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Phytochemical and Therapeutical Valorisation of *Galium tunetanum* Poiret.

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Title: Phytochemical and therapeutical valorisation of *Galium tunetanum* Poiret

Summary:

Galium tunetanum Poiret is one of dense pre-existent forest vestige belong to Rubiaceae is a North African endemic species constitute an enigma toward their phytochemicals and therapeutic proprieties. Areal part of the plant material is harvested from Megriss – Setif - Algeria, dried in shade, and reduced to a fine powder; then the major groups of secondary metabolites were extracted and quantified. The evaluation of antioxidant activities of species were carried out with DPPH, β-Carotene bleaching test and reducing power assay. Whereas, the antimicrobial capacity was tested against six bacterial strains, three fungi and one yeast. The anti-inflammatory activity was investigated *in vitro* and *in vivo* by human red blood cell (HRBC) membrane stabilization method, the denaturation of protein *in vitro*, Xylene-induced ear oedema in mice and cotton pellet-induced granuloma. The analgesic effect of the flavonoids extracted from *Galium tunetanum* Poiret was studied using hot plate and acetic acid induced writhing tests. The antiulcer activity of tannins were carry out by the preventive and curative test by studying various parameters. All of these therapeutic activities were completed by the acute toxicity study of methanolic extract. Polyphenols and saponins can be good antioxidant agents. However, they were less active or inactive against strains used in antimicrobial activity. Phenolic compounds demonstrate good capacities to be used against inflammatory diseases, especially the flavonoids that are nontoxic and efficacy against this kind of diseases at low doses. Those flavonoids have also potent analgesic activity and demonstrate the utility of these molecules on central mechanism of pain inhibition. Moreover, tannins provide protection against gastric mucosal damage induced by either ethanol/HCl or absolute ethanol, and the methanolic extract was found to possess significant wound healing activity.

Key words: Megriss, *Galium tunetanum*; secondary metabolites; antioxidant; anti-inflammatory; analgesic; anti-ulcer; healing wounds

العنوان: تثمين الخصائص الكيميائية والعلاجية لنبات الجويسئة *Galium tunetanum Poiret*

ملخص

تعتبر نبتة الجويسئة (*Galium tunetanum Poiret*) احدى النباتات المتبقية من أثار الغابات الكثيفة التي وجدت في المنطقة حيث تنتمي إلى الفصيلة الفَويّة المستوطنة شمال أفريقيا وتشكل هذه النبتة لغزا من حيث خصائصها الكيميائية و العلاجية.

تم اخذ عينات المادة النباتية من جبل مقرس بسطيف -الجزائر حيث جففت في الظل، سحقت ثم تم استخلاص المركبات الكيمائية الثانوية، تم تثمين وتقييم الأنشطة المضادات للأكسدة بواسطة عدة اختبارات منها اختبارDPPH، اختبار بيتا-كاروتين واختبار إرجاع القوة. أما فيما يخص دراسة النشاطية المضادة للميكروبات، فقد تم تقديرها باستعمال ستة سلالات بكتيرية وثلاث فطريات من بينها خميرة وتم التحقق أيضا من النشاطية المضادة للالتهابات باستعمال عدة اختبارات منها اختبار ثبات اغشية خلايا الدم الحمراء (HRBC)، اختبار فقدان طبيعة البروتين في المختبر، اختبار الزيلين المسبب لذمة الأذن لدى الفئران واختبار القطن المسبب للورم. وقد درس أيضا التأثير المسكن للفلافونويدات المستخلصة من *Galium tunetanum* Poiret عن طريق اختبار الصفيحة الساخنة واختبار حمض الخليك. أما فيما يخص دراسة القرحة المعدية فتم استخدام جزيئات الدباغة كاختبار وقائي وعلاجي من خلال دراسة معايير مختلفة، إضافة الى دراسة مدى قدرة المستخلص المثيلي في علاج الجراح وفي النهاية تم دراسة السمية الحادة للنبتة.

تعتبر جزيئات البوليفينول والصابونين المستخرجة من *Galium tunetanum* Poiret من المضادات للأكسدة الجيدة في حين كانت جميع الجزيئات المستخلصة أقل نشاطا أو غير نشطة ضد سلالات الميكروبات المستخدمة. وقد أظهرت مركبات الفينولية قدرات جيدة في علاج الأمراض الالتهابية وبالأخص مركبات الفلافونويد بجرعات منخفضة وغير سامة إضافة ان لها نشاط مسكن قوي في تثبيط الآلية المركزية للألم. اما فيما يخص جزيئات الدباغة فهي توفر حماية ضد تضرر الغشاء المخاطي للمعدة المحدث باستعمال الايثانول / حمض الهيدروكلوريد أو الايثانول المطلق. كما أظهرت النتائج أن للمستخلص المثيلي قدرات معتبرة في التئام الجراح.

الكلمات الدالة :جبل مقرس، جويسئة، مركبات الايض الثانوية، المضادة للأكسدة، المضادة للالتهاب، المسكنة للألم، المضادة للقرحة المعدية، المعالجة للجراح.

Titre : Valorisation phytochimique et thérapeutique de *Galium tunetanum* Poiret

Résumé

Galium tunetanum Poiret est un vestige des forêts préexistantes, une espèce endémique d'Afrique du Nord qui constitue une énigme par ses propriétés phytochimiques thérapeutiques. Le matériel végétal est récolté de la montagne de Megriss – Sétif – Algérie , séché à l'ombre, réduit en poudre fine pour être soumis à l'extraction et à la quantification des grands groupes de métabolites secondaires.

L'activité antioxydante est réalisée avec tous les extraits à l'exception des alcaloïdes en utilisant le test de DPPH, le test de blanchiment de β-carotène et le pouvoir réducteur. Par ailleurs, la capacité antimicrobienne de la plante est testée avec six souches bactériennes et trois champignons y compris une levure. L'activité anti-inflammatoire a été étudiée in vitro par le test des globules rouges HRBC (procédé de stabilisation de la membrane du sang humain) et la dénaturation des protéines ; et in vivo par l'œdème, induit par le xylène, au niveau de l'oreille des souris et le granulome, induit par les pastilles de coton stérile, chez les rats.

L'effet analgésique de l'extrait des flavonoïdes de *Galium tunetanum* Poiret est étudié en utilisant le test de la plaque chauffante et le test de contorsion induit par l'acide acétique. En revanche, l'activité antiulcéreuse de l'extrait des tanins a été effectuée par le test préventif et curatif en étudiant différents paramètres et enfin, l'activité cicatrisante de l'extrait méthanolique. L'étude est complétée par le test de la toxicité aiguë.

Les polyphénols et les saponines peuvent être de bons agents antioxydants. Cependant, toutes les molécules extraites de *Galium tunetanum* Poiret sont moins actives ou inactives contre les souches microbiennes et fongiques utilisées. Les composés phénoliques possèdent en outre, de bonnes capacités inflammatoires ; en particulier les flavonoïdes qui sont non toxiques et efficaces à faibles doses contre ce type de maladies. Les flavonoïdes possèdent également une activité analgésique puissante, ce qui démontre l'utilité de ces molécules sur le mécanisme central de l'inhibition de la douleur. En outre, l'extrait des tanins offre une protection contre les dommages de la muqueuse gastrique induite par l'éthanol / HCl et l'éthanol absolu. Enfin, l'extrait méthanolique a une activité de cicatrisation potentielle. L'étude de la toxicité aiguë montre que cette espèce peut présenter des effets indésirables mais non mortels.

Mots clés : Megriss, *Galium tunetanum* ; métabolites secondaires ; antioxydant ; antiinflammatoire ; analgésique ; antiulcéreux ; cicatrisant

Abbreviation:

µM	Micro mole
AA	Arachidonic acid
ALP	Alkaline phosphatase,
ALT	Alanine aminotransferase,
ANOVA	Analyse of variance
AP-1	Activator protein 1
AST	Aspartate transaminase
ATCC	American type culture collection
BHT	Hydroxy-toluene Butylene
CK	Creatinine kinase.
COX-2	Cyclo-oxygenase 2
DHA	Docosahexenoic acid
DL_{50}	Lethal Dose for killing a half of population.
DMSO	Dimethylsulfoxide
DPPH	2-2-Diphényl-1-picryl-hydrazyl
EC_{50}	half-maximal effective concentration
EPA	Eicosapentaenoic
FPP	Farnesyl pyrophosphate
GC/MS	Gas chromatography/mass spectrometry
HCT	Haematocrit,
HGB	Haemoglobin,
HPA	Hypothalamus-pituitary-adrenal
IC50	Half-maximal inhibitory concentration
IIS	Innate immune system
IS	Immune system
LOX-12	Lipoxygenase -12
LOX-15	Lipoxygenase-15
LOX-5	Lipoxygenase-5
LTB4	Leukotrienes B4
LXs	Lipoxins
MCHC	Mean corpuscular haemoglobin concentration,
MCV	Mean corpuscular volume,
MPV	Mean platelet volume.
NCCB	Netherlands Culture Collection of bacteria
NF-kB	Nuclear factor- kappaB
PBS	Phosphate buffer saline
PGF2α	Prostaglandin F 2 alpha
PGs	Prostaglandins
PLT	Platelet,
PMNs	Polymorphonuclear neutrophils
RBC	Red blood cells,
ROS	Reactive Oxygen Species
ROW	Relative organ weights
SD	Standard error deviation
SNS	Sympathetic nervous system
WBC	White blood cell,
WHO	World Health Organization.

Table of contents

I – Introduction 1

II – Bibliographic review 5

II – 1 Location and characterization of physical environment 5

II – 1 – 1 Geographic situation 5

II – 1 – 2 Geology 5

II – 1 – 3 Geomorphology 7

II – 1 – 4 Pedology 7

II – 1 – 5 Hydrology 8

II – 1 – 6 Climate 8

II – 2 Plant description 9

II – 2 – 1 Botanical description 9

II – 2 – 2 Phytosociology membership 10

II – 3 Secondary metabolites 10

II – 3 – 1 Chemical types of secondary metabolites 10

II – 3– 1 – 1 Terpenoids 11

II– 3– 1 – 2 Phenolics 15

II – 3 – 1 – 2 Nitrogen Containing Secondary Metabolites 24

II – 3 – 2 Global derivatives biosynthetic pathway of secondary metabolites 25

II – 4 Toxicology 26

II – 5 Common diseases 27

II – 5 – 1 Oxidative stress 27

II – 5 – 2 Microbial diseases 28

II – 5 – 3 The inflammatory process 30

II – 5 – 4 Pain sensation and pathology 31

II – 5 – 5 Peptic ulcer diseases 32

II – 5 – 6 Healing wounds process 32

III –1 Materials 35

III – 1 – 1 Plant materials 35

III – 1 – 2 chemicals 35

III – 1 – 3 Animals 35
III – 2 Methods & experiments 35
III – 2 – 1 Secondary metabolites extraction 35
III – 2 – 1 – 1 Preparation of methanol extract 35
III – 2 – 1 – 2 Preparation of aqueous extract 35
III – 2 – 1 – 3 Extraction of the essential oil 35
III – 2 – 1 – 4 Extraction of flavonoids 36
III – 2 – 1 – 5 Extraction of Tannins 36
III – 2 – 1 – 6 Extraction of saponins 36
III – 2 – 1 – 7 Extraction of alkaloids 37
III – 2 –2 Quantification of major secondary metabolites groups 37
III – 2 – 2 - 1 Essential oil analysis 37
III – 2 – 2 – 2 Determination of Total Phenolics Content 38
III – 2 – 2 - 3 Determination of total flavonoids content 38
III – 2 – 2 – 4 Determination of total tannins content 38
III – 2 – 2 – 5 Quantitative Estimation of Alkaloids 38
III – 2 – 2 – 6 Determination of chlorophylls a and b 39
III – 2 – 2 – 7 Determination of carotenoids 39
III – 2 – 3 Antioxidant activity 39
III – 2 – 3 – 1 DPPH Assay 40
III – 2 – 3 – 2 β-Carotene linoleic acid bleaching assay 40
III – 2 – 3 – 3 Reducing power 41
III – 2 – 4 Antimicrobial activity 41
III – 2 – 4 – 1 Test strains and culture media 41
III – 2 – 4 – 2 Antibacterial Activity 42
III – 2 – 4 – 3 Antifungal activity 42
III – 2 – 5 *In vitro* anti-inflammatory activity 42
III – 2 – 5 – 1 Inhibition of proteins denaturation 43
III – 2 – 5 – 2 The Human Red Blood Cell (HRBC) membrane stabilization method 43

III – 2 – 5 – 3 Heat induced haemolysis 44

III – 2 – 6 Acute toxicity 44

III – 2 – 6 – 1 Acute oral toxicity 44

III – 2 – 6 – 3 Relative organ weights and histopathology 45

III – 2 – 7 *In vivo* anti-inflammatory activity 46

III – 2 – 7 – 1 Xylene-induced ear oedema in mice 46

III – 2 – 7 – 2 Cotton pellet-induced granuloma in rats 46

III – 2 – 8 Analgesic activity 46

III – 2 – 8 – 1 Mouse writhing test 46

III – 2 – 8 – 2 Hot plate method 47

III – 2 – 9 Antiulcerative activity 47

III – 2 – 9 – 1 Preventive effects of tannin extract against acute gastric lesions 47

III – 2 – 9 – 2 Curative effects of tannin extract against acute gastric lesions 48

III – 2 – 9 – 3 Calculation of ulcer index and Percentage ulcer inhibition 48

III – 2 – 9 – 4 Collection of Gastric Juice 49

III – 2 – 9 – 5 Estimation of Total Acidity 49

III – 2 – 9 – 6 Estimation of pepsin activity 49

III – 2 – 10 Healing wounds activity 50

III – 2 – 10 – 1 Preparation of ointment by fusion method 50

III – 2 – 10 – 2 Excision wound model 50

III – 2 – 11 Statistical analysis 50

IV – Results & Discussion 53

IV – 1 Secondary metabolites yield 53

IV – 2 Secondary metabolites quantification 53

IV – 2 – 1 Polyphenols quantification 53

IV – 2 – 2 Total chlorophyll (a + b) content 55

IV – 2 – 3 Alkaloids quantification 55

IV – 2 – 4 Carotenoids quantification 56

IV – 2 – 5 Chemical composition of the essential oils 56

IV – 3 Antioxidant activity 61

IV – 3 – 1 DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) assay 61

IV – 3 – 1 – 1 Methanolic and aqueous extracts 61

IV – 3 – 1 – 2 Flavonoids extract 62

IV – 3 – 1 – 3 Tannins extract 63

IV – 3 – 1 – 4 Saponins 63

IV – 3 – 1 – 5 Essential oil 64

IV – 3 – 2 β-Carotene linoleic acid bleaching assay 65

IV – 3 – 2 – 1 Methanolic and aqueous extracts 65

IV – 3 – 2 – 2 Flavonoids extract 65

IV – 3 – 2 – 3 Tannins extract 66

IV – 3 – 2 – 4 Saponins 67

IV – 3 – 2 – 5 Essential oils 67

IV – 3 – 3 Reducing power assay 68

IV – 3 – 3 – 1 methanolic and aqueous extracts 68

IV – 3 – 3 – 2 flavonoids extract 69

IV – 3 – 3 – 3 Tannins extract 70

IV – 3 – 3 – 4 Saponins 70

IV – 3 – 3 – 5 Essential oils 71

IV – 4 Antimicrobial activity 72

IV – 4 – 1 Antibacterial activity 72

IV – 4 – 2 Antifungal activity 72

IV – 5 *In vitro* Anti-inflammatory activity 75

IV – 5 – 1 Protein denaturation 75

IV – 5 – 2 Effect of extracts on hypotonicity induced haemolysis of HRBCs 77

IV – 5 – 3 Heat induced haemolysis 78

IV – 6 *In vivo* acute toxicity of *Galium t:* 80

IV – 6 – 1 Physical observation and mortality 80

IV – 6 – 2 Histopathological examination 83

IV – 6 – 3 Haematological and biochemical parameters 87

IV – 6 – 4 LD_{50} Value 89

IV – 7 The effect of flavonoids in acute and chronic inflammatory animal Models 90

IV – 7 – 1 Xylene-induced ear oedema in mice 90

IV – 7 – 2 Effect of flavonoids of *Galium t* on cotton pellet-induced granuloma in rats 92

IV – 8 Analgesic activity of flavonoids extract 93

IV – 8 – 1 Acetic Acid Induced Writhing Assay 93

IV – 8 – 2 Hot Plate Method 95

IV – 9 Anti-ulcerative activity of tannins extract 97

IV – 9 – 1 Preventive effect 97

IV – 9 – 2 Curative effect 99

IV – 10 Healing wounds activity of methanolic activity 101

V – Conclusion & perspectives 106

VI - Bibliography 110

List of figures

Figure 1: Location carte of the mountain of Megriss extracted from topographic carte: Paper of Kharatta and Setif with 1/25000 6
Figure 2: Photos of Galium tunetanum Poiret 9
Figure 3: Parent hydrocarbons of terpenes (isoprenoids) 11
Figure 4: Diterpenes phytol and cembrene 13
Figure 5: Skeletons of triterpenes: oleanane 14
Figure 6: Chemical structures of (a) α-carotene; (b) β-carotene; (c) lycopene;(d) β-cryptoxanthin; (e) lutein; (f) zeaxantin; (g) astaxanthin; (h) canthaxanthin and (i) fucoxanthin. H. 15
Figure 7: Classification of families of phenolic compounds 16
Figure 8: The monolignols, p-coumaryl, coniferyl, and sinapyl alcohol 19
Figure 9: Different flavonoids classes 19
Figure 10: Structures of some flavonoid families 20
Figure 11: Structures of some isoflavonoids families 21
Figure 12: Structures of some neoflavonoids families 21
Figure 13: Structures of some minor flavonoid families 22
Figure 14: Classification of the tannins 23
Figure 15: Overview of plant secondary metabolism showing the interrelationships between the different pathways. TCA, tricarboxylic acid 26
Figure 16:Basic curve of damage–response framework of microbial pathogenesis 30
Figure 17: Total chlorophyll (a + b) content in methanolic extract and in dry matter of *Galium t.* 55
Figure 18: DPPH scavenging activity of Galium t extracts 64
Figure 19: Scavenging effects of essential oil of Galium t on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. 64
Figure 20: β-Carotene bleaching test of Galium t extracts 67
Figure 21: Reducing power assay of Galium t extracts. 69
Figure 22: Reducing power of essential oil of Galium t. 71
Figure 23: Effect of methanolic extract of Galium t on tissue vital organs during 15 days of treatment 85
Figure 24: Dark colour of vital organs after post mortem of dead rats 86
Figure 25: Probity analysis according to Finney methods. 90

Figure 26: The percentage inhibition of the pain in hot plate test in mice of flavonoids extract of Galium t. 97
Figure 28: Gastric lesion observed in control group induced by HCl-Ethanol 98
Figure 28: Gastric lesion observed in group treated with tannin extract of Galium t 98
Figure 30: Excision circular wound after 3 day treated with Galium t methanolic extract ... 103
Figure 30: Excision circular wound after 12 day treated with Galium t methanolic extract . 103

List of table

Table 1: Different coumarin types and their pharmacological properties. 17
Table 2: Major Active Oxygen Species. 28
Table 3: Yield of secondary metabolites extracted from Galium t. 53
Table 4: Concentration of polyphenols, flavonoids and tannins extracted by different solvents from Galium t . 54
Table 5: Concentration of alkaloids (mg EA/GDM) 56
Table 6: Chemical composition of essential oil of Galium t. 57
Table 7: Inhibition of linoleic acid oxidation of flavonoids extracts of Galium t by the β-carotene bleaching method (%) 66
Table 8: EC_{50} value for reducing power of standard and tested extracts 72
Table 9: Bacterial inhibition zones in millimetre for standard, control and extracts of Galium t 74
Table 10: Fungal inhibition zones in millimetre for standard, control and extracts of Galium t 75
Table 11: The percentage inhibition of protein denaturation (%). 76
Table 12: IC_{50} from the percentage inhibition of protein denaturation. 76
Table 13: The percentage inhibition of hypotonicity induced haemolysis of HRBCs (%) 78
Table 14: IC_{50} from the percentage inhibition of hypotonicity induced haemolysis of HRBCs (%) 78
Table 15: The percentage inhibition of heat induced haemolysis of HRBCs (%) 79
Table 16: Acute toxicity of methanolic extract of Galium t in rats 82
Table 17: Difference of body weight before and after treatment 82
Table 18: Post mortem result for acute toxicity of methanolic extract of Galium t administered intraperitoneally (i.p) to wistar albino rats 84
Table 19: Relative organ weights (ROW) of control and rats treated with methanolic extract of Galium t measured during the acute toxicity study 86
Table 20: Haematological values of control and rats treated with methanolic extract of Galium t measured during the acute toxicity study. 87
Table 21: Clinical biochemistry values of control and rats treated with methanolic extract of88
Table 22: Statistic regression for the calculus of DL50 90
Table 23: Effect of flavonoids extract of Galium t on xylene-induced ear oedema in mice. 91

Table 24: Effect of flavonoids extract of Galium t on cotton pellet-induced granuloma in rats. 92
Table 25: Effect of flavonoids extract of Galium t on acetic acid - induced writhing test in mice. 94
Table 26: Effect of flavonoids extract of Galium t on hot plate test in mice (time in seconds). 96
Table 27: Preventive effect of tannins extracted from Galium t in HCl/ethanol-induced ulcer in rats. 98
Table 28: Curative effect of tannins extracted from Galium t in ethanol-induced ulcer in rats 100
Table 29: Evaluation of Galium t methanolic extract and MYCOCIDE® ointments on wound healing by excision wound method in rat. 103

Introduction

I – Introduction

In aiming to recover the floristic biodiversity in area of Setif - Algeria, the study of its vegetal patrimony supply a very important data base for selecting choice in context of territories management. In reference to a previous study carry out in the mountain of Megriss, owing to high biogeographic value of their endemic plants and the various threats burdensome on the site, the recommendation of phytochemical study contribute significantly to reveals exhaustive classification and organization of vascular phytocenosis.

Hence, *Galium tunetanum* Poiret is one of dense pre-existent forest vestige characteristic of class of *Quercetea pubescentis* and belong to *Rubiaceae* is a North African endemic species constitute an enigma toward their phytochemical and therapeutical proprieties which determine its use.

Plants produce an amazing diversity of low molecular weight compounds. Although the structures of close to 50 000 have already been elucidated (De Luca and St Pierre, 2000), there are probably hundreds of thousands of such compounds. Only a few of these are part of 'primary' metabolic pathways (those common to all organisms). The rest are termed 'secondary' metabolites; this term is historical and was initially associated with inessentiality but, here, a 'secondary' metabolite is defined as a compound whose biosynthesis is restricted to selected plant groups (Mol et *al*., 1998; Dudareva and Pichersky, 2000), constitute a source of pharmaceutics substance.

There are numbers of synthetic drugs and antibiotics available for this purpose but it create several unwanted effect. It is already reported that herbal medications are more effective, nontoxic in nature, non-resistant to microorganism, more available, affordable and cheap over the conventional medicine (Farahpour and Habibi, 2012). From this point of view, there is a very much growing interest in research field to discover new potential herbal medicine for treatment of infectious (Rojas et *al*., 2003) and non-infectious diseases.

In recent years, much attention has been devoted to natural antioxidant and their association with health benefits (Arnous et *al*., 2001). Plants are potential sources of natural antioxidants. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive (Lu and Foo, 1995). Generation of free radicals or reactive oxygen species

(ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Mandal et *al*., 2009).

Antioxidants are molecules that are capable of neutralizing the harmful effects of the ROS through the endogenous enzymatic defence system such as the superoxide dismutase, glutathione peroxidase and catalase in human system (Valko et *al*., 2006).

Nowadays, an increasing number of infectious agents are becoming more resistant to commercial antimicrobial compounds (Hancock et *al*., 2012). The necessity to develop new drugs requires varied strategies, among them, the bioprospection of secondary metabolites produced by medicinal plants (Benko-Iseppon and Crovella, 2010; Dionisi et *al*., 2012).

The resistance which certain microorganisms have developed against antibiotics initiated antimicrobial investigations and different applications of essentials oils or plants against a wide range of bacteria (Gram-negative and Gram-positive) including antibiotic resistant species, fungal species and yeast (Nelson, 1997; Hammer et *al*., 1998; Hammer et *al*., 1999; Jimenez-Arellanes et *al*., 2003).

Many non-infectious diseases are known to be treated by herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role as therapeutic remedies in many developing countries (Ody, 1993; Khan et *al*., 2008).

Drugs which are in use presently for the management of pain and inflammatory conditions are either narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids e.g. hydrocortisone. All of these drugs present well-known side and toxic effects. It is well documented that these non-steroidal anti-inflammatory drugs (NSAIDs) produce intestinal tract ulcers (With potential internal bleeding) in 10- 30 % of long-term users, and erosions of the stomach lining and intestinal tract in 30-50 % of cases (Hayliyar et *al*., 1992).

Peptic ulcer disease is one of the most common gastrointestinal disorders, which causes a high rate of morbidity particularly in the population of non-industrialized countries (Falk, 2001).

A number of drugs including proton pump inhibitors and H_2 receptor antagonists are available for the treatment of peptic ulcer, but clinical evaluation of these drugs has shown incidence of relapses, side effects, and drug interactions. This has been the rationale for the development of new antiulcer drugs and the search for novel molecules has been extended to herbal drugs that

offer better protection and decreased relapse. Drugs of plant origin are gaining popularity and are being investigated for a number of disorders, including peptic ulcer. (Dey and Dey, 2002)

Wounds are inescapable events of life, which arise due to physical or chemical injury or microbial infections. The healing of wounds often deviates from a normal course and under-healing, over-healing or failure of wounds to heal is common (Biswas and Mukherjee, 2003).

This study was divided into three parts, the first one treat some bibliographic elucidations of the studied phenomena like secondary metabolites and common diseases. The second part explain the used materials, the experimental procedures and the different technics used in this study. The third part was devoted to treat the obtained results clarified with tables, photos and graphics, finely discussed.

The purpose of this study is to develop floristic biodiversity of Setif, in researching a new species, which contain new molecules for beneficial use, especially in therapeutic domain.

The choice of the mountain of Megriss is based on their high biogeographic value of their endemic plants by using our phytosociologic research performed on July 2002 (Gaamoune, 2002).

The recommendation of phytochemical and therapeutic studies contributed significantly to reveal the exhaustive organization of vascular phytocenosis of the mountain.

The choice of the species *Galium t* is based firstly, on our phytosociologic study, after statistic treatment and the determination of phytosociologic classes and the floristic list. Secondary, because is a North African endemic species constitute a source of phytochemicals and therapeutic proprieties. And finally, this study provides the opportunity to enhance the endemic flora of the region to set choices in the context of land management.

Bibliographic review

II – Bibliographic review

II – 1 Location and characterization of physical environment

II – 1 – 1 Geographic situation

The Mountain of Megriss is located in the northern part of high plains of Setif at twenty kilometres of the city of Setif at the coordinates:

X: 5° 18’ 20" and X’: 5° 24' 7”

Y: 36° 18' 30” and Y’:36° 21’ 54”

The Mountain is bordered in the northwest by Djebel Soumer, in the East by Draa Tizi-Messaoud and Madjen El-Hanache, in the southeast by Draa-Zenouba and Kef El-Keroucha, in the south by the mountain of Takouka and in south-west by Ain-Abessa (Figure 1).

II – 1 – 2 Geology

The paleogeographic and structural evolution of the Mountain show the presence of three geologic units (Vila, 1976; Vila and Obert, 1977):

- **The quaternary**

The quaternary is marked by different type of mass of fallen rocks:

- Mass of fallen rocks with marly gangue observed in the around the Mountain
- The ancient mass of fallen rocks located in the north side, it is a flow of solifluxion dated perhaps of the old quaternary.

- **The Numidian nape**

It is visible from the tabular mass, not very fractured, from the mountain of Megriss until truncate masse in the base of Chouf Karoun (in south-west), the foliation of Kherrata and Djebel Taourirt (in the south-west area). Essentially formed of clay and a layer of sandstone.

