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TOPIC

**Study of the phytochemical composition and
biological activities of *Rhamnus alaternus* and
*Retama sphaerocarpa***

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DEDICATION

I dedicate this dissertation to:

My dearest parents

My brother, sisters and their families

All my family

All my professors and teachers

My friends and colleagues

Everyone who loves nature and science

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ABSTRACT

Rhamnus alaternus L (Rhamnaceae) commonly known as M'lilez and *Retama sphaerocarpa* (Retama) known as R'tem are two medicinal plants growing in the Mediterranean Basin including Algeria. The aim of this study was to evaluate the phytochemical composition by HPLC-MS and GC-MS and to study the biological activities of extracts (methanolic, aqueous and traditional) prepared from different parts of these two plants. For *R.alaternus* results the flavonoid investigation showed that the composition of the plant is dominated with the presence of flavonols derivatives (quercetin, kaempferol and rhamnetin derivatives). The extracts showed a good antioxidant/free radical scavenger activity, hence in FRAP and ORAC tests the results of the bark methanolic extract were ($1.71 \pm 0.07 \text{ Fe}^{2+}/\text{g}$; $6.55 \pm 0.03 \text{ mmol TE/g}$, respectively), Furthermore, the bark methanolic extract was the best in cytotoxicity test because it reduced significantly the proliferation of U937 cancer cells ($6.39 \mu\text{g/ml}$), and oppositely produced less PBMC cell death ($220.35 \mu\text{g/ml}$), PE Annexin V test showed that *R.alaternus* methanolic extract induce apoptosis to U937 cancer cells in a dose dependent manner. Regarding *R sphaerocarpa* results the isoflavones (daidzein, genistein, Glycitein derivatives) dominated the composition of the extracts and the alkaloids study showed that *R. sphaerocarpa* stems methanolic extract contains quinolizidine alkaloids; the main components were cytosine and retamine. In another hand a good antioxidant activity was found, hence in FRAP and ORAC the results of the fruits methanolic extract were ($0.65 \pm 0.05 \text{ Fe}^{2+}/\text{g}$; $7.31 \pm 0.07 \text{ mmol TE/g}$, respectively). All the extracts of *R. sphaerocarpa* showed a good activity in advanced glycation end-products (AGEs) formation assay; the methanolic extract of the stems was the most active ($\text{IC}_{50} = 9.36\%$), this extract was tested for acute toxicity and was found to be slightly toxic ($\text{LD}_{50} = 2488.86 \text{ mg/kg}$). It was used also for antidiabetic activity in rats and showed at the end of the experiment at the dose 50 mg/kg a significant decrease in feed and water intake, blood glucose level ($75 \pm 3.60 \text{ mg/dl}$), plasma triglycerides and cholesterol levels of diabetic rats ($48.66 \pm 4.72 \text{ mg/dl}$, $89.00 \pm 3.00 \text{ mg/dl}$, respectively). Similarly the hematological parameters studied were improved and the effect was not dose dependent. As a conclusion *R. alaternus* and *R. sphaerocarpa* especially bark and stems (respectively) can be regarded as good sources of bioactive compounds. The results confirm that the decoction, usually employed in traditional medicine of these plants is of a therapeutic importance related directly to their content in phytochemical active compounds.

Key words: *Rhamnus alaternus*; *Retama sphaerocarpa*; flavonoids; alkaloids; antioxidants, cytotoxicity; antidiabetic.

RESUME

Rhamnus alaternus L (Rhamnaceae) communément connue sous le nom M'lilez et *Retama sphaerocarpa* (Retama) connue sous le nom R'tem sont deux plantes médicinales croissant dans le bassin méditerranéen incluant l'Algérie. Le but de cette étude est l'évaluation de la composition phytochimique en utilisant les méthodes: HPLC-MS and GC-MS, ainsi que l'étude des activités biologiques des extraits (methanolic, aqueux et traditionnelle) préparés à partir de différents organes de ces deux plantes. Pour les résultats de *R.alaternus* l'analyse des flavonoïdes a montré que la plante est dominée par la présence des dérivés des flavonols (quercétine, kaempférol et rhamnétine). Les extraits ont montré une bonne activité antioxydante, ainsi dans les essais de FRAP et ORAC les résultats de l'extrait methanolic de l'écorce étaient (1.71 ± 0.07 Fe²⁺/g; 6.55 ± 0.03 mmol TE/g, respectivement). D'autre part; l'extrait methanolic de l'écorce a présenté les meilleurs résultats dans le test de cytotoxicité réduisant de façon significative la prolifération des cellules cancéreuses U937 (6.39 µg/ml), et de manière opposée il a produit moins de mort chez les cellules mononucléaires de sang périphérique (220.35 µg/ml). PE Annexin V test montre que cet extrait provoque une apoptose des cellules cancéreuses (U937) dépendante de la dose. Concernant les résultats de *R.sphaerocarpa* les dérivés des isoflavones (daïdzéine, génistéine, glycitéine) dominent la composition des extraits. L'étude des alcaloïdes a montré que l'extrait methanolic des rameaux contient les alcaloïdes quinolizidiniques et les principaux composants sont cytosine et retamine. Les extraits de *R.sphaerocarpa* ont démontré une bonne activité antioxydante, ainsi les résultats de l'extrait des fruits dans les essais FRAP et ORAC étaient (0.65 ± 0.05 Fe²⁺/g; 7.31 ± 0.07 mmol TE/g, respectivement). De même tous les extraits ont montré une bonne activité dans le test de la formation des produits de glycation avancée, cependant l'extrait methanolic des rameaux était le plus actif (IC₅₀ 9.36%). Ce même extrait a été utilisé pour le test de la toxicité aiguë et il a été trouvé comme légèrement toxique (LD₅₀ = 2488.86 mg/kg). Cet extrait a été aussi utilisé pour l'évaluation de l'activité antidiabétique sur des rats, et il a montré une bonne activité se manifestant à la fin de l'expérience à la dose 50mg/kg par une diminution significative dans la consommation d'aliments et d'eau, ainsi qu'une réduction de la glycémie (75 ± 3.60 mg/dl) et des triglycérides et du cholestérol plasmatiques des rats diabétiques (48.66 ± 4.72 mg/dl, 89.00 ± 3.00 mg/dl, respectivement). De façon similaire les paramètres hématologiques étudiés ont été améliorés, et l'effet n'est pas dose dépendant. En conclusion, *R. sphaerocarpa* et *R. alaternus* particulièrement les parties rameaux et écorce peuvent être considérés comme de bonnes sources de composés bioactifs. Les résultats confirment que la décoction, habituellement utilisée dans la médecine traditionnelle de ces plantes est d'une importance thérapeutique directement liée à leur composition phytochimique.

Mots clés: *Rhamnus alaternus*; *Retama sphaerocarpa*; flavonoïdes; alcaloïdes; antioxydants, cytotoxicité; antidiabétique.

ملخص

تعتبر النبتتين *Rhamnus alaternus* L (Rhamnaceae) المعروفة بـ: مليز و *Retama sphaerocarpa* (Retama) المعروفة بـ: رتم من النباتات الطبية التي تنمو بمنطقة البحر الأبيض المتوسط بما في ذلك الجزائر. الهدف من هذا البحث هو دراسة التركيب الكيميائي باستعمال طرق التحليل بواسطة HPLC-MS و GC-MS و تقييم النشاطية البيولوجية للمستخلصات (الميثانولي، المائي و التقليدي) المحضرة من أعضاء مختلفة من النبتتين. في ما يخص نتائج نبتة *R. alaternus* فقد أظهر التحليل الكيميائي أن مشتقات مركبات الفلافونول (quercetin, kaempferol و rhamnetin) هي المهيمنة على التركيب الكيميائي للنبتة. كما أظهرت كل المستخلصات نتائج جيدة في النشاطية المضادة للأكسدة، حيث أن المستخلص الميثانولي للحاء في تجربتي FRAP و ORAC قدم النتائج التالية (0.07 ± 1.71 مليمول $Fe^{2+}/غ$ ، 6.55 ± 0.03 مليمول TE/غ على التوالي)، في تجربة أخرى، كان المستخلص الميثانولي للحاء هو الأفضل في اختبار السمية الخلوية لأنه خفض بشكل كبير من انتشار الخلايا السرطانية U937 (6.39 ميكروغ/مل)، وبشكل معاكس سبب أقل نسبة موت لخلايا الدم (العادية) وحيدة النواة (220.35 ميكروغ/مل)، هذا وقد بينت تجربة PE Annexin V أن المستخلص تسبب في الموت المبرمج للخلايا السرطانية U937. أما نتائج نبتة *R. sphaerocarpa* فقد هيمنت مشتقات الإيسوفلافون (daidzein, genistein و glycitein) على التركيب الكيميائي للثمار و الأغصان، أما دراسة القلويدات فقد بينت أن المستخلص الميثانولي لأغصان نبتة *R. sphaerocarpa* غني بالقلويدات الكينوليزيدينية و المهيمنة منها هي (cytisine و retamine). كما أظهرت المستخلصات نتائج جيدة في النشاطية المضادة للأكسدة، حيث أن المستخلص الميثانولي للثمار أظهر في تجربتي FRAP و ORAC النتائج التالية (0.05 ± 0.65 مليمول $Fe^{2+}/غ$ ، 0.07 ± 7.31 مليمول TE/غ على التوالي)، من جهة أخرى كل مستخلصات *R. sphaerocarpa* كان لها نشاط جيد في اختبار (AGEs)، و كان المستخلص الميثانولي هو الأكثر نشاطا ($IC_{50}=9.36\%$). استعمل هذا المستخلص لدراسة السمية الحادة، وقد بينت النتائج أن لديه سمية قليلة ($LD_{50}=2488.86$) واستعمل هذا المستخلص كذلك في دراسة النشاطية المضادة للسكري، حيث أظهر في نهاية التجربة عند الجرعة 50 مع/كغ انخفاضاً كبيراً في شراهية الحيوانات للأكل و استهلاك الماء مع انخفاض كذلك في مستوى السكر في الدم (3.60 ± 75 دل/مع)؛ وكذلك انخفضت الدهون ثلاثية الجلسريد و مستويات الكوليسترول في البلازما للجرذان المصابة بداء السكري (4.72 ± 48.66 مغ/دل، 3.00 ± 89.00 مغ/دل، على التوالي)، كما لوحظ تحسن في مستويات المؤشرات الدموية المدروسة، و لم يكن التأثير متوقفاً على الجرعة. كخلاصة *Rhamnus alaternus* خاصة للحاء و *Retama sphaerocarpa* خاصة الأغصان هي مصادر جيدة للمركبات النشطة حيويًا. وتؤكد النتائج أن الاستخدام الطبي التقليدي للنبتتين (غلي النبات)، هو ذو أهمية تتعلق مباشرة بالتركيب الفيتوكيميائي لهما.

الكلمات المفتاحية: *Rhamnus alaternus*، *Retama sphaerocarpa*، الفلافونويدات، القلويدات، مضادات الأكسدة، السمية الخلوية، مضاد السكري.

ABBREVIATIONS

AAD	amino-Actinomycin D
AAPH	2,2'-azobis (2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AGEs	advanced glycation end products
AUC	area under the curve
CE	catechin
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
DAD	diode array detector
ESI	electrospray ionisation
ET	electron transfer
FBS	fetal bovine serum
FCS	fetal calf serum
FID	flame ionization detector
FL	fluorescein
FRAP	ferric reducing/antioxidant power
GaE	gallic acid
GC	gas chromatography
HAT	hydrogen atom transfer
Hb	hemoglobin
HPLC	high-performance liquid chromatography
IC ₅₀	half maximal inhibitory concentration
LD ₅₀	median lethal dose
MS	mass spectrometry
NMR	nuclear Magnetic Resonance
OGTT	oral glucose tolerance test
ORAC	oxygen radical absorbance capacity
OCDE	organisation for Economic Co-operation and Development

PDA	photo diode array
PE	Phycoerythrin
PBMCs	human peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCV	hematocrit
QE	quercetin equivalents
RBC	red blood cells
ROS	reactive oxygen species
RPMI	roswell Park Memorial Institute medium
RSD	relative standard deviation
RSM	<i>Retama sphaerocarpa</i> stems methanolic extract
STZ	streptozotocin
TE	trolox equivalent
TEA	trimethylamine
TEAC	trolox equivalent antioxidant capacity
TLC	thin layer chromatography
TPTZ	2,4,6-tripyridyl-s-triazine
t_R	retention time
U937	human monocytic leukemia cells
UV-vis	ultraviolet visible
VWR	company involved in the distribution of laboratory products
WBC	white blood cells

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INTRODUCTION

INTRODUCTION

The biomolecular diversity of natural products still represents a valuable starting point for the development of new drug formulations, and in this vision the sustainable management of traditional medicinal plant resources remains an important aspect, being as in the past and recent history of drug discovery inexorably bound to the plant kingdom (Kingston, 2011, Newman and Cragg, 2007). Indeed, the experience of traditional medicine is precious in identifying possible target plant species or particular parts of them.

Rhamnus alaternus L. (Rhamnaceae) grows in the Mediterranean region, and is commonly found in Algeria, where it is known locally as “M’lilez”. The aerial parts of this plant are used in some North African countries for the treatment of liver complications such as jaundice (Boukef, 2001). Many antioxidant compounds were identified in *Rhamnus alaternus* like anthraquinones such as emodin, chrysophanol and flavonoids such as Kaempferol 3-*O*-bisorhamninoside (3) (Bhourri *et al.*, 2011b; Ben Ammar *et al.*, 2009). Experimental studies have demonstrated that extracts and purified compounds from aerial parts of *R. alaternus* possess different biological activities, including antibacterial, antimutagenic, antigenotoxic, antioxidant/free radical scavenging, and cytotoxic activities, and are able to modulate cellular gene expression (Ben Ammar *et al.*, 2007a-b; 2008a-b; 2009).

Retama sphaerocarpa (L.) Boiss widely found within the Mediterranean area both in wet and dry climates (Quezel and Santa, 1963). In Algeria and according to survey got from people, the stems of *Retama* were used traditionally to cure rabies in both humans and animals, to prepare a type of chewing tobacco commonly called "Chemma". Several papers in literature account for the composition of different extracts from *R. sphaerocarpa*, the majority of them dealing with the analysis of its alkaloids. Phytochemical studies on *Retama sphaerocarpa* report the presence of a particular class of alkaloids: the quinolizidine alkaloids, which are known to possess a wide range of pharmacological and toxicological properties (El-Shazly *et al.*, 1996). *Retama sphaerocarpa* was shown to possess antimicrobial and cytotoxic activities (López-Lázaro *et al.*, 2000; Louaar *et al.*, 2007). It is notified that other species from *Retama* genus but not *Retama sphaerocarpa* were studied for their diuretic, hypoglycemic (Maghrani *et al.*, 2005a-b), antioxidant, antiviral (Edziri *et al.*, 2010), antihypertensive (Eddouks *et al.*, 2007), and anti-inflammatory (Bremner *et al.*, 2009) activities.

The purpose of this study can be listed as the following:

- Preparation of different extracts by maceration and decoction from *R.alaternus* and *R. sphaerocarpa*.
- Characterisation of flavonoids profile of the extracts by means of high-performance liquid chromatography-diode array detector/electrospray ionisation mass spectrometry (HPLC-DAD/ESI-MS) analysis.
- Alkaloids determination in *R. sphaerocarpa* stems methanolic extract by means of gas chromatographic (GC) separation coupled to a mass spectrometer (MS) and a flame ionization detector (FID).
- Evaluation of the antioxidant capacity of *R.alaternus* and *R. sphaerocarpa* extracts using a battery of five different simple redox-based assays differing in the mechanisms involved and the chemical environment used. The Folin-Ciocalteu assay; the bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[•]); the trolox equivalent antioxidant capacity (TEAC) assay; the ferric reducing/antioxidant power (FRAP) assay; and, the oxygen radical absorbance capacity (ORAC) assay.
- The study of antiproliferative effect of *R. alaternus* extracts on cultures of human monocytic leukemia U937 cells, and of human normal peripheral blood mononuclear cells.
- Evaluation of apoptosis/necrosis effect of *R. alaternus* using phycoerythrin-labeled annexin V (PE Annexin V) and 7-Amino-Actinomycin D (7-AAD) flow cytometry assay.
- Determination of the antiglycation activity of *R. sphaerocarpa* extracts by advanced glycation end products (AGEs) formation assay.
- Estimation of the acute toxicity of *R. sphaerocarpa* in *Albino Wistar* rats through the calculation of the corresponding LD₅₀ value.
- Investigation of the effect of *R. sphaerocarpa* after long-term treatment of normal and streptozotocin induced diabetes rats, the biochemical and hematological parameters were analyzed for all animals.

Chapter 01

LITERATURE REVIEW

Chapter 01

LITERATURE REVIEW

1. *Rhamnus alaternus* L.

1.1. Arabic vernacular names

- Aoud el kheir; méliles; mlîlâ, Quaced ((Quezel and Santa, 1963; Beloued, 2005).

1.2. Berber names

- Ajroudj; Khalis n'imidekh; Amliles ((Quezel and Santa, 1963; Beloued, 2005).

1.3. Taxonomy

Kingdom: Plants

Subkingdom: Tracheophytes

Branch: Spermatophytes

Sub-Branch: Angiosperms

Class: Dicotyledons

Order: Rhamnales

Family: Rhamnaceae

Genus: *Rhamnus*

Specie: *Rhamnus alaternus* L. (Quezel and Santa, 1963).

1.4. Botany and ecology

Rhamnus alaternus is a native Mediterranean, dioecious, evergreen shrub or tree, 2-6 m in height. It is a cosmopolitan plant but mostly found in temperate and tropical regions; forest, scrub and Algerian tell (Tsahar *et al.*, 2002). The stems have reddish bark and pubescent branches, the leaves are rounded, compact and alternating. Flowering occurs between February and April, and fleshy fruit appear between April and August (Quezel and Santa, 1962-1963). Unripe fruits are green, which turn red, then black when ripe (Spichiger *et al.*, 2004). Its fleshy fruits are an important source of nutriment they are regularly consumed by birds and small mammals, it is a predominantly entomophilous species, and the seeds are dispersed by ants (Aronne and Wilcock 1994; Gómez *et al.*, 2003; Arroyo *et al.*, 2009). *R. alaternus* is one of the species commonly used

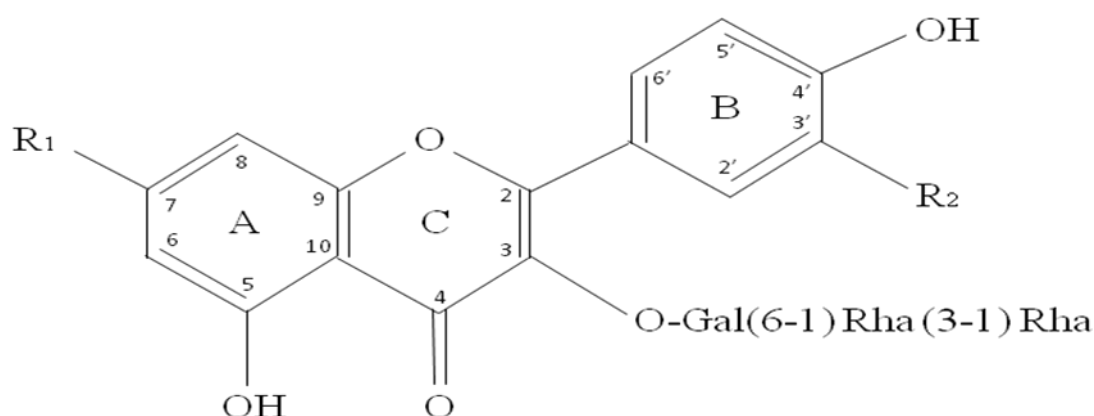
in reforestation programs in the Mediterranean, due to its ability to survive in xeric environments (Gulias *et al.*, 2004).

1.5. Traditional applications

In North Africa especially in Algeria, Tunisia and Morocco, *R. alaternus* have diverse uses, but the doses must be with precaution. The infusion of the bark or leaves of *R. alaternus* are mostly used to cure jaundice and liver troubles, for this 10g of root or trunk bark are mixed with half a liter of water, boiled for 10 minutes, filtered, and then two cups are drunk per day (Beloued, 2005). In the other hand, the fruits of this plant are used as purgative and laxative (bellakhdar, 1997), for this: two grams of crushed fruits are mixed with a quarter of water, the mixture is boiled, and then to be drunk in a fasting morning (Beloued, 2005). The wood of the plant was used to have a yellow coloration of fabric and wool (bellakhdar, 1997).

1.6. Phytochemical composition

The genus *Rhamnus* contains abundant phenolic substances, especially anthraquinones but also tannins and flavonoids (Tsahar *et al.*, 2002). *R. alaternus* leave, and bark of the root and trunk are rich in anthraquinones (Paris and Moyse, 1976-1981). Four anthraquinone aglicones were identified in the above ground parts of *R. alaternus* (emodin, chrysophanol, alaterin and physcion); emodin is the most abundant (Tsahar *et al.*, 2002). Three triglycoside flavonoids were isolated from the leaves of *R. alaternus* (kaempferol 3-O-bisorhamninoside (1), rhamnocitrin 3-O-bisorhamninoside (2) and rhamnetin-3-O-bisorhamninoside (3) (Figure 1) along with apigenin, kaempferol and quercetin. (Ben Ammar *et al.*, 2009).



Compound name	R ₁	R ₂
1- Kaempferol 3-O-b-isorhamninoside	OH	H
2- Rhamnocitrin 3-O-b-isorhamninoside	OCH ₃	H
3- Rhamnetin-3-O-bisorhamninoside	OCH ₃	OH

Figure 1. Chemical structure of the flavonoids (1-3) isolated from *R. alaternus* leaves (Ben Ammar *et al.*, 2009).

1.7. Biological activities

Previous studies have shown potent antioxidant, free radical scavenging, antimutagenic and antigenotoxic activities of crude extracts from *R. alaternus* (Chevolleau *et al.*, 1992; Ben Ammar *et al.*, 2005; 2008a-b). It was also reported other biological activities of *R. alaternus* extracts: antibacterial and antiproliferative (Kosalec *et al.* 2013; Ben Ammar *et al.*, 2007a; 2008b; 2011). In human cells, extracts of *R. alaternus* leaves modulate the expression levels of genes implicated in both DNA repair and oxidative defense systems (Ben Ammar *et al.*, 2007a-b). It was proved that kaempferol 3-O-b-isorhamninoside and rhamnocitrin 3-O-b-isorhamninoside isolated from *R. alaternus* Induce apoptosis in human lymphoblastoid cells (Bhouri *et al.*, 2011a; 2012).

2. *Retama sphaerocarpa* (L.) Boiss.

2.1. Arabic vernacular name

- R'tem (Quezel and Santa, 1963).

2.2. Berber names

- Tillugwât; Îllugwât; Allugû, Talggû (Quezel and Santa, 1963; Bellakhdar, 1997).

2.3. Taxonomy

Kingdom: Plants

Subkingdom: Tracheophytes

Branch: Spermatophytes

Sub-Branch: Angiosperms

Class: Dicotyledons

Order: Fabales

Family: Fabaceae (leguminosae)

Genus: *Retama*

Specie: *Retama sphaerocarpa* (L.) Boisse (Quezel and Santa, 1963).

2.4. Botany and ecology

From *Retama* genus three species are growing in Algeria, *Retama monosperma*, *Retama raetam*, and *Retama sphaerocarpa* (Belmokhtar and Kaid-Harche, 2012; Boulila *et al.*, 2009). *R. sphaerocarpa* is a perennial shrub up to 5m height with a simple modular structure consisting of several branches (figure 2), its open structure allows adequate amount of light to pass through it (Fungairiño *et al.*, 2005; Padilla and Pugnaire, 2009). Its small, ephemeral leaves are shed within a couple of weeks, the flowers are small (5-6mm) of yellow color and appear from April to May, while the fruits are of a hard cover and appear in winter (January–February) (Domingo *et al.*, 1997; 1998; 1999; Pugnaire *et al.*, 1996). Shrubby legumes of *Retama* genus are endemic to the Mediterranean Basin and they are distributed in the various Mediterranean climates (from humid to arid) and ecosystems including coastal, dunes, maquis, and also deserts, since *Retama* shrubs are tolerant to extreme drought conditions (Martin-Cordero *et al.*, 2000; Quezel and Santa, 1962-1963). *Retama* species are of ecological interest for dune stabilization, soil fixation, and revegetation of semi arid ecosystems (Caravaca *et al.*, 2003). An important trait of shrub legumes that contributes to their adaptation to low fertile (nitrogen-deficient) soils, and to improve fertility, is their capacity to establish N₂-fixing symbiosis with rhizobia, which are the soil bacteria that can fix N₂ in the nodules formed with legumes (Boulila *et al.*, 2009). *R. sphaerocarpa* has a remarkable capacity to withstand drought owing to its crown architecture and its deep root system, which can penetrate to depths of 25m (Haase *et al.* 1996), providing access to deep water sources (Caravaca *et al.*, 2005).

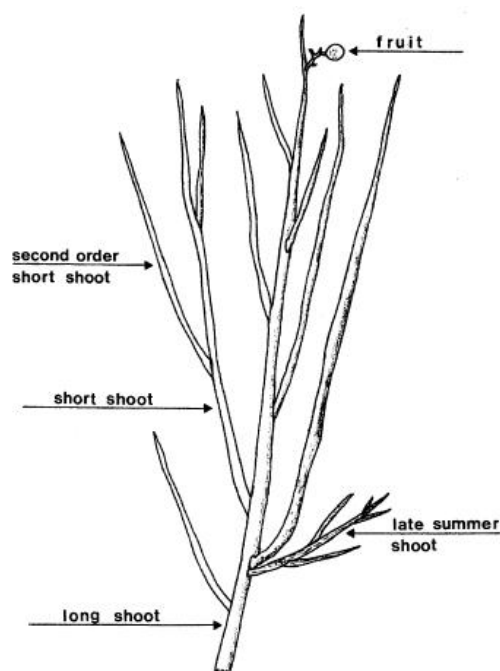


Figure 2. Sketch of a shoot of *Retama sphaerocarpa* (Haase *et al.*, 2000).

2.5. Traditional application

Retama species including *R. sphaerocarpa* have same traditional applications. They are mostly known to cure rabies and to reduce blood glucose level in the medicinal folk traditions of the east of Algeria (Louaar *et al.*, 2005; Djeddi *et al.*, 2013), in Morocco *Retama* is largely recommended by traditional herbal healers for diabetes control (Maghrani *et al.*, 2005b).

2.6. Phytochemical composition

Phytochemical studies have shown that *R. sphaerocarpa* contains quinolizidine alkaloids (Figure 3). El-Shazly and his coworkers (1996) detected the following alkaloids: cytisine, retamine, sparteine, Ammodendrine, 5,6 dehydrolupanine, lupanine, Anagryne, the phytochemical structures of the most abundant alkaloids in *R. sphaerocarpa* are shown in figure 4. Also phenolics were isolated from the aerial parts of the plant and they are: Retamatrioside, rhamnazin, 6-methoxypseudobapteenin, genistein 8-C-glucoside, genistein 7-O-b-glucoside, 7-hydroxy-6'-methoxy-3',4'-methylenedioxyflavone 7-O-β-glucoside, genistein, daidzein, rhamnazin-3-O-β-glucopyranosyl-(1-5)-α-arabinofuranoside (López-Lázaro *et al.*, 1998; Martín-Cordero *et al.*, 1999; 2000; Louaar *et al.*, 2005; 2007). Fatty acids were detected in *R. sphaerocarpa* stems and grain, whereas sterols were detected in stems and monoglycerides in grains (Touati *et al.*, 2015).

