated diterpenes/ E,E-farnesyl acetone	[81]
Seeds	Cardenolides	Frugoside /Caroglaucigenin/ Carotoxigenine /Calotropin	[107]
Fruit		Coroglaucigenin/ uzarigenin	[84]

R : Root ; RB : Root Bark

Polyphenol content like flavonoids also varies in different parts of *C.procera* plant; where the Rutin is a major form of flavonoids present in this species with highest amount found in latex with 9.7 %, followed by 7.6 % in flowers, 5.0 % in leaves, 4.8 and 1.7 % stem and roots respectively [108]. In addition to this, Mudarine considered one of the most principal cardioactive alkaloid constituent found in leaves [99].

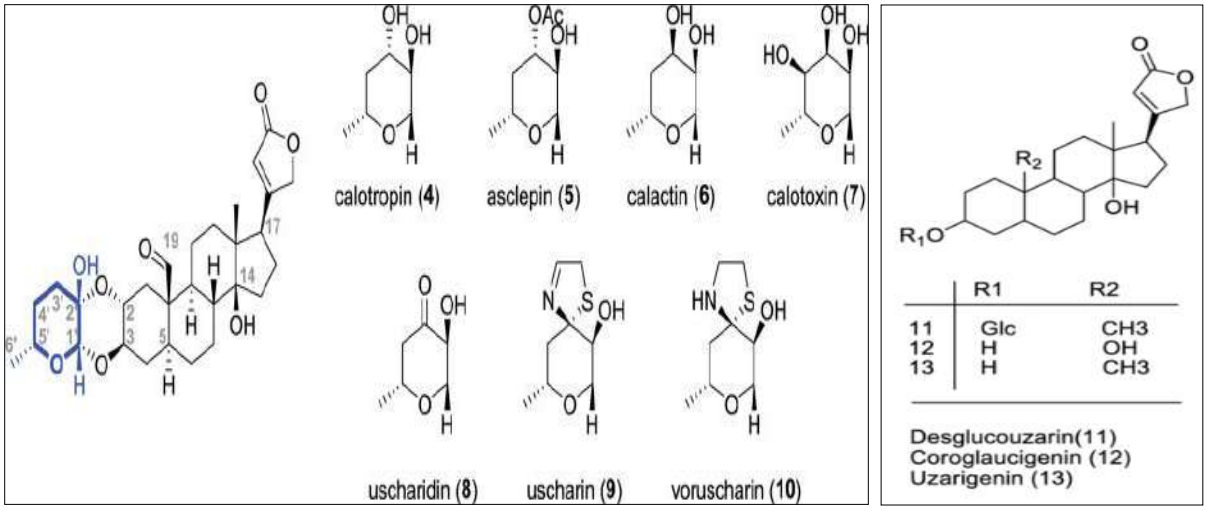
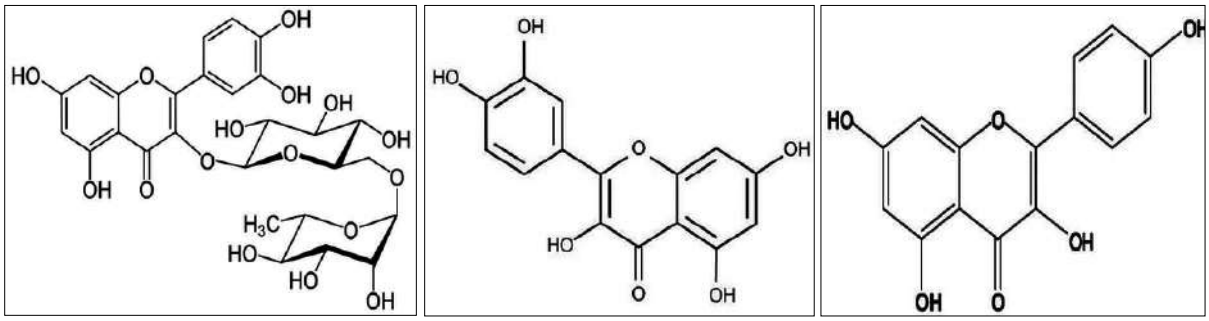
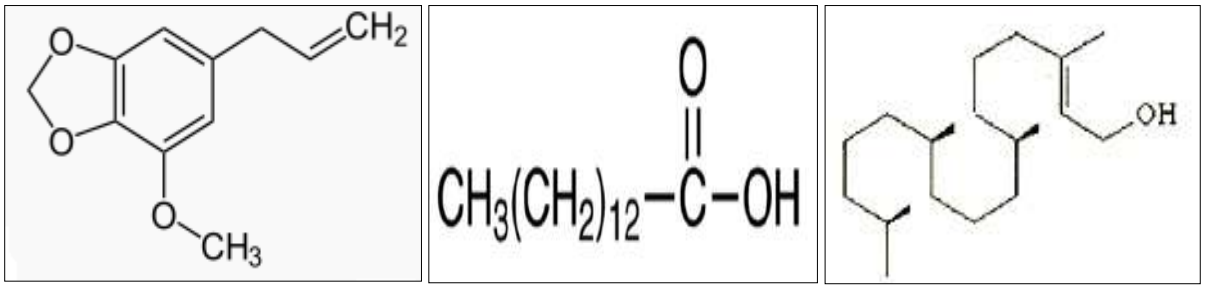


Figure II.4. Examples of isolated Cardiac glycosides structures of *C. procera*.



Rutin **Quercetin** **Kaempferol**

Figure II.5. Examples of isolated Flavonoids structures of *C. procera*.



Myristicin **Myristic Acid** **E-phytol**

Figure II.6. Examples of Essential oil structures of *C. procera*.

II.8. Cytotoxicity and Pharmacological applications

The widespread use of the *C. procera* plant in traditional medicine has been highlighted by scientists and researchers to search more scientifically on the compounds that give this plant the power in treating various ailments around the world.

In this regard, several researchers and studies have been carried out from ancient times to the present day have reported that *C. procera* have high medicinal or pharmacological activity due to its high content of biologically active compounds including cardiac glycosides, alkaloids, terpenes, resins, saponins ,lipids, flavonoids, tannins, and steroids [109,105,110,111].

II.8.1. Latex

- Cardiac glycosides present in latex have Anti-proliferative activity which inhibits the proliferation of MCF-7 cells through cytotoxicity, apoptosis, and autophagy [112]. Anthelmintic effects against Haemonchus contortus by damaging its cuticle and causing ultrastructural changes [113].
- Antioxidant and anti-apoptotic activities against the toxicity of 4-Nonylphenol [114], anti-inflammatory [115].
- Antitumor effect using Chitinase enzyme present in the latex [116].
- Insecticidal and larvicidal activity [117].
- Cardiovascular effect via reduced the elevated markers enzyme levels in serum and heart homogenates [118].

II.8.2. Leaf

- Leaf extracts of *C. procera* have an antihyperglycemic potential it’s reduce blood glucose to a significant level [119].
- Anti-helmintic activity [120], anti-microbial activity [2].
- Anti-bacterial potential against Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis, and Escherichia coli [121].

II.8.3. Stem

- In addition to that, anti-inflammatory and gastromucosal protective effect of the stem bark of C. procera has also been observed [122].
- Stem-leaves extract showed anticancer effect and anti-proliferative activity against HCT- cancer cell line [123].

II.8.4. Flower

The flowers mostly have flavonoids compounds [124] among other Quercetin-3-rutinoside with D-arabinose, glucose, glucosamine and L-rhamnose [104,105], which exhibit:

- Hepatoprotective activity, anti-inflammatory, antipyretic, analgesic, and antimicrobial effects and larvicidal activity [125].
- The ethanolic extracts of the flower have been reported to possess an antimalarial activity [126].

II.8.5. Root

- The roots ethanolic extract showed strong anti-implantation (inhibition 100%) and uterotropic activity for albino rats [127].
- Root bark Oxypregnane oligoglycosides compounds, has an anti-tumor effect against U373. glioblastoma and anti-cancer cytotoxic against PC-3 prostate cancer cell lines [101].
- Antiulcer activity [128].

II.9. *C. procera* in industry

C. procera is a rich source of natural renewable with low density fibers (**Figure II.2.D**), Through recent studies on the properties of this fiber, it was found that composed of 64 % cellulose, 19.5 % hemicelluloses, and 9.7 % of lignin [129].

- It also has a high absorption power of crude oil, through these characteristics; fiber can be used for the removal of contaminants due to oil spill [130,131,132].
- According to **Bassu (2020)** study due to its antimicrobial characteristics the fiber from *C. procera* can substitute cotton (Gossypium sp.) wool for surgical or stuffing purposes [133].
- **Synthesis of Nanoparticles:** Cerium oxideNPs and iron NPs produced from *C. procera* flower and leaf extracts found to be strong efficient against bacteria and fungi with cost-effective, and eco-friendly properties [134-135].
- **Phytoremediation:** because it contains many heavy minerals, this plant can be used as a phytomonitoring tool for evaluating minerals in the environment, by regulation of cellular homeostasis via redox signaling [136-137].
- This has been proven in many recent studies, Fruits and leaf powder of *C. procera* were found to adsorb the colorant dyes (Acid red 73 and Congo Red dye) used in dyeing processes, which are pollute aquatic life [138-139].

II.10. Conclusion

C. procera has been used for years to prevent and treat many diseases in traditional medicine. Although it is considered as a toxic plant characterize by the production of white poisonous latex, in the literature a series of studies confirms that *C. procera* is a rich source of biologically active compounds which has positive effects on cardiovascular diseases, and associated with anticancer, antifungal and insecticidal, anti-inflammatory properties. Therefore, further studies must be carried in order to identify *C. procera* compound(s) and to understand the mechanism of action responsible for this potential.

Part two:

Experimentation

Chapter I:

I.A. *Ethnopharmacological survey: Diseases and treatment*

I.A.1. Introduction

The chemical composition of traditional herbal preparations is very complex; in which at certain dose and/or under uncontrolled conditions of use present an intrinsic toxicity, hence medicine can become a threat to human health. In this perspective and in the context of the socio-economic and ethno-botanical work carried out on traditional medicine and practice in Algeria, we propose to contribute the ethno-pharmacological study of the of some toxic species of our Sahara as part of the development of natural resources in the arid zones of Algeria. The first chapter of the experimental part aiming to document the traditional uses of *C. procera*, and to evaluate the importance of this plant in folk medicine in Djanet province (Tassili N'Ajjer, Southern Algeria), largely, used in the treatment of numerous human diseases. We investigated the efficiency of this plant with great scrutiny concerning the methods of preparation of each part of the plant, and we checked the treated diseases as well as the side effects after applying the treatment. From another perspective, the study aims to assess the efficacy and toxicity of the plant.

I.A.2. Characteristics of the survey study and the experimental site

I.A.2.1. Geographical location

The Tassili N'Ajjer is a vast plateau in the Central Sahara; it is a mountainous area where unique cave paintings and drawings found in this immense rocky desert led to the creation of a national park in 1972. In 1982, the park is classified as a world heritage site and declared a biosphere reserve in 1986 [140]. Djanet city is located in the extreme southeastern part of the Algerian Sahara, about 2.200 km from Algiers [141]. It is located in the region of Tassili n'Ajjer (24°33'N.; 9°29' E.), at an altitude of 1094 m, limited by the Libyan border to the east, the Nigerian border to the south and north by the wilaya of Tamanrasset, while in the southwest; there is the commune of Bordj El Haouas and the Wilaya (Town) of Illizi [142].

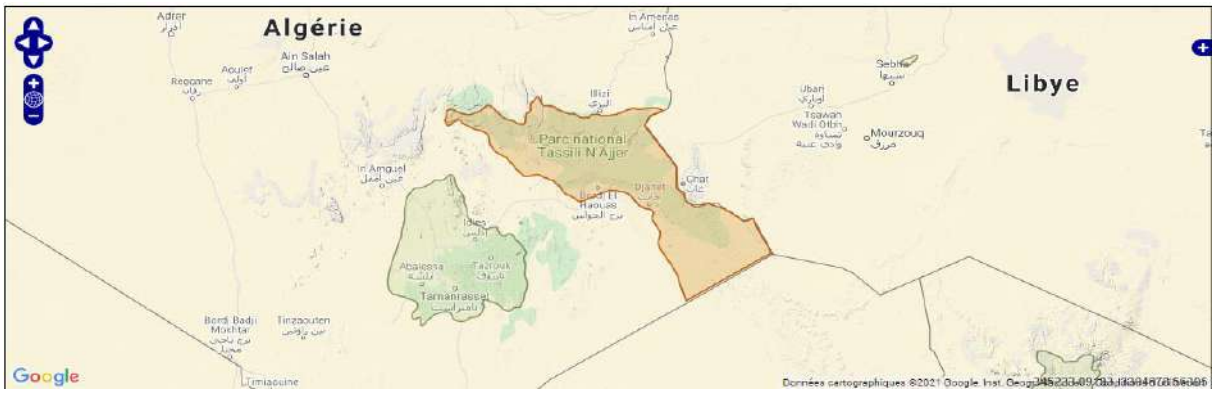


Figure I.A.1. The geographic location of the survey and sampling site [143].

I.A.2.2. Climatic characteristics

Tassili N'Ajjer is characterized by a desert climate and the plateau is mostly hyper-arid, with sub-arid microclimates suitable for the survival of a relict Mediterranean flora. The rainiest months are: March, January and April [144] where the annual rainfall amount varies between 15 and 25 mm per year [145], to what, is closely linked the growth of annual plants. Overall, this area is dominated by two main climatic seasons, a temperate season extending from October to April, which is characterized by daily temperatures between -1°C at night and +35 °C during the day; and a dry season, all the rest of the year, with this daily temperature interval [+15, +47] °C [146].These temperatures are, exclusively, pleasant in the Saharian regions [144].

I.A.2.3. Soil characteristics

Tassili N'Ajjer characterized by rugged mountainous terrain and desertic plateau of black rocks and white sands which constitute the Erg [146], Also The wadis considered the commonest geomorphological features in Tassili N'Ajjer in which differ from deep sandy soils to rocky substrate through to gravelly-sandy and gravelly floors [147].

I.A.3. Ethnopharmacological survey

This study was launched in May 2020 in Djanet, in order to get a comprehensive overview on the different traditional uses of *C. procera* in the Saharian pharmacopoeia, and to index the traditional medicinal knowledge linked to the use of this species. The data sheet is presented in **Figure I.A.2**.

<table>
<tr><td colspan="8">SECTION A:</td></tr>
<tr><td colspan="2">Gender</td><td>Age</td><td>Area</td><td colspan="4">Educational level</td></tr>
<tr><td>M</td><td>F</td><td rowspan="2">….</td><td rowspan="2">….</td><td>Primary</td><td>Secondary</td><td>University</td><td>Illiterate</td></tr>
<tr><td>….</td><td>….</td><td></td><td></td><td></td><td></td></tr>
<tr><td colspan="8">SECTION B</td></tr>
<tr><td colspan="4">Local Names …….</td><td colspan="4">Utilization (type of disease) ….</td></tr>
<tr><td colspan="8">Used part(s)</td></tr>
<tr><td>Latex</td><td>Leaf</td><td>Stem</td><td>Stem bark</td><td>Fruit</td><td>Flower</td><td>Root</td><td>Whole plant</td></tr>
<tr><td colspan="8">Mode of use</td></tr>
<tr><td>Decoction</td><td>Infusion</td><td>Maceration</td><td>Fumigation</td><td>Powder</td><td>Cream</td><td>Bath</td><td></td></tr>
<tr><td colspan="4">Outcome……..</td><td colspan="4">Side effects ……..</td></tr>
<tr><td colspan="8">SECTION C Other uses ……..</td></tr>
</table>

Figure I.A.2. Questionnaire sheet of survey.

For this, 100 surveys were carried out, the local language spoken by the interlocutor was Tamahaq; thus, it was necessary to be assisted by a local translator to complete all the data. Each survey sheet contains three parts of information;

- Section A was related to the interviewed personal information (area, gender, age, region, educational level),
- while section B focused on the plant such as local names, used organ, preparation methods and mode of administration, treatment duration; treated diseases, outcome and side effects;
- Section C included questions about other uses of this plant (outside the field of treating physical diseases). Collected data resume in **Table I.A.1**.

Overall, the majority of surveyed people were familiar with this saharian plant; knowing that it has several local names, in Tamahaq it is called Tourha or Tourjah and in Arabi (Kranka, Uchar). This species was identified and confirmed by Professor Ammar Eddoud a botanist in the department of Biology and Agronomy in the University of Ouargla.

I.A.4. Results and Discussion

I.A.4.1. According to gender

In this study, 100 persons were interviewed. Among the affected population who use the plant in traditional therapy, women dominate the practice by 54%, probably it returns to the cultural traditions of Touareg community, where women are more occupied by the health of their families than men and they are more patient and engaged to look for a solution to any social problems, especially the medical ones. They are more attached to nature and beneficially adapted to its resources. In addition, the easiness transmission of the traditional uses of the plant between women plays a big role. Overall, these values are similar and/or too close to those obtained in previous national ethnopharmacological researches realized on medicinal use of the plants [148,149].

I.A.4.2. According to age

Consulted persons were between 12 and 80 years old, so the average age of the study is 35.32 years old; the use of the plant is widespread in all age appointed groups, these ages were divided to categories, we noticed that younger people [12–30] and [30–50] years old represented the main users among local herbalists with 42% and 43% respectively; they used this plant much more compared to the older people (> 50 years) with 15%. In agreement with **Boudjelal et al (2013)**, the present study confirm the acquisition of knowledge and its

transmission from generation to another; it validated, also, that elderly people used less medical treatment by plants [150]. Indeed, the use of medicinal plants by older people does not represent a great therapeutic value [151].

I.A.4.3. According to the level of study

It was noted that the socio-cultural level in this region affects, greatly, the use of this plant in medication. The survey showed in **Figure I.A.3** that illiterates were the most prevalent (28%), because the knowledge on folk medicine is transmitted orally from one generation to the next by their ancestors; followed by people with a secondary education (26%) then people with a university education, they occupy the third place with a rate of 22% and in the last, people with average and primary education, representing respectively 13% and 11%.

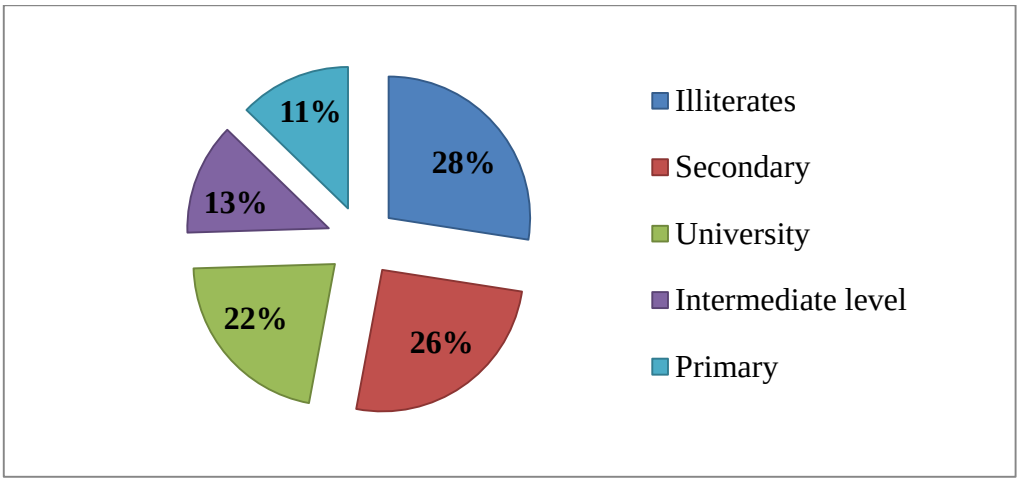


Figure I.A.3. Frequency use of *C. procera* according to the study level.

I.A.4.4. According to organ used

Concerning our question on the used part of the plant, all parts were noted used for treatment, including: latex, stems, leaves, roots, flowers, fruits, stem bark and whole plant. Latex and leaves are the most commonly used with a percentage of 45.69% and 25.82% respectively, followed by the stems with a rate of 13.9%. However, the use of the rest of the plant parts was very limited; it was estimated to 5.96 % for roots, 3.97% for flowers, 3.31% for the whole plant; fruits and stems bark are also used in a similar low percentage estimated to 0.66% (**Figure I.A.4).**

According to **Bitsindou (1996)**, the high frequency use of leaves can be explained by the ease and speed of harvesting, but also by the fact that they are the site of photosynthesis

and frequently the storage of secondary metabolites responsible of the biological properties [152], as well as the benefits felt by the populations [153].

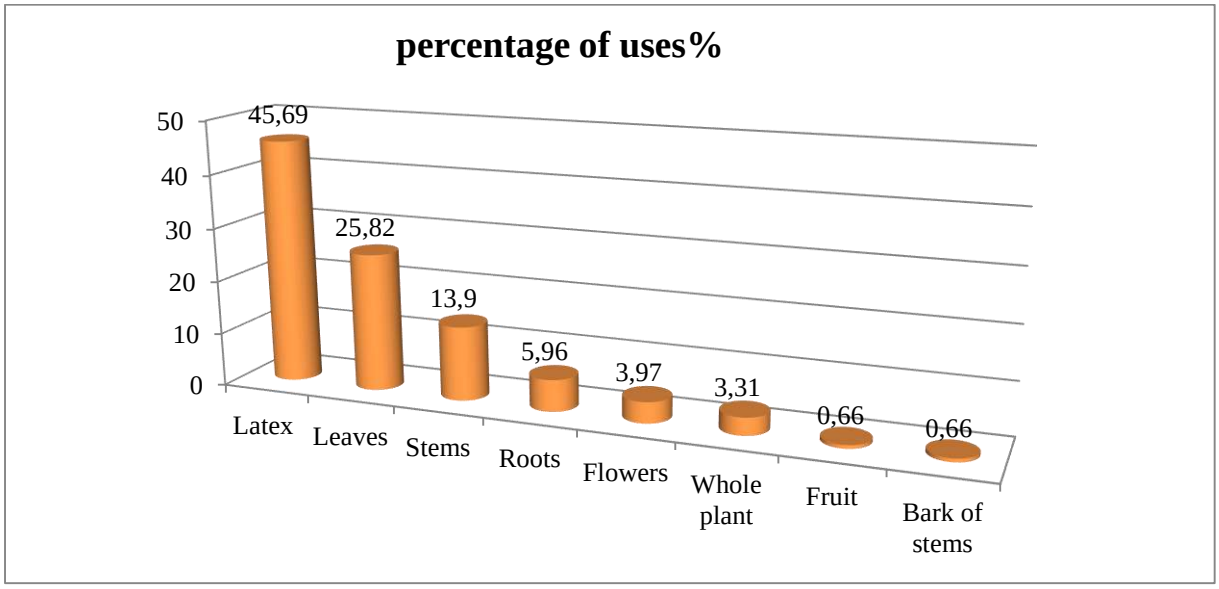


Figure I.A.4. Uses of different parts of *C. procera* in Algeria.

Definitely, these differences in the use of the plant organs are explained by the difference in concentration of the active constituents and the deference in chemical components between all its parts that is proven by many previous chemical investigations on this plant. Effectively, *C. procera* contains cardenolide, proceragenin, the root bark contains benzoylinesolone and benzoylisolinelone; leaves and stalk enclose calotropin and calotropagenin; the flower includes calotropenyl acetate and multiflavenol. While, the latex contains uzarigenin and terpenol ester [154].This applies to all other plants; many different parts of plants contain totally different phytochemical substances [19].

I.4.5. According to the preparation method

Medicinal uses of *C. procera* in Djanet are numerous with different preparation methods present in **Figure I.A.5** , since the plant contains latex, most common used methods are either by using it raw (29.41%) or by cooking it and using it like rubber (27.73%), the plant is used fresh or dried; sometimes with water in the form of decoction (21%) or infusion (5.88%), poultice (5.88%), maceration (5.04%) and sometimes it is used as a powder with milk or honey (3.36%), mixed with olive oil (1.68%), to be used as an ointment. the traditional methods detail of use this plant in treating diseases shows in **Table I.A.1**.

Table I.A.1. Traditional knowledge and practices used in treating diseases with *C. procera* in Djanet.

Part	Mode of preparation	Mode of administration	Diseases and Illness
Latex	-Fresh latex	-Cataplasm	- Scorpion and Snake poison - Warts, Dimple (human skin) - Ticks, Scabies (animal skin) -Dermatose.
	-Cooked with water until it becomes rubbery	-Cataplasm	-Toothache, Hemorrhoids
	-Diluted latex - Latex Powder	-Oral -Ear drops -Oral -Ointment	-Digestive disorders, Constipation -Eliminates bacteria from the ear -Abortive -Hemorrhoids, infected wounds
Leaves	- Decoction - Maceration -Infusion/Decoction	-Oral -Oral -Oral/body baths	-Intestinal worms -Gastralgia, Cardiovascular disease. -Fever, Rheumatic Pain -Wounds, Bleeding
	-Decoction / infusion /maceration	- Cataplasm	-Diabetes, Cold diseases, bee stings
	-Powder with vaseline	-Ointment	- Burns
	-Powder mixed with honey	-Oral	-Whooping cough
	- Fumigation	- Inhalation	- Epilepsy, asthme - Colds -Harmful insects, Pathogenic Bacteria
Stems/Bak of stems	- Decoction	- Nasal instillation	-Colds, Fever, Nasal mucosa
	-Fumigation	-Inhalation	-Fever, Epilepsy
	-Decoction -Powder with olive oil	- body baths - Ointment	- Burns
	-Decoction / Maceration	-Oral	-Diabetes, Cough
Roots / Flowers / Fruits	-Decoction /infusion (roots)	-Oral -Body baths -Decoction	-Fever -Wounds and Bleeding
	-Decoction / infusion / Powder with water (roots+flowers)	-body lotions - Ointment	-Arthritis and Knees, Epilepsy - Wound
	- Decoction (flowers)	-Oral	-Liver disease -Kidney and Urinary tract Infection
	-Decoction / infusion / maceration	-Oral/body baths	-Dermatoses

	(flowers + roots + fruits)		
Whole plant	- Decoction	-Oral	- Urinary tract, Bladder and Kidneys
	-Infusion	-Oral	-Cardiovascular disease
	-Fumigation		- Harmful insects, Pathogenic Bacteria

Active ingredient amount can be affected by the preparation of the medication, time and season of the plant's harvest, as well as the type of soil in which it grows, can also influence its effectiveness [155].

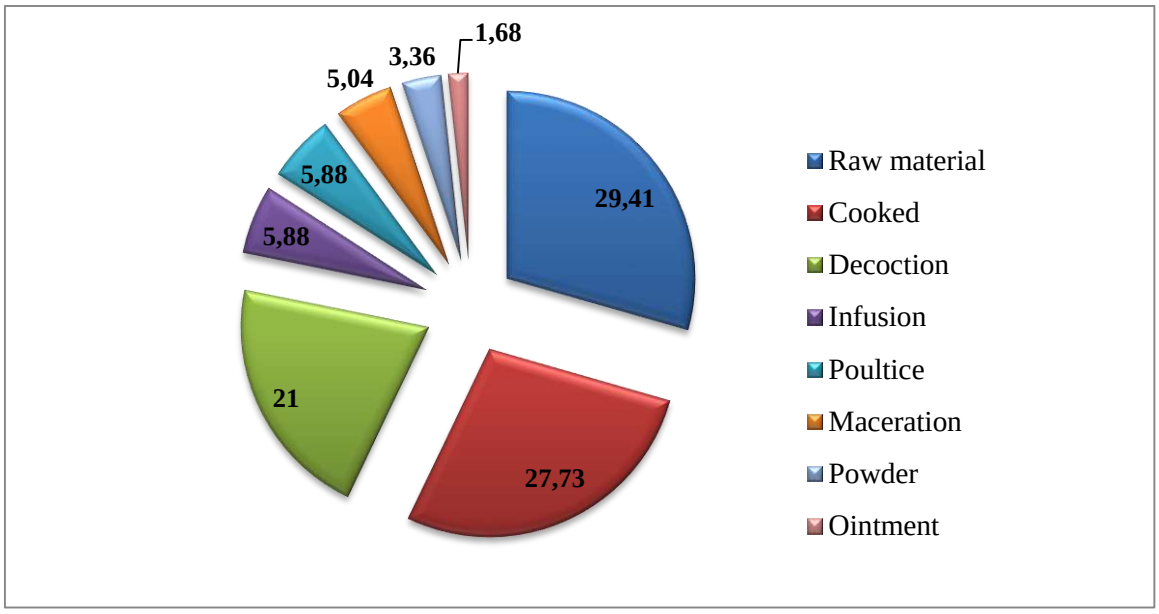


Figure I.A.5. Importance of *C. procera* according to the preparation method.

In fact, the medicinal plants are used for many reasons; among them is the effectiveness of plant extracts in treating diseases compared to modern medicines in addition to their low price [156]. In Tassili N'Ajjer, and since it is a remote desert area, diseases are often associated with very harsh climatic conditions and lifestyle, that is why the local population have relied on plant treatment since ancient times.

I.A.4.6. According to treated diseases

Among the most ailments, often mentioned and treated by *C. procera*, it can be especially stated that 34.14% to 35.77% of interviewed persons were treated for the high body temperature, poisonous scorpions, snakes and harmful insects **Figure I.A.6**.

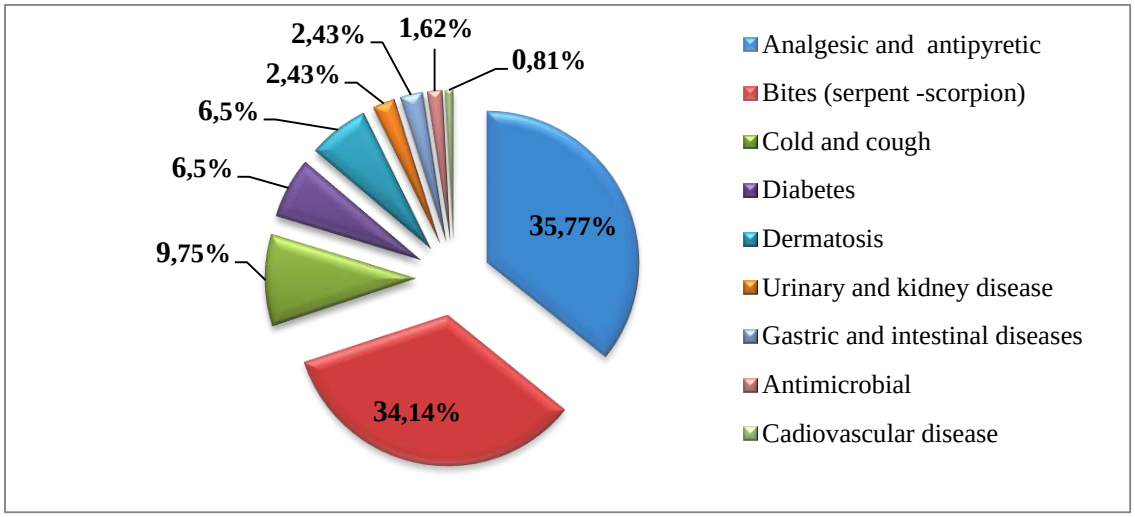


Figure I.A.6. The different diseases treated by *C. procera.*

According to **Abbiw (1990**), *C. procera* has a reputation for repelling scorpions and may be grown near dwellings for this purpose [157]. Common *C. procera* constituents such as β-sitosterol and quercetin have been shown to be anti-inflammatory agents responsible for the alleviation of snake-bite effects and scorpion poisoning especially with sufficient doses [158]. Also, **Punia (2013)** confirmed that the toxic glycoside calactin (powerful bacteriolytic enzyme) present in *C.procera* latex its concentration increases after an insect attack as a defensive mechanism [159]. Moreover, ethanol extract of *C. procera* aerial parts have a significant analgesic, antipyretic, neuromuscular and anti-inflammatory activity [65]. These support the high rates of using this plant by the Touareg to treat fever, pain and muscle spasms that we found.