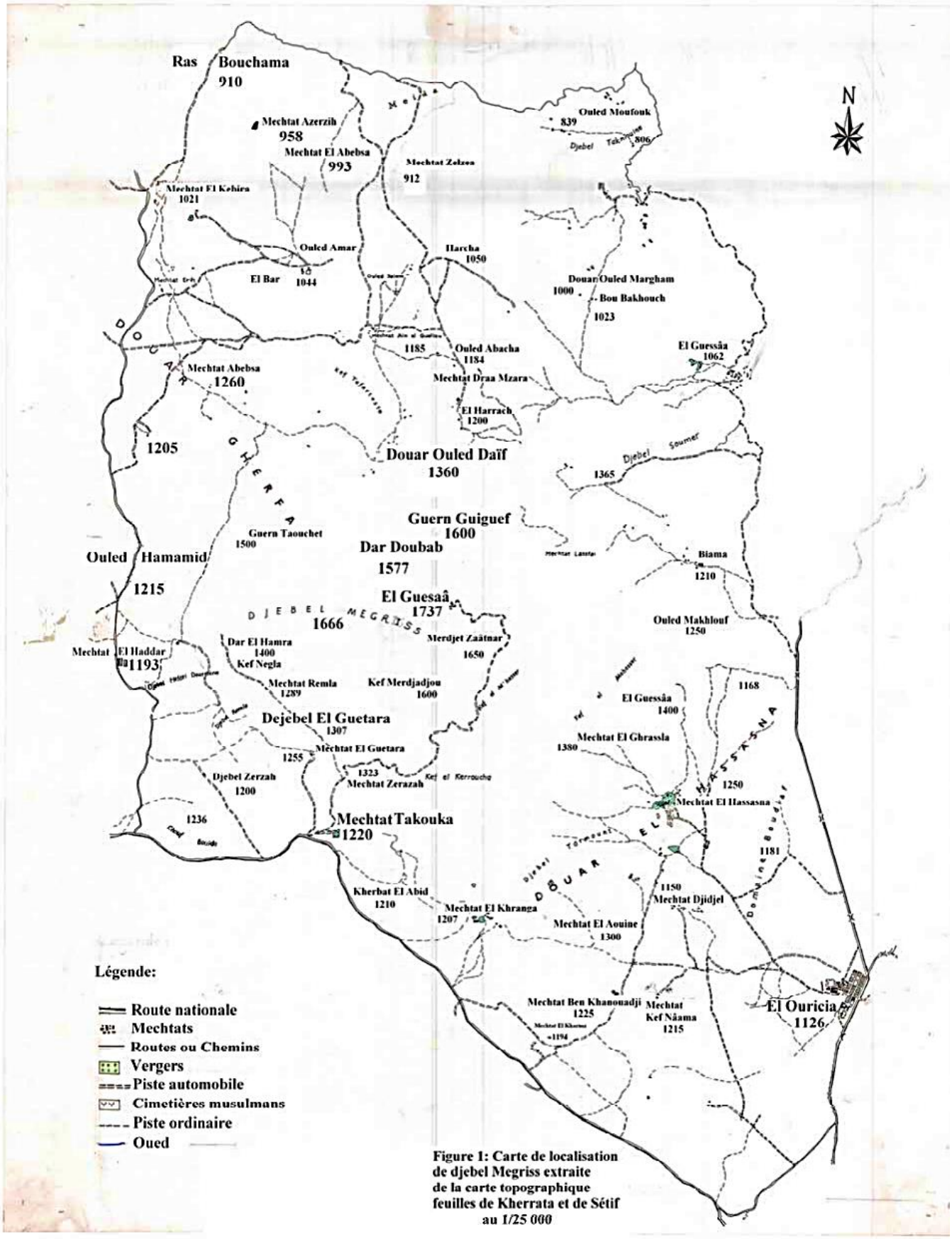


Figure 1: Carte de localisation de djebel Megriss extraite de la carte topographique feuilles de Kherrata et de Sétif au 1/25 000

Figure 1: Location carte of the mountain of Megriss extracted from topographic carte: Paper of Kharatta and Setif with 1/25000 (Boulaacheb, 2009).

- **The Tellian nape**

It has the smallest dimension, pinned under the contact of the foot of Numidian in the northeast of the mountain of Megriss. It is make up of marl belong to three series: the inferior Oligocene, the superior Lutetian and Priabonian and the superior Senonian.

II – 1 – 3 Geomorphology

According to Boulaacheb (2009), the mountain of Megriss is very rugged. The slopes are very strong, they reaching 90% at the cliffs. This type of slope favours runoff and water erosion. Therefore, the vegetal cover is influenced, in some places, the soil is completely devoid of vegetation.

Southern slope is much more exposed to thermal variations around the freezing point than North Slope. On the southern slopes, the aggressiveness of the gel is particularly important in snow finite period. The southern slopes of the Megriss is warmer than the north side and this is proved by the flowering plant species on the southern flank that starts before flowering species northern flank. Some cliffs are completely bare. The ridges are devoid of vegetation and only a few species can exist there, the effects of wind and frost are more important.

The large rocky outcrop that characterizes the landscape of the mountain of Megriss realizes the runoff, water and wind erosion.

II – 1 – 4 Pedology

According to Lahmar et *al*. (1993), the soils of the mountain Megriss are of two types: Vertisols and leached soils**.**

- **Prevailing vertices soils**

These are heavy soils, rich in swelling clays .They show large shrinkage cracks when dry. They are developed either on heavy earth materials (marl, clay), nor the product of erosion. They are found both in flat or depressed areas on slopes. Vertisols developed on clay materials foothills of the mountain of Megriss are little or no carbonate.

- **The leached soils**

These soils are limited to sandstone on the heights. These are developed soils, low to medium thick, containing a leached sandy horizon, massive, neutral, based on a horizon of accumulation of clay, slightly acidic and having locally signs of waterlogging. In the mountain of Megriss, bedrock prominently covers the massive. We can deduce that this soils are azonal.

II – 1 – 5 Hydrology

The hydrographic network of Megriss show the richness of the water area. Many cross the Wadis, the most important are the Wadi El Bordj in the north and Wadi El Hader in the west. The water regime is quite variable throughout the year, it is linked to rainfall patterns. The strongest flow is recorded in winter and melting snow in early spring. These wadis may be in water until July, when water stagnates in the deeper parts.

The numerous rocky outcrops calcareous clay leaves little opportunity for water to infiltrate deeply. The flow of water remain on the surface, this increases runoff. However, a small part of the water seeps into the sandy soil. The rippling water is recovered either by the numerous temporary ponds located below cliffs or by rivulets.

The secondary network, meanwhile, is dense enough, especially in spring, as water from snowdrifts (slush) feeds many small sources, temporary ponds and streams (Boulaacheb, 2009).

II – 1 – 6 Climate

The average annual rainfall is 503 mm, the first snowfall is observed in late October, and the snowpack settles permanently in December. The snow continues until mid-spring (April), at this time of year, the temperature rise and the heating of the soil cause melting of snow.

The seasonal pattern is of the type: winter, spring, fall, summer (WSFS). The annual average humidity is 68%, the highest humidity is recorded in winter (83.9%), the fall (69.9%) and spring (70%); a significant percentage of moisture is observed in summer (48.3%) (Boulaacheb, 2009).

However, average annual temperatures are 10 ° C. August is the warmest months (26.4 ° C) and January is the coldest one (-0.6 ° C). The temperature range, with a value of 27 ° C, make the mountain of Megriss in the semi-continental climate. Further, the dry period is three months (June, July, and August), with high temperatures during the months of July and August. This

climate classify the mountain of Megriss, in a bioclimatic sub humid conditions with cold winter.

II – 2 Plant description

II – 2 – 1 Botanical description

According to Quezel and Santa (1963), *Galium tunetanum* Poiret (Figure 2) is an endemic species of North Africa belong to the Rubiaceae; can be found in Iberian Peninsula according to Ortega-Olivencia and Devesa (2007), it is a perennial herb, lignified at the base, multicauline, pubescent, without strong aculeus, green, blackened or not drying. Stems (20-50 cm × 0.5-2 mm), erect or ascending, simple but with short axillary branchlets, opposite leaves and small (aspect of fasciculate), or branches developed, foliose, leaves larger than axils; internodes sub-equal basal media and 2.7 times or greater than the leaves, superior to 4 times higher, with hairs (0.05-0.1 mm), usually retrorse, with hairs (0.2-0.5 mm) (Ortega-Olivencia and Devesa, 2007).

Leaves (6-14 × 0.5-0.9 mm), in whorls of 7-10 (6 on lateral branches), sessile, erect, reflexed, linear or filiform, acute and mucronate (0.3-0.7 mm) with edge, almost cylindrical and revolute, leathery, with one discreet nervure, discolours, bright green bundle, grey-green on the underside when visible; with hairs 0.05-0.2 mm, underside, when visible(Ortega-Olivencia and Devesa, 2007).

Inflorescence (4.5 to 24 cm), paniculiform, narrowly pyramidal, multiflora, with shaft and woolly branches, they opposed, with sub-equal or lower peduncle bracts, partial inflorescences in composite peaks, lax and stalked, the last order with 2-3 flowers. Inflorescence bracts first order (3-13 × 0,5-0,9 mm), whorls 4-9, patents, deciduous, sometimes linear-lanceolate, sub flat, pubescent; in the second (2,7-5,8 × 0,4-0,7 mm), in whorls of 2 – 6, similar to the above. Bracteoles, if any, of (1.3-2.7 × 0.3-0.8 mm), 1-2 knot, (Ortega-Olivencia and Devesa, 2007).

Figure 2: Photos of *Galium tunetanum* Poiret

sub-equal or greater than the pedicels, linear-lanceolate, pubescent generally flat.

Flowers hermaphrodite, tetramer; pedicels (0.7-2 × 0.1-0.15 mm), smaller than the diameter of the corolla, divaricated in the fruit, pubescent or densely white-villous, with hairs (0.2-0.4 mm). Corolla (3.4-3.7 mm) diameter, rotate, with hairs (0.15- 0.3 mm), pale yellow; (0.3-0.4 mm) tube; lobes (1.4-1.7 mm), triangular-ovate or oblong, acute or sub-acute, sometimes apiculum to 0.2 mm, incurved or patent; stamens 4; filaments 0.5-0.7 mm; anthers (0.35-0.6 (0.7) mm), oblong, yellowish. Ovary (0.6-0.7 mm), ovoid or oblong, densely white-hirsute with hairs (0.15 - 0.4 mm); style up to 0.6 mm; is globular stigmas. Mericarps (0.9-1.2 mm), oblong or more or less ovoid; brownish villous, with hairs 0.3-0.5 mm, smooth or somewhat rough (Ortega-Olivencia and Devesa, 2007). Fruits hairless (Quezel and Santa, 1963).

II – 2 – 2 Phytosociology membership

This plant prefer the forests and the brushwood, and according to Boulaacheb (2009), even can be found in matorral of *Quercus ilex* , lawn of *Lagurus ovatus* and *Filago germanica* and meadow, testified of the pre-existent dense forest and belong according to the same source to the phytocociologic class of *Quercetea pubescentis.*

II – 3 Secondary metabolites

The characteristic feature of higher plants is their capacity to synthesize an enormous variety of organic molecules, the so-called secondary metabolites (Balandrin et *al*., 1985; Curtin, 1983).

Plant secondary metabolites differ from the ubiquitous primary metabolites (e.g. carbo-hydrates, proteins, fats, nucleic acids) in that their distribution is limited, often only being produced by specific plants or groups of plants. Even within individual populations and within individual plants themselves secondary metabolites vary quantitatively and qualitatively. An appreciation of this variability is essential when trying to interpret studies using non-characterized (chemically and biologically) plants and extracts (Marriott, 2000).

II – 3 – 1 Chemical types of secondary metabolites

In plants, an enormous variety of secondary metabolites has been described, their number amounting to more than 100 000 (Hadacek, 2002). A simple classification of secondary

metabolites includes three main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates) (Agostini-Costa et *al.*, 2012).

II – 3– 1 – 1 Terpenoids

Terpenoids represent a large and important class of natural products with more than 30,000 different structures. Terpenoids (consisting of C_5 (isoprene) ‘‘building blocks’’) are known for their wide commercial applications, such as flavour and fragrance additives (Julsing et *al.*, 2006).

A vast majority of the different terpenes structures produced by plants as secondary metabolites that are presumed to be involved in defence as toxins and feeding deterrents to a large number of plant feeding insects and mammals (Gershenzon and Croteau, 1991). Below, several examples will draw from the major subclasses (Figure 3)

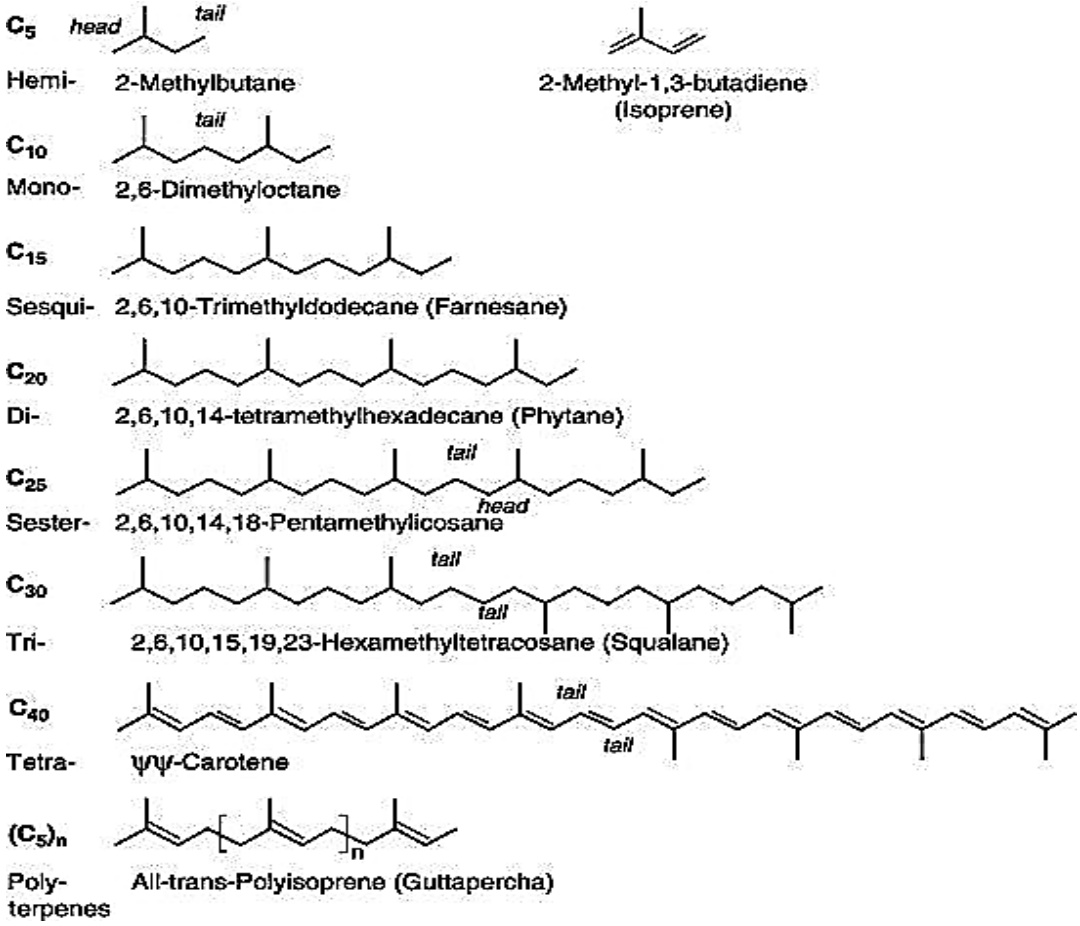


Figure 2: Parent hydrocarbons of terpenes (isoprenoids) (modified from Eberhard, 2006)

- **Monoterpenes (C_{10})**

The monoterpenoids which are composed of the condensation of two isoprene units, are important components of essential oils (Gould, 1997). They are widely distributed in nature, most of which have been found in higher plants.

However, a number of halogenated derivatives have been isolated from marine organisms and have been found in defence and pheromonal secretions of insects. Monoterpenes have intensely strong odours and they are the most common volatile compounds in plants responsible for fragrance and flavour.

Therefore, monoterpenes have a great commercial interest for food industry as well as perfume and fragrance industry (Robbers et *al*., 1996). Monoterpenes may be linear (acyclic) or contain rings

- **Sesquiterpenes (C_{15})**

Sesquiterpenes are colourless lipophilic compounds. Biosynthesis in plants is from three isoprene units, and occurs via farnesyl pyrophosphate (FPP), in the endoplasmic reticulum (Yu and Utsumi, 2009).

Sesquiterpenes consist of a 15 carbon backbone, and whilst diverse in their structure, the majority, and the most functional forms are cyclic. The large number of sesquiterpene synthases (Bennett et *al*., 2002; Cheng et *al*., 2007; Picaud et *al*., 2007) coupled with the fact that a single synthase may produce numerous products and further modifications after sesquiterpene synthesis, such as oxidation and glycosylation take place, (Lange and Lee, 1987; Little and Croteau, 2002; Schnee et *al*., 2002) result in a vast number of varied structures, many similar synthases may produce the same products, in different ratios which affect the metabolite profile of a plant and can be used to classify closely related species or subspecies.

Regulation of the pathways is highly controlled in some species where sesquiterpenes are produced as a stress response (Zhang et *al*., 2012). Sesquiterpenes may be acyclic or contain rings, including many unique combinations.

- **Diterpenes and sesterterpenes**

The diterpene compounds arise from geranylgeranyl diphosphate, and present 20 carbon units in their basic skeletal type. One of the simplest and most important of the diterpenes is phytol (Figure 4), a reduced form of geranylgeraniol, which forms the lipophilic side-chain of the chlorophylls (Vetter and Schröder, 2011).

Cyclization reactions of geranylgeranyl diphosphate led to many structural types of diterpenoids, presenting a large range of polarity nature, from apolar hydrocarbons such as cembrene (Figure 4) (Villanueva and Setzer, 2010).

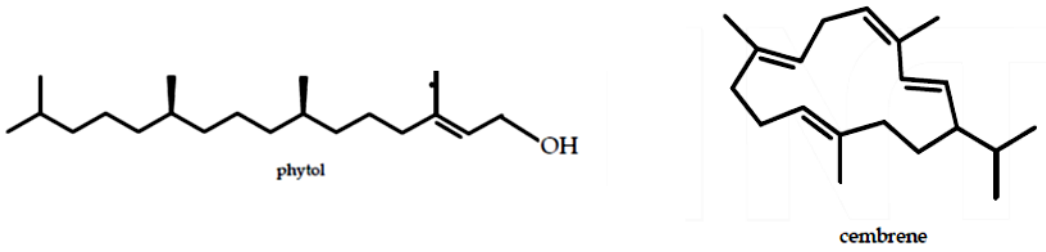


Figure 3: Diterpenes phytol and cembrene

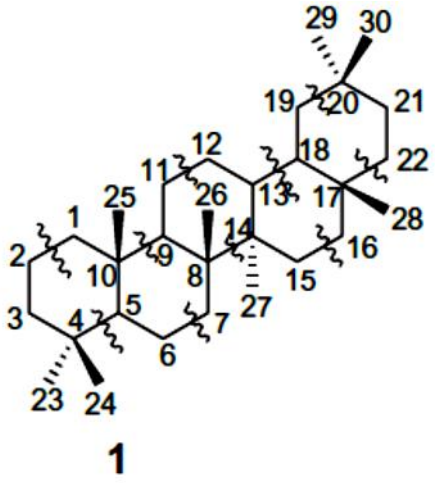
Sesterterpenes (C_{25}) may be the least common group of terpenoids. This class of compounds arises from geranylfarnesyl diphosphate, which by cyclization can give rise to various skeletal types, presenting different oxidation levels and several biological activities (Agostini-Costa et *al*., 2012).

- **Triterpenes (C_{30})**

Triterpenes are a class of natural products present in all organisms, especially in plants (Maria et *al*., 2012). Triterpenoids constitute a wide, biologically interesting group of terpenoids and include a large structural diversity of secondary metabolites with more than 100 carbon skeletons (Figure 5) identified from terrestrial and marine living organisms (Mahato et *al*., 1992).

This class of natural products, including triterpenes, steroids, limonoids, quassinoids, and triterpenoidal and steroidal saponins, consists of over 30,000 compounds isolated and identified (Dzubak et *al*., 2006). Most of triterpenic skeletons are tetracycles, containing three six-membered and one five-membered rings, and pentacycles, with either four six-membered and

one five-membered rings or five six-membered rings. However, acyclic, mono-, di-, tri-, and hexacyclic scaffolds, have also been isolated and identified from natural sources. The term triterpene refers to three monoterpenes and consequently to 30 carbons grouped in six isoprenyl units. Depending on the plant species, secondary metabolites belonging to this family are mostly stocked in the mitochondria, microsomal or chloroplasts of cells (Nes and Heftmann, 1981). These components and their glycosylated homologs play crucial roles in protecting the plant against insects, fungi, and bacteria (Heftmann, 1975).



1

Figure 4: Skeletons of triterpenes: oleanane

- **Tetraterpenes (Carotenoids)**

Carotenoids form one of the most important classes of plant pigments and play a crucial role in defining the quality parameters of fruit and vegetables (Van den Berg et *al*., 2000). Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms but not animals.

In plants, they contribute to the photosynthetic machinery and protect them against photo-damage. Fruits and vegetables constitute the major sources of carotenoid in human diet (Mangels et *al*., 1993; Agarwal and Rao, 2000; Johnson, 2002).

Carotenoids are classified according to the structure as follows:

- The hydrocarbon carotenoids which are known as carotenes example β-carotene

- The oxygenated carotenoids which are derivatives of these hydrocarbons known as xanthophylls, examples of these compounds are a zeaxanthin and lutein (hydroxy), spirilloxanthin (methoxy), echinenone (oxo), and antheraxanthin (epoxy) (Goodwin, 1980) (Figure 6).

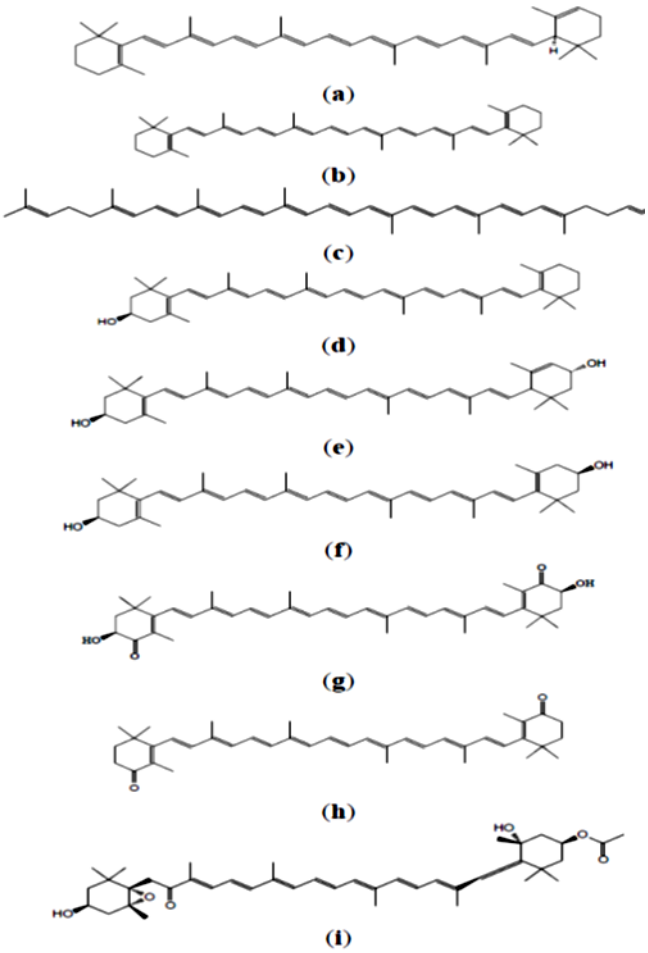


Figure 5: Chemical structures of (a) α-carotene; (b) β-carotene; (c) lycopene;(d) β-cryptoxanthin; (e) lutein; (f) zeaxantin; (g) astaxanthin; (h) canthaxanthin and (i) fucoxanthin. H (Tanaka et *al* ., 2012).

II– 3– 1 – 2 Phenolics

As stated by Harborne (1989), the term "phenolic" or "polyphenol" can be precisely defined chemically as a substance which possesses an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, etc.). As a general rule, the terms phenolics and polyphenols refer to all secondary

natural metabolites arising biogenetically from the shikimate-phenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols.

However, polyphenols are among the most widespread class of metabolites in nature, and their distribution is almost ubiquitous. It is estimated that 100,000 to 200,000 secondary metabolites exist (Metcalf, 1987). The phenolic compounds are categorized as secondary metabolites, and their function in plants is poorly understood (Figure 7) (Ghasemzadeh and Ghasemzadeh, 2011).

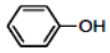
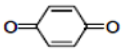
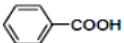
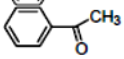
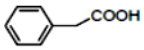
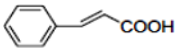
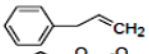
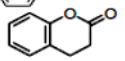
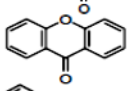
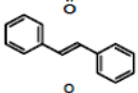
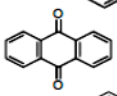
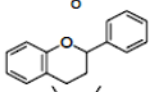
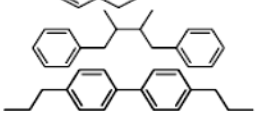
Carbon numbers	*Class*	*Basic structure*	*Sources*
C_6	Simple phenols		
	Benzoquinones		
C_6-C_1	Benzoic acid		Cranberry, cereals
C_6-C_2	Acetophenones		Apple, apricot, banana, cauliflower
	Phenylacetic acid		
C_6-C_3	Cinnamic acid		Carrot, citrus, tomato, spinach, peaches, cereal, pears, eggplant
	Phenylpropene		
	Coumarins		Carrot, celery, citrus, parsley
	Chromones		
C_6-C_4	Naphthoquinones		Nuts
C_6-C_1-C_6	Xanthones		Mango, Mangosteen
C_6-C_2-C_6	Stilbenes		Grapes
	Anthraquinones		
C_6-C_3-C_6	Flavonoids		Widely distributed
$(C_6$-$C_3)_2$	Lignans, neolignans		Sesame, rye, wheat, flax
$(C_6$-$C_1)_n$	Hydrolysable tannins	Heterogeneous polymer composed of phenolic acids and simple sugars	Pomegranate, raspberry
$(C_6$-$C_3)_n$	Lignins	Highly crosslinked aromatic polymer	

Figure 6: Classification of families of phenolic compounds

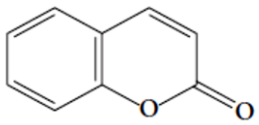
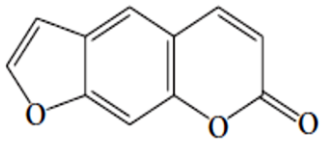
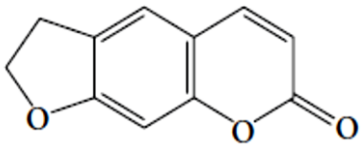
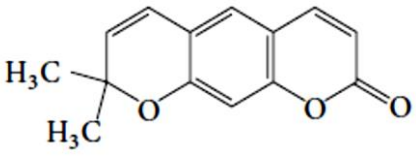
- **Coumarin**

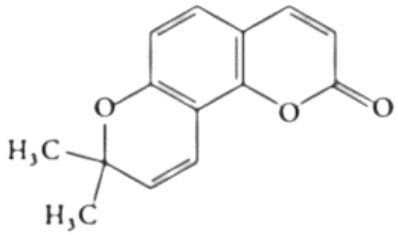
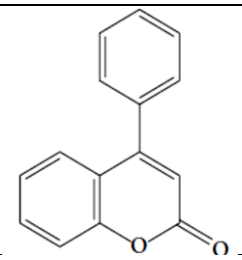
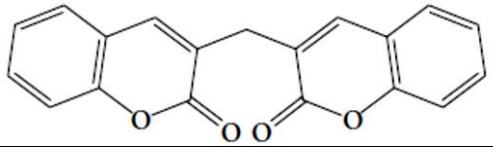
Coumarins owe their class name to 'Coumarou', the vernacular name of the Tonka bean (*Dipteryx odorata* Willd. *Fabaceae*), from which coumarin itself was isolated in 1820 (Bruneton, 1999).

Coumarin is classified as a member of the benzopyrone family of compounds, all of which consist of a benzene ring joined to a pyrone ring (Ojala, 2001). The structure of coumarin consists of an aromatic ring linked to a condensed lactone ring (Keating and Kennedy, 1997). Coumarin is a natural volatile active compound found in many plants. At ambient temperature, it is a white crystal with a smell similar to that of vanilla and melting point of 341-344K (Muray, 1991)

Natural coumarins are mainly classified into six types based on the chemical structure of the compounds (Table 1)

Table 1: Different coumarin types and their pharmacological properties (Venugopala et *al*., 2013).

Type of coumarin	General chemical structure	Example
1. Simple coumarins		Coumarin Esculetin Osthole
2. Furano coumarins		Imperatorin Psoralen
3. Dihydrofurano coumarins		Anthogenol Felamidin
Pyrano coumarins are of two types		
4a. Linear type		Grandivittin Agasyllin

4b. Angular type		Inophyllum A, B, C, E, P, G1, andG2
Type of coumarin	**General chemical structure**	**Example**
4. Phenyl coumarins		Isodispar B, dispardiol B, mammea A/AB cyclo E
5. Bicoumarins		Dicoumarol

- **Lignin**

Lignin is the generic term for a large group of aromatic polymers resulting from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (Boerjan et *al*., 2003; Ralph et *al*., 2004). These polymers are deposited predominantly in the walls of secondarily thickened cells, making them rigid and impervious.

In addition to developmentally programmed deposition of lignin, its biosynthesis can also be induced upon various biotic and abiotic stress conditions, such as wounding, pathogen infection, metabolic stress, and perturbations in cell wall structure (Can˜ o-Delgado et *al* ., 2003; Tronchet et *al* ., 2010).