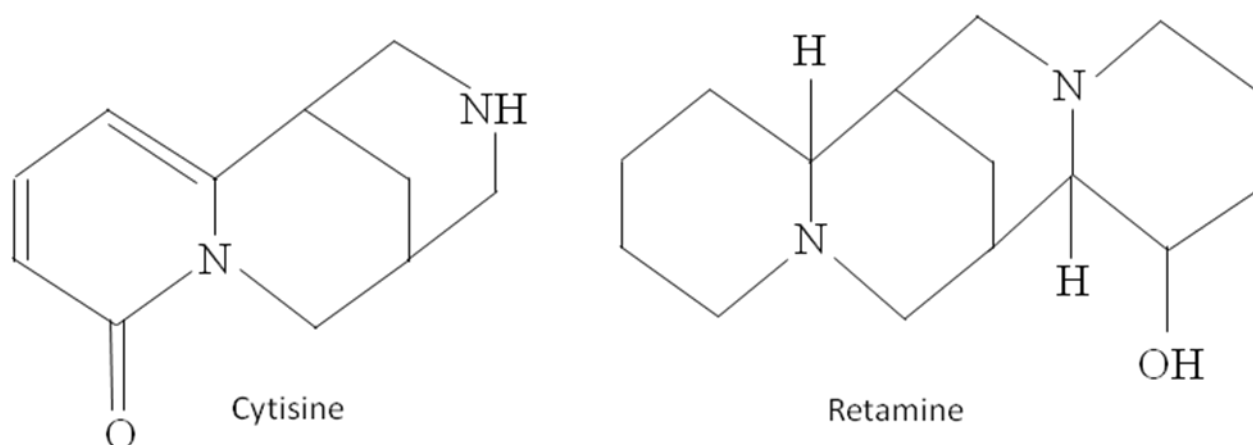


Figure 3. Structures of Cytisine and Retamine (Orhan *et al.*, 2007).

2.7. Biological activities

Diverse biological activities were attributed to *R. sphaerocarpa*; hence flavonoids such as rhamnazin extracted from the aerial parts of this plant showed a cytotoxic activity and produced a dose-dependent inhibition of cell growth (López-Lázaro *et al.*, 2000). The leaves of *Retama sphaerocarpa* were reported to have antimicrobial properties against *Staphylococcus aureus* (Louaar *et al.*, 2007). Moreover, several studies investigated Retama Genus for various pharmacological effects, including hypoglycemic, diuretic (Maghrani *et al.*, 2005b), antioxidant, antiviral (Edziri *et al.*, 2010), antihypertensive (Eddouks *et al.*, 2007) and anti-inflammatory (Bremner *et al.*, 2009) activities.

3. Antioxidants

3.1. The concept of free radicals and antioxidants

A free radical is a chemical species that has an odd number of electrons, highly unstable and active toward chemical reactions (Jensen, 2003; Wu *et al.*, 2013) and seek to stabilize themselves by “stealing” electrons from other chemicals or compounds (including proteins, carbohydrates, lipids and DNA) (Talaulikar and Manyonda, 2011). The free radical species are called reactive oxygen species (ROS). They are generated naturally in the human body and additional free radicals can be developed when we’re exposed to environmental toxins, pollutants and others, radicals derived from oxygen represent the most important class of such species generated in living systems (Valko *et al.*, 2004). ROS includes radicals such as anion superoxide

($\text{O}_2\bullet^-$), hydroxyl radical ($\bullet\text{OH}$), nitric oxide (NO), and other species like hydrogen peroxide (H_2O_2) (Sen *et al.*, 2010).

An antioxidant is a compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate (Matkowski, 2008). Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron-“stealing” reaction (Taulikar and Manyonda, 2011). The antioxidants can have various mechanism of action for instance: the scavenging of reactive oxygen species (ROS); prevention of ROS formation and chain breaking effects (Gopi *et al.*, 2014). The antioxidant compounds can be recycled in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances (Blassan *et al.*, 2014).

3.2. Natural antioxidants

The human organisms possess antioxidant defense systems (endogenous) that deal with reactive oxygen species; it is divided into two major groups: enzymatic antioxidants and non-enzymatic antioxidants (Yapo *et al.*, 2013). Despite its remarkable efficiency, this endogenous antioxidant system does not suffice. humans depend on various types of antioxidants that are present in the diet to maintain free radical concentrations at low levels like: vitamins C and E that are generic names for ascorbic acid and tocopherols (Barros *et al.*, 2011), other vitamins are also important like vitamin E and vitamin K (Vervoort *et al.*, 1997; Carocho and Ferreira, 2013). Phenolic compounds are also an important antioxidants present in plants they are secondary plant metabolites possessing an aromatic ring bearing one or more hydroxyl groups (Balasundram *et al.*, 2006). They are considered as the most widely occurring groups of phytochemicals, and are of considerable physiological and morphological importance in plants (Popa *et al.*, 2008; Naczka and Shahidi, 2006), many of the phenolic compounds are considered as strong antioxidants like flavonoids that the antioxidant properties is conferred to their phenolic hydroxyl groups attached to ring structures. Some of the most important flavonoids are catechin, quercetin and kaempferol (Rice Evans *et al.*, 1996; Prochazkova *et al.*, 2011).

3.3. Detection of antioxidants in plants

The presence of antioxidants in plants can be detected by different methods like spectrophotometric experiments, or by more specified complex analysis like High performance liquid chromatography (HPLC) and gas chromatography (GC). However, it is impossible to get all the antioxidant compounds in a plant by one or two types of experiments because there are

other conditions to take in consideration, like the preparation of plante samples, the type of solvent, the extraction method and others. Recent technological advances and the development of new methods to improve the production, detection, isolation and/or characterization have revolutionized the screening of bioactive compounds such as antioxidants (Gil-Chávez *et al.*, 2013). Indeed, in recent publications the chromatographic determination of antioxidants predominates, frequently accompanied by bioactivity-guided fractionation, spectral structure elucidation of isolated chemicals by MS or NMR. The use of hyphenated techniques such as LC-MS and LC-NMR is considered to be the best means for structure determination when novel compounds are present in vitro that have not been known from intact plants (Tian *et al.*, 2005; Sanchez-Sampedro *et al.*, 2007; Matkowski, 2008).

4. Diabetes mellitus (DM)

The word ‘diabetes’ is derived from the Greek word “Diab” (meaning to pass through, referring to the cycle of heavy thirst and frequent urination); ‘mellitus’ is the Latin word for “sweetened with honey” (presence of sugar in the urine) (Tiwari *et al.*, 2013). This disease can be defined as a syndrome characterized by a state of chronic hyperglycemia, DM and its complications like the cardiovascular disorders are the fourth most important causes of mortality and the principal cause of irreversible blindness (Perez *et al.*, 1998). The incidence of this disease affects 1–2% of the population worldwide (Ahmed, 2005). DM can be divided into two types that are: type 1 diabetes also known as Insulin-dependent DM and Type 2 diabetes mellitus, formerly called non-insulin-dependent DM (American diabetes association, 2008; Li and Hölscherb, 2007).

4.1. Oxidative stress and DM

Different studies assessed the role of the oxidative stress in the prognosis of diabetes and diabetic complications (Baynes and Thorpe 1999; Giacco and Brownlee 2010). Generally, these studies attribute the high oxidative stress level in diabetes to the imbalance between the production of the reactive oxygen species and the decline in the endogenous antioxidants in different tissues. The magnitude of such imbalance contributes to the severity of the oxidant-mediated damage present in the diabetic context (Halliwell, 1994). Brownlee suggested the model that reactive oxygen species initiate the development of diabetic complications in his unifying hypothesis of diabetes. He hypothesized that the overproduction of the reactive oxygen species (ROS) like superoxide anion $O_2^{\bullet-}$, in the mitochondrial electron-transport chain of glucose treated

cells may alter important biochemical reactions responsible for diabetic complications development (Brownlee, 2005).

4.2. Plants as a natural medicine used for therapy of DM

Before the discovery of antidiabetic drugs, diabetic patients used medicinal plants and traditional medicine (Sewell and Rafieiann, 2014). Plants may operate through different mechanisms that may affect for example the blood sugar, Insulin, or also the pancreatic β cells (Bailey and Day, 1989).

The ethnobotanical information reports about 800 plants that may possess antidiabetic potential (Grover *et al.*, 2002; Jung *et al.*, 2006). Medicinal foods are prescribed widely even when their biologically active compounds are unknown, because of their effectiveness and availability (Dewanjee *et al.*, 2009; Patel *et al.*, 2012b). The World Health Organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective, with less or no side effects and are considered excellent candidates for oral therapy (Shokeen *et al.*, 2008).

Finding the promising candidates for the treatment of diabetes mellitus could be achieved through the evaluation of the anti-diabetic properties of different medicinal plant extracts, their fractions and isolated components, followed by preliminary phytochemical screening, and tests of their probable toxicities (Nasri and Shirzad, 2013; Bnouham *et al.*, 2006). Although various compounds with different mechanism of actions have been shown to reduce blood glucose, however, antioxidant activity of these plants has a crucial role in their antidiabetic actions (Nasri and Rafieian, 2014; Bahmani *et al.*, 2014). The phytoconstituents that may act positively against diabetes are belonging to different classes of metabolites in plants such as quinolizidine alkaloids; flavonoids; terpenoids and others. These constituents may be considered as a promising source of hypoglycemic agents with minimal side effects (Sarkhail *et al.*, 2010; Mohammadi *et al.*, 2010; Patel *et al.*, 2012). To recognize the most active candidates, pharmacological evaluation will be determined through measuring different biochemical parameters such as serum glucose, insulin, hemoglobin, lipid profile, serum urea and creatinine, plasma aspartate transaminase and alanine transaminase. The antidiabetic properties will also be further confirmed through microscopical examinations of the pancreatic sections (Baradaran *et al.*, 2013).

Chapter 02

MATERIALS AND METHODS

Chapter 02

Materials and methods

1. Plants

The bark and leaves of *Rhamnus alaternus* were collected in 2010 from Teniet En Nasr (Bordj Bou Arreridj, Algeria), the climate of this region is semi arid with a cold winter and a hot summer and knows as a forest place characterized by the abundance of trees and herbaceous plants (DPSB-BBA, 2014). whereas *Retama sphaerocarpa* fruits and stems were collected in 2011 from Ras El Aioun (Batna, Algeria) and this region is characterized by a semi-arid climate, the temperature change between day and night; summer and winter, hence in summer it can reach up to 38°C as maximum especially in August, whereas in winter it may go down into 0°C especially in January; the quantity of rain that falls is between 200-350 Mm per year considered very few for vegetation growing; (Messaoudene, 2009). The identification of the two plants was based on the work of Quezel and Santa (1963), and validated by botanists in the Department of Ecology and Plant Biology at Setif 1 University (Algeria). The plants (figure 4) were dried and stored at room temperature until use.

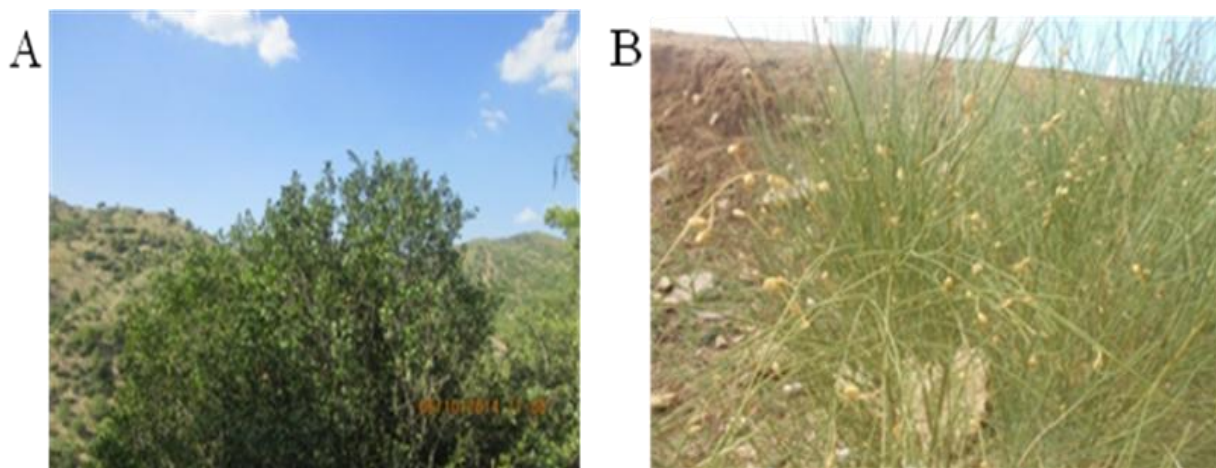


Figure 4. Plants pictures in their environment. **A:** *Rhamnus alaternus* in Teniet En Nasr; **B:** *Retama sphaerocarpa* in Ras El Aioun. (2014).

2. Animals

Male and female Wistar rats weighing between 150 and 180g were obtained from Pasteur Institute (Algiers, Algeria). They were housed under standard environmental conditions (temperature $28\pm 1^{\circ}\text{C}$ photoperiod: 12 h light and 12 h dark cycle). The animals were allowed free access to water and ad libitum standard laboratory diet.

3. Chemicals

Unless otherwise stated, all reagents and solvents were of analytical grade and used without further purification. High-performance liquid chromatography (HPLC) grade methanol, fluorescein (FL) sodium nitrite, Folin-Ciocalteu phenol reagent and hydrochloride acid were purchased from Carlo Erba (Milan, Italy). High-performance liquid chromatography grade water and acetonitrile, aluminum chloride anhydrous and potassium peroxodisulfate were from VWR. Pure reference standards daidzein and genistein were purchased from Extrasynthese (Lyon, France). All other reagents, if not specified, were purchased from Sigma-Aldrich (Milan, Italy).

4. Extraction procedure

The extraction procedure is summarized in figure 5. The methanolic and aqueous extracts from *R. alaternus* bark and leaves and *R. sphearocarpa* fruits and stems were prepared by maceration. Briefly, drug samples were powdered by a mill and 50g of the powdered drugs were mixed with 500ml of methanol or water and then left for 24 hours under occasional stirring. Furthermore, a traditional aqueous extract was prepared from both plants by decoction: Boiling 10g of the powdered drug in 500ml of water for 10min (Beloued, 2005), then to obtain the dried extracts, the mixtures were filtered, and the filtrates were evaporated to dryness under vacuum by using a rotavapor (Büchi). The residues were kept into brown vial until use (Gnanaprakash *et al.*, 2010; Thanabhorn *et al.*, 2006). For the chemical and biochemical assays, the appropriate volumes of methanol or water were used to redissolve the methanolic, aqueous and traditional extracts respectively. All solutions were freshly prepared before experiments and used immediately.

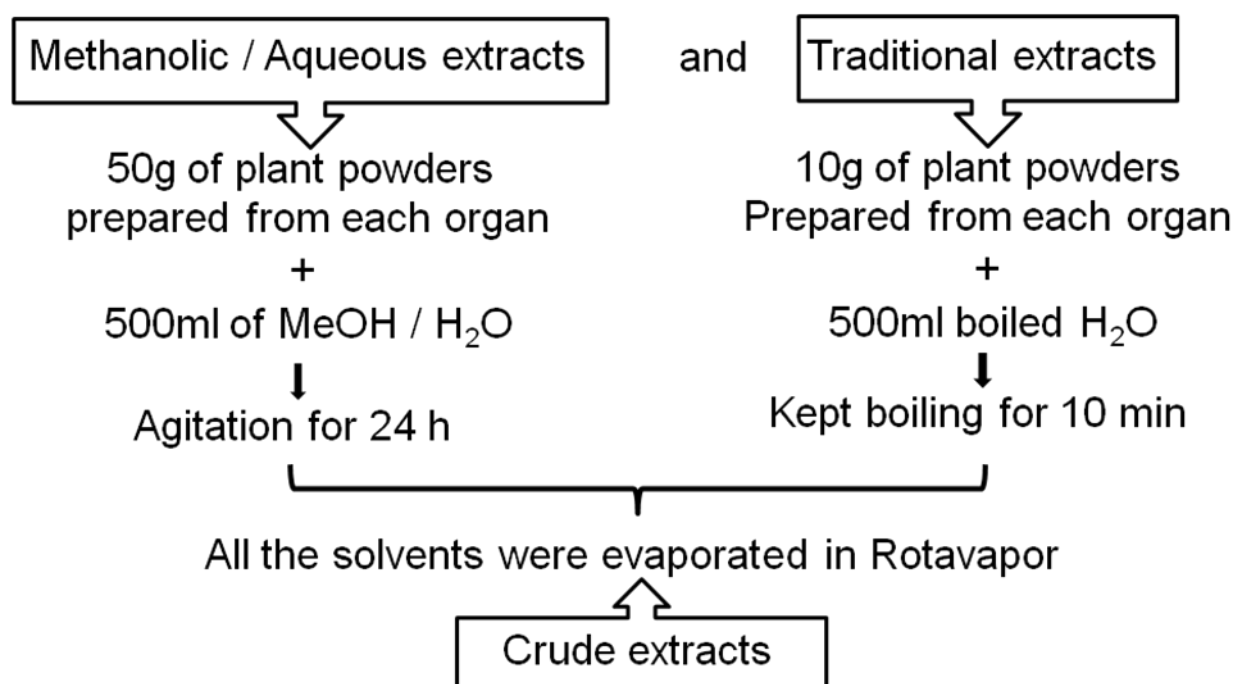


Figure 5. Extraction procedure

5. Folin-Ciocalteu colorimetric method

The antioxidant capacity (expressed as total phenols content) of the extracts was determined by means of the Folin-Ciocalteu reagent (Spagna *et al.*, 2002; Cimino *et al.*, 2007). 50µl of the solutions containing different concentrations of the extracts to be tested were added to 450µl of deionized water; 500µl of Folin-Ciocalteu reagent and 500µl of 10% aqueous sodium carbonate solution; samples were then maintained at room temperature for 1h. Absorbance was measured at 786nm (UV-Vis Spectrophotometer, Shimadzu Japan) against the blank containing 50µl of the same solvent used to dissolve the extracts. Total phenolic content was expressed as µg of gallic acid equivalents per mg of extract, using calibration curve prepared with gallic acid standard solutions. Each determination was performed in duplicate and repeated at least two times.

6. Total flavonoids content

The flavonoid content was measured using the colorimetric assay with little modification in the procedure reported by Lenucci and coworker (2006). Aliquots (50µl) of the methanol/water extracts were diluted with distilled water to a final volume of 0.5ml, and 30µl of 5% NaNO₂ were added. After 5min the mixture was added with 60µl of 10% AlCl₃, consequently after 6min, with

200µl of 1M NaOH and 210µl of distilled water. Absorbance was recorded at 510nm using a UV- visible spectrophotometer (Shimadzu, Japan) and flavonoid content was expressed as µg of catechin equivalents (CatE) per mg of extract. Each determination was carried out in duplicate and repeated at least two times.

7. Total flavonols content

The content of flavonols was determined by the method described by Tomaino and coworker (2010). Aliquots (125µl) of the solutions containing the different extracts to be tested were mixed with 125µl of AlCl_3 (2mg/ml), and 750µl of sodium acetate (50mg/ml). The absorbance at 440nm was detected by a spectrophotometer after 2.5h. Total flavonol content was expressed as µg of quercetin equivalents (QE) per mg of dried extract. All determinations were carried out in duplicate and repeated at least two times.

8. Determination of condensed tannins

In presence of concentrated H_2SO_4 , condensed tannins were transformed by the reaction with vanillin to anthocyanidols. The experiment is previously described by Tounsi and coworkers (2009). 50µl of each extract appropriately diluted was mixed with 3ml of 4% methanol vanillin solution and 1.5ml of H_2SO_4 . After 15 min, the absorbance was measured at 500nm. Condensed tannin contents of the extracts (three replicates per treatment) were expressed as ng catechin equivalents (CE) per milligram of extract through the calibration curve of catechin.

9. Thin layer chromatography (TLC) analysis of the extracts

Thin layer chromatography (TLC) analyses were performed as previously described by Sakulpanich and coworker (2009). The TLC fingerprints were performed on a precoated aluminum plate of silica gel 60 F₂₅₄ (10x20cm) using ethyl acetate: methanol: water (100:17:13) as the mobile phase. The developing distance was 8.0cm. After removing the plate from the chamber, the plate was dried with an air dryer, and sprayed with 10% alcoholic potassium hydroxide solution. Anthraquinones show pink spots.

10. Flavonoid analysis by high-performance liquid chromatography-diode array detector/electrospray ionisation mass spectrometry (HPLC-DAD/ESI-MS)

In this section two methods were used; the first was for *R. alaternus* bark and *R. sphaerocarpa* fruits extracts and the second for *R. sphaerocarpa* stems aqueous extract and *R. alaternus* leaves methanolic and aqueous extracts.

The solutions containing the different extracts (*R. alaternus* bark and *R. sphaerocarpa* fruits extracts) to be tested (10mg/ml) were filtered through a 0.45µm membrane filter (Whatman, Clifton, NJ, USA). HPLC-DAD/ESI-MS analyses of flavonoids were performed on a Prominence LC system (Shimadzu, Milan, Italy) consisting of a CBM-20A controller, two LC-20AD pumps, a CTO-20AC, a DGU-20A₃ degasser, a SIL-20AC autosampler, a SPD-M20A photo diode array (PDA) detector, and a quadrupolar mass analyzer (LCMS-2020), equipped with an electrospray ionization (ESI) interface, operated in the negative mode. Data acquisition was performed by Shimadzu LabSolution software ver. 5.10.153.

Separation was carried out on an Ascentis Express C18 column, 15 cm x 4.6 mm i.d. packed with 2.7 µm partially porous particles (Supelco, Bellefonte, PA, USA). The injection volume was 5µl, and the mobile phase consisted of water/acetic acid (0.075%) at pH=3 (solvent A) and acetonitrile/acetic acid (0.075%) (solvent B), respectively in the following linear gradient mode: 0min, 0% B; 60min, 40% B; 70min, 100% B; 71min, 0% B. The mobile phase flow rate was 1.0ml/min, and it was splitted to 0.2ml/min prior to MS detection. PDA wavelength range was 190-400nm and the chromatograms were extracted at 280nm

The quantitative determination of each compound was carried out by means of the external standard method using kaempferol; quercetin, Apigenin; Luteolin; Genistein; Daidzein as reference compounds in a concentration range of 1-150ppm, The results were obtained from the average of three determinations and are expressed as mg/g dried extract ± percent relative standard deviation (%RSD).

For the analytical determinations of *R. sphaerocarpa* stems aqueous extract and *R. alaternus* leaves methanol and aqueous extracts; variable aliquots of the powders were accurately weighted and solved in HPLC grade water immediately prior analyses to obtain at least three samples for each extract (10mg/ml). Quantification was carried out using daidzein derivatives (correlation coefficient $R_2 = 0.9998$), whilst the other signals were quantified using genistein ($R_2 = 0.9999$). Analyses were always done in triplicate. LC-UV-vis-DAD-MS experiments were performed on a

Waters instrument equipped with a 1525 Binary HPLC pump, a Micromass ZQ with a ESI Z-spray source, and a 996 Photo Diode Array Detector (DAD). The extracts were analysed using solvent system A (2.5% formic acid in water) and B (2.5% formic acid in acetonitrile) with the following gradient: 0min: 5% B; 10min: 15% B; 30min: 25 % B; 35min: 30% B; 50min: 90% B; then kept for 7min at 100% B. The solvent flow rate was 1ml/min, the temperature was kept at 25°C with a column oven (Hitachi L-2300, VWR International, Milan, Italy), and the injector volume selected was 20µl.

DAD analyses were carried out in the range between 800 and 190nm, setting the detector at 245 and 260nm for isoflavones and 350nm for flavonols. Collected data were processed through a MassLynx v. 4.00 software (Waters S.p.A. Milano, Italy).

11. Gas chromatographic (GC) separation coupled to a mass spectrometer (MS) and a flame ionization detector (FID) analyses of alkaloids in *R. sphareocarpa* stems methanolic extract (RSM)

For the GC/MS analyses, a small aliquot (50mg) of the methanolic extract was put in an 8ml amber vial and 1ml HCl was added. The solution was vigorously shaken (250rpm) for 10 minutes at room temperature. Then the pH value was brought to 8 with TEA (triethylamine), 1ml CH₂Cl₂ was added and the two layers left to separate for 5 minutes. The organic layer was then removed and dried over anhydrous Na₂SO₄. For the GC/MS analyses, 4µl of the sample was taken and injected. Gas chromatographic (GC) analyses were run on a Hewlett-Packard gas-chromatograph mod. 5890, equipped with a flame ionization detector (FID). GC-FID analyses were carried out in the following analytical conditions: ZB-5 capillary column (30m × 0.25mm i.d. × 0.25µm film thickness); helium as carrier gas; injector and detector temperature were set at 250 and 300°C, respectively. Oven temperature was programmed as follows: 0min. 100°C, then kept at 100°C for 1min; from 100 to 150°C at 5°C/min, then kept at 150°C for a further 2min; from 150 to 300°C at 3°C/min, then kept at 300°C for a further 10 minutes.

Gas chromatography-mass spectrometry (GC-MS) was carried out on the same gas chromatograph connected to a Hewlett-Packard mass spectrometer model 5971A, ionization voltage 70eV, electron multiplier 1700V; ion source temperature 180°C. Gas chromatographic conditions were the same as above.

The identity of components was based on their GC retention time, and computer matching of spectral MS data with those from Wiley 275 library, and the comparison of the fragmentation patterns with those reported in literature (El-Shazly *et al.*, 1996).

12. Antioxidant activity

12.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) test

The free radical-scavenging capacity of the extracts was determined by the DPPH assay (Rapisarda *et al.*, 1999; Morabito *et al.*, 2010), a method based on the reduction of the stable radical 1,1-diphenyl-2-picrylhydrazyl. The reagent mixture consisted in 1.5ml of 100mM DPPH• in methanol, to which 37.5µl of various concentrations of the extracts to be tested were added; an equal volume of the solvent employed to dissolve the extracts was added to control tubes. After 20min of incubation at room temperature, the absorbance was recorded at 517nm in a UV-Vis spectrophotometer (Shimadzu, Japan). Results were expressed as mmol trolox equivalents/g of dried extract, using calibration curve prepared with trolox as standard solutions. Each determination was performed in duplicate and repeated at least two times.

12.2 ABTS^{•+} radical assay

The ability of the extracts under study, to scavenge the ABTS^{•+} radical was evaluated as described by Morabito and coworkers (2010). Briefly, this method determines the capacity of antioxidants to quench the ABTS^{•+} radical. The ABTS^{•+} radical cation was produced by the oxidation of 1.7mM ABTS^{•+} (2,2'-azinobis-(3-ethylbenzothiazine- 6-sulfonic acid) with potassium persulphate (4.3mM final concentration) in water. The mixture was allowed to stand in the dark at room temperature for 12-16h before use, and then the ABTS^{•+} solution was diluted with phosphate buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7±0.02 at 734nm. 50µl of a solution containing different concentrations of the extracts to be tested or of the vehicle alone (methanol or water) were added to 2ml of the ABTS^{•+} solution, and the absorbance was recorded at 734nm in a UV-Vis spectrophotometer (Shimadzu, Japan) after allowing the reaction to stand for 6min in the dark at room temperature. Each determination was performed in duplicate and repeated at least two times. Results were expressed as mmol trolox equivalents/g of dried extract, using calibration curve prepared with trolox standard solutions.