9.75% of interviewed persons treat colds and cough diseases, diabetes and dermatoses with 6.5% rate each. Latex has been used in leprosy, eczema, inflammation, cutaneous infections, syphilis, and malarial, low hectic fevers and as abortive [128]. Analgesic and antioxidant effect, these features enables to decrease diabetic neuropathy pain [160,161]. Additionally, 2.43% of the reviewed people reported that the plant is used for gastric intestinal, urinary and kidney diseases**.** The stems were used in diuretics preparation, stomach tonic, anti-diarrhoetics, anthelmintic and many other diseases [71].

Touareg of Djanet indicated the activity of *C. procera* against microbes and in cardiovascular diseases but only by minor percentages, 1.62% and 0.81% respectively. The results obtained by **Kareem et al (2008)** showed that *C. procera* leaves and latex showed an antimicrobial and fungicidal effect [2].

On the light of this ethnopharmacological survey in Djanet, it was revealed that *C. procera* is used alone or in combination with other species to treat common diseases; it has a

great reputation as a medicinal plant in the whole world and, especially, in folk Algerian medicine.

Many preliminary phytochemical screenings studies carried out in different parts of *C. procera* indicated that *C. procera* is rich in active ingredients that give it much medicinal potential, which explains their widespread use in folk medicine [89,162,163,164,165,166].

I.A.4.7. Side effects of *C. procera* treatment

At the end of this survey, a great deal of information was gathered about the folk practices and uses of the plant that indicates its importance in herbal medicine. However, the frequent use leads to its side effects, especially in the case of increased doses. *C. procera* is known as a toxic plant, it is avoided by grazing animals; the latex is used by tribes to poison arrows used for hunting, and any contact with human eye could cause ocular toxicity and/or loss of vision and photophobia [81]. On the light of the present survey, some side effects, exposed to Touareg summarizes in **table I.A.2**.

Table I.A.2. Side effects of *C. procera* traditional applications.

Side effects of external use	Side effects of internal use
Skin diseases (loss of skin color like vitiligo)	Oral acidity
Latex causes eye pain and can lead to blindness	Stomach irritation and Diarrhea
Sneezing and allergies	Muscle spasm
Inflammation	Vomiting and nausea
Skin burns	Slow heart rate (Affects cardiac functions)
Acne	Pulmonary and hepatic insufficiency

Among the 98% of people who confirmed the effectiveness of *C. procera*, 47% reported the success of the treatment without side effects.

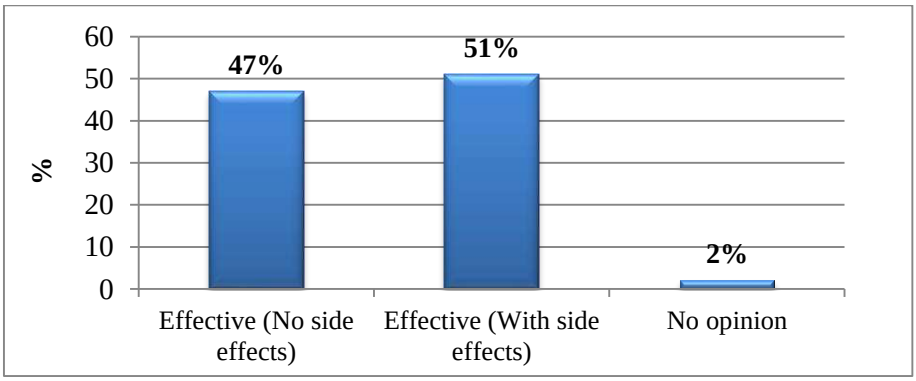


Figure I.A.7. Judgment of the questioned population about the efficiency of *C. procera.*

In fact, *C. procera* preparations should be used with caution, by a trained medical practitioner to avoid and/or control its harmful effects when its medicinal benefits are needful. Although, it is a poisonous this species is considered as an unlimited source of medicine for the local people in Djanet.

Chapter I:

I.B. Preliminary investigation

I.B.1.Introduction

Because *C.procera* had been shown numerous medicinal benefits, this requires research on the bioactive compounds into this plant.

The present study focuses on the investigation of the phytochemical composition existing in the crude latex and the aerial parts (leaves+ stem) of *C. procera* from Algerian desert (Djanet).

I.B.2. Material and Methods

I.B.2.1. Plant material

C. procera aerial parts (leaves, stems and flowers) are collected from Djanet at Tasili N'Ajjer region on October 2019; it has been identified and confirmed by Dr Ammar Eddoud from the Department of Agronomy at the University of Ouargla.

I.B.2.2. Preparation of plant material

First of all, the leaves and stems of *C. procera* were rinsed with water, in order to remove dust and sand, and then they were air dried in shed for 40 days in properly ventilated room under fan in ambient conditions, to keep as much as possible the entire composition at its original state. After that, the vegetal material was reduced to powder and stored in tightly closed glass vials away from light and moisture for further studies.

I.B.2.3. Preparation of plant extracts

The maceration of 60 g aerial parts powder was carried out with 400 ml of three solvents; firstly, Petroleum Ether (Etp), then Dichloromethane (DCM) and finally Ethanol (EtOH 96%) succesively; for one hour and half with constant stirring for each solvent; while, latex was used in its raw state. The solutions were filtered then concentrated with a rotary evaporator to obtain three crud extracts; they were conserved at -18 °C, until use.

I.B.2.4. Determination of extraction yields (Y)

Yields of extractions were determined for the three dried aerial parts of *C. procera* by calculating the following ratio:

$Y (\%) = (Mext/Mdm) \times 100$

Where; Y: the yield in (%).

Mext: the mass of the dried extract (g).

Mdm: the dry mass of the vegetal sample (g).

I.B.2.5. Preliminary phytochemical tests

According to the protocol described by **Khandelwal (1995)**; **Evans and Trease (2002)**; **Kokate et al (2009)**; **De et al (2010)**, prepared samples were analyzed to determine the presence of tannins, alkaloids, saponins, cardiac glycosides, steroids, terpenoids and phenolic compounds [167,168,169,170]. The results are presented in the **Table I.B.1**.

I.B.2.5.1. Tests for Alkaloids

a) Mayer's test: 1ml of each extract was mixed with a few drops of Mayer's reagent (Potassium Mercuric Iodide Solution). Cream color precipitate indicates the presence of alkaloids.

b) Wagner's test: To 1ml of each extract was mixed with equal volumes of Wagner's reagent (Iodine in potassium iodide). Formation of reddish-brown precipitate indicates the presence of alkaloids.

c) Dragendorff's reagent test: 2 ml of Dragendorff's reagent was added to 1ml of each extract and mixed. Then 2 ml of dilute HCl was added. Orange colored precipitate indicates the presence of alkaloids.

d) Ferric chloride test: To 1-2 ml of all the extracts, add few drops of neutral ferric chloride solution. Deposition of yellow precipitate indicates the presence of alkaloids.

II.2.5.2. Tests for Phenols

Ellagic acid test: The extracts were treated with few drops of 5% (w/v) glacial acetic acid followed by 5% (w/v) $NaNO_2$ solution. Muddy brown color Formation indicates the presence of phenols.

I.B.2.5.3. Tests for Flavonoids

a) Zinc-HCl reduction test: To all extracts; add a pinch of Zinc dust with few drops of Conc. HCl. Deep red color indicates the presence of flavonoids.

b) Shinoda's test: Add a small piece of magnesium paper to all extracts with few drops of conc. HCl carefully along the walls of the tube. Appearance of red color indicates the presence of flavonoids.

I.B.2.5.4. Tests for Tannins

a) Ferric chloride test: Formation of blackish precipitate indicates the presence of tannins when few drops of $FeCl_3$ solution were added to all extracts.

b) Gelatin test: The extracts were treated with few drops of gelatin solution. White precipitate indicates the presence of tannins.

I.B.2.5.5. Tests for Saponins

5ml of each extract is taken in a test tube and shaken vigorously to obtain a stable froth. To this frothing solution, 5-6 drops of olive oil was added. Formation of an emulsion indicates the presence of saponins.

I.B.2.5.6. Test for Terpenoids

1% HCl was added to all extracts and allowed to stand for 5-6 hours; these were later treated with 1ml of Trim-Hill reagent (a solution of 10 ml of acetic acid, 1 ml of 0.2% copper sulphate in water and 0.5 ml of concentrated hydrochloric acid) and heated in a boiling water bath for 5-10 minutes. Bluish green color indicates the presence of terpenoids.

I.B.2.5.7. Test for Sterols

a) Salkowski test: 5 ml of chloroform was added to 1-2 ml of all the extracts then 1 ml of conc. H_2SO_4 was added carefully and mixed. The formation of reddish color in the lower layer indicates the presence of steroids.

I.B.2.5.8. Tests for Glycosides and Cardiac glycosides

a) Conc. H_2SO_4 test: To 1ml of the extracts, 1ml of conc. H_2SO_4 was added and allowed to stand for 2 min. a reddish color precipitate indicates the presence of glycosides.

b) Keller Killiani test: 1ml of the extracts were dissolved in 1ml of glacial acetic acid and cooled, after that, 2-3 drops of $FeCl_3$ were added. To this solution 2ml of conc. H_2SO_4 was added carefully. Appearance of reddish-brown colored ring at the junction of two layers indicates the presence of cardiac glycosides.

I.B.3. Results and Discussion

I.B.3.1. Extraction yields (Y)

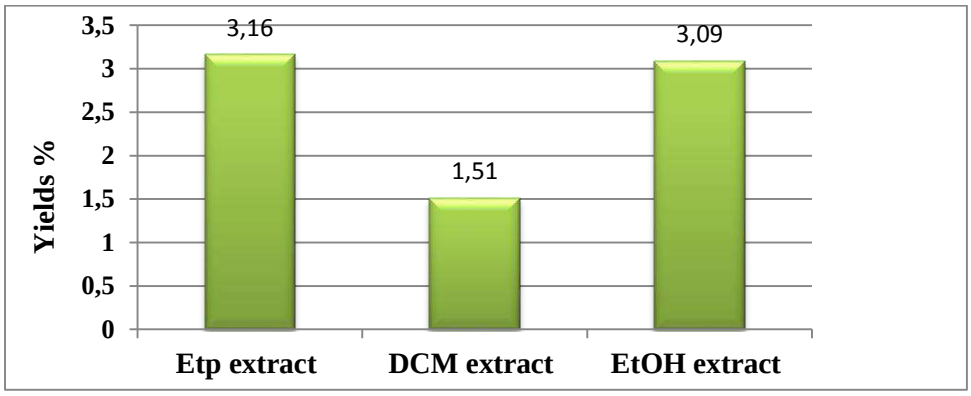


Figure I.B.1. Graphic representation of extracts yields.

The yields of the aerial parts extraction by maceration were estimated to 3.16% for Etp extract, 1.51% to DCM extract, and 3.09% to EtOH extract. Generally, the extraction yields depend on the extraction method, the solvent used for extraction, and most important on the richness of the species in second metabolites [171]. In fact, the differences in the extract yields from plant materials might be ascribed to the presence of various compounds, with different chemical characteristics and polarities that might or not be soluble in a particular solvent [172].

I.B.3.2. Phytochemical screening

The phytochemical screening results are illustrated in the **Table I.B.1**.

Table I.B.1. Results of preliminary phytochemical screening of aerial parts extracts and crude latex from *C. procera.*

Chemical group	Test	Etp extract	DCM extract	EtOH extract	Crude Latex
Alkaloids	Wagner	/	/	/	+
	Mayer	+	+	-	+
	Dragendroff	+	+	-	+
	FeCl3	+	+	-	+
Phenols	Ellagic acid	-	+	-	+
Flavonoids	Shinoda	-	-	+	/
	Zn-HCl	-	-	-	-
Tanins	$FeCl_3$	-	+	-	-
	Test gelatine	+	-	+	/
Saponins		-	-	-	-
Terpenoids		+	+	+	+
Steroïds	Salkowski	+	+	+	+
Glycosides	Conc. H_2SO_4	+	+	+	+
Cardiac glycosides	Keller Killiani	+++	+++	+++	+++

(-) absence, (+) presence, (/) not determined,

The results in **Table I.B.1** revealed the richness of *C.proccera* in natural products, on one hand, the three extracts contain Tanins, Terpenoids, Steroids, Glycosides and Cardiac Glycosides; whereas Alkaloids are present only in Etp and DCM extracts; Polyphenols in DCM and Flavonoids in EtOH extracts; while, Saponins were totally absent. On the other hand, its latex comprises alkaloids; phenols, terpenoids, steroids, glycosides and a large quantity of cardiac glycosides; however, flavonoids, tannins, saponins were not detected. According to **Figueiredo et al (2008**), numerous factors may influence the variation of the secondary metabolites composition of plants extracts such as environmental conditions, extraction methods and geographic variations [173]. Besides, previous reports indicated that *C. procera* contains various classes of secondary metabolites, including cardenolides, alkaloids, tannins, triterpenes and flavonoids, as it is known, all of these natural products exhibited diverse biological activities such as cytotoxic, anti-cancerous, anti-inflammatory and analgesic activities [174,175,176].

The abundance of the latex in the green parts of *C. procera* makes the plant popularly known, a fact reinforces the idea that this latex is produced and accumulated as a defense strategy against insects, viruses and fungi [177].

Globally, the present findings about *C. procera* components indicate its biological power and explain the widespread uses of the species in traditional medicine; these bioactive compounds probably contribute, among others, to the insecticidal nature of the studied extracts [65,109,111]. Phytochemical properties of *C. procera* reported in this part are in agreement with the study performed by **Kawo et al (2009)** on its potential use in folk practices [178].

I.B.4.Conclusion

Through the present study, we achieved an interesting data set. It is revealed that the plant shows many pharmacological proprieties by treating various diseases. This is due to the richness of this plant with active compounds, which was shown by the phytochemical tests

In addition to previous studies, we consider our work as a scientific contribution to maintain knowledge about this medicinal plant, specifically in Tassili N'Ajjer. The obtained results will serve as a database for further focused research on *C. procera*.

Chapter II:

Volatile compounds: extraction and characterization: Phytochemical study

II.1.Introduction

C. procera is considered one of the most famous plants that humans have used since ancient times in the treatment of many human or animal diseases.

C. procera (Asclepiadaceae) is one of the famous plants that humans have used in the treatment of many human or animal diseases because of its numerous observed medicinal benefits. The findings have aroused our curiosity to search the depths of this plant in terms of what it might contain.

Since, no literature information is available on the volatile fraction of Algerian *C. procera* Ait; the present study focuses in this part on the identification and the comparison of the chemical volatile constituents from hydro-distilled extract of dried vegetal material.

II.2. Material and Methods

II.2.1. Extraction of the Volatile portions

400 g of crushed dried aerial parts (leaves, stems) and flowers were subjected separately to hydro-distillation using distilled water in Clevenger-type apparatus for 5 h. The distillated product was extracted with n-hexane to obtain two volatile samples: (VDA) from dried aerial parts and (VDF) from dried flowers; both were passed through anhydrous sodium sulphate Na_2SO_4 to remove residual water. Each Sample had distinct fragrance and it was conserved at -18°C until use.

II.2.2. Gas Chromatography/ Mass Spectrometry (GC/ MS) Analysis

Samples were subjected to analysis using Shimadzu GC-MS (TQ 8040 NX) equipped with an auto-injector; (30 m x 0.25 mm ID x 0.25 μm) column; the sample was injected with split ratio at 10.0; helium was used as carrier gas at 60 kPa inlet pressure; Oven temperature was set from 70 °C for 1 minutes, 120 to 180 °C for 5 min, 220 °C to 280 °C for 5 min. The injector temperature was 280 °C. Ion source temperature 200°C; interface temperature 280 °C; solvent cut time 3min with relative detector gain mode 0.86 kV ; scan MS ACQ mode; mass range of m/z 40-500.

II.2.3. Identification of the chemical compositions

Identification of volatile components was performed based on the comparison of their retention index with those in the literature in addition to the comparison of their mass fragmentation patterns with those in the NIST 17 and W11N17MAIN1 library.

II.3.Results and Discussion

II.3.1. Separation and Characterization of *C.procera* volatiles component by GC/MS

Volatile compounds of dried aerial parts (VDA) and dried flowers (VDF) of *C. procera* were extracted by hydrodistillation method. A transparent liquid sample with a strong fragrance was obtained for each; both were analyzed by means of gas chromatography coupled with mass spectrometry. The separation results of the chemical composition of **(**VDA) and (VDF) are represented on the chromatograms (**Figure II.1** and **Figure II.2**).

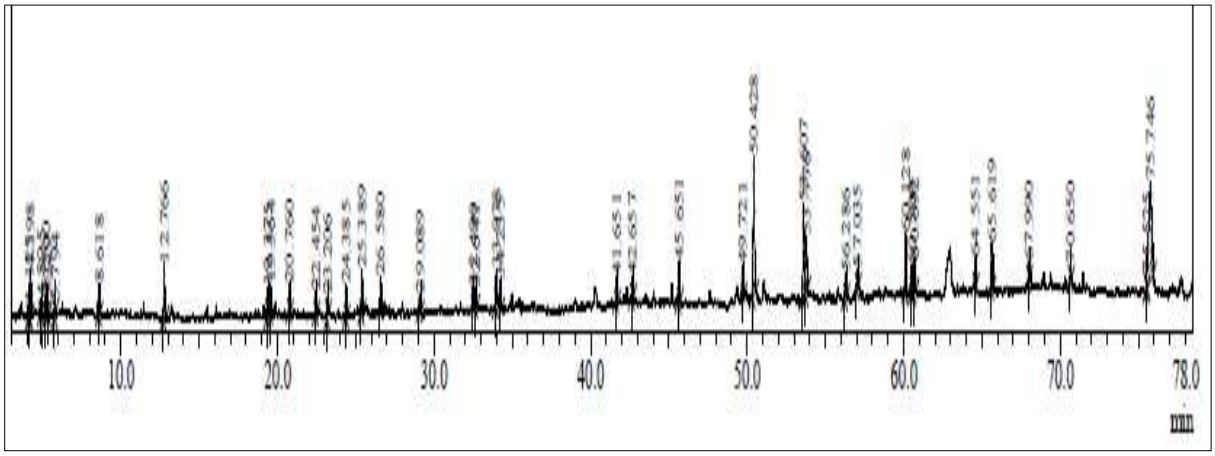


Figure II.1. Chromatogram obtained for VDA by GC-MS.

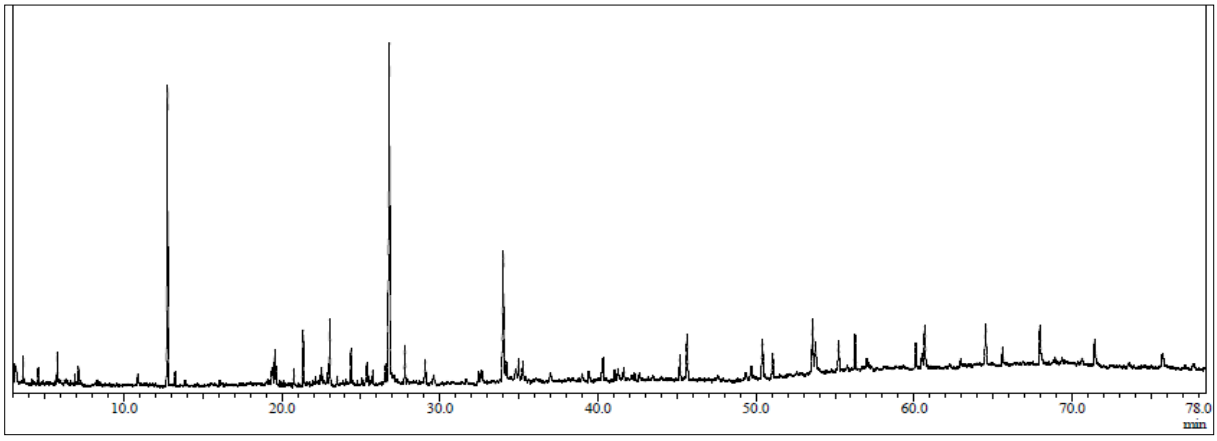


Figure II.2. Chromatogram obtained for VDF by GC-MS.

GC-MS revealed that VDA and VDF are rich in volatiles. Overall, Twenty two compounds were identified in the sample of dried aerial parts and Sixteen in dried flowers one, corresponding to 54.41% and 61.91% of their total composition, respectively. Characterizations of substances are confirmed by comparison of their retention indexes and mass spectra with those of standards; the chemical composition results with the percentage of each detected compound are transcribed in **Table II.1**.

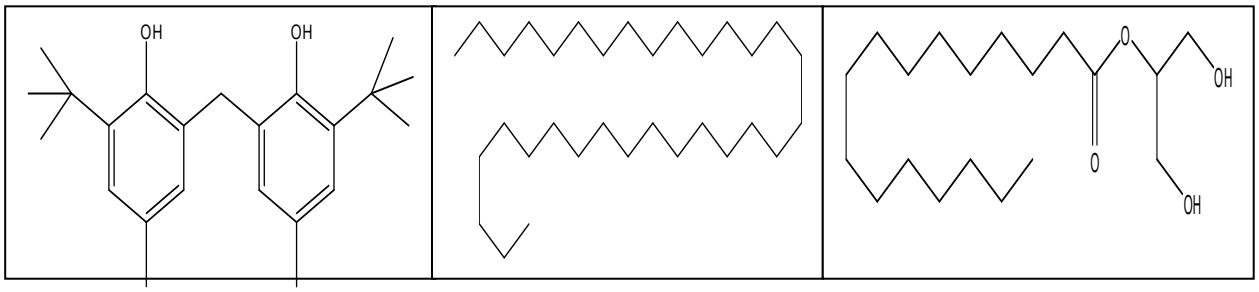
Table II.1. Results of GC-MS Analysis.

N°	RT (min)	Identified compound	VDF (%)	VDA (%)
1	4.12	Formamide, N,N-diethyl-	-	0.45
2	4.19	2-pinene	-	1.48
3	4.89	β-Pinene	-	0.40
4	5.19	Acetamide, N,N-diethyl-	-	0.79
5	5.32	α-phellandrene	-	0.29
6	5.67	O-Cymene	-	0.19
7	6.88	Guaiacol	0.27	-
8	8.49	1,2-Propanedione, 1-phenyl-	-	0.37
9	10.87	Nonanic acid	0.35	-
10	12.75	p-Vinylguaiacol	14	3.67
11	19.11	Artonil	-	0.28
12	19.87	2,4-Di-tert-butylphenol	-	0.48
13	20.08	Cycloheptanol,2-methylene	0.16	-
14	21.34	Dodecanoic acid	2.25	-
15	22.45	Hexadecane	-	1.07
16	23.02	Benzophenone	2.05	-
17	23.20	Apiol	-	0.90
18	25.75	Tridecanoic acid, 12-methyl-, methyl ester	0.46	-
19	26.80	Myristic acid	20.37	-
20	27.78	Tetradecanoic acid, ethyl ester	1.80	-
21	32.49	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	-	1.14
22	34.00	Palmitic acid	7.86	1.84
23	34.23	Decane, 1-iodo-	-	1.68
24	43.51	14- β.-H-pregna	-	0.53
25	50.42	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	2.75	12.97
26	53.58	Dotriacontane	3.26	10.27
27	53.76	Palmitoylglycerol	1.73	4.37
28	55.23	Bis(2-ethylhexyl) phthalate	1.73	-
29	57.02	Tetracosane	-	1.09
30	60.11	Tetrapentacontane	2.05	7.88
31	60.51	Octadecanoic acid, 2,3-dihydroxypropyl ester	0.82	2.27
Total identified			61.91 %	54.41 %

RT: *Retention Time.*

II.3.2. Volatiles of *C. procera* (VDA)

The most abundant compound identified in VDA is Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- with 12.97%; followed by Dotriacontane(10.27%) ; Tetrapentacontane(7.88%); and 2- Palmitoylglycerol (4.37%).

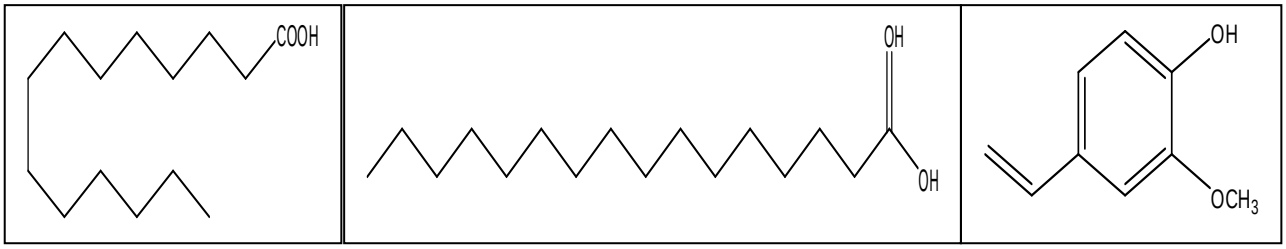


Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl- **Dotriacontane** **2- Palmitoylglycerol**

Figure II.3. Structures of the main compounds present in VDA.

II.3.3. Volatiles of *C. procera* (VDF)

Regarding VDF, 20.37% was the amount of its major compound: Myristic acid, followed by *p*-vinylguaiacol (14%); Palmitic acid (7.86%); Dotriacontane (3.26%) and (2.25%) Dodecanoic acid. These results are supported by the findings of **Wahba a**nd **Khalid (2018)**, who reported that Myristic acid of Egyptian *C. procera* was the major constituent detected in various organs of the plant [92].



Myristic acid ***p*-vinylguaiacol** **Palmitic acid**

Figure II.4. Structures of the main compounds present in VDF.

As it can be noticed, some common constituents to VDA and VDF samples were detected, namely: *p*-Vinylguaiacol; Palmitic acid; Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-; Dotriacontane; 2- Palmitoylglycerol ; Tetrapentacontane, Octadecanoic acid, and 2,3-dihydroxypropyl ester.

The dried *C. procera* aerial parts produced other volatile organic compounds that include Formamide, N,N-diethyl-;2-Pinene; β-Pinene; Acetamide, N,N-diethyl-; α-phellandrene; O-Cymene; 1,2-Propanedione, 1-phenyl-; Artonil ; 2,4-Di-tert-butylphenol; Hexadecane; Apiol; 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; Decane, 1-iodo-;14-β-H-pregna; Tetracosane; Octadecanoic acid, 2,3-dihydroxypropyl ester .

The results also showed the presence of Guaiacol ; Nonanic acid; Cycloheptanol,2-methylene; benzophenone; Tridecanoic acid, 12-methyl-, methyl ester; Tetradecanoic acid, ethyl ester; in VDF.

Successfully, GC-MS investigation revealed the presence of a variety of chemical families, as both VDA and VDF profiles exposed the presence of terpenes and oxygenated terpenes, aromatic alcohol (benzyl alcohol), amides and saturated linear chain fatty acid, halogen compounds and carbonyls like ketones.

The obtained results confirm previous investigations from Egypt, Saudi, and Nigeria carried out on the volatile part of this species. Where the oxygenated terpenes were the most present in the essential oils of Saudi and Egyptian *C. procera* with 70.83% and 66.22% respectively [109].

It could also be noted that **Kubmarawa** and **Ogunwande (2008)** results have also reported that Nigerian *C. procera* leaf oil contained phytol (33.6 %), and myristic acid (31.2 %) as major constituents [72].

However, the geographical characterizations differences and environmental factors influenced significantly the quality and the quantity of the identified components in a plant volatile portion [72,92].

Figure II.5 provides information on the chemical families present in the VDA of *C. procera*; they are classified into five chemical classes. Indeed, 54.41% of its volatiles consist 74.5% of miscellaneous compounds, 14.97% Non-terpene aldehydes/ketones/esters compounds, 4.33% Monoterpenes, 3.38% Fatty acids and 2.79 % Nitrogen derivatives.

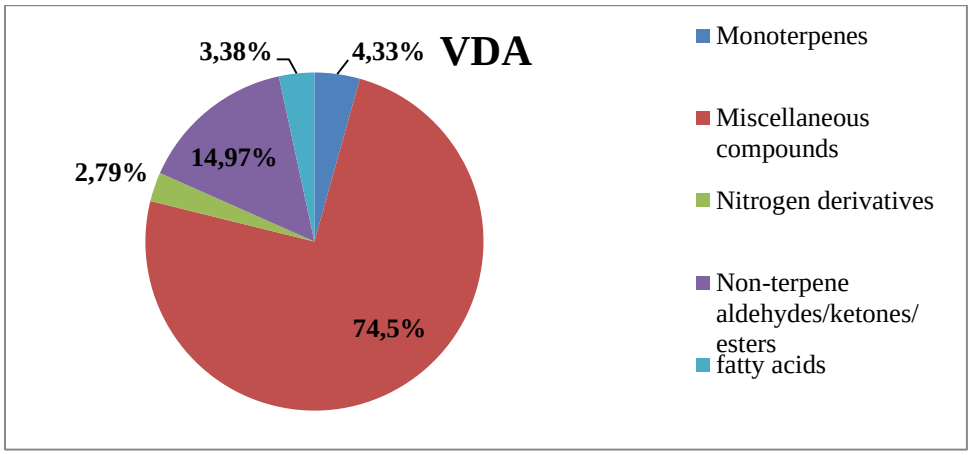


Figure II.5. Chemical classes present in VDA sample.

The chemical families representing 61.91% of VDF of *C. procera* are categorized into four classes as it is shown on **Figure II.6**. Fatty acids with 49.79% showed predominance in this sample, followed by 39.12% miscellaneous compounds, 10.33% non-terpene with different classes: aldehydes/ketones/ esters and finally 0.74% oxygenated sesquiterpenes.

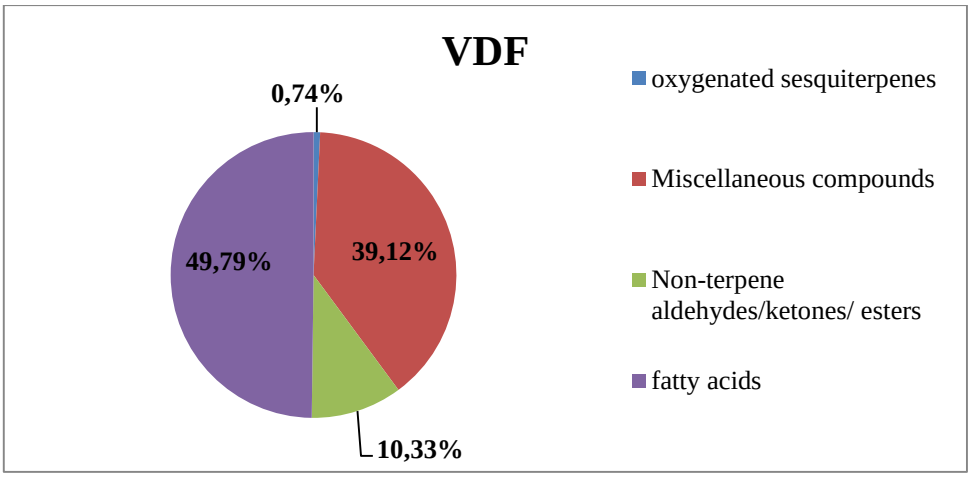


Figure II.6. Chemical classes present in VDF sample.

According to **Moronkola et al (2011)**, both leaf and stem of *C. procera* contain octadecenamide and its saturated form long chain fatty acids and amides, sulfurate, halogen compounds and ketones [7]. By comparing the volatile previous fractions, VDA were more numerous and more diversified in terms of chemical classes than VDF, this difference can be explained by the different presence of enzymes responsible for the qualitative and quantitative composition of volatile substances in each part of the plant.

Plants are of an intrinsic worth through their bio-substances; they can occur at several levels. Recent studies confirm that some plant essential oils exhibit antimicrobial, antioxidant, anti-inflammatory, anti-allergic, and anticancer properties [179,180]. It is well established that plant essential oils have also gained momentum due to their insecticidal activities [181].

In literature, *C.* genus volatiles proved its role as antimicrobial, antibacterial and antifungal agents [182]. In addition, **Singhi et al (2004**) recorded insecticidal activity on female Aedes aegypti mosquitoes [183]. Furthermore, the study realized carried out by **Sagheer et al (2014)**, showed that *C. procera* volatiles highly affected all the stages of red flour development beetle [184].

II.4. Conclusion

The present study revealed the richness of *C. procera* with volatiles compounds among others certainly there are strong bio-insecticidal and/or repellent compounds which can contribute to the development of new strategies to control insect pests.