The main building blocks of lignin are the hydroxycinnamyl alcohols (or monolignols) coniferyl alcohol and sinapyl alcohol, with typically minor amounts of *p*-coumaryl alcohol (Figure 8)

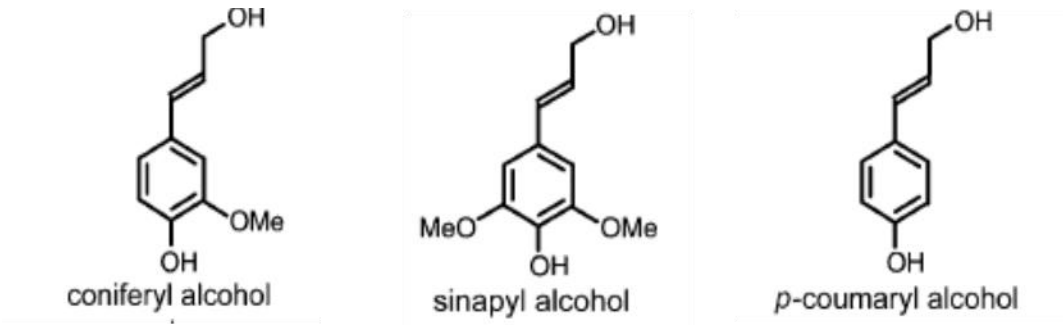


Figure 7: The monolignols, p-coumaryl, coniferyl, and sinapyl alcohol (Boerjan et *al*., 2003)

- **Flavonoids**

The term "flavonoid" is generally used to describe a broad collection of natural products that include a C_6-C_3-C_6 carbon framework, or more specifically a phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes:

- The flavonoids (2-phenylbenzopyrans) (Figure 9 N°1),
- Isoflavonoids (3-benzopyrans) (Figure 9 N° 2),
- The neoflavonoids (4-benzopyrans) (Figure 9 N° 3).

These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.

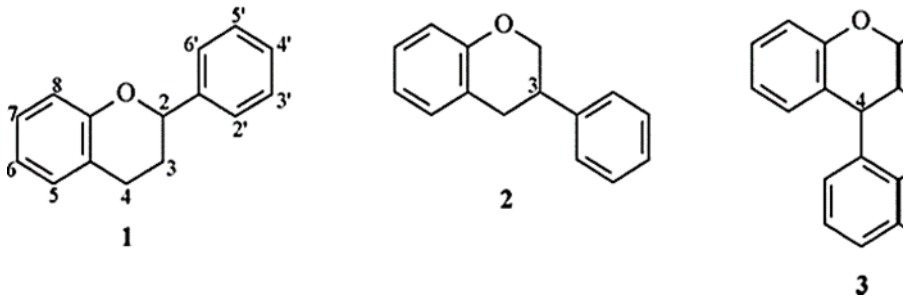


Figure 8: Different flavonoids classes

- **Phenylbenzopyrans (C_6-C_3-C_6 Backbone)**

Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups (Figure 10)

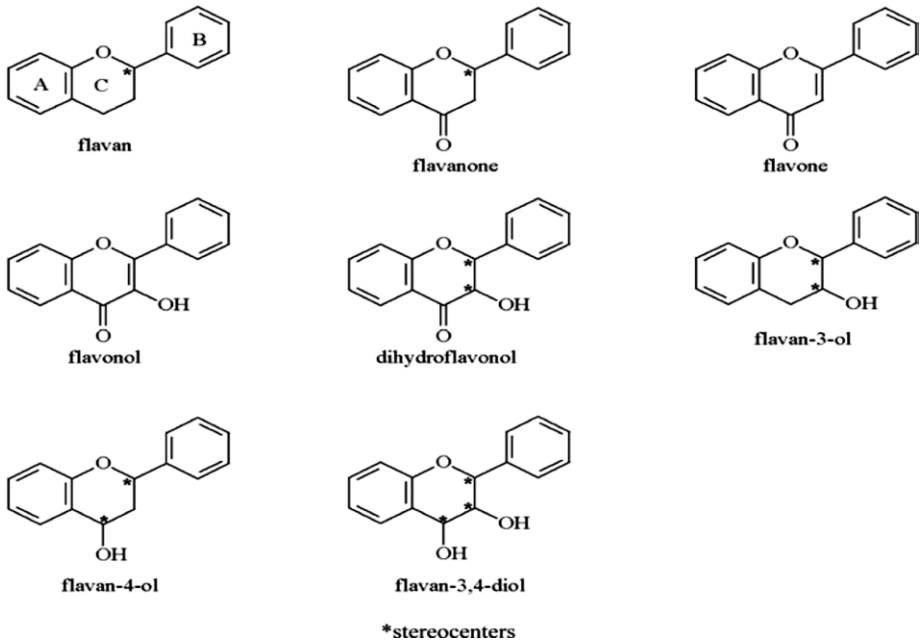


Figure 9: Structures of some flavonoid families (Muray, 1991)

- **Isoflavonoids**

Isoflavonoids are a large subclass of flavonoids having a 15-carbon (C_6–C_3–C_6) backbone arranged as a 1, 2-diphenylpropane skeleton. There are over 1000 structures of isoflavonoids and their derivatives and over 5000 structures of flavonoids in total (Reynaud et *al*., 2005). These isoflavonoids are biologically active secondary metabolites produced by most of the leguminous plants and have been reported to be produced by non-leguminous plants as well. Isoflavonoids are derived from the basic 3-phenylchroman backbones of isoflavones by various modifications, such as methylation, hydroxylation, or polymerization. These modifications lead to simple isoflavonoids such as isoflavanones, isoflavans, and isoflavonols as well as more complex structures such as rotenoids, pterocarpans, and coumestans (Dewick, 1993) (Figure 11).

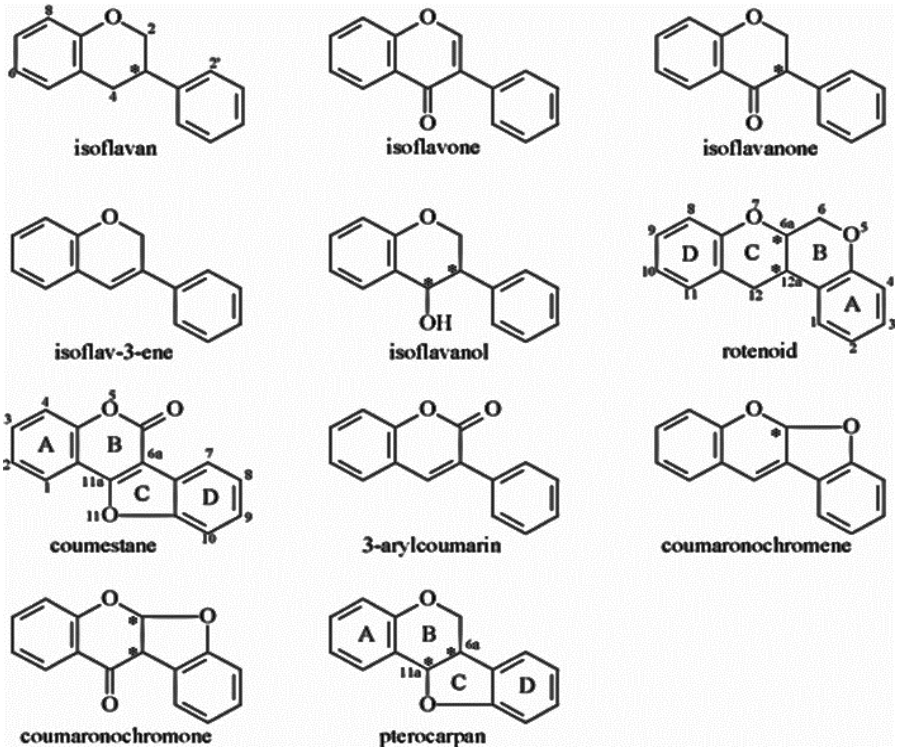


Figure 10: Structures of some isoflavonoids families (Muray, 1991)

- **Neoflavonoids**

The term neoflavonoids refers to a group of C-15 naturally occurring compounds, which are related structurally and biogenetically to the flavonoids and to the isoflavonoids.

In the higher plants the neoflavonoids have limited taxonomic distribution, being found in the *Guttiferae*, the *Papilionoideae* (subfamily *Leguminosae*) and more recently being identified in the *Rubiaceae*, the *Passifloraceae* and the *Polypodiaceae*. The naturally occurring neoflavonoids have been grouped together in accordance with their structural types and source (Donnelly, 1985) (Figure 12)

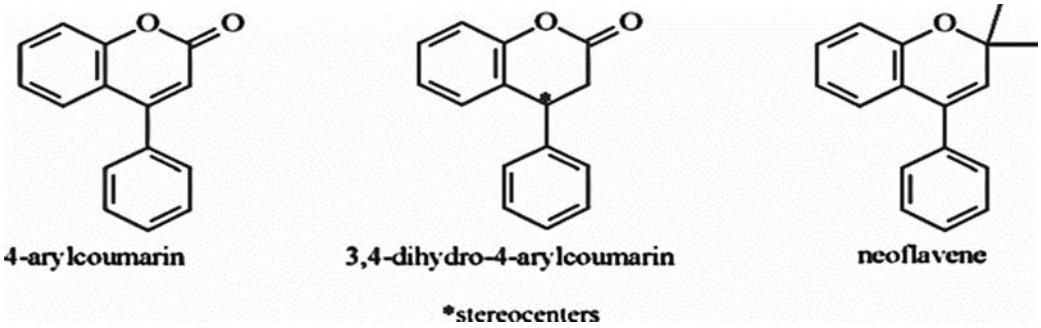


Figure 11: Structures of some neoflavonoids families (Muray , 1991)

- **Minor Flavonoids**

Flavanones, chalcones and dihydrochalcones are biochemically related compounds of restricted occurrence and for this reason; they are described as minor flavonoids (Harborne and Mabry, 1982) (Figure 13)

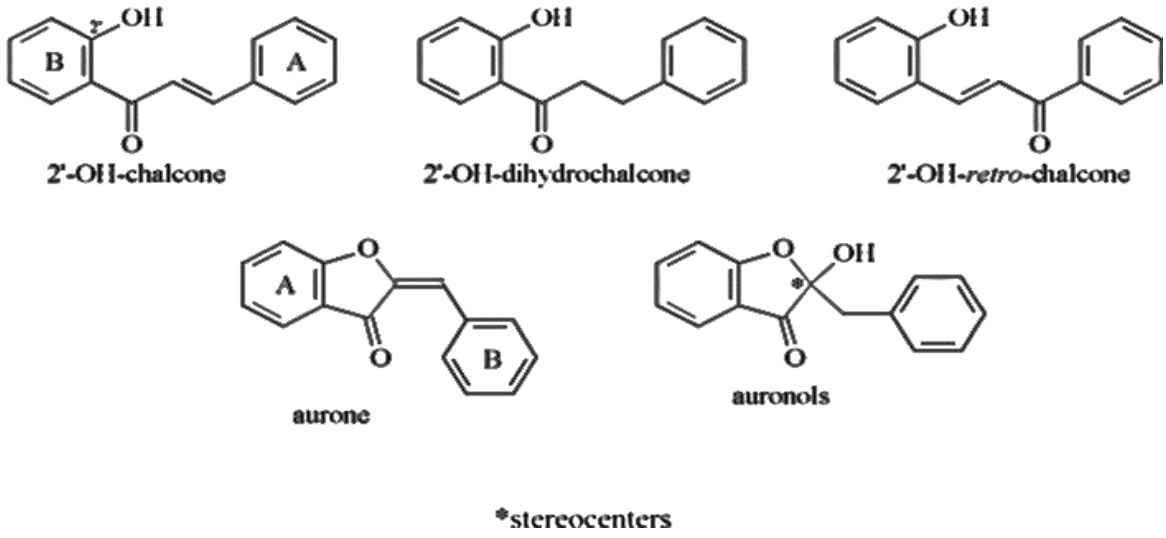


Figure 12: Structures of some minor flavonoid families (Muray , 1991)

Flavanones have a saturated C- ring whereas chalcones as well as dihydrochalcones have an open structure and a carbon skeleton numbered in a different way than other flavonoids (Itagaki et *al*., 1966).

- **Tannins**

Tannins are polyphenols sometimes called plant polyphenols (Haslam, 1989); although originally the name tannin was given to the plant extracts exhibiting astringency, without knowing their chemical structures (Okuda et *al*., 1985).

The term tannin refers to the use of tannins in tanning animal hides into leather; however, the term is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with proteins and other macromolecules. Tannins have molecular weights ranging from 500 to over 3000 (Bate-Smith, 1962).

Based on their structural characteristics it is therefore possible to divide the tannins into four major groups: gallotannins, ellagitannins, complex tannins, and condensed tannins (Figure 14).

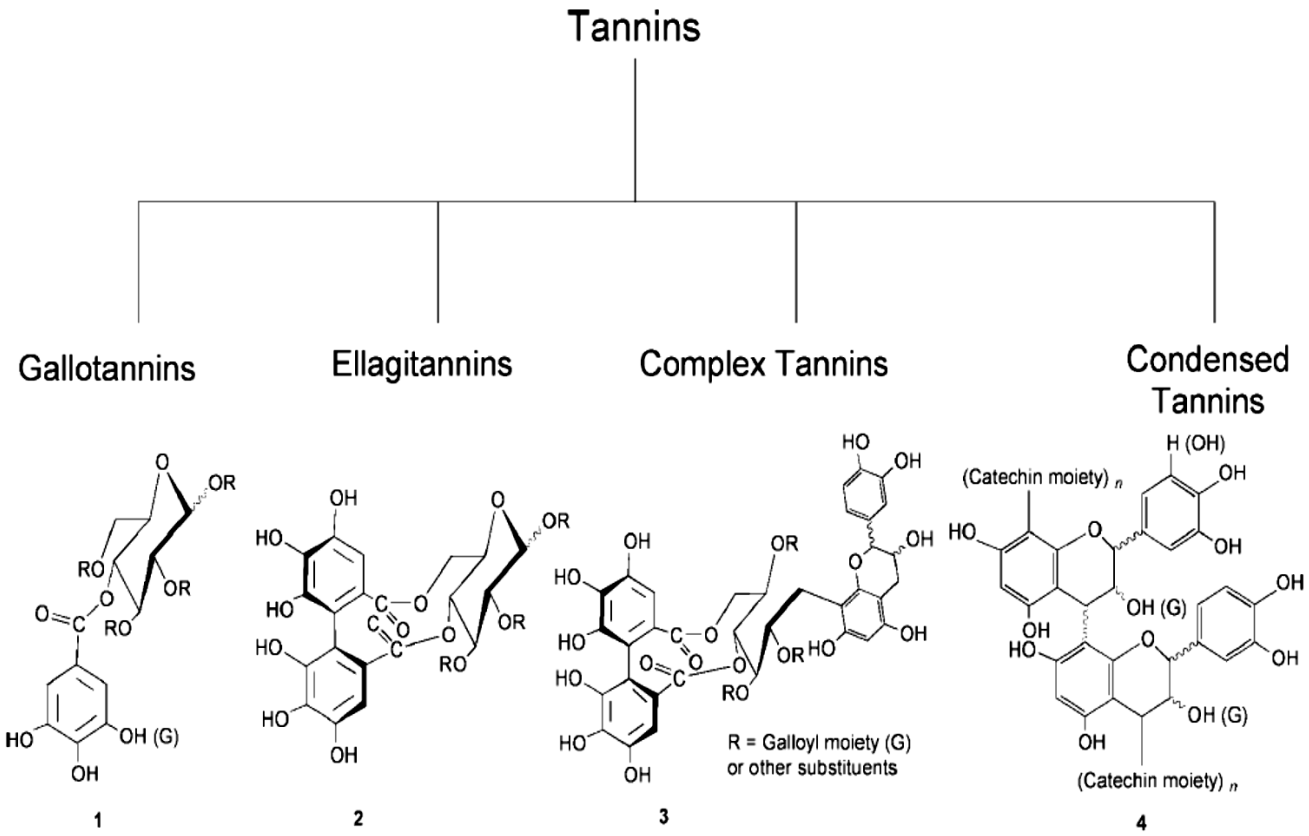


Figure 13: Classification of the tannins *(Khanbabaee and Van Ree, 2001)*

(1) Gallotannins are all those tannins in which galloyl units or their meta-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units.

(2) Ellagitannins are those tannins in which at least two galloyl units are C–C coupled to each other, and do not contain a glycosidically linked catechin unit.

(3) Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit.

(4) Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin (Khanbabaee and Van Ree, 2001).

II – 3 – 1 – 2 Nitrogen Containing Secondary Metabolites

Over 14,000 nitrogen--containing secondary metabolites have been described so far: Alkaloids, amines, nonprotein amino acids, cyanogenic glycosides, and glucosinolates are the main compounds in this group. A few secondary metabolites of high molecular weight should also be considered in this context: some plants accumulate peptides and proteins (protease inhibitors, amylase inhibitors) that play a role in antimicrobial and antiherbivore defence. Their biosynthesis can be induced upon wounding or microbial attack (Wink, 2004).

- **Alkaloids**

Alkaloids are defined as basic compounds synthesized by living organisms containing one or more heterocyclic nitrogen atoms, derived from amino acids (with some exceptions) and pharmacologically active. The class name is directly related to the fact that nearly all alkaloids are basic (alkaline) compounds.

Alkaloids constitute a very large group of secondary metabolites, with more than 12,000 substances isolated. A huge variety of structural formulas, coming from different biosynthetic pathways and presenting very diverse pharmacological activities are characteristic of the group (Brielmann et *al.*, 2006).

Alkaloids can be classified in terms of their biological activity, their chemical structure, or more accepted according their biosynthetic pathway (Warzecha et *al*., 2000).

The alkaloids have been divided into three major classes depending on the precursors and the final structure:

- The true alkaloids are derived from amino acids, are basic and contain nitrogen in a heteroyclic ring, e.g. nicotine and atropine. Common alkaloid ring structures include the pyridines, pyrroles, indoles, pyrrolidines, isoquinolines and piperidines.
- The pseudoalkaloids are basic but are not derived from amino acids, e.g. caffeine and solanidine.
- The protoalkaloids are derived from amino acids, are basic but the nitrogen is not in a heterocycle, e.g. the phenylethylamine derived alkaloids such as mescaline (Bennett and Wallsgrove, 1994).

II – 3 – 2 Global derivatives biosynthetic pathway of secondary metabolites

The building blocks of secondary metabolites are derived from primary metabolism and are effectively tapped off from the processes of photosynthesis, glycolysis and the citric acid cycle (Figure 15). The most important building blocks are derived from acetyl-CoA, shikimic acid, mevalonic acid and deoxyxylulose.

Some of the important classes of secondary metabolites derived from these pathways include:

- The anthraquinones from the acetate pathway;
- The alkaloids and phenylpropanoids (e.g. lignans, aromatic essential oils, and coumarins) from the shikimate pathway;
- A vast array of terpenoids and steroid metabolites, which include the saponins, terpenoid essential oils and carotenoids, derived from the mevalonate and deoxy- xylulose pathways.

Other building blocks based on amino acids are also frequently employed. From this very modest number of building blocks a vast array of secondary compounds is synthesized. This range is achieved by for example combining building blocks of the same type, using mixtures of different building blocks (e.g. flavonoids are formed from combinations between the acetate and shikimate pathways) and incorporating one or more sugar units, e.g. saponins (Dewick, 2002).

NAPRALERT, which is a database of the world literature of natural products, lists over 135 000 isolated and characterized natural products (Cordell, 2000) , which is probably just a small percentage if it is considered that only an estimated 5-15% (Marais et *al*., 2006) of the terrestrial species of plants have been investigated for the presence of secondary metabolites.

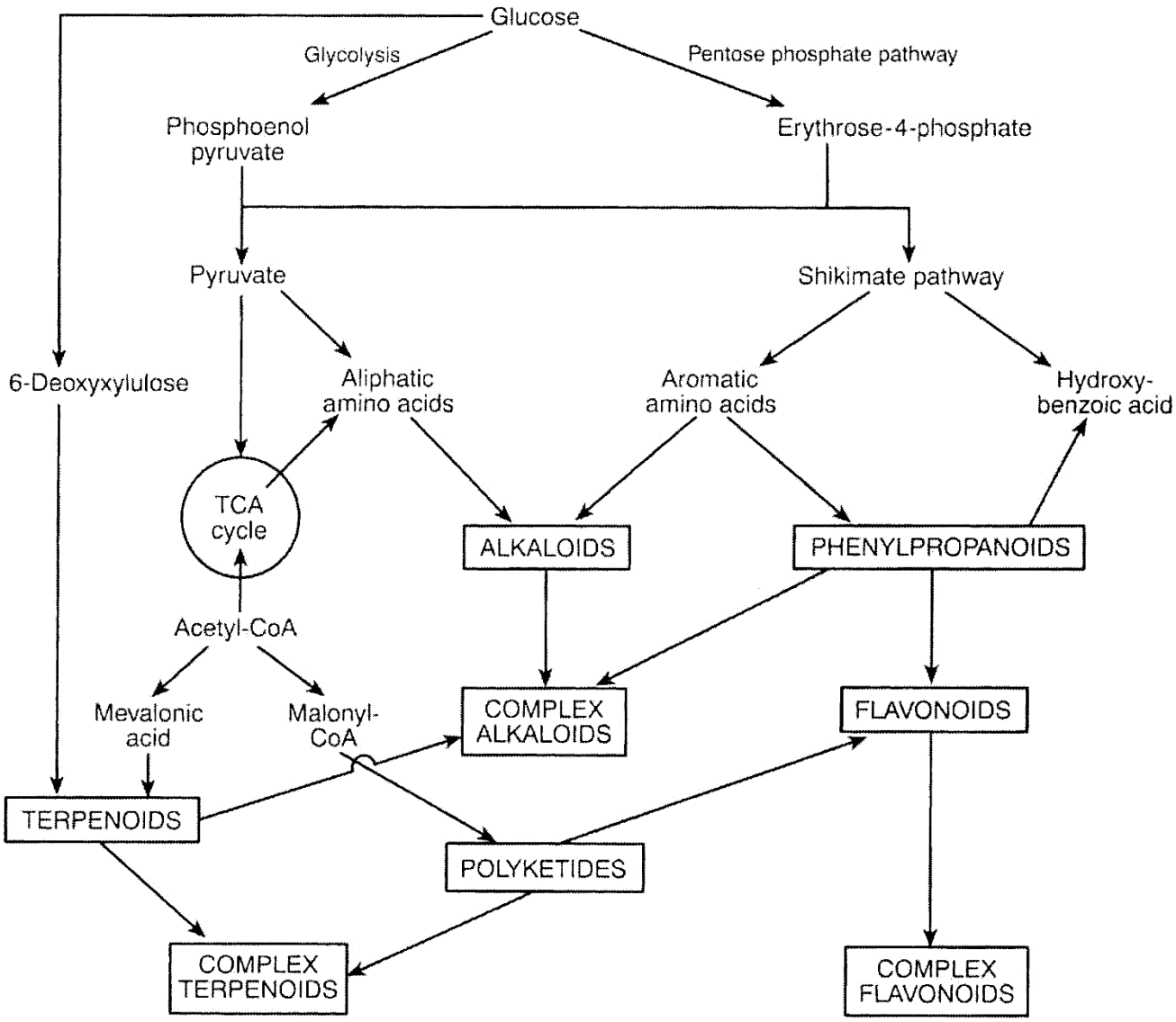


Figure 14: Overview of plant secondary metabolism showing the interrelationships between the different pathways. TCA, tricarboxylic acid (Edwards and Gatehouse, 1999).

II – 4 Toxicology

A thousand years ago an extensive use of plants as medicines have been reported and were initially taken in the form of crude drugs such as tinctures, elixirs, poultices, powders, and other herbal formulations (Gullo et *al*., 2006).

In recent years, there is a growing interest in herbal therapy. Data on scientific screening of plant extracts, whether crude or purified, appears to be accumulating gradually but steadily. Literatures on antidiarrheal, antimalarial and antitrypanosomal activities of plants-based

products support this claim (Nok, 1999; Abdullahi et *al*., 2001; John et *al*., 2001; Atawodi et *al*., 2003).

The major contributory factors to this growing interest include rising costs of orthodox medications, low therapeutic index of synthetic compounds and the growing incidence of drug resistance (Onyeyilli and Egwu, 1995; Seed, 2000).

A number of studies have reported the toxic effects of herbal medicines (Gamble, 1957; Calixto, 2000; Jaouad et *al*., 2004; Taziebou et *al*., 2008). Studies of medicinal plants using scientific approaches showed that various biological components of medicinal plants exhibit a variety of properties and can be used to treat various ailments (Nair and Henry, 1983; Mali et *al*., 2004). Toxicological tests have shown that many plants currently used are highly toxic when given either acutely or subchronically (Raman and Lau, 1996; Basch et *al*., 2003) and for this reason, research is carried out in order to determine the toxicity of medicinal plants. (Phillipson and Wright, 1991).

Although modern medicine may be available in developing countries, phytomedicines have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (Farnsworth and Soejarto, 1991).

II – 5 Common diseases

There are many common and important diseases, which can affect Algerian public health, and our investigation was based on the most popular ones:

II – 5 – 1 Oxidative stress

Cells continuously produce free radicals and reactive oxygen species (ROS) as part of metabolic processes (Jackson, 2000). Oxidative stress is defined as a "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them."(Yoshikawa and Naito, 2002).

Table 2: Major Active Oxygen Species (Yoshikawa and Naito, 2002).

$O_2^{\bullet -}$	Superoxide radical
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
$^1O_2$	Singlet oxygen
$HOO^{\bullet}$	Hydroperoxyl radical
LOOH	Alkylhydroperoxide
$LOO^{\bullet}$	Alkylperoxyl radical
$LO^{\bullet}$	Alkoxyl radical
ClO^-	Hypochlorite ion
$Fe^{4+}O$	Ferryl ion
$Fe^{5+}O$	Periferryl ion
$NO^{\bullet}$	Nitric oxide

Oxidative stress contribute to many pathological conditions including:

- Cancer and neurological disorders (Toshniwal and Zarling, 1992; Lyras et *al*., 1997; Sayre et *al*., 2001; Jenner, 2003),
- Atherosclerosis, hypertension and ischemia/perfusion (Kukreja and Hess, 1992; Kerr et *al*., 1999; Dhalla et *al*., 2000; Kasparova et *al*., 2005),
- Diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease (Asami et *al*., 1997),
- Asthme (Andreadis et *al*., 2003 ; Comhair et *al*., 2005a; Comhair et *al*., 2005b ; Ercan et *al*., 2006 ; Dut et *al*., 2008; Fitzpatrick et *al*., 2009).

ROS can be divided into two groups, free radicals and nonradicals. Molecules containing one or more unpaired electrons and thus giving reactivity to the molecule are called free radicals. When two free radicals share their unpaired electrons, nonradical forms are created. The three major ROS that are of physiological significance are superoxide anion ($O_2^{\cdot}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2). ROS are summarized in table 2 (Miller et *al*., 1990).

II – 5 – 2 Microbial diseases

One of the most fascinating aspects of the microorganisms with which we share the earth is that, despite all of the benefits they provide, they also contribute significantly to human misery as pathogens. The vast majority of microorganisms that associate with humans cause no harm.

In fact, they provide many benefits to their human hosts. There is little doubt that a diverse microbial biota living in and on humans is an important part of human well-being.

However, humankind is also plagued by nearly 2000 different microbes that can cause various types of disease (Cowan, 2012). Disease-causing bacteria are heterotrophs that use the organic matter of living cells as food. Bacteria and other kinds of organisms that are capable of causing harm to their host are called pathogens. Only a small minority of bacteria fall into this category.

Pathogenic bacteria can cause disease in several ways. Many are normally harmless commensals but cause disease when their populations increase to excessively high numbers. Other bacteria invade the healthy tissue of their host and cause disease by altering the tissue's normal physiology. Many other illnesses are caused by toxins or poisons produced by bacteria. In general, toxins cause tissue damage, fever, and aches and pains (Enger et *al*., 2012).

Fungi can cause significant number of human diseases represented by pathogens, capable of infecting healthy people, or opportunistic invaders, which are normally no virulent in healthy people but could be disseminated to deep tissue and cause fatal disease in unhealthy people. (Chakrabarti , 2005; Reedy et *al*., 2007).

Fungal diseases can be broadly classified on the basis of causative agents as:

- Dermatophytosis,
- Histoplasmosis,
- Blastomycosis,
- Coccidiomycosis,
- Candidiasis,
- Cryptococcosis,
- Aspergillosis,
- Hyalohyphomycosis
- Zygomycosis (Sullivan et *al*., 2005)

The damage–response framework is based on three tenets that are both self-evident and incontrovertible:

1) Microbial pathogenesis is the outcome of the interaction between two entities, namely a host and a microbe;
2) The relevant outcome of host–microbe interaction in a given host is damage in the host

3) Host damage can reflect the action of microbial factors, the host response, or both.