12.3 Ferric reducing/antioxidant power (FRAP) assay

The ferric reducing ability of the extracts under study was evaluated according to the method described by Cimino and coworkers (2013), with some modifications. The method is based on the reduction of a ferric–tripyridyltriazine complex to its colored ferrous form in the presence of antioxidants. The fresh working solution (FRAP reagent) was prepared by mixing

2.5ml of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (prepared in 40mM HCl), with 25ml of 0.3M acetate buffer (pH 3.6), and 2.5ml of 20mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution, and then warmed (preheated) at 37°C before use. 50 μl of a methanolic or water solution containing different concentrations of the extracts to be tested, or of the vehicle alone (methanol or water), were added to 1ml of FRAP reagent, and the absorbance was measured at 593nm in a spectrophotometer (Shimadzu, Japan) after incubation at 20°C for 4min. A standard curve was prepared using various concentrations of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Each determination was performed in duplicate and repeated at least two times, and results were expressed as mmol Fe^{2+} /g of dried extract.

12.4 Oxygen radical absorbance capacity assay using fluorescein (ORAC-FL) assay

The ORAC-FL assay was performed as described by Dávalos and coworkers (2004). This assay is based upon the effect of peroxy radicals generated from the thermal decomposition of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) on the signal intensity from the fluorescent probe fluorescein (FL) in the presence of an oxygen-radical absorbing substance. The reaction was carried out in 75mM phosphate buffer (pH 7.4), and the final reaction mixture was 2ml. An aliquot of the solution containing different concentrations of the extracts to be tested (200 μl), and 1.2ml of FL solution (70nM, final concentration) were placed in cuvette. The mixture was pre-incubated for 15min at 37°C in the spectrofluorimeter. Freshly prepared AAPH solution (600 μl ; 12mM, final concentration) was rapidly added. Fluorescence was recorded every minute for 80min at 37°C; the fluorescence conditions were as follows: excitation at 485nm, and emission at 520nm. A blank containing FL and AAPH with only the solvent employed to dissolve the extracts, different calibration solutions using Trolox (1-7.5 μM , final concentration) as reference antioxidant, were also carried out in each assay. All the reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC was calculated by subtracting the AUC of the blank. The final ORAC values were determined by linear regression equation of trolox concentrations, and expressed as mmol Trolox equivalents/g dried extract.

13. Cell culture

13.1 Human monocytic leukemia cells U937

Human monocytic leukemia cells U937, were cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco), 1% L-glutamine, and 1% Penicillin-Streptomycin. The cultures were maintained in a humidified incubator with 5% CO₂ at 37°C.

13.2 Human peripheral blood mononuclear cells (PBMC) isolation

PBMC were isolated from heparinised blood from healthy volunteer donors by centrifugation on a Ficoll–Hypaque (Bio-Lynx, Gibco) density gradient. Cells were washed 2x with phosphate buffered saline (PBS) (pH 7.2) supplemented with 2% fetal calf serum (FCS) and re-suspended in the growth medium consisting of RPMI-1640 supplemented with 10% FCS, and 1% penicillin/ streptomycin (Awah *et al.*, 2010).

13.3 Trypan blue dye exclusion assay

PBMCs were seeded in 48-well cell culture plates at the density of 5.0×10^4 cells/well; U937 cells were seeded in 48-well cell culture plates at the density of 4.0×10^4 cells/well. Medium containing various concentrations of the extracts (from 0.5 to 100 µg/ml) or taxol (from 5 to 100 µg/ml) were applied onto culture wells, and the cells were further cultured for 24h. Control cells were treated with 0.1% DMSO. Taxol was used as reference cytotoxic compound. After 24 h, cells were collected and cell number was counted in the hemocytometer by the trypan blue exclusion assay (Cimino *et al.*, 2014); for this 10 µl of the cells suspension was removed and mixed with an equal volume of trypan blue dye solution, and cell viability was determined by counting the unstained cells under light microscopy. Data were calculated as percent of cell viability by the following formula:

$$\% \text{ cell viability} = (\text{Number of cells in treated samples} / \text{Number of cells in control}) * 100$$

The average of cell viability obtained from triplicate determinations at each concentration was plotted as a dose response graph. The 50% inhibition concentration (IC₅₀) of the extracts or of taxol was determined as the drug concentrations that reduced cell number by 50% in treated compared to untreated cultures.

13.4 Evaluation of apoptosis/necrosis with annexin V-PE and 7-AAD flow cytometry assay

Flow cytometry studies by use of phycoerythrin-labeled annexin V (PE Annexin V) and 7-Amino-Actinomycin D (7-AAD) staining were performed into the U937 cells previously treated with *Rhamnus alaternus* bark methanolic extract for 24h. Briefly U937 cells were cultured with and without extract (50, 100, 200, 300, 400µg/ml) for 24h. After treatment, cells were harvested, washed twice with cold PBS, re-suspended in annexin binding buffer, stained simultaneously with 5µl of PE Annexin V and 5µl of 7-AAD. then the cells were gently vortexed and incubated for 15 minutes at room temperature (20-25°C) in the dark in according to instructions of producer (BD Bioscience, Euroclone), cells without exposure to the extract served as controls. Afterward, the samples were analyzed in flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA), the final data were analyzed using the BD FACSDiva software.

14. Acute oral toxicity study of *R. alaternus* bark methanolic extract

The acute oral toxicity study of *Rhamnus alaternus* bark methanolic extract was conducted using test guide-lines on acute oral toxicity test 423 according to OCDE (2001) and as reported by Florence and his collaborators; 2014. Eighteen Wistar rats fasted overnight, but allowed free access to water ad libitum were randomly assigned into the following three groups of six rats of either sex (three males and three females). Control received distilled water and two groups received the extract at the doses of 2000mg/kg and 5000mg/kg. The rats were not fed for 3h following administration. The signs of toxic effects and/or mortality were observed 8h after administration then, for next 14 days (Florence *et al.*, 2014).

15. Advanced glycation end products (AGEs) formation assay

The formation of advanced glycation end products (AGEs) was assessed as previously described by Harris and coworkers (2011), with some modifications. Bovine serum albumin (1mg/ml) was incubated with 100mM glucose/100mM fructose, in 100mM sodium phosphate monobasic monohydrate buffer (pH 7.4) with methanol and/or distilled water vehicle (negative control), or with *Retama sphaerocarpa* fruits or stems extracts (experimental treatment), or with quercetin (positive control). Multiple concentrations of each extract (0.02-0.3µg/ml) or quercetin (5-10µg/ml) were tested in quadruplicate. The samples were prepared in sterile tubes and incubated for 7 days at 37°C on a mechanical shaker. Following incubation, fluorescence was

measured at excitation and emission wavelengths of 355nm and 460nm respectively in spectrofluorophotometer (RF-5301PC; SHIMADZU).

The corrected fluorescence readings (F) for the negative control (F_{negative}) and experimental treatments ($F_{\text{experimental}}$) were used to determine the percent inhibition of AGEs formation as follows:

$$\% \text{ inhibition} = [(100 - (F_{\text{experimental}} * 100)) / (F_{\text{negative}})]$$

IC₅₀ values were defined as the amount of extract or compound ($\mu\text{g/ml}$) required to reduce AGEs formation by 50%.

16. Acute oral toxicity study of *R. sphaerocarpa* stems methanolic extract (RSM)

The Wistar rats were randomly assigned into five groups of six animals in each and were given the extract orally at the doses of 2000mg, 3000mg, 3500mg, 4000mg, and 5000mg/kg body weight. Behavior and toxic signs were observed continually 8h after administration then, for next 14 days. The number of dead animals in each group was counted graphically by probit analysis to estimate the lethal dose responsible of 50% of animal's death (LD₅₀) (Félicien *et al.*, 2013).

17. Evaluation of RSM effect on normal healthy rats

This study was carried out using the method cited by Karuppusamy and Thangaraj (2014), with some modifications. At the end of the fasting period, taken as zero time (0h), blood was collected from the tail vein of the animals and glucose was estimated using a glucometer (On Call Plus, USA). The animals were then randomly divided into three groups of six animals each. Group I served as control and received distilled water. Groups 2 and 3 and 4 received RSM orally at the dose of 20, 50mg and 100mg/kg. Blood glucose levels were determined using a glucometer (On Call Plus, USA) in 1, 2, 3 and 4h following the treatment.

18. Evaluation of RSM effect in oral glucose tolerance test

The oral glucose tolerance test was performed following the method described by Karuppusamy and Thangaraj (2014), with some modification, after healthy rats were divided into four groups of six animals each: group 1 served as control received only vehicle (distilled water) and groups 2; 3 and 4 received RSM orally at the doses: 20mg; 50mg and 100mg/kg, respectively. All the animals were given glucose (2g/kg) 60min after dosing. Blood samples were

collected from the tail vein and glucose level was estimated using a glucometer just prior to (0h) and at 30, 60, 90 and 120min after the glucose loading.

19. Evaluation of RSM effect in streptozotocin (STZ)-induced diabetic rats

19.1. Induction of diabetes to the rats

Diabetes was induced as described by Oyedemi and coworkers (2011a), with some modification. Overnight fasted male Wistar rats were treated with a single intraperitoneal injection of freshly prepared solution of streptozotocin (55mg/kg of body weight) dissolved in physiological saline solution. The injection volume was 1ml/kg. Control rats were injected with same physiological solution only. After 96h of injection, the blood glucose levels were measured using a glucometer (On Call Plus, USA). The animals with blood glucose levels above 250mg/dl were considered to be diabetic and used for the experiment.

19.2. Experimental design

The animals were divided into eight groups of six rats, the experiment was carried out in a daily treatment for 15 days, RSM was dissolved in distilled water, and all the groups were organized as the following:

Group I: normal control rats administered drinking water;

Group II: normal rats administered RSM (50mg/kg);

Group III: normal rats administered RSM (100mg/kg);

Group IV: normal rats administered glibenclamide (10mg/kg);

Group V: diabetic control rats administered drinking water;

Group VI: diabetic rats administered RSM (50mg/kg);

Group VII: diabetic rats administered RSM (100mg/kg);

Group VIII: diabetic rats administered standard drug glibenclamide (10mg/kg).

19.3. Effect of RSM on feed and water intake of the rats

Feed and water intakes were measured everyday at the same hour during the experimental period.

19.4. Determination of hematological parameters

The hematological parameters tested include red blood cells (RBC), hemoglobin (Hb), white blood cells (WBC) and hematocrit (PCV); the estimation was done using Medonic (Beckman coulter) for three samples in each group at the laboratory of Mouhab pharmacy (Setif).

19.5. Determination of biochemical parameters

Plasma triglycerides and plasma total cholesterol analyses for normal and diabetic rats were done spectrophotometrically for three samples in each group using standard kits at the laboratory of Mouhab pharmacy (Setif). Blood glucose level was estimated using a glucometer on days 1, 5, 10 and 15 of extract administration. The other biochemical parameters (triglycerides and cholesterol) were determined on day 15; for this the blood was withdrawn from the retro-orbital plexus of the eye under diethyl-ether anesthesia just before the sacrifice of the animals (Shirwaikar *et al.*, 2004).

20. Statistical analysis

All experiments concerning the antioxidant/radical scavenger capacity of the extracts under study were carried out in duplicate and repeated at least two times except for the LD₅₀ study. All values were expressed as mean \pm standard error (SD) and were analyzed by One-way analysis of variance (ANOVA) followed by the Tukey HSD test, and statistically significant findings were considered at P-value<0.05 and P-value<0.01, the final results were also expressed as equivalent of the standards used in each experiment. The LD₅₀ lethal dose of *R. sphaerocarpa* that killed 50% of the rats was calculated using probit analysis. IC₅₀ of AGE formation assay and the 50% inhibition concentration (IC₅₀) of the extracts and taxol implicated on U937 cancer cells and PBMCs normal cells were calculated using the software PHARM/PCS, Version 4, based on the Pharmacologic calculation (Tallarida and Murray, 1982).

Chapter 03

RESULTS AND DISCUSSION

Chapter 03

Results and discussion

1 *Rhamnus alaternus* L.

1.1. Extraction yields

The yields of the extraction method for *R. alaternus* plant are summarized in tables 1. From the results it can be notified that the traditional extracts of the bark and leaves gave the best yields (27.75%; 21.5% respectively) followed by the the leaves and stems methanolic extracts (17.4%; 14.48% respectively). The aqueous extracts seem to be less in yield (14.20%; 7.6%) for bark and leaves respectively. The high yield of the traditional extract can be explained by the fact that the traditional method of preparation is based on heating the water (decoction), and thus some compounds that require high temperature can be dissolved more in this extract. Dai and Mumper (2010) reported that high temperature of water could extract phenolic compounds in high amounts. However, the methanol gave also good yields; previous studies reported that pure methanol is one of the most effective solvents for recovering antioxidant components (Anwar *et al.*, 2011; Azizah *et al.*, 1999).

Table 1. Extraction yields of *Rhamnus alaternus* bark and leaves.

<i>Rhamnus alaternus</i> organs	Extracts	Yields (%)
Bark	Methanolic	14.48
	Aqueous	14.20
	Decoction	27.75
Leaves	Methanolic	17.4
	Aqueous	7.6
	Decoction	21.5

1.2. Total flavonoids, total flavonols and total tannins contents

In the following work figure 6 shows the catechin calibration curve used for the determination of total tannins content and table 2 summarize our results of the quantitative

determination of total flavonoids, total flavonols and total tannins of methanolic, aqueous and traditional extracts of *R. alaternus* bark and leaves. *R. alaternus* appear to be rich in phenolic compounds especially a large presence of flavonoids was notified. The methanolic extract of the bark showed a significant ($P<0.01$) higher amount of flavonoids, flavonols and tannins (142.69 ± 1.80 CE/mg, 51.17 ± 0.41 QE/mg, 231.64 ± 3.57 CE/mg of extract, respectively) as compared to the other bark extracts. Whereas among the leaves extracts the traditional one presented higher amount of tannins (25.39 ± 2.17 CE/mg of extract), but the methanolic leaves extract was found to be significantly more rich in total flavonoids and total flavonols (101.06 ± 4.26 CE /mg; 86.14 ± 4.36 QE/mg of extract, respectively). Comparing the two plant parts, the amounts differed between the bark and leaves in the sense that sometimes appearing more in the bark and sometimes more in the leaves. However, the high amount of flavonoids is present in the methanolic extract of the bark and also of the leaves comparing to other type of extracts, this may be due to the low water solubility of flavonoids, needing an organic solvent to be extracted from the vegetable matrix. Previous studies reported that *Rhamnus* species are rich in various phenolic substances, such as flavonoids (Ben Ammar *et al.*, 2008a), chalcones (Pandey *et al.*, 2008), naphthalene derivatives (Ng *et al.*, 2007). Other studies also reported that solvents with different polarity had significant effects on total phenolic contents (Xu and Chang, 2007).

Table 2. Total flavonoids, total flavonols and total tanins contents in *R. alaternus* bark and leaves

<i>Rhamnus alaternus</i> plant parts	types of extracts	Total flavonoids $\mu\text{g CE/mg}$ of extract	Total flavonols $\mu\text{g QE/mg}$ of extract	Total tanins ng CE/mg of extract
Bark	Methanolic	142.69 ± 1.80	51.17 ± 0.41	231.64 ± 3.57
	Aqueous	$34.46 \pm 0.94^{**}$	$24.09 \pm 0.85^{**}$	$42.78 \pm 0.37^{**}$
	Decoction	$86.30 \pm 3.88^{**}$	$12.03 \pm 0.88^{**}$	$32.29 \pm 1.94^{**}$
Leaves	Methanolic	101.06 ± 4.26	86.14 ± 4.36	12.05 ± 0.84
	Aqueous	$15.11 \pm 0.80^{**}$	$2.06 \pm 0.07^{**}$	$3.22 \pm 0.62^{**}$
	Decoction	$78.85 \pm 3.67^{**}$	$32.47 \pm 2.09^{**}$	$25.39 \pm 2.17^{**}$

Results were expressed as mean \pm SD of three experiments. **Versus methanolic extract of each plant part $P<0.01$; SD: Standard deviation; CE: Catechin equivalent; QE: Quercetin equivalent.

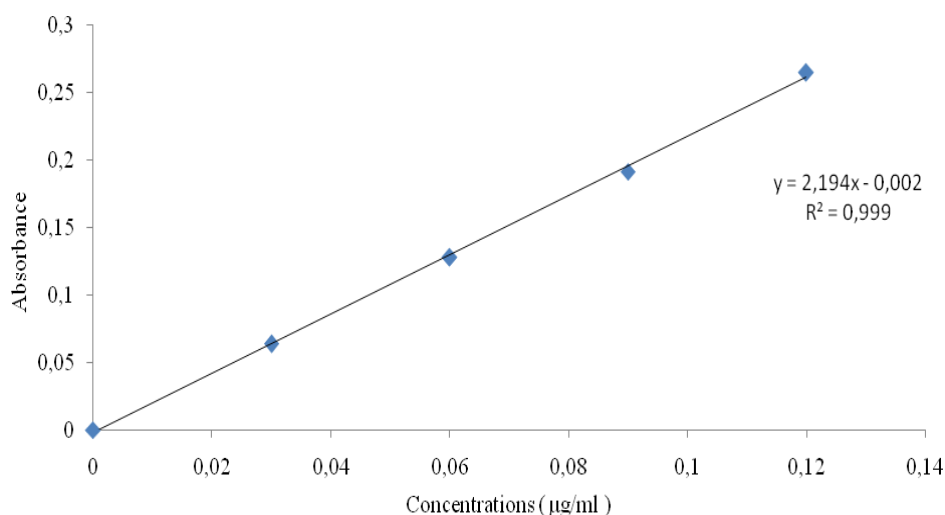


Figure 6. Calibration curve of catechin used for the determination of condensed tannins

1.3. Anthraquinones identification by TLC analysis

Rhamnus alaternus bark and leaves extracts were submitted to a simple preliminary TLC analysis. The fingerprint showed many pink spots in the plates of *R. alaternus* bark and leaves extracts (figure 7) indicating the presence of anthraquinones, from the first view the bark appear to be richer in anthraquinones then the leaves because there are more spots and at many different points in the developing distance of the three extracts. Whereas in the leaves extracts, the traditional one have more remarkable appearing spots, hence the TLC test confirm to us the presence of anthraquinones in the Algerian *R. alaternus*. This finding is truly proved by many other deep works but on *R. alaternus* collected from other countries, like Kosalec and his coworkers (2013) that studied the bark of *R. alaternus* collected from Croatia. In addition, other works on Lebanese *R. alaternus* come out to find many anthraquinones; we cite in this case the work of Abou Chaar and his coworkers (1980). It is important to have further studies to specify anthraquinones of the Algerian *Rhamnus alaternus* plant, and other Algerian *Rhamnus* species.

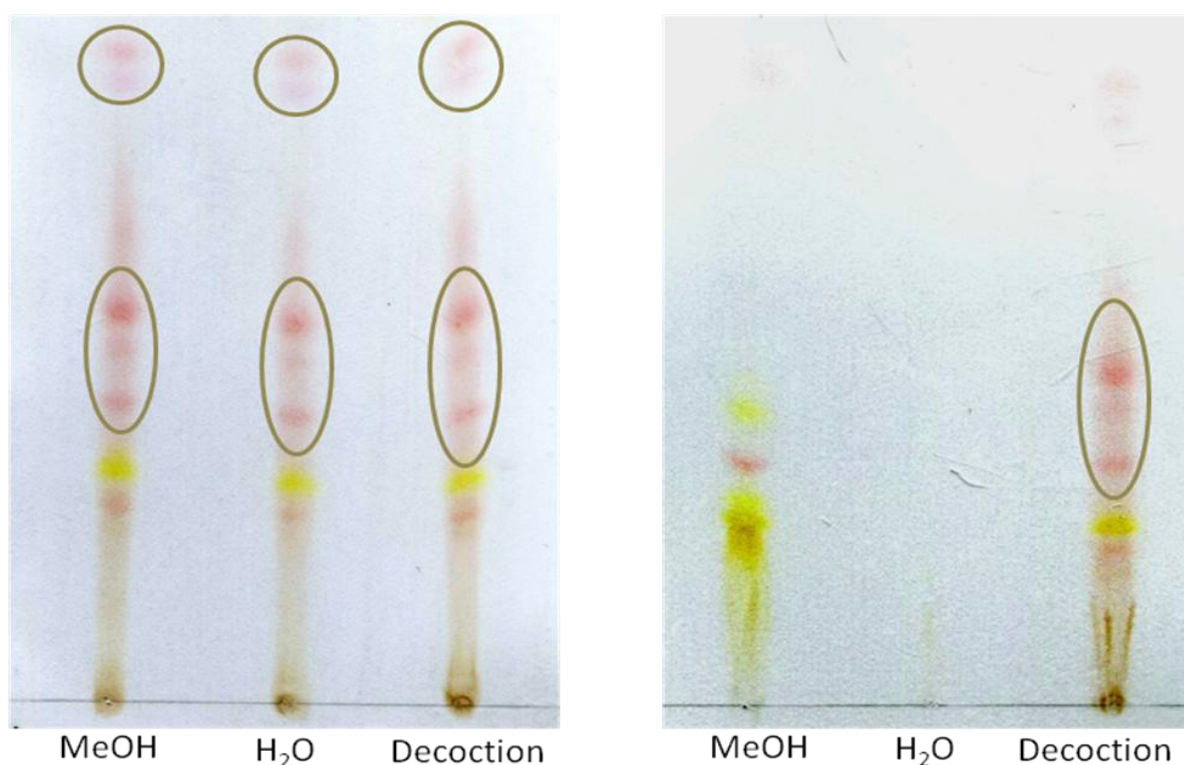


Figure 7. TLC fingerprint of A: *R. alaternus* bark extracts and B: *R. alaternus* leaves extracts sprayed with 10% alcoholic potassium hydroxide solution. MeOH: Methanolic extract; H₂O: Aqueous extract.

1.4. Identification and quantification of flavonoids

Characterization of the flavonoid content in *Rhamnus alaternus* bark extracts (Methanolic, aqueous and traditional) and leaves extracts (methanolic and aqueous) was accomplished by HPLC–DAD–ESI–MS. To our knowledge, no metabolic fingerprint studies have been done on this species so far. So, we report here for the first time the complete secondary metabolic fingerprint of *R. alaternus* leaves polar extract presented in figure 7 and table 3. The resulting chromatograms are shown in figure 9 and 10; the peak's numbers in the chromatograms correspond to the names in tables 4 and 5. Concerning the three extracts of the bark they showed a pretty similar flavonoid fingerprint with rhamnetin hexoside as main compound being of (9.25 ± 1.6 ; 6.57 ± 1.6 ; 4.85 ± 1.6 mg/g extract) respectively for methanolic, aqueous and traditional extracts. The second major compound was Kaempferol hexoside presenting up to (6.14 ± 3.0 mg/g) in the bark methanolic extract, other compounds were also present as quercetin derivative, rhamnocitrin hexoside. The three extracts conversely differed in the quantitative content, in fact, the methanolic extract presented a higher flavonoid content with respect to the aqueous (17.88 mg/g extract) and the traditional (14.21 mg/g extract) extracts. These data confirm those

already obtained by the spectrophotometric assay based on AlCl_3 -flavonol complexation showing that the flavonol content found in the methanolic extract was significantly higher than that measured in the aqueous extract and in the traditional one.

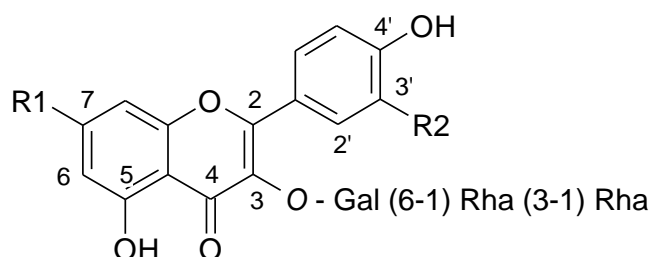


Figure 8. The chemical structures of the compounds aglycones found in *R. alaternus* leaves extracts, R1 and R2 are nominated in table 3 (Boussahel S., 2013; C.N.R; 2010).

Table 3. The chemical nomination of R1 and R2 appearing in figure 9. Peak numbers refer to figure 8.

Peak number	R ₁	R ₂
1	OH	OH
2	OH	H
4 (7)*	OCH ₃	OCH ₃
5	OCH ₃	H

*aqueous extract

Table 4. HPLC/MS identification and quantification of flavonoids contained in methanolic, aqueous and traditional extracts obtained from *Rhamnus alaternus* bark.

Peak	t _R (min)	Compounds (expressed as)	mg/g extract ± % RSD		
			Methanolic	Aqueous	Decoction
1	24,8	Quercetin hexoside	0.72±1.4	0.70±0.8	0.59±2.6
2	26.5	Kaempferol hexoside	6.14±3.0	4.70±2.8	3.90±2.4
3	29.0	Rhamnocitrin hexoside	1.93±2.5	1.11±0.4	1.00±2.7
4	35.5	Rhamnetin hexoside	9.25±1.6	6.57±1.6	4.85±1.6
5	36.9	Kaempferol derivative	1.97±2.0	1.58±4.5	1.30±4.2
6	40.3	Quercetin derivative	1.98±2.4	1.20±1.2	0.99±2.9
7	41.3	Quercetin derivative	2.40±4.5	2.02±3.4	1.58±1.1

Data are expressed as mean of three experiments±RSD. RSD: Relative standard deviation.

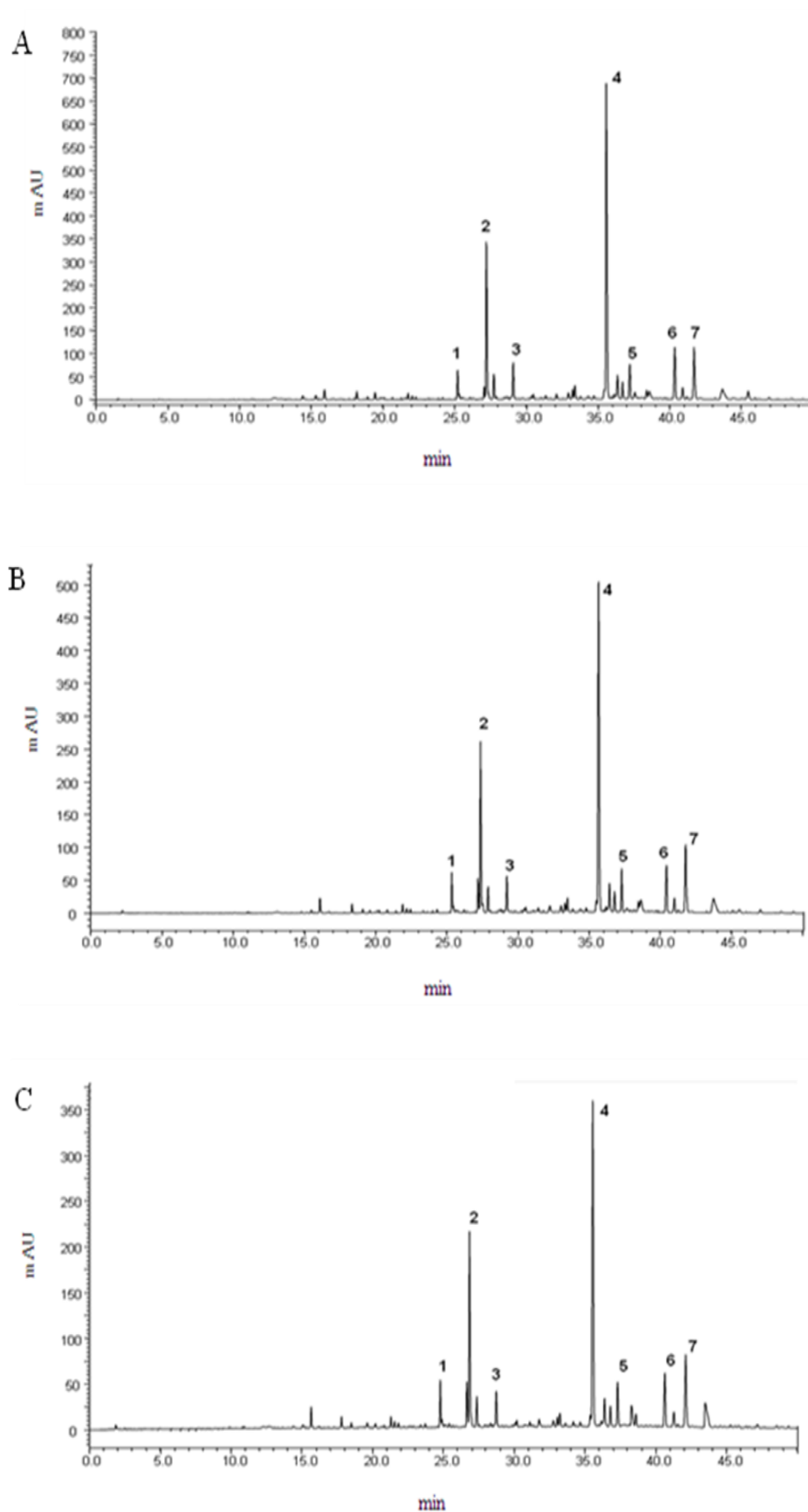


Figure 9. HPLC/MS representative chromatograms of flavonoids from *Rhamnus alaternus* bark (extracted at 280nm). A: methanolic extract, B: aqueous extract and C: traditional extract. The peaks numbers correspond to the names in table 4.