Chapter III:

Phenolic compounds:

Phytochemical study

III.1. Introduction

During the last decade phenolic compounds have received widespread attention due to their numerous benefits for human health. For example, their direct and indirect antioxidant properties help in the precaution of oxidative stress-related to disorders such as cancer and cardiovascular diseases [185,186], these categorized into many different groups, among them, the simple phenols, phenolic acids, condensed and hydrolyzable tannins, coumarin and flavonoids [187]. Previous studies showed the radical-scavenging properties of phenolic acids and flavonoids in plant foods are associated with Cardioprotective effects, antiviral, anti-carcinogenic and anti-inflammatory activities [188,189,190]. All *C. procera* organs showed variable phenolic amount according to seasonal variation; so that the highest content was recorded for samples of mature leaf, stem and apical bud, in winter [191].

In this context, the objectives of this chapter attempt to identify and quantify the phenolic constituents of *C. procera* ethyl acetate and n-Butanol fractions since, to the best of our knowledge, no phytochemical investigation had been carried out previously in Algeria.

III.2. Material and Methods

III.2.1. Phenolic compounds extraction

Based on the powder prepared from the previous chapter, 400 g of dried aerial parts leaves and stems powder of *C. procera* was macerated in methanol 99.9% (3 ×2 L) with agitation at room temperature to enhance the extraction; the maceration was repeated 3 times with renewal of the solvent and lasts in each case 24 hours. The first extract recovered was filtered, dry concentrated under reduced pressure at a moderate temperature (approximately 40° C) to obtain (10.97 g) crude extract.

The residue was suspended in water, and then liquid-liquid extractions were applied firstly with petroleum ether (Etp), chloroform ($CHCl_3$), ethyl acetate **(EtAc)**, and n-Butanol **(n-BuOH)** (500 ml ×3) for each. Each of the four organic phases were separately collected, filtered and finally evaporated to dryness under vacuum.

EtAc and **n-BuOH** fractions were stored at −18 °C in an amber glass vial for analysis. **Figure III.1** represents the flowchart of the different steps of the extraction by maceration according to the protocol described by **Oraibi** and **Hamad (2018)** [90].

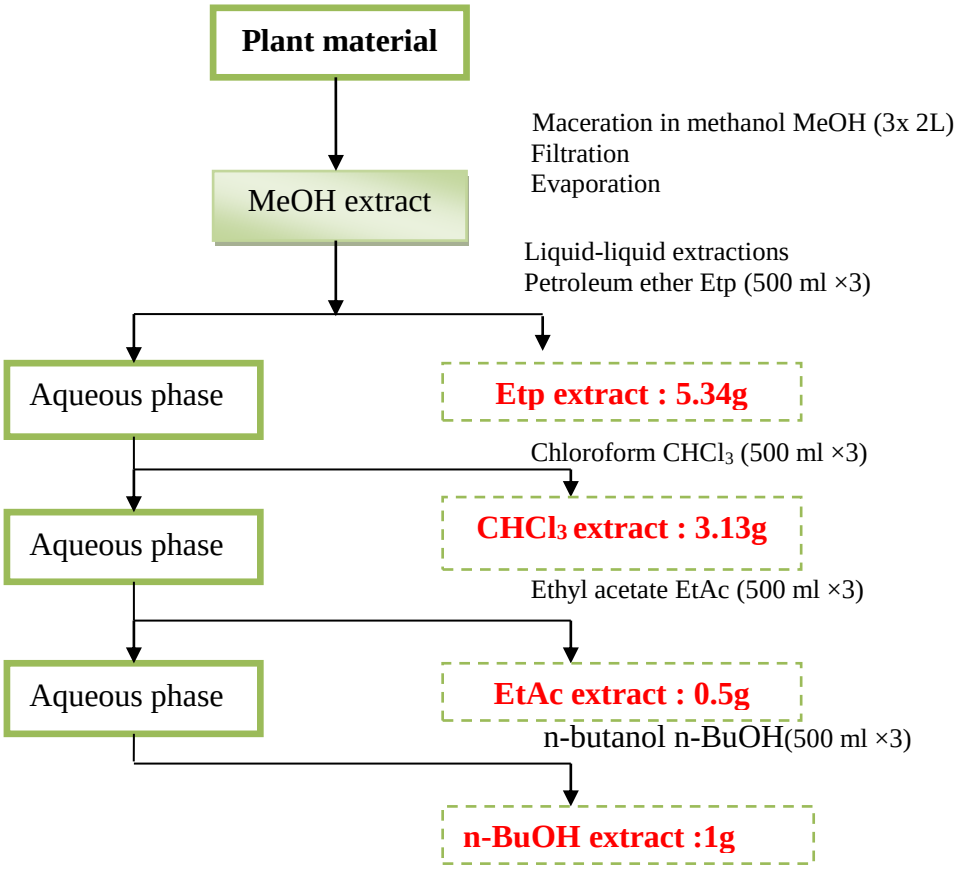


Figure III.1. Diagram of protocol extraction using maceration.

III.2.2. Equipments and Chemicals

TLC plates (60 F254 silica gel plate on aluminum support) were from Merck (Darmstadt, Germany), HPLC (Agilent ChemStation), HPLC (Shimadzu Nexera-i LC-2040C 3D + HPLC device was used for quantitative analysis), LC-MS/MS (Shimadzu Corporation Lab solutions, Kyoto, Japan, Model: LCMS 8040, Triple Quadrupole), FTIR-8300 (Shimadzu), NMR (Bruker BioSpin GmbHAvance with TCI cyroprobe operating at 400MHz), Binocular magnifier (KARL KOLAB Scientific Technical Supplies D-6072 Dreieich. West Germany).

All analytical grade solvents used were from BIOCHEM Chemopharma, except Methanol gradient HPLC grade, LiChrosolv Reag.Ph Eur, Germany.

III.2.3. Authentic standards

Standards were available for comparison with **EtAc** fraction: (+) Rutin trihydrate, 97% was obtained from Alfa Aesar GmbH& Co KG (Germany), and Gallic acid (99%) from BIOCHEM Chemopharma (Quebec).

III.2.4. Qualitative analysis

The phytochemical investigation was done according to the protocol described in the previous Chapter **I.B**.

III.2.4.1. Ellagic acid's test for phenols

EtAc fraction was treated with few drops of 5% glacial acetic acid followed by 5% Sodium nitrite solution. Appearance of muddy brown color indicates the presence of phenols.

III.2.4.2. Shinoda's test for flavonoids

Few drops of concentrate HCl were carefully added to **EtAc** fraction with some pieces of magnesium, appearance of red color indicates the presence of flavonoids.

III.2.5. Preparation of sample and standards

For TLC and HPLC analysis; **EtAc** fraction, standards Gallic acid and Rutin were prepared by dissolving 0.01 mg in 1 ml of Methanol (MeOH) HPLC grade, As for HPLC analysis of **n-BuOH** fraction, 1 mg in 1 ml of Methanol (MeOH) HPLC grade was dislolved then subjecting them to ultrasonication for 5 minutes, to ensure homogeneous mixtures.

III.2.5. Thin layer chromatography (TLC) Analysis

The thin layer chromatography analyzes was carried out in the normal phase to check how many components are present within the **EtAc** fraction, and to identify their compounds compared with a known compounds, drops of **EtAc** fraction and solutions of standards were put on TLC plate's baseline using different systems of solvents to obtain the best separation.

The solvent systems for the different classes of compounds are as follows, (the proportions are given by volume):

- Chloroform / Ethyl acetate (9:1 v/v).
- Chloroform/Methanol (5:5 v/v).
- Ethyl acetate/ Acetic acid/water (8.1.1 v/v).
- Chloroform **$CHCl_3$**/ Ethyl acetate **EtAc**/ Methanol **MeOH**/ Formic acid **FA** (7: 1.5: 1.5: 1 v/v). In our case, this was the best system for separating **EtAc** compounds.

Concerning the visualization of separated components, dried TLC plate's evaporated with Neu's reagent (10 ml of solution A (1 g of 2-aminoethyldiphenylboric acid and 100 ml of methanol) and 8 ml of solution B (5 g of PEG 4000 and 100 ml of ethanol)), heated at 100°C for 5 min then checked under UV light at 366 and 254 nm as illustrates in **Figure III.3** under UV light at 365 nm, flavonoids appear as bright yellow, green or orange and blue fluorescent spots [192].

III.2.6. Preparative thin layer chromatography (PTLC) analysis

Using silica gel preparative TLC, two well-separated fluorescent spots (compound (1) and compound (2)) were isolated from **EtAc** fraction dissolved in MeOH. The plates were developed with $CHCl_3$/EtAc/ MeOH/ FA (7:1.5:1.5:1 v/v) mobile phase and examined under UV lamp. Two well-separated fluorescent spots; compound **(1)** and compound (**2)** were appearing in **Figure III.4**. They were scraped out and collected then extracted with MeOH for further experimental (HPLC, LC-MS, FT- IR, NMR) analysis.

III.2.7.Analytical High Performance Liquid Chromatography (HPLC) analysis

I) EtAc fraction analysis

In order to ensure the purity of the isolated compounds, samples of **20** µl of **EtAc** fraction, compound **(1)** and compound **(2)** were filtered through 0.45 µm filters then injected into analytical HPLC system using the following conditions:

- The mobile phase was made of phase A: 1% acidified H_2O, C: MeOH.
- The running gradient was as follows: flow C= 5 % to 95 % (0 – 55 min), C= 5 % (3 min).
- The elution conditions were: flow rate was set at 1.0 ml/min at 117 bar pressure, and operating temperature of 40 °C.
- Compounds separation was carried out on Agilent ChemStation C18 (25 cm x 4.6 mm, 5 µm) column, at max column temperature 60˚C.
- The effluent was monitored at 254 nm by UV detector with 400 max bar pressure.

The eluted compounds peaks were qualitatively characterized according to their UV retention times, **Figure III.5** illustrates the **EtAc** fraction profile and **Figure III.6** illustrates the isolated compound **(1)** and **(2)** profiles.

II) n-BuOH fraction analysis

Chromatographic separation was conducted also in order to identify and quantify the components of **n-BuOH** fraction, with gradient elution mode using the following conditions:

- A Phenylhexyl C_6 column (150 mm × 4.6 mm, 3 µm, GL Sciences InterSustain Made in Japan).
- The flow rate of 1.0 mL/min at 30 °C.
- The mobile phase was 0.1% acidified H_2O (A) and acetonitrile (B)
- Gradient elution: **B =** 5% to 9.5% (0.01–7 min), **B** = 9.5% to 17% (7–20 min), **B** = 17% – 40% (20–35 min), **B** = 40% – 0% (35– 40 min).

- **n-BuOH** sample and the 15 standards were filtered through 0.45 µm filters injected into the device as 10 µL.
- The effluent was monitored at 254/277 nm by UV detector.

The quantitative determination was performed for phenolic components, by using external calibration with standards.

- The 15 standards are: gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, salicylic acid, rutin, chicoric acid, apigenin-7-O-glucoside, cinnamic acid, quercetin, naringenin. **Figure III.7** illustrates **n-BuOH** fraction profile.

The concentrations of the phenolic compounds were expressed by mg/ml extract illustrates in **Table III.2.**

III.2.8. Infrared Spectroscopic (FT-IR) analysis

KBr discs of compounds **(1)** and **(2)**, standard Rutin and Gallic acid were subjected to FT-IR analysis in this range of scanning: 4000- 400 cm^{-1} at a resolution of 8 cm^{-1}. The spectra of the isolated compounds **(1)** and **(2)** were compared with the standards spectra, the results are recorded in **Figure III.9** and **Figure III.10**.

III.2.9.Nuclear Magnetic Resonance Spectrometry (NMR) analysis

^{1}H-NMR and ^{13}C-NMR analysis of both compounds used their solutions in deuterated Methanol (MeOD), and as an internal standard Tetramethylsilane (TMS) was used. The chemical shift values (δ) and the coupling constants (*J*) were reported in ppm and Hz unit respectively.

III.2.10. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The Quantitative and qualitative analysis of isolated compounds **(1)** and **(2)** was also confirmed by LC-MS system using the following conditions:

- The mobile phase was filtered through a 0.22 µ membrane, injection volume: 10 uL.
- Ionization: ESI (Positive), Acquisition Mode: MRM, Ion Interface voltage:-3.50 kV, (Dwell Time) 100.0.
- Ambient CDL Temperature: 250º C, Block Temperature: 400º C.
- Detector Sampling Frequency: 2 Hz, Detector Start Time-End Time: 0.00- 5.00 min.
- Nebulizing Gas Flow: 3.00 L/min, Drying Gas Flow: 15.00 L/min.

III.3. Results and Discussion

III.3.1. Extraction yields (Y)

In this study, the phytochemical screening results of *C. procera* aerial parts extract showed the presence of large number of compounds. Therefore, it was fractionated gradually with Etp, $CHCl_3$, **EtAc** and **n-BuOH**, providing respectively 5.34g, 3.13g, **0.50g** and 1g to give the following fractions yields successively, (1.33%), (0.78%), (0.12%), (0.25%). The yields were determined relative to plant dry matter as shown in **Figure III.2**.

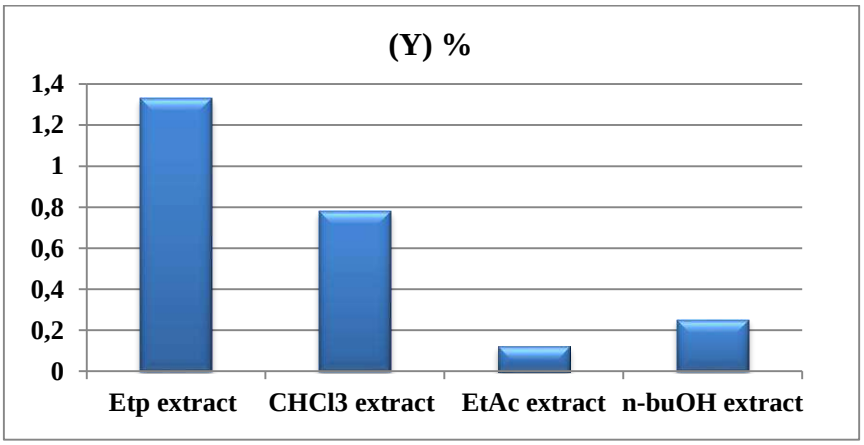


Figure III.2. Yield (Y %) extracts.

The variation in yields is probably due to the nature of compounds classes, generally present in plants in different amounts; furthermore, extrinsic parameters related to extraction and growing conditions such as temperature, duration, soil nature,etc frequently could influence such results [121,193].

III.3.2. Phytochemical results

Ellagic acid and Shinoda's tests indicated that **EtAc** fraction presented important quantities of phenols and flavonoids. In order to identify them, the fraction was subjected to further purification. In fact, previous studies reported the presence of phenols in *C.* genus especially, in the **EtAc** extract of *C.gigantea* root bark and *C. procera* leaves and bark [121,194].

III.3.3. TLC Analysis results

For quick identification method, analytical TLC of **EtAc** fraction were monitored under UV light at 366 and 254 nm using Chloroform: Ethyl acetate: Methanol: Formic acid (70: 14: 14: 10 v/v) as a mobile phase, it is considered one of the best solvent systems used for phenolic compounds separation, particularly flavonoids [192].

Using standards, this qualitative result revealed the presence of Rutin (compound 1) and Gallic acid (compound 2). Once the TLC plates sprayed with Neu's reagent then exposed to UV-Vis light at 366 nm, compound 1 (Rf: 0.18) was intensely orange and compound 2 (Rf: 0.62) was strongly blue as presents in Table **III.1.**

Table III.1. Rf and colors of isolated compounds.

mobile phase $CHCl_3$/ EtAc /MeOH/FA(7:1.5:1.5:1 v/v) at 366 nm		
	Rf	Neu's reaction color
compound **(1)**	**0.18**	Orange
Rutin standard	**0.18**	Orange
compound **(2)**	**0.62**	Blue
Gallic acid standard	**0.63**	Blue

However, at 254 nm compound **(1)** presented an intensely yellow color but compound **(2)** showed a black color; in fact, they were the same colors observed for Gallic acid and Rutin standards, but in less intensity. **Figure III.3** represents the chromatogram of **EtAc** fraction and standards.

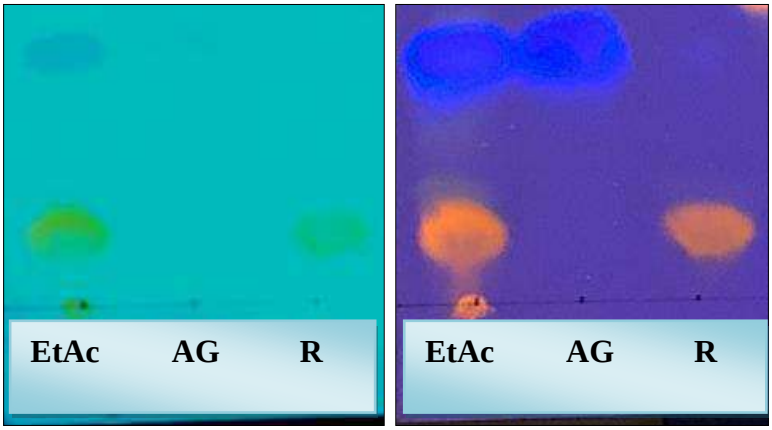


Figure III.3. TLC plate of **EtAc** fraction comparing with standard Gallic acid **(AG)** and Rutin **(R)** at 366/254 nm using Neu's reagent.

III.3.4. PTLC Analysis results

Figure III.4. Represent the chromatogram of purified **EtAc** fraction compounds by PTLC.

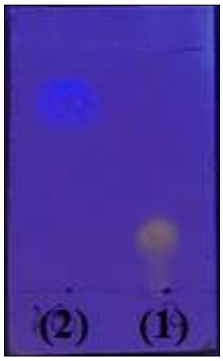


Figure III.4.TLC plate of isolated compound **(1)** and **(2)** at 366 nm using Neu's reagent.

Isolated compounds were obtained in form of colored crystals; compound **(1)** ones were yellow (50 mg) and those of compound **(2)** were white (98mg). To confirm their purity, they were analyzed by HPLC.

III.3.5. HPLC/DAD Analysis results

I) Analysis results of EtAc fraction

Figure III.5 illustrates **EtAc** fraction profile which clearly shows that it contains two compounds: the first one (90.38%) at 7.13 min and the second (9%) at 29.72 min.

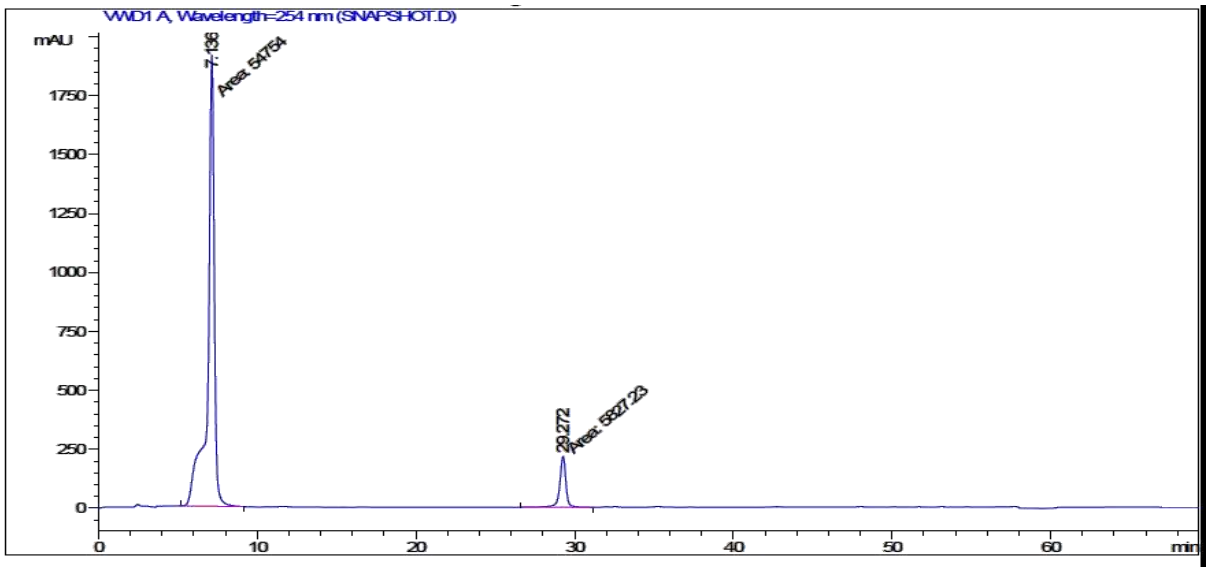


Figure III.5. HPLC chromatogram of **EtAc** fraction at λ=254 nm.

As it is revealed on **Figure III.6**, HPLC chromatograms show obviously one major peak with another less concentrated. The first compound refers to Rutin and the second one to Gallic acid that were purified from EtAc fraction of *C. procera* aerial parts.

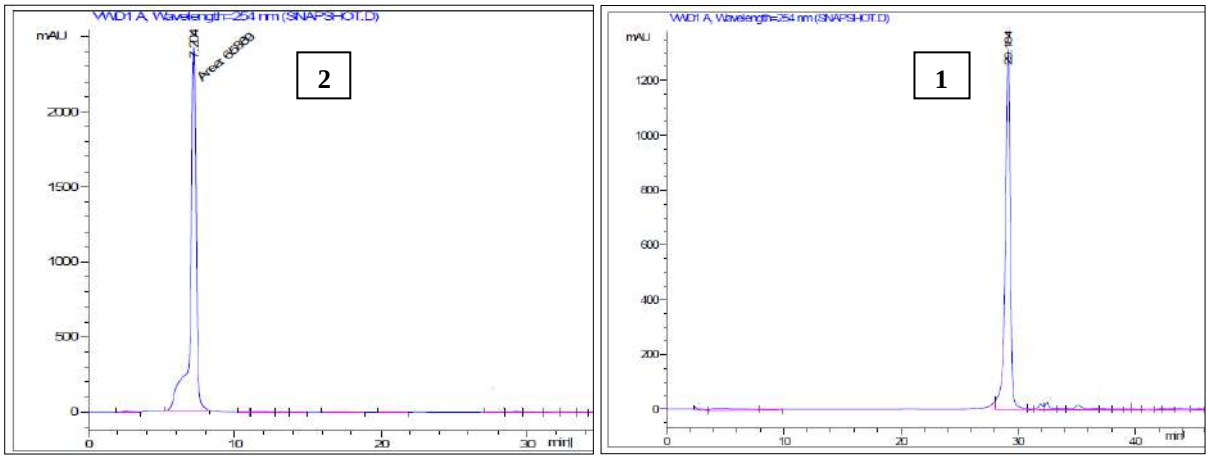


Figure III.6. HPLC chromatogram of isolated compound **(1)** and compound **(2)** at λ=254 nm.

TLC and HPLC analysis information about EtAc fractions are consistent with **Patel et al (2014)**, they stated that both *C. procera* and *C. gigantea* have significant phenolics and flavonoids content in their leaves, flower and root [195].

II) Analysis results of n-BuOH fraction

Figure III.7 illustrates the phenolic profiles of **n-BuOH** fraction, while the phenolic concentrations are reported in **Table III.2**.

The chromatogram obviously shows that the **n-BuOH** fraction rich in active ingredients where three phytocompounds have been identified as Rutin (Rt =23.20min), Quercetin (Rt =31.98min), and Epicatechin (Rt =14.22).

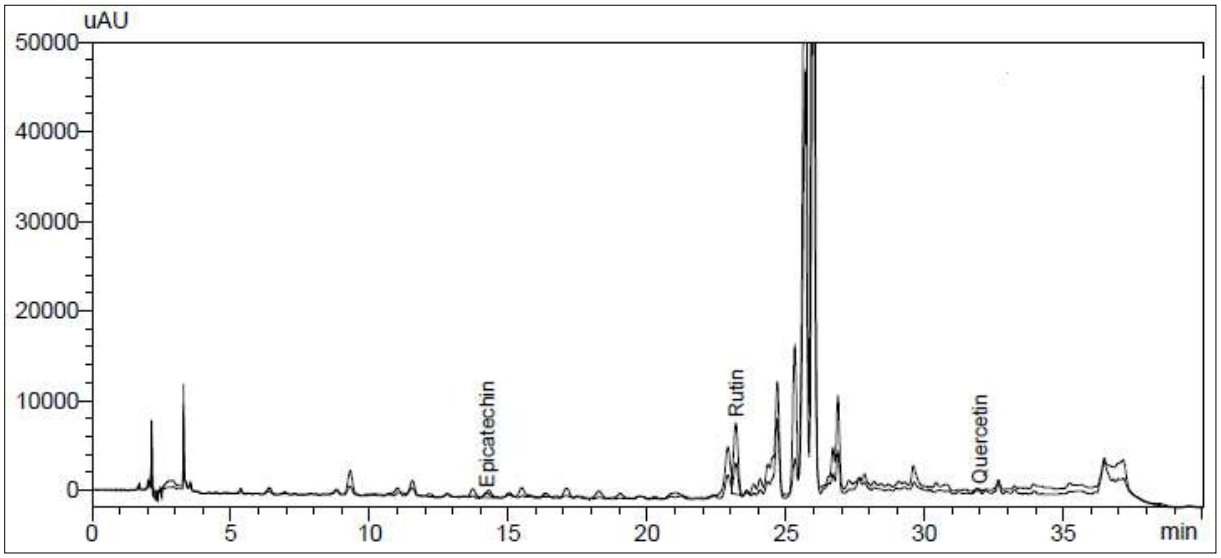
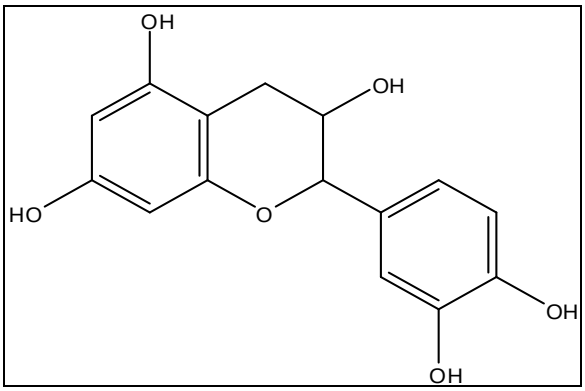


Figure III.7. HPLC chromatogram of **n-BuOH** fraction at λ=254/277 nm.

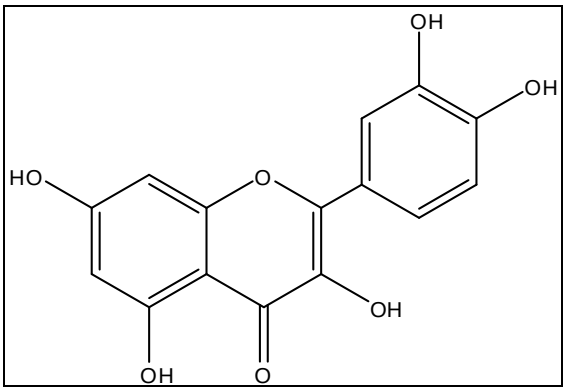
Table III.2 represents the concentrations of the three detected flavonoids in **n-BuOH** fraction where Rutin was the most concentrated with 0.2228 % followed by Epicatechin with 0.08 l % and the lowest value was recorded for Quercetin with 0.0565 %.

Table III.2. Flavonoid concentrations of **n-BuOH** fraction

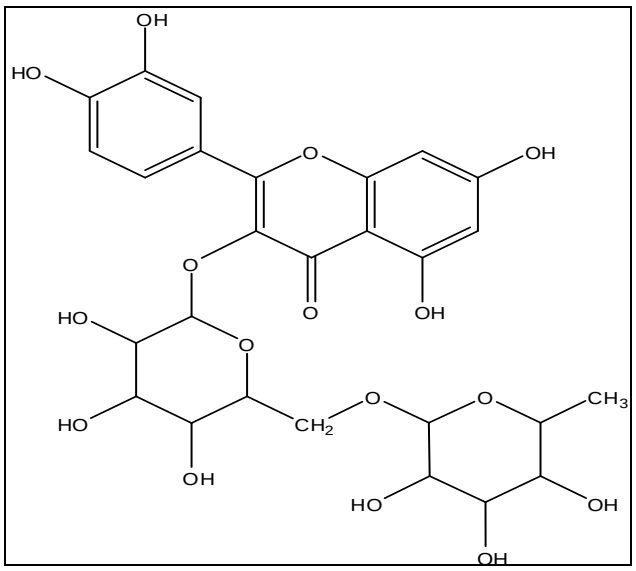
Compounds	Rt (min)	**(%)**	λ (**nm)**
Epicatechin	14,228	0.08 l	277
Rutin	23,201	0.2228	254
Quercetin	31,988	0.0565	254



Epicatechin



Quercetin



Rutin

Figure. III.8. Structures of the three identified compounds from **n-BuOH** fraction.

The results of **n-BuOH** fraction analysis are in combination with previously published reports, where Quercetin and its derivatives have been reported among the most abundant flavonoids in *C. procera* [196,197]. Also, it has been demonstrated by **Tour** and **Talele (2011)** study that the hydroalcoholic extract of *C. procera* stem bark contains 0.19% w/w of Epicatechin [122]. As it was mentioned the content of each compound in the plant could change according to geographical origin and the extraction method [121].

III.3.6. FT-IR Analysis results

Comparisons of the purified samples with standards' (Rutin and Gallic acid) IR spectrums are shown on **Figure III.9** and **Figure III.10**.

First of all, the isolated compounds were analyzed by IR (KBr). Compound **(1)** spectrum displays the following vibrations V*max* (cm^{-1}): 3409 and 3321(O-H stretching), 2935.5 (CH), 2661.6 and 2720 (aromatic CH bonding), 1631.7 (C=O), 1596.9 (C=C vibration of aromatic groups), 1462 (C=O), 1384.8 (C-OH), 1114.8 (C-O) [162].

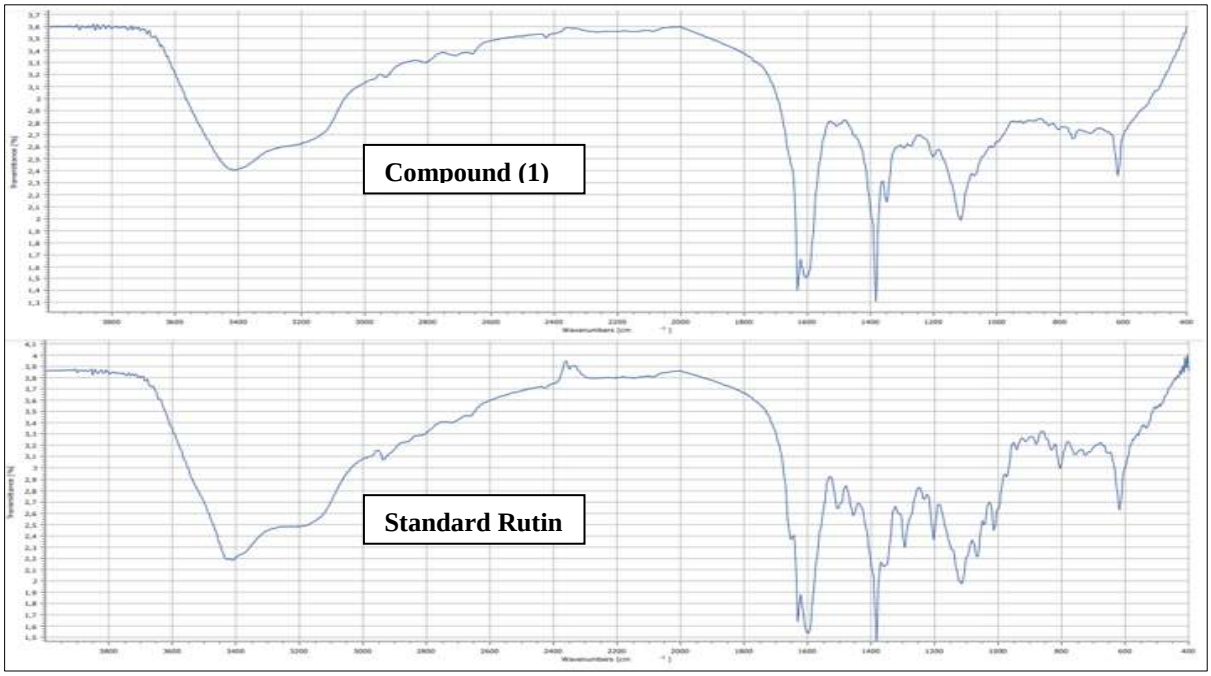


Figure III.9. FT-IR spectrum of compound **(1)** and standard Rutin.