Damage is not a static outcome, but can change as a function of the immune response or time. When damage is plotted as a function of the host response, host–microbe interaction can be represented by a parabola, whereby maximal damage occurs in situations of either weak or strong responses (Figure 16) (Casadevall and Pirofski, 1999).

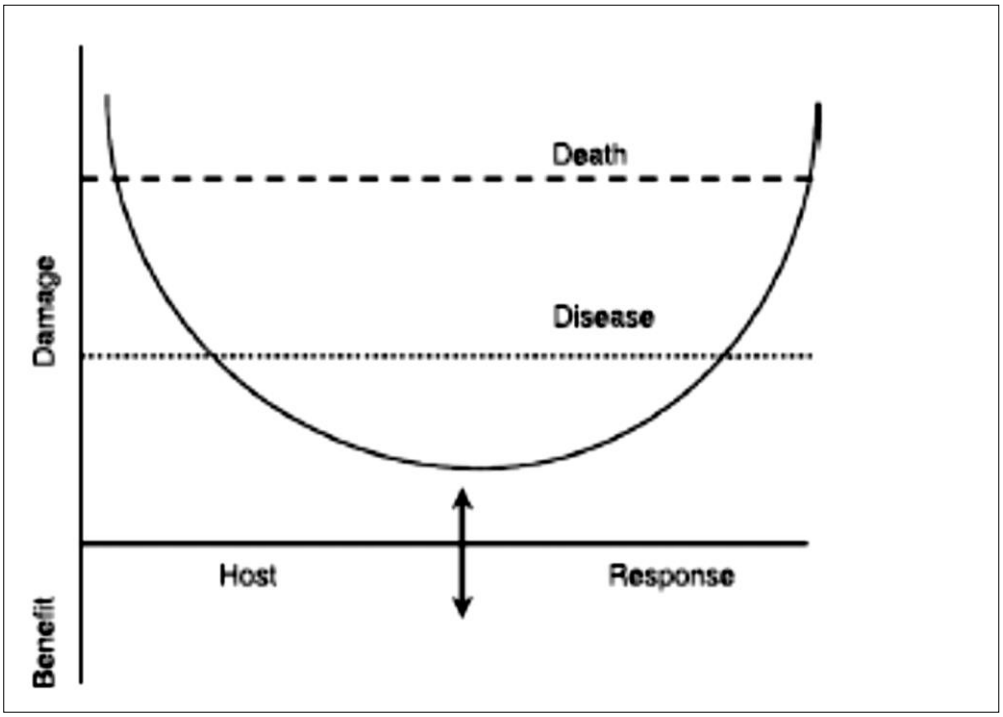


Figure 15:Basic curve of damage–response framework of microbial pathogenesis (Casadevall and Pirofski, 1999)

II – 5 – 3 The inflammatory process

Based on visual observation, the ancients characterised inflammation by five cardinal signs, namely redness (rubor), swelling (tumour), heat (calor; only applicable to the body' extremities), pain (dolor) and loss of function (functio laesa). The first four of these signs were named by Celsus in ancient Rome (30–38 B.C) and the last by Galen (A.D 130–200) (Hurley, 1972). More recently, inflammation was described as "the succession of changes which occurs in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroy its structure and vitality" (Sanderson, 1871), or "the reaction to injury of the living microcirculation and related tissues (Spector and Willoughby, 1963). The local process can be divided into three phases (Serhan , 2007):

- **Initiation phase:** Pro-inflammatory eicosanoids like leukotriene B4 (LTB4) and prostaglandins (PGs) initiate the inflammatory response. PMNs generate LTB4 and PGE_2 from precursor AA with the use of lipoxygenase-5 (LOX-5) and cyclo-oxygenase 2 (COX-2).
- **Resolution phase:** This phase starts with the Eicosanoid Switch to resolution. When the PGE_2 and/or PGD_2 level is equal to the level of LTB4, the PMNs activate the switch from pro-inflammatory to anti-inflammatory eicosanoids production by limiting the production of LOX-5. This switch is responsible for the production of anti-inflammatory lipoxins (LXs) from AA through activation of lipoxygenase -12 (LOX-12), lipoxygenase-15 (LOX-15) and acetylated COX-2 (Gilroy et *al*., 1999; Bannenberg et *al*., 2005).

  This last mechanism has been found to be responsible for the production of more stable aspirin-triggered LXs (ATLs) with a longer half-value period (Serhan et *al*., 1995). Other resolving metabolites that support LXs are resolvins, (neuro)protectins and maresins produced from respectively EPA and DHA (Serhan, 2007; Serhan et *al*., 2009) . A second substantial increase of COX-2 activity will produce anti-inflammatory PGs (PGD2 and PGF2a) during this phase (Willoughby et *al*., 2000)
- **Termination phase:** This phase starts when the Stop Signal takes place. This happens when sufficient anti-inflammatory mediators such as LXs are available to stop the pro-inflammatory process (Gilroy et *al*., 1999; Bannenberg et *al*., 2005;). LXs are capable of inhibiting both PMN infiltration and the activity of cytotoxic cells of the ISS, inducing phagocytosis to clear debris by non-cytoxic macrophages and attenuating an accumulation of the pro-inflammatory transcription factors, i.e. nuclear factor- kappaB (NF-kB) and activator protein 1 (AP-1) (Maddox and Serhan, 1996; Maderna and Godson , 2005) .

II – 5 – 4 Pain sensation and pathology

Pain is an unpleasant sensory and emotional experience usually triggered by stimulation of peripheral nerves and often associated with actual or potential tissue damage (Cutrer, 2001).

Pain sensations could arise due to:

- Inflammation of the nerves, e.g., temporal neuritis.
- Injury to the nerves and nerve endings with scar formation, e.g., surgical damage or disk prolapse.
- Nerve invasion by cancer, e.g., brachial plexopathy.
- Injury to the structures in the spinal cord, thalamus, or cortical areas that process pain information, which can lead to intractable pain e.g., spinal trauma.

- Abnormal activity in the nerve circuits that is perceived as pain, e.g., phantom pain with cortical reorganization (Kopf and Nilesh, 2010).

II – 5 – 5 Peptic ulcer diseases

Peptic ulcer disease appears to be a heterogeneous group of disorders having in common a hole in the gastroduodenal mucosa in the presence of gastric acid, but differing in the pathophysiology of ulceration (Fresto, 1988).

Although the pathophysiology of ulcer disease has not yet been clearly elucidated, scientists have moved away from assigning acid the dominant role in ulcer causation and now recognize distinct and identifiable causes. The two most common causes of peptic ulcer are infection with the bacterium *Helicobacter pylori* (H. pylori) and the use of non-steroidal anti-inflammatory drugs (NSAIDs) (Soll, 1990; Graham and Go, 1993; Soll et *al*., 1993).

II – 5 – 6 Healing wounds process

Wounds are inescapable events of life, which arise due to physical or chemical injury or microbial infections. The healing of wounds often deviates from a normal course and under-healing, over-healing or failure of wounds to heal is common (Biswas and Mukherjee, 2003). Wound healing is a dynamic process involving biochemical and physiological phenomena that behave in a harmonious way in order to guarantee tissue (Robards et *al*., 1999). Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production (Lambole and Upendra, 2012). Later, the epithelial tissue is regenerated. Wound healing consists of an orderly progression of events that re-establish the integrity of the damaged tissue (Krishna and Upendra, 2012). There are three main phases of wound healing: inflammatory, proliferative and remodelling phase. The inflammatory phase begins immediately after injury with vasoconstriction that favours and releases inflammatory mediators. The proliferative phase is characterized by granulation tissue formation mainly by fibroblasts and angiogenesis. The remodelling phase is characterized by reformulation and improvement in the components of the collagen fibre that increases the tensile strength (Sourav et *al*., 2012).The process of wound healing may be hampered by the presence of free radicals, which can damage wound surrounding cells, or by microbial infection (Houghton et *al*., 2005). The moist wound area is very much susceptible to microbial growth and it delays the healing process remarkably.

Although the healing of wound is a natural biological process but according to severity treatment is recommended to increase the rate of healing and minimize the microbial growth

around the wounded area. There are numbers of synthetic drugs and antibiotics available for this purpose but it create several unwanted effect. It is already reported that herbal medications are more effective, nontoxic in nature, non-resistant to microorganism, more available, affordable and cheap over the conventional medicine (Farahpour and Habibi, 2012). From this point of view, there is a very much growing interest in research field to discover new potential herbal medicine for treatment of infectious diseases along with their diverse medicinal importance, as it is safer in concern (Rojas et *al*., 2003).

Materials and Methods

III –1 Materials

III – 1 – 1 Plant materials

The areal parts of *Galium t* were collected from the mountain of Megriss Setif -Algeria in May 2014, determined in our laboratory.

III – 1 – 2 chemicals

The used products are shown in table 1 in appendix, the non-mentioned products were obtained from Sigma Aldrich.

III – 1 – 3 Animals

Experiments were conducted using adult male Wistar albino rats (180 – 220 g) except the acute toxicity (the animals weights were mentioned in table 2 in appendix) and Swiss mice (20 – 30 g). Animals were housed in standard metal cage, kept under ambient temperature (20-25°C) and illuminated environment approximately of 12:12 hours dark/light cycle. They were provided with standard food pellet and tap water until the night before sacrifice.

III – 2 Methods & experiments

III – 2 – 1 Secondary metabolites extraction

III – 2 – 1 – 1 Preparation of methanol extract

The method of Neda et *al*. (2010), was used with a slight modification. Briefly, air-dried herbs were powdered and macerated in 80% methanol (MeOH) for 24 hours, 48 hours and 72 hours at room temperature (1:10 w/v, 100 g dried herb). After maceration, the extracts were collected, filtered and evaporated to dryness under vacuum. The dry extract was stored at a temperature of − 18 °C for later use.

III – 2 – 1 – 2 Preparation of aqueous extract

The method for preparing aqueous extract from dried plant has been already described by Predrag et *al*. (2005). Dried plant material (25 g) was stirred in 250 mL of distilled water for 15 minutes at 90 ° C followed by rapid filtration through four layers of gauze and then by a more delicate filtration through Whatman filter paper #1. The resulting filtrate evaporated to dryness under vacuum. The powder was stored at – 18 °C until required.

III – 2 – 1 – 3 Extraction of the essential oil

For the extraction of essential oils, 100 g of dry plants *Galium t* are soaked in distilled water for 24 hours, a diethyl ether layer was placed in Clevenger-type apparatus at the beginning of

the extraction to maximize performance of extraction. The system subjected to hydrodistillation for 3 hours. The obtained oils were stored at − 18 °C in sealed brown glass vials until tested.

III – 2 – 1 – 4 Extraction of flavonoids

The dried aerial plant matrix was milled into coarse powder, then (100g) were defatted three times with petroleum ether (25% w/v) for 3 hours, at 50 °C.

The powder was taking up again three times with 70% ethanol (raw material: solvent ratio was 1: 10) for 90 minutes at 100 °C. The extracts were pooled and concentrated in vacuum to collect the aqueous residue (100 mL), which was extracted with chloroform, and then acidified with 20% H_2SO_4 (pH = 5) and extracted with ethyl acetate. The appearance of an interphase precipitate was observed upon extraction with ethyl acetate. The ethyl acetate fraction and the interphase precipitate were taken as a two flavonoids fraction for our experiments (He-Long et *al*., 2010; Chirikova *et al*., 2010).

III – 2 – 1 – 5 Extraction of Tannins

For tannins extraction, two methods were used for comparison:

- Barrau et *al*. (2005) have described the first method of extraction. Briefly, 10 g of dry plant material were reduced to powder, extracted by maceration in 100 mL of 30% ethanol in water at 60 °C for 2 hours. The fraction was concentrated and dried in a vacuum oven at 45 °C and kept at – 18 °C when not in use.

- Mohamad Ibrahim et *al*. (2005) have described the second method. Powdered materials (20 g) was macerated in 200 mL of acetone for 24 hours; the supernatant was then separated from the residue by filtration using Whatman #1 filter paper, the fraction was concentrated and dried to a constant weight in a vacuum oven at 45°C and the residues obtained was stored at – 18°C .

III – 2 – 1 – 6 Extraction of saponins

They were extracted according to the method worked out by Bouchelta et *al*. (2005).

Dry aerial parts powdered were delipidated during 2 hours by 150 mL of n-hexane. After elimination of the organic phases, the precipitates obtained were macerated in 50 mL of absolute ethanol under magnetic agitation at the ambient temperature during 24 hours. The ethanolic phase was evaporated at 40 °C by the rotavapor. The dry residues were extracted three times by 50 mL from distilled water/petroleum ether mixture (V/V) heated at 50 °C in water

bath during 30 minutes. The aqueous phases were mixed then treated by 10 mL of n-butanol during 30 minutes. The organic phases, evaporated at 40 °C, were weighed and stored at – 18 °C for ulterior use.

III – 2 – 1 – 7 Extraction of alkaloids

They were obtained by triple liquid–liquid extraction according to the method of Harbone, (1998) with a slight modifications. Briefly, 10g of powdered aerial parts of *Galium t* were extracted with 100 mL of absolute ethanol during 24 hours. The ethanolic extract was then evaporated under vacuum at 40 °C by rotavapor.

The aqueous phase was taken again by 20 mL of chloroform and acidify by HCl (5%) to pH = 3; they were let rest during 30 minutes at the ambient temperature.

The acid aqueous phase was extracted again by 20 mL of chloroform, basified by $NaHCO_3$ (5%) to pH= 9 and let rest during 15 minutes at the ambient temperature.

The chloroformic phases were mixed and evaporated to a dry residues, made up of total alkaloids extract, was weighed then stored at – 18 °C for ulterior use.

III – 2 –2 Quantification of major secondary metabolites groups

III – 2 – 2 - 1 Essential oil analysis

Essential oil analysis was carry out in LEXVA ANALITIQUE laboratory France by Dr. Gilles FIGUEREDO.

The essential oil was analysed on a Hewlett-Packard gas chromatograph Model 6890, equipped with a DB5 MS column (30m X 0.25mm, 0.25µm), programming from 50 °C (5 minutes) to 300°C at 5 °C/minute, 5 minutes hold. Hydrogen as carrier gas (1.0 mL/minute); injection in split mode (1: 60); injector and detector temperature, 280 and 300°C respectively. The essential oil is diluted in hexane: 1/30. The MS working in electron impact mode at 70 eV; ion source temperature, 230°C; mass spectra data were acquired in the scan mode in m/z range 33- 450.

Retention indices were determined relative to the retention times of a series on n-alkanes (C_8 to C_{26}) with linear interpolation. The identification of the components was based on comparison of their mass spectra with those of NIST mass spectral library (Masada, 1976; NIST, 2002) and those described by Adams (2001), as well as on comparison of their retention indices either with those of authentic compounds or with literature values.

III – 2 – 2 – 2 Determination of Total Phenolics Content

For total polyphenols determination, the Folin Ciocalteu method was used (Li et *al*., 2007). The sample (0.2 mL) was mixed with 1 mL of the Folin Ciocalteu reagent previously diluted with 10 mL of deionized water. The solution was allowed to stand for 4 minutes at 25 °C before 0.2 mL of a saturated sodium carbonate solution (75 mg/mL) was added. The mixed solution was allowed to stand for another 120 minutes before the absorbance at 765nm was measured. Gallic acid was used as a standard for the calibration curve. The total phenolics content was expressed as mg equivalent of Gallic acid per grams of dry matters (mg EAG/GDM).

III – 2 – 2 - 3 Determination of total flavonoids content

The flavonoids content in the extracts were estimated by the Aluminium chloride solution according to the method described by Bahorun et *al.* (1996).

Briefly, 1 mL of the methanol solution of each extract was added to 1 mL of 2 % $AlCl_3$ in methanol. After 10 minutes, the absorbance was determined at 430 nm. quercetin was used as a standards. Results were expressed as mg equivalent of quercetin per grams of dry matters (mg EQ/GDM).

III – 2 – 2 – 4 Determination of total tannins content

Tannin content was evaluated using the haemoglobin precipitation assay.

An aliquot of 0.5 mL of each extracts was mixed with 0,5 mL of haemolysis bovine blood (4 mL of blood was mixed with 425mL of distilled water to reach an absorbance of 1,6 at 578 nm against a blank of distilled water).

The mixture was incubated at 25 °C for 20 minutes, centrifuged at 480g for 10 minutes and measured at 578 nm (Hagerman and Butler, 1978). Tannic acid at various concentrations (100-600 µg / mL) was used in the same manner as the samples. Results were expressed as mg equivalent of tannic acid per grams of dry matters (mg EAT/GDM)

III – 2 – 2 – 5 Quantitative Estimation of Alkaloids

Briefly, to 1mL of each extract, 5 mL phosphate buffer (pH 4.7) and 5 mL BCG solution were added. The mixture was shacked with 4 mL of chloroform, and then the extracts were collected in a 10-mL volumetric flask and diluted to adjust volume with chloroform.

The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without extracts. Atropine was used as a standard material. Results were expressed

as mg equivalent of Atropine per grams of dry matters (mg EA/GDM) (Hemachakradhar and Pavan, 2013).

III – 2 – 2 – 6 Determination of chlorophylls a and b

To quantify total chlorophylls (a + b) the method of Lisiewska et *al*. (2004), was adopted. Briefly, dry plants material and methanolic extract were extracted with diluted acetone (80%). The absorbance of acetone chlorophyll solutions were measured spectrophotometrically at 644 nm and 662 nm, against a blank (80% acetone).

Total chlorophyll (a + b) content was calculated using the following equations and given in mg/g:

$$Chla = ((9{,}784 \times A662)-(0{,}99 \times A644)) \times V/1000 \times m$$

$$Chlb = ((21{,}42 \times A644)-(4{,}65 \times A662)) \times V/1000 \times m$$

Where;

A: represents the measured absorbance of chlorophyll extracts at wave lengths.

V: is the total volume of 80% acetone chlorophyll extracts (mL).

m: is the mass of plant material used for quantification.

III – 2 – 2 – 7 Determination of carotenoids

Total carotenoids content in the extracts were determined by a spectrophotometric assay described by Youngmin et *al*. (2006). Approximately, 5 mL of extract were mixed with equal volume of distilled water and 15 mL of hexane/acetone/methanol (50/25/25, v/v) solution. The mixture was then homogenized with a Polytron and centrifuged at 3000 rpm (940g) for 10 minutes.

The absorbance of the top layer of hexane was measured at 450 nm using a spectrophotometer. Total carotenoids content of the samples were calculated as l g β-carotene per 100 g of sample using an extinction coefficient of $E\ {}^{1\%}_{1cm}$=2505 (De Ritter and Purcell, 1981). . Results were expressed as mg equivalent of β-carotene per grams of dry matters (mg β-carotene /GDM)

III – 2 – 3 Antioxidant activity

The antioxidant activity was carry out with all extracts except alkaloids, using three tests as below:

III – 2 – 3 – 1 DPPH Assay

The donation capacity of extracts was measured by bleaching of the purple-coloured solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato et *al*. (1998). One millilitre of the extract at different concentrations was added to 0.5 mL of a DPPH-methanolic solution (78 µg/ mL). The mixture was shaken vigorously and left standing at room temperature for 30 minutes in the dark. BHT was used as a standard.

The absorbance of the resulting solution was then measured at 517 nm. The antiradical activity was expressed as IC_{50} (micrograms per millilitre), the antiradical dose required to cause a 50 % inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity. The ability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = [(A_0 - A_1)/ A_0] \times 100$$

Where;

A_0: is the absorbance of the control at 30 minutes

A_1: is the absorbance of the sample at 30 minutes. (Bettaieb et *al*,. 2011).

III – 2 – 3 – 2 β-Carotene linoleic acid bleaching assay

The method of Miller, (1971), was adopted. β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid and 200 mg of the tween 40 emulsifier mixture. After evaporation of the chloroform under vacuum, 100 mL of oxygen-saturated distilled water were added with vigorous shaking. Next, 4mL of this mixture is transferred into test tubes containing different concentrations of the samples (2 mg/mL for extracts and 350 µL portions of the essential oil prepared at 25% equal to 156, 65 mg/mL).

As soon as the emulsion is added to each tube, the zero time point absorbance was measured at 470 nm. The emulsion is incubated for 2 hours at 50 °C. A blank, devoid of β-carotene, is prepared for background subtraction (Krishnaiah et *al*,. 2010). BHT is used as standards.

The antioxidant activity of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula (1):

$$Inhibition\ (\%) = [(At - Ct)] / [(C_0 - Ct)] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Where;

At: was the absorbance values measured for the test sample after incubation for 120 minutes.

Ct: was the absorbance values measured for the control after incubation for 120 minutes.

C_0: was the absorbance values for the control measured at zero time during the incubation (Bettaieb et *al*,. 2011).

III – 2 – 3 – 3 Reducing power

The reducing power was determined according to the method of Oyaizu, (1986). To 2.5 mL of each extract, 2.5 mL of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide were added. The mixture was incubated at 50 °C for 20 minutes.

After cooling, 2.5 mL of 100 mg/mL trichloroacetic acid were added, the mixture was centrifuged at 200g for 10 minutes. The upper layer (5 mL) was mixed with 5mL of deionized water and 1mL of 1 mg/mL ferric chloride. The absorbance was measured at 700nm against a blank.

A higher absorbance indicates a higher reducing power. EC_{50} value (mg extract/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid and BHT were used as standards (Huang and Mau, 2006).

III – 2 – 4 Antimicrobial activity

The antimicrobial activity was carry out with all the extracts.

III – 2 – 4 – 1 Test strains and culture media

Strains of bacteria were obtained from the American Type Culture Collection and Netherlands Culture Collection of bacteria. Six bacterial strains were tested: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, *Shigella flexheri* NCCB1406, *Klebsiella pneumoniae* ATCC700603, *Proteus mirabilis* ATCC35659, *Staphylococcus aureus* ATCC25923. Two fungi: *Aspergillus niger* 2CA936 and *Aspergillus flavus* NRRL 3357; and one yeast: (*Candida albicans* ATCC1024) was also tested.

Muller Hinton agar, the potato dextrose agar and Sabouraud were used for culture of bacteria, fungi and yeast respectively.

III – 2 – 4 – 2 Antibacterial Activity

Agar disc diffusion method was employed for the determination of antimicrobial activities of *Galium t* secondary metabolites (Agar disc diffusion method adopted by NCCLS, 1997 and 1999). Briefly, the suspension of the tested microorganisms (0.1 mL containing 10^7 cells) was spread on the solid media plates. Chromatography paper 3MM discs (6 mm in diameter) were impregnated with 10 µL of different concentration of the extracts and placed on the inoculated plates. These plates were incubated at 37 °C for 24 hours. Gentamicin (10 µg/disc) was used as a standards and DMSO as a control.

The antibacterial activity was determined by measuring of inhibition zone diameters (mm) and evaluated according the parameters suggested by Alves et *al*. (2000):

- <9 mm, inactive ;
- 9–12 mm, less active ;
- 13–18 mm, active;
- >18 mm, very active.

III – 2 – 4 – 3 Antifungal activity

The antifungal activity was tested by disc diffusion method with modifications (Agar disc diffusion method adopted by NCCLS, 1999). The potato dextrose agar plates were inoculated with each fungal culture (*Aspergillus niger* 2CA936, *Aspergillus flavus NRRL 3357*).

The spore suspension was prepared in an emulsion of 0,5 % tween 80 adjusted to a concentration of 2-3 × 10^6 spores/mL, corresponding to 0.15 to 0.17 absorption at 530 nm (Yazdani et *al*., 2012). Whereas, *Candida albicans* ATCC1024 suspension is obtained in physiological saline solution (0.9 %) from culture in Sabouraud 24 hours at 37 °C before, adjusted to 10^5 CFU / mL. 100 µL of suspension was placed over agar in Petri dishes and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 µL of each samples at different concentrations. Nystatin 100µg, Clotrimazon 50 µg and Amphotericin 100 µg were used as a standards and DMSO as control. Inhibition zones were determined after incubation at 27 °C for 48 hours.

III – 2 – 5 *In vitro* anti-inflammatory activity

The *In vitro* anti-inflammatory activity was realized with the phenolic compounds (polyphenols represented by methanolic extract, flavonoids represented by the ethyl acetate phase and total tannins extract) following the tests below:

III – 2 – 5 – 1 Inhibition of proteins denaturation

The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations of extracts so that final concentrations became 100, 200, 400, 500 and 800 µg/mL.

A similar volume of double distilled water served as the control. Next, the mixtures were incubated at 37 ± 2ºC for 15 minutes and then heated at 70 °C for five minutes.

After cooling, the absorbance was measured at 660 nm using the vehicle as a blank. Diclofenac sodium in the final concentrations of 100, 200, 400, 500 and 800 µg/mL was used as the reference drug and treated similarly for the determination of absorbance (Chandra et *al*,. 2012).

The denaturation of protein inhibited by the extract and standard were expressed as percentage using the formula:

Percentage of inhibition = (Control – Test)/Control × 100 (Mizushima and Kobayashi, 1968).

III – 2 – 5 – 2 The Human Red Blood Cell (HRBC) membrane stabilization method

To prepare the HRBC suspension, fresh whole human blood (10 mL) was collected and transferred into the centrifuge tubes. These lasts were centrifuged at 3000 rpm for 10 minutes thrice and washed with equal volume of normal saline each time. The volume of blood was measured and reconstituted as 10 % v/v suspension with normal saline.

The principle involved here was stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The mixture contain 1mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hypo saline (0.36 %), 0.5 mL HRBC suspension (10 % v/v) and 0.5 mL of plant extracts or standard drug (diclofenac sodium) at various concentrations (50, 100, 250, 500, 1000, 2000 µg/mL). The control was distilled water instead of hypo saline to produce 100 % haemolysis.

The mixtures were incubated at 37 °C for 30 minutes and centrifuged at 2500 rpm for 5 minutes. The absorbance of haemoglobin content in the suspensions were estimated at 560 nm. The percentage of haemolysis of HRBC membrane can be calculated as follows:

Haemolysis (%) = (Optical density of Test sample / Optical density of Control) ×100

However the percentage of HRBC membrane stabilization can be calculated as follows:

Protection (%) = 100 – [(Optical density of Test sample / Optical density of Control) × 100] (Seema et *al*., 2011).

III – 2 – 5 – 3 Heat induced haemolysis

HRBC membrane stabilization test was performed by the following described method (Sadique et *al*., 1989). The reaction mixture 2 mL consists of 1 mL of test sample solution and 1 mL of 10% HRBC suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug.

All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 °C for 30 minutes, at the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula:

$$Percentage\ Inhibition = [(A\ of\ Control - A\ of\ Sample)/A\ of\ Control] \times 100.$$

III – 2 – 6 Acute toxicity

III – 2 – 6 – 1 Acute oral toxicity

The acute toxicity (LD_{50}) of methanolic extract of *Galium t* was determined using modified Özbek et *al*. (2004), Néné-Bi et *al*. (2008) and Konan et *al*. (2012) methods in rat by peritoneal route. Forty eight animals were randomly allocated into six heterogenic groups of eight animals each. Group I (Control): animals were injected with vehicle (distilled water), remaining groups (II, III, IV, V and VI) were injected with 1000, 3000, 6000, 9000 and 12 000 mg/ kg of body weight by methanolic extract respectively (table 2 in appendix).

The animal's behaviours were observed for 1, 2, 3, 4, 5, 6, 24, 48, and 72 hours and the number of dead animals were recorded until the 15^{th} days after injection.

Both methods of Dragstedt and Lang (1957) and probity using Bio stat professional 2007 build 3.2 (El Allaoui et *al*., 2011), were adopted to determine the LD_{50}.

In Dragstedt and Lang (1957) method, DL_{50} was determined by the following formula:

$$DL_{50} = \frac{50(X2-X1) + X1Y2-Y1X2}{Y2-Y1}$$

X2: upper dose surrounding DL
X1: lower dose surrounding DL
Y1: Percentage of mortality corresponding to X1
Y2: Percentage of mortality corresponding to X2 (Sangare et *al*., 2012)

III – 2 – 6 – 2 Haematological and biochemical analysis

On the necropsy day, blood was withdrawn through decapitation of all rats under diethyl ether anaesthesia. The blood was placed into EDTA tubes for haematological assay and in heparin tubes for clinical biochemistry determination.

The blood for haematological assay was immediately analysed in CHU Setif haematological laboratory for the measure of white blood cell (WBC), Haemoglobin (HGB), Red blood cell (RBC), Haematocrit (HCT), Mean cell volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC) and Platelets (PLT).

The blood in the heparin tubes were centrifuged at 3000 rpm at 4°C for 10 minutes. The serums was then kept at -20°C until analysis for clinical biochemistry measurements in CHU Setif biochemistry laboratory. Total protein, Albumin, Alanine Bilirubin, Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Uric acid (AU), Creatinine, Glucose, Cholesterol and Total Triglycerides (TG) were analysed.