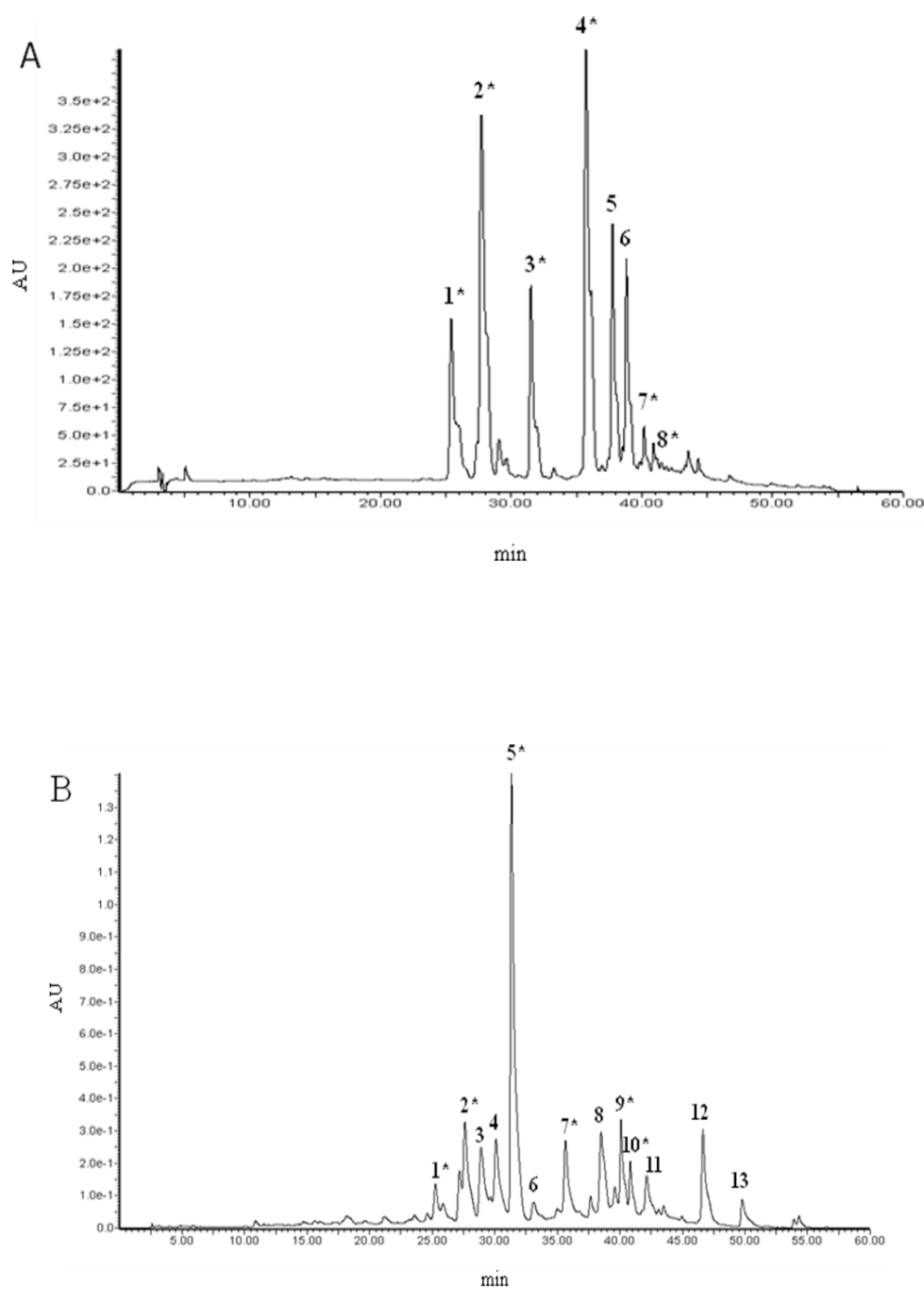


Figure 10. HPLC-PDA representative chromatograms of flavonoids from *Rhamnus alaternus* leaves (visualized at at 350nm). A: methanolic extract, B: aqueous extract. For peaks identification see table (the common peaks between the two extracts are highlighted), the peaks numbers correspond to the names in table 5.

Table 5. HPLC/Uv-vis-DAD/MS identification and quantification of flavonoids contained in the methanolic and aqueous extracts obtained from *R. alaternus* leaves.

Peaks	Names of the compounds	Area %	
		Methanolic	Aqueous
1	Quercetin 3- <i>O</i> -[α -L-rhamnopyranosyl (1 \rightarrow 3)- <i>O</i> - α -L-rhamnopyranosyl (1 \rightarrow 6)]- β -D-galactopyranoside	11.04	2.74
2	Kaempferol 3- <i>O</i> -[α -L-rhamnopyranosyl (1 \rightarrow 3)- <i>O</i> - α -L-rhamnopyranosyl (1 \rightarrow 6)]- β -D-galactopyranoside	27.60	13.38
3 (5)*	Kaempferol derivative	9.67	31.41
3	Quercetin derivative	-	5.43
4 (7)*	Rhamnetin 3- <i>O</i> -[α -L-rhamnopyranosyl (1 \rightarrow 3)- <i>O</i> - α -L-rhamnopyranosyl (1 \rightarrow 6)]- β -D-galactopyranoside	29.63	9.78
5	Rhamnocitrin 3- <i>O</i> -[α -L-rhamnopyranosyl (1 \rightarrow 3)- <i>O</i> - α -L-rhamnopyranosyl (1 \rightarrow 6)]- β -D-galactopyranoside	10.29	-
6	Quercetin derivative	9.38	-
6	Rhamnocitrin derivative	-	1. 83
7 (9)*	Kaempferol derivative	1.32	4.84
8 (10) *	Rhamnetin derivative	1.06	2.28
8	Rhamnetin derivative	-	7.21
11	Quercetin	-	3.27
12	Kaempferol	-	9.55
13	Rhamnetin	-	3.19

*Aqueous extract

Regarding *R.alaternus* leaves extracts, 8 peaks were tentatively identified in the chromatogram of *R. alaternus* leaves methanolic extract (figure 9). Hence peaks 2, 4 and 5 are kaempferol 3-*O*-[α -L-rhamnopyranosyl(1 \rightarrow 3)-*O*- α -L-rhamnopyranosyl(1 \rightarrow 6)]- β -D-galactopyranoside; rhamnetin 3-*O*-[α -L-rhamnopyranosyl(1 \rightarrow 3)-*O*- α -L-rhamnopyranosyl(1 \rightarrow 6)]- β -D-galactopyranoside and rhamnocitrin 3-*O*-[α -L-rhamnopyranosyl(1 \rightarrow 3)-*O*- α -L-rhamnopyranosyl(1 \rightarrow 6)]- β -D-galactopyranoside, respectively. In agreement with the work of Ben Ammar and coworkers (2009), we also identified peak 1 as quercetin 3-*O*-[α -L-rhamnopyranosyl(1 \rightarrow 3)-*O*- α -L-rhamnopyranosyl(1 \rightarrow 6)]- β -D-galactopyranoside. All the other peaks appear to be kaempferol, quercetin and rhamnetin derivatives. The major compound in the leaves methanolic extract was found to be rhamnetin 3-*O*-[α -L-rhamnopyranosyl(1 \rightarrow 3)-*O*- α -L-rhamnopyranosyl(1 \rightarrow 6)]- β -D-galactopyranoside (29.63 %) (Table 5). In the other hand the chromatographic pattern from *R. alaternus* leaves aqueous extract (figure 9) has several similarities with the previous one of *R. alaternus* leaves methanolic extract, including the presence of peaks 2 and 4 numbered in the chromatogram of the methanolic extract as 2 and 7 and identified according to the literature (Ben Ammar *et al.*, 2009). Flavones (quercetin, kaempferol and rhamnetin derivatives) kept dominating *R.alaternus* aqueous extract, which is also characterized by the presence of their corresponding aglycones (peak 13, 11 and 12, respectively).

Comparing between bark and leaves extracts we observed a similarity in the flavonoid composition characterized by the large presence of quercetin, kaempferol and rhamnetin derivatives, in all the extracts of both plant parts, further studies on the chemical composition of the traditional leaves extract is important. Recent papers also reported the presence of highly hydroxylated flavonoids (kaempferol, quercetin and rhamnetin) in the polar extracts from *R.alaternus* leaves (Ben Ammar *et al.*, 2009).

1.5. Antioxidant/radical scavenging activity

The extracts from *R. alaternus* bark and leaves were evaluated for antioxidant/radical scavenging capacity; for this we used a battery of different chemical assays for measuring the reducing capacity of the test extracts. The figures 11 and 12 respectively demonstrate the calibration curves for: gallic acid used in folin-Ciocalteau experiment and trolox used in DPPH assay, whereas figures 12 and 13 respectively shows the calibration curves of : trolox used in TEAK test and FeSO₄·7H₂O used in FRAP assay. The full results of antioxidant experiments are summarized in tables 6 and 7.

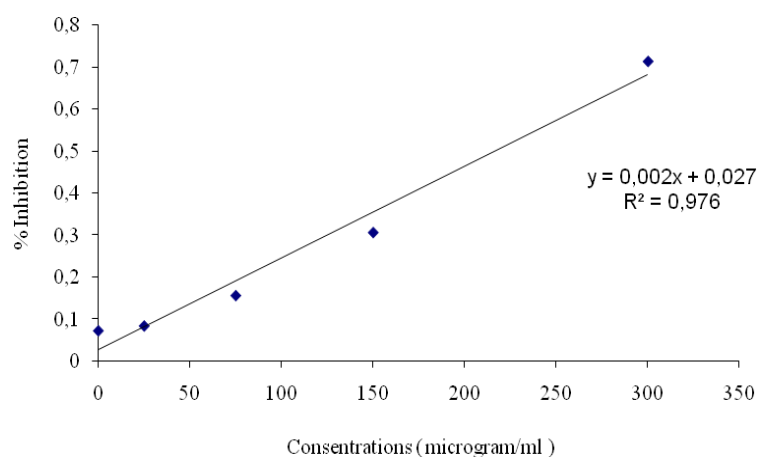


Figure 11. Gallic acid calibration curve used in folin-Ciocalteau experiment

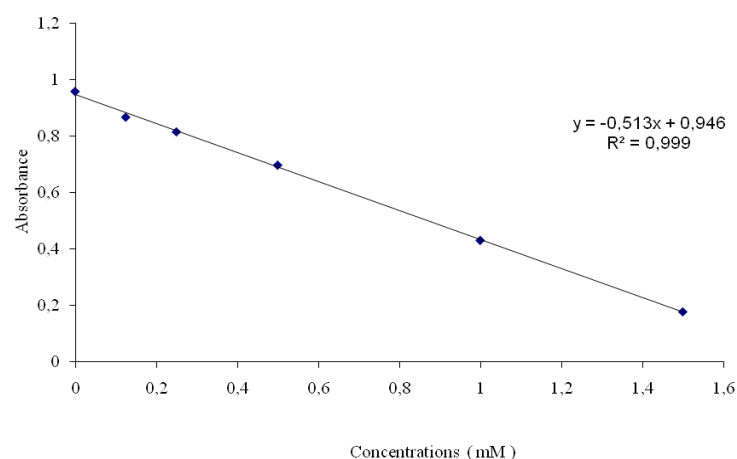


Figure 12. Trolox calibration curve used in 1,1-diphenyl-2-picrylhydrazyl (DPPH•) test

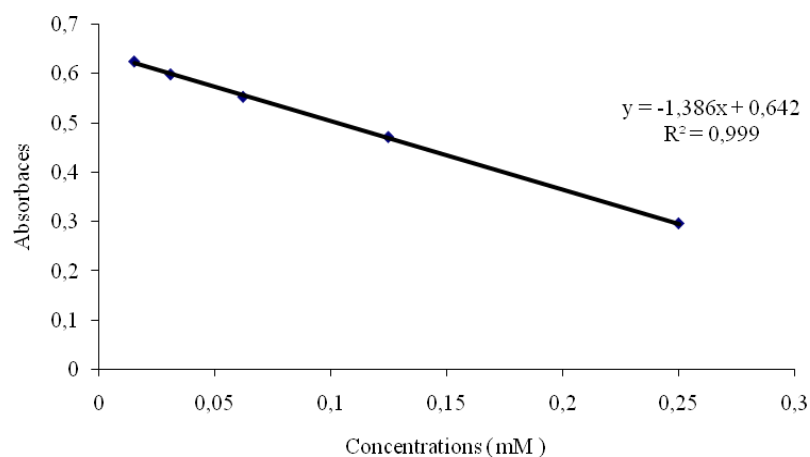


Figure 13. Trolox calibration curve used in ABTS•+ radical assay

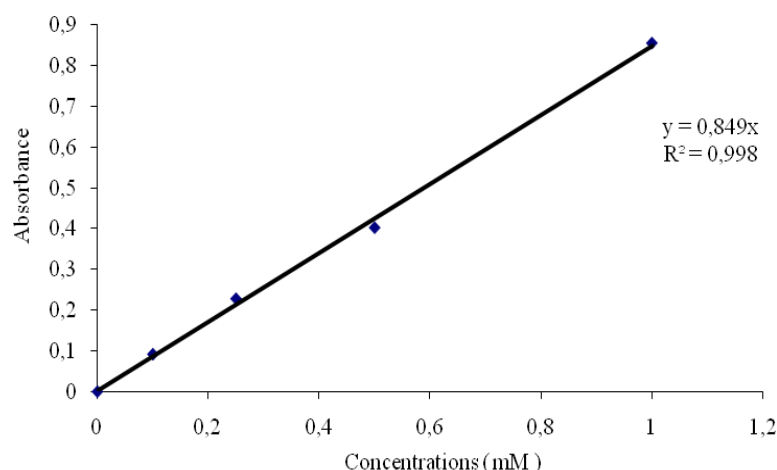


Figure 14. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ calibration curves used in ferric reducing/antioxidant power (FRAP) assay.

Table 6. Total phenolics content and antioxidant activity of *Rhamnus alaternus* bark extracts measured by means of different *in vitro* tests.

Tests	<i>Rhamnus alaternus</i> bark extracts		
	Methanolic	Aqueous	Decoction
Folin μg GaE/mg extract	190.227 ± 12.117	$162.722 \pm 0.603^*$	$181.557 \pm 4.034^{**}$
DPPH mmol TE/g extract	0.607 ± 0.108	0.392 ± 0.050	0.542 ± 0.077
TEAC mmol TE/g extract	0.754 ± 0.001	0.648 ± 0.002	0.661 ± 0.004
FRAP mmol Fe^{2+} /g extract	1.714 ± 0.068	$1.240 \pm 0.052^*$	$1.263 \pm 0.056^*$
ORAC mmol TE/g extract	6.548 ± 0.027	$5.268 \pm 0.123^*$	$3.960 \pm 0.093^*$

Results were expressed as mean \pm SD of three experiments. * Versus methanolic extract $P < 0.01$; ** Versus aqueous extract $P < 0.01$. GaE: gallic acid equivalent; TE: trolox equivalent; SD: standard deviation; DPPH: 2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing/antioxidant power; TEAC: trolox equivalents antioxidant capacity; ORAC: oxygen radical absorbance capacity.

Table 7. Total phenolics content and antioxidant activity of *Rhamnus alaternus* leaves extracts measured by means of different *in vitro* tests.

Tests	<i>Rhamnus alaternus</i> leaves extracts		
	Methanolic	Aqueous	Decoction
Folin μg GaE/mg extract	$116.031 \pm 8.617^{**}$	$117.456 \pm 1.564^{**}$	163.706 ± 14.787
DPPH mmol TE/g extract	0.358 ± 0.06	0.143 ± 0.038	0.509 ± 0.079
TEAC mmol TE/g extract	0.518 ± 0.034	$0.192 \pm 0.018^*$	0.529 ± 0.014
FRAP mmol Fe^{2+} /g extract	0.728 ± 0.0582	$0.245 \pm 0.029^*$	0.574 ± 0.057
ORAC mmol TE/g extract	$6.143 \pm 0.295^{**}$	$4.158 \pm 0.498^{**}$	181.269 ± 9.7387

Results were expressed as mean \pm SD of three experiments. * Versus methanolic extract $P < 0.01$; ** Versus traditional extract $P < 0.01$. All other abbreviations are cited in table 6.

The three extracts of *R. alaternus* bark showed significant antioxidant capacities, with few differences between them in DPPH (0.61 ± 0.11 mmol TE/g; 0.39 ± 0.05 mmol TE/g; 0.54 ± 0.08

mmol TE/g extract) and TEAC (0.52 ± 0.03 mmol TE/g; 0.19 ± 0.02 mmol TE/g; 0.53 ± 0.01 mmol TE/g) respectively for methanolic, aqueous and traditional extracts. Conversely in folin, FRAP and ORAC assays the methanolic extract appeared significantly more active (190.23 ± 12.12 μ g GaE/mg; 1.71 ± 0.07 mmol Fe^{2+} /g; 6.55 ± 0.03 mmol TE/g respectively) than the other two extracts. In addition all the extracts of *R. alaternus* leaves showed also a good potential of antioxidant activity similar to that of the bark extracts. Observing the leaves results we didn't mark any significant difference between the traditional and methanolic extracts in all the assays except in ORAC and folin assays the traditional extract was more remarkable (163.71 ± 14.79 mmol TE/g; 181.27 ± 9.74 mmol TE/g of extract respectively) and presented significant difference with both methanolic and aqueous extracts. Whereas the aqueous extract was the less active in the group. In general, the methanolic extract of the bark seems to be the most active between all the extracts.

It is widely accepted that to characterize the properties of antioxidant agents, different validated benchmark methods are needed. One has to point out that the antioxidant activity measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay. Among our assays ORAC is a hydrogen atom transfer (HAT) based method, total phenols assay by Folin-Ciocalteu reagent, DPPH, FRAP and TEAC are single electron transfer (ET) based methods (Apak *et al.*, 2007).

It is generally considered that the primary mechanism of the radical scavenging activity of flavonoids is hydrogen atom donation, although they may also act by single-electron transfer. Thus our findings, taken together, suggest that the antioxidant activity of *R. alaternus* bark and leaves extracts may be due, at least partially, to their content in flavonoids.

1.6. Cytotoxicity of *R. alaternus* bark extracts

Cytotoxicity of *R. alaternus* bark and leaves extracts was evaluated in a leukemic cancer cell line (U937) and in normal peripheral blood mononuclear cells PBMCs. This method gives an indication of potential usefulness in a clinical setting, for which selectivity in favor of the cancer cell line being the more susceptible is required (Houghton *et al.*, 2007). After 24h of treatment of both types of cells with increasing concentrations of *R. alaternus* bark and leaves extracts; the viability was examined using the trypan blue assay; a simple and good method for the indication of gross membrane integrity. When staining the cells with the trypan blue dye, the viable cells stay intact therefore they do not take up the trypan blue dye, whereas the nonviable cells have the characteristic to take up the dye because they have a damaged membrane. Under microscope, we

can differ the dead cells from the viable ones, since the dead cells show a dark color due to the trypan blue entrance inside it (figure 15).

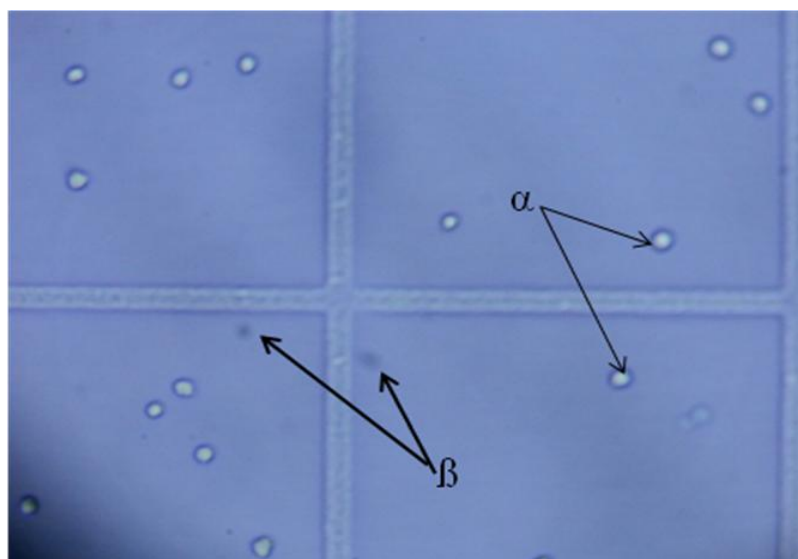


Figure 15. Morphology of U937 cancer cells seen under microscope after staining with trypan blue dye and placed in the hemocytometer. α : viable cells; β : dead cells.

The percent of viability of U937 cancer cells and PBMCs normal cells is plotted against the different concentrations of the extracts used as well as for taxol as shown in figures 16; 17 and 18.

The taxol used as a control antitumor agent was very active in our experiment even at the lowest concentrations. As we can see in figure 16, at 5 μ g/ml already taxol reduced U937 cell viability to 25.83%, and at the dose 100 μ g/ml the U937 cells were almost suppressed; the viability percent of the cells was only (9.14%). This show how much taxol is strongly toxic against cancer cells, but meanwhile at the concentrations 12.5 μ g/ml; 50 μ g/ml and 100 μ g/ml taxol affect also significantly the viability of PBMCs normal cells (53.48%; 43.36% and 38.46% respectively).

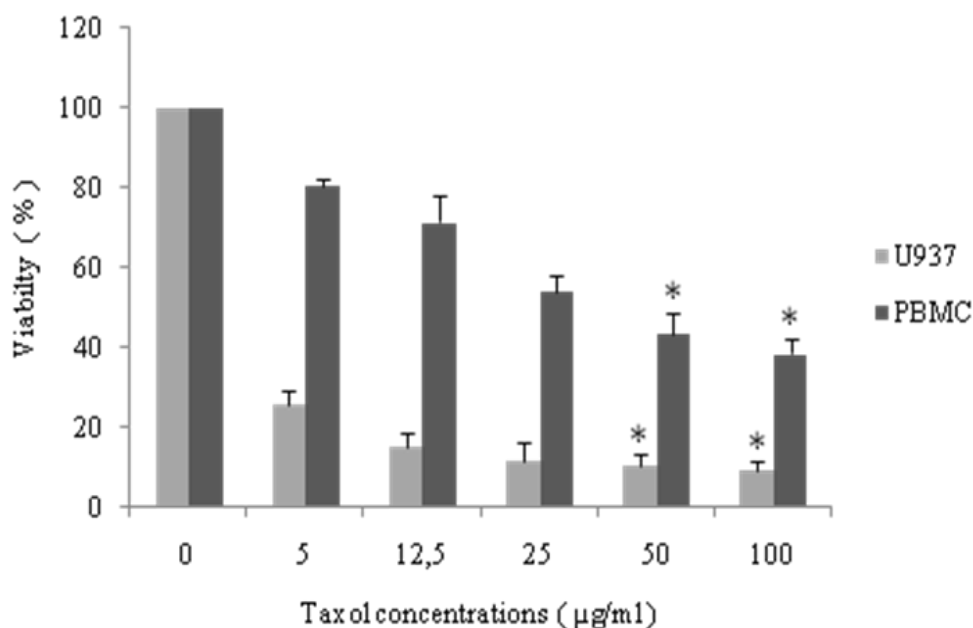


Figure 16. Effect of taxol at different concentrations on U937 and PBMCs cell viability. Results are expressed as mean \pm SD of three repetitions, and considered statistically significant at *: $P < 0.05$ when compared with the lower concentration (5 µg/ml).

Concerning the results of the viability percent of *R.alaternus* bark extracts (figure 17); the methanolic one induced a marked decrease in cell viability of U937 cancer cells in a dose dependent manner (as compared to its lower concentration). In fact at the lowest dose (0.5 µg/ml) the percent of viability was (72.83%) and at highest dose (12.5 µg/ml) it decreased to (35.87%). The other noticeable result is that this extract have no significant effect on PBMCs cells at all the doses tested, hence at the lowest dose (0.5 µg/ml) the viability percent of PBMCs was (97.78%), whereas at highest dose (12.5 µg/ml) it was (82.22%). Contrarily the aqueous and traditional bark extracts induced cell death in a concentration-dependent manner to both types of cells; we marked for PBMCs cells at the dose (12.5 µg/ml) a viability percent of (75.80% and 62.90%) for aqueous and traditional extracts respectively, this viability was reduced to (25.80% and 22.58% respectively) at the dose (100 µg/ml).