Regarding the IR (KBr) data recorded for compound **(2)** it shows the presence of a band at 3232.5 cm^{-1} indicating hydroxyl groups (O-H), at 2426 cm^{-1} for (aromatic CH) bond, 1631.7 cm^{-1} for carbonyl groups (C=O), The absorption bands 1608.5, 1446.5, 1384.8, 1238.2, 1029.9 cm^{-1} respectively indicating the presence of benzene cycle attached with three –O-aryls directly, 717.5 cm^{-1} confirmed substituted benzene [189].

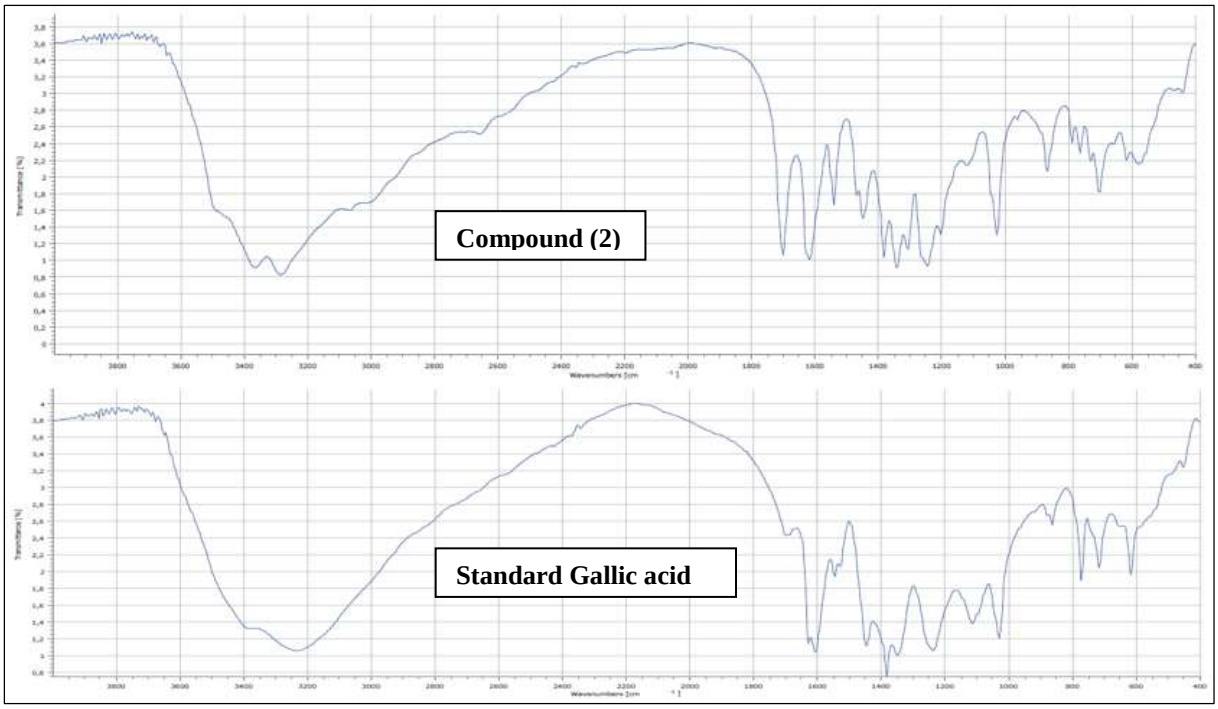


Figure III.10. FT-IR spectrum of compound **(2)** and standard Gallic acid.

III.3.7. NMR Analysis results

III.3.7.1. Structural elucidation of compound (1)

Registered ^{1}H-NMR spectrum (400 MHz in MeOD, ppm) of isolated compound **(1)** showed all resonances of hydroxylic aromatic groups: OH (C-4'), OH (C-3'), OH (C-7) and OH (C-5) from 8 to 12.5 ppm; aromatic CH moieties (C-6', C-5', C-2', C-6 and C-8) appeared from 6 to 8 ppm; the aliphatic hydroxylic groups of the sugar units could be observed from 4 to 6 ppm, rutinose CH signals were detected from 3 to 4 ppm in particular, positions H-(C-6''), H-(C1''), H-(C-1''') were well-separated as double signals appearing at 5.23, 3.93, and 1.26 ppm as presents in **Figure III.11.A**. The assignment of the resonances was performed according to the literature [198,199].

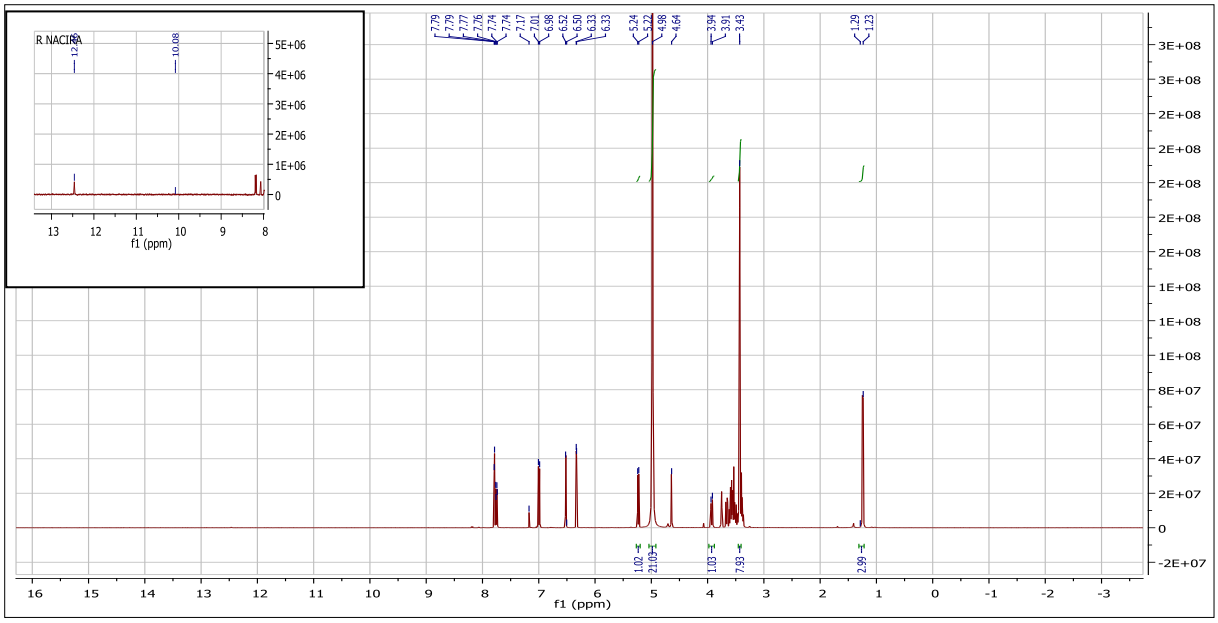


Figure III.11.A. The ^{1}H-NMR spectrum of isolated compound **(1)**.

Whereas, on ^{13}C-NMR (MeOD, 400 MHz, δ ppm) spectrum were present the following 27 carbon signals: 18.74 (C-6’’’), 69.40 (C-6’’), 70.56 (C-5’’’), 72.25 (C-2’’’), 72.96 (C-3’’’), 73.09 (C- 4’’), 74.78 (C-4’’’), 76.58 (C-2’’), 78.08 (C-5’’), 79.04(C-3’’), 95.71 (C-8), 100.79 (C-6), 103.27 (C-1’’’), 105.57 (C1’’), 106.48 (C-10), 116.90 (C-2’), 118.54(C-5’), 123.79 (C-6’), 124.40 (C-1’), 136.48 (C-3), 146.70 (C-3’), 150.67 (C-4’), 159.37 (C-9), 160.19 (C-2), 163.85 (C-5),166.90 (C-7), 180.28 (C-4).

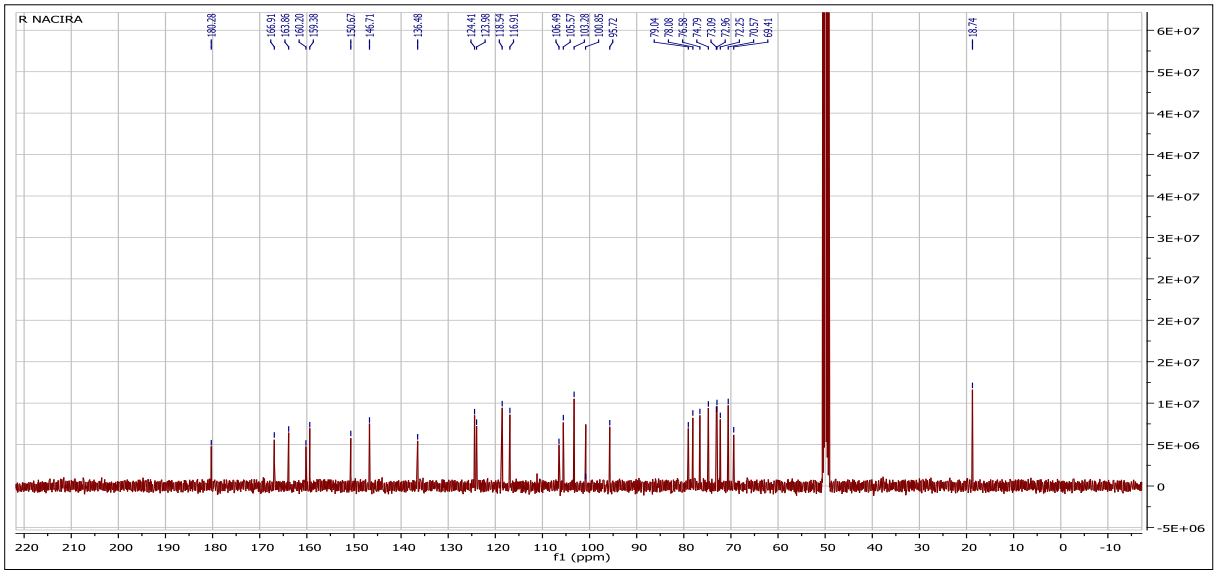


Figure III.11.B. The ^{13}C -NMR spectrum of isolated compound **(1)**.

^{13}C-NMR data of isolated compound **(1)** with ^{13}C-NMR of Rutin previously reported [200,201], are presented in **Table III.3**.

Table III.3. ^{13}C-NMR data for isolated and standard Rutin.

Position	Compound (1)	Standard Rutin
C-9	159.37	158.5
C-3	136.48	135.7
C-4	180.28	179.4
C-5	163.85	163.0
C-6	100.79	100.0
C-7	166.90	166.0
C-8	95.71	95.0
C-2	160.19	159.4
C-10	106.48	105.7
C-1'	124.40	123.7
C-5'	118.54	117.8
C-3'	146.70	145.9
C-4'	150.67	149.9
C-2'	166.90	116.1
C-6'	123.79	123.2
C1''	105.57	104.8
C-2''	76.58	75.8
C-5''	78.08	77.2
C-2'''	72.25	71.5
C-3''	79.04	78.2
C-6''	69.40	68.6

C- 1’’’	103.27	102.5
C- 4’’	73.09	72.3
C-3’’’	72.96	72.2
C-4’’’	74.78	74.0
C-5’’’	70.56	69.8
C-6’’’	18.74	18.0

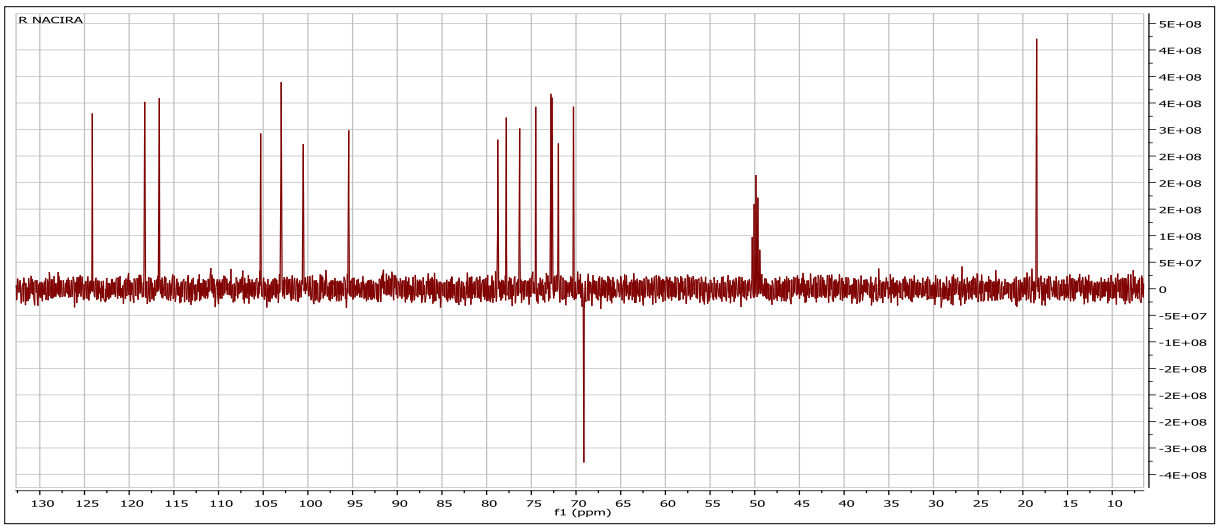


Figure III.11.C. The dept 135-NMR spectrum of compound **(1)**.

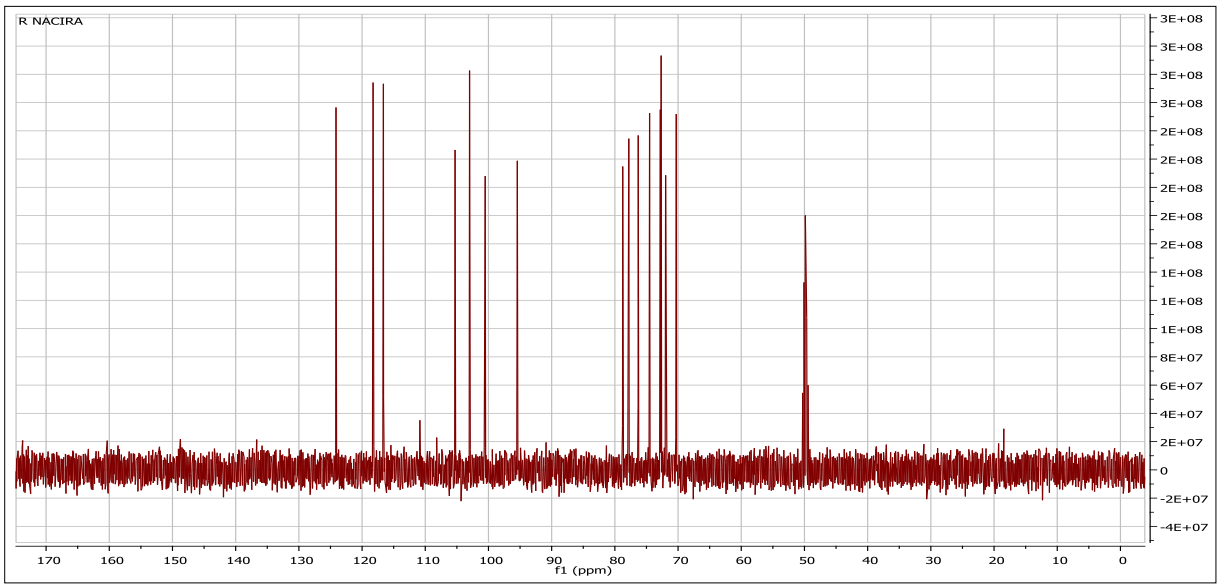


Figure III.11.D. The dept 90-NMR spectrum of compound **(1)**.

The dept 135-NMR spectral data shown in **Figure III.11.C** confirms the presence of the only **CH2 (C-6'')** group in compound **(1)**. Besides, the dept 90-NMR spectral data shown in **Figure III.11.D** confirms the existence of 16 **(CH)** groups.

Thus, it can be confirmed that compound **(1)** is characterized as 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin $C_{27}H_{30}O_{16}$) (**Figure III.12)**.

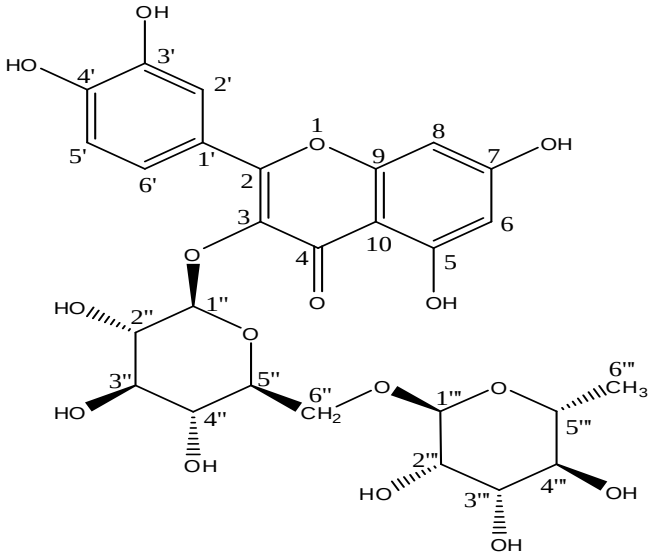


Figure III.12. 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin).

III.3.7.2. Structural elucidation of compound (2)

As for the elucidation of compound **(2)**; ^{1}H-NMR and ^{13}C-NMR spectrums (400 MHz in MeOD, ppm) were employed. Effectively, ^{1}H-NMR spectrum (**Figure III.13.A)** shows the presence of an aromatic singlet at δH 7.08 (2H, s), at δH 4.89 (3H, s) a broad singlet confirming the presence of three OH-aryls.

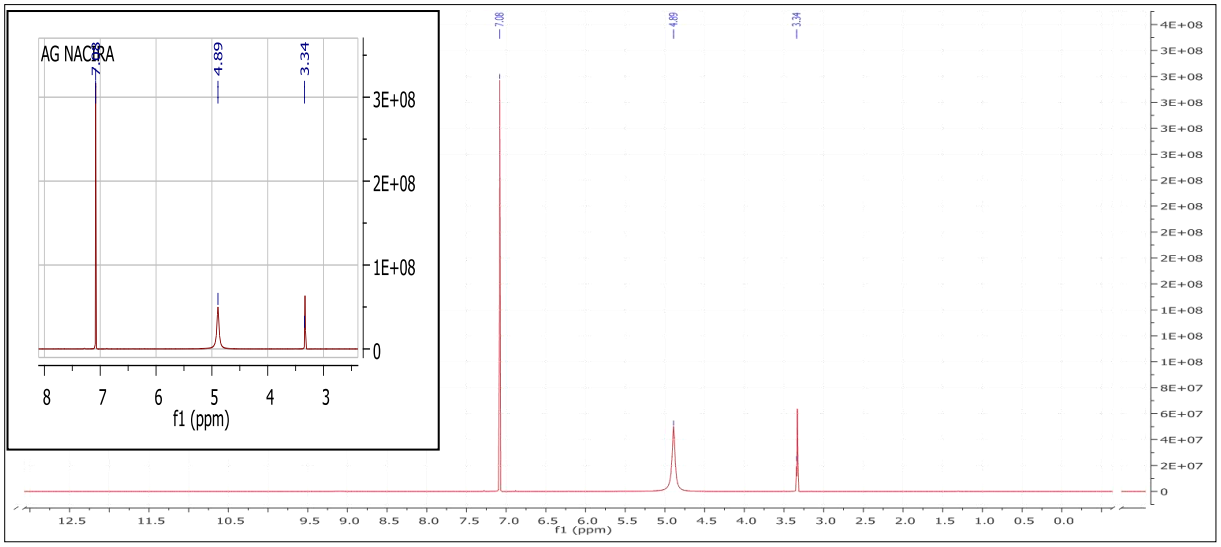


Figure III.13.A.The ^{1}H-NMR spectrum of isolated compound **(2)**.

^{13}C-NMR spectrum in **Figure III.13.B** and **Table III.4** illustrates the obtained data (compound **2**) compared with those of Gallic acid isolated from chloroform fraction of syzygium litorale stem bark [202].

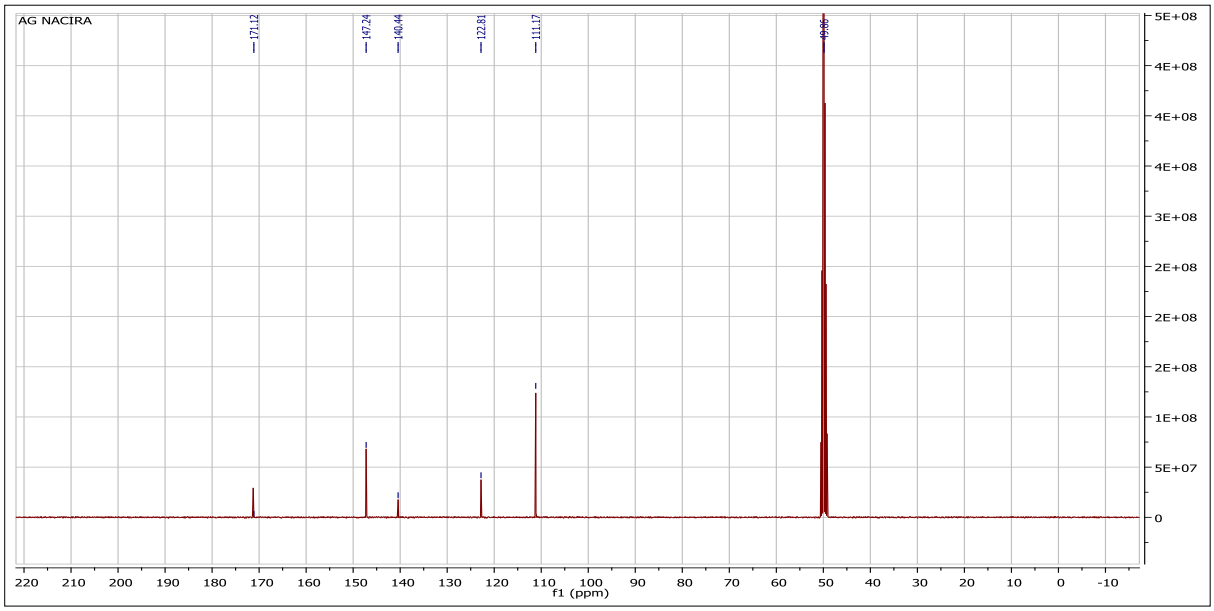


Figure III.13.B. ^{13}C-NMR spectrum of isolated compound **(2)**.

Table III.4. ^{1}H- NMR and ^{13}C-NMR data for isolated and standard Gallic acid [202].

Position	**Compound (2)**		standard **Gallic acid**	
	δC (400 MHz)	δH (400 MHz)	δC (400 MHz)	δH (600 MHz)
2(6)	111. 16	7.08(s)	110.37	7.06 (s)
1	122.80		122.04	
4	140.43		139.63	
3(5)	147.23		146.44	
7	171.24		170.45	

It was characterized as 3, 4, 5-trihydroxybenzoic acid (Gallic acid $C_7H_6O_5$). **Figure III.14** illustrates its structure.

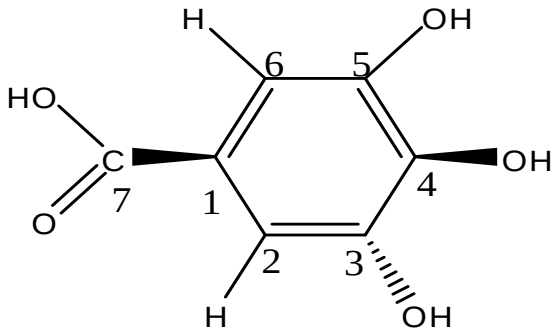


Figure III.14.3, 4, 5-trihydroxybenzoic acid structure (Gallic acid)

The present study provides a detailed report on the isolation and structure elucidation of 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin) and 3,4,5-trihydroxybenzoic acid (Gallic acid) from ethyl acetate fraction of *C. procera* aerial parts from Algeria.

These results were consistent with those found by **Oraibi** and **Hamad (2018**), they separated and purified Rutin, Quercetin and Kaempferol from *C. procera* ethyl acetate extract in Iraq [90]. Furthermore, the comparison study conducted by **Gholamshahi et al (2014)**, in Jiroft and Bam regions of Kerman, Iran, which analyzed total phenols in *C. procera* leaves and latex extracts, showed that Gallic acid content were high in leaves extracts with 9.72 and 9.02 mg Gallic acid/g dry weight (in Bam and Jiroft plants, respectively) [203].

III.3.8. LC-MS Analysis result

Liquid Chromatography-Mass Spectrometry (LC-MS) method is a powerful with high sensitivity and selectivity; generally, used for the identification and quantification of easily ionizable phenolic compounds [204].

Therefore, the characteristic fragmentation pattern of isolated Rutin in positive ion mode was carried out by careful examination of ESI-MS spectrum. The deprotonated ion at m/z 611 $[M+H]^+$ showed abundant ions at m/z 303.2 (loss of rutinose moiety $C_{12}H_{22}O_9$). However, the characteristic fragmentation pattern of isolated Gallic acid in negative ion mode was neutral losse of COOH, the typical MS spectrum of the deprotonated ion at m/z 169 $[M-H]^-$ produced ions at m/z 125.10.

Over all, the MS spectra and fragmentation patterns of isolated Rutin and Gallic acid are shown in **Figure III.15** and **Figure III.16** respectively, which were identified by comparing the fragment ions with those in the literature [205,206].

Figure III.15. LC-MS spectrum of isolated Rutin ion 303.2 m/z.

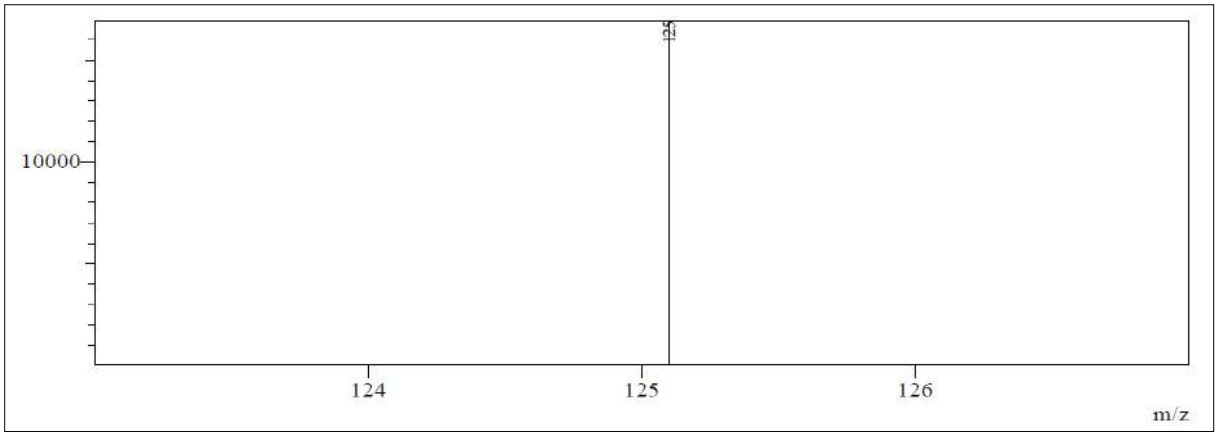


Figure III.16.LC-MS spectrum of isolated Gallic acid ion 125.1 m/z.

III.4. Conclusion

The identification and quantification of ethyl acetate and n- Butanol fractions phenolic compounds from *C. procera* aerial parts have been determined using TLC, PTLC, HPLC, IR, NMR and LC-MS data. The long process of purification achieved to the characterization of two compounds isolated from ethyl acetate that are identical with those reported for the flavonol glycosides: quercetin 3-O- rutinoside (Rutin) and the phenolic acid: 3,4,5-Trihydroxybenzoic acid (Gallic acid). While in the n- Butanol fraction three phenolic compounds were identified, namely: Epicatechin, Rutin, and Quercetin.

Chapter IV:

Alkaloid compounds:

Phytochemical study

IV.1. Introduction

C. procera seems to be well known by its richness in important pharmaco-phytochemicals; among others, the famous amino acids namely: neutral (hydrophobic and hydrophilic), basic, acidic and essential categories [207]. These compounds helped to establish the scientific basis of folklore tradition and belief behind the use of *C. procera* in the treatment of various diseases [208].

The analytical study in present chapter is based on the phytochemical tests that have confirmed the presence of alkaloids in the species. It aimed to extract the total fixed alkaloids and the volatile ones from *C. procera* aerial parts, using different extraction methods and to determine the chemical profile of the obtained extracts. Therefore, different steps of separation and physico-chemical analysis were used for the realization of this part of work.

IV.2. Material and Methods

IV.2.1. Total Fixed Alkaloids (TFA) extraction

The plant powder, previously prepared was degreased using petroleum ether (Etp) for 72 h with agitation, to ensure the entire elimination of fats and pigments.

IV.2.1.1. Extraction of TFA by (Stas-Otto) method

The first extraction method used for the extraction of **TFA** takes place in an alkaline medium; it is also called the **Stas-Otto** method [19].

First of all, 1 Kg of degreased powder was alkalized with 40 ml NH_4OH (0.5 N) for 24 hours; after that, it was extracted by Soxhlet extractor using 7 L DCM (8 cycles in a 250 ml Soxhlet extractor and 16 cycles in a 500 ml Soxhlet extractor). Secondly, liquid-liquid extractions were applied to the final DCM extract by means (300 ml x 3) of sulfuric acid solution (H_2SO_4 0.5 N), to separate the alkaloids in their salt form. In a third step, the acidic phase was then made alkaline with NH_4OH 0.5 N solution (300 ml x 3) to reach pH = 9; followed by a series of liquid–liquid extraction that were carried out using Separatory Funnel, using 700 ml of ethyl ether. Finally, the organic fractions were dehydrated with anhydrous sodium sulphate Na_2SO_4 (10 g), filtered and dry concentrated, then stored at -18 °C.

Figure IV.1 represents the flowchart of the different steps of TFA extraction by **(Stas-Otto)** method.

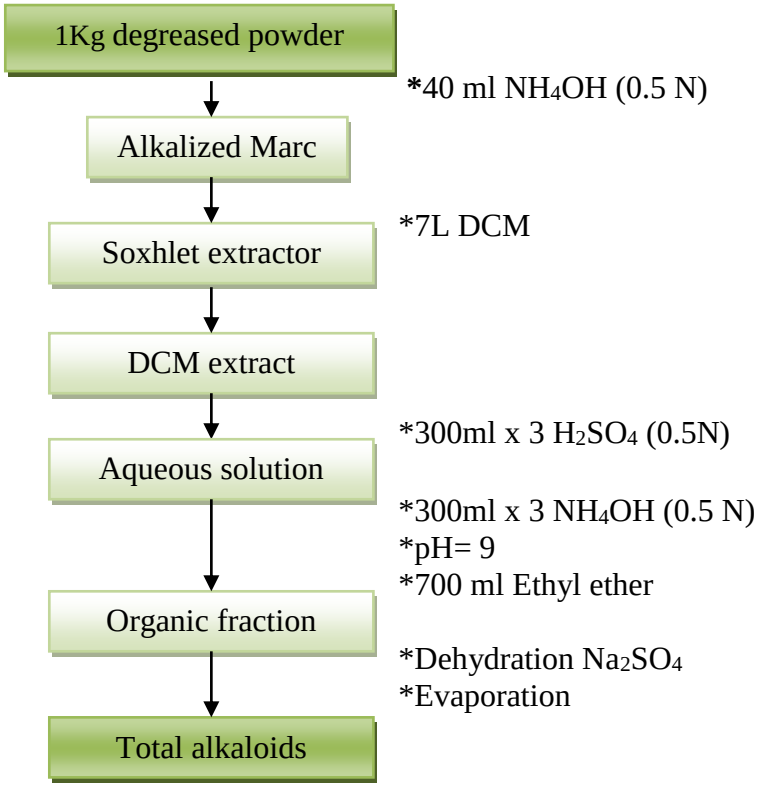


Figure IV.1. Flowchart of TFA extraction in alkaline medium by (Stas-Otto) method.