III – 2 – 6 – 3 Relative organ weights and histopathology

Animals were sacrificed for autopsy to excise liver, kidney, heart, and spleen, these organs were rinsed in 0.9 % saline, weighed and relative organ weights (ROW) were calculated as the formula:

$$ROW = \frac{Absolute\ organ\ weight\ (g)\ X\ 100}{Body\ weight\ on\ the\ day\ of\ sacrifice\ (g)}$$

Tissue pieces of vital organs (heart, liver, spleen and kidneys) were fixed in 10 % paraformaldehyde, to be transferred to CHU – Setif pathological anatomy laboratory for paraffin histology and processed in paraffin embedding as per the standard protocol. Each tissue were stained with haematoxylin and eosin, and observed for possible histopathological damages.

III – 2 – 7 *In vivo* anti-inflammatory activity

III – 2 – 7 – 1 Xylene-induced ear oedema in mice

The experiment was based on a previously described method of Junping et *al.* (2005). Four groups of five mice each were injected intraperitoneally by *Galium t* flavonoids (10mg/kg and 30 mg/kg) one hour before xylene application. Dexamethasone (30 mg/kg) was given as a reference anti-inflammatory drug and distilled water (10 mL/kg) given to control animals.

Ear oedema was induced by applying carefully a drop of xylene (0.03 mL) to the anterior and posterior surfaces of the right ear. The left ear remained untreated and considered as control One hour after xylene application, the animals were sacrificed under ether anaesthesia and 6 mm punches were made in the right and left ears of each mouse using a borer. Each ear punch was weighed and differences between the weight of the right and left ear punches of mice were recorded.

III – 2 – 7 – 2 Cotton pellet-induced granuloma in rats

The effect of *Galium t* flavonoids extract on cotton pellet-induced granuloma in rats was conducted as the method described by Ismail et *al.* (1997).

Fifteen rats were divided into three groups of five animals each. Granulomatous lesions were induced by surgically inserting sterile cotton pellets (15 ± 1 mg) subcutaneously in both axilla regions of each rat following a single incision, which was thereafter closed by interrupted sutures.

After implantation of pellets, flavonoids extract of *Galium t* (30 mg/kg) was injected intraperitoneally once daily for seven consecutive days. Dexamethasone (4 mg/kg) was also given daily to standard group while the control group received the same volume of distilled water (10 mL/kg).

On day eight, the cotton pellets were dissected out under ether anaesthesia, cleaned of extraneous tissue, and dried at 50 °C to a constant weight. The mean weights for different groups were determined. The increase in dry weight of the pellets was taken as the measure of the granuloma formation.

III – 2 – 8 Analgesic activity

III – 2 – 8 – 1 Mouse writhing test

The protocol established by Koster et *al*. (1959), Salawu et *al*.(2008) and Chirikova et *al.* (2010) was adopted. The mice were randomly divided into three groups of six animals each.

Doses of 100 mg/kg of flavonoids extract was administered to the first group while the remaining two groups received distilled water 10 mL/kg and Diclofenac sodium 10 mg/kg respectively.

All treatments were administered orally, and after 30 minutes 10 mL/kg of 0.6% acetic acid solution in normal saline were injected intraperitoneally. The numbers of writhes were counted for 15 minutes after acetic acid injection.

Percentage of protection against acetic acid induced writhing was calculated using the formula:

$$Pourcentage\ de\ protection = ((Wc\text{-}Wt)/\ Wc) \times 100$$

Where,

Wc: Mean values of number of writhing in control group

Wt : Mean values of number of writhing in the test groups.

III – 2 – 8 – 2 Hot plate method

Experimental animals of either sex were randomly selected and divided into three groups designated as group-I, group-II and group-III consisting of five mice in each group for control, standard and treated group respectively.

Each group received a particular treatment i.e. control (1% Tween-80 solution in water, 10mL/kg, p.o.), standard (Diclofenac sodium 10 mg/kg, p.o.) and the test sample (flavonoids extract of 100 mg/kg, p.o.).

The animals were positioned on hot plate kept at a temperature of 55±0.5 °C (Zulfiker et *al*., 2010). Reaction time was recorded when animals licked their fore or hind paws, or jumped. Reaction time was recorded at 0, 30, 45 and 60 minutes after oral administration of the samples (Eddy and Leimback, 1953; Kulkarni, 1999; Toma et *al*., 2003).

III – 2 – 9 Antiulcerative activity

III – 2 – 9 – 1 Preventive effects of tannin extract against acute gastric lesions

The antiulcerative activity was investigated using fifteen male Wistar rats divided into three groups, the first group was orally administered with 10 mL/ kg of water (control), the second group with sample suspension containing 200 mg of tannins extract of *Galium t* and the third one with 80 mg/ kg omeprazole, 30 minutes before gastric ulcer induction.

The gastric mucosal lesions that lead to acute gastric ulcer were induced by oral administration of 1.5 mL of 150 mM HCl/ethanol (40:60, v/v) solution (Mizui and Doteuchi, 1983).

Animals were sacrificed under anaesthesia 60 minutes after and the stomachs were removed, opened along the greater curvature, rinsed with physiological saline solution and stretched on polystyrene boards.

The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system (Digimizer version 4.0.0.0). Percentage of the total lesion area (haemorrhagic sites) to the total surface area of the stomach except the forestomach was defined as the ulcer index.

III – 2 – 9 – 2 Curative effects of tannin extract against acute gastric lesions

Male Wistar rats weighing about 180-220 g were divided into three groups of at least 6 rats. Ulcers were induced in all rats by oral gavages of 1 mL of absolute ethanol each. Group I was the control received 1 mL water only, rats in group II were treated with 200 mg /kg of tannins extract of *Galium t* and rats in group III were treated with 30 mg / kg of omeprazole for each of 8 successive days (Süleyman et *al*., 2002). .

On 9th day, all animals were sacrificed under anaesthesia, the stomachs were dissected out and opened along the greater curvature. Stomachs were gently rinsed with water to remove the gastric contents and blood clots for subsequent ulcer scoring.

III – 2 – 9 – 3 Calculation of ulcer index and Percentage ulcer inhibition

Ulcer index has been calculated by adding the total number of ulcers per stomach and the total severity of ulcers per stomach (Devendra et *al*., 2010). A score for the ulcer was made as follows:

- 0: normal coloured stomach.
- 0.5: red coloration.
- 1: spot ulcers.
- 1.5: haemorrhagic streak.
- 2: ulcers.
- 3: perforation

Mean ulcer score for each animal was expressed as ulcer index**.** The percentage of ulcer inhibition was determined as follows:

$$Inhibition\ of\ Ulcer\ Index\ (\%) = \frac{[Control\ mean\ index - Test\ mean\ index]}{Control\ men\ index} \times 100$$

III – 2 – 9 – 4 Collection of Gastric Juice

The stomach was excised carefully by keeping the oesophagus closed and opened along the greater curvature and the luminal content was removed. The gastric contents were collected and centrifuged at 1000 rpm for 10 minutes; the volume of the supernatant was expressed as mL/100 g of body weight and the centrifuged samples were decanted and analysed for gastric volume, pH and total acidity (Jaikumar et *al*., 2010).

III – 2 – 9 – 5 Estimation of Total Acidity

It was measured by the Method of Hawk, (1965). Briefly, 1mL of supernatant liquid was pipette out and diluted to 10 mL with distilled water, pH of this solution was noted with the help of pH meter. The solution was titrated against by 0.01N sodium hydroxide using Phenolphthalein reagent as indicator. The end point was titrated when the solution turned to orange pink. The volume of NaOH was noted, which corresponds to total acidity. Acidity was expressed as formula (Vinothapooshan and Sundar, 2010).

$$Acidity = \frac{Volume\ of\ NaOH \times Normality \times 100\ \ (mEq/1)}{0,1}$$

III – 2 – 9 – 6 Estimation of pepsin activity

Aliquots of 20 µL of the gastric contents were incubated with 500 µL of albumin solution (5mg/mL, 0.06 N Hydrochloric acid) at 37 °C for 10 minutes. The reaction was stopped with 200 µL of 10% trichloroacetic acid and the samples were centrifuged at 1500 rpm for 20 minutes. The supernatant was alkalinized with 2.5 mL of 0.55 M sodium carbonate, 400 µL of 0.1 N Folin reagent was added to the tubes, which were then incubated for 30 minutes at room temperature. The absorbance of the sample was determined at 660 nm. A standard curve of tyrosine was used for the determination of the concentration of pepsin. Pepsin content of the gastric fluid was expressed as µg of tyrosine/mL (Eq.tyr) (Smeeta and Subhash, 2013).

III – 2 – 10 Healing wounds activity

III – 2 – 10 – 1 Preparation of ointment by fusion method

(a) Preparation of simple ointment

Wool fat 2 mg , Hard Paraffin 2 mg, Cetostearyl alcohol 2 mg , White Soft Paraffin 34 mg. Each ingredient was mixed and heated gently with stirring then cooled. The base was then packed in a wide mouth container.

(b) Preparation of 5 % ointment

Methanolic extract of *Galium t* was added (5% w/ w) slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container (Gerald et *al*., 1994; Clark, 1996).

III – 2 – 10 – 2 Excision wound model

In excision wound, three groups of animals containing six in each group (the group I was considered as control, the group II served as the reference standard treated with 0.25 % w/w *MYCOCIDE*® ointment and the group III animals were treated with the 5% w/w methanolic extract).

Hairs were removed from the dorsal thoracic central region of anaesthetised rats. One excision wound was inflicted by cutting away a 250 mm^2 full thickness about 5 mm of skin from a predetermined area; the wound was left undressed to the open environment. Then the ointments were applied as stated above.

The calculation of percent reduction in wound area as the flowing equation:

Wound contraction (%) = (Healed area/ total area)×100 (Biswas et *al*., 2004)

Wound healing property was evaluated by wound contraction percentage. The wound area was measured each three day during fifteen days, by placing a transparent paper over the wound and tracing it out (Werner et *al*., 1994), area of this impression was calculated using the Digimizer version 4.0.0.0.

III – 2 – 11 Statistical analysis

All tests were carry out in triplicate and the results were expressed as the mean ± standard deviation. Statistical differences were assessed using one-way ANOVA , Newman-Keuls Multiple Comparison, t test of Student and Fisher test to determine whether there were any significant differences ($P < 0.001$ or $P < 0.05$).

The choice of statistic treatment was based on results type. Data were subjected to analysis using the Microsoft Excel 2013, Graphpad prism 5 Demo and coStat version 6.400.

Results & Discussion

IV – Results & Discussion

IV – 1 Secondary metabolites yield

The extraction of secondary metabolites from *Galium t* offered practically important quantities (Table 3).

Table 3: Yield of secondary metabolites extracted from *Galium t*.

Plant Compounds or extracts	Weight of extracts (g)	Percentage (%)
Methanolic extract	2,2	22
Ethanolic extract	30.61	30.61
Aqueous extract	2.87	19.13
Essential oils from aerial part	0,6912	1,3824
Essential oils from flowers	0.6695	0.9557
Flavonoids extract (Ethyl acetate fraction)	1.1	4.4
Flavonoids extract (aqueous interphase)	4.3	17.2
Tannins extract by acetone	2.1	10.5
Tannins extract by ethanol 30%	0.6	3
Alkaloids extract	0.312	3,12
Saponins extract	0.021	0.21
Low polarity molecules	1.5	1.5

IV – 2 Secondary metabolites quantification

IV – 2 – 1 Polyphenols quantification

Galium t plant is a good source of phenolic compounds. In table 4, is summarized the quantities of polyphenols, flavonoids determined according to method of Bahorun et *al*. (1996), using quercetin as reference molecules and tannins. These compounds are known to exist in genus *Galium* but their chemical, biological and pharmacological properties have not yet been investigated.

In this investigation, acetone and 30% ethanol were used to derive tannin extract from *Galium t.* The haemoglobin precipitation assay reported that an acetone was believed to be a better solvent for tannin extraction in comparison to 30% ethanol solvent. It may be that acetone in the extraction solvent increases the total yield by inhibiting interactions between tannins and proteins during extraction (Hagerman, 2002), or even by breaking hydrogen bonds between tannin-protein complexes (Porter, 1989). Consequently, tannins extract according the method of Barrau et *al*. (2005) was completely removed from the experiments.

Table 4: Concentration of polyphenols, flavonoids and tannins extracted by different solvents from *Galium t.*

Plant Compounds or extracts	Concentration of Extracted Compounds (µg / mL)		
	Concentration of Polyphenols	Concentration of flavonoids (Quercetin equivalent)	Concentration of tannins
Extract	Mean±SD	Mean ±SD	Mean±SD
Flavonoids (ethyl acetate fraction)	111.56±2,82	49.30±0,16	-
Acetone extract	-	-	431,23± 23,83
Ethanol 30 % extract	-	-	82,35±5,47
Flavonoids aqueous interphase	69.46±2,16	18.04±1,77	-
Methanolic extract	58.94±0,99	18.04±1,77	116,04±32,30
Aqueous extract	65.26±0,99	13.18±0	62,90±5,38

According to Vlase et *al*. (2014), the amount of the total polyphenols reach in *Galium mollugo* 3.46 ± 0.13 g/100 g, *Galium verum* 2.6 ± 0.12 g/100 g, *Galium aparine* 2.4 ± 0.24 g/100 g and *Galium odoratum* 1.4 ± 0.35 g/100 g. Moreover, the content of flavonoids, reach in *Galium mollugo* 6.93 ± 0.44g/100 g, *Galium verum* 5.21 ± 0.24 g/100 g, *Galium odoratum* 2.81 ± 0.54g/100 g and *Galium aparine* 1.60 ± 0.53g/100 g. These data confirm the richness of *Galium t* in phenolic compounds.

IV – 2 – 2 Total chlorophyll (a + b) content

Both chlorophyll a and b contents were at least 10 fold higher in methanolic extract than in dry matter (Figure 17). Total quantity of chlorophylls a and b reach 24.62 % of methanolic extract, that's why, apolar organic solvents must be used to remove these chlorophylls.

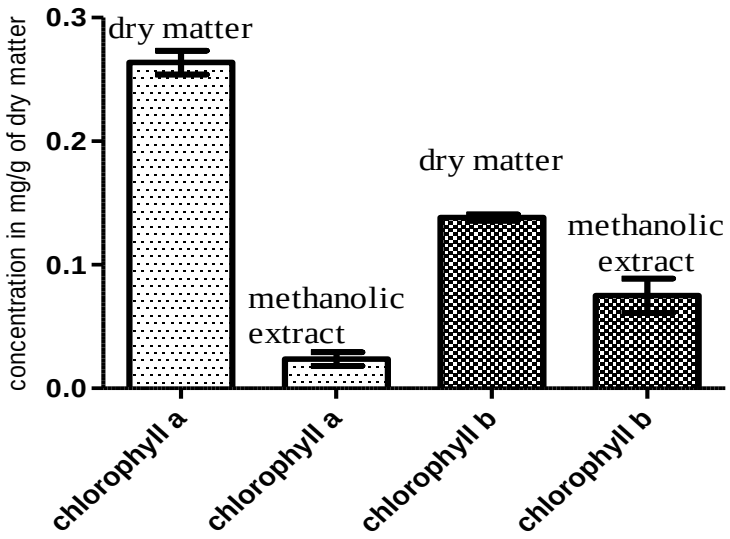


Figure 16: Total chlorophyll (a + b) content in methanolic extract and in dry matter of *Galium t.*

IV – 2 – 3 Alkaloids quantification

Farias (2006), reported that *Rubiaceae* family presents a large diversity of substances, with emphasis on production of bioactive alkaloids. The alkaloids in plant material naturally exhibit a variety of physics, biological as well as medicinal properties (Rahman et *al.*, 2011). The lowest yield by weight of any medicinally useful alkaloid ever produced on a commercial basis is 0.003 % which was extracted from the *Catharanthus roseus* (Gonzales and Tolentino, 2014). Hence, 0.714 % yield in *Galium t* is more enough that can be used as medicinal plants (Table 5).

Table 5: Concentration of alkaloids (mg EA/GDM)

Extract	Mean$\pm SD$
Aqueous extract	4,97±0,25
Methanolic extract	7,14±0,46
Alkaloids extract	10,26±0,71

IV – 2 – 4 Carotenoids quantification

The results showed a mean total carotenoids content of 38,65 ±6,60 mg β-carotene Eq/GDM in methanolic extract, assessed very important in comparison with seeds of *Galium aparine* which content 0.5 mg /GDM (Butnariu and Samfira, 2013) and 132.2 mg/ GDM of total carotenoids content in *Cucurbita maxima* (Shi et *al.*, 2013).

IV – 2 – 5 Chemical composition of the essential oils

The hydrodistillation of dried *Galium t* gave yellowish essential oils. The identified compounds were shown in table 6.

The GC/MS analysis of the essential oil of *Galium t* led to the identification of 83 different components (Figure 6 in appendix). The major components were found to be:

- Fatty acid 32,32 % (with the predominance of hexadecanoic acid 18,53%, Tetradecanoic acid 4,65% and Dodecanoic acid 2,51%),
- Alkane 17,06 %
- Terpene compounds 10,55%, with monoterpene 2,31 %, diterpene 5,49%, sesquiterpene 0,33 % and other terpenene 2,42% (the major molecules were : Phytolisomere 5,28 %, Carvacrol 0,67% and beta caryophyllene 0,33%),
- Aromas 8,33 % (the predominance of Styrene 2,14 %, Ethyl benzene 1,68%, acetophenone 1,09%, benzene acetaldehyde 1,06% and farnesyl acetone 0,93 %),
- Carboxylic acids 6.86 % (the predominance of linoleic acid 6,32%),
- Phenolic compounds 6,43 % (the predominance of Butylated hydroxytoluene 5,68% and 4-vinyl-2-methoxy-phenol 0,52%),
- Esters 6,03% (the predominance of Ethyl linoleate 3,50% and methyl linoleate 2,31%)

- Ketones 5.82 % (the predominance of 2-Pentadécanone-6,10,14-triméthyl 3,01% and Dihydroédulan I 1,28%),
- Aldehydes 3.79 % (the predominance of Hexanal 0,96% and 2-Hexenal trans 0,89%),
- Hydrocarbons 1,12 %,
- Benzopyran 0,63 %,
- Ether 0,39%,
- Benzene 0,28%,
- Alcohol 0,25%
- Phenyl propanoids 0.16 %.

Table 6: Chemical composition of essential oil of *Galium t.*

Tr	**Molecules**	**% FID**
2.29	Toluene	0.092
2.57	1,3,5-Cycloheptatriene	0.412
3.10	Sec-Butyl ether	0.160
3.16	Hexanal	0.398
3.93	2,4-Dimethyl-1-heptene	0.086
4.24	2-Hexenal trans	0.369
4.36	Ethylbenzene	0.694
4.64	1,3,5,7-Cyclooctatetraene	0.093
5.00	Styrene	0.885
5.64	Cumene	0.074
6.23	Isocumene	0.116
6.42	Benzaldehyds	0.092
6.95	Furan, 2-pentyl	0.150
7.08	Hexanoic acid + heptadienal (unidentified isomer)	0.065
7.36	2,4-Heptadienal	0.142
7.66	Limonene	0.099

7.94	Benzene acetaldehyde	0.437
8.10	branched hydrocarbon	0.056
8.19	Gamma-Terpinene	0.103
8.31	Acetophenone	0.449
8.41	Benzofuran	0.312
8.62	Terpinolene	0.130
8.88	Linalool	0.334
8.96	Nonanal	0.252
9.08	phenyl ethyl alcohol	0.102
9.72	Nonadienal (unidentified isomer)	0.055
9.77	Phenylacetaldehyde	0.144
9.83	Nonene-1-al	0.073
10.03	Benzofuran, 2-methyl	0.262
10.20	Octanoic Acid	0.203
10.32	Methyl salicylate	0.037
10.38	Alpha terpineol	0.092
10.44	Safranal	0.043
10.52	Decanal	0.100
10.67	4-vinylphenol	0.098
10.73	Beta-Cyclocitral	0.099
11.60	Nonanoic acid	0.804
11.73	Dihydroedulan I	0.527
11.80	Dihydroedulan II	0.155
11.84	Carvacrol	0.278

11.99	4-vinyl-2-methoxy-phenol	0.213
12.55	Eugenol	0.065
12.66	Gamma-Nonalactone	0.052
12.83	Decanoic acid	0.573
13.16	Diphenyl ether	0.026
13.23	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)butan-2-one	0.147
13.32	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)butan-2-one isomer	0.249
13.47	Beta caryophyllene	0.138
13.77	Géranyl acetone	0.250
13.90	2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	0.145
13.96	Undecanoic acid	0.158
14.18	Beta-E-Ionone	0.298
14.41	n C15H32	0.044
14.48	BHT	2.344
15.24	Dodecanoic acid	1.036
15.27	Gamma-Undecalactone	0.101
15.45	Oxyde de caryophyllène	0.211
15.58	n C16H34	0.084
15.73	Tetradecanal	0.061
16.69	n C17H36	0.100
17.43	Tetradecanoic acid	1.921
17.75	n C18H38	0.067
18.18	2-Pentadecanone-6,10,14-trimethyl	1.243
18.76	n C19H40	0.072

18.86	Farnesyl acetone	0.386
19.20	Linoleta Methyl	0.952
19.25	Isophytol	0.282
19.59	Hexadecanoic Acid	7.650
19.68	Ethyl hexadecanoate	0.056
19.73	nC20H42	0.087
20.65	n C21H44	0.292
20.75	Phytol isomer	2.180
21.05	Linoleic acid	2.608
21.08	Ethyl linoleate	1.444
21.27	octadecanoic acid	1.158
21.54	nC22H46	0.235
22.40	n C23H48	0.487
23.22	nC24H50	0.603
24.06	nC25H52	1.010
24.80	nC26H54	0.611
25.53	n C27H56	0.879
26.25	nC28H58	0.674
26.95	nC29H60	1.028

n: unkawn molecules

Ilina et *al*. (2011), report that air-dried *Galium hercynicum* herb content 0.01% of essential oils with a total of 47 compounds and 0.01% for *Galium humifusum* with a total of 75 compounds. Furthermore, Ilina et *al*. (2009) found a total amount of volatile compounds in *Galium verum* flowers equal to 2.74% with 21 compounds. These data reveal the importance of *Galium t* essential oils.

IV – 3 Antioxidant activity

IV – 3 – 1 DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) assay

IV – 3 – 1 – 1 Methanolic and aqueous extracts

The scavenging effects of methanolic and aqueous extracts increased with their concentrations to similar extents (Figure 18). IC_{50} values of BHT, methanolic and aqueous extracts were 34,01±1,10 µg/mL, 54,45±1,93 µg/mL and 50,18±0,82 µg/mL respectively. Their comparison using ANOVA One-way analysis of variance and Newman-Keuls Multiple Comparison test with $P < 0,05$ demonstrate that there is no significant difference, which mean that these extracts have a good free radical scavenging abilities and can be used as a radicals inhibitors or scavengers.

Lower IC_{50} value indicates stronger ability of the extract to act as DPPH scavenger while the higher IC_{50} value indicates the lower scavenging activity of the scavengers as more scavengers were required to achieve 50 % scavenging reaction.

The DPPH radical scavenging capacities of methanolic and aqueous extracts have good free radical scavenging abilities and can be used as a radical's inhibitors or scavengers, acting possibly as primary antioxidants. Many studies indicate a linear relationship between total phenolics and antioxidant activity (Kim et *al.*, 2003a; Kim et *al*., 2003b; Djeridane et *al.*, 2006). Methods used for the assessment of phenolic compounds and antioxidant capacity are based on redox properties, that is why there should be found some correlation between content of total phenolic compounds and antioxidant capacity as measured by the DPPH• radical scavenging methods, but this mechanism depends on the other factors like the kind of component or pH of the reaction. Methanolic and aqueous extract of *Galium t* contain high concentration of phenolic compounds specially the flavonoids and tannins, and this may explain these results.

Neda et *al*. (2010), assessed that *Galium verum* collected from two different mountains was able to reduce the stable radical DPPH• to the yellow coloured DPPH-H, reaching 50% reduction with an IC_{50} of 3,10 µg/mL and 8,04 µg/mL. However, Kahkeshani et *al*. (2013) indicate that *Galium odoratum* methanolic extract displayed antioxidant activity with IC_{50} values of 148 µg/mL. Thus, *Galium t* occupying a moderate position between the other *Galium genera.*

IV – 3 – 1 – 2 Flavonoids extract

The scavenging effect of flavonoids extracted from *Galium t* on the DPPH radical was related to the amounts of substance added. The scavenging effects of three phases on the DPPH radical increased in the order acetate phase < total flavonoids extract < aqueous interphase, with IC_{50}: 22, 17±2,49 µg/mL , 39,10±5,04 µg/mL and 53,44±7,70 µg/mL respectively. Scavenging effects of flavonoids extract of *Galium t* on (DPPH) radical are showed in Figure 18. Statistical comparison using ANOVA One-way analysis of variance and Newman-Keuls Multiple Comparison test with $P < 0,05$ indicate that there is a significant difference between both ethyl acetate phase, aqueous interphase and the BHT, but there is no difference between a total flavonoids extract and BHT.

The ethyl acetate phase exhibit a low value of IC_{50}, lower than BHT which makes this phase better than standard, hence, this phase present a significant difference due to the high continence of flavonoids. Nevertheless, the aqueous interphase marks a high value of IC_{50} because of the low concentration of flavonoids, which indicate a lower scavenging activity. Total flavonoids, which are the mixture of the two phases, exhibit a feeble antagonism with a beneficial effect by a similar value of IC_{50} with the BHT. The role of antioxidants in the inhibition of antioxidant processes occurring in living organisms consists of: scavenging free radicals and quenching singlet oxygen, disconnection of radical reactions, chelate metals which catalyse the oxidation process, inhibition of certain enzymes (eg., oxidases). Flavonoids are active in all these processes (Nijveldt et *al*., 2001). The effectiveness of flavonoids in DPPH radical scavenging depends largely on their structure, hydrophobicity, biological and oxidative activity.

The ability and disconnection of radical chain reactions by flavonoids is mainly dependent on the presence in B ring of at least two *o*-hydroxyl groups. It enables the formation of intramolecular hydrogen bond between hydroxyl groups, which increases the stability of the phenoxyl radicals (Majewska et *al*., 2011).

Zhang et *al*. (2012), isolate from *Artemisia sphaerocephala* five flavonoids and investigate their DPPH radical scavenging, found a strong inhibition with IC_{50} values of 9.61±0.08, 8.62±0.14, 16.12 ± 0.34, 16.45±0.12, and 21.93 ± 0.65 µM, which make the flavonoids extract of *Galium t* better than isolated flavonoids molecules.

IV – 3 – 1 – 3 Tannins extract

Tannins extracted from *Galium t* using the method of Mohamad Ibrahim. et *al.* (2005), was the best method to investigate the antioxidant activity. The scavenging effects of tannin from *Galium t* on the DPPH radical show a low effect with IC_{50} estimated 132,64±7,36 µg/mL (Figure 18). Hagerman et *al*. (1998), provided insights into the mechanism of procyanidin as the potential antioxidants, which showed that hydroxyl groups were important factors for free radical scavenging by tannins. Nevertheless, tannins extract was very weak against BHT.

As known, acetone is used for chlorophylls extraction and these pigments absorbed maximally at 401 - 455 nm or 630 - 696 nm, which interfere with DPPH radical; both substance absorb at 515 nm and caused a lower radical scavenging activity.

Benmehdia et *al*. (2013), reported that IC_{50} value of tannins extracted from *Aristolochia clematitis* L. roots was 196 µg/mL. Whereas, Zhang and Lin (2008) assessed that IC_{50} value of tannins extracted from *Canarium album* stem bark was 54.80±0.50 µg/mL. Thus, it can be concluded that tannins extract of *Galium t* was moderately weak antioxidant substance.

IV – 3 – 1 – 4 Saponins

In DPPH assay, the saponins are more effective to scavenge hydroxyl radicals. The saponins of *Galium t* have a significant scavenger effect comparing with other group of molecules. Its scavenging effect increases with concentration, as illustrated in figure 18.

The scavenger effect of saponins is relatively low with IC_{50} (84.93 ± 4.92 µg/mL) than BHT (34,01±1,10 µg/mL) and the statistical treatment by "student" and "Fisher test" for the with $P < 0.05$ shows that there is no significant difference, this means that saponins are able to scavenge DPPH radical.

In general, small molecules that have a better access to radical site have a higher apparent antioxidant activity in the DPPH assay than big molecule, and the saponins may act by their sugar part. This signify that saponins in *Galium t* have strong and effective antioxidant activity based on DPPH radical scavenging.