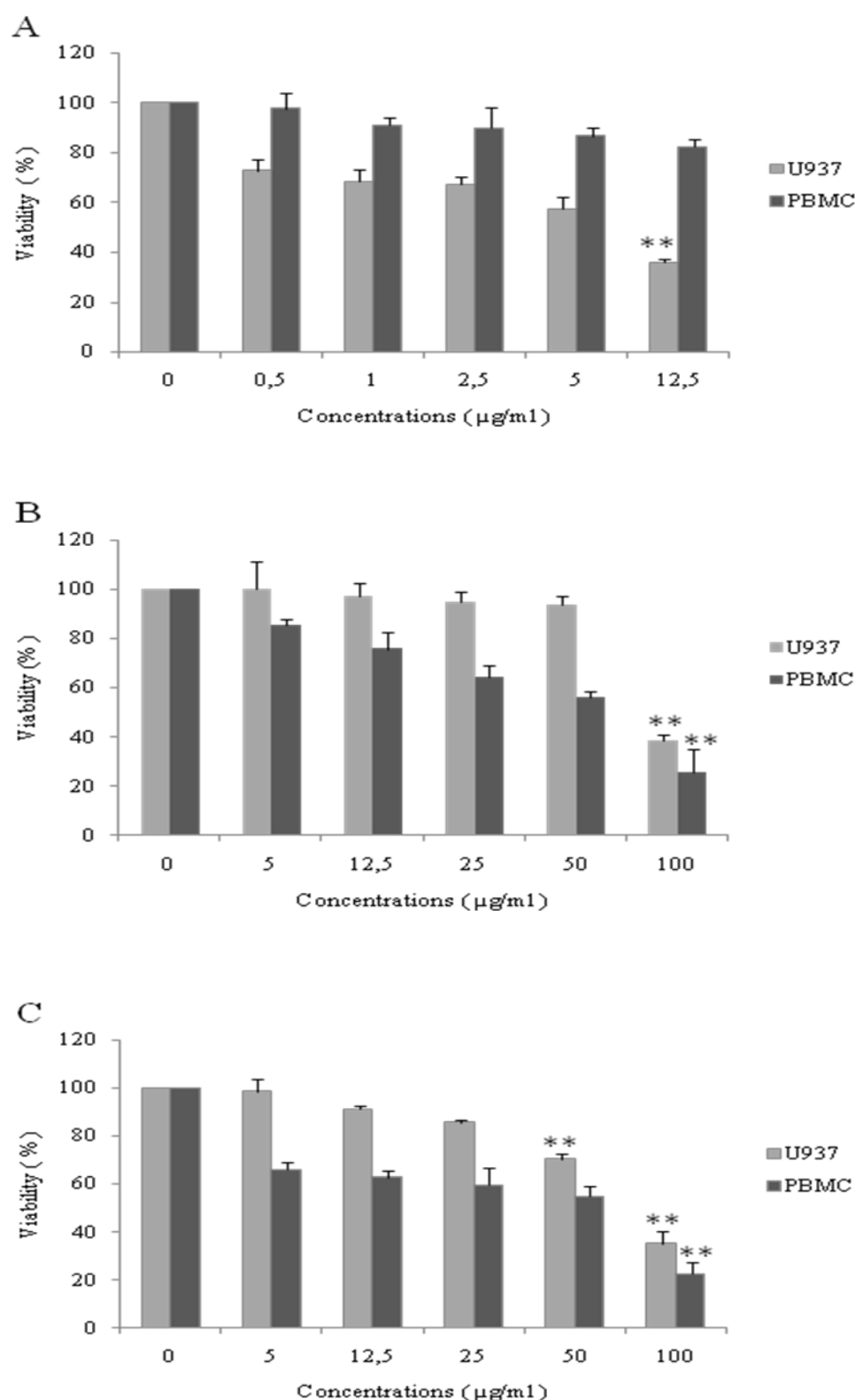


Figure 17. Effect of *R. alaternus* bark extracts at different concentrations on U937 and PBMCs cell viability. A: methanolic extract; B: aqueous extract and C: decoction extract; the results are expressed as mean±SD of three repetitions, and considered statistically significant at **: $P < 0.01$ when compared with the lower concentration (0.5µg/ml) or (5µg/ml).

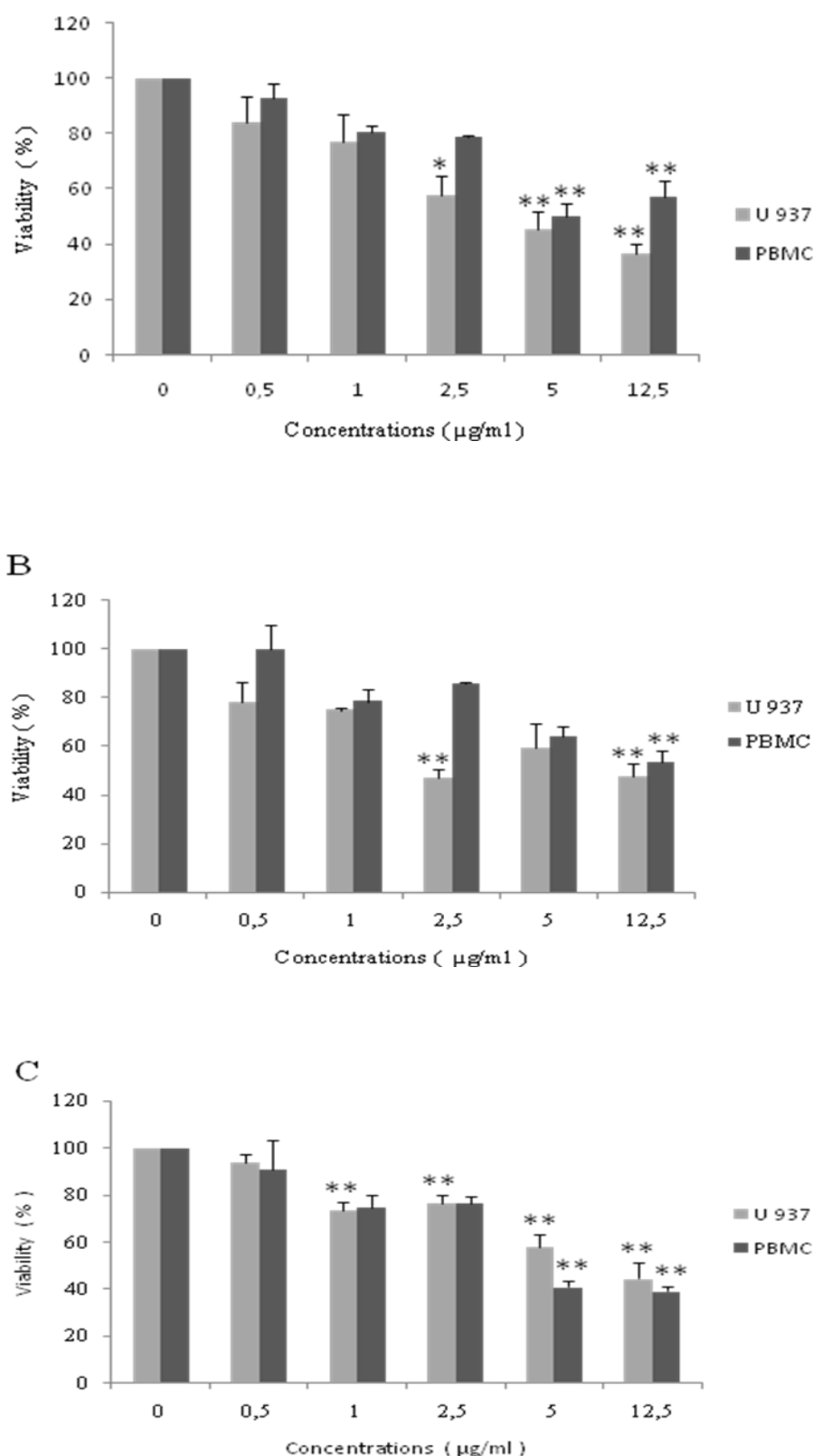


Figure 18. Effect of *R. alaternus* leaves extracts at different concentrations on U937 and PBMCs cell viability. A: Methanolic extract; B: aqueous extract and C: decoction extract; the results are expressed as the mean \pm SD of three repetitions, and considered statistically significant at *: P<0.05 and **: P<0.01 when compared with the lower concentration (0.5 $\mu\text{g/ml}$).

Regarding the results of the leaves extracts showed in figure 18; the methanolic extract induced a clear significant decrease in cell viability to U937 and PBMCs cells in a concentration-dependant manner. Hence at the dose (1µg/ml) the cells were at (76.85%) of viability for U937 and at (80.36%) of viability for PBMCs, this viability decreased at the dose (12.5µg/ml) to (36.36%) for U937 and to (57.14%) for PBMCs. While the effect of the aqueous and traditional leaves extracts was not dose dependant but reduced significantly the percent of viability of the two types of cells at some doses espacially at the highest dose (12.5µg/ml), where the percent of cell viability treated with the traditional extract was (44.62% and 39.28% respectively) for U937 and PBMCs cells. At the same dose (12.5µg/ml), cells treated with the aqueous extract marked a viability percent of (47.93% and 53.57%) respectively for U937 and PBMCs.

The figure 19 show the morphology of PBMCs normal cells and U937 cancer cells, as seen under inverted microscope after 24h of treatment with control (no dose), with taxol and with *R. alaternus* bark methanolic extract at the doses (5µg/ml, 12,5 µg/ml, respectively). The PBMCs cells of the treated groups at the chosen doses appear to have the same morphology as that of the control group; this may support in part the fact that many cells are still viable. Whereas in U937 cancer cells as we can see in figure 19 (E, F), we marked a difference in cell's shape when comparing between the treated and the control cells, this is probably due to necrosis or apoptosis occurring to the cells. However, this suggestion stays only a hypothesis that needs specific assays to identify whether the cells are suffering from any injury.

The concentration inducing the 50% of cell death (IC_{50}) to PBMCs normal cells and to U937 cancer cells was calculated after 24h of exposure for all the extracts tested and the data are shown in table 8. Concerning the bark extracts in normal PBMCs cells the IC_{50} of the traditional bark extract was 29.35µg/ml, when the aqueous bark extract was tested, the concentration inducing the 50% of cell death was similar (IC_{50} 38.81 µg/ml) to that of the traditional bark extract, even if at less extend. Conversely, the methanolic bark extract exerted only a weak cytotoxic effect on normal PBMCs, with IC_{50} (220.35µg/ml) which is a clear indication that normal human cells may be more resistant to this extract. In U937 cancer cells IC_{50} values of the *R. alaternus* methanolic bark extract was the lowest (6.39µg/ml) comparing to the two other bark extracts that showed (76.74 µg/ml and 84.65 µg/ml respectively) for aqueous and traditional extracts. These results suggest that methanolic bark extract suppressed potently the proliferation of U937 cells, while the same cells exposed to traditional and aqueous bark extracts was not significantly reduced.

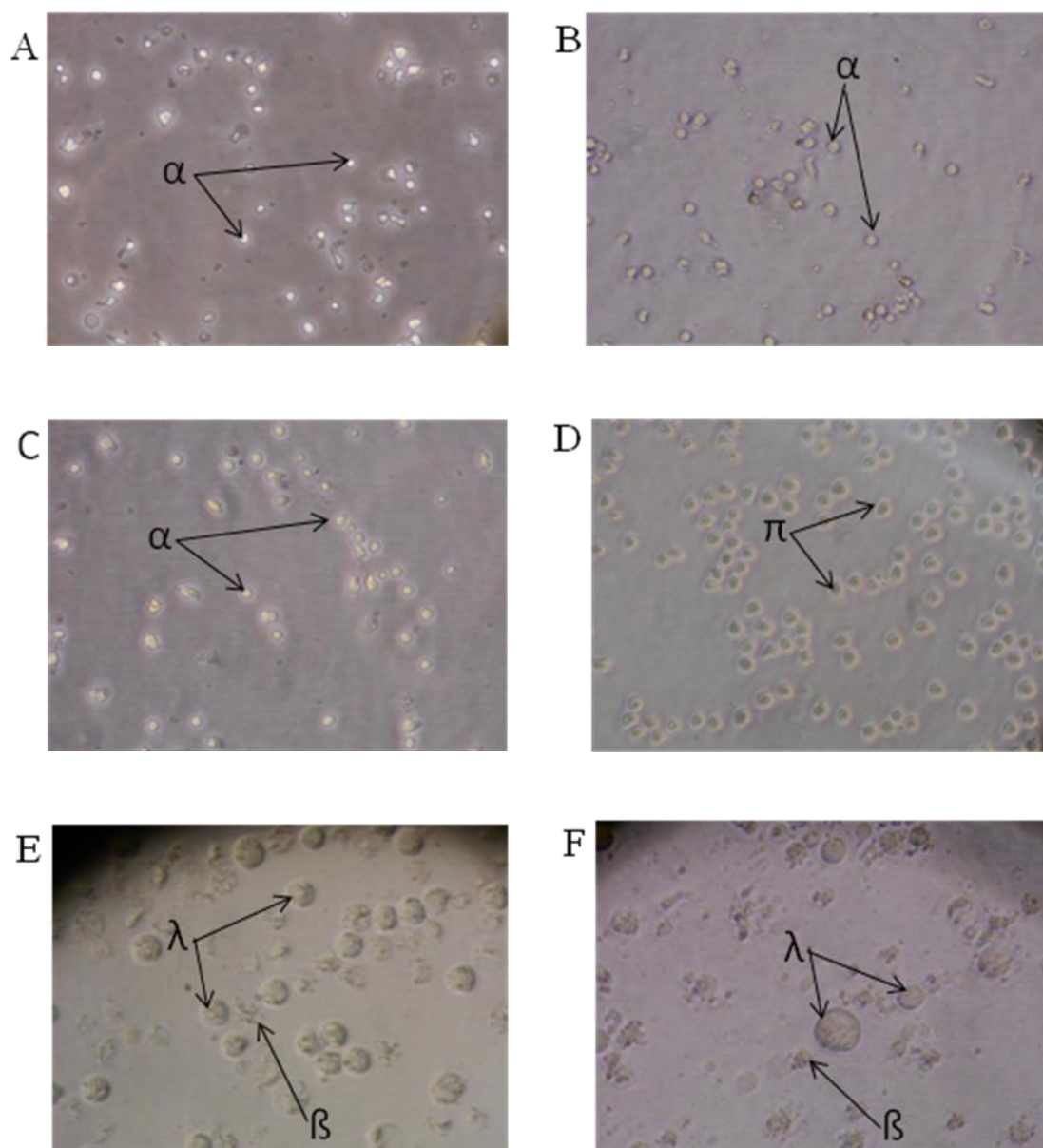


Figure 19. Morphology of U937 cancer cells and PBMCs normal cells after 24 hours of treatment with *R. alaternus* bark methanolic extract. A: PBMCs control cells; B: PBMCs cells treated with *R. alaternus* extract (12.5 μ g/ml); C: PBMCs cells treated with taxol (5 μ g/ml); D: U937 control cells; E: U937 cells treated with *R. alaternus* extract (12.5 μ g/ml); F: U937 cells treated with taxol (5 μ g/ml). α : PBMCs cells morphology; π : U937 normal cells; λ : swelling U937 cells; β : fragments. All the cells were observed under inverted microscope and photographed (Nikon) in same magnification (40x).

Regarding *R. alaternus* leaves extracts, the traditional one induced potentially more cell death of PBMCs then all the other extracts as the IC₅₀ value was (5.277 μ g/ml), also both methanolic and aqueous leaves extracts provoked notable cell death to normal cells with an IC₅₀ of (11.596 μ g/ml and 13.647 μ g/ml) respectively. The effect of leaves extracts on the proliferation of U937 cancer cells was remarkably clear; the methanolic leaves extract reduced in the best the growth of cancer

cells, since the IC_{50} was about (4.665 μ g/ml), also the aqueous and traditional extracts had a good effect, the IC_{50} was (7.802 μ g/ml and 8.619 μ g/ml, respectively).

We conclude that the leaves extracts presented lower IC_{50} for both PBMCs and U937 compared to bark extracts, so they are more toxic to both types of cells and the differences notified between the extracts are probably related to the different classes of compounds present in this extracts.

Table 8. IC_{50} values (concentration eliciting 50% inhibition) for *R. alaternus* extracts and taxol applied to PBMCs and U937 cells. Cells were treated with various concentrations of the extract and taxol, and the cell number was counted after 24h of exposure.

		IC_{50} μ g/ml (Confidence Limits)	
<i>R. alaternus</i> extracts		PBMCs	U937
Bark	Methanolic	220.35 (186.64-260.41)	6.39 (4.10-9.95)
	Aqueous	38.81 (18.06-53.39)	76.74 (58.56-98.75)
	Decoction	29.35 (17.59-53.52)	84.65 (57.39-101.17)
Leaves	Methanolic	11.596 (3.053-44.042)	4.665 (3.529-6.166)
	Aqueous	13.647 (4.131-45.003)	7.802 (1.818-33.475)
	Decoction	5.277 (2.277-15.528)	8.619 (2.941-25.236)
Taxol		19.46 (12.35-22.54)	2.47 (1.48-3.35)

The criteria of cytotoxicity for a crude extract, established by the U.S. National Cancer Institute (NCI) in the preliminary assays, report IC_{50} values of 10–20 μ g/ml for a total/crude extract as cytotoxic, 20 to \leq 50 μ g/ml as moderately cytotoxic, and <10 μ g/ml as strongly cytotoxic (Ellithey *et al.*, 2014). The IC_{50} values of U937 cells treated with *R. alaternus* bark methanolic extract and leaves extracts was found to be in the range <10 μ g/ml, so we can consider them as active toward U937 cancer cells. On the contrary, the traditional and aqueous bark extracts can be considered as poorly cytotoxic for U937 cells since the IC_{50} values are in the range (20 to \leq 50 μ g/ml). Furthermore, these data are also interesting as they suggest that the methanolic bark extract possess a cytotoxic activity specifically on cancer cells (U937), while the traditional and aqueous bark extracts are moderately toxic on PBMCs and the three leaves extracts are cytotoxic to normal peripheral blood mononuclear cells. Subsequently, another experiment was done to evaluate the possible reason for the decreased cell viability.

1.7. Apoptosis analysis by flow cytometry

In our study apoptotic and/or necrotic in U937 treated cells were investigated by flow cytometry assay after applying the two-color variation of PE annexin V and cell viability dye 7-AAD, on U937 cells treated previously for 24 hour with *R. alaternus* bark methanolic extract and the results are offered in the cytograms of the figure 20. The way cells bind to annexin V (x axis) and/or 7-AAD (y axis) can discriminate viable, intact cells from apoptotic and/or necrotic cells. Hence, the lower left quadrant of each panel (Q3) shows the viable cells which exclude 7-AAD and are negative for PE-Annexin V binding, the upper right quadrants (Q2) contain the non-viable, necrotic cells, positive for PE-Annexin V binding and for 7-AAD uptake; the lower right quadrants (Q4) represent the apoptotic cells, PE-Annexin V positive and 7-AAD negative.

Using this strategy, we can explain our results; the majority of the untreated intact U937 cell population (Control) was at (99.8%) composed of healthy, viable cells, showing annexin V and 7-AAD double negative staining. The U937 cells treated with the extract at the dose (50µg/ml) were similar to the control group; the population was at (92.1%) intact and viable. Whereas U937 cells treated with the extract at higher doses, they showed the appearance of two distinct populations in a concentration dependent manner: one presenting annexin V single positive expression, which was assigned for early apoptotic (Figure 20 Q4). The other showing annexin V and 7-AAD double positive staining; that we have referred as late apoptotic cells (Figure 20 Q2).

In assistance with figure 20, the figure 21 shows the percent of early and late apoptosis at the different doses of the extract. The appearance of these two populations was progressive and dose dependent; hence starting from the dose: 100µg/ml we marked that about (45.0%) of the cell population was in late apoptosis; (50.0%) was in early apoptotic and only (4.5%) was healthy. Then a significant increase of late apoptotic going to (91.3%) was clearly appearing at the dose 300µg/ml and just (0.2%) of cell population stayed intact and healthy as well as for the dose 400µg/ml. these results lead to suggest that *R. alaternus* bark methanolic extract could induce apoptotic response in U937 cells.

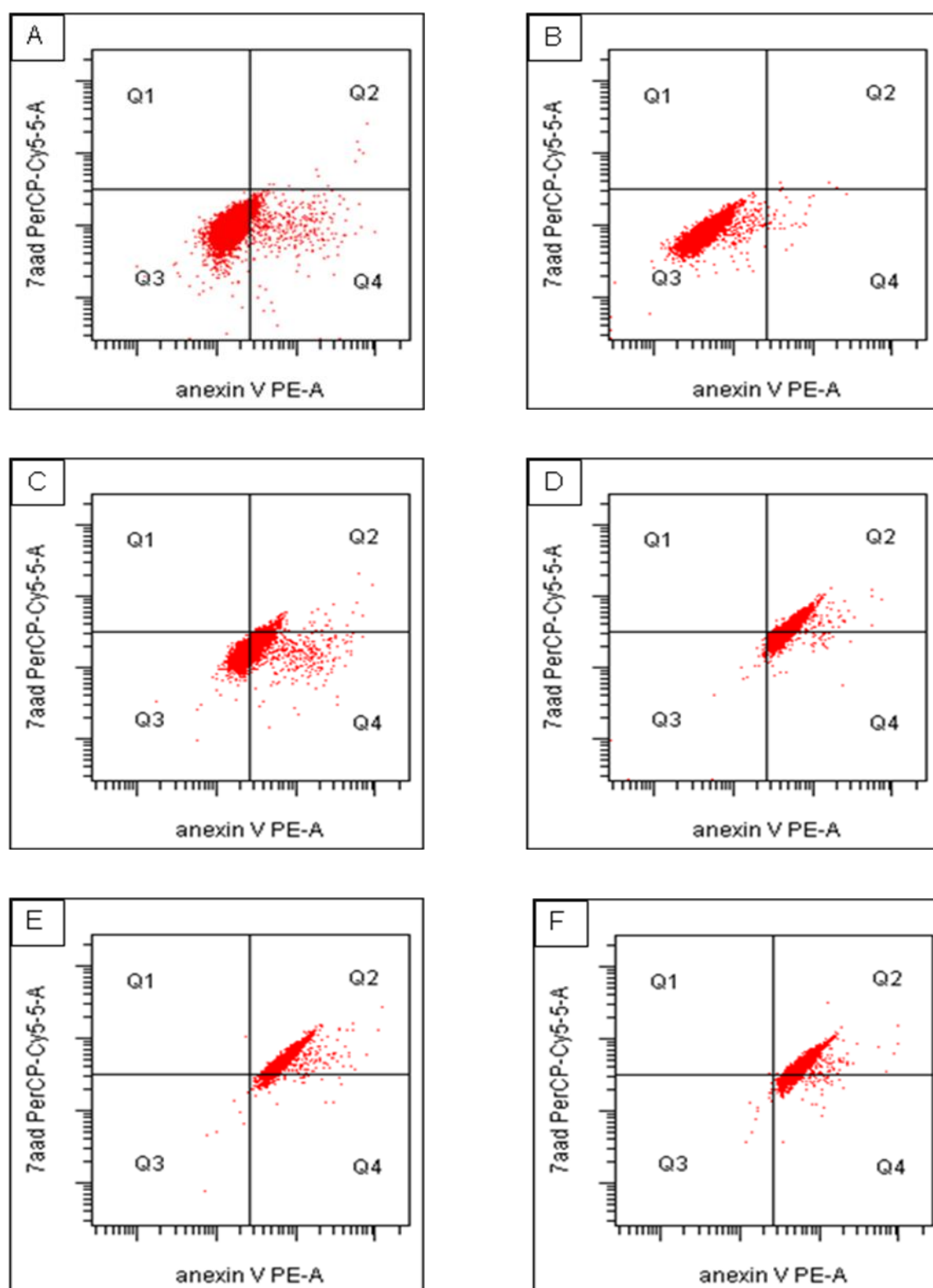


Figure 20. Flow cytometry analysis of *R. alaternus* bark methanolic extract effect on U937 cells. A: untreated control population; B; C; D; E and F are the exponentially growing U937 cells exposed to *R. alaternus* bark methanolic extract at the following concentrations: 50; 100; 200; 300 and 400 µg/ml, respectively. For 24h and were labeled with annexin-V and 7-AAD and subjected to flow cytometric analyses to identify subpopulations undergoing early (lower right quadrant /Q4); late (upper right quadrant/Q2) apoptosis or necrosis (upper left quadrant/Q1).

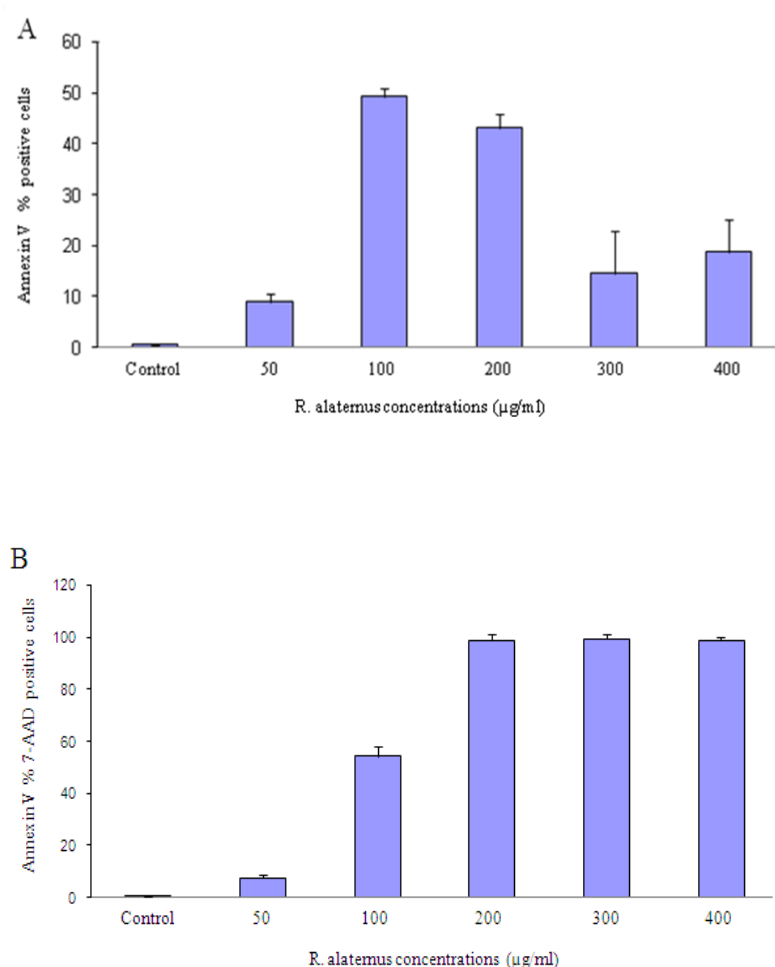


Figure 21. Apoptosis induction to U937 cells by *R. alaternus* bark methanolic extract. A: percent of early apoptosis; B: percent of late apoptosis, each bar presents the mean percentage \pm SD of cells detected in each quadrant.

The measurement of Annexin V binding to the cell surface as indicative for apoptosis was performed in conjunction with 7-AAD dye exclusion to establish integrity of the cell membrane. In this type of experiment the cell viability is preserved, allowing the simultaneous detection of intact cells, cells undergoing apoptosis, and dead cells resulting from apoptotic and/or necrotic processes in the same sample and at the same time (Da Silva *et al.*, 2014). The difference between apoptosis and necrosis of cell death is that during the initial stages of apoptosis the cell membrane remains intact. While at the very moment that necrosis occurs the cell membrane loses its integrity and becomes leaky allowing the entrance of molecules as large as 7-AAD, which is used in this experiment to mark necrotic cells (Vermes *et al.*, 1995).

Kaempferol and rhamnocitrin glycosides from *R. alaternus* have been demonstrated to induce apoptosis in human lymphoblastoid cells TK6, (Bhourri *et al.*, 2011b). Quercetin can induce

caspase-dependent cell death in U937 cells (Lee *et al.*, 2006), thus, we can suppose that the high cytotoxic effect of *R. alaternus* bark methanolic extract is due to kaempferol, rhamnocitrin, and quercetin derivatives contained in these extract. In addition, the methanolic and aqueous leaves extracts that marked a high cytotoxicity in trypan blue assay contain the same listed compounds; this can explain their cytotoxicity toward U937 cells. We suggest that most probably the traditional leaves extract contain amounts of kaempferol, rhamnocitrin and quercetin as it reduced significantly the U937 cell growth, as to the other two extracts of the bark, they do not contain a sufficient amount of these compounds to obtain an evident cytotoxic effect on leukemia cells. On the other hand, they may contain other hydrophilic compounds toxic for normal PBMCs, which may be also present more specifically in the traditional leaves extract as it was highly toxic for PBMCs, and thus a further analysis of the flavonoid in the traditional extracts is required.