IV.2.1.2. Extraction of TFA in acid medium

The extraction of total alkaloids in an acid medium was carried out according to the protocol of **Makkar et al (2007)** with slight modifications [209]. So, 1 Kg degreased powder was macerated, under agitation, with 3 L of 15% acetic acid (in methanol MeOH); after that, collected filtrate was made alkaline with NH_4OH (0.5 N) until PH = 9. The alkaline solution obtained was carried out with a liquid–liquid extraction, in a Separatory Funnel, with 1.5 L DCM, and the organic fractions were dehydrated with anhydrous sodium sulphate Na_2SO_4 (10 g), filtered, dry concentrated and stored at -18 °C.

Figure IV.2 represents the flowchart of the different steps of TFA extraction in acid medium.

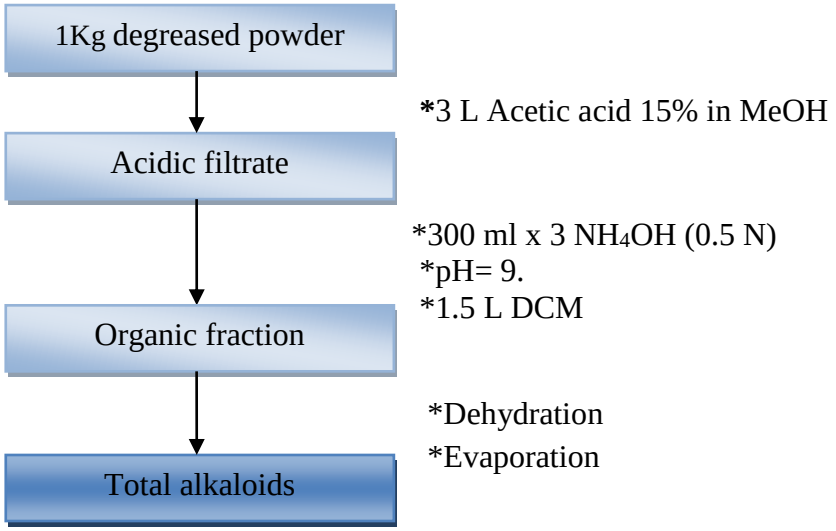


Figure IV.2. Flowchart of TFA extraction in acid medium by maceration

IV.2.2. Thin-layer chromatography (TLC) analysis

In order to choose the best separation system for TFA extract obtained from acidic medium process, thin layer analyzes were carried out using silica gel aluminum plates (Merck)(60 F254, 0.25mm). The plates were eluted in glass cuvette saturated with several solvent systems (eluent).

More than twenty solvent systems were used for the separation of compounds classes, some of them are cited as follows; the proportions are given by volume:

- Dichloromethane/Ethanol (5 : 5v/v)
- Toluene/Ethyl acetate / Diethylamine (7 :2 :1v/v/v)
- Dichloromethane/Acetone/ Diethylamine (5 :4 :1v/v/v)
- Cyclohexane/Dichloromethane/ Diethylamine (5 :4 :1v/v/v)
- Methanol/ Dichloromethane (5:5v/v)
- Dichloromethane/ Ethyl acetate (2:8v/v)
- Dichloromethane/ Methanol/ Diethylamine (9:1:0.1v/v/v)
- Methanol/ Dichloromethane/ Ammonium hydroxide (5:5:0.1v/v/v)
- Ethanol/ Dichloromethane/ Ammonium hydroxide (5:5:0.1v/v/v)
- Dichloromethane/ Diethylamine/ Ethyl acetate (6:3:1v/v/v)
- Toluene/ Acetone/ Ethanol/ Ammonium hydroxide (3:4:2:1v/v/v/v)
- Ethyl acetate/ Ethanol/ water (5:4:1v/v/v)

The observation of the TLC plates was carried out directly (visible) and also under UV light (at 254 and at 356 nm); before and after revelation with **Dragendroff** reagent.

IV.2.2.1. Dragendroff reagent

For the visualization of separated compounds, TLC plates were revealed with Dragendroff's reagent. A positive result was confirmed by the apparition of an orange color. Dragendorff reagent is usually prepared by mixing of 5 ml of aqueous **A** solution composed of 0.8 g of $BiONO_3$ and 10 ml of glacial acetic acid with 5 ml of aqueous **B** solution composed of 20 g KI powder these, resulting a orange homogeneous solution [210].

IV.2.3. Purification of TFA by column chromatography (CC)

Several types of stationary and mobile phases can be used in glass columns, the length and diameter of the column were chosen according to the quantity of sample to be purified. In our case, **5g** of TFA extract obtained in acid medium was mixed with 1g of silica gel then pulverized until a homogeneous powder was obtained.

The powder was placed on the top of a column, and coarsely fractionated on a flash chromatography column (ϕ= 4.5 cm, L= 60 cm) filled with silica gel (300 g) as stationary phase prepared in 100% DCM and gradually enriched with EtOH up to 100% EtOH, finally, 100% MeOH was added to the column.

The collected fractions were grouped according to their chromatographic profile similarity on thin layer plates; these ones were developed with different mobile phase systems, and visualized under a UV lamp, at 254 and at 366 nm. The column progression is shown in **Table IV.1**.

Table IV.1. Chromatographic fractionation of the **TFA** of *C. procera* extract

Fractions	**Elution system**		**Grouped fractions**	**Name of grouped fractions**	**Mass (mg)**
	DCM%	EtOH%			
1-5	100	0	1-3	AI	153.1 mg
	100	0	4-5	AII	80.0mg
6-10	75	25	6	BI	64.9mg
	75	25	7	BII	42.09mg
	75	25	8-10	BIII	**2087.5mg**
11-15	50	50	11-14	CI	**1513.1mg**
	50	50	15	CII	604.0mg

16-20	25	75	16-18	DI	153.5mg
	25	75	19-20	DII	40.4mg
21-25	0	100	21-25	EI	197.01 mg
26-30	100% MeOH		26-30	F	50mg

The collected fractions were grouped according to the results of the analysis by TLC and revealed using Dragendroff reagent, to determine the alkaloid spots as shown on **Figure IV.3.A** and **Figure IV.3.B**.

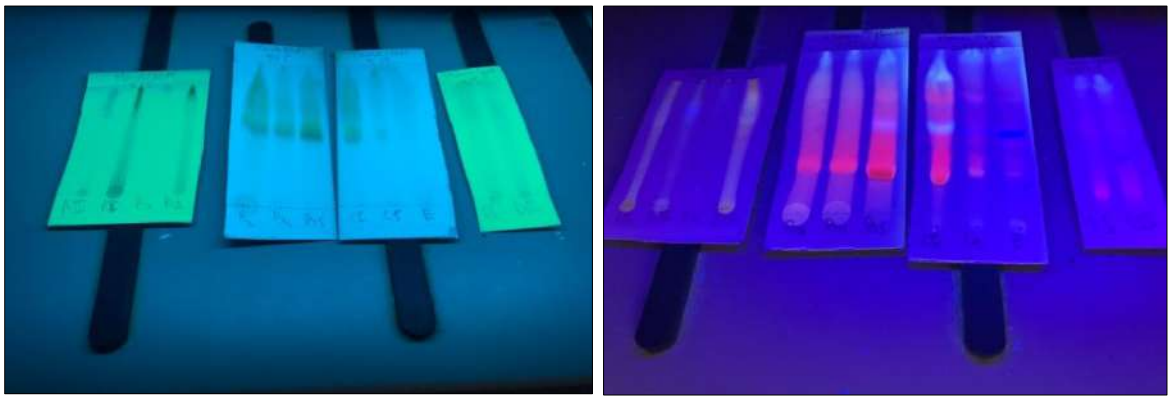


Figure IV.3.A.TLC plates of grouped fractions at 254 /366nm.

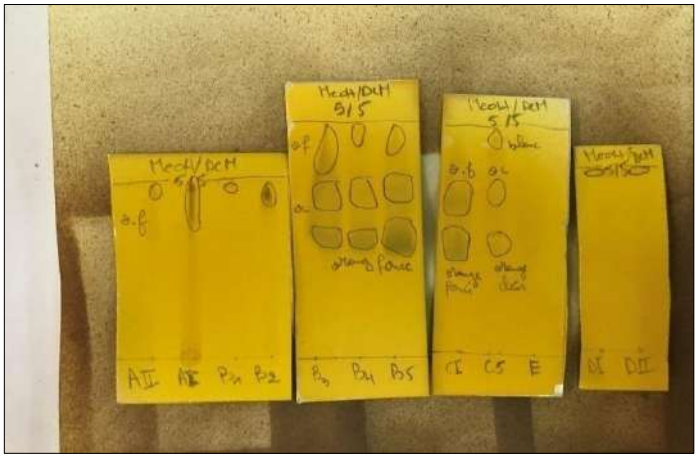


Figure IV.3.B.TLC plates of grouped fractions revealed with Dragendroff reagent.

IV.2.4. Separation and purification of AI fraction

IV.2.4.1. Vacuum liquid chromatography (VLC) fractionation

AI fraction (153.1 mg) was then purified on a vacuum liquid chromatography column (ϕ=1cm, L=10cm), 7g of silica gel were used as stationary phase, and as a mobile phase,

DCM (100%) gradually enriched with Acetone, Ethanol, Methanol, Water and Acetic acid, respectively. Regular checks at each fractionation step are carried out by TLC to obtain 7 sub-fractions. **Table.IV.2** summarizes the sub-fractionations and their masses.

Table.IV.2. Flowchart of AI fraction purification steps.

Grouped s-fractions of AI	**Mass (mg)**
AI1	67.8
AI2	7.3
AI3	7.2
AI4	2.4
AI5	42.7
AI6	//
AI7	7.4

IV.2.4. 2. Analytical HPLC analysis of AI sub-fractions

AI1, AI2, AI3, AI4 fractions(prepared by dissolving 1 mg in 1 ml of Methanol (MeOH) HPLC grade) have been analyzed using YL-Clarity series analytical HPLC-DAD; column Ascentis® C18-AB5µ (25 cm x 4.6 mm, 5 µm) at 35°C; injected volume 10 uL. All analyzes were carried out with a gradient of Acetonitrile (HPLC grade) and water, (ACCN/H_2O). The flow rate was fixed at 0.5 ml/min, and the detection was done at these λ maxes: 280, 254, and 366 nm. Analysis time was 40.00 min. **Figure IV.9** illustrates the profiles of the four sub-fractions.

IV.2.5. Separation and purification of BIII fraction

IV.2.5.1. Vacuum liquid chromatography (VLC) fractionation

Fraction **BIII (2087.5mg)** was purified on a column (ϕ=3cm, L=80cm) filled with 150 g of silica gel as stationary phase. Afterwards, the elution was carried out using a mixture of: DCM-Acetone-EtOH; starting by 100% DCM in which gradual volumes of Acetone were added until 100% Acetone; Then, from 100% Acetone up to 100% EtOH gradually. As a last step, a proportion of 100% MeOH was used; to note, regular checks were carried out by TLC

at each fractionation step. During the elution, it was collected 10 and 20 ml fractions for each system (43 systems) to obtain 342 fractions at the end; as it is shown in **Table IV.3.**

Table IV.3. Chromatographic fractionation of **BIII** fraction.

Elution system		**Grouped fractions**	**Name of grouped fractions**	**Mass (mg)**
DCM (ml)	**Acetone (ml)**			
200	0	1-17	BIII1	222.1
195	5	18-26		
		27-28	**BIII2**	**185.0**
190	10	29-31		
		32-47	**BIII3**	**267.7**
185	15	48-57	BIII4	77.2
180 ↓ 5% **115**	**20** ↓ 5% **85**	58-187	BIII5	385.1
110	90	188-197	BIII6	52.3
105	95	198-207		
100	100	208-217	BIII7	48.1
90	110	218-227		
85	115	228-237	BIII8	100.8
80	120	238-247		
75	125	248-257		
70	130	258-267		
		268-277	BIII9	32.7
65	135	278-287		
60	140	288-297	BIII10	20.7

55	145	298-307		
50	150	308-317	BIII11	27.5
0	100	318-322	BIII12	140.4
Acetone (ml)	**Ethanol (ml)**			
95	5	323	BIII13	25.8
90	10	324	BIII14	204.6
85	15	325		
80	20	326	BIII15	83.1
75	25	327		
70 ↓ 5% **50**	**30** ↓ 5% **50**	328-331	BIII16	145.8
100% Ethanol		332-341	BIII17	58.6
100 % Methanol		342		

IV.2.5.2. Purification of BIII2 and BIII3 by column chromatography (CC)

Among the 17 sub-fractions aforementioned, we have chosen two fractions **BIII2** and **BIII3** (the weightiest and the least complicated) to complete the purification by Sephadex ® LH-20 column.

For this, the elution was achieved in an isocratic mode using a mixture of DCM/ MeOH 50/50 % on column (ϕ=1 cm, L=10 cm) containing 2 g of Sephadex as stationary phase.

At the beginning, 50 sub-fractions were collected from each fraction; they were grouped according to the similarity of their chromatographic profile revealed by TLC plates developed with different mobile phase systems, and visualized under UV light at 254 and 366 nm. Lastly, the number was reduced to 5 sub fractions from **BIII2**, and 6 sub fractions from **BIII3** as presented in **Table IV.4.**

Table IV.4. Chromatographic fractionation of **BIII2 and BIII3** sub-fractions.

Grouped s-fractions of BIII2	Mass (mg)	Grouped s-fractions of BIII3	Mass (mg)
$BIII2_1$	23.9	**$BIII3_1$**	3.9
$BIII2_2$	59.7	**$BIII3_2$**	5.7
$BIII2_3$	18.2	**$BIII3_3$**	42.4
$BIII2_4$	4.3	**$BIII3_4$**	190.8
$BIII2_5$	50	**$BIII3_5$**	3.3
/	/	**$BIII3_6$**	4.9

IV.2.6. Separation and purification of CI fraction

IV.2.6.1. Purification of CI by vacuum liquid chromatography (VLC)

Regarding fraction CI (1513.1mg), it was purified on a column (ϕ=3cm, L=80cm) containing 100g of silica gel. For this, the elution was achieved in a gradient mode using a mixture of: DCM-Acetone-EtOH-MeOH starting by 100% DCM in which gradual volumes of Acetone were added until 100% Acetone; Then, from 100% Acetone up to 100% EtOH gradually. As a last step, a proportion of 100% MeOH was used.

During the elution, it was collected 50 to 200 ml fractions for each system (12 systems) to obtain 47 fractions at the end; as it is shown in **Table IV.5.**

Table IV.5. Chromatographic fractionation of **CI** fraction.

Elution system		**Grouped fractions**	**Name of grouped fractions**	**Mass (mg)**
DCM (ml)	**Acetone**(ml)			
200	0	1-4	**CI1**	509.5
195	5	5		
		6-8	**CI2**	112.5
150	50	9	**CI3**	252.3
100	100	10		
0	200	11-13		
Acetone(ml)	**Ethanol(ml)**			
195	5	14-15	**CI4**	184.2

150	50	16-17		
100	100	18-19	**CI5**	125.4
50	150	20-21	**CI6**	114.6
0	200	22		
		23	**CI7**	7.8
Ethanol(ml)	**Methanol (ml)**			
100	100	24	**CI8**	//
100%Methanol		25-47	**CI9**	53.2

IV.2.6.2. Purification of CI5 by column chromatography (CC)

Among **CI** fractions we have chosen **125.4 mg** of **CI5 sub-fraction** (the weightiest and the least complicated) to complete the purification by column (ϕ=1cm, L=10cm) containing 7g of silica gel as stationary phase, the elution was done in gradient mode using the following solvents: Petroleum ether- DCM- Ethyl acetate-Acetone-EtOH-MeOH .

In the beginning, 64 sub-sub-fractions were collected, regrouped later according to the similarities of their chromatographic profile on TLC plates to collect at the end 7 s-sub fractions as illustrates in table **Table IV.6**.

Table IV.6. Chromatographic fractionation of **CI5 sub-fraction.**

Grouped s-fractions of CI5	**Mass (mg)**
$CI5_1$	10.4
$CI5_2$	23
$CI5_3$	9.1
$CI5_4$	2
$CI5_5$	77.7
$CI5_6$	2.8
$CI5_7$	//

IV.2.6.3. Purification of $CI5_2$, $CI5_5$ by column chromatography (CC)

To complete the purification, **23 m**g of $CI5_2$ s-sub-fraction and **77.7 mg** of $CI5_5$ s-sub-fraction were subjected on Sephadex ® LH-20 column (ϕ=1cm, L=10cm), in an isocratic elution mode, using a mixture of DCM and MeOH 50/50%.

Using TLC plates, **3** s-sub fractions from $CI5_2$ and **9** s-sub fractions from $CI5_5$ were collected, as illustrates **Table IV.7**.

Table IV.7. Chromatographic fractionation of $CI5_2$ and $CI5_5$ s-sub-fractions.

grouped ss-fractions of $CI5_2$	Mass (mg)	grouped ss-fractions of $CI5_5$	Mass (mg)
$CI5_{21}$	19.9	$CI5_{51}$	10.9
$CI5_{22}$	//	$CI5_{52}$	5
$CI5_{23}$	2.3	$CI5_{53}$	2.1
		$CI5_{54}$	7.5
		$CI5_{55}$	8.6
		$CI5_{56}$	3
		$CI5_{57}$	10.3
		$CI5_{58}$	22.6
		$CI5_{59}$	//

IV.2.7. Separation and purification of DI fraction

IV.2.7.1. Purification of DI by vacuum liquid chromatography (VLC)

153.5 mg of fraction **DI** was then purified on a column of (ϕ=1cm, L=10cm) containing 7g of silica gel as stationary phase, the elution was done in gradient mode using a DCM-EtOH- MeOH as a mobile phase; starting by 100% DCM in which gradual volumes of EtOH were added until 100% EtOH, then from 100% EtOH up to 100% MeOH gradually. Regular checks at each fractionation step was carried out by (**TLC**) to obtain at the end 5 sub-fractions as it is shown in **Table IV.8**.

Table IV.8. Chromatographic fractionation of **DI** fraction.

grouped s-fractions of **DI**	Mass (mg)
DI_1	9.6
DI_2	18.8
DI_3	52.7
DI_4	15.1
DI_5	5.1

IV.2.8. Identififcation and quantification of $BIII2_5$, $CI5_{58}$ s-sub-fractions

In order to identify and quantify **$BIII2_5$**, **$CI5_{58}$** fractions (prepared by dissolving 1 mg in 1 ml of Methanol (MeOH) HPLC grade); Shimadzu Nexera-i LC-2040C 3D plus HPLC device was used for quantitative analysis. Chromatographic separation was conducted with gradient elution mode by using the following conditions:

- Compounds separation was carried out on reverse phase C18 colomn 4.6 x 250 mm, 5 µm (UP) (LC Column, phenomenex).
- The mobile phase was made of phase A: 0.1% acidified H2O, B: MeOH.
- The elution conditions were: flow rate was set at 1.0 ml/min at 30 °C.
- The gradient elution was as follows: flow **B** = 5–15% (0.01–5 min), **B** = 15–25% (5–15 min), **B** = 25–50% (15–25 min), **B** = 50–70% (25–35 min), **B** = 70–0% (35–40min), **B** = 0% (40–45 min).
- The filtered samples (0.45 µm filters) from each fraction and Alkaloids standards were injected into the device as 10 µL.
- The effluent was monitored at 284 nm, 258 nm by UV detector.

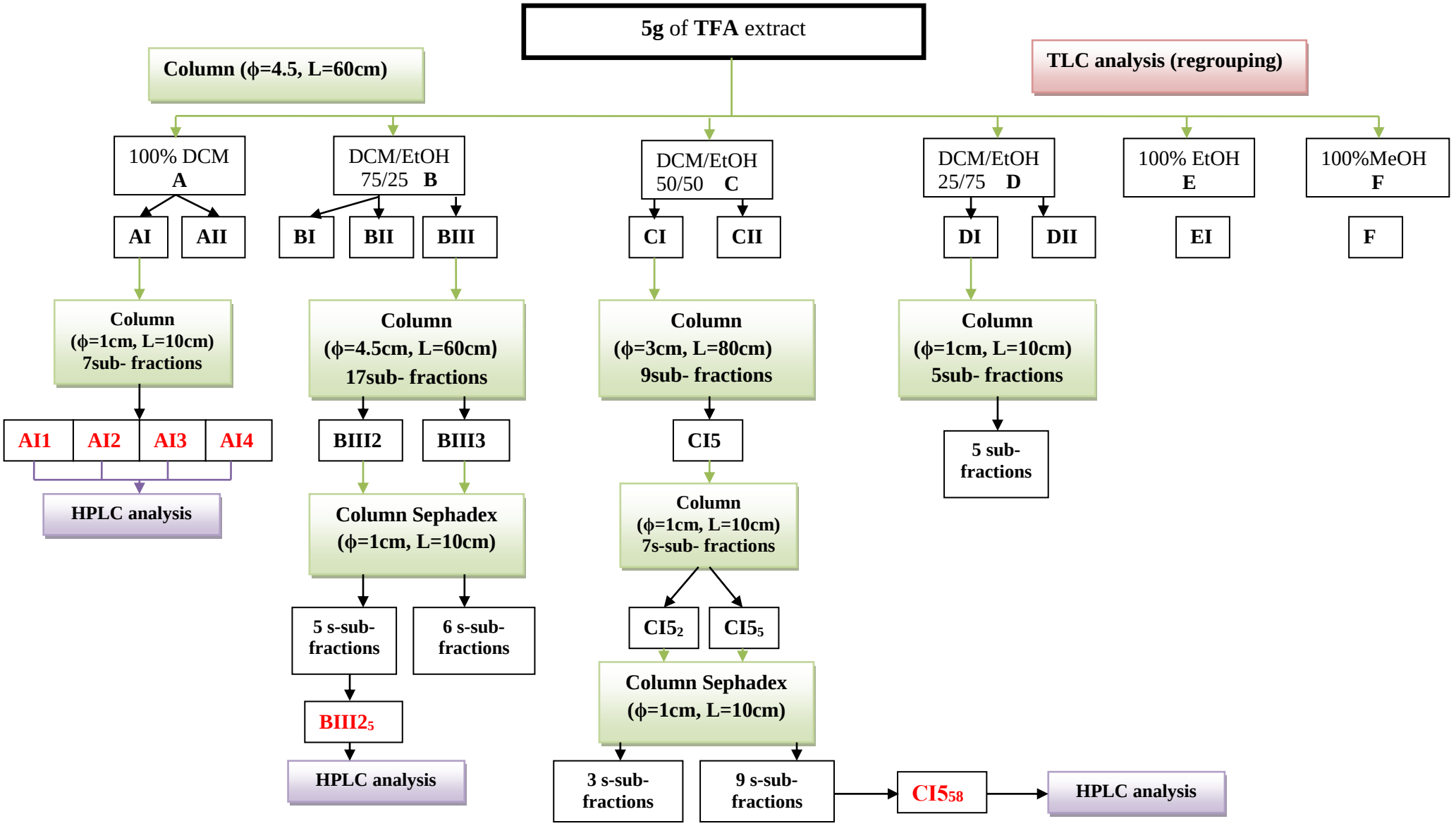


Figure IV.4. Summary organigram of TFA purification steps

IV.2.9. Extraction of Volatile Alkaloids from *C. procera* hydrolats (VAH)

The hydrolats (aqueous phase) obtained after extraction of volatile component were re-extracted using alkaloids extraction in acid and alkaline medium method. Actually, 500 ml Sulfuric acid (H_2SO_4 0.5N) were mixed with the hydrolats to ensure the transformation of the alkaloids in their salt form; next, 1 L of NH_4OH 0.5 N solution was added to reach pH= 9. Afterwards, a sequence of liquid-liquid extraction was carried out using 700 ml Dichloromethane (DMC). Later, the organic fractions were dehydrated with Na_2SO_4 (1g), filtered, dry concentrated and then stored at -18 °C. **Figure IV.5** represents the flowchart of the different steps of **VAH** extraction in acid and alkaline medium.

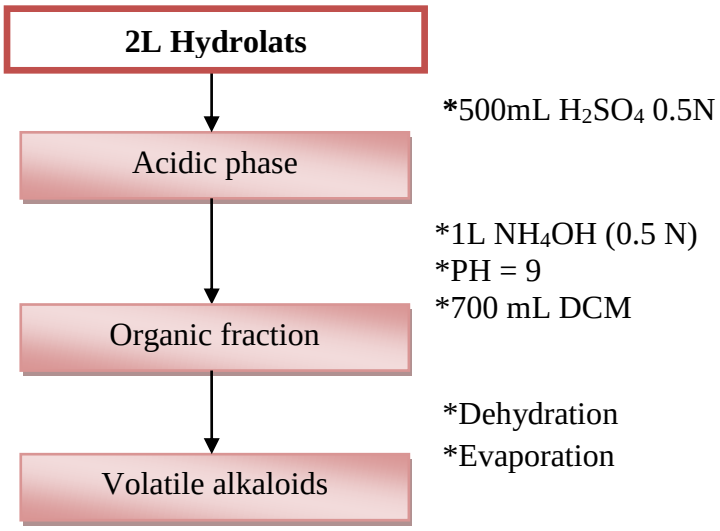


Figure IV.5. Flowchart of VAH extraction

IV.2.9.1. Thin-layer chromatography analysis of VAH

TLC analysis was carried out in the normal phase on silica gel aluminum plates Merck (60 F254, 0.25mm), with numerous solvent systems to select the best possible separation. Examples of these ones are named as follow:

- Dichloromethane/Ethanol (5: 5 v/v)
- n-Hexane/ Ethanol (9 :1 v/v);
- n-Hexane/ DCM (9 :1 v/v); n-Hexane/ DCM (5 :5 v/v)
- Methanol/ Dichloromethane (5:5 v/v)
- Dichloromethane/ Ethyl acetate (8:2 v/v)
- Petroleum ether/ Ethyl acetate (8:2 v/v)
- Methanol/ Dichloromethane/ Ammonium hydroxide (2:7:1 v/v/v)
- Ethanol/ Dichloromethane/ Ammonium hydroxide (2 :7:1 v/v/v)

The observation of the TLC plates with n-Hex/DCM **(**5:5v/v) were carried out directly (visible) and under UV light (254 and 356 nm), before and after revelation with **Dragendroff** reagent.

Figure IV.6.TLC plate of VAH fractions at 366 nm.

IV.2.9.2. Chromatographic fractionation of VAH by column chromatography (CC)

VAH extract (**0.1g)** was placed on the top of a Pastor pipet filled with 1g silica gel and coarsely fractionated with 100% n-Hexane to n-Hex/DCM 50/50 until 100% DCM. At the end, two fractions were collected, **VAH1** with a yellow color and **VAH2** with a reddish-brown color **(Annex 1)**.

IV.2.9.3. Gas Chromatography-Mass Spectrometry analysis of VAH1 and VAH2

To the best of our knowledge, the first successful report on the estimation of alkaloids by Gas Chromatography was established by **Brochmann-Hanssen** and **Svendsen (1962)** [211].

In this study, **VAH1** and **VAH2** were subjected to analysis using Shimadzu GC-MS (TQ 8040 NX) model mass spectrometer equipped with auto-injector; (30 m x 0.25 mm ID x 0.25 μm) column; column Flow :1.00 ml/min. The chromatographic conditions were the following, the sample was injected with split ratio at 40.0; Helium was used as carrier gas at 60 kPa inlet pressure. Temperatures (T°)of the different parts were set as follow: Oven T° was from 70 °C for 1 min, 290 °C for 20 min, the injector T° at 250 °C; ion source T° at 200°C; interface T° at 290 °C. Moreover, solvent cut time 2min with relative detector gain mode 0.84 kV; scan MS ACQ mode; mass range of m/z 35-650.

IV.2.9.4. Identification of components

The identification of **VAH1** and **VAH2** volatile components obtained by GC-MS were based on one hand, on the comparison of their retention indices relative to the series of n-hydrocarbons calculated according to Van Den Dool equation with those of standards; on another hand, between comparing of the compounds mass spectra with their standards homologues in NIST 17 and W11N17MAIN1 library.

IV.3. Results and Discussion

IV.3.1. Determination of extraction yield

The calculated yields were somehow different; it is probably due to the type of extraction, pH and the nature of the solvent used. In fact, the mass of the extract obtained by the methanol acid medium extraction was 17.2084 g representing a yield equal to 1.72%, while the mass of the extract obtained by the extraction in alkaline medium was 1.3148 g with an extraction yield of 0.13%, the results are represented in **Figure IV.7**.

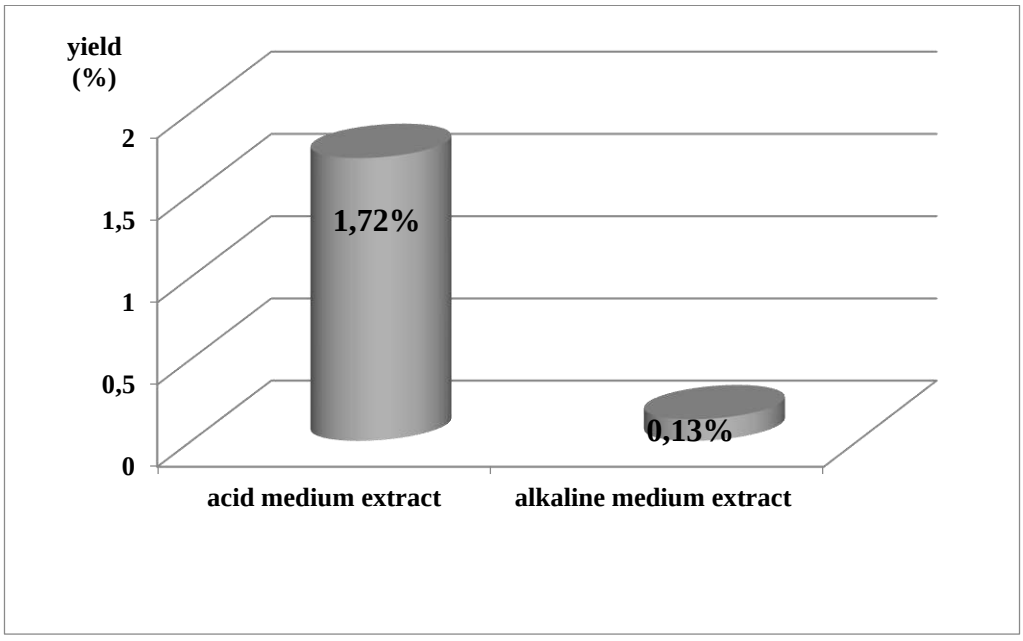


Figure IV.7. Graphical representation of the extraction yields.

According to the recorded results, it should be noted that the differences observed between the alkaloid contents obtained by applying two extraction methods could be explained by the nature and thus the solubility of alkaloids in solvents. In addition, the yield of the natural products extraction seems to be linked to the geographical origin, the conditions and the duration of storage of the plant material and the prolonging time of extraction [193].

IV.3.2. TLC Analysis results

The **TFA** extract obtained from methanol in the acid medium is presented as a honey powder, soluble in methanol. First analysis by TLC showed it rich in alkaloids; this extract reacted with **Dragendroff's** reagent, displaying an orange fluorescence under UV at 365 nm suggesting an alkaloid type structure.

In line with these results, **Garba** and **Okeniyi (2012)** study have demonstrated that *C. procera* contain high concentration of total alkaloids estimated at 0.7 g [212].