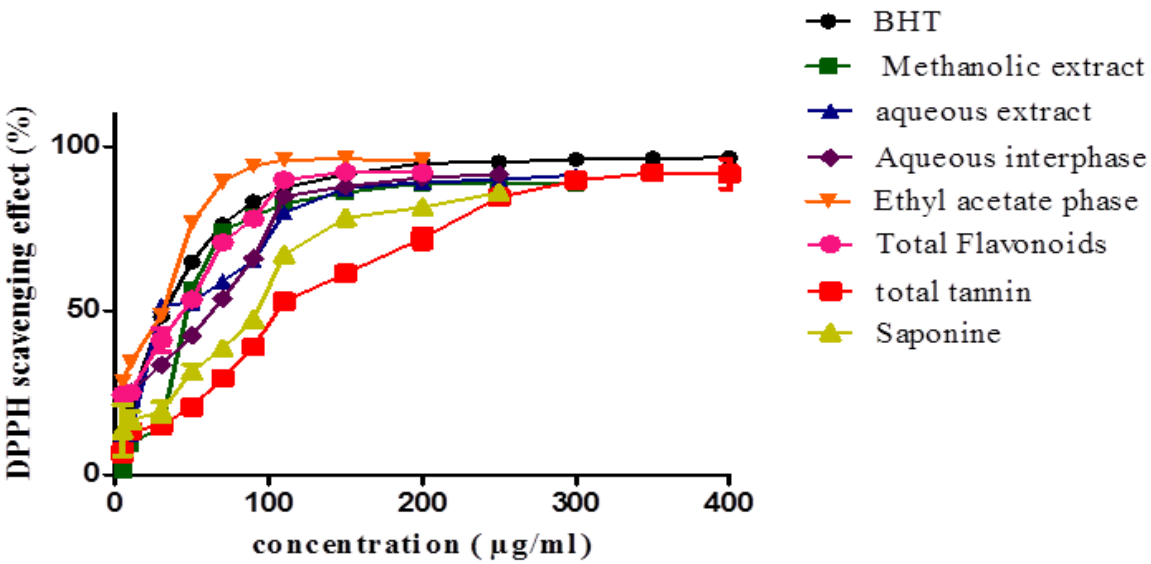


Figure 17: DPPH scavenging activity of *Galium t* extracts

IV – 3 – 1 – 5 Essential oil

In the DPPH assay, the radical scavenging ability of the essential oil and the BHT was measured spectrophotometrically (Figure 19).

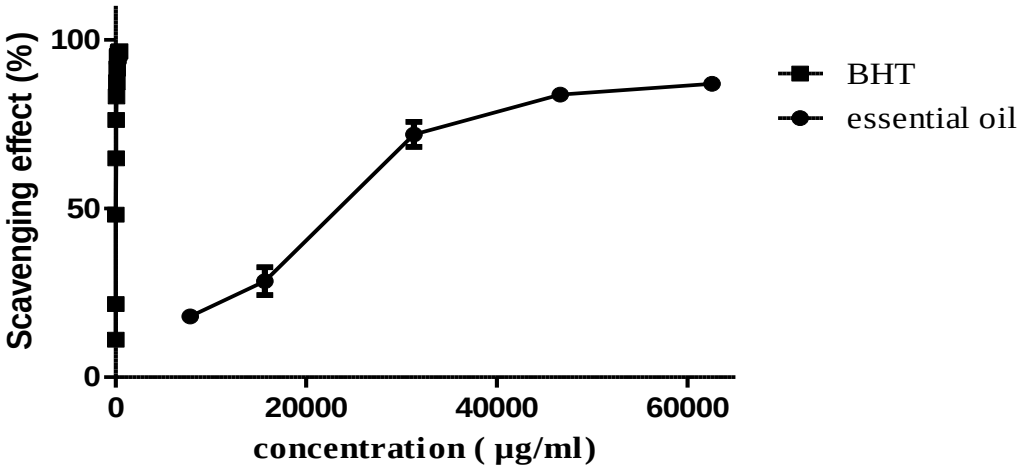


Figure 18: Scavenging effects of essential oil of *Galium t* on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical.

In general, the essential oil was able to reduce the stable radical DPPH to the yellow colour of DPPH-H with an IC_{50} value of 23,17±2,64 mg/mL, showed very weak activity in comparison with BHT which exhibit high antioxidant activity with IC_{50} values of 3.4×10^{-2} mg/mL.

This result is explained by chemical composition of essential oil, which contain phenolic compounds, but the presence of other molecules may antagonize the processes and reduce their antioxidant capacities. This makes the essential oil of *Galium t* less active against free radicals.

IV – 3 – 2 β-Carotene linoleic acid bleaching assay

IV – 3 – 2 – 1 Methanolic and aqueous extracts

Lipid peroxidation inhibitory activity of methanolic and aqueous extracts expressed as percent inhibition relative to the control were 80,83±10,74 % and 93,95±4,53 % respectively, lower than standards which was 107,48±16,14% (Figure 20).

Statistic treatment of result indicate that there is no difference between extracts and BHT.

The presence of an antioxidant can hinder the extent of β-carotene destruction by "neutralizing" the linoleate free radical and any other free radicals formed within the system (Kamath and Rajini, 2007). Antioxidant capacity was classified as high (>70%), intermediate (40–70%) or low (<40%) levels of oxidation inhibition (Hassimotto et *al.*, 2005).

The greatest inhibition potential of lipid oxidation found in the aqueous extract occurred probably due to the greater removal of antioxidants with lipophilic compounds, provided by the high concentration of carotenoids; while the β-Carotene bleaching method is suitable for lipophilic compounds.

Bousselsela et *al*. (2012), evaluate the inhibition potential of lipid oxidation of *Hertia cheirifolia* aqueous extract of 67.56%, largely lower than aqueous extracts of *Galium t*. Even, Bouaziz et *al*. (2015), were found the inhibition potential of lipid oxidation of *Myrtus communis* L in chloroform extract (91.19%±0.51%) followed by ethyl acetate extract (90.73%±1.90%), aqueous extract (89.54%±1.20%) and methanol extract (79.13%±1.20%). These results are lower than inhibition potential of lipid oxidation found in the aqueous extract of *Galium t*.

IV – 3 – 2 – 2 Flavonoids extract

As shown in figure 20, the absorbance of the standard at 470 nm attain a value of 1,107±0,039 after 120 minutes, while the extracts decreased to a range of 0,396±0,055 for the ethyl acetate phase, 0 for the aqueous interphase and 0,512±0,043 for total flavonoids. These results indicate that all the phases can weakly or cannot inhibit oxidation of linoleic acid.

The calculated percentage of inhibition capacities of the extracts is given in table 7 in which is shown a low aptitudes to inhibit oxidation of linoleic acid for all flavonoids phases in comparison with BHT.

Table 7: Inhibition of linoleic acid oxidation of flavonoids extracts of *Galium t* by the β-carotene bleaching method (%)

Standards/ extracts	(%) $\pm SD$
BHT	107.48 ±16.14
Ethyl acetate phase	32,58±7,97***
Aqueous interphase	0***
Total flavonoids	46,40 ±5,79***

*$P < 0.001$, *** a highly significant difference.*

All phase of flavonoids extracts of *Galium t* cannot accomplish the reaction as BHT with a high concentration of substance, expressed as lower percentage of inhibition capacities, and the mixture of the two phases present a little synergic effect,

In spite that polar compounds like ascorbic acid are well known antioxidants, the β-carotene bleaching test did not show antioxidative properties. This interesting phenomenon has been described as the polar paradox (Kulisica et *al.*, 2004). Polar antioxidants remaining in the aqueous phase of the emulsion are more diluted in the lipid phase and are thus less effective in protecting linoleic acid β-carotene undergoes rapid depolarization in the absence of an antioxidant (Siramon and Ohtani, 2007). This may explain the weakness of flavonoids extracts of *Galium t*. These data was confirmed by Formagio et *al*. (2014), which gave 4.13 ± 1.42 % for ascorbic acid as percentage of inhibition capacities.

IV – 3 – 2 – 3 Tannins extract

The decrease in absorbance of β-carotene in the presence of tannins with the coupled oxidation of β-carotene and linoleic acid is show in figure 20. The tannin extract reach a percentage of 61, 17±4, 50 %** of protection of β-carotene from bleaching after 120 minutes.

The acetone extract seems approve the moderate to strong power to inhibit linoleic acid oxidation, which indicates the power of tannins from *Galium t* to trap free radical but not like BHT.

IV – 3 – 2 – 4 Saponins

Figure 20 depicts the inhibition of β-carotene bleaching by saponins of *Galium t* As can be seen, at *2.0 mg.mL*$^{-1}$ concentration, antioxidant activity of the saponins was measured as 70.99 ±7.91 %, lower than BHT which was 107.48±16.14 %, but strong according to Hassimotto et *al*. (2005). The saponins extracted from *Galium t*, can protect the β-carotene molecules from the attack of the free linoleic acid radical formed upon the abstraction of a hydrogen atom from one of its methylene groups, which lost the double bonds and therefore takes its characteristic orange color.

IV – 3 – 2 – 5 Essential oils

Antioxidant activity of the oil was measured as 46,17±0,66 %, in this test system, oxidation of linoleic acid by the BHT was 107,48±16,14 % , indicates pronounced weakness with a moderate power. Essential oil of *Galium t* can be primary antioxidant, but the presence of linoleic acid in the composition of the essential oils may affect their capacities (Figure 20).

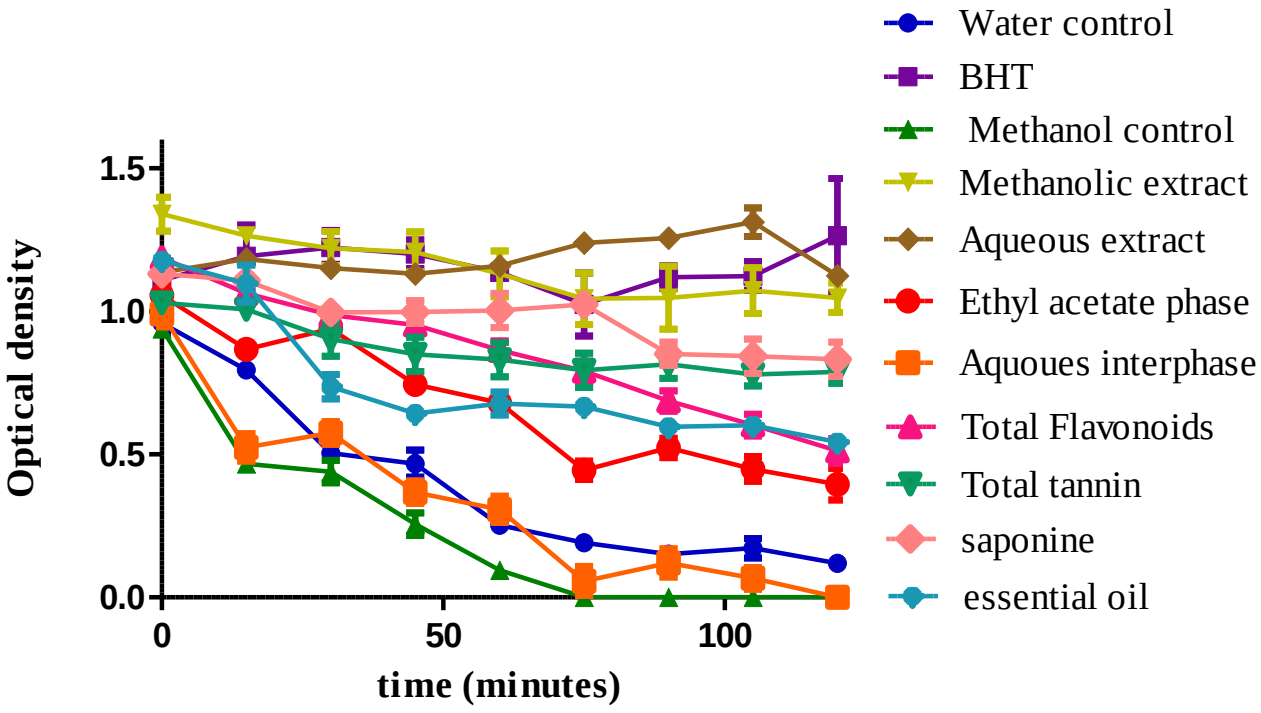


Figure 19: β-Carotene bleaching test of *Galium t* extracts

IV – 3 – 3 Reducing power assay

IV – 3 – 3 – 1 methanolic and aqueous extracts

The reducing power of methanolic and aqueous extracts of *Galium t* expressed as the slope (obtained by linear regression analysis) of the curves (Figure 21); a higher slope corresponds to a stronger reducing power of the extract. The EC_{50} of the extracts is very important (Table 8) but lower capacity than ascorbic acid and BHT.

Most non enzymatic antioxidant activity, such as the scavenging of free radicals and the inhibition of peroxidation, is mediated by redox reactions (Zhu et *al*., 2002). The reducing capacity of the extract is another significant indicator of antioxidant activity. (Olayinka et *al*., 2010).

The reductive potential measure the ability of sample to act as electron donor and, therefore, reacts with free radicals converting them to more stable products and thereby terminates radical chain reactions (Gürsoy et *al*., 2012).

In this test the Fe^{2+} has been known to accelerate formation of hydroxyl radicals via the Fenton reaction, leading to occurrence of many diseases (Halliwell, 1996; Juntachote and Berghofer, 2005). It is reported that chelating agents that form σ-bonds with a metal ion, are effective secondary antioxidants since they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Kumar and Ganesan, 2008)

The presence of antioxidants in the samples results in the reduction of the ferric cyanide complex to the ferrous form, which can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. The increased absorbance at 700 nm indicates an increase in reducing power of samples (Lih *et* al,. 2001).

Higher slope corresponds to a stronger reducing power of an extract, observed in aqueous extract then methanolic extract, and the EC_{50} of the extracts are very important , but lower capacity than ascorbic acid and BHT.

According to Ilias et *al*. (2011), the EC_{50} *Olea europaea* L olive methanolic extract was 30,54 ± 0.029 µg/mL, lower than *Galium t* methanolic extracts. However, the needle methanolic extract of *Juniperus oxycedrus subsp. oxycedrus* reach 290 ± 18.3 µg/mL (Chaouche et *al*., 2013) and the standard reach 53.67 ± 2.4 µg/mL in the same source. Thus, methanolic extract display a moderate capacities to chelating Iron ions.

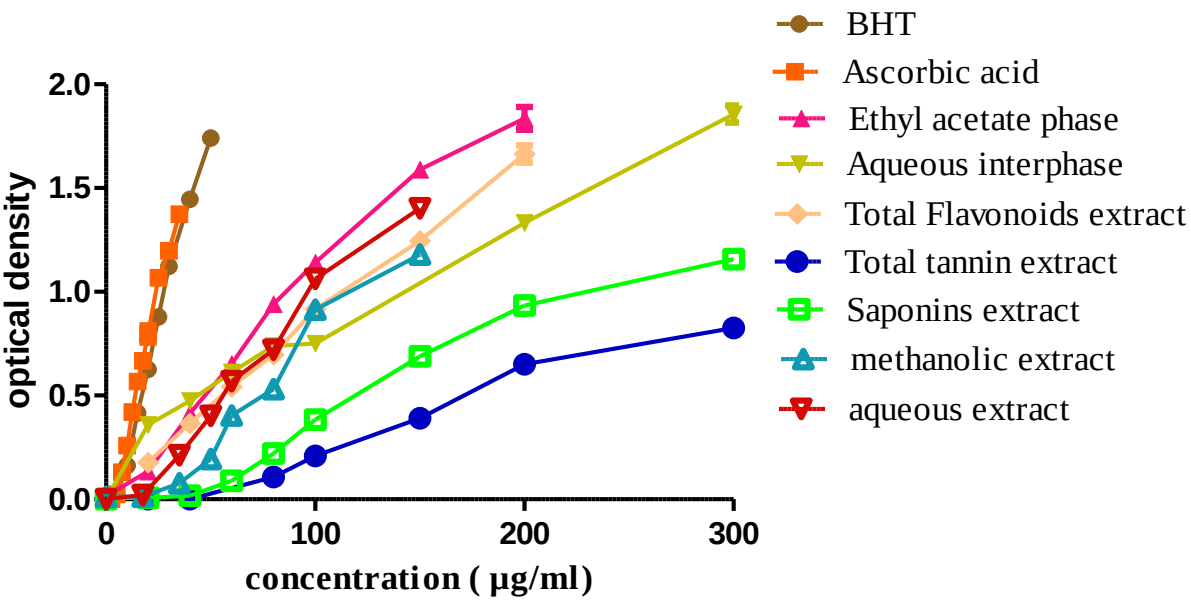


Figure 20: Reducing power assay of *Galium t* extracts.

IV – 3 – 3 – 2 flavonoids extract

Ethyl acetate phase and aqueous interphase exhibited the highest reducing power followed by the total flavonoids extract but not satisfactory in comparison with BHT and ascorbic acid (Figure 21). EC $_{50}$ values of the flavonoids phases (Table 8) indicate the important capacities of flavonoids to reduce ferric cyanide complex to the ferrous.

The extracts that showed comparable absorbance readings with ascorbic acid and BHT are considered to have high reducing power. However, the flavonoids extracts of *Galium t* show low capacities to reducing Fe^{3+} to Fe^{2+} in comparison with BHT and ascorbic acid, but an acceptable value of EC_{50}. It was confirmed that the hydroxyl groups at C-3' and C-4' of the B-ring to be more active in reducing iron concentration (Moran et *al* ., 1997).

For Belyagoubi-Benhammou et *al*. (2014), EC_{50} of ascorbic acid was evaluated of 0.06 ±0.002 mg/mL, weaker than ethyl acetate extract. Further, reducing power of *Salvia mirzayanii* ethyl acetate fraction was 169.04± 7.8 µg/mL (Moein et *al*., 2008). Thus, express the strength of the flavonoids extract of *Galium t*.

IV – 3 – 3 – 3 Tannins extract

Tannin extract of *Galium t* show a low capacity to reduce the ferric cyanide complex (Figure 21 and Table 8). The increasing optical density value indicates increasing trend of reducing power. Tannin seems to act as an antioxidant by complexing iron, forming complexes with Fe $^{+3}$ (Fe^{+2} n−TA) (Lopes et *al*., 1999). Tannins have the ability to chelate metal ions such as Fe(II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation (Karamac´ et *al* ., 2006).

Glahn et *al.* (2002), observed that Gallic acid, similar to tannic acid, can bind Fe. They concluded that inhibition of Fe absorption by phenolic compounds is likely to be due to the binding of Fe, thus making the iron unavailable for absorption. Furthermore, Osman et *al.* (2014), report that tannins extracted from *Acacia nilotica, subspecies nilotica (Ann).* Make an IC_{50} of 129 ± 1.7 µg/mL, *Acacia nilotica subspecies tomentosa (Ant)* with an IC_{50} of *56.5 ± 0.96 µg/mL* and *Acacia nilotica subspecies adansonii, (Ana,)* with IC_{50} of 113.2 ± 6.9 µg/mL.

Hence, tannin from *Galium t* can be a good trapper of free radicals and complexing iron ion by chelating Fe^{+2}. Tannin would protect DNA from oxidative damage. It is tempting to propose the use of tannin for prevention and/or experimental therapy of disorders related to iron-mediated oxidative stress, such as colorectal cancer (Nelson, 1992; Weinberg, 1994).

IV – 3 – 3 – 4 Saponins

Strong reducing agents formed Perl's Prussian blue color and absorbed at 700 nm. Figure 21, showed the reducing activities of saponins extract of *Galium t* in comparison with ascorbic acid and BHT. The higher absorbance of the reaction mixture, the higher would be the reducing power. The EC_{50} values of saponins are demonstrated in table 8, indicate a low ability of saponins to reduce the complex of ferric ferrous cyanide.

Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are important because of their pharmacological actions. Saponins exhibit diverse biological functions (Xuy et *al.*, 2001). These bioactive agents contain many ring structures with reducing capability responsible for the reducing power observed in the present investigation (Hemalatha and Rajeswari, 2013). Nevertheless, saponins extract of *Galium t* was weak may interfere with others groups of molecules.

IV – 3 – 3 – 5 Essential oils

The reducing power of the essential oil of *Galium t* increases by increasing the concentration of the essential oil with low power electron donation of reactive free radicals (Figure 22).

The reducing power of essential oil of *Galium t* is 12.82 ± 1.00 mg / mL to provide an optical density of 0.5, which remained significantly lower than BHT and ascorbic acid to give the same color intensity (Table 8). This trend can be attributed to the eventual effect of essential oil of *Galium t* that contains lower amounts of reductant that may react with radicals to stabilize and make end to radical chain reactions but no so pronounced.

As shown in the three different assays, the *Galium t* essential oil exhibited a low antiradical, attributed to the presence alkane and fatty acid which constitute 49, 39 % but phenolic compounds with 6.43 % of its composition. This is sufficient to reduce free radicals and make the essential oil of *Galium t* a moderate to weak antioxidant product.

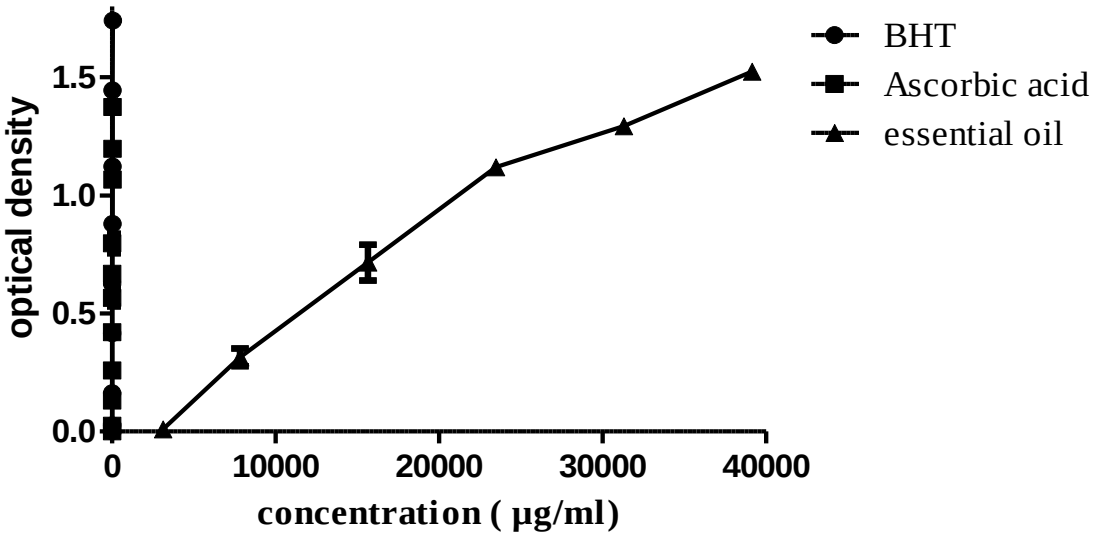


Figure 21: Reducing power of essential oil of *Galium t.*

Table 8: EC_{50} value for reducing power of standard and tested extracts

Plant extracts and standard compounds	Ec_{50} (µg/mL)$\pm \boldsymbol{Sd}$
BHT	16,06±0,18
Ascorbic acid	14,47±0,28
Methanolic extract	94,10±0,74***
Aqueous extract	74,43±1,38***
Ethyle acetate phase	46,24±0,42***
Aqueous interphase	53,12±1,02***
Total flavonoids extract	56,97±0,67***
Total tannins extract	176,29±1,11***
Saponins extract	118,89±0,67***
Essential oils	12823,33±1001,54***

*$P < 0.001$, *** a highly significant difference.*

IV – 4 Antimicrobial activity

IV – 4 – 1 Antibacterial activity

Results obtained in the present study relieved that the tested metabolite group possess a low potential microbial activity against *Escherichia coli, Salmonella typhimurium, Shigella flexheri, Klebsiella pneumoniae, Proteus mirabilis and Staphylococcus aureus.* Gentamicin and DMSO were used as standard and control respectively; the results were summarized in table 9.

IV – 4 – 2 Antifungal activity

Results of the antifungal activity relieved that the tested compounds possess a low antifungal activity against *Aspergillus niger* 2CA936, *Aspergillus flavius* NRRL3357 and *Candida albicans* ATCC1024. Nystatin, Clotrimazon and Amphotericin were used as standards and DMSO was used as control, the results were resumed in table 10.

Plants have developed natural defense mechanisms to protect themselves long before the man played an active role in protecting them. It is known that plants synthesize a variety of groups

of bioactive compounds in plant tissues as secondary metabolites (Santos et *al*., 2009). The use of plants to heal diseases, including infectious one, has been extensively applied by people. Data from the literature reveal the great potential of plants for therapeutic treatment, in spite of the fact that they have not been completely investigated (Nascimento et *al*., 2000).

Considering the parameter suggested by Alves et *al*. (2000), all extracts are less active or inactive against strains used in this experiment and the minimal inhibitor concentration is not necessary to be determined. Secondary metabolites of *Galium t* are so far to be considered as antibiotics or used as antiseptic.

According to Jan et *al.* (2015), *Galium t ricornutum subsp. longipedanculatum* demonstrate a high antifungal activity with 24 mm and 55 mm against *Candida albicans* ATCC 2091 and *Aspergillus flavus* ATCC 32611 respectively. Also, *Galium odoratum* gave an inhibition zone of 16 ± 0.05 and 14 ± 0.05 mm against *Staphylococcus aureus* and *Escherichia coli* respectively, but *Galium mollugo* and *Galium aparine* were less or completely inactive (Vlase et *al*., 2014).

Table 9: Bacterial inhibition zones in millimetre for standard, control and extracts of *Galium t*

Tested compounds	*Escherichia coli* ATCC 25922	*Salmonella typhimurium* ATCC 13311	*klebsella pneumoniae* ATCC700603	*Shigella flexheri* NCCB1406	*Proteus mirabilis* ATCC35659	*Staphylococcus aureus* ATCC25923
standard	18,50±0,41	19,17±0,24	24,67±0,47	24,33±0,47	35,33±0,47	27,67±0,47
Control	NI	NI	NI	NI	NI	NI
methanolic extract (50µg)	*9,67±0,47*	NI	NI	NI	NI	NI
methanolic extract (100µg)	9,17±0,62	NI	NI	NI	NI	NI
methanolic extract (200µg)	NI	NI	NI	NI	NI	9,00±0,82
aqueous extract	NI	NI	NI	NI	NI	NI
flavonoids(ethyl acetate phase) 100µg	NI	NI	7,33±0,47	NI	NI	9
flavonoids(ethyl acetate phase) 200µg	NI	8,67±0,24	NI	NI	NI	9
flavonoids (aqueous interphase) 100µg	NI	NI	NI	NI	NI	7,33±0,47
flavonoids (aqueous interphase) 200µg	NI	NI	NI	NI	NI	7,33±0,47
Tannins extract (100µg)	NI	NI	9,67±0,47	NI	NI	NI
Tannins extract (200µg)	NI	NI	8,67±0,47	NI	NI	NI
alkaloids extract (100 µg)	NI	NI	NI	NI	NI	NI
alkaloids extract (200 µg)	NI	NI	NI	NI	*10,67±0,47*	NI
Saponins extract (100 µg)	NI	NI	NI	NI	NI	NI
Saponins extract (100 µg)	NI	NI	NI	NI	NI	*9,67±0,47*
Essential oils 25 %	9,17±0,62	9,67±0,47	9,00±0,00	9,33±0,47	9,17±0,62	8,83±0,85
Essential oils 50 %	10,83±0,62	NI	9,00±0,00	9,67±0,47	9,17±0,62	10,50±0,71
Essential oils 75 %	9,50±0,71	9,33±0,47	8,67±0,94	9,67±0,47	9,33±0,47	9,33±0,47

NI: No inhibition

Table 10: Fungal inhibition zones in millimetre for standard, control and extracts of *Galium t*

Tested Compounds	*Aspergillus niger* 2CA936	*Aspergillus flavius* NRRL3357	*Candida albicans* ATCC1024.
Nystatin	9,40±0,22	15,53±0,79	9,29±0,19
Clotrimazon	15,85±0,32	23,86±1,15	44,28±0,49
Amphotericin	17,55±0,14	16,20±1,19	15,58±0,12
control	NI	NI	NI
methanolic extract (100µg)	NI	NI	NI
methanolic extract (200µg)	7,78±0,68	NI	NI
aqueous extract	NI	NI	NI
flavonoids(ethyl acetate phase) 200µg	7,47±0,80	NI	NI
alkaloids extract (100 µg)	NI	NI	NI
alkaloids extract (200 µg)	9,20±0,95	NI	NI
Essential oils 25 %	NI	NI	11,25±0,32
Essential oils 50 %	NI	NI	11,36±0,59
Essential oils 75 %	NI	NI	NI

NI: No inhibition

IV – 5 *In vitro* Anti-inflammatory activity

IV – 5 – 1 Protein denaturation

Denaturation of proteins is a well-documented cause of inflammation. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation (Deattu et *al*., 2012). The inhibitory effects of different concentrations of Diclofenac and *Galium t* extracts on protein denaturation are shown in table 11.

Diclofenac sodium (100 - 800 µg/mL) demonstrate a significant inhibition of denaturation of egg albumin in concentration dependent manner; however methanolic extract, flavonoids

extract and tannins extract score a maximum of inhibition at 400-500 µg/mL, and becomes inefficacy on protein protection at high concentration observed a drop in percent inhibition of protein denaturation. This was further confirmed by comparing their IC_{50} values (Table 12).

Table 11: The percentage inhibition of protein denaturation (%).