1.8. Acute toxicity test

The study of acute toxicity was carried out using *R. alaternus* bark methanolic extract because it was the best in results among the studied extracts. The oral injections to the rats of a single dose (2000mg/kg or 5000mg/kg of body weight) prepared using *R. alaternus* bark methanolic extract, did not affect the behavior of the animals, some diarrhea was observed only in the first day, but the animals came back to normal, and no toxic signs nor mortality were recorded. The oral LD₅₀ value of *R. alaternus* methanolic extract must be greater than 5000mg/kg, so from this result and according to the classification of Diezi, (1989), we conclude that *Rhamnus alaternus* bark is considered as non-toxic.

2 *Retama sphaerocarpa* (L.) Boiss

2.1. Extraction yields

The yields of the extraction for *R. sphaerocarpa* plant are summarized in tables 9. The traditional extract of the stems presented the best yield (31.7%) followed by fruits traditional extract (13.4%). Then the aqueous and methanolic extracts of the stems gave approximately same yields (13.17%; 13.15% respectively). The good yield of the traditional (by decoction) extracts may be due to the heating method that can allow more molecules to dissolve in the water (Dai and Mumper 2010).

Table 9. Extraction yields of *Retama sphaerocarpa* fruits and stems extracts.

<i>Retama sphaerocarpa</i> organs	Extracts	Yields (%)
Fruits	Methanol	9,24
	Aqueous	6.5
	Decoction	13.4
Stems	Methanol	13.15
	Aqueous	13.17
	Decoction	31.7

2.2. Total flavonoids, total flavonols, total tannins contents and anthraquinones identification

In our study the TLC fingerprint assay didn't detect the presence of anthraquinones in *R. sphaerocarpa* extracts. In another hand, we determined the amount of total flavonoids, total flavonols and total tannins of the three different extracts (methanolic, aqueous and traditional) prepared from the fruits and stems of *R. sphaerocarpa*, Catechin calibration curve used in condensed tannins assay is presented previously in figure 6 and the results are shown in table 10.

Concerning the fruits extracts the methanolic one has higher amount of flavonoids and flavonols (42.65 ± 0.35 $\mu\text{g CE /mg}$ and 26.17 ± 0.75 $\mu\text{g CE /mg}$ of extract, respectively), then the other fruit extracts, whereas in total tannins assay the traditional fruit extract was significantly better ($18.20 \pm 0.16\text{ng CE/mg}$ of extract). In another hand comparing between the stems extracts the traditional one contained a superior significant amount of flavonoids (100.99 ± 1.33 $\mu\text{g CE /mg}$ of extract) but the methanolic extract was the best ($P < 0.01$) in total flavonols and tannins

(15.90±0.24 µg CE /mg; 4.66±0.13 ng CE/mg of extract, respectively). In general the fruits and stems are close in the results of the total contents studied. Other studies showed that Retama contain a variety of chemical compounds including flavonoids, alkaloids and others (El-Shazly *et al.*, 1996; Louaar *et al.*, 2007; Djeddi *et al.*, 2013).

Table 10. Total flavonoids, flavonols and total tannins contents in *R. sphaerocarpa* fruits and stems.

<i>Retama sphaerocarpa</i> plant parts	types of extracts	Total flavonoids µg CE /mg of extract	Total flavonols µg QE/mg of extract	Total tannins ng CE/mg of extract
Fruits	Methanolic	42.655 ± 0.348	26.174 ± 0.749	5.475 ± 0.171*
	Aqueous	4.807 ± 0.306**	3.923 ± 0.291**	7.584 ± 0.250*
	Decoction	32.820 ± 0.979**	13.623 ± 0.584**	18.203 ± 0.161
Stems	Methanolic	37.243 ± 2.930*	15.898 ± 0.240	4.657 ± 0.134
	Aqueous	11,960 ± 0,515*	4.299 ± 0.015**	1.208 ± 0.082**
	Decoction	100.988 ± 1.327	4.256 ± 0.001**	2.492 ± 0.152**

Results were expressed as mean±SD of three experiments. * Versus traditional extract, $P<0.01$ ** Versus methanolic extract $P<0.01$ for each plant part separately; SD: standard deviation; CE: catechin equivalent; QE: quercetin equivalent.

2.3. Identification and quantification of flavonoids

In our study the identification of the flavonoid composition of *R. sphaerocarpa* extracts of the fruits (methanolic, aqueous and traditional) and stems aqueous extract was done by HPLC–DAD–ESI–MS as described in methods. Concerning *R.sphaerocarpa* fruits extracts the figure 22 show the resulting chromatograms and the table 11 present the quantitative determination of the flavonoid content. The identified compounds in *R. sphaerocarpa* fruits extracts were mainly isoflavones an interesting and biologically active subclass of flavonoids, then there are also flavonols with less amount, the methanolic extract seems to contain the highest amount of total isoflavones (11.2mg/g of extract) and total flavonols (4.35mg/g of extract). These results confirm the one already obtained by the spectrophotometric assay cited above and in literature; recent papers reported the isolation and structure elucidation of several flavonoids (mainly flavonols and isoflavones) from *R. Sphaerocarpa* (Akkal *et al.*, 2010; Louaar *et al.*, 2005, 2007; López-Lázaro *et al.*, 2000; Martin-Cordero *et al.*, 1999).

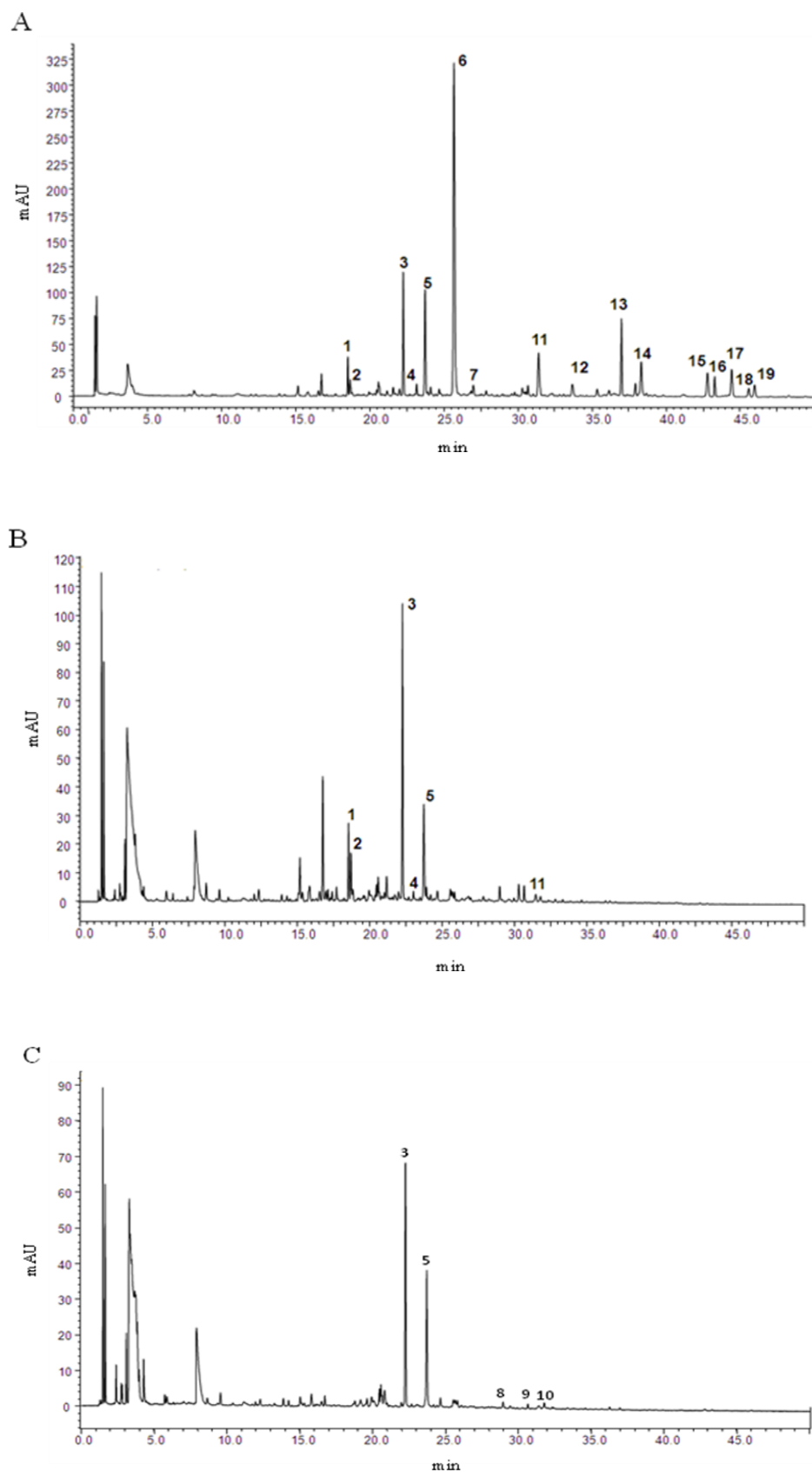


Figure 22. HPLC-PDA representative chromatograms of flavonoids from *R. sphaerocarpa* fruits (extracted at 280nm). A: methanolic extract, B: aqueous extract and C: decoction extract. For peak identification, see table 11.

Table 11. HPLC-PDA/ESI-MS identification and quantification of flavonoids contained in methanolic, aqueous and traditional extracts obtained from *R. sphaerocarpa* fruits.

Peak	t_R (min)	Compounds (expressed as)	mg/g extract \pm % RSD		
			Methanolic	Aqueous	Decoction
1	18.3	Genistein-di-hexoside	0.67 ± 1.0	0.40 ± 1.6	-
2	18.5	Apigenin-di-hexoside	0.43 ± 3.6	0.46 ± 2.3	-
3	22.1	Genistein-di-hexoside	1.63 ± 2.0	1.54 ± 1.1	1.17 ± 2.4
4	23.1	Quercetin-hexoside	0.32 ± 2.5	0.22 ± 2.5	-
5	23.7	Genistein-hexoside	1.50 ± 0.3	0.55 ± 0.4	0.60 ± 1.2
6	25.6	Daidzein-derivate	4.58 ± 1.9	-	-
7	27.0	Genistein	0.38 ± 1.5	-	-
10	30.7	Isorhamnetin-hexoside	-	-	0.02 ± 0.2
11	31.3	Apigenin-derivative	1.52 ± 2.2	0.08 ± 1.8	-
12	33.4	Genistein hexosyl-malonate	0.50 ± 1.8	-	-
13	37.0	Isorhamnetin-hexoside	1.04 ± 0.8	-	-
14	38.3	Quercetin-derivative	0.66 ± 2.2	-	-
17	44.4	Kaempferol-derivative	1.59 ± 2.0	-	-
18	45.6	Quercetin-malonyl-di-hexoside	0.34 ± 2.5	-	-
19	45.8	Isorhamnetin-di-hexoside	0.40 ± 0.5	-	-

Data are expressed as mean of three experiments \pm relative standard deviation (RSD).

In the fruits methanolic extract, 14 compounds were identified. The daidzein derivative was the main one (4.58mg/g of extract) followed by Genistein-di-hexoside (1.6mg/g of extract) and kaempferol derivative (1.59mg/g of extract). In the fruits aqueous extract 6 compounds were identified: one flavonol named quercetin-hexoside (0.22mg/g of extract) and five isoflavones the main compound among them is genistein-di-hexoside (1.54mg/g of extract). Whereas only three compounds were identified in the fruits traditional extract: two isoflavones that are genistein-di-

hexoside representing the main compound (1.17mg/g of extract) and genistein hexoside (0.60mg/g of extract), and one flavonol that is isorhamnetin-hexoside (0.02mg/g of extract).

Regarding the aqueous extract from *R sphaerocarpa* stems, we report for the first time a secondary metabolic fingerprint of the aqueous extract from the stems of *R. sphaerocarpa* presented in figure 23 and the chromatogram is presented in the figure 24. Among the 30 peaks (including minor components) appearing in the chromatogram, 9 major peaks were tentatively identified by means of their Uv-vis spectra, mass spectra, and relative retention times, the corresponding names of the identified compounds are presented in table 12.

The chromatogram in the figure 24 appears to be dominated by the presence of nine major peaks (1-9). A first preliminary analysis of the chromatogram based on the UV-vis spectra of these signals allowed to hypothesize the strong presence of isoflavones in the extract. The majority of the peaks in the chromatogram were identified as derivatives of daidzein, genistein and glycitein, which are known to be quite common in the leguminosae family (Kaufman *et al.*, 1997). Three different 7-*O*-glucoside derivatives are present in our extract (peaks: 3, 4 and 6 of the chromatogram). We were able to identify peaks number 1 and 5 as daidzein 8-*C*- glucoside and genistein 8-*C*- glucoside, respectively, Louaar and his coworkers (2007) also isolated and characterized a C-glycoside from *R. sphaerocarpa*.

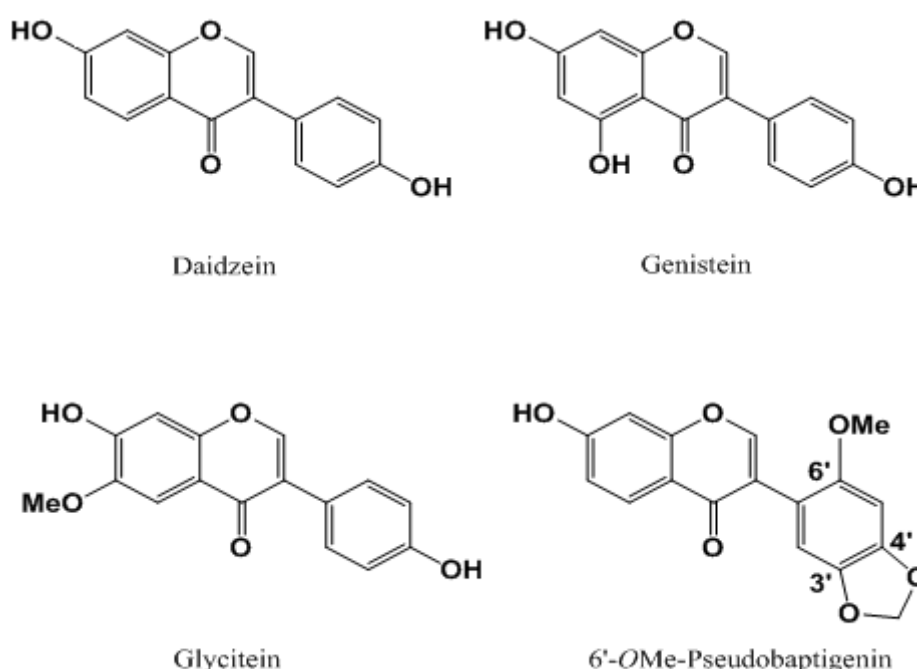


Figure 23. Isoflavones aglycones found in the aqueous extract of *Retama sphaerocarpa* stems (C.N.R; 2010).

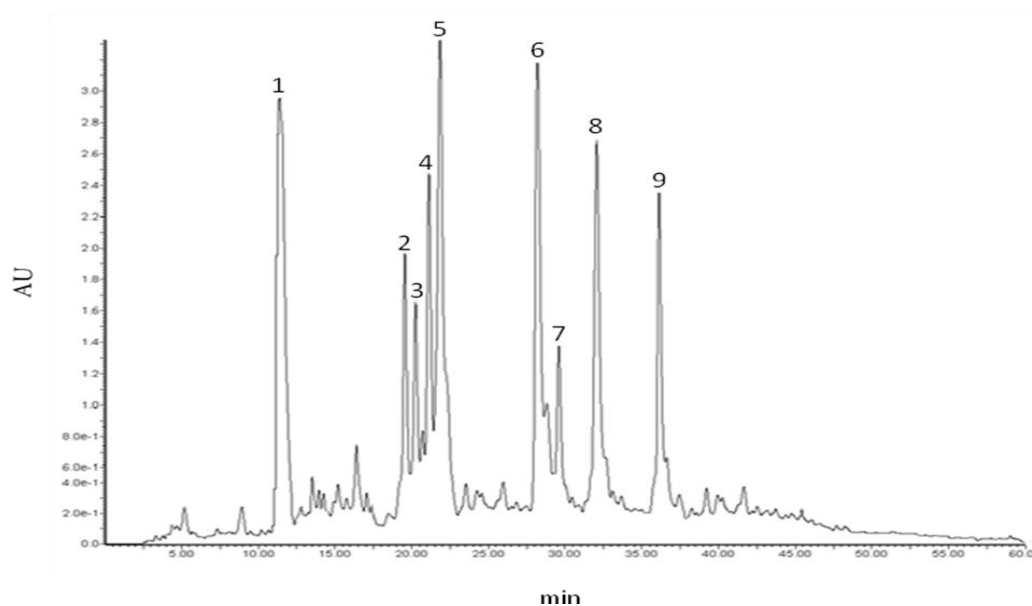


Figure 24. HPLC profile of *Retama sphaerocarpa* aqueous extract (visualised at 260nm); tentative identification of peaks present on the chromatogram, Peaks numbers refers to table 12.

Table 12. HPLC/MS quantification of flavonoids contained in the aqueous extract obtained from *R. sphaerocarpa* stems.

Peak	Peak name	MW	mg/100 mg extract
1	Daidzein 8-C- hexoside	416	6.12
2	Genistein 7-O-xylosyl-8-C-glucoside	564	0.68
3	Glycitein 7-O- glucoside	446	0.05
4	Daidzein 7-O-glucoside (daidzein)	416	0.38
5	Genistein 8-C-glucoside	432	0.70
6	Genistein 7-O-glucoside (genistein)	432	0.52
7	Daidzein glycosyl-malonate	502	0.27
8	6'-OMe-pseudobaptigenin-glucoside	474	1.56
9	Genistein glucosyl-malonate	518	0.18

MW=Molecular weight

Interestingly, we have found, genistein 7-O-xylosyl-8-C-glucoside (peak: 2) as recently reported by Akkal *et al* (2010). We could find also 6'-OMe-pseudobaptigenin 6' methoxy-7-O-glucoside (peak: 8). Pseudobaptigenin was reported for the first time to occur in *Tephrosia maxima* (Venkata Rao *et al.*, 1984) then in *R. sphaerocarpa* itself (López-Lázaro *et al*, 1998; Louaar *et al.*, 2007). Although there are various papers reporting the presence, in the same extract, of flavonols

derivatives like vicenin 2, vitexin, quercetin and rhamnazin (Louaar *et al*, 2005; Martin-Cordero *et al.*, 1999). We could not find them in detectable amounts.

Peaks number 7 and 9 presented UV-vis spectra with shape and absorption maximum wavelengths typical of that of isoflavones daidzein and genistein (MW= 270) this assignment hypothesis was confirmed by the corresponding mass spectra. In another hand; the comparison of our results with analytical data found in literature (Wu *et al*, 2004; Klejdus *et al.*, 2001) led to the final identification of peaks: 7 and 9 as glycosyl-malonate conjugates. Actually, the presence of this kind of conjugation is quite common in other plants of the fabaceae family, e.g. in soy (Romani *et al.*, 2003; Wu *et al*, 2004), red clover (Klejdus *et al.*, 2001), lupin (Bednarek *et al*, 2001) and Medicago (Farag *et al.*, 2007). Nevertheless, to our knowledge, no previous reports exist about their presence in *R. sphaerocarpa*.

Quantatively, we observe that the aqueous extract of *R. sphaerocarpa* is dominated by the 8-C-glycoside of daidzein (6.12mg/100mg extract), which alone counts for more than 50% of the total isoflavone amount (table 12), followed by pseudobaptigenin-glucoside (1,56mg/100mg). The amount of all other derivatives remains below the value of 1mg/100mg infusion extract.

We have reported the complete secondary metabolic fingerprint of *Retama sphaerocarpa* stems methanolic extract for the first time in a separate work (not published data reported in: Bouusahel, 2010). As we are in continues work with those data, we need to mention them briefly; *R. sphaerocarpa* stems methanolic extract like the extracts studied here, was found also to be dominated by the presence of several isoflavones (daidzein, genistein, glycitein, together with their derivatives (mainly glycosides, glycoside acetates, and glycoside malonates) (chromatogram and compounds structures were reported). The daidzein derivative was the compound with highest percentage (32%) in the stems methanolic extract followed by genistein glucoside (12.7%).

2.4. GC/MS/FID analysis of alkaloids in *R. sphaerocarpa* stems methanolic extract

The examination of the methanolic extract from the stems of *Retama sphaerocarpa* (RSM) by GC/MS showed the presence of the following 9 alkaloids: β -isosparteine; 11, 12-dehydrosparteine; ammodendrine; retamine; cytisine; 5,6 -dehydrolupamine; lupamine; anagryne; baptifoline; 1,7-oxoretamin reported in the chromatogram of the figure 25 and the amounts are reported in the table 13. The peak assignments were done according to El-Shazly and his coworkers (1996) and supported by MS experimental evidences. The cytisine is the major

alkaloid found in the stems methanolic extract representing up to (23.58%) followed by retamine (18.11%), the aqueous extract of *R. sphaerocarpa* stems was subjected also in our separated previous work (not published data) to the alkaloid analysis using the same method as described here, the results were reported in (Boussahel, 2010); the aqueous extract showed the presence of the same alkaloids as the methanolic extract except 1,7-oxoretamin was not identified in the aqueous extract. Retamine classed the second major compound in the methanolic extract, dominated the aqueous extract presenting up to (44%), whereas ammodendrine was the second principal peak in the aqueous extract.

Martin-Cordero and his coworkers (1993), have extracted nine alkaloids from the stems and leaves of *R. sphaerocarpa* of which six are the same found in our work, moreover these group of researchers found that retamine was the major alkaloid. Retamine in a recent paper was found also as a major component of the dichloromethane stems extract from *Retama sphaerocarpa* (Touati *et al.*, 2015). El-Shazly and coworkers (1996) reported the presence of eight different quinolizidine alkaloids from the stems of *R. sphaerocarpa*, of which only six perfectly match with our findings. The alkaloids β -isosparteine and 11,12-dehydrosparteine, for instance, are reported by the same authors to occur in considerable amounts in *R. raetam* and not in *R. sphaerocarpa*; whilst baptifoline, (0.38% of total alkaloids in the methanolic extract and 3.10% of total alkaloids in the aqueous extract) was present only as traces in El-Shazly and coworkers paper (1996). These discrepancies may originate from the different solvent used for the extraction but also the plant was collected from different lands. In recent papers that studied other *Retama* species like *Retama monosperma*; cytisine, Ammodendrine, and anagryrine that we detected in *R. sphaerocarpa* were the major components (Fdil *et al.*, 2012). As well retamine, anagryrine, cytisine were previously isolated from the 75% ethanol extract of the aerial part of *Retama raetam* (Abdel-Halim *et al.*, 1992), taking all together we confirm that *Retama sphaerocarpa* is a good source of quinolizidine alkaloids that are proved to be important therapeutically.

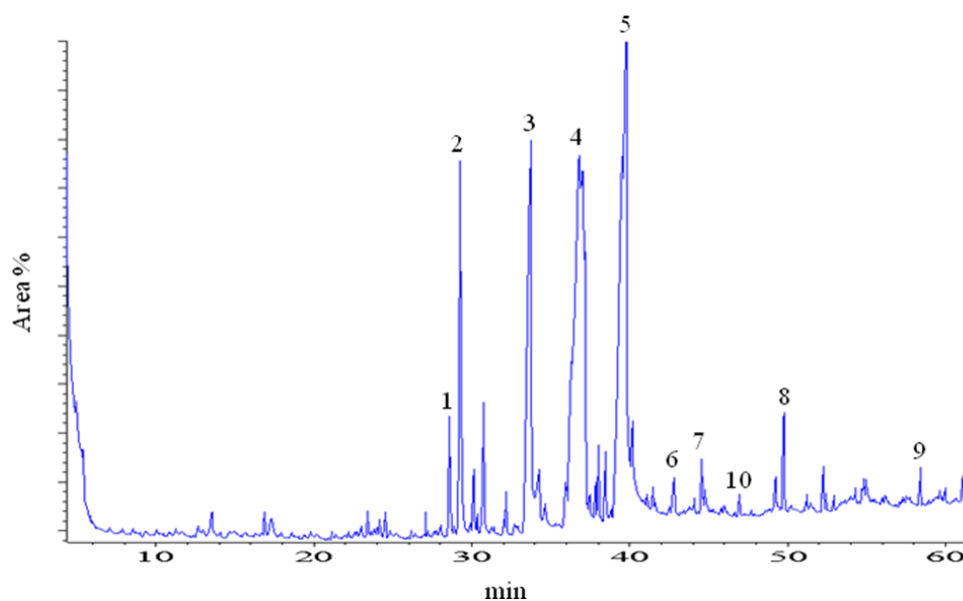


Figure 25. GC/FID profiles of RSM, numbers refer to table 13.

Table 13. Alkaloids found in RSM. Numbers correspond to figure 26.

Peak number	Compound name	Area %
1	β -isosparteine	1.48
2	11,12-dehydrosparteine	5.50
3	Ammodendrine	1.36
4	Retamine	18.11
5	Cytisine	23.58
6	5,6-dehydrolupamine	0.7
7	Lupamine	0.64
8	Anagyrine	1.11
9	Baptifoline	0.38
10	1,7-oxoretamine	0.53

2.5. Antioxidant/radical scavenging activity

Different antioxidant assays were performed for *R. sphaerocarpa* stems and fruits extracts. The figures 26, 26, 27 and 29 demonstrate all the calibration curves used in antioxidant activity, whereas the corresponding results are shown in tables 14 and 15.

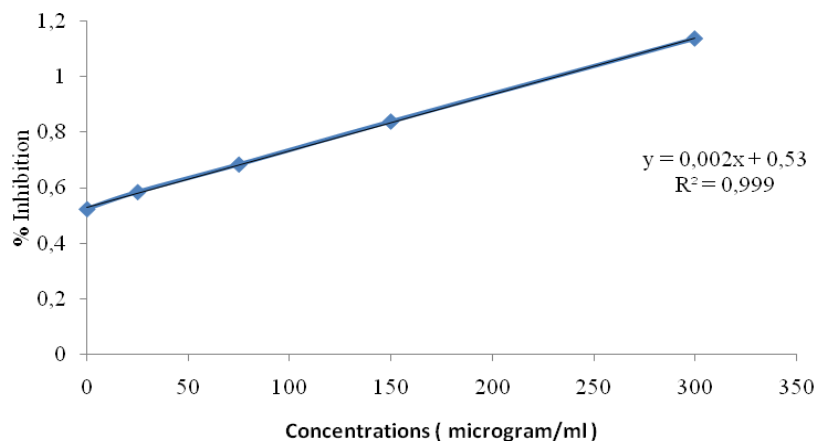


Figure 26. Gallic acid calibration curve used in folin-Ciocalteau experiment.