IV.3.3. Analytical HPLC profiles of AI sub-fractions

HPLC qualitative analysis of **AI** sub-fractions provided the following chromatograms:

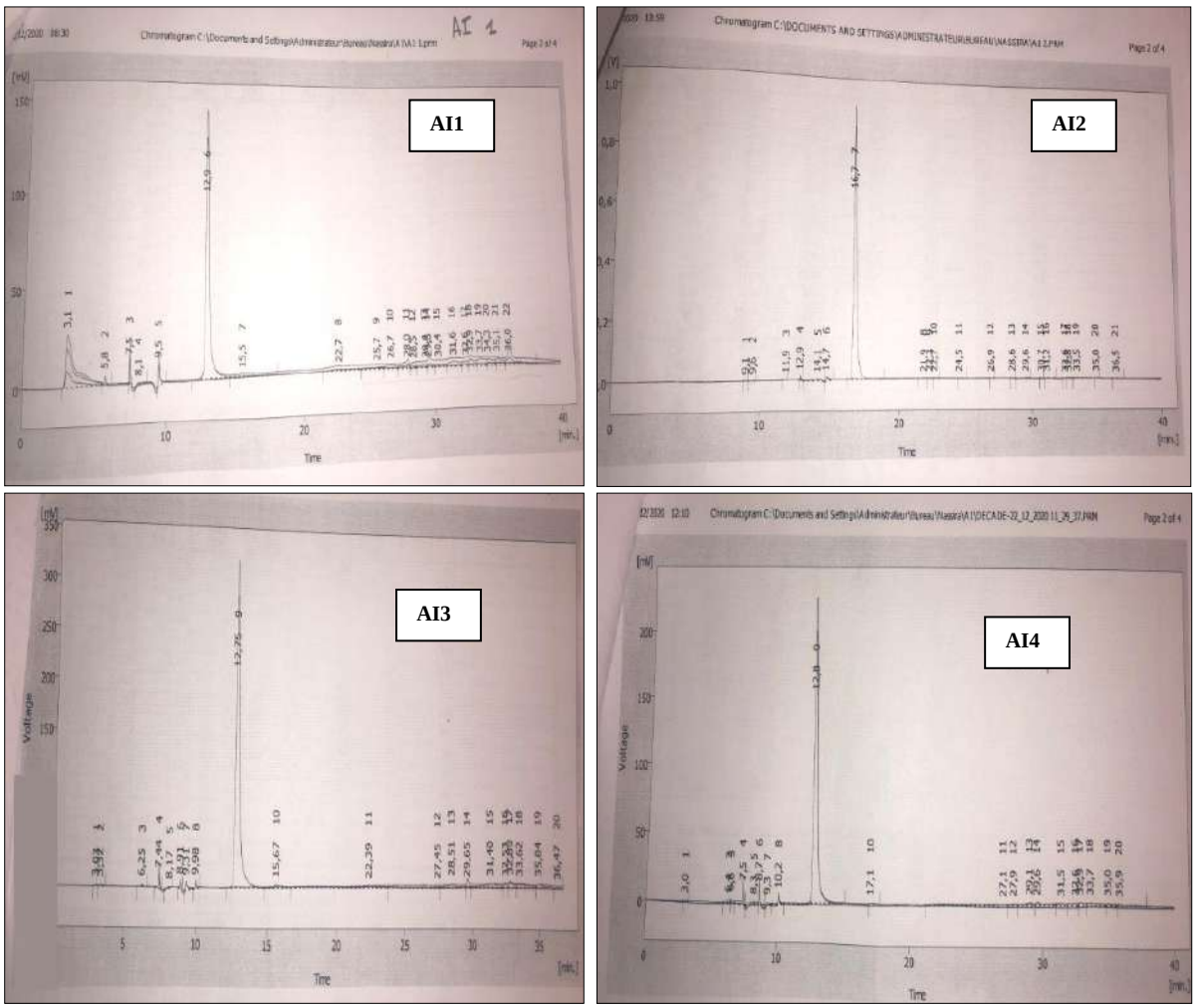


Figure IV.8. HPLC chromatograms of **AI1, AI2, AI3, AI4** sub- fractions (at λ=254 nm).

According to **AI1, AI2, AI3, AI4** chromatograms, one major peak was observed in every sub-fraction, where in **AI2** and **AI4** sub-fractions are considered as the most purified fractions.

IV.3.4. Identification and quantification of $BIII2_5$, $CI5_{58}$ fractions results

Concerning **$BIII2_5$**, **$CI5_{58}$** fractions, their qualitative analysis results furnished with the chromatograms in **Figure IV.9** and **Figure IV.10** respectively.

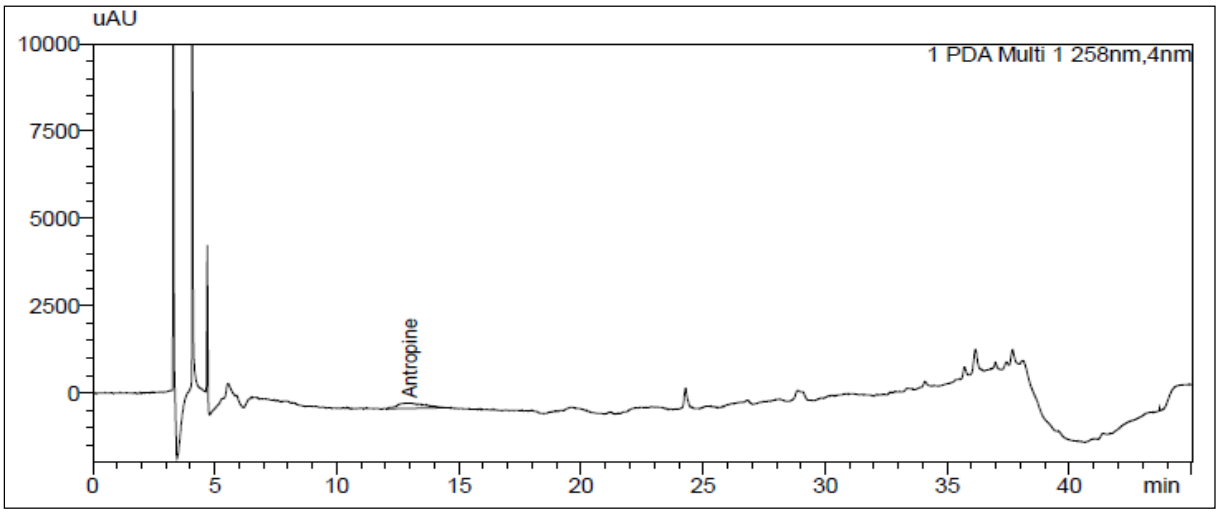


Figure IV.9. HPLC chromatogram of **$BIII2_5$ sub-fraction** at λ=258 nm.

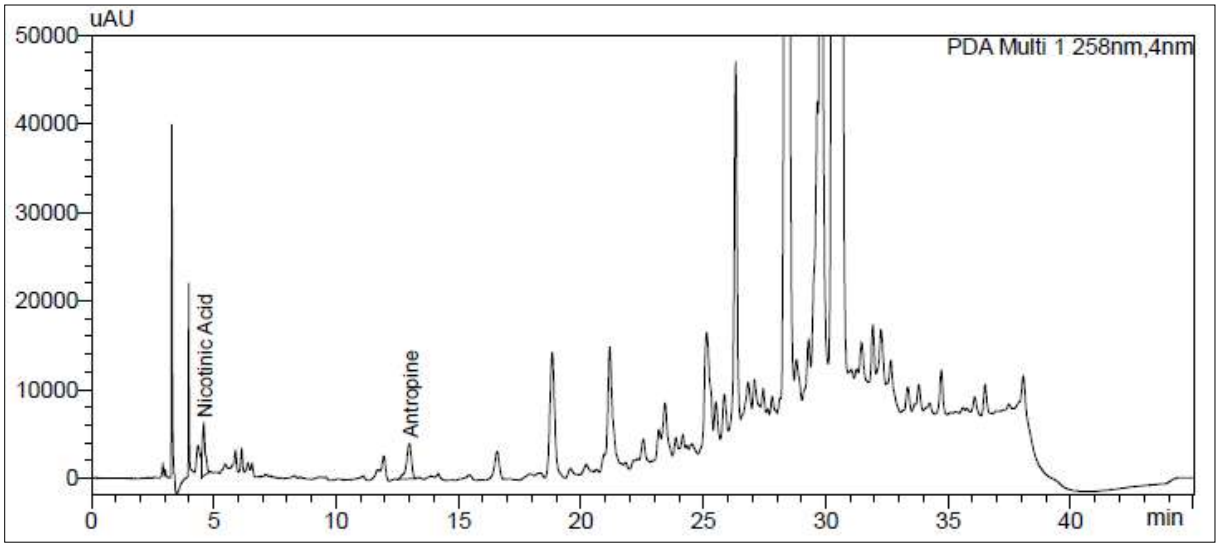


Figure IV.10. HPLC chromatogram of **$CI5_{58}$ sub-fraction** at λ=258 nm.

Rendering to the chromatograms of **$BIII2_5$**, **$CI5_{58}$** sub-fractions after several purification steps; we note that **$BIII2_5$**, **$CI5_{58}$** sub-fractions were a mixture of compounds contains very large peaks and very small ones considered as traces of compounds among them the presence of Atropine in **$BIII2_5$** (Rt =12.8 min) and in **$CI5_{58}$** (Rt =12.99 min) at 258 nm. The alkaloid content was found to be as Atropine with 3.67 mg (7.34%) in **$BIII2_5$** and 1.58 mg (6.99%) in **$CI5_{58}$** sub-fractions.

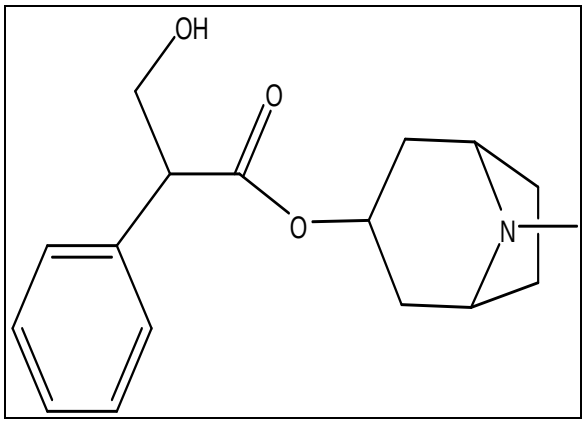


Figure IV.11. Structures of Atropine identified from **BIII2$_5$, CI5$_{58}$ sub-**fractions.

As it is previously mentioned, it is known that the most poisonous parts of *C. procera* are leaves, stem, latex is also characterized by neurotoxic and anticholinergic responses that lead to toxicity or death [213]. Probably, the presence of alkaloids in these parts especially in latex, such as atropine, gives the plant its toxic character. These findings are in accordance with **Mishra (2018)** who reported that the tropane alkaloids like atropine are considered as anticholinergics [214].

IV.3.5.Characterization VAH of *C. procera* by GC/MS

As mentioned previously, **VAH1** was colored yellow and a reddish-brown was the color of **VAH2.** Both sub-fractions exposed a strong fragrance, and were analyzed for their volatile constituents by means of Gas Chromatography coupled with mass spectrometry GC/MS.

The characterization results of the volatile component of **VAH1** and **VAH2** by GC-MS are represented by the chromatograms in **Figure IV.12.A** and **Figure IV.12.B** respectively.

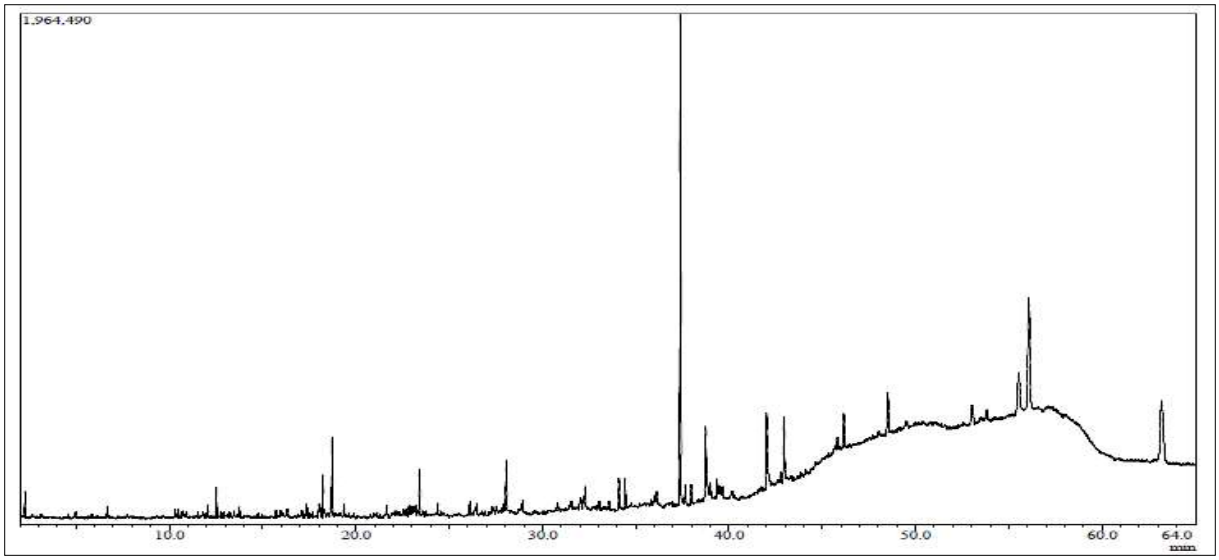


Figure IV.12.A. Chromatogram obtained by GC-MS for **VAH1**.

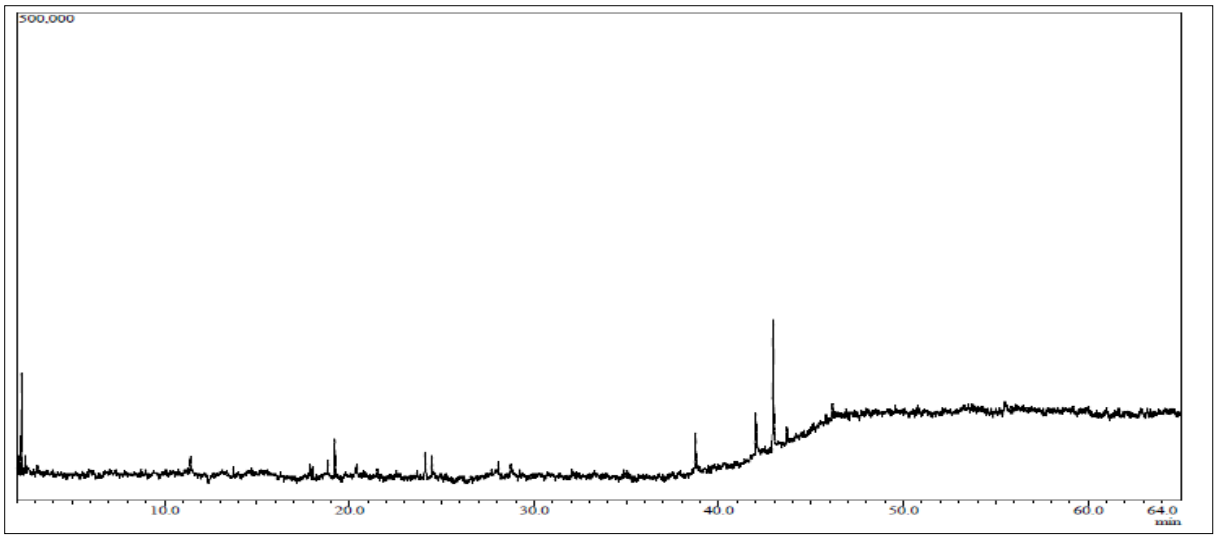


Figure IV.12.B. Chromatogram obtained by GC-MS for **VAH2**.

IV.3.6. GC-MS analysis results of VAH1

In general, and without taking in account the unidentified **17** compounds **(24.8%)**, the analysis allowed the identification of **32** compounds from **VAH1** sub-fraction corresponding to **69.63 %** of the total area. They were characterized by the comparison of their retention indices and their mass spectra with those of standards and with reported data. The chemical compounds and their percentages are transcribed in **Table IV.9**.

Table IV.9. Chemical composition of **VAH1** provided by GC-MS.

RT	Identified compound	(%)
2.25	Toluene	0.57
12.06	Tetradecane, 5-methyl-	0.43
12.51	Dodecane, 4,6-dimethyl-	0.84
17.34	2,6,10-Trimethyltridecane	0.47
18.65	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.40
18.72	2,6-Di-tert-butyl-4-methyl-phenol	2.43
21.65	Heneicosane	0.49
23.00	Methanone, (1-hydroxycyclohexyl)phenyl-	0.68
23.19	Decane, 1-iodo-	0.43
23.83	Eicosane	3.34
26.10	1-Dodecanamine, N,N-diethyl-Dodecyldiethylamine	0.53
26.46	Dotriacontane	1.15
27.39	Tetracosane	1.28
28.05	5,5-Diethylpentadecane	3.75
32.06	dl-Chimyl alcohol	0.70
33.57	1-Docosanol, acetate	0.44
34.10	Hexadecanoic acid, 2-hydroxyethyl ester	1.19
34.42	N,N-dimethylhexadecanamide	2.12
35.18	2-Methylhexacosane	0.78
35.85	2-Methylhentriacontane	0.47
36.00	Nonacosane	0.58
36.93	Triacontane, 1-iodo-	0.36
37.39	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	18.17
37.65	Octadecanoic acid, 2-hydroxyethyl ester	0.87

38.75	Palmitin	4.97
39.42	1,2-Benzenedicarboxylic acid, 3-nitro-	0.48
39.58	Tetrapentacontane	2.34
42.00	Stearin	5.06
42.76	Hexacontane	0.64
42.95	13-Docosenamide, (Z)-	3.27
53.03	γ -Sitosterol	1.66
56.07	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	9.13

The most abundant compound in **VAH1** is Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- with **18.17%**; followed by Phenol, 2,4-bis(1,1-dimethylethyl)-,phosphite (3:1) **9.13%** ; Stearin **5.06%**; Palmitin **4.97%**. **Figure IV.13** provides information on the chemical families present in the VAH1 of *C. procera* classified into six chemical classes.

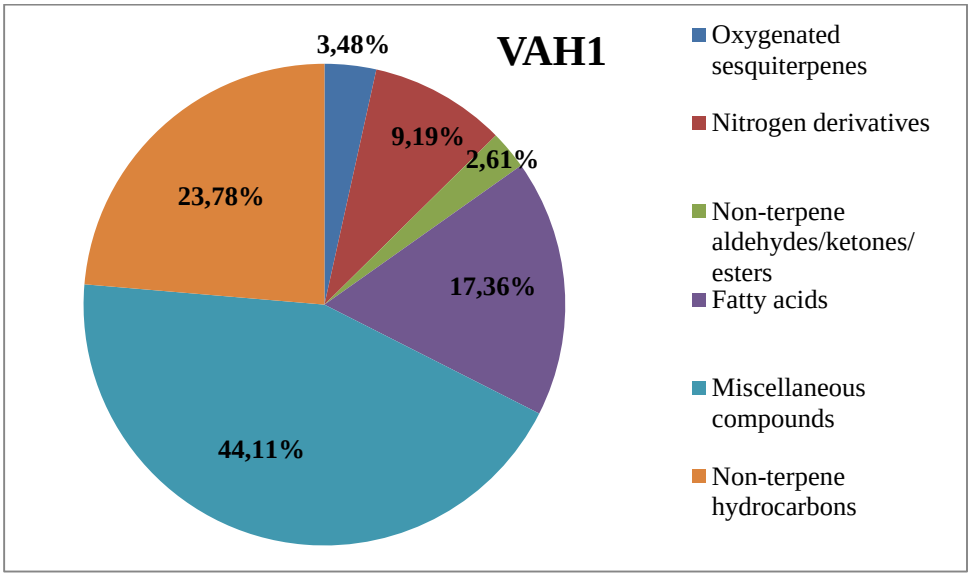
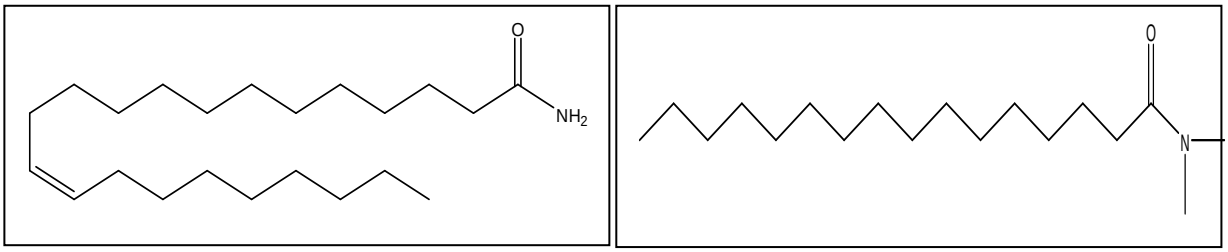


Figure IV.13. The chemical families present in **VAH1** sub- fraction.

VAH1 sub-fraction showed a predominance of miscellaneous compounds with **44.11%**, followed by **23.78%** hydrocarbons, **17.36%** fatty acids. Besides, nitrogen derivative compounds represented **9.19%** of the sample. Oxygenated sesquiterpenes recorded their presence with **3.48%** and aldehydes/ketones/ esters with **2.61%**.

IV.3.7. Volatile alkaloids and nitrogen derivative of VAH1

The nitrogen derivative of **VAH1** estimated with **9.19%** of total identified compounds, where the most abundant compounds consist of **3.27 %** 13-Docosenamide, (Z)- ; **2.12%** N,N-dimethylhexadecanamide, however, the residual percentage represents less proportions as for 1-Dodecanamine,N,N-diethyl-Dodecyldiethylamine (**0.53%)** and 3-nitro-1,2-Benzenedicarboxylic acid (**0.48%)**, while volatile alkaloids were totally absent in the sample .



13-Docosenamide, (Z)- **N,N-dimethylhexadecanamide**

Figure IV.14. Structures of the main nitrogen derivative compounds in **VAH1.**

IV.3.8. GC-MS analysis results of VAH2

12 compounds were identified from **VAH2** sub-fraction corresponding to **76.58%** of the total area, without taking in account the six unidentified compounds **(23.42%).**

The chemical compounds and their percentages results obtained from the **VAH2** chromatogram are recorded in **Table IV.10**.

Table IV.10. Chemical composition of **VAH2** provided by GC-MS.

RT	Identified compound	(%)
2.25	Toluene	5.76
11.40	Isoquinoline	3.72
17.90	2-Hexanoylfuran	3.44
18.85	Methanone, dicyclohexyl-	3.43
19.20	Dihydroactinolide	6.37
24.10	Imidazole, 5-phenyl-1,3,4-trimethyl-	4.83
24.47	Myristic acid	3.97
28.06	Methyl melissate	4.13

38.74	2-Palmitoylglycerol	7.63
41.99	Glyceryl Monostearin	9.97
42.94	13-Docosenamide, (Z)-	19.78
43.69	Squalene	3.55

According to GC-MS results, the highest amount constituting the sub- fraction **VAH2** is characterized as 13-Docosenamide, (Z) - with **19.78 %**; the other main constituents are; Glyceryl Monostearin **(9.97%),** followed by 2-Palmitoylglycerol (**7.63%)** and Dihydroactinolide (**6.37%**). **Figure IV.15** provides information on the chemical families present in the **VAH2** classified into six chemical classes.

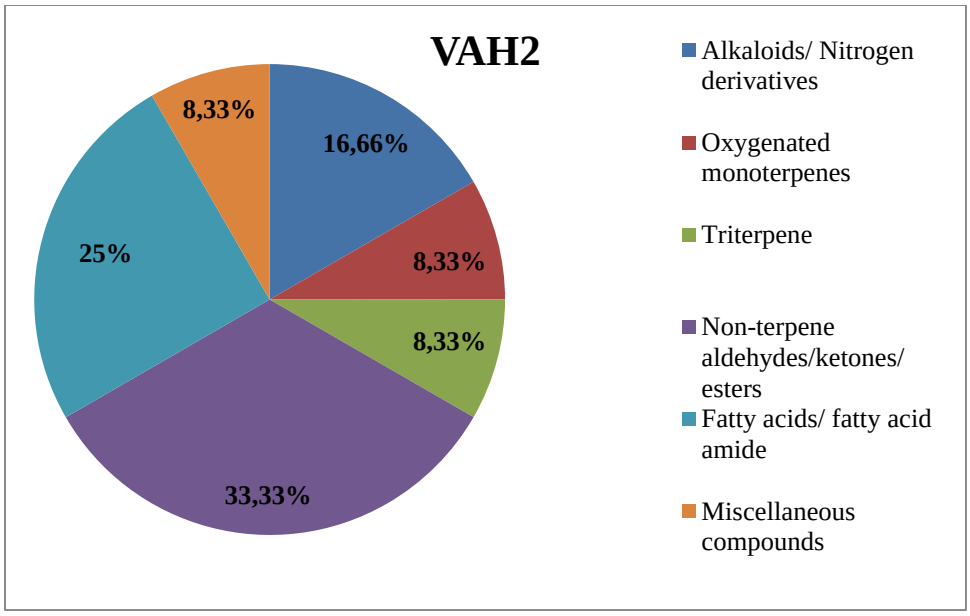
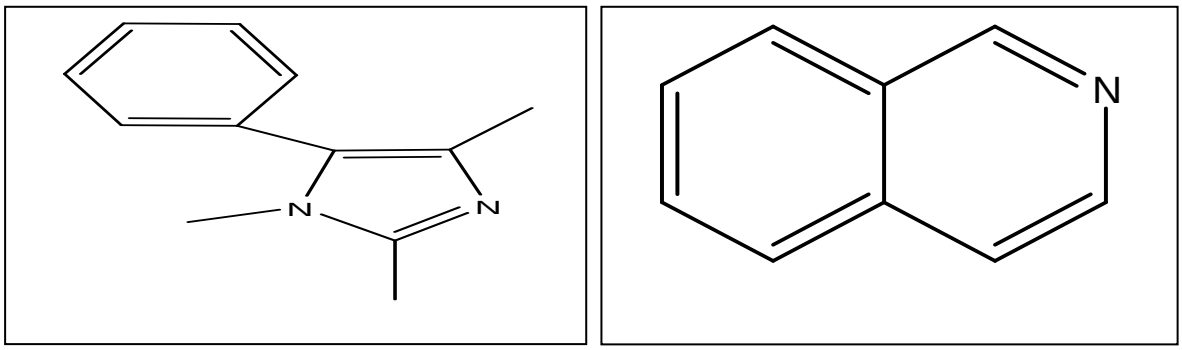


Figure IV.15. The chemical families present in **VAH2** sub- fraction.

Indeed, **VAH2** sub-fraction predominant of **33.33%** aldehydes/ketones/esters compounds, other abundantly occurring compounds included **25%** fatty acids/ fatty acid amide, alkaloids and nitrogen derivatives compounds with **16.66%**, oxygenated monoterpenes, triterpene and miscellaneous compounds in the same proportions, estimated at **8.33%**.

IV.3.9.Volatile alkaloids and nitrogen derivatives of VAH2

Volatile alkaloids and nitrogen derivative obtained from **VAH2** estimated at **9.19%** of the total identified compounds, where 13-Docosenamide,(Z)- was found to be the major nitrogen derivative compound (**19.78%)** followed by Imidazole, 5-phenyl-1,3,4-trimethyl (**4.83%**) and Isoquinoline **(3.72%**) as volatile alkaloid compounds.



Imidazole, 5-phenyl-1,3,4-trimethyl- **Isoquinoline**

Figure IV.16. Structures of the main volatile alkaloid compounds in **VAH2**

Recently, the significant role of plant hydrolats has also received growing attention; due to its widespread use in several fields, especially in aromatherapy, food industry and cosmetic production [215].

According to our results, *C. procera* hydrolats are revealed potentially rich in alkaloids and nitrogen derivatives. Previous studies [216,217,218] have confirmed that the N, N-dimethylamides have been patented as insect repellents and they have proved effectiveness as insecticides and antimicrobial agent against Staphylococcus aureus. Beside, **Kim et al (2018)** suggest that 13-Docosenamide and (Z)-Erucamide may have preventive effects against memory deficits related to Alzheimer's disease by modulation of cholinergic functions [219]. Furthermore, Imidazoles and isoquinolines alkaloids may have the potential to be used as an effective antioxidant, antimutagenic, anticarcinogenic and antimicrobial agents [220].These findings support the use of this plant in traditional medicine and confirm the results reached by **Russell et al (2011)**, who confirmed that **i**n addition to the toxins [221], *C. procera* has a different defense mechanism such as the use of irritating volatiles, especially to repel desert grazing animals.

IV.4.Conclusion

Overall, important information has been provided, by the present study, about the fixed and volatile alkaloids of *C. procera* (Ait) aerial parts. Additionally, these natural

products constitute a potential alternative source to be used as raw materials in many different products purpose especially the pharmaceutical field.

Chapter V:

Biological study:

Bio-insecticidal activity

V.1. Introduction

Recently, plant extracts represent a bio-alternative of insecticides to protect crops, because they are safe. In addition to their role in controlling pests and diseases, they produce less toxic residues compared to chemical products, and are less dangerous to human health. The present chapter aims to evaluate the toxicity effect of the chemical composition of *C. procera* (Etp, DCM, EtOH) extracts against *Parlatoria blanchardi* Targioni-Tozzetti1892 (Hemiptera: Diaspididae) and *Tribolium castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae), also to evaluate its Latex effeteness comparing to the commercial insecticide (TCHOKE), the aim was also a comparative study between the Rutin and Gallic acid effeteness towards *P. blanchardi.*

V.2. Material and Methods

V.2.1. Plant material

The vegetable samples of *C. procera* leaves, stems and latex were collected from Djanet at Tasili N'Ajjer (Southeastern Algerian Sahara) on October 2019. The Latex was conserved at -18°C until use.

V.2.2. Insects

Two species of insects; the first one consists of white Scale *P. blanchardi* individuals taken from the middle leaves of the date palm, locally called Deglet Nour, cultivated in one of Metlili Oasis in Ghardaia province, Algeria.

The second one includes the white flour beetle colonies of *T. castaneum*. Initial stock culture was obtained from Agricultural Cooperative for Seeds and Derivatives, at Metlili. *P. blanchardi* samples were identified by Dr. Hayet Saggou; while, *T. castaneum* individuals were determined by Dr. Abdellah Kemassi; both are from the department of agronomic science at the University of Ouargla.

V.2.3. Preparation of samples

Petroleum Ether (Etp), Dichloromethane (DCM) and Ethanol (EtOH) extracts, where the extraction methods are previously described in Chapter I.B, were comparatively tested for their insecticidal and repellency effects. Whereas the crude Latex, commercial insecticide (Tchoke), isolated Rutin and Gallic acid were comparatively tested for their insecticidal effects.

V.2.4. Repellent activity

V.2.4.1. Insect preparation

T.castaneum beetles were reared in glass containers (0.5 liters) with a mixture of wheat flour, and yeast extract (9:1 w/w) as food; they were covered with a perforated lid for ventilation. The cultures were maintained in the laboratory at 29- 32°C and 70–80% RH. Subsequently, adult insects (7-10 days old) were collected for the bio-assay [222].

V.2.4.2. Doses and treatments

In order to assess the repellent activity of *C. procera* extracts against *T. castaneum* adults, each tested sample (EtOH, DCM, Etp extracts) was diluted in acetone into four concentrations (5, 3, 1.5 and 0.6 mg/ml) and absolute acetone was used as a control [223,224,225].

The applied repellency test was adopted from the area preference method of **McDonald et al (1970)**, with slight modifications. Filter paper discs (Whatman N°3 diameter 9 cm) were used by cutting each into two halves; one half was treated uniformly with 500 µl of one of the extracts concentrations, the second one was treated only with Acetone as a control [226].

To evaporate the solvent completely, treated and control halves were left to air-drying. Afterwards, each treated half disc was carefully fixed together and placed in Petri dishes by attaching the treated half to the control half with tape; for each replicate, twenty *T. castaneum* adults were introduced in the middle of each Petri dish, and each concentration was replicated three times: after 1h, 3h and 5 h counting from the beginning of the test. The repellency percentage (RP) of each extract was calculated using the following formula:

RP (%) = (Nc-Nt)/(Nc+Nt)×100 where:

Nc: Number of insects on control half.

Nt: Number of insects on treated half.

V.2.5. Insecticidal activity

V.2.5.1. Insect preparation

Sufficient pieces (9 pieces in each dose) of palm leaflets equal to 2 cm from highly infested and non-infested leaves by *P.blanchardi* were prepared for the treatment; each piece was placed under the binocular magnifier to count (from 11 to 44 individuals) the number of living individuals (male, female, 1st and 2nd fixed larva). After that, 3 pieces of leaflets were putted in glass Petri dishes that were containing soaked cotton with distilled water to keep them moist **(Annex 10)**.