Tested Compounds	Concentration (100 µg/mL)	Concentration (200 µg/mL)	Concentration (400 µg/mL)	Concentration (500 µg/mL)	Concentration (800 µg/mL)
Diclofenac sodium (inhibition %)	35,57± 12,26	42,43± 10,62	82,84± 2,74	87,50± 2,40	90,13± 3,71
Methanolic extract (inhibition %)	24,67± 12,65	26,95± 12,94	31,97± 14,88	24,15± 11,12	25,60± 8,70
Flavonoids extract (inhibition %)	0	14,99± 11,55	52,84± 9,38	36,72± 7,86	40,47± 12,61
Tannins extract (inhibition %)	5,31± 7,51	16,56± 13,58	24,94± 8,32	55,87± 8,90	39,11± 9,45

Table 12: IC_{50} from the percentage inhibition of protein denaturation.

Tested Compounds	*IC_{50} (µg/mL)*
Diclofenac sodium	*201,04 ±53,77*
Methanolic extract	*4826,72±281,71****
Flavonoids extract	*360,62 ±32,18****
Tannins extract	*486,59 ±90,00****

***: *a highly significant difference with P < 0,001*

This mean that the secondary metabolites of *Galium t* haven't a capacity to protect proteins from denaturation induced by heating like Diclofenac, but a maximum inhibition of 52,84± 9,38 % for flavonoids extract and 55,87± 8,90% for tannin extract is very important to notice.

Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo* (Opie, 1962; Umapathy et *al.*, 2010). Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Madan et *al.*, 2011). Agents that can prevent protein denaturation

therefore, would be worthwhile for anti-arthritis drug development (Chandra et *al.*, 2012). Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory drugs) etc., have shown dose dependent ability to thermally induced protein denaturation (Mizushima and Kobayashi, 1968).

Ability of *Galium t* extracts to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activities. The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by the tested extracts and standard drug but the statistic treatment show that extracts are less efficient than Diclofenac sodium and less toxic, however their inhibition percentage are so important at small precise dose which make this molecules more suggested for inflammatory and arthritic diseases.

According to Chandra et *al*. (2012), *Coffea arabica* Linn. (Family: Rubiaceae) possessed IC_{50} value of 40 µg/mL and diclofenac sodium was found to be 625 µg/mL. Even, Sowjanya Kumar Reddy et *al*. (2013) found IC_{50} value of diclofenac sodium 485 µg/mL and that of methanol extract of *Lepidium sativum* Linn, seeds of 770 µg/mL. Thus, flavonoids and tannins extracts were largely lower and consequently better.

IV – 5 – 2 Effect of extracts on hypotonicity induced haemolysis of HRBCs

Data from table 13 show that methanolic extract and flavonoids extract of *Galium t* significantly ($p \leq 0.001$) inhibited lysis induced by water. This is confirmed by the high percentage inhibition of haemolysis obtained for doses of 500, 1000, and 2000 µg/mL. However, tannin behave another orientation, they became harmful from a concentration of 1000 µg/mL by inducing haemolysis.

Statistical comparison of results between the IC_{50} of standard drug and the extracts are shown in table 14.

Table 13: The percentage inhibition of hypotonicity induced haemolysis of HRBCs (%).

Concentration (µg/mL)	50	100	250	500	1000	2000
Methanolic extract (%)	36,82±6,76	78,65±3,87	95,66±0,90	97,99±0,07	99,34±0,25	95,70±0,46
Flavonoids (%)	51,24±2,89	58,98±2,94	53,19±3,66	64,42±5,12	97,21±0,51	98,04±0,18
Tannins (%)	99,74±0,27	98,47±0,22	80,60±4,43	73,62±1,18	73,25±0,52	17,31±3,44
Diclofenac sodium (%)	68,13±1,40	78,14±2,50	90,10±0,60	93,92±0,95	97,74±1,29	98,68±0,77

Table 14: IC_{50} from the percentage inhibition of hypotonicity induced haemolysis of HRBCs (%)

Tested compounds	Ic_{50} (µg/mL)
Diclofenac sodium	13,06±2,45
Methanolic extract	64,88± 5,30***
Flavonoids extract	72,33±6,18***
Tannins extract	33,67± 0,08***

***: *a highly significant difference with P < 0,001*

IV – 5 – 3 Heat induced haemolysis

Besides, in heat-induced conditions, the samples were found to inhibit lysis of erythrocyte membrane (Table 15). The percentage protection was recorded as 50,79±7,65 % for methanolic extract , 54,45 ±11,18 % for the flavonoids extract at the concentrations of 1000 µg/mL . However, the maximum of protection was registered in case of tannin extract at 200 µg /mL by 92,76 ± 2,73 %, and have the same orientation in protein denaturation test and hypotonicity induced haemolysis of HRBCs method.

Table 15: The percentage inhibition of heat induced haemolysis of HRBCs (%)

Concentration (µg/mL)	50	100	150	200	400	1000
Aspirin (%)	66,21 ±1,75	74,82 ±4,13	49,92 ±4,75	48,83 ±2,72	-	-
Methanolic extract (%)	-	-	-	2,85 ±4,03***	4,03 ± 5,69***	50,79 ±7,65***
Flavonoids (%)	-	-	-	0	0	54,45 ±11,18***
Tannins (%)	-	-	-	92,76 ±2,73**	5,32 ± 0,79***	3,52 ±3,14***

***: *a highly significant difference with P < 0,001*

It is relevant from the present study that secondary metabolites of *Galium t* protected the human erythrocyte membrane against lysis induced by hypotonic solution and heat. The lysosomal enzymes released during inflammation produce a variety of 122 disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC membrane are similar to lysosomal membrane components (Rajendran and Lakshmi, 2008).

Compounds with membrane stabilizing properties are well known for their ability to interfere with release of phospholipases that trigger the formation of inflammatory mediators (Aitadafouri et *al*., 1996).

A possible explanation for the stabilizing activity of *Galium t* extracts could be an increase in the surface area to volume ratio of the cells which could be brought about by an expansion of membrane or shrinkage of the cell, and an interaction with membrane proteins (Mahat and Patil, 2007; Iranloye et *al*., 2011).

Moreover, it has also been shown that the deformability and cell volume of erythrocytes is closely related to the intracellular content of calcium (Shinde et *al*., 1999; Gambhire et *al*., 2009). Hence, it may be speculated that the cytoprotective effect on erythrocyte membrane may be due to the ability of *Galium t* extracts to alter the influx of calcium into the erythrocytes.

Another explanation may be said that, the possible mode of action of the extracts and standard anti-inflammatory drugs could be connected with binding to the erythrocyte membranes with

subsequent alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the haemolysis of red blood cells.

In some research work, it has been reported that, some chemical constituents present in the extracts may exert the same mechanism, which are well known for their anti-inflammatory activity (Sandhya et *al*., 2010).

Both *in vitro* and *in vivo* study in experimental animals, demonstrated that flavonoids exert extensive stabilizing effects on lysosomes (Van-Cangeghen, 1972; Middleton, 1996) while tannin and saponins have the ability to bind cations and other biomolecules, and are able to stabilize the erythrocyte membrane (El-Shabrawy et *al*., 1997; Oyedapo et *al*., 2004).

From the above investigation, it was concluded that the secondary metabolites of *Galium t* has significant membrane stabilization property comparable to the standard drug Diclofenac Sodium except tannins extract, which act by a low concentration and become harmful by augmenting concentration may be by the osmotic pressure or by the presence of other molecules which have heamolytic action.

Khalili et *al.* (2014), investigate the antihaemolytic activity of *Galium verum* aerial parts and found the value of IC_{50} of 1.32 µg /mL. However, Neelambika and Leelavathi (2014), gave a value of IC_{50} of the standard *Ibuprofen* equal to 479.0±17.4 µg/mL. Thus, enhance the secondary metabolites of *Galium t* for antihaemolytic proprieties.

IV – 6 *In vivo* acute toxicity of *Galium t:*

IV – 6 – 1 Physical observation and mortality

Galium t methanolic extract administered by intraperitoneal injection up to the dose of 3000 mg/kg caused no deaths after 24h. The different pharmacotoxic effects, which can be retained as toxicity element of the vegetal product are:

- Decrease in spontaneous behaviours normal in all the groups after 24 hours of injection and prolonged until the end of experience, observed in the groups injected by the doses 6000, 9000 and 12000 mg/kg.
- Anorexia for the groups of 6000, 9000 and 12000 mg/kg.
- Piloerection in all the groups after 24 hours of injection.
- Cutaneous necrosis and coat loss in all groups at different degrees.
- Ocular oedema and eye paleness for the groups of 6000, 9000 and 12000 mg/kg.

- Unilateral paraplegia observed in the groups of 9000 and 12000 mg/kg.
- Putrefaction and Cannibalism after 96 hours observed in the groups of 9000 and 12000 mg/kg
-

However, mortality appear at 12,5 % in group of 3000mg/kg after 96 hours, 50% in group of 6000 mg after 48 hours, 75 % of death was observed in group of 9000 mg/kg (three rats at 48 hours, one rat after 11 days and two rats in the 14 day). Finally, the group injected with 12000mg/kg were decimated (Table 16). The anorexia was confirmed by the body weight loss after 15 days of treatment observed in the groups 6000, 9000 and 12000 mg/kg (Table 17).

Toxicity studies in appropriate animal models are therefore commonly used to assess the potential health risk to humans. Such toxicity studies assess the hazard, namely the basic toxicity of the substance, and the risk is determined by considering the probability of exposure to a particular hazard at certain levels (Klaassen and Eaton, 1991).

Phytochemicals are thought to have a positive or negative effect on an animal. Tannins and flavonoids are thought to have both proxidant and antioxidant effects on the body. While the antioxidant protects the tissues and organs, the proxidant damages the tissues and organs.

The weight changes of the animals during the period of observation, which was more visible at higher doses, suggest the presence of tannins and other phenolics, which are thought to interfere with absorption of nutrients making them unavailable and thereby reducing feed intake (Sofowora, 1993), this explain the anorexia and the loose of body weights.

Table 16: Acute toxicity of methanolic extract of *Galium t* in rats

Group	Decreased Motor activity	Anorexia	Piloerection	Cutaneous Necrosis And Coat loss	Ocular oedema and pale eyes	Unilateral paraplegia	Putrefaction	Cannibalism	Mortality
Control	-	-	-	-	-	-	-	-	-
1000 mg/kg	-	-	-	1 cm	-	-	-	-	-
3000 mg/kg	-	-	+	3 cm	-	-	-	-	12,5 %
6000 mg/kg	++	+	+	Diffuse in abdomen	+	-	-	++	50%
9000 mg/kg	+++	++	+	Diffuse in abdomen	+	++	++	+++	75%
12000 mg/kg	+++	+++	+	Diffuse in abdomen	+	+++	++	+++	100%

+ denotes Mild effect, ++denotes important effect, +++denotes severe effect, − denotes Zero effect

Table 17: Difference of body weight before and after treatment

Groups	Before Treatment		After Treatment	
	Average	± SD	Average	± SD
Control	152,38	26,87	173,38	25,23
1000 mg/Kg	143,13	18,48	145,63	15,91
3000 mg/Kg	161,13	20,91	170,43	63,00
6000 mg/Kg	143,25	27,79	153,50	79,38
9000 mg/Kg	136,00	27,40	121,00	55,56
12000 mg/Kg	148,63	23,58	-	-

The dietary saponins have significant effects on all phases of metabolism, from the ingestion of feed to the excretion of wastes (Cheeke, 1996). Dietary saponins depressed growth, feed consumption (Sim et *al*., 1984;Terapunduwat and Tasaki, 1986; Potter et *al*., 1993; Jenkins and Atwal, 1994).

These negative effects have been ascribed to several properties of saponins such as:

- Reduced feed intake caused by the astringent and irritating taste of saponins (Oleszek et *al*., 1994);
- Reduction in intestinal motility (Klita et *al*., 1996);
- Reduction in protein digestibility (Shimoyamada et *al*., 1998)
- Damage to the intestinal membrane and inhibition of nutrient transport (George et *al*., 2002);
- Production of active metabolites (Wakabayashi et *al*., 1998).

This expresses the intestinal swollen with enteritis in groups treated with high concentration of *Galium t* methanolic extract.

By other side , acute poisoning of pyrrolizidine alkaloids is characterised by haemorrhagic necrosis (Peterson and Culvenor, 1983; International Programme on Chemical Safety, 1989; Huxtable , 1989; Prakash et *al*., 2005), and this describe cutaneous necrosis and coat loss in injection spot which become very important in groups injected with higher concentration of extract until touching the totality of peritoneum.

IV – 6 – 2 Histopathological examination

Post mortem demonstrate a swollen intestines with enteritis, a diffuse inflammation in a belly with a green colour and a putrid smell; however a dark colour of organ indicate a congestion observed in the groups 9000 and 12000 mg/kg (Figure 24, Table 19).

Histopathological examination of control and treated groups showed normal structure with a conserved architecture in all vital organs with the exception of congestive vessel (Figure 23) become important according to the injected dose (Table 18).

Table 18: Post mortem result for acute toxicity of methanolic extract of *Galium t* administered intraperitoneally (i.p) to wistar albino rats

Organ	Control		1000 mg/Kg		3000 mg/Kg		6000 mg/Kg		9000 mg/Kg		12000 mg/Kg	
	Con	Organ Weight	Con	Organ Weight	Con	Organ Weight	Con	Organ Weight	Con	Organ Weight	Con	Organ Weight
Liver	+	5,56 ±0,56	+	5,11 ±0,96	++	7,00 ±0,61	++	6,63 ±0,39	+++	6,34 ±0,04	+++	4,06 ±0,75
Spleen	+	0,56 ±0,15	-	0,77 ±0,21	++	1,05 ±0,20	++	1,45 ±0,40	+++	0,64 ±0,29	+++	0,39 ±0,15
Heart	-	0,67 ±0,05	+	0,68	+	0,77 ±0,07	++	0,68 ±0,12	+++	0,62 ±0,11	+++	0,43 ±0,02
Kidneys		1,25 ±0,01	-	1,23 ±0,10	-	1,68 ±0,35	+	1,24 ±0,21	+	1,25 ±0,03	+	1,06 ±0,20

Con: congestion, + denotes Mild effect, ++denotes important effect, +++denotes severe effect, − denotes Zero effect.

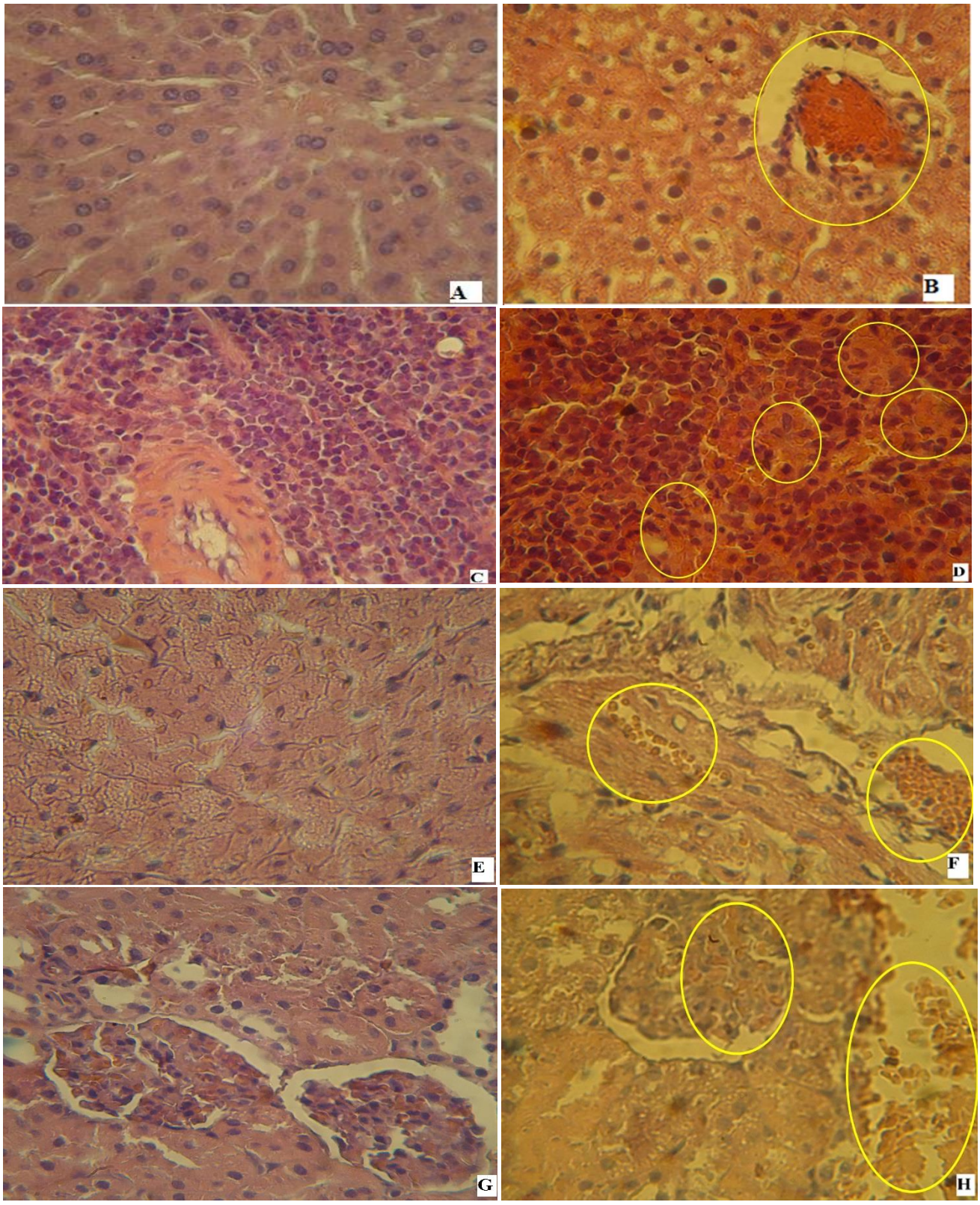


Figure 22: Effect of methanolic extract of *Galium t* on tissue vital organs during 15 days of treatment*: (*A) control liver, (C) control spleen, (E) control heart (G) control kidney (B) liver tissue of 12000 mg/kg treated group (D) spleen tissue of 12000 mg/kg treated group (F) heart tissue of 12000mg/kg treated group (H) kidney tissue of 12000mg/kg treated groups. Magnification (×100) (yellow circle indicate congestion).

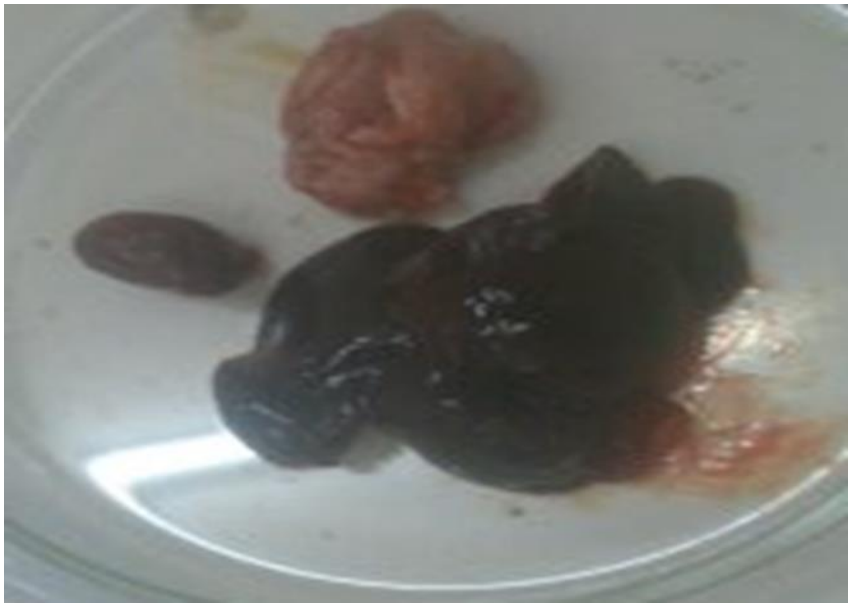


Figure 23: Dark colour of vital organs after post mortem of dead rats

Table 19: Relative organ weights (ROW) of control and rats treated with methanolic extract of *Galium t* measured during the acute toxicity study

Animals groups	Liver	Spleen	Heart	Kidneys
Control	3,21±0,26	0,32±0,07	0,38±0,02	0,72±0,00
1000 mg/kg	3,51±0,66	0,53±0,15*	0,46±0,00	0,84±0,07
3000 mg/kg	4,10±0,29*	0,62±0,10**	0,45±0,03	0,99±0,17
6000 mg/kg	4,13±0,02*	0,42±0,15	0,40±0,06	0,81±0,01
9000 mg/kg	3,36±0,51	0,32±0,10	0,36±0,01	0,88±0,13
12000 mg/kg	5,56±0,27***	1,22±0,27***	0,57±0,08**	1,04±0,14*

****: highly significant difference; ** a very significant difference; * a significant difference with P < 0,05*

Organ weight also is an important index of physiological and pathological status in animals. The relative organ weight is fundamental to diagnose whether the organ was exposed to the injury or not. The heart, liver, kidneys, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicant (Dybing et *al*., 2002). In this study, a significant changes were observed in gross anatomy of systemic organs of treated groups, with a maximum ROW observed in groups treated with 12000 mg/kg in response to hematologic disorders consist of

an important congestion in all vital organ resulting of the important haemorrhagic inflammation in peritoneum except kidneys.

IV – 6 – 3 Haematological and biochemical parameters

Haematological and biochemical parameters are presented in tables 20 and 21 respectively. The haematological parameters record a decrease in the number of red blood cells, haemoglobin and haematocrit (anaemia), platelet thrombocytopenia. Moreover, this anomaly reach the maximum in the group treated with 6000 mg/kg

Table 20: Haematological values of control and rats treated with methanolic extract of *Galium t* measured during the acute toxicity study.

Haematological parameters	Control	1000 mg/kg	3000 mg/kg	6000 mg/kg	9000 mg/kg	12000 mg/kg
RBC (10^6/mm^3)	8,09±0,31	5,39±0,42	3,93±2,78	2,78±2,87*	5,99±1	4,93±0,76
WBC (10^3/mm^3)	6,17±1,46	5,69±2,76	3,85±3,20	3,10±3,47	6,20±0,4	4,07±0,82
PLT (10^3/mm^3)	504,50±87	353±238,59	370,75±276,4	182±188,61	819±17	930,33±212,23
HCT (%)	45,68±1,23	38,1±1,6	20,97±14,8*	15,67±16,25*	34,5±6,3	29,33±3,20
HGB (g/dL)	16,22±0,43	10,83±0,49	7,55±5,21	5,80±5,89	11,4±2,1	9,9±1,23
MCHC (g/dL)	35,45±0,69	37,01±0,21	37,4±2	38,40±2,69	33±0,1	33,9±0,57
MCV (µm^3)	56,58±2,37	55,54±2,09	53,3±0,41	57,60±5,68	59,5±1,1	59,8±3,14
MPV (µm^3)	6,87±0,24	7,11±0,23	3,85±3,20	6,95±0,15	7,3±0,1	7,37±0,21

*Values are expressed as mean ± standard deviation, significant value, RBC; red blood cells, WBC; white blood cell, PLT; platelet, HCT; haematocrit, HGB; haemoglobin, MCHC; mean corpuscular haemoglobin concentration, MCV; mean corpuscular volume, MPV; mean platelet volume. ***: highly significant difference; ** a very significant difference; * a significant difference with P < 0,05*

However, Plasma glucose did not record any significant decrement whatever the injected doses. Concerning the hepatic damage markers, the transaminases make a proportional decrease considering the injected dose, a proportional increase of alkaline phosphatase, a proportional decrease in total protein until the dose of 6000 mg/kg and the bilirubin mark a little increase in treated groups. On the other hand, there was a change in renal check-up, especially uric acid that increase in treated groups. Regarding lipid profile, there was a little increase of triglycerides and cholesterol considering the injected dose.

Table 21: Clinical biochemistry values of control and rats treated with methanolic extract of *Galium t* measured during the acute toxicity study.

Biochemical indicators	Control	1000 mg/kg	3000 mg/kg	6000 mg/kg	9000 mg/kg	12000 mg/kg
Glucose (g/l)	1,16± 0,1	1,11±0,17	1,28±0,21	1,19±0,09	1,16±0,13	0,97±0,14
ALT (UI/l)	35,5±8,20	32,25±11,16	29,60± 5	23,25±8,61	21,33±6,13	23,75±1,3
AST (UI/l)	127±20,01	130,63±35,77	128,60±26,1	104,33±18,57	-	-
ALP (UI/l)	172,25±60,07	176,88±83,68	223,4±19,25	242±72,83	276,33±4,64	359,67±136,9
CK (MG/L)	6,475±0,66	5,43±0,50	5,14±1,56	5,68±0,84	4,23±0,69	5,4±0,71
Uric Acid (mg/dL)	8,25±0,83	21,38±6,84*	10,80±3,06	9±3,81	12±4,32	12,5±4,61
Total Protein (g/L)	67,5±4,09	52,63±4,27	58,40±7,53	54,25±4,49	63,67±6,13	70±7,78
Bilirubin (mg/dL)	9,5±1,50	17,13± 6,17	7,40±3,2	7,25±3,27	8,33±6,18	10,75±4,71
Triglycerides (g/L)	0,62±0,04	0,89± 0,30	0,82±0,18	1,1±0,48	0,77±0,17	0,75±0,25
Cholesterol (g/L)	0,47±0,07	0,50±0,05	0,48±0,07	0,45±0,11	0,51±0,05	0,64±0,12

*Values are expressed as mean ± standard deviation, ALT; alanine aminotransferase, AST; aspartate transaminase, ALP; alkaline phosphatase, CK; creatinine kinase. ***: highly significant difference; ** very significant difference; * significant difference with P < 0,05*

Measurement of haematological parameters can be used to investigate the extent of deleterious effect of foreign compounds including plant extracts on the blood constituents of an animal. It can also be used to explain blood relating functions of chemical compounds and plant extracts (Oyedemi et *al*., 2001).

Taken all data together, the results in this study showed that the acute administration of *Galium t* methanolic extract affect slightly some biochemical and haematological parameters.

Saponins have long been known to have a lytic action on erythrocyte membranes (Glauert et *al.*, 1962), which explain the decreasing of number of red blood cells (slight anaemia). Moreover, Bowman and Rand (1980) stated that alkaloid substances caused haemolysis in various types of cells, especially RBC, and followed by decline in their number drastically.

Therefore, the combining action of saponins and alkaloids provoke the observed alterations in several blood components (red blood cell, haemoglobin, haematocrit and platelet).

The biochemical parameters were used as indices for assessing organ dysfunction or damage as could arise in toxicity especially the liver, plays an important role in many metabolic processes; any disturbance in the liver would affect the normal level of measurable biochemical parameters in this organ. AST, ALT and ALP, which are marker enzymes present in high concentrations in the liver. When liver cells are inflamed or damaged, these enzymes leak into the blood stream leading to a rise in the plasma level of these enzymes (Nkosi et *al*., 2005).

Galium t methanolic extract act upon the alkaline phosphatase consequently causing its increasing, that is related to reduction of food intake and not an organ dysfunction. Moreover, the increase of uric acid confirm this suggestion.

Concerning the proportional increase of lipid profile in blood were explained by the cannibalism observed in animal treated with high dose of extract may be due to the countenance of alkaloids which act as drugs or hallucinogenic; but this hypothesis must be confirmed by other tests.

The maximum of anomalies were observed in groups treated with 6000mg/kg, this may indicate that the maximum of molecules absorption localized at this concentration beyond which, others processes interfere to decrease the intensity of disorders.

IV – 6 – 4 LD_{50} Value

The mortality rate increased within 24 hours and the percentages of mortality were converted to probits (Table 22, Figure 25). The determination of LD_{50} value was obtained by two methods:

- **Graphic method of Funney:** After 24 hours, the mortality rate was determined. This experiment permitted to obtain a LD_{50} value of 6424, 5 mg/kg by weight.
- **Calculation method of Dragsted and Lang (1957):** This experiment permitted to determine a LD_{50} value of 6600 mg/kg by weight.

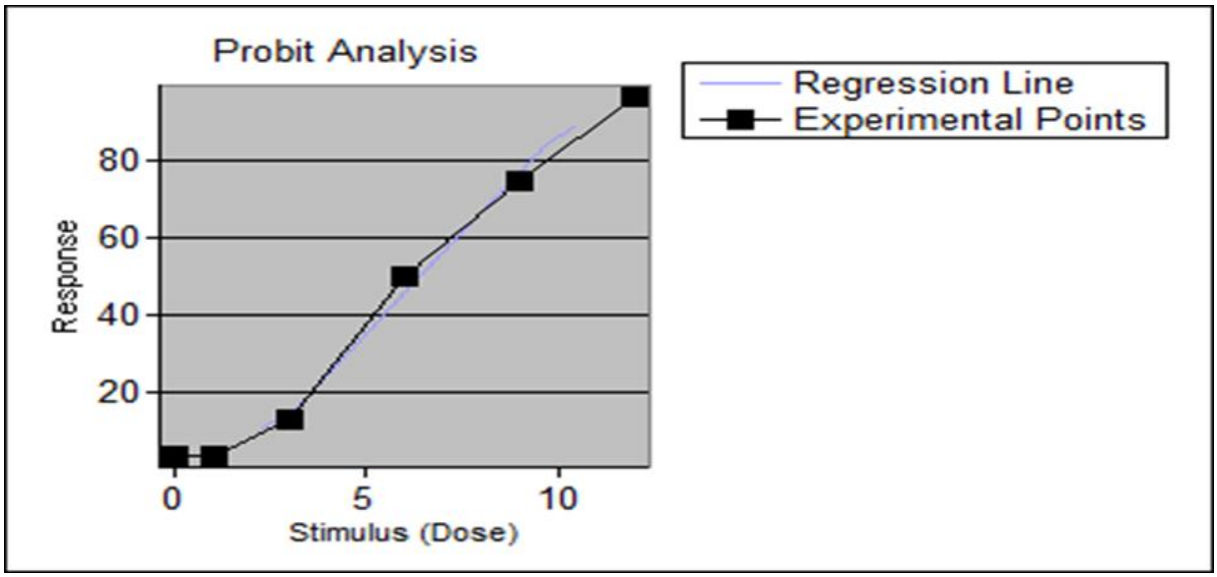


Figure 24: Probity analysis according to Finney methods.