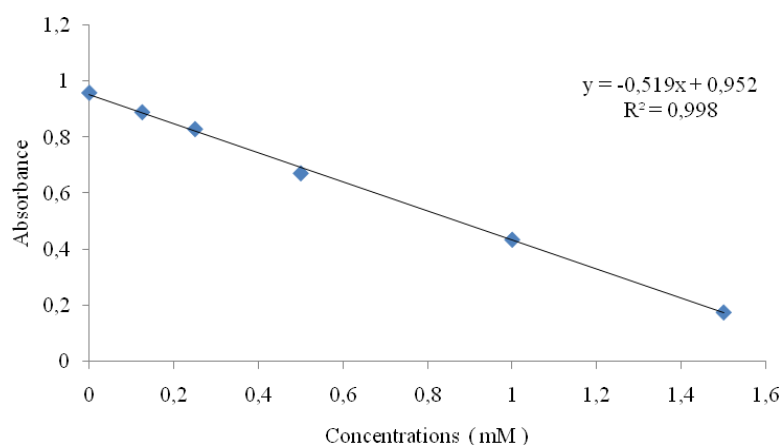


Figure 27. Trolox calibration curves used in 1,1-diphenyl-2-picrylhydrazyl (DPPH•) test

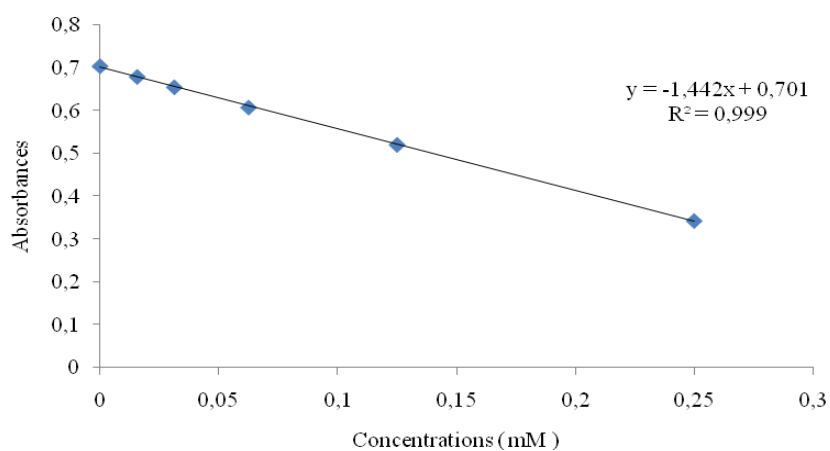


Figure 28. Trolox calibration curves used in ABTS•+ radical assay.

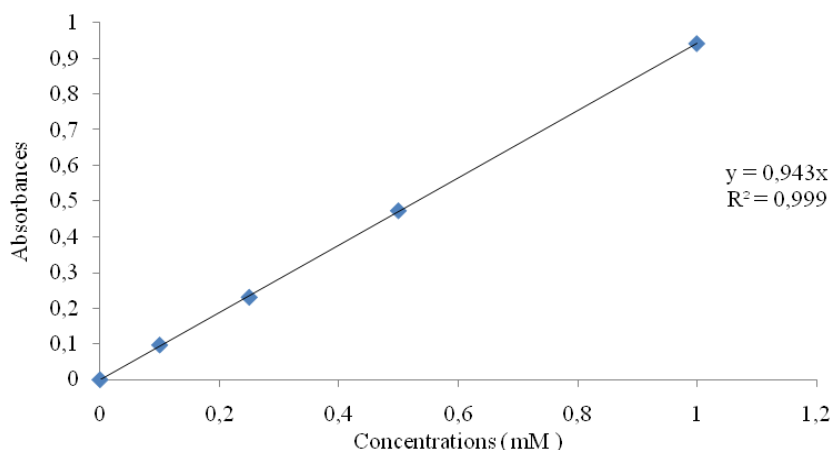


Figure 29. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ calibration curves used in ferric reducing/antioxidant power (FRAP) assay.

It has been reported that bioactive plant extracts with high levels of phenolic and flavonoid compounds exhibit strong anti-oxidant activity (Zhao *et al.*, 2014). Therefore, the folin–Ciocalteu method was performed for the identification of total phenolic content. The results indicate that the methanolic extract of the stems showed a significant high amount of phenolic compounds (241.87 ± 16.37 GaE/mg extract) as compared to all the tested extracts, followed by the traditional and methanolic extract from the fruits (152.77 ± 3.47 μg GaE/mg and 140.87 ± 8.40 μg GaE/mg of extract). Additionally FRAP assay was performed as it is considered different from other assays because there are no free radicals involved but the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The results showed that the methanolic and traditional extracts of the fruits together displayed the highest reducing capacity (0.65 ± 0.05 mmol Fe^{2+} /g; 0.60 ± 0.04 mmol Fe^{2+} /g of extract, respectively) as compared to all the tested extracts, and they are followed by the methanolic extract of the stems (0.49 ± 0.02 mmol Fe^{2+} /g extract). In the two assays of direct free radical inhibition (DPPH• and TEAC); the ability of our extracts to reduce the blue/green ABTS⁺, and the purple DPPH showed a pretty similarity in the results without any significant difference between the studied fruits and stems extracts. In DPPH assay, the results of the fruits extracts were (0.239 ± 0.022 mmol TE/g; 0.16 ± 0.09 mmol TE/g and 0.22 ± 0.08 mmol TE/g of extract) for methanolic, aqueous and traditional extracts respectively. Whereas in TEAC experiment the results of the stems extracts were (0.38 ± 0.02 mmol TE/g; 0.34 ± 0.02 mmol TE/g; 0.31 ± 0.02 mmol TE/g of extract). The two radical scavenging assays (DPPH• and ABTS⁺); measure the ability of the antioxidants in a sample to scavenge a pre-formed radical. The DPPH• radical is stable at formation, DPPH in powder form is simply dissolved in methanol and the resulting purple solution is added to the sample. In contrast the ABTS⁺ radical cation is created

by reacting ABTS with the oxidant and the reaction proceeds during incubation to form the radical cation (Wootton-Beard *et al.*, 2011).

Table 14. Total phenolic content and antioxidant activity of *R. sphaerocarpa* fruits extracts measured by means of different *in vitro* tests

tests	<i>Retama sphaerocarpa</i> fruits extracts		
	Methanolic	Aqueous	Decoction
Folin µg GaE/mg extract	140.875 ± 8.404	76.573 ± 2.498**	152.772 ± 3.471
DPPH mmol TE/g extract	0.239 ± 0.022	0.157 ± 0.094	0.221 ± 0.079
TEAC mmol TE/g extract	0.39 ± 0.038	0.328 ± 0.06	0.30 ± 0.005
FRAP mmol Fe ²⁺ /g extract	0.652 ± 0.053	0.218 ± 0.012**	0.605 ± 0.043
ORAC mmol TE/g extract	7.314 ± 0.066	4.034 ± 0.053**	6.548 ± 0.027

Results were expressed as mean±SD of three experiments. **Versus methanolic extract $P < 0.01$, GaE: gallic acid equivalent; TE: trolox equivalent; SD: standard deviation; DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing/antioxidant power; TEAC: trolox equivalents antioxidant capacity; ORAC: oxygen radical absorbance capacity.

Table 15. Total phenolic content and antioxidant activity of *R. sphaerocarpa* stems extracts measured by means of different *in vitro* tests.

tests	<i>Retama sphaerocarpa</i> stems extracts		
	Methanolic	Aqueous	Decoction
Folin µg GaE/mg extract	241.875 ± 16.369	198.256 ± 16.378**	122.2 ± 8.202**
DPPH mmol TE/g extract	0.110 ± 0.018	0.13 ± 0.033	0.122 ± 0.022
TEAC mmol TE/g extract	0.383 ± 0.017	0.342 ± 0.02	0.31 ± 0.022
FRAP mmol Fe ²⁺ /g extract	0.478 ± 0.023	0.221 ± 0.049**	0.286 ± 0.0276**
ORAC mmol TE/g extract	4.228 ± 0.086*	10.147 ± 0.318*	54.820 ± 0.9352

Results were expressed as mean±SD of three experiments. *: Versus traditional extract $P < 0.01$; **: Versus methanolic extract $P < 0.01$; all other abbreviations are cited in table 14.

As DPPH and ABTS-based assays use non-physiological radicals, we performed ORAC assay because it reflect physiological perturbations and detect chemical change in a fluorescent molecule caused by a free radical attack. The results showed that the traditional extract of the stems marked the highest activity (54.82±0.935 mmol TE/g extract), followed by the stems aqueous extract (10.147±0.3183 mmol TE/g extract) than the fruits methanolic extract (7.31±0.07 mmol TE/g extract). The antioxidant activity observed in *Retama sphaerocarpa* fruits and stems is a result of a mixture of active compounds present in the extracts. The antioxidant activity is due mainly to the redox properties of the compounds, which allow them to act as reducing agents or hydrogen atom donors (Tosun *et al.*, 2011). The main compounds responsible of *R. sphaerocarpa* activity are suggested to be phenolics. Structurally, phenols comprise an aromatic ring bearing one or more hydroxyl substituents; they range from simple molecules to highly polymerized

compounds (Bravo, 1998). The active polyphenolic antioxidants in *R. sphaerocarpa* are supposed to be isoflavones and flavones, since HPLC/MS analysis confirmed their large abundance in our extracts especially isoflavones. Many previous studies linked the beneficial properties of isoflavones to their antioxidant activity. The isoflavones like daidzein and genistein with their corresponding glycosides were isolated by other researchers from plants and tested for their antioxidant activities, the results showed that the effect is dependent on the system used in the experiment and the chemical structure of these compounds (Arora *et al.*, 1998; Guo *et al.*, 2002; Lee *et al.*, 2005). In addition to what preceded, in HPLC-PDA/ESI-MS identification and quantification of flavonoids we have notified that the fruits traditional extract contains very less isoflavones and flavones in comparison with the fruits methanolic extract even this, all the studied antioxidant assays as we have seen shows that the traditional extract of the fruits have a potentiality similar to that of the methanolic one, this is a practical result that confirm the presence of non-flavonoids compounds in the traditional extract responsible of its high activity. Our suggestion is reinforced by the results of folin–Ciocalteu assay, which indicate a high level of phenolics in the traditional fruits extract that can be other than flavonoids, adding to this, total tannins assay showed that the traditional extract contained statistically higher amount of tannins as compared to all other tested extracts. Other than flavonoids, polyphenolic compounds such as anthocyanins and tannins are considered to have antioxidant activity (Zhou *et al.*, 2011; Hosu *et al.*, 2014). In another hand the differences in antioxidant activity between the extracts is expected since previous works confirmed that the solubility of phenolic compounds is governed by the type of solvent (polarity (Djeridane *et al.*, 2006).

2.6. Advanced glycation end products (AGE) formation assay

In the present study, *Retama sphaerocarpa* fruits and stems extracts were assessed for inhibitory effects on in vitro AGE formation through fluorometric detection of fluorescent AGEs. Quercetin, a known AGE inhibitor, served as a positive control. All the results are expressed as IC₅₀ that represent the amounts of extract or quercetin (µg/ml) required for reducing AGEs formation by 50% and they are presented in table 16, also the representative response of the extracts and the standard is presented graphically in figures 30 and 31.

The extracts exhibited like the standard concentration-dependent inhibition of fluorescent AGE formation. The standard quercetin (IC₅₀ 5.42µg/ml) and the methanolic extract of the stems (IC₅₀ 9.36µg/ml), showed the highest inhibition of AGE formation, followed by the methanolic extract of the fruits (IC₅₀ 38.6µg/ml), the traditional extracts of both fruits and stems presented a very

close and moderate inhibition (IC_{50} 81.33 μ g/ml; 84.026 μ g/ml, respectively). Whereas the fruits and stems aqueous extracts were less active in comparison with all the studied extracts.

Table 16. IC_{50} values (concentration reducing 50% of AGE formation) of *R. sphaerocarpa* fruits and stems extracts and quercetin (positive control).

<i>R. sphaerocarpa</i> extracts		IC_{50} μ g/ml (Confidence limits)
Fruits	Methanolic	38.6 (29.13-51.17)
	Aqueous	283.54 (237.35-338.72)
	Decoction	81.33 (51.95-127.34)
Stems	Methanolic	9.36 (5.98-14.65)
	Aqueous	179.68 (76.76-420.60)
	Decoction	84.026 (29.93-235.90)
Quercetin		5.42 (4.84-6.06)

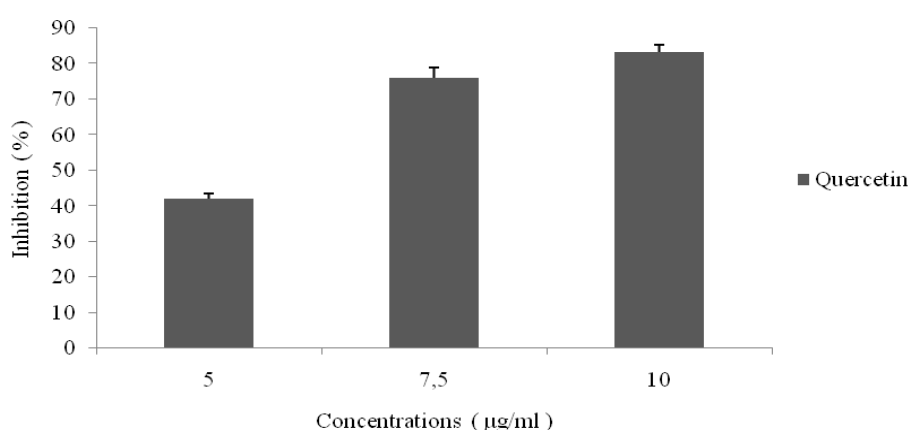


Figure 30. Effect of the standard quercetin on *in vitro* formation of fluorescent AGEs. Results are expressed as the mean \pm SD of the percent inhibition relative to negative control versus the different concentrations of the standard.

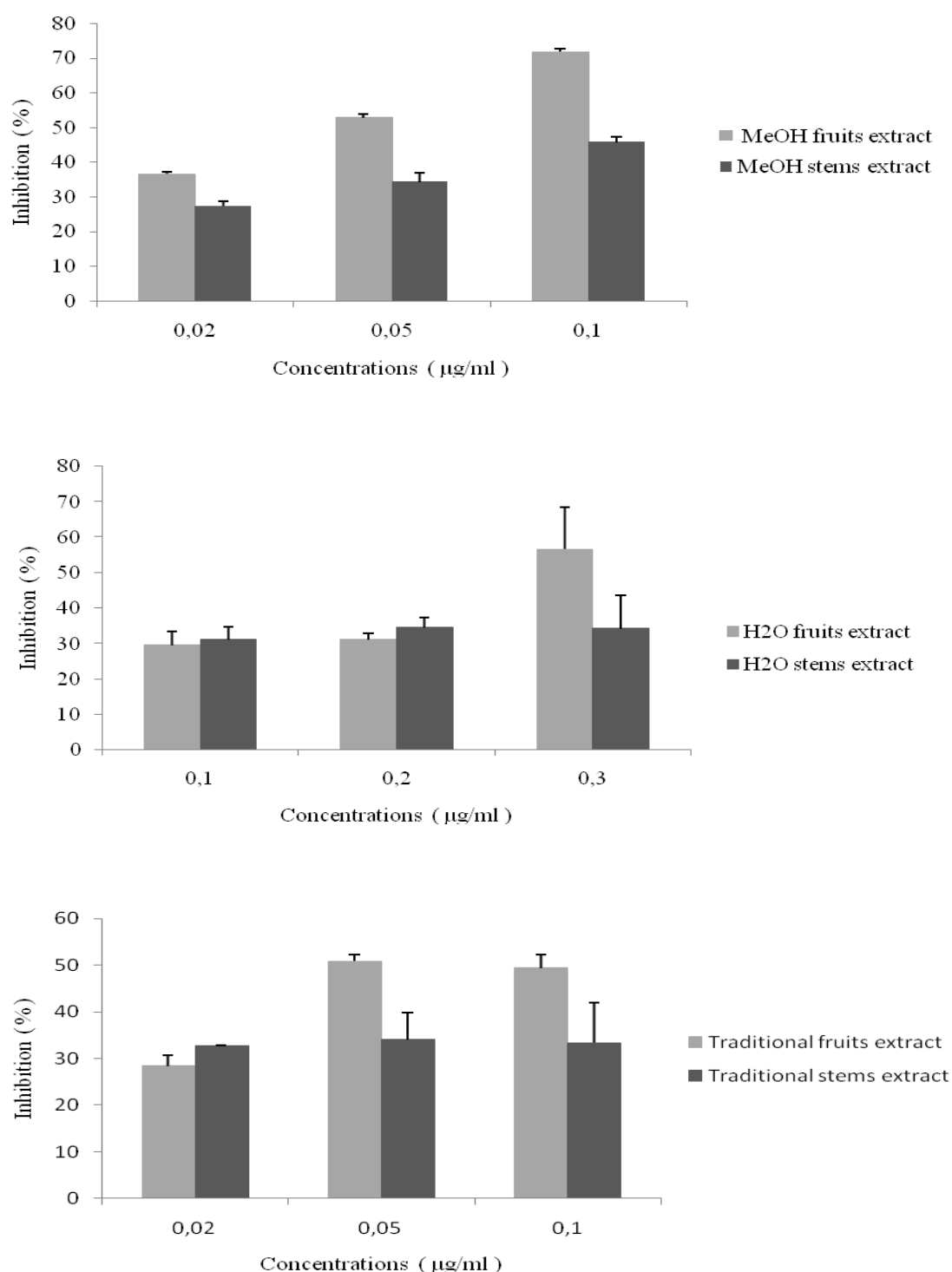


Figure 31. Effect of *Retama sphaerocarpa* fruits and stems extracts on *in vitro* formation of fluorescent AGEs. Results are expressed as the mean \pm SD of the percent inhibition relative to negative control versus the concentrations of MeOH: methanolic, H₂O: aqueous and decoction fruits and stems extracts.

Nakagawa and coworkers (2002) supported in their work the theory that oxidative reactions have significant influence in AGEs formation and the possibility that antioxidants would be AGEs

inhibitors. In our studies, the results of antioxidant activity indicate that the most active among the extracts are classed in this order: the fruits methanolic extract, fruit traditional extract and stems methanolic extract. In folin–Ciocalteu assay, the methanolic extract of the stems was markedly higher from all studied extracts in term of polyphenols content followed by the fruits methanolic and traditional extracts exactly. Like in this test, the same three extracts are the more active; thus, the stems methanolic extract is showing the highest inhibitory effect on AGEs, followed by the methanolic and traditional extracts of the fruits.

Combining these data, we conclude that the antiglycation activity might be partly attributed to phenolics considered as antioxidant compounds present in our extracts. In addition, since the methanolic extract of the stems was particularly the most rich in total phenolic content and most potent in AGEs formation assay. This leads to suggest that the active compounds can be either flavonoids as detected in our work by HPLC analysis or non-flavonoids because phenolic compounds are a much diversified group of phytochemicals that some of them can be present in the extracts. Other studies on several natural extracts/compounds such as polyphenolic-rich extracts, flavonoids, saponins, tannins, anthraquinones, polysaccharides showed their antioxidant and antiglycation activities (Zhang *et al.*, 2011; Bi *et al.*, 2012; Ramkissoon *et al.*, 2013; Anusiri *et al.*, 2014).

2.7. Acute oral toxicity of *R. sphaerocarpa* stems methanolic extract (RSM)

In this study, we evaluated the acute toxicity of *R. sphaerocarpa* stems methanolic extract in *Albino Wister* rats after an oral injection. At this aim five groups of Wistar rats, each comprise 6 animals were treated with RSM at different doses. The results indicate that the appearance of intoxication signs and/or mortality of the rats are dose dependent. In fact, at the lowest dose (2000mg/kg), none of the animals died, but some signs of intoxication start to appear about four hours after treatment; two rats showed troubles in their body movement but came back to normal after 24 hours. Higher doses provoked a regular dose-dependent increase in intoxication symptoms and in the number of died animals; thus at the dose (3000mg/kg) one animal suffered from a continuous hard trouble in body movement that finished by his death after three days. We observed at the other higher doses that the rats are restraint and their body movements trouble continually and more seriously until it make them incapable to control any of their mouvements. This means each time the animal wants to move his body trouble uncontrollably leading to the death of three, five and six animals at the doses (3500mg/kg, 4000mg/kg and 5000mg/kg,

consecutively), this happened for a period of 2 days, after that, everything was restored to survivors.

To calculate the median acute toxicity value (LD_{50}) the method of Miller and Tainter (1944) was implicated. For this the doses injected were transformed to log-dose and the percentage of mortality was calculated from the number of deceased rats in each group, and then transformed to probit, the dead percentage for 0 and 100 were corrected before the determination of probits, the table 17 summarize the cited steps. The probit values are plotted against log-doses as shown in figure 32, and then the dose corresponding to probit 5, that represent graphically $\text{Log-}LD_{50}$ is calculated. The results indicate that ($\text{Log } LD_{50}=3.541$) and ($LD_{50}=3479.05\pm228.78$ mg/kg), with 95% confidence interval of (3250.27-3707.84 mg/kg). Based on the classification of Diezi (1989), which considers substances with LD_{50} between 500 and 5000mg/kg bodyweight slightly toxic, *R. sphaerocarpa* LD_{50} falls in this range, being slightly toxic in acute ingestion.

Table 17. Results of probits determination

Dose mg/kg	Log dose	Observed effect		Expected effect	
		Probit	Corrected %	Probit	Corrected %
2000	3.3	0	0	1.098	0
3000	3.48	4.03	16.7	4.0068	16.1
35000	3.54	5	50	4.9764	48.9
4000	3.6	5.97	83.3	5.946	82.7
4500	3.65	7.23	98.7	6.754	96

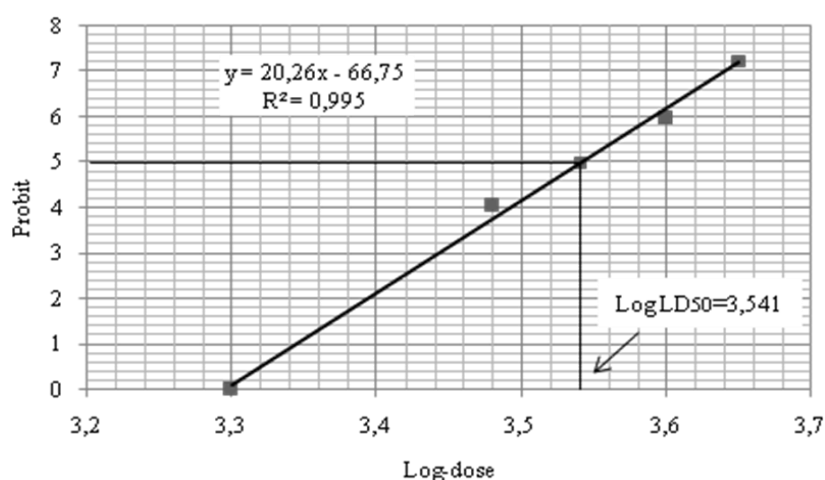


Figure 32. Adjusted probit toxicity (log-dose) curve of RSM in rats, (n=6 animals per dose).

In our previous separated studies (not published data) reported in Boussahel, (2010), we have tested the acute toxicity of the aqueous extract prepared from *R. sphaerocarpa* stems. The results showed an LD₅₀ equal to 2488.86mg/kg calculated with the same method, when comparing the two extracts we observe that *R. sphaerocarpa* stems methanolic extract is more safe than the stems aqueous extract, however both extracts are classed in the same category (slightly toxic). The symptoms appearing on animals are particularly related to trouble in the body movement, this leads to suggest that a neurological problem occurred in the rats because of the extract oral injection. In literature a treatment period of many days with high daily doses; the isoflavones genistein, daidzein (detected in our extract): can be toxic to the reproductive tract in female rats (Lamartiniere *et al.*, 2002; Carbonel *et al.*, 2011). The literature did not show as far as we know any study of the effect on the neural system related to isoflavones injection. Whereas various pharmacological and toxicological properties have been attributed to quinolizidine alkaloids and the plants of leguminosae family are a major source of this kind of alkaloids (Maximo *et al.*, 2006; Wink and Witte, 1985; Schmeller *et al.*, 1994). The quinolizidine alkaloids that we have identified above using GS/MS are reported in literature to exert a neurological effect, Resta and coworkers (2008) reported that in mammals, quinolizidine intoxication is characterized by trembling, shaking, excitation, and convulsion. They reported also that spartein show moderate acute oral toxicity due to neurological effects leading to the loss of motor co-ordination and muscular control. These are mostly the same symptoms observed in our acute oral toxicity, thus, we suggest that most probably the slight toxicity effect of *Retama sphaerocarpa* is due to the presence of quinolizidine alkaloids in this plant.

2.8. Effect of RSM on normoglycemic rats

After the treatment of normal healthy rats with a single graded doses of RSM (20mg/kg; 50mg/kg; 100mg/kg of body weight); changes in the blood glucose level of each group of animals was followed during a 4h period, the results are demonstrated in figure 33.

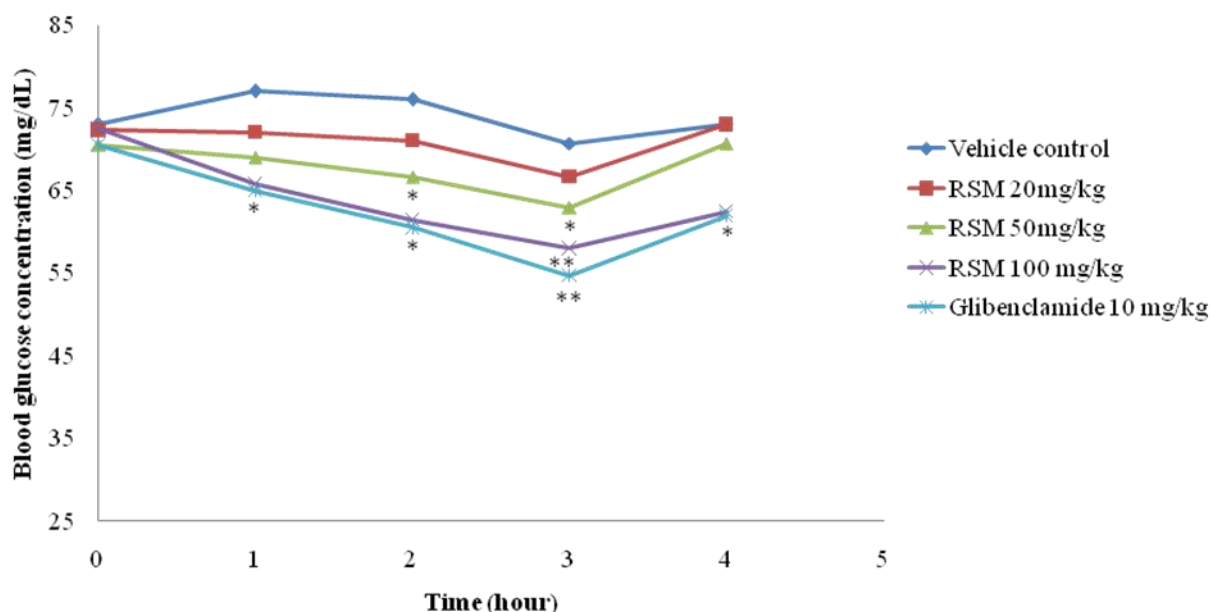


Figure 33. Effect of RSM on blood glucose levels in normoglycemic rats. Each value is expressed as Mean of six observations. *: $P<0.05$; **: $P<0.01$, when compared with corresponding values of the vehicle control group.

At 0h the blood glucose level of all the rats was in the range [70-73 mg/dl], hypoglycemia was observed starting from 1h and the effect was dose dependent, in fact at 1h the doses 20mg/kg and 50mg/kg didn't show any effect (72mg/kg; 69mg/kg respectively). Whereas the dose 100mg/kg presented a slight reduction of blood glucose level (65.75mg/dl). The lowest dose 20mg/kg reduced slightly the blood glucose level only at the third hour (66.67mg/dl), whereas the higher doses reduced significantly the blood glucose level in a dose dependent way. At 2 and 3 hours for 50mg/kg (66.67mg/dl; 63.66mg/dl respectively) and at 1, 2 and 3 hours for 100mg/kg (65.75mg/dl; 61.5mg/dl and 58mg/dl) as compared to control.