V.2.5.2. Doses and treatments

DCM and Etp extracts were solubilized in distilled water with drops of tween; while, EtOH extract was dissolved only in distilled water. EtOH, DCM, Etp extracts were applied with the 10 following concentrations (0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 4 and 5 mg/ml);

Different aqueous concentrations were prepared, first ones for crude latex and Organophosphoric insecticide (TCHOKE as a commercial name) as follow: 15, 11.25, 7.5, 3.75, 0.15, 0.075, 0.03, 0.01, and 0.009 µl/ml; the others for the isolated Rutin and Gallic acid to obtain these five doses from each compound: 0.5, 0.4, 0.3, 0.2, 0.1 mg/ml. Besides, the distilled water was used as a negative control.

Effectively, each sample was uniformly sprayed (1 ml/dish) on the entire surface of the leaflets and each treatment was replicated 3 times. As for the incubation, it was at 30 °C and lasted 24 hours.

V.2.6. Statistical analysis

Probit analysis was conducted to estimate lethal dose (LD_{50}) where the transformation percentages of the mortalities corrected in Probit and doses transformation in decimal logarithm were used [227]. The transformations allow, through the Excel software, establishing the regression lines Y = ax + b, (where Y represents the corrected mortality Probit) and regression coefficients (r2).

Consequently, the result is valid if control mortality is less than 5%; however, if the mortality after exposure is between 5% and 20%, it should be corrected using Abbott's formula. If the control mortality is >20% the bioassay is too redone [228,229].

The results were expressed by means ± standard deviation (SD), LD_{50} were also calculated.

V.3. Results and Discussion

V.3.1. Repellent activity

The treatment of *T. castaneum* adults by different concentrations of EtOH, DCM, Etp extracts provided variable repellencies that increased with their concentrations recorded through diverse periods 1h, 3h, 5h as shown in **Table V.1** and **Figure V.1**.

Table V.1. Repellency of *C. procera* extracts against *T. castaneum* adults over time.

Extract	Dose mg/cm²	1h	3h	5h	Total Repellency%
EtOH extract	5	76,66 ±5.77	90±10	96.66±5.77	87.77±10.18
	3	63.33±23.09	70±10	70±10	67.77± 7.55
	1.5	26.66±5.77	56.66±5.77	60±26.45	47.77±11.94
	0.6	13.33±15.27	16.66±11.54	23.33±11.54	17.77±2.15
DCM extract	5	60±00	66.66±5.77	70±10	65.55± 5.01
	3	30±10	33.33±5.77	36.66±5.77	33.33±2.44
	1.5	6.66±5.77	13.33±15.27	23.33±25.16	14.44±9.69
	0.6	00±00	3.33±5.77	3.33±5.77	2.22±3.33
Etp extract	5	46.66±15.27	50±20	56.66±5.77	51.11±7.24
	3	20±26.45	26.66±25.16	40±30	28.88±2.50
	1.5	6.66±5.77	9.66±0.57	16.66±28.86	11±15.05
	0.6	-6.66±5.77	-3.33±5.77	00±00	-3.33±3.33

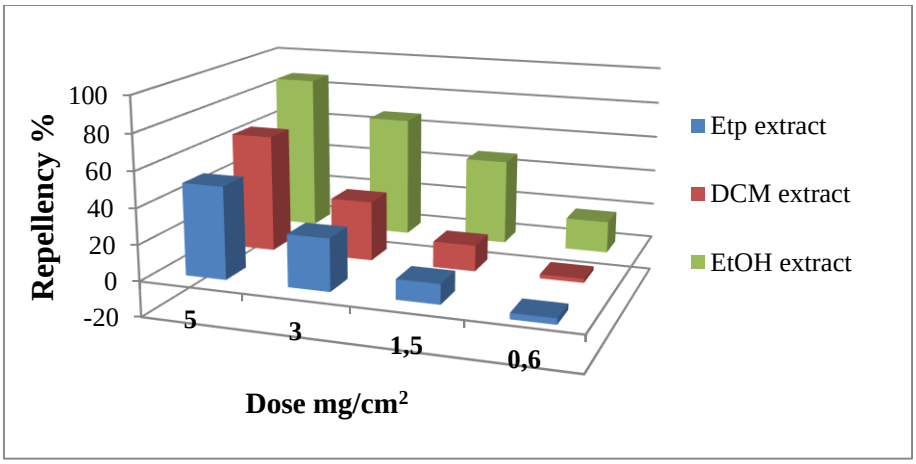


Figure V.1. Total repellency of *C. procera* extracts against *T. castaneum* adults

The highest repellent activities were recorded at 5mg/cm² for all extracts, wherein EtOH sample showed the great efficient on *T. castaneum* adults; effectively, after the first hour its repellency was estimated to 76,66 ±5.77, but after 5 h of exposure it reached 96,66 ± 5.77%. While, DCM and Etp extracts, at the same interval of time, recorded 70.00±10% and 56.66±5.77%, respectively.

In agreement with **Habib** and **Karim (2016)** and **Acheuk (2020**), the ethyl acetate extract of the same genus (*C. gigantean*) showed effectiveness on *T. castaneum* increased respectively with exposure time [222,230]. Distinctly, Etp extract was the opposite on repelling *T. castaneum* adults compared to the two first extracts; In fact, at 0.6 mg/cm² and after the first hour the repellency was evaluated to -6.66 ±5.77%; it was considered as the lowest repellency, afterward the insect repellent stopped completely over time.

This result demonstrated that the Etp extract showed a repellent toxicity against *T. castaneum* that decreased with time. Similarly, **Alam et al (2009)** reported that the Etp extract of *C. gigantea* behaved *T. castaneum* in reverse over time [232].

V.3.2. Insecticidal Study

V.3.2.1. Etp, DCM, EtOH extracts

Twenty four hours after spraying the Etp, DCM, EtOH extracts, an appreciable insecticidal potential was shown on larvae and adult *P.blancharidi*; contrarily, the leaflets treated by distilled water did not present any effects; this result was confirmed also in many previous studies [232,233,234] which reported the high anti-insect ability of the plant. The insecticidal activities of the tasted samples are presented in **Figure V.2**.

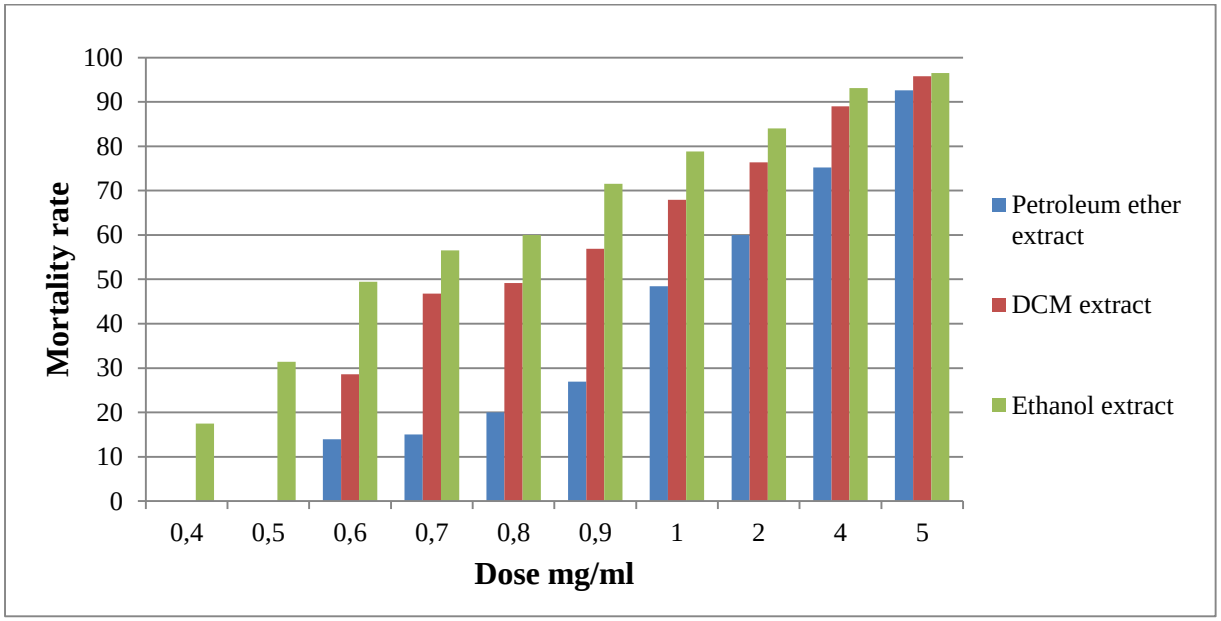


Figure V.2. Concentration-response relationships for the toxicity effect of *C. procera* extracts against *P.blanchardi*.

The results showed that the tested extracts had significant insecticidal efficiency against *P.blanchardi*; indeed, this Toxicity significantly increased with increasing of doses.

In fact, these results are in agreement with a study carried out by **KO et al (2009**) that reported an increased insecticidal efficiency on *S. zeamais* and *T. castaneum* with the enhancement of *L. cubeba* essential oil concentration [235].

Actually, the mortalities caused by the three extracts vary between 13.91% and 96.53%, where EtOH extract provided the highest mortality rate evaluated to 96.53 ± 0.01% at 5 mg/ml; while the lowest percentage (13.91 ± 0.01%) was presented by the Etp extract at 0.6mg/ml. The resulted findings consolidate those of **Ogbulie et al. (2007)** which concluded the efficiency of EtOH as a solvent to extract bioactive principles from a plant [236].

The transformation of mortality percentages in both Probit and decimal logarithm of all the doses, allowed establishing the regression lines and regression coefficient (r2) of the three extracts, this bioassay data was conducted to estimate (LD_{50}); **Figure V.3** represent the LD_{50} samples.

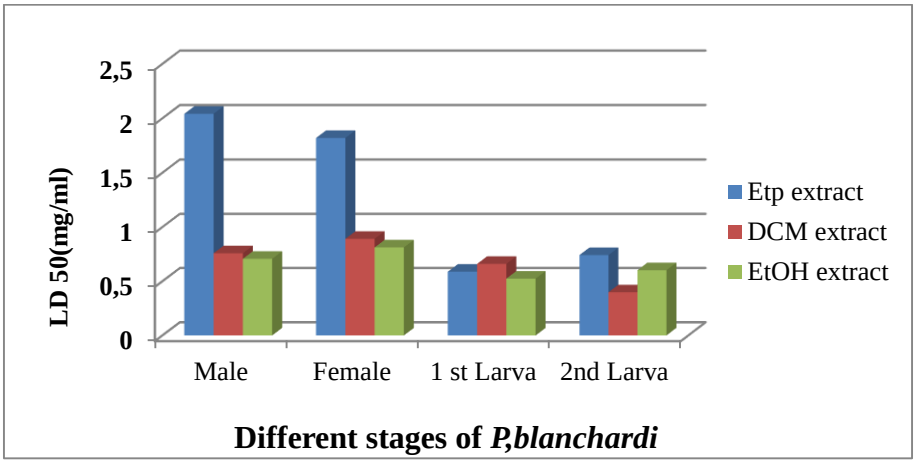


Figure V.3. LD_{50} of *C. procera* extracts against *P. blanchardi* at different stages.

Regarding *P. blanchardi* stages, each stage indicated an acceptable effect of EtOH extract on both male and female adults, 0.70 and 0.81 mg/ml respectively, but the 1st larva individuals were more susceptible than adults with LD_{50} equal to 0.52 ml/mg; while the 2nd larva of *P. blanchardi* were the most affected by DCM extract where the lethal dose value was estimated to 0.39 mg/ml.

On the other side, the present experiment shows that the adults individuals of *P. blanchadi* are the most resistant of all stages to all *C. procera* extracts, especially to Etp extract which shows the highest LD_{50} (2.04 for male and 1.81 for female) compared to the other extracts.

Over all, the larval stages were more sensitive to the treatment than adults. According to **Belkhiri (2010)**, the treatment with Spirotetramate product inhibited the key enzyme in fatty acid biosynthesis which blocked molting in larvae and stops their passage to the next

stage, because of the reduced number of eggs laid and the high mortality rate of larvae, there was a decrease in live adults [237].

Despite the short duration of treatment on adults and larvae *P. blanchardi*, it was deduced that these extracts are potentially toxic against *P. blanchardi* stages, whereas EtOH extract proved to be more harmful than the other extracts.

These finding suggest that the presence of different phytochemicals in EtOH, DCM and Etp extracts. According to **Khan et al (2019**), whole *C. procera* plant extracts caused mortality of larva, reduced the number of eggs, and inhibited the oviposition of Rhipicephalus microplus Canestrini (Ixodida: Ixodidae) [238]. It is similarly, **Moursy (1997)** have proved the insecticidal and larvicidal properties of *C. procera* plant [239].

The results of the two experiments indicated that EtOH extract was most potential and can be used as alternate potential to synthetic insecticides. Globally, the variable registered effect of the three extracts on *T.castaneum* adults and *P.blanchardi* adults and larvae, might be explained by the differences marked on the phytochemical composition of the tested samples (phytochemical investigation in previous **Chapter II**); where the weak toxicity and repellency of Etp extract could be related to the absence of flavonoids and phenolic compounds present in both DCM and EtOH extracts which exhibited high toxic and repellent properties.

Although, **Robertson et al (2017)** have another opinion; they noted that the relationship between concentration and insect repellent activity of extracts was probably due to the distribution heterogeneity of the compounds in each spray; consequently, they exhibit different effect [240]. However, other researches [241,242] concluded that plant extracts contain many secondary metabolites acting as repellents, feeding deterrents and toxins. In fact, they are included in the defense system of plants against herbivores, pests and pathogens.

V.3.2.2. Crude Latex and commercial insecticide

A comparison between the crude Latex and commercial insecticide (TCHOKE) is presented in **Figure V.4.**

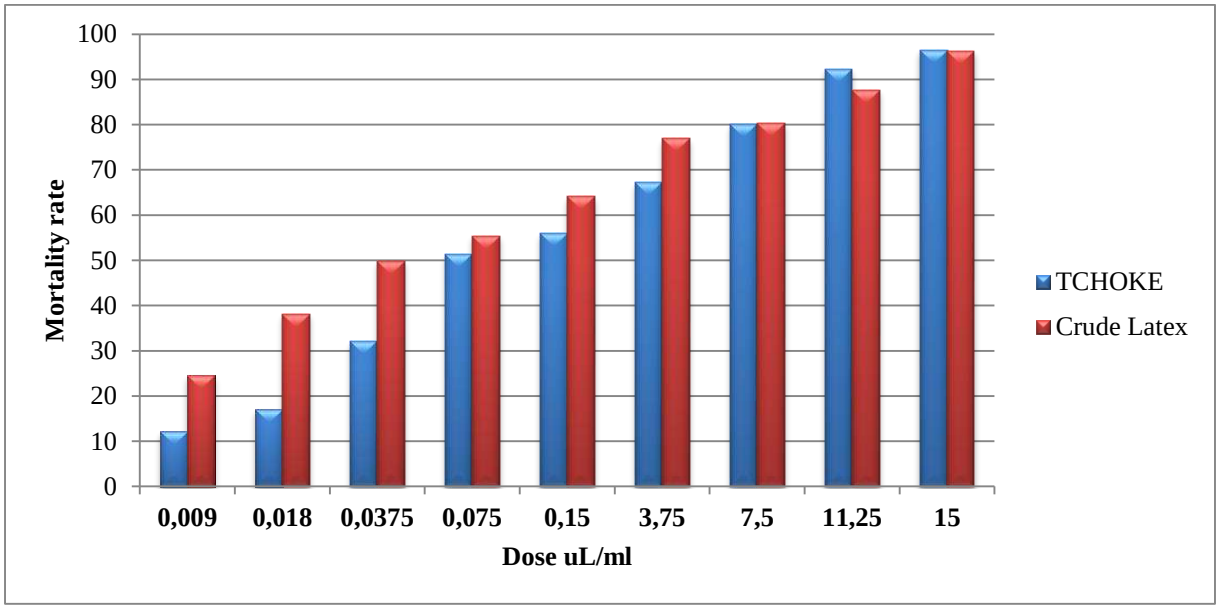


Figure V.4. Comparison between the toxicity effects of *C. procera* Latex and TCHOKE against *P. blanchardi.*

Through the insecticidal rats shown above on **Figure V.4**, it could be noted that the mortality rate of *P.blanchardi* started progressively from 0.009 µl/ml up to 15 µl /ml.

Firt of all, it is appearing that at 15µl/ml and at 7.5µl/ml the latex and the commercial insecticide showed very similar toxicity on the insects.

On another hand, at lower concentrations: 0.0375, 0.018 and 0.009 µl/ml, *C. procera* latex exhibited the highest rates (49.905±0%), (38.09±1.35%), (24.58 ±2.67%), respectively compared to the commercial insecticide (32.095±3.77%), (17.075±1.08%), (12.20± 0.48%).

These interesting results confirmed the efficiency of *C. procera* latex on *P. blanchardi* larvae and adults compared to the commercial insecticide (Tchoke).

Besides, **Singhi et al (2004)** results prove that different aqueous concentrations of this plant affected the gravid female Aedes aegypti mosquitoes and this behavior continued till three gonotrophic cycles [183]**.**

Furthermore, the Study realized carried out by **Markouk et al (2000)** showed that *C. procera* latex caused 50% mortality against *Anopheles labranchiae* Falleroni (1926) at 28 ppm [243].

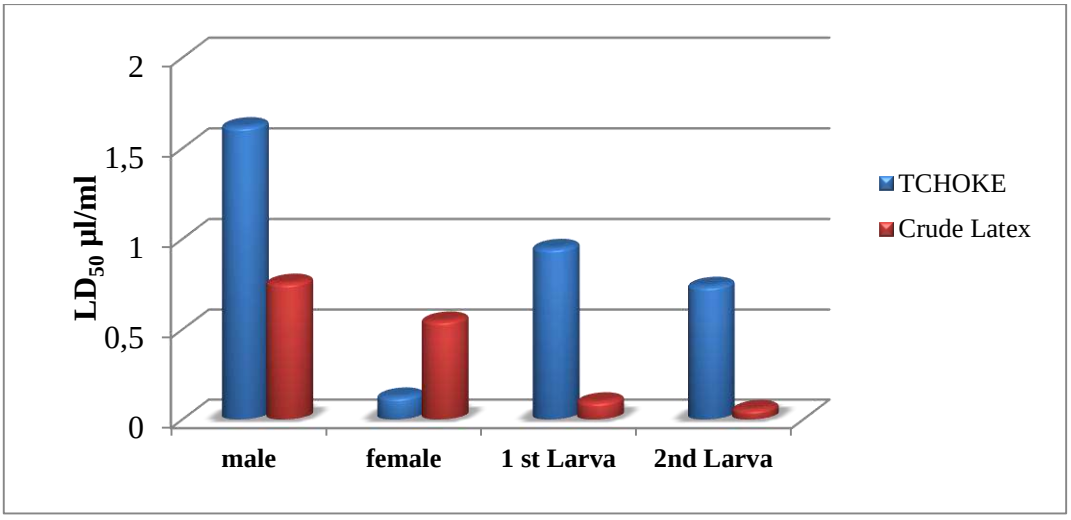


Figure V.5. LD_{50} of *C. procera* Latex and TCHOKE against *P. blanchardi* at different stages.

Referring to LD_{50} results, it is clear that the latex sample is potentially very toxic on all stages of *P. blanchardi*; particularly, on the 1st and 2nd larva; their corresponding LD_{50} were 0.1 and 0.057 µl / ml, respectively. Whereas, the LD_{50} values on male and female insects were 0.75 and 0.54 µl/ml respectively; besides, the commercial insecticide showed remarkable results but its action on females was the most important with LD_{50} equal to 0.12 µl/ml.

Similarly, **Shahi et al (2010**) found that latex is a strong killer against both *Culex quinquefasciatus* and *Anopheles stephensi* larvae compared to other *C. procera* extract [244]. Another study showed that the *C. procera* latex contained the larvicidal compounds which, after 5 min, caused 100% mortality in 3rd stage larvae of *Aedes aegypti* (Linn) [117].

The results showed that the latex possesses insecticidal and larvicidal activities on *P. blanchardi*, even more than commercial insecticide.

V.3.2.3. Isolated Rutin and Gallic acid

The insecticidal study has evaluated five treatments containing: 0.5, 0.4, 0.3, 0.2, 0.1mg/ml of isolated Rutin and Gallic acid, as **Figure V.6** present.

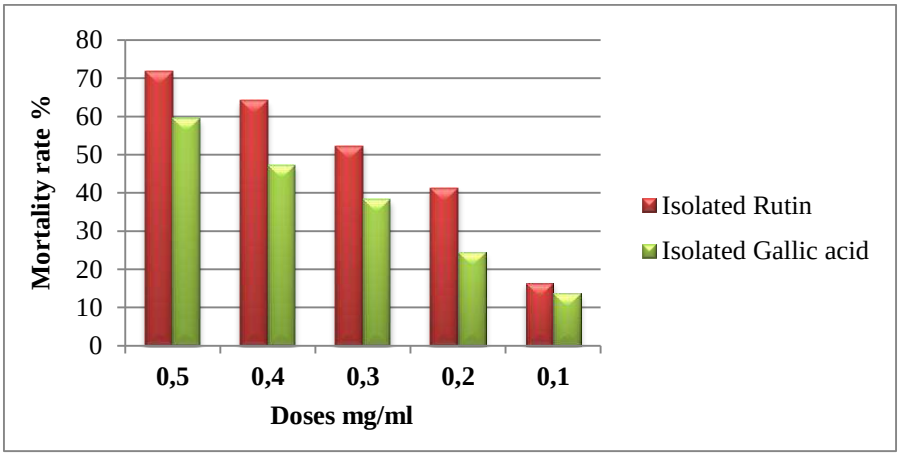


Figure V.6. The toxicity effect of isolated Rutin, Gallic acid against *P. blanchardi*.

Effectively, the mortalities vary between 71.73% and 13.59%; at 0.5 mg/ml isolated Rutin provided the highest mortality rate on *P. blanchardi* stages evaluated to 71.73± 3.13% while 59.44 ± 3.82% was recorded for Gallic acid at the same concentration; the lowest percentages (16.46 ± 1.38%) and (13.59 ± 0.89%) were presented by Rutin and Gallic acid respectively at 0.1 mg/ml. Probably, oxygen radicals formed from phenolic compounds oxidation were the cause of the compounds toxicity; in fact, these radicals disrupt the ability of membrane integrity and the metabolism in the gut epithelium, precipitating proteins from feeding deterrence, digestion inhibition, digestive enzymes [245]. In particular, even at low doses, flavonoids can be feeding deterrents for phytophagous insects [246].

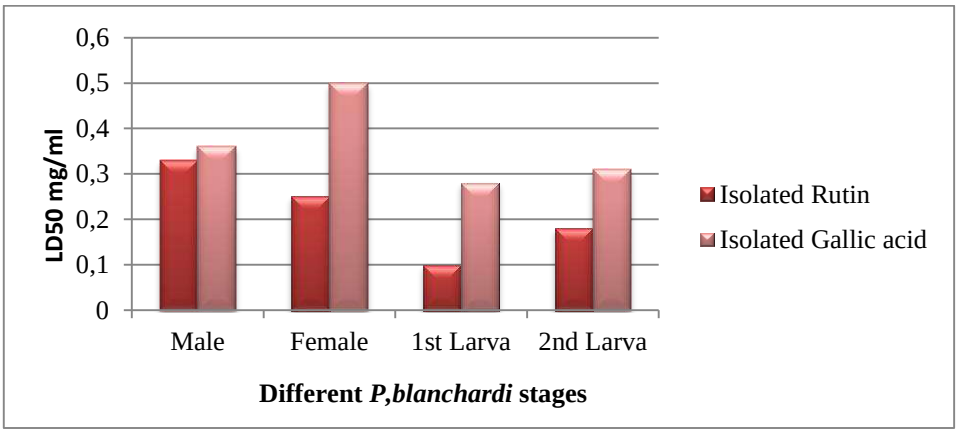


Figure V.7.LD_{50} of isolated Rutin, Gallic acid against *P. blanchardi*.

Overall, an acceptable toxicity effect was recorded for isolated rutin and gallic acid on *P. blanchardi* stages. Nevertheless, it is interesting to note that isolated rutin was more toxic than gallic acid towards all fixed larval stages; but, larvae were more susceptible than adults.

Regarding the sensitivity of male individuals, a similar effect was recorded for treatment with Rutin and Gallic acid with an LD50 equal to 0.33 mg/ml and 0.36 mg/ml respectively. Whereas the female stages were more resistant to treatment with gallic acid than with rutin with an LD50 equal to 0.5 mg/ml **(Figure V.7)**.

The study carried out by **Piubelli et al (2006)** to evaluate the biological and physiological activity of rutin on populations of A. gemmatalis proved that Rutin have deleterious effects which reduced food intake by A. gemmatalis population, whereas, the larvae from the resistant population were more negatively influenced by Rutin even at low concentration of Rutin (0.65%) to the insect diet [247].

The obtained results have demonstrated that rutin and Gallic acid had a potent insecticidal and larvicidal effect towards *P. blanchardi* adults and larvae.

V.4. Conclusion

The chemicals produced by the green parts of *C. procera* were evaluated for its toxicity against *T. castaneum* adults and *P.blanchardi* adults and larvae stages

The obtained results have demonstrated that *C. procera* aerial part had a potent repulsive and insecticidal effect, it can be concluded that the plant compounds could be used as insect economic control strategies, as well as bio-insecticide substitute in which it's potential would help to decrease the negative impact of chemical insecticides, harmful to humans and the environment. However, other studies must be carried out to accurately determine the action mechanism level of these chemicals towards pest behavior.

General conclusion and perspectives

The present study represents a contribution to the analysis of the chemical composition of *C. procera,* a widespread toxic plant in Djanet province (Tassili N'Ajjer, Southern Algeria). Therefore, various analytical techniques were used after a survey (in sito) of their traditional knowledge and uses; furthermore, an assessment of the effectiveness of the explored chemical composition was accomplished against several discomforts encountered in the agri-food and toxicological sector.

The first experimental chapter was divided into two parts; the first one was an investigation aiming to document the traditional uses of *C. procera*, and to evaluate the importance of this plant largely used in folk medicine. An interesting data set was achieved at the end of survey which revealed that the plant contains many pharmacological proprieties by treating various diseases.

Through these results, the second part of this chapter focused on the phytochemical tests on *C. procera* aerial parts extracts and latex which made it possible to highlight the main families of secondary metabolites belonging to potentially active families; they show the great richness in Alkaloids, Phenols, Flavonoids, Sterols and Triterpenes, Glycosides and Cardiac glycosides.

The second experimental chapter, pointed to to identify and compare the chemical volatile constituents from hydro-distilled extract of dried *C. procera* aerial parts (VDA) and dried flowers (VDF) by GC-MS analysis and total area, Twenty four compounds (54.41%) were identified from VDA sample mainly consist of Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- with 12.97%; followed by Dotriacontane (7.38%) ; Tetrapentacontane(4.74%); 2- Palmitoylglycerol (4.37%).

In addition, the analysis of VDF led to the identification of seventeen constituents representing approximately 61.91% of its entire chemical composition. The predominant compounds were Myristic acid (20.37%), p-Vinylguaiacol (14%) ; Palmitic acid (7.86%); Dotriacontane(3.26%) and Dodecanoic acid (2.25%).

The third experimental chapter represents a detailed report on the phenolic composition of the Ethyl acetate and n-Butanol fractions of *C. procera* aerial parts using TLC, PTLC, HPLC, LC-MS, FT-IR and NMR, these allowed the identification and the characterization of two phenolic compounds, namely: 3, 3', 4', 5, 7-pentahydroxyflavones-3 -rutinoside (Rutin) and 3,4,5-trihydroxybenzoic acid (Gallic acid) from ethyl acetate fraction, while in the n- Butanol fraction three flavanoids were identified, namely: Epicatechin, Rutin, and Quercetin.

In the fourth experimental chapter, on one hand, the extraction of the total fixed alkaloids (TFA) from C. *procera* aerial parts and the identification using HPLC led to identify the Atropine from BIII25 with 7.34% and $CI5_{58}$ with 6.99%.

On the other hand, the volatile alkaloids VAH1 and VAH2 of the dried aerial parts hydrolats obtained from the hydrodistillation and purification using CC were analyzed by GC-MS.

The analysis allowed the identification of 32 compounds from VAH1 where the nitrogen derivative compounds estimated at 9.19% of the total identified compounds. In witch, their most abundant compounds consist of 13-Docosenamide, (Z) - (3.27 %); N, N-dimethylhexadecanamide (2.12%), 1-Dodecanamine, N, N-diethyl- Dodecyldiethylamine (0.53%) and 1, 2-Benzenedicarboxylic acid, 3-nitro-(0.48%), while volatile alkaloids were totally absent in the sample .

Twelve compounds were identified from VAH2, where the volatile alkaloids and nitrogen derivative estimated with at 16.66 % of the total identified compounds, where 13-Docosenamide, (Z) - was found to be the major nitrogen derivative compound with (19.78%), and as volatile alkaloid; Imidazole, 5-phenyl-1, 3, 4-trimethyl- (4.83%) and Isoquinoline with (3.72%).

In the last chapter, the application part of our work was devoted to the study of the bio-insecticide effect:

- ✓ To explore the insecticidal and repellency effects of *C. procera* EtOH, DCM, Etp the aerial parts extract, against *P.blanchardi targ* (larvae and adults stages) and *T. castaneum* adults, in which the bioassay data, analyzed by Probit analysis, showed that *C. procera* extracts caused an appreciable mortality, especially EtOH extract (LD_{50}: 0.65 ± 0.01 mg/ml), on different stages of *P.blanchardi*, and variable repellency on *T.castaneum* adults which ranged between 96, 66 ± 5.77%, achieved by 5mg (EtOH)/ml extract after 5 hours, and -6.66 ±5.77% recorded by 0.6 mg (Etp)/ml after 1h.
- ✓ Comparative study between the Latex and a commercial insecticide (TCHOKE), which clearly showed that latex was potentially very toxic on all *P. blanchardi* stages, in particular on the 1^{st} and 2^{nd} larva; with LD_{50} equal to 0.1 and 0.057 µl / ml, respectively even more than the commercial insecticide .

- ✓ The insecticidal study has evaluated also the isolated Rutin and Gallic acid, it is interesting to note that isolated Rutin was more toxic than Gallic acid towards all fixed larval stages; in particular on the 1st larva with LD_{50} equal to 0.1 mg/ml.

Overall, the current study have demonstrated that *C. procera* is a rich source of secondary metabolites (polyphenols, alkaloids,..) among others certainly there are strong bio-insecticidal and/or repellent compounds which can contribute to the development of new strategies to control insect pests and decrease the negative impact of chemical insecticides harmful to humans and the environment.