Table 22: Statistic regression for the calculus of DL50 [software Bio stat] (2007; 3.2)

DL (lethal concentration) $_{50}$	6,4245	DL (letal concentration) $_{50}$ standard Erreur	0,9238
DL_{50} LCL (the lowest lethal concentration)	4,5134	DL_{50} UCL (the upper lethal concentration)	8,3356
Beta	0,3124	Alfa	2,9924
standard Beta Erreur	0,0729	-	
DL_{10}	2,3226	DL_{16}	3,2242
DL_{84}	9,6247	DL_{100}	11,2248

The LD_{50} value of *Galium t* methanolic extract determined according to the method of Dragsted & Lang (1957) and Graphic method of Funney were 6600 mg/kg by weight and 6424, 5 mg/kg mg/kg by weight, respectively. These values were statistically very close. According to the classification of Diezi (1989), *Galium t* methanolic extract was no toxic substance.

IV – 7 The effect of flavonoids in acute and chronic inflammatory animal Models

IV – 7 – 1 Xylene-induced ear oedema in mice

The effect of flavonoids extract of *Galium t* on xylene-induced ear oedema in mice is recapitulated in table 23. The intraperitoneal injection of 10 mg/kg of flavonoids extract one hour after xylene application, significantly ($P < 0.001$) inhibited the development of ear oedema

better than 30 mg/ kg of dexamethasone. However, the same dose of flavonoids extract make 60.05 % of inhibition.

Table 23: Effect of flavonoids extract of *Galium t* on xylene-induced ear oedema in mice.

Tested Compounds	Difference by mg	Inhibition (%)
Dexamethasone	2.83 ±0.79	41.46
Flavonoids extract 10mg/kg	2.76± 0.89	42.84
Flavonoids extract 30mg/kg	1.93 ±0.41	60.05
Control	48,40±9,37	-

Statistical treatment of results shows that there is currently not a significant difference between the standard drug and flavonoids extract. The xylene-induced ear oedema model is useful for the evaluation of anti-inflammatory topical steroids or for non-steroidal antiphlogistic agents, especially those inhibiting phospholipase A_2 (Kwang-Ho et *al*., 2008).

Histopathologically, severe vasodilation, edematous changes of the skin and infiltration of inflammatory cells are detected as signs of acute inflammation after topical application of xylene (Kou et *al*., 2003), marked increases in ear weight were detected because of the acute inflammation response. These increases in ear weight have been used as valuable markers for anti-inflammatory effects (Al-Majed et *al*., 2003; Ojowele, 2005).

The xylene-induced inflammation caused the release of pro-inflammatory mediators from sensory neurons that act on peripheral target cells, such as mast cells and other immune cells, producing neurogenic inflammation characterized by warmth, redness and oedema (Richardson and Vasko, 2002).

Suppression of this response is a likely indication of antiphlogistic effect (Parveen et *al*., 2007), and the results clearly showed that flavonoids extracted from *Galium t* exerted an important antiphlogistic effect against xylene-induced ear edema.

The percent of inhibition of the formation of ear oedema of dexamethasone (10mg) in the experiment carry out by Sowemimo et *al*. (2013), was 50.20 % approximately similar to 10 mg of flavonoids extract of *Galium t* injected to mice. Furthermore, Bagad et *al*. (2013), display a

percent of inhibition of 66.6 % for 50 mg/kg of Diclofenac. Therefore, *Galium t* flavonoids were better than synthetic anti-inflammatory drugs.

IV – 7 – 2 Effect of flavonoids of *Galium t* on cotton pellet-induced granuloma in rats

Flavonoids extract of *Galium t* and standard drug was evaluated by cotton pellet induced granuloma formation to understand its potential in chronic inflammatory phase**.** Flavonoids extract of *Galium t* inhibited the granuloma induced by cotton pellets in rats (Table 24) by a percentage of 36,19 %. However, the standard drug (Dexamethasone) produces maximum activity by inhibiting the dry weight of cotton pellet by 71,45 %.

Table 24: Effect of flavonoids extract of *Galium t* on cotton pellet-induced granuloma in rats.

Tested Compounds	Difference by mg	Inhibition (%)
Dexamethasone 16 mg/kg	9,25±1,48	71,45
Flavonoids extract 30mg/kg	20,68 ±3,70	36,19**
Control	32,4± 0,27	-

****: highly significant difference; ** very significant difference; * significant difference with* $P < 0{,}001$.

The anti-inflammatory effect of the extracts was further investigated by the cotton pellets induced granuloma formation in rats, which is a model for chronic inflammation. The cotton pellet-induced granuloma is widely used to assess the transudative and proliferative components of chronic inflammation (Winter and Porter, 1957). It consists of 3 phases:

1) A transudative phase, defined as the increase in the wet weight of the pellet that occurs during the first 3 hours.
2) An exudative phase, defined as plasma leaking from the bloodstream around the granuloma that occurs between 3 and 72 hours after the implantation of pellet.
3) A proliferative phase, measured as the increase in the dry weight of the granuloma that occurs between 3 and 6 days after the implantation (Swingle and Shideman, 1972).

Moreover, the implanted material induces a host inflammatory response and modulates the release of inflammatory mediators, which finally leads to tissue proliferation and granular formation (Remes and Williams, 1992; Tang and Eaton, 1995; Hu et *al*., 2001).

The extract exhibited significant anti-inflammatory activity in the cotton-pellet granuloma test. This reflected its efficacy to inhibit the increase in the number of fibroblasts and synthesis of

collagen and mucopolysaccharides during granuloma tissue formation (Arrigoni-Martellie, 1977). The extract may act by inhibiting cellular migrations involved in this inflammation model (Pedernera et *al*., 2010).

Flavonoids with a percentage of inhibition of 36.19% estimated very important comparing with result of Dongmo et *al.* (2013), and many other researches, when this percentage represents the standard. In this case, the high concentration of standard fact those flavonoids do not shake like dexamethasone.

IV – 8 Analgesic activity of flavonoids extract

Pain is defined as a subjective, unpleasant, physical and psychological experience observed as a result of the stimulation of identifiable nerve fibers with defined pathways to the brain via the spinal cord (Palecek and Willis, 2005).

Pain often results from tissue damage that stimulates nociceptive receptors (nociceptive pain) but pain may also occur without nociception; here it could be as a result of damage to neural structures (neuropathic pain or neuralgia). While the former is often acute, self- limiting after healing and responds easily to analgesics, the latter is very difficult to treat, there may or may not be evidence of injury, causes chronic pain and will persist long after the initial injury has healed (Juan and Lembeck, 1974; Somasundaram et *al*., 2000).

IV – 8 – 1 Acetic Acid Induced Writhing Assay

Acetic acid induced writhing in mice attributed visceral pain finds much attention of screening analgesic drugs (Hasan et *al*., 2010).

The writhing test has long been used as a screening tool for the evaluation of analgesic properties of new substances. Table 25 shows the pain behavior of writhing response of mice of analgesic activities of Diclofenac sodium and flavonoids extract of *Galium t*.

Table 25: Effect of flavonoids extract of *Galium t* on acetic acid - induced writhing test in mice.

Tested Compounds	Average contortion	Inhibition en %
Diclofenac sodium 10mg/kg	6±1,15	83,33
Flavonoids extract 100mg/kg	12,8±1,77**	64,44**
Control	36±3,11	-

****: a highly significant difference; ** a very significant difference; * a significant difference with P* < 0,001.

The control animal showed 36±3,11 writhing count/30 minutes but, animal treated with Diclofenac sodium caused significant reduction of writhing count, from 36±3,11 to 6±1,15 ($p<0.001$). Animals treated with flavonoids extract of *Galium t* reduced the writhing count from 36±3,11 to 12,8±1,77.

The results suggested flavonoids extract and Diclofenac sodium had analgesic action and showed significant ($p<0.001$) reduction of pain in comparison with control group. *Galium t* flavonoids, demonstrate a very important analgesic activity (64,44%) in comparison with Diclofenac sodium (83,33%).

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid (Ahmed et *al*., 2006), via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte et *al*., 1988).

In other words, the acetic acid induced writhing has been associated with increased level of PGE_2 and PGF2α in peritoneal fluids as well as lipoxygenase products (Derardt et *al*., 1980).

The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria et *al*., 2008).

The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte et *al*., 1988; Ferdous et *al*., 2008).

Flavonoids are known to target prostaglandins which are involved in the late phase of acute inflammation and pain perception (Bittar et *al*., 2000; Santa-Cecília et *al*., 2011) acting mechanism similar to the non- steroidal anti-inflammatory agents, such as indomethacin and diclofenac sodium.

These results strongly suggest that flavonoids extract of *Galium t* possesses peripheral analgesic effect and its mechanism of action may be mediated through inhibition of local peritoneal receptors or arachidonic acid pathways, involving cyclo-oxygenases and/or lipoxygenases.

Morinda officinalis (Rubiaceae*)* root methanolic extract produce at 200 mg/kg an analgesic effect of 37.8±1.30 % of writhing inhibition (Choi et *al*., 2005), and flavonoids extracted from *Pentas lanceolata* Leaves (Rubiaceae) at 200 mg/kg make an inhibition percentage of 19.05 % (Suman et *al*., 2014) widely weaker than flavonoids extract of *Galium t* . Hence, *Galium t* count between the best species of Rubiaceae possesses peripheral analgesic effect.

IV – 8 – 2 Hot Plate Method

The results of the effect of Diclofenac sodium and flavonoids extract of *Galium t* on the hot plate method at 0 minute. 30 minutes, 45 minutes and 60 minutes is presented in table 26. Diclofenac sodium and flavonoids extract of *Galium t* was capable of increasing the latency period of pain induced by heating of the plate (Figure 26).

The results show that there was no significant difference in the pain reaction time during the pre-drug testing time. After drug and extract administration, comparing the pre and post drug pain reaction time showed that the reference drug (Diclofenac sodium 10 mg/kg) and the flavonoids extract of *Galium t* (100 mg/kg) significantly ($p<0.001$) increased the pain reaction time, but the extract producing a better effect than the reference drug.

Flavonoids extract reached a maximum of 263.16% after 45 minutes to drop to 151.05% after one hour, remains incomparable with any other drug of this kind.

Hot plate test was also assayed to characterize the analgesic activity of extract. Nociceptive reaction toward thermal stimuli in mice is a well-validated model for detection of opiate analgesics as well as several types of analgesics drugs from spinal origin (Alhaider et *al*., 1991).

The result of the hot plate test showed that flavonoids extract of *Galium t* significantly increased the pain reaction time of the mice on hot plate better than diclofenac sodium.

According Turner (1965), hot plate test is a model for assaying effects of drugs on central pain. It is an establish fact that any agent that causes a prolongation of the hot plate latency using this test must be acting centrally (Thamizh mozhi et *al*., 2013).

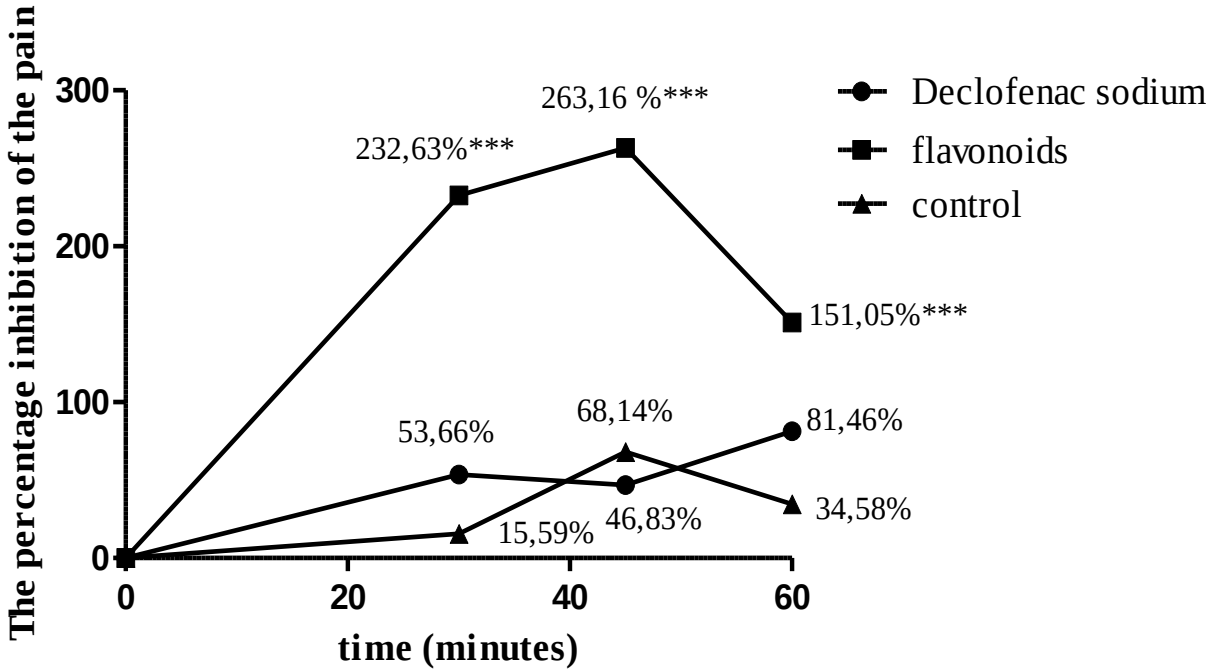
The nociceptors seem to be sensitized by sensory nerves. This model may minimize the involvement of endogenous substances such as PGs.

Non-steroidal anti-inflammatory drugs such as Diclofenac sodium used in this study are known to inhibit cyclooxygenase enzymes I and II which are implicated in the production of inflammation mediating agent prostaglandin (PGE_2) from arachidonic acid (Parmar and Ghosh, 1978; Dhara et *al*., 2000; Wu , 2002).

The model of analgesic activity exhibited by flavonoids extract was better than Diclofenac sodium, which suggests may be mediated by cyclooxygenase I and II inhibition (Chandra et *al*., 2012). Moreover, Iniyavan et *al*. (2012), display 75.248 % of pain inhibition obtained from Pentazocine. This confirm the powerful analgesic of flavonoids extract of *Galium t*.

Table 26: Effect of flavonoids extract of *Galium t* on hot plate test in mice (time in seconds).

Tested Compounds	0	30 minutes	45 minutes	60 minutes
Diclofenac sodium	4,6±4,1	6,3±2,41	6,02±2,51	7,44±4,48
Flavonoids	3,4±3,8	12,64± 2,79	13,8±4,16	9,54±3,08
Control	6±5,9	6,82±1,84	9,92±4,62	7,94±3,27



****: a highly significant difference; ** a very significant difference; * a significant difference with P* < 0,001

Figure 25: The percentage inhibition of the pain in hot plate test in mice of flavonoids extract of *Galium t.*

IV – 9 Anti-ulcerative activity of tannins extract

IV – 9 – 1 Preventive effect

The antiulcer activity of tannins extracted from *Galium t* in HCl/ethanol-induced gastric lesion model, is shown in table 27. Results showed that rats pre-treated with omeprazole or tannins extract before being given HCl/ethanol solution had significantly reduced areas of gastric ulcer formation compared with ulcer control group (Figure 27- 28).

Pre-treatment of animals with tannins extract (200mg/kg) significantly reduced formation of ulcers induced by HCl/ethanol mixture, the percentage inhibition being 75,30±17,64 % in comparison with standard drug (80 mg/kg) which make 83,80±7,20 % of protection. Statistical treatment of results show that there is no significant difference between the groups treated with omeprazole and those treated with tannins extract.

Table 27: Preventive effect of tannins extracted from *Galium t* in HCl/ethanol-induced ulcer in rats.

Tested Compounds	Ulcer Index	Inhibition En %
Omeprazole	0,15±0,02	83,80±7,20
Tannins extract 200mg/Kg	0,16±0,09	75,30±17,64
Control	0,66±0,27	-

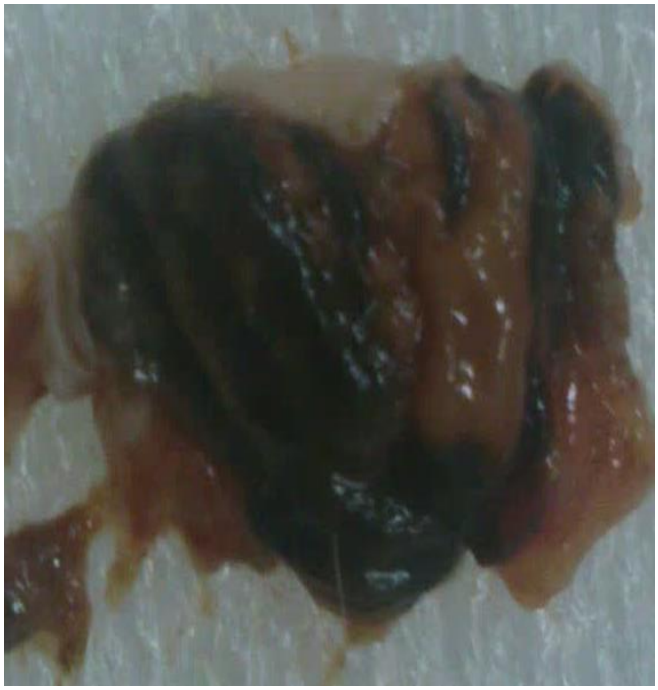


Figure 27: Gastric lesion observed in control group induced by HCl-Ethanol

Figure 27: Gastric lesion observed in group treated with tannin extract of *Galium t*

HCl-Ethanol induced gastric ulcer was employed to study the cytoprotective effect of plants extracts. HCl-Ethanol induced gastric damage ranging from endothelial microvascular damage to development of macroscopic gastric mucosal lesions, which is attributed mainly to the inhibition of biosynthesis of cytoprotective PG resulting in overproduction of leukotrienes and other products of the 5-lipoxygenase pathway (Nasuti et *al*., 2006). These agents break the mucosal barrier, provoke an increase in gastric mucosal permeability to H^+ and Na^+ ions reducing the transmucosal potential difference and induce formation of erosions and ulcers (Ashoka and Shashidhar, 2011).

The antiulcer proprieties observed in preventive effect study might be due to a possible relationship between protection of mucosal injury, inhibition of acid secretion and the antioxidant nature of tannins extract of *Galium t*.

The plant extract possess antisecretory, cytoprotective and proton pump inhibition mechanism. This study indicates that tannins extract of *Galium t* has a potential antiulcer activity. However, this result must be confirmed by the curative effect study below.

IV – 9 – 2 Curative effect

The results of the curative effect of tannins extracted from *Galium t* were shown in the table 28. Oral administration of tannins extract (200 mg/kg) for 8 days significantly ($p<0.05$) reduced the ulcer index in ethanol induced experimental ulcer in rats, with percentage of 56,79±8,17%; this result was comparable with standard drug which attain the percentage inhibition of 48,77±5,85 % and the statistical comparison confirm this results.

Peptic ulcers are caused when the natural balances between the aggressive factors of acid, pepsin, defensive mechanisms of mucus, bicarbonate, mucosal turnover and blood supply (mucosal barrier) are disturbed (Piper and Stiel, 1986) .

Baron et *al*. (1980), reported that acid and pepsin are relatively less important as causative agents and that a defect in the defensive mechanism of gastric mucosa is the first step toward ulcer formation (Marhuenda et *al*., 1993).

Studies suggest that the ethanol damage to the gastrointestinal mucosa starts with microvascular injury, namely disruption of the vascular endothelium resulting in increased vascular permeability, oedema formation and epithelial lifting (Szabo et *al*., 1995).

Ethanol induced gastric injury is associated with significant production of oxygen free radicals leading to increased lipid peroxidation, which causes damage to cell and cell membrane (Cheesman, 1993). The antioxidants being reported to play a significant role in the protection of gastric mucosa against various necrotic agents (Trivedi and Rawal, 2001).

Administration of antioxidants inhibits ethanol-induced gastric injury in rat (Ligumsky et *al*., 1995). That is why; tannins extract of *Galium t* was tested against the peptic ulcer. It is speculated that the gastroprotective effect exerted by this extract, could be attributed to its antioxidant property.

Further, ethanol induced gastric lesion formation may be due to stasis in gastric blood flow which contributes to the development of the haemorrhage and necrotic aspects of tissue injury. Alcohol rapidly penetrates the gastric mucosa apparently causing cell and plasma membrane damage leading to increased intracellular membrane permeability to sodium and water. The massive intracellular accumulation of calcium represents a major step in the pathogenesis of gastric mucosal injury. This leads to cell death and exfoliation in the surface epithelium (Raju et *al*., 2009).

Our result demonstrate that the tannins extract of *Galium t* reduced significantly the ulcer induced by ethanol. The extract at a dose of 200 mg/kg significantly decreased the ulcer index, pepsin activity and total acidity when compared to their respective controls, indicate the healing of the induced ulcer.

The anti-ulcer activity shown by the tannins extract in ethanol-induced ulcers suggests that the extract has a cytoprotective effect, i.e. it protects the gastric mucosa by mechanisms other than gastric acid secretion (Robert, 1979). Such mechanisms include inhibition of leukotrienes (Roger et *al*., 1986), pepsinogen (Konda et *al*., 1991) and substance P (Karmeli and Okone, 1991), free radical scavenging (Szabo et *al*.,1985), increasing gastric mucosal blood flow (Kauffman , 1981), increasing the protective glycoprotein content and thereby strengthens the gastric mucosa (Horowitz, 1977) and prevention of oxidation of the mucosal xanthine dehydrogenase (Sinclair, 1990).

Table 28: Curative effect of tannins extracted from *Galium t* in ethanol-induced ulcer in rats

Treatment	Dose (mg/kg) P.O	Volume of gastric juice (ml/100g)	pH	Total Acidity (meq/L)	Ulcer index	Inhibition %	Pepsine (µg/ml) Eq.Tyr
Control	10 mL/kg	1.84 ±0,14	2.80 ±0.14	70.16 ±0,30	9,00±1,22	-	67.96 ±1.71
Standard Omeprazole	50	1,25±0,05	5,24 ±0,25*	22,5 ±7,5	4,83±0,20	48,77±5,85	11,15±2,90
Tannins	200	1,10±0,00	4,28±0,40	25,63±4,84	3,88±0,89	56,79±8,17	10,14±3,27

****: a highly significant difference; ** a very significant difference; * a significant difference with P* < 0,001

Other species of Rubiaceae like *Ixora coccinea* gave a protection of 54.48 % at 200 mg/kg (Babu et *al*., 2014), *Gardenia gummifera* at concentration of 150 mg/kg can inhibit the formation of duodenal ulcer by 44.61% (Pradeep Kumar et *al*., 2015) and *Nauclea latifolia* make 69.20 % of protection at 200 mg/kg (Balogun et *al*., 2014).

These results suggest that the tannins extract of *Galium t* were beneficial in the treatment of gastric lesions. Further studies to elucidate the mechanism of action are recommended.

IV – 10 Healing wounds activity of methanolic activity

The area of wound measured on the days 0, 3, 6, 9, 12 and 15 after surgery in all groups revealed interesting results (Table 29). Angiogenesis, which is important factor in the first days of healing, was significantly higher in MYCOCIDE® ointment group than treated group and control. Strikingly, epithelisation in control was important at the third day of healing.

On the 6^{th} day, epithelisation in treated group was higher than other groups and wound healing was proceeding towards the proliferative phase. However, standard group still in the inflammatory phase of healing.

On the 9^{th} days, the standard group wound healing was significantly better compared with treated group and control. But, on the 12^{th} days, the progression of wound healing in the treated group was better than other groups. However, a nearly complete epithelisation was observed in treated group regarding others groups at 15^{th} day.

Statistic treatment show that there is no significant difference between all groups in process of wound healing. However, rapidity of wound epithelisation indicate a difference between the groups. On Day 3 there were no differences among the groups but three days later at Day 6 there were significant differences between treated group (57,29±15,09 %) and the other groups (33,14±5,87 % for standard group and 51,82±11,19 % for control). no differences among the groups were observed on the 9th day of the study. Whereas, On the 12^{th} days a significant difference was seen between treated group (90,53±1,53 %), standard (82,25±6,22%) and control (83,15±0,57%).

Finally, on the last day of the experiment, the effectiveness of the treatment with methanolic extract ointment was completely obvious showed a huge contraction in wound size (98,76±1,53 %) compared to the other groups and a very significant difference between all

groups were observed (85,92±1,42 for control and 92,40±0,35 for MYCOCIDE® ointment group).

Wound healing is a fundamental response to tissue injury that results in restoration of tissue integrity. This is mainly achieved by the synthesis of the connective tissue matrix. Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength (Fernandez et *al*., 2002).

Wound healing involves a complex and superbly orchestrated interaction of cells, extracellular matrix and cytokines (Gupta et *al*., 2008). Collagenation, wound contraction and epithelisation are crucial phases of wound healing. The phases of inflammation, macrophagia, fibroblasia and collagenation are intimately interlinked. Thus, intervention at any one of these phases using drugs could eventually either promote or inhibit one or all phases of healing (Vinothapooshan and Sundar, 2010).

In excision wound healing model, the methanol extract showed significant increase in percentage closure by enhanced epithelialization (figure 29 – 30). This enhanced epithelialization may be due to the effect of *Galium t* extracts on enhanced collagen synthesis and/or by chelation of the free radicals and reactive species of oxygen, promoting contraction of the wound and increasing the formation of capillary vessels (Fernandez et *al*., 2002); and fibroblasts and including keratinocyte proliferation, but do not act on the differentiation towards cornified cells (Deters et *al*., 2001).

Table 29: Evaluation of *Galium t* methanolic extract and MYCOCIDE® ointments on wound healing by excision wound method in rat.

Post wounding days	Wound area (mm^2) (mean ± SEM) and percentage of wound contraction		
	Mycocide® ointment	Methanolic extract ointment	Control
0	216±42,71	256±33,82	216,50 ±18,26
3rd	181,52±52,80 **(16,72±11,95%)**	242,24 ±34,95 **(5,35±4,91%)**	182,40 ±17,08 **(15,81 ±0,75%)**
6 th	153,81+28,84 **(33,14 ±5,87%)**	109,10 ±42,34* **(57,29±15,09 %)***	104,73±26,03 **(51,82±11,19%)**
9 th	42,60±9,28 **(80,01±4,17%)**	63,09±8,14 **(74,68±5,69%)**	54,37± 5,20 **(74,91±0,50%)**
12 th	36,21±8,87 **(82,25±6,22%)**	24,18±4,72* **(90,53±1,53%)***	36,58±4,38 **(83,15±0,57%)**
15 th	16,40±3,19 (**92,40±0,35%)**	3,44±4,34 **(98,76±1,53%)****	30,50± 3,99 **(85,92 ±1,42%)**

****: a highly significant difference; ** a very significant difference; * a significant difference with* $P < 0{,}001$

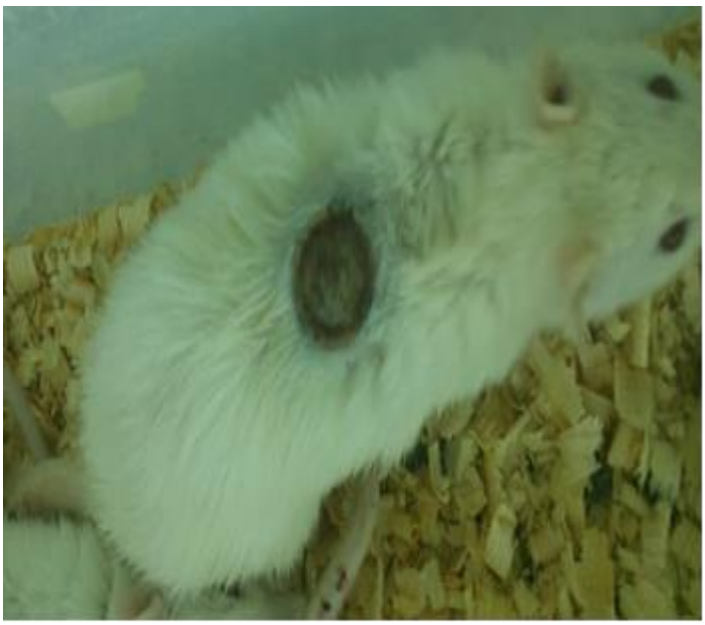


Figure