The decline in blood sugar level reached his maximum in this experiment at the third hour for the extracts and also for glibenclamide treatments, than the blood glucose level start to rise and restore gradually for all treatments by 4 hour, it was (70.67mg/dl and 62.5mg/dl respectively) for the doses 50mg/kg and 100mg/kg.

We conclude that the doses 100mg/kg and 50mg/ml in this order are compared in their effect favorably well with that of glibenclamide drug, while the dose 20mg/ml didn't show any remarkable results. Maghrani and his coworkers (2003) studied the effect of the single dose 20mg/ml prepared from the aqueous extract of *R. reteam* leaves on the blood glucose level of normal rats. Their results indicated that the blood glucose level dropped significantly ($p<0.01$) and was more pronounced 4 and 6 hour after injection. Taking together the results we conclude

that different species and different organs can react more or less on the blood glucose level of normal rats and we confirm that *R. sphaerocarpa* have a hypoglycemic effect on normal rats, in the first 4 hours after injection.

2.9. Effect of RSM on oral glucose tolerance in normal rats

In our study the oral glucose tolerance test was carried out to evaluate the hypoglycemic activity of three doses from RSM in glucose loaded hyperglycemic rats and the results of this test are shown in figure 34. The oral administration of the glucose load at (2g/kg) in the control rats produced a rapid increase in their blood glucose level. Hence it was at 0min (72.5mg/dl) and became at 30min (125mg/dl), then it start to decrease gradually being (114mg/dl) at 60min and (94.75mg/dl) at 120min. Glibenclamide drug reduced the blood glucose level significantly ($P<0.01$) during the study period, it was (94.25mg/dl and 66.5mg/dl respectively) for 30min and 120min.

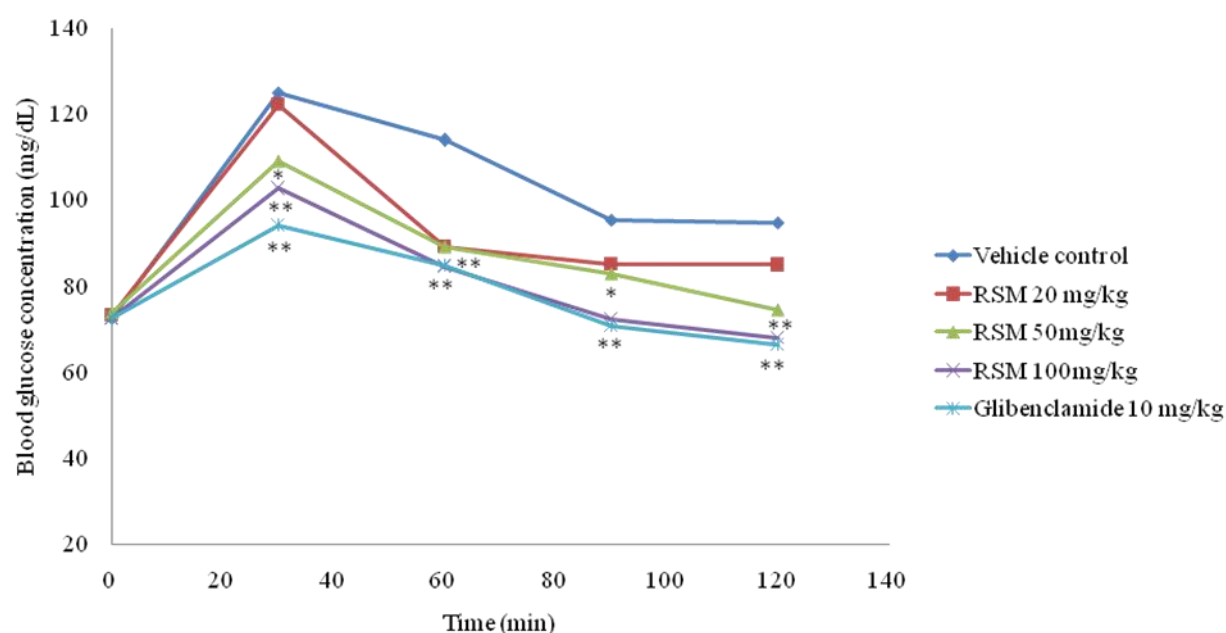


Figure 34. Effect of RSM on glucose tolerance in rats. Each value is expressed as Mean of six observations. *: $P<0.05$; **: $P<0.01$ when compared with corresponding values of the vehicle control group.

The administration of different doses of RSM, 60min prior to glucose loading reduced significantly the rise of blood glucose level when compared to the control. At 30 minutes the dose 20mg/ml did not show hypoglycemic activity when compared with control group (122.25mg/dl),

whereas the doses 50mg/ml and 100mg/ml had a significant ($P<0.05$) hypoglycemic effect at the same cited time being of (109.25mg/dl and 102.75mg/dl respectively). At 60min after glucose leading the reduction was significant ($P<0.01$) at all RSM doses used (89.25 mg/dl; 89.25mg/dl and 84.5mg/dl respectively) for 20mg/kg; 50mg/kg and 100mg/kg. The higher doses (50mg/ml and 100mg/kg) presented more lowering effect in a dose dependent manner during the 120 minutes of experiment. The doses 100mg/ml and 50mg/ml in this order showed the maximum improvement in glucose tolerance test. The change in blood glucose levels in response to an oral glucose load has long been used clinically for the diagnosis of diabetes mellitus and in research to evaluate the effectiveness of hypoglycemic agents (Krisanapun *et al.*, 2009); we conclude that *R. sphaerocarpa* could be a good candidate for high glucose treatment as the traditional medicine reported.

2.10. Effect of RSM on blood glucose level in normal and STZ-induced diabetic rats

Normal and confirmed diabetic rats were assigned into different groups, the animals of one group between all were administered daily with glibenclamide drug and the other groups were treated orally with RSM at two different doses (50mg/kg and 100 mg/kg). To know if RSM have a hypoglycemic effect, the blood glucose level was measured each five days for the 15 days of treatment, the figure 35 shows the results of all the measurement.

We notified that during the treatment period, the normal animals injected with RSM presented a normal variation in blood glucose level as compared to normal untreated control. Hence in the 5 day the glucose levels of normal rats injected with RSM were (68.25 ± 6.18 mg/dl and 69.5 ± 2.89 mg/dl respectively) for 50mg/kg and 100mg/kg then in 15 day, the glucose levels were (71.5 ± 3.70 mg/dl 77.75 ± 7.13 mg/dl and respectively). This is an indication that at these doses and at a period of 15 days; RSM is able to maintain the blood glucose level of normal animals, while the glibenclamide reduced the glucose level of normal rats significantly during all the treatment period espacialy at the last day of experiment (39.25 ± 3.20 mg/dl). Our results in this point come different from those reported by Maghrani and his coworkers (2003), because in their study the blood glucose level of normal rats injected daily with *R. reteam* at the dose 20mg/ml showed a significant reduction in blood glucose level during the 15 days of their experiment.

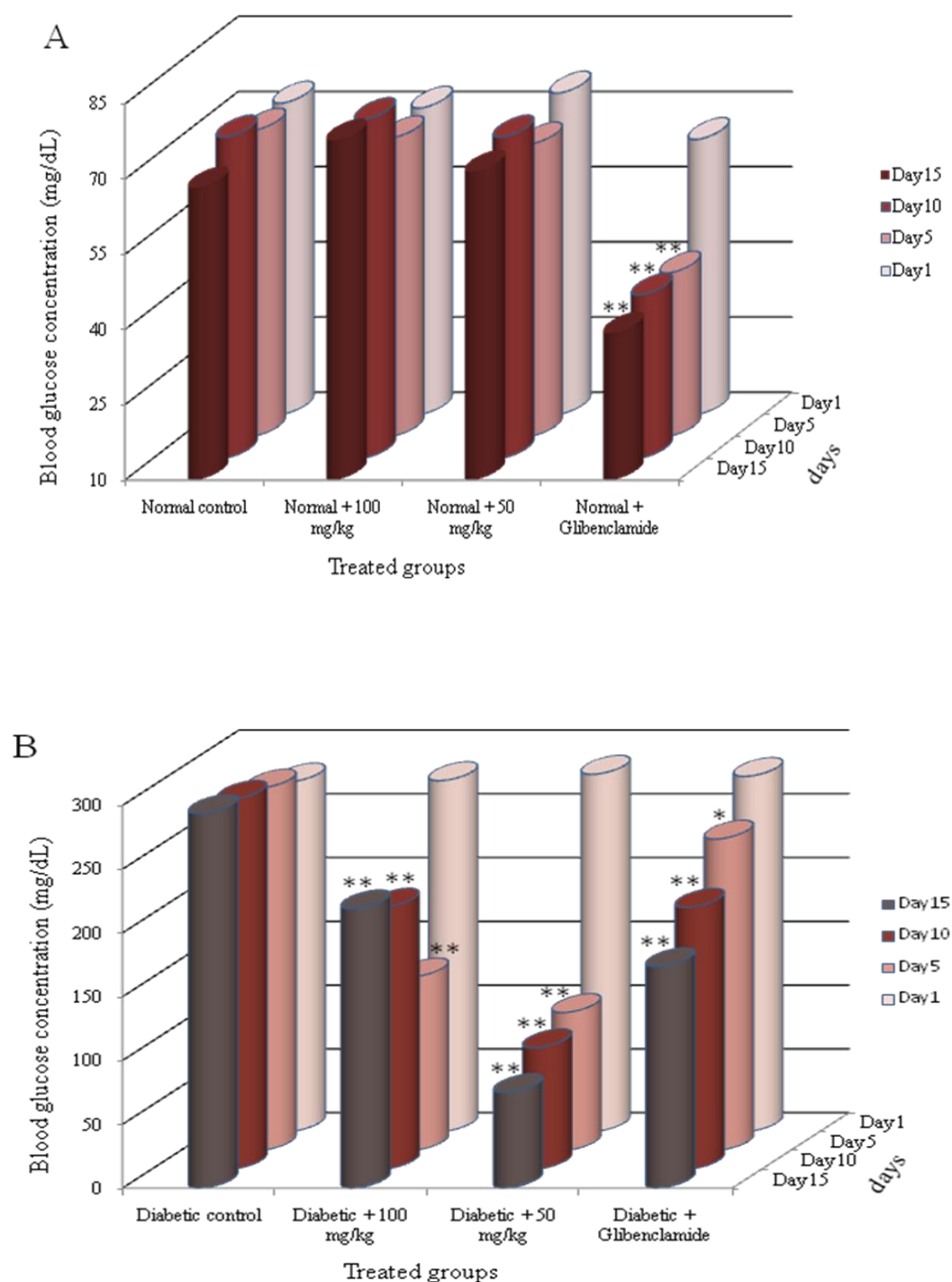


Figure 35. Effect of RSM on blood glucose level of A: normal and B: diabetic rats. Each value is expressed as mean of six observations. (*: $P < 0.05$ and **: $P < 0.01$) as compared with diabetic and normal control groups,

In another hand the injection of RSM reduced starting from 5 day significantly ($P < 0.01$) the high level of glucose in diabetic rats (108 ± 7.94 mg/dl and 136 ± 1.00 mg/dl respectively) for the doses 50 and 100 mg/kg as compared to diabetic untreated rats (284 ± 1.00 mg/dl). At the dose 50 mg/kg the decrease was related to duration of treatment, thus in the 10 and 15 days the glucose levels were

(95.33±8.08mg/dl and 75±3.60mg/dl respectively). Whereas at the dose 100mg/dl the effect was not dependent on the duration of treatment and the reduction of blood level was at maximum in the 5-day and come to rise again in the 10 and 15 days (205±5.57mg/dl and 219±6.00mg/dl respectively). The glibenclamide drug also reduced significantly ($P<0.01$) the blood glucose level during the period of treatment (244±22.54mg/dl; 205.33±5.51mg/dl and 173.67±4.93mg/dl respectively) in the 5; 10 and 15 days.

Our results go in accordance with the results of Maghrani and coworkers (2005a), they found also that the most significant effect of *R. reatam* was reached on day 15 and the blood glucose level was normalized after two weeks of daily treatment with *R. reteam*. In accordance with them also, our study show that the level of blood glucose continue to increase in the diabetic control animals (293mg/dl at 15 days) due to the aggravation of diabetic state in absence of any hypoglycemic treatment. When comparing between the results of the doses 50 and 100mg/kg we observed that the effect of RSM was not dose dependent, since the dose 50mg/kg had more lowering effect than 100mg/ml and not only this but also at the day 15 the blood glucose level of diabetic rats receiving the dose 50mg/ml was compared favorably well with that of normal control non-diabetic animals.

STZ-induced hyperglycemia has been described as an utilizable experimental model to study the activity of antidiabetic agents (Karuppusamy *et al.*, 2014). Induction of diabetes in the experimental rats was confirmed by the presence of a high blood glucose level and the control of glucose elevation is the first step toward a sustained reduction in hyperglycemia that will decrease the risk of developing micro vascular diseases and reduce their complications (Kim and Hyun, 2006). Our results demonstrated that RSM is able to give good hypoglycemic effect on diabetic rats at lower doses (<100mg/ml), thus it is a good candidate for reducing glucose level.

2.11. Effect of RSM on feed and water intake of normal and diabetic rats

The level of water and feed intake for both normal and diabetic rats (treated and untreated) are shown in table 18. The water intake of normal rats during the 15 days was about (139±4.07 ml), and that of normal treated rats was (112.5±10.86 ml and 125.81±1.89 ml) for the doses 50mg/kg and 100mg/kg respectively. We observe that the animals treated with RSM did not mark a big difrence in water intake as compared to control. The same thing was observed for the food; the normal untreated rats ate about (89.41±4.12 mg) per day and the normal animals treated ate about (83.85±8.50 mg and 82.06±3.49 respectively) for the doses 50mg/kg and 100mg/kg.

The increase in the feed and water intake are well known symptoms markers of diabetes in both human and animal models which are direct consequence of insulin deficiency (Swantson *et al.*, 1990), in fact the diabetic untreated rats were showing a very high intake of the water (924 ± 45.01 ml) and feed (197.71 ± 14.36 mg). However, the animals treated daily with RSM doses marked a significant reduction ($P < 0.01$) in the water intake being of (593 ± 42.32 ml and 776.5 ± 72.81 ml) respectively for 50mg/kg and 100mg/kg and in feed intake (117.08 ± 22.48 mg and 132.24 ± 11.62 mg respectively) as compared with the diabetic untreated rats. The reducer effect was better than that of glibenclamide that was (824 ± 78.41 ml and 169.49 ± 16.53 mg) for water and feed intake respectively, and the effect was not found to be dose dependent because also here the dose 50mg/kg gave better results than the dose 100mg/kg.

Our results of the change in body weight, the feed and water intake and the non-related dose dependent effect are similar with those reported in the work of Oyedemi and coworkers (2011a); who demonstrate the effect on STZ-induced diabetic rats of another plant than ours, but from the same family (leguminosae).

Table 18. Effect of RSM on: water and feed intakes of normal and diabetic rats. Values are the mean \pm SD of 6 rats in each group; (*: $P < 0.05$ and **: $P < 0.01$) as compared with diabetic control group.

Treatment	Water intake (ml)	Feed intake (gram)
Normal control	139 ± 4.07	89.41 ± 4.12
Normal + RSM (50mg/kg)	112.5 ± 10.86	83.85 ± 8.50
Normal+ RSM (100mg/kg)	125.81 ± 1.89	82.06 ± 3.49
Normal + standard glibenclamide (10 mg/kg)	117.31 ± 8.88	86.26 ± 6.37
Diabetic control	924 ± 45.01	197.71 ± 14.36
Diabetic + RSM (50mg/kg)	$593 \pm 42.32^{**}$	$117.08 \pm 22.48^{**}$
Diabetic + RSM (100mg/kg)	$776.50 \pm 72.81^{**}$	$132.24 \pm 11.62^{**}$
Diabetic + standard glibenclamide (10 mg/kg)	$824 \pm 78.41^{*}$	169.49 ± 16.53

2.12. Effect of RSM on haematological parameters of normal and diabetic rats

After RSM treatment of both normal and STZ-induced diabetes rats, the blood was withdrawn from the retro-orbital plexus of three animal's eyes from each group for analysis of red blood cells (RBC); white blood cells (WBC); hemoglobin (Hb) and hematocrit (PCV). The results are summarized in tables 19 and 20. The treatment of normal rats with RSM at the dose 100mg/kg; showed normal values of RBC ($8.43 \pm 0.63 \times 10^6/\text{mm}^3$), WBC ($7.23 \pm 0.70 \times 10^6/\text{mm}^3$) PCV ($48.33 \pm 1.53\%$) and Hb (14.33 ± 0.91 g/dL). Regarding the dose 50mg/kg and glibenclamide

three parameters were in the normal range RBC, PCV and Hb, while the white blood cells (WBC) presented a significant decrease in the groups treated with 50mg/kg being of ($7.93 \pm 0.35 \times 10^3/\text{mm}^3$) and in glibenclamide group ($7.88 \pm 0.33 \times 10^3/\text{mm}^3$) as compared to control.

Table 19. Effect of RSM on some hematological parameters of normal rats

Parameters	Normal Control	Normal + RSM (50mg/kg)	Normal + RSM (100mg/kg)	glibenclamide group
RBC ($\times 10^6/\text{mm}^3$)	8.10 ± 0.81	8.61 ± 0.36	8.43 ± 0.63	8.18 ± 0.43
WBC ($\times 10^3/\text{mm}^3$)	7.27 ± 0.32	$7.93 \pm 0.35^*$	7.23 ± 0.70	$7.88 \pm 0.33^*$
PCV (%)	48.67 ± 1.15	49.50 ± 0.71	48.33 ± 1.53	48.25 ± 2.22
Hb (g/dL)	14.05 ± 0.07	14.86 ± 0.64	14.33 ± 0.91	14.16 ± 0.65

(*: $P < 0.05$) as compared with normal control group. RBC: Red blood cells; WBC: White blood count; PCV: Hematocrit; Hb: Hemoglobin.

The same parameters were analyzed for diabetic rats untreated and treated with RSM and glibenclamide. We observed a significant decrease in all studied parameters of the diabetic untreated group ($6.21 \pm 0.44 \times 10^6/\text{mm}^3$; $5.35 \pm 0.35 \times 10^3/\text{mm}^3$ respectively) for RBC and WBC. When comparing with normal control reported in table 18, the significant reduction of this parameters might be due to a state of anaemia in the body of diabetic rats, because the alteration of this parameters are known to cause anaemia condition (Balasubraimanian *et al.*, 2009).

Table 20. Effect of RSM on some hematological parameters of diabetic rats

Parameters	Diabetic Control	Diabetic + RSM (50mg/kg)	Diabetic + RSM (100mg/kg)	glibenclamide group
RBC ($\times 10^6/\text{mm}^3$)	6.21 ± 0.44	$7.15 \pm 0.33^*$	$7.11 \pm 0.22^*$	$6.98 \pm 0.15^*$
WBC ($\times 10^3/\text{mm}^3$)	5.35 ± 0.35	$6.83 \pm 0.20^{**}$	$6.52 \pm 0.17^{**}$	$6.77 \pm 0.12^{**}$
PCV (%)	37.3 ± 2.4	$49.55 \pm 2.90^{**}$	$45.65 \pm 1.77^{**}$	$47.75 \pm 1.20^{**}$
Hb (g/dL)	12.55 ± 0.21	$14.30 \pm 0.28^{**}$	$14.13 \pm 0.32^{**}$	$13.55 \pm 0.21^{**}$

(*: $P < 0.05$ and **: $P < 0.01$) as compared with diabetic control group. All other abbreviations are listed in table 18.

However, the administration of RSM and glibenclamide to diabetic rats marked a significant ($P < 0.01$) increase of the studied parameters when comparing with diabetic untreated rats, especially at the dose 50mg/kg of RSM; the increase was the best and the nearest to normal control; presenting for PCV (49.55 ± 2.90 %) and for Hb (14.30 ± 0.28 g/dL). The dose 100mg/kg showed also a good improvement in the parameters.

The assessment of haematological parameters could be used to reveal the deleterious effect of foreign compounds including plant extracts on the blood constituents of animals (Oyedemi *et al.*, 2011a), they are used also to determine possible alterations in the levels of biomolecules

(Magalhaes *et al.*, 2008). The occurrence of anaemia in diabetes mellitus has been reported (Oyedemi *et al.*, 2011b); that is why we tested these parameters. The effect of RSM is due to its chemical composition, we have demonstrated above that RSM is an extract rich of diverse chemical compounds of an important therapeutic action. These compounds are mainly isoflavones, flavones, tannins and alkaloids and maybe others not detected in our study, add to this RSM with its compounds mixture is combining both antioxidant and antiglycation activities and natural antioxidants showed very significant antidiabetic potential against various pharmacological experimental systems (Sellamuthu *et al.*, 2012). Though, the action mechanism of this plant in diabetic rats is not investigated in this study. However, it may be attributed to the ability of plant extract to lower lipid peroxidation level that causes haemolysis of erythrocytes (Ashafa *et al.*, 2009). Streptozotocin is a well known chemical that suppresses the immune system by damaging white blood cells (WBC) and certain organs in the body (Oyedemi *et al.*, 2011a); hence the intraperitoneal injection of streptozotocin into rats significantly reduced the WBC. After plant extract administration at both doses, WBC were significantly improved, this is due may be to the presence in the extract of active compounds able to stimulate the production of white blood count.

2.13. Effect of RSM on plasma lipids in normal and STZ-induced diabetic rats

The plasma triglycerides and total cholesterol were tested in three samples for each group for both normal and STZ-induced diabetic rats untreated and treated with RSM. The results are summarized in table 21. We observed in RSM normal treated groups with the dose 100mg/kg a significant reduction in both plasma triglycerides (46.67 ± 5.77 mg/dL), and cholesterol (48.67 ± 2.08 mg/dL) levels, as compared to the normal untreated control group, while only the cholesterol decreased significantly ($P < 0.05$) in normal animals treated with glibenclamide (53.00 ± 1.00 mg/dL). The diabetic control rats showed a significant high level ($P < 0.01$) of plasma triglycerides and cholesterol (159.25 ± 14.64 mg/dL and 147.67 ± 5.03 mg/dL) comparing with normal untreated control. but the diabetic groups administered with RSM 50mg/kg showed a significant decrease ($P < 0.01$) in both plasma triglycerides (48.66 ± 4.72 mg/dL) and total cholesterol (89.00 ± 3.00 mg/dL) levels as compared to diabetic control group. The results of diabetic glibenclamide group were close to those of normal control. The effect of RSM was not dose related because the dose 50mg/kg was far better than 100mg/kg as it reduced more significantly the plasma lipids of diabetic rats. Thus, it is reasonable to conclude that RSM could modulate blood lipid abnormalities.

Ideal treatment for diabetes, in addition to glycemic control, should have a favorable effect on lipid profile (Kesari *et al.*, 2007). Our results come in accordance with those reported by Maghrani and his coworkers (2004). They demonstrated that the aqueous extract of *R. raetam* induced a significant decrease of plasma cholesterol and triglycerides levels in STZ diabetic rats after long-term (repeated) administrations. In addition to this Maghrani and his coworkers (2004), demonstrated that *R. raetam* did not affect insulin secretion in both normal and STZ rats and they conclude that *R. raetam* extract reduced plasma cholesterol and triglycerides levels without stimulating insulin secretion. For us to know if *R. sphaerocarpa* is stimulating insulin or not like its analogue *R. raetam*, it will be important to do further studies on the effect of *R. sphaerocarpa* on the secretion of insulin.

Table 21. Effect of RSM on plasma lipids in normal and STZ-induced diabetic rats

Treatments	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Normal control	72.00 ± 8.89	61.50 ± 2.12
Normal + RSM (50mg/kg)	53.33 ± 4.16*	48.67 ± 4.16*
Normal+ RSM (100mg/kg)	48.67 ± 2.08***	46.67 ± 5.77*
Normal + standard glibenclamide (10 mg/kg)	53.00 ± 1.00*	60.67 ± 6.03
Diabetic control	147.67 ± 5.03	159.25 ± 14.64
Diabetic + RSM (50mg/kg)	89.00 ± 3.00**	48.66 ± 4.72**
Diabetic + RSM (100mg/kg)	119.00 ± 4.58**	103.5 ± 7.78**
Diabetic + standard glibenclamide (10 mg/kg)	77.33 ± 2.52**	70.00 ± 5.57**

*: P<0.05 and ***: P<0.01 as compared to normal untreated rats; **: P<0.01 as compared to diabetic control rats.

Recent studies have shown that compounds with combined antioxidant and antiglycation properties are more effective in treating diabetes mellitus (Duraismy *et al.*, 2003; Xi *et al.*, 2008); it is the case of *R.sphaerocarpa* stems methanolic extract that showed good antioxidant, antiglycation and good antidiabetic effect. Our studies indicate that the methanolic extract of *R. sphaerocarpa* stems contains a range of active pharmacological agents, which include alkaloids, flavonoids and tannins. In previous authors studies the oral administration of flavonoid fractions from plants caused a hypoglycemic effect better than alkaloid fractions (Lú *et al.*, 2009). Flavonoid rich extracts was proved also to have hypolipidemic effects on STZ-induced diabetic rats (Sharma *et al.*, 2008; Bansal *et al.*, 2012). In other studies, a flavone derivative showed an Anti-hyperlipidemic action (Guo *et al.*, 2006). Quercetin and flavones were found to have an antidiabetic effect related to their chemicals structure (Coskun *et al.*, 2005; Torres-Piedra *et al.*, 2010), the isoflavones daidzein and genistein (that are main component in our extract) were found in previous studies to reduce glucose (Hintz and Ren, 2004). Thus, the significant

antidiabetic effect of *R. sphaerocarpa* methanolic stems extract may be due to the presence of flavonoids that are known for their antidiabetic effect, or maybe the effect is due to more than one antihyperglycemic principle. Since the extract contain surly other components like alkaloids and tannins, but also the effect can be due to the synergistic effects of all the active components in the extract.

CONCLUSION

CONCLUSION

From this study we conclude that *Rhamnus alaternus* is an important medicinal plant, that contain a considerable amounts of bioactive compounds, the bark of the plant more famous in traditional medicine was more potent then the leaves. The flavonoids secondary metabolites in the plant and important pharmaceutical agent were largely present in the extracts of *R. alaternus* especially: kaempferol, rhamnocitrin, and quercetin derivatives. All the extracts of the plant showed a good antioxidant activity due to its chemical composition. Not only this but the methanolic extract from the bark possess the ability to decrease specifically the growth of cancer cells with a dose dependent apoptosis effect to U937 cancer cells, in another hand the results of acute toxicity showed that this plant is safe for use. Further studies are warranted to separate the active compounds from *R. alaternus* to use them in medicines and health amelioration.

In another hand *Retama sphaerocarpa*, is also considered as an important therapeutic agent; it includes a mixture of interesting bioactive compounds belonging mainly to the classes: flavonoids and quinolizidine alkaloids. The water (aqueous, traditional) and methanolic extracts from this plant combine antioxidant with antiglycation activities and the aqueous extract is a good antidiabetic agent reducing the glucose and plasma lipids profile, this come in accordance with the traditional medicine that reported the effectiveness of Retama in the reduction of blood glucose level. The biological activities of *R. sphaerocarpa* could be due to the presence of the various phytoconstituents detected in the phytochemical screening, which alone or in synergism can impart the therapeutic effect. Meanwhile it is important to mention that *R. sphaerocarpa* was found in our studies as a slightly toxic plant, so the use of this plant should be with precaution especially at high doses. *R. sphaerocarpa* with its activities can be a source for new researches about isolation of new natural herbal antidiabetic drugs.

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