The results of this work can equally be supplemented by:

- ➢ Purification and characterization of alkaloids.
- ➢ Evaluation of the insecticidal effect of the alkaloid extracts and fractions.
- ➢ Evaluation of the insecticidal effect of the volatile fractions.
- ➢ Explore other biological activity including anticancer activity of isolated alkaloids**.**

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Scientific article

B. Nacira, B. Mebarka, L. Yacine, H. Mohamed, H. Roukia and H. M. Mahfoud. Isolation and structural elucidation of phenolic components from *C. procera* (ait) and evaluation of insecticidal Activity. J. Anim. Plant Sci ISSN: 1018-7081., 33 (4) 2023. https://doi.org/10.36899/JAPS.2023.4.0690

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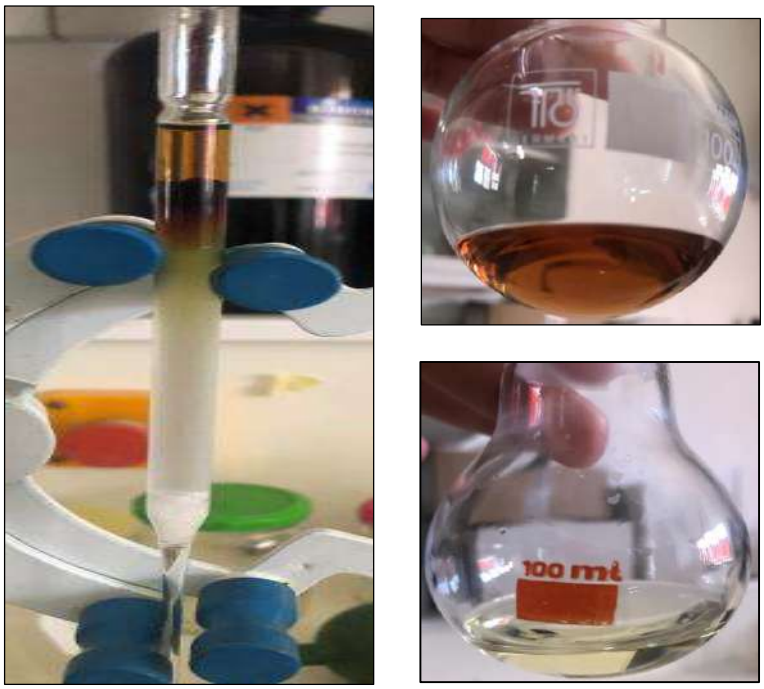
Poster Communication

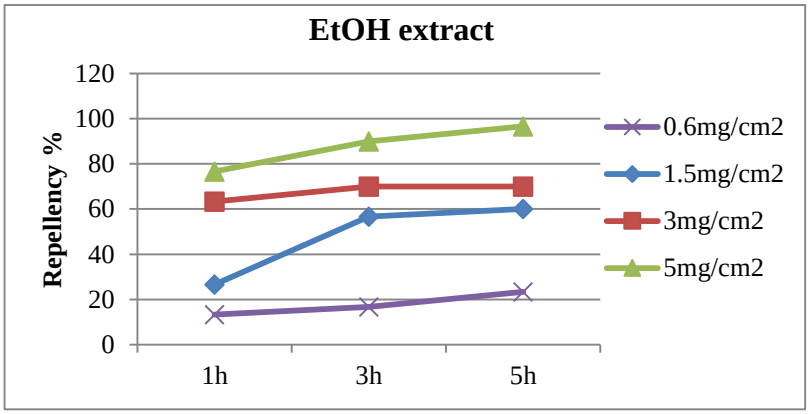
Bellaouar Nacira, 1st International Conference on Chemical Masters and Environment, Preservation.IC-CMEP'22, 09-10 March, Ouargla, Algeria

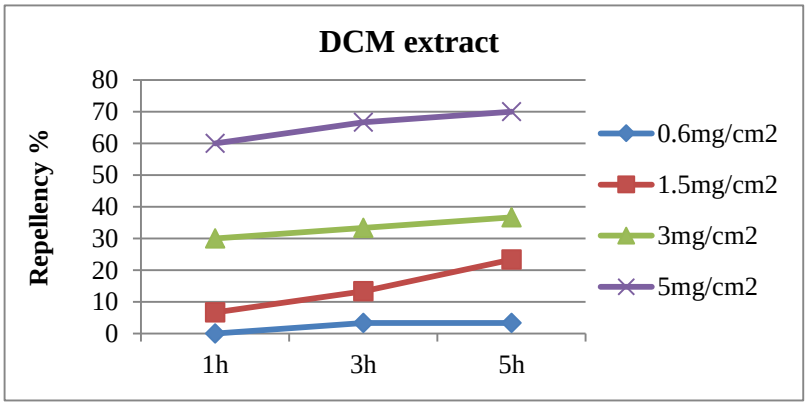
Annexes

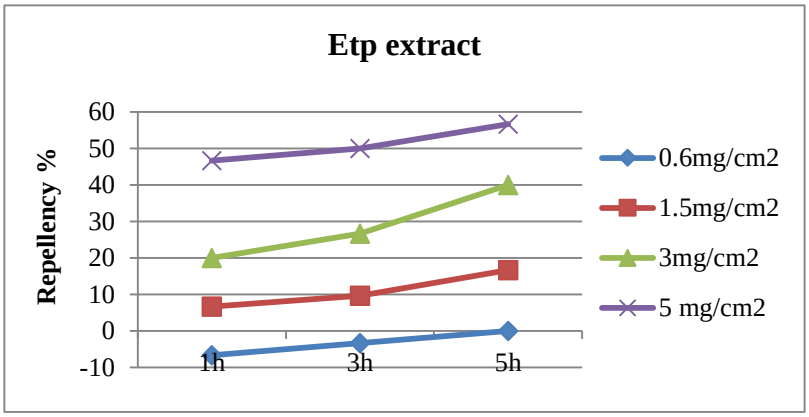
Annex 01. Chromatographic fractionation images of VAH fraction.



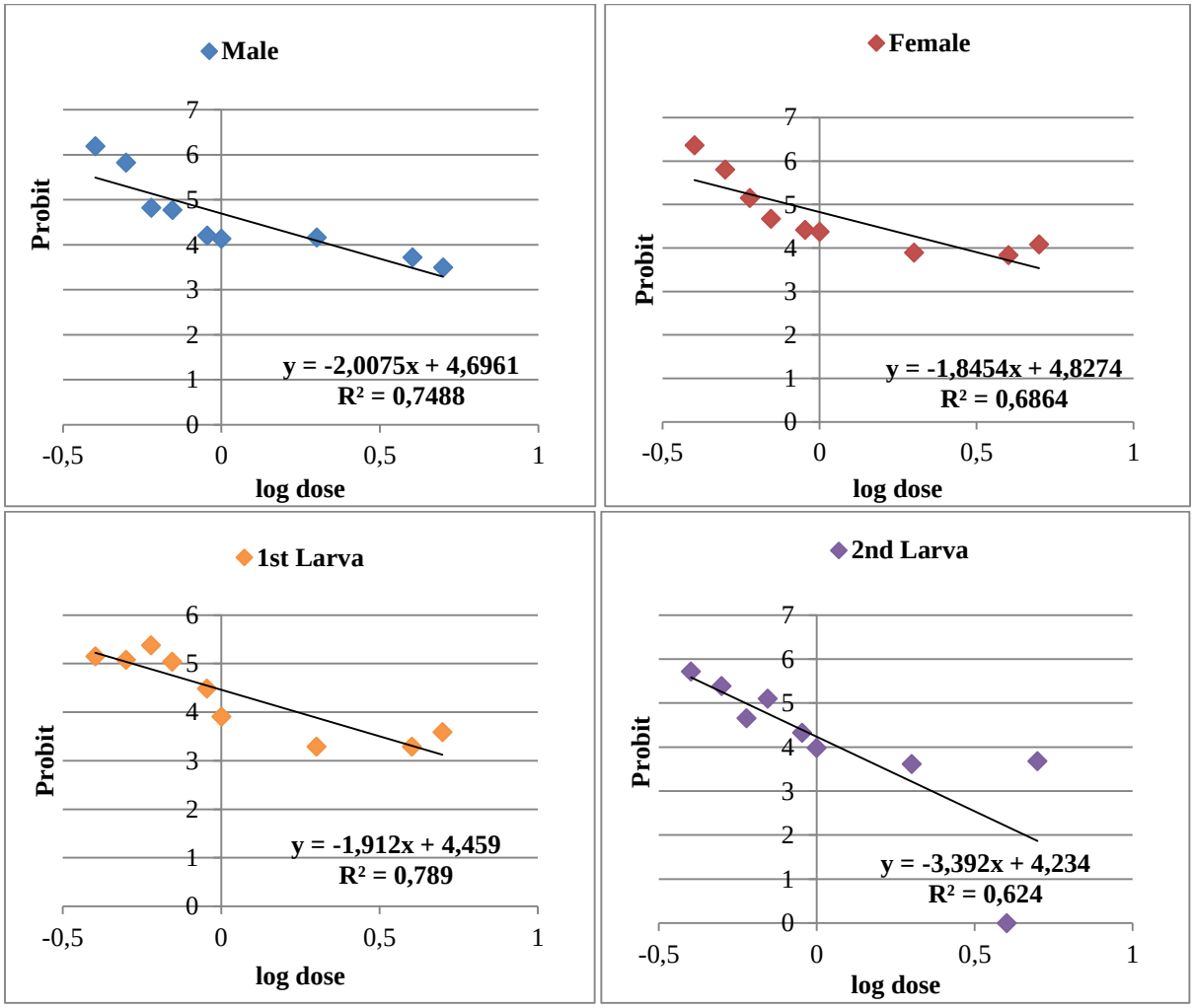
Annex 02. Post-treatment periods of EtOH, DCM, Etp extracts of *C.procera*.



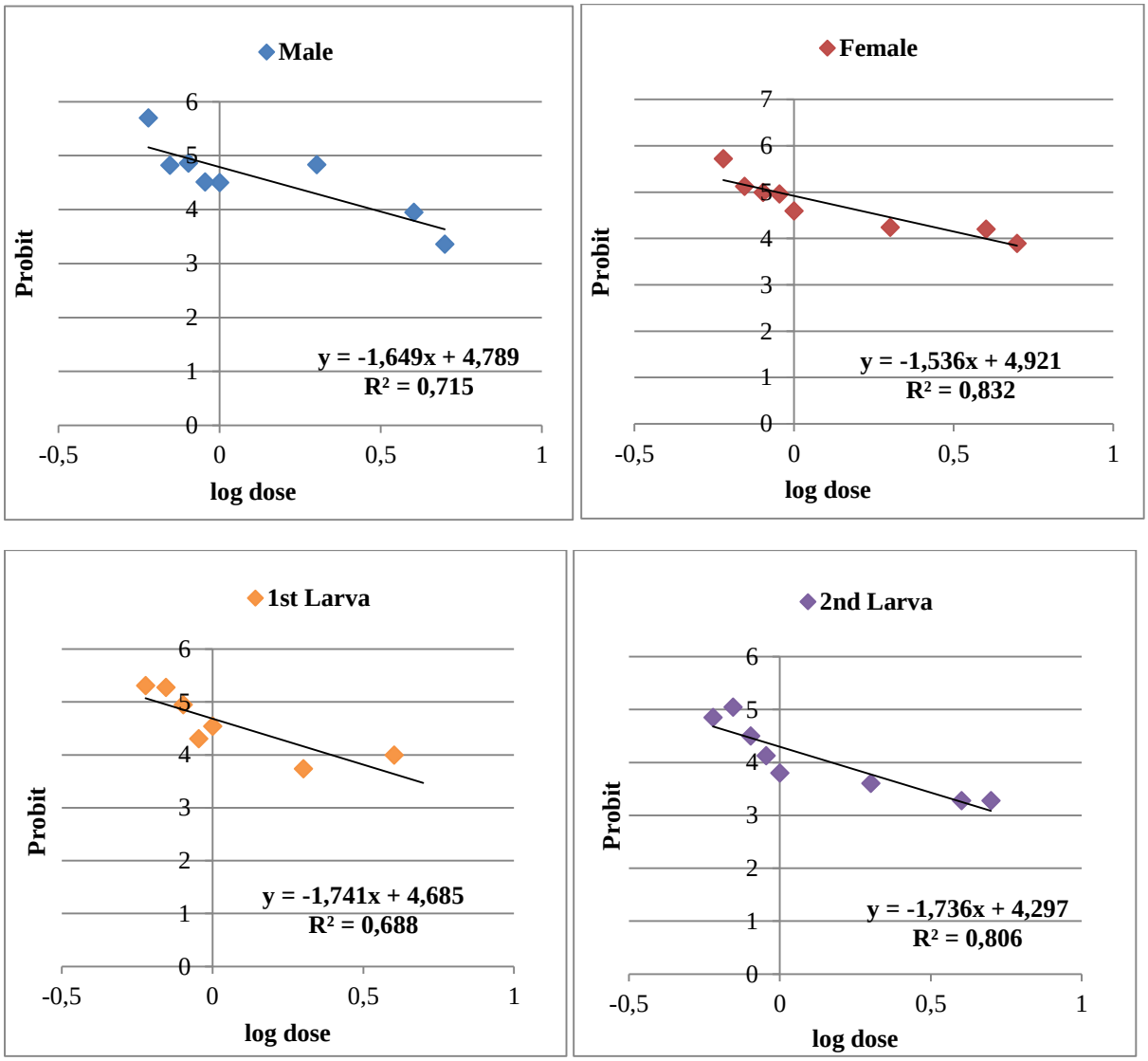




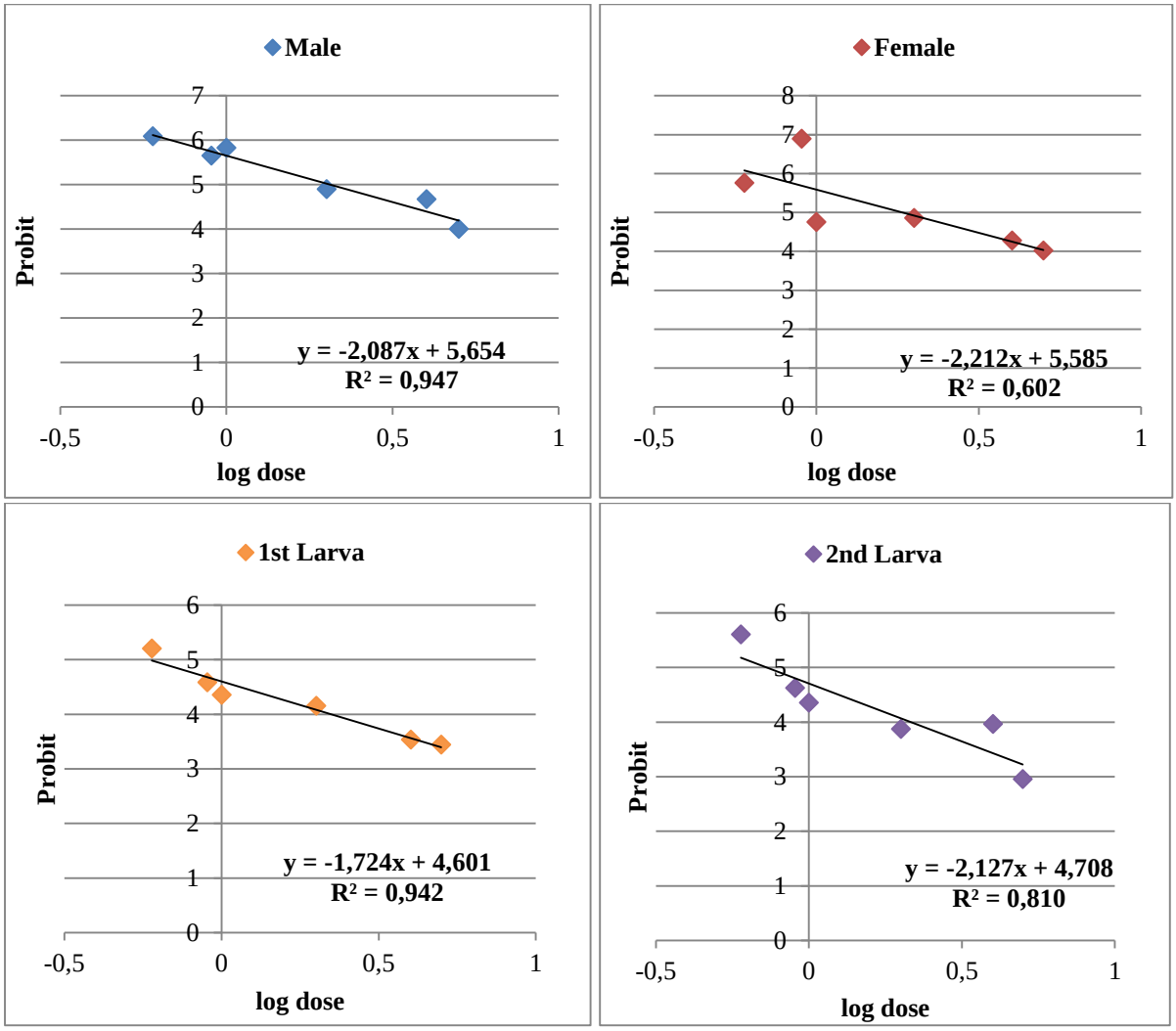
Annex 03. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of the EtOH extract doses of *C. procera*



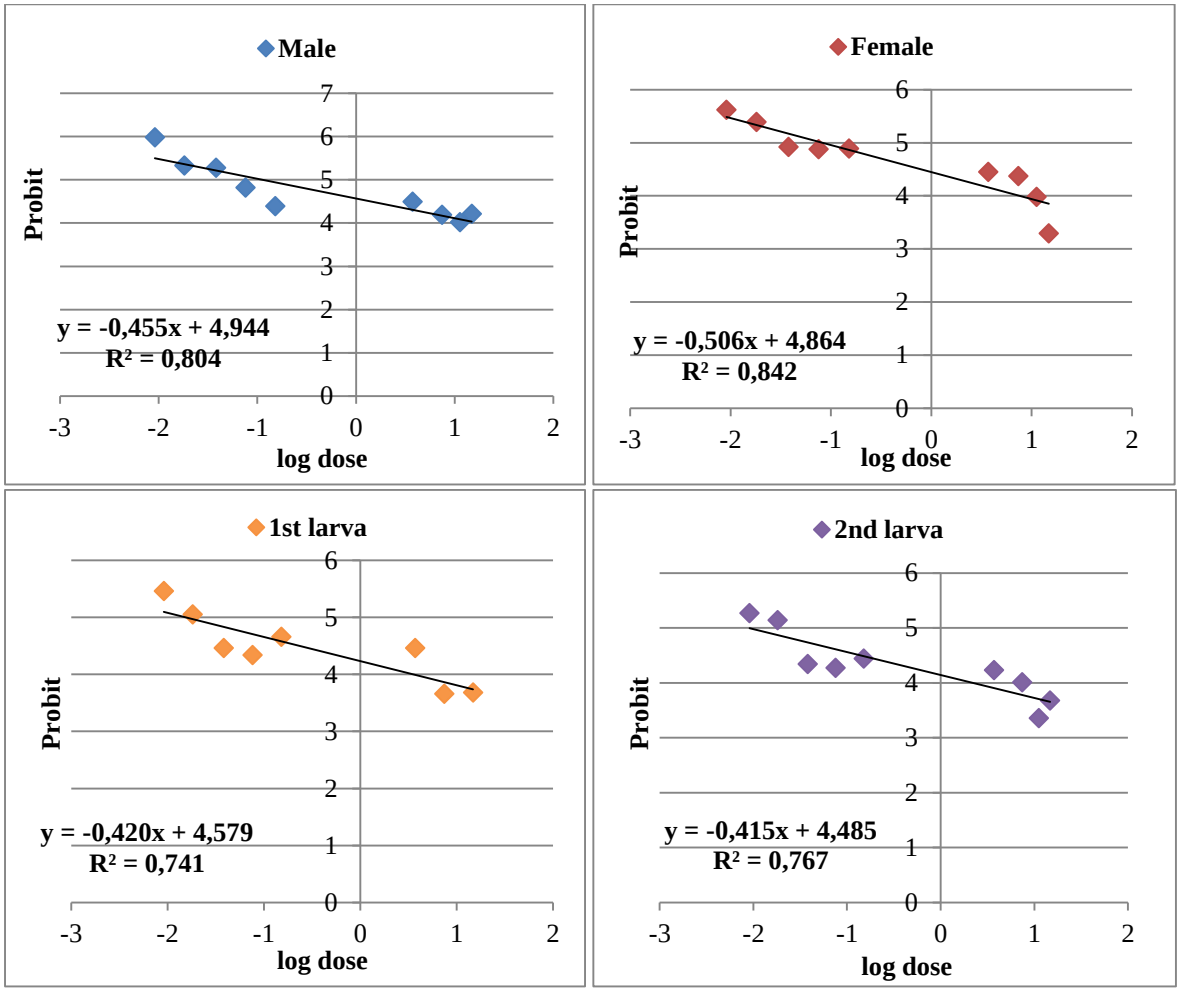
Annex 04. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of the DCM extract doses of *C. procera*



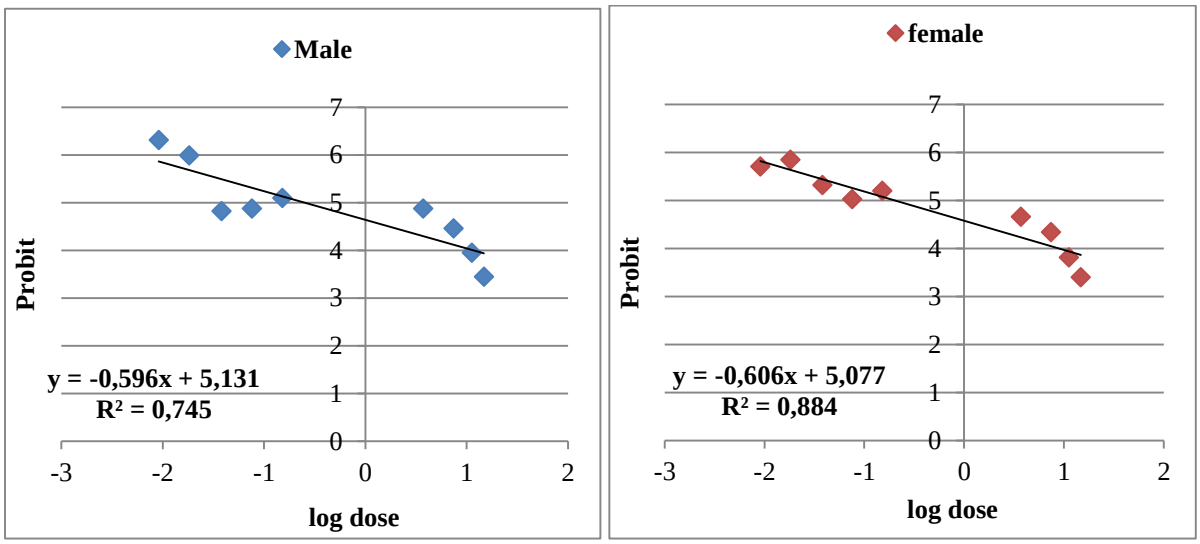
Annex 05. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of the Etp extract doses of *C. procera*

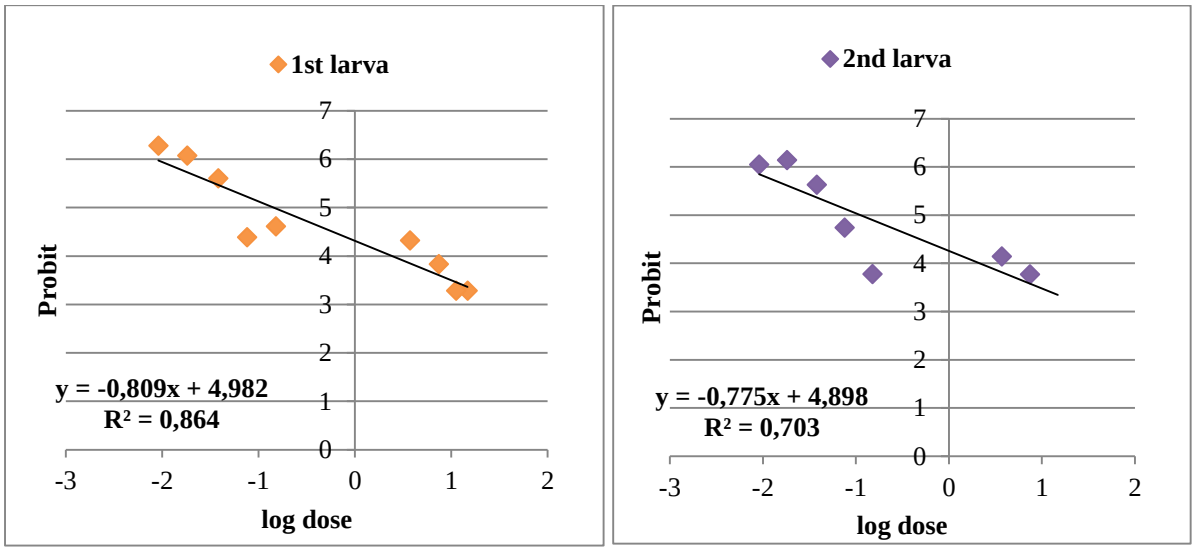


Annex 06. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of the latex doses of *C. procera*

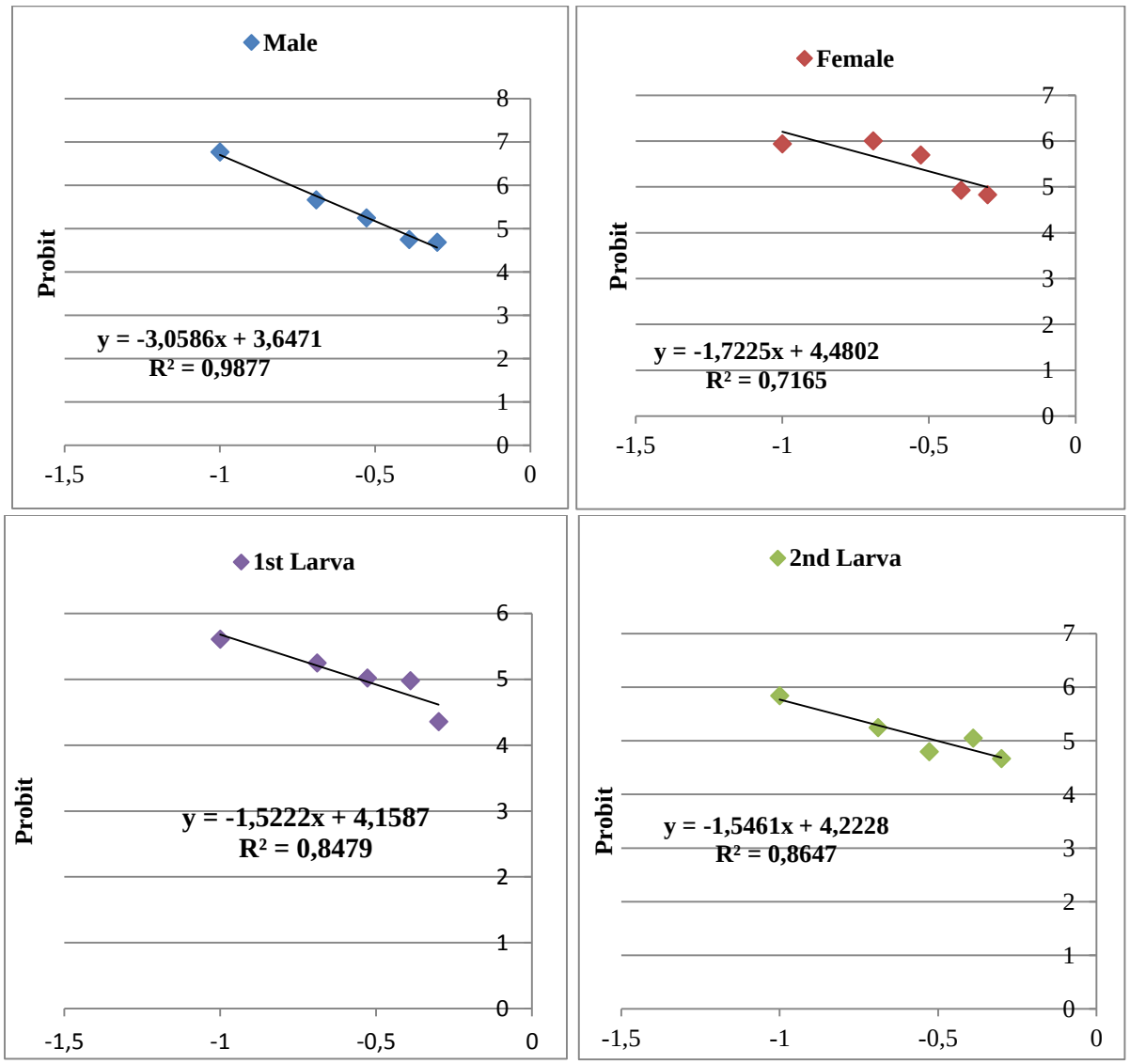


Annex 07. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of the commercial insecticide doses

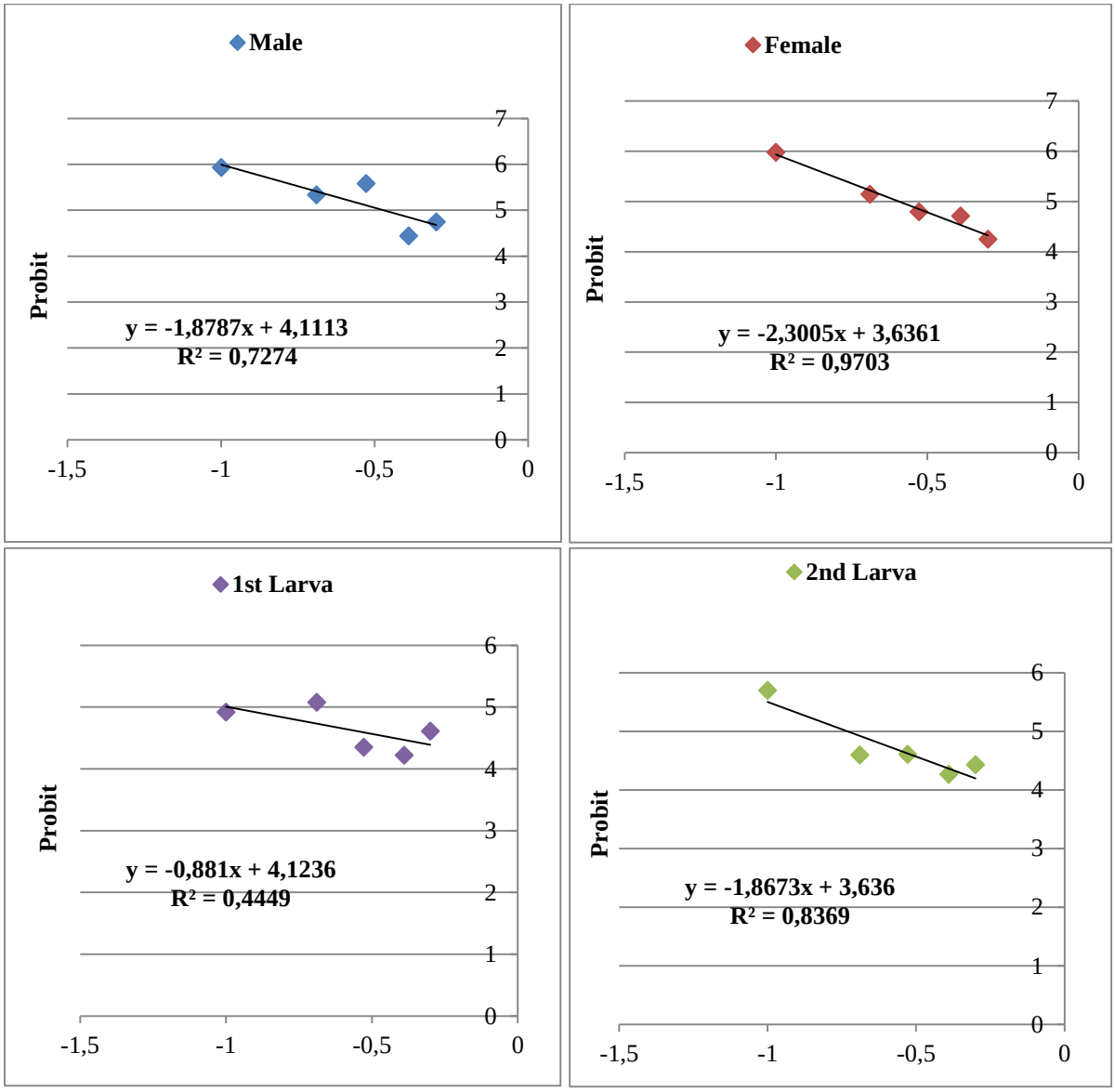




Annex 08. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of isolated Gallic acid doses



Annex 09. Relationship between the probit mortality of *P. blanchardi* individuals and the decimal logarithm of isolated Rutin doses



Annex 10. Sample of data used for insecticidal part **(individials number)**

Individuals numbres	**Male**	**Female**	**1st Fixed larva**	**2nd Fixed larva**
EtOH/ DCM/ Etp extracts	125	146	145	223
Latex and TCHOKE	150	254	47	30
Rutin and Gallic acid	145	325	178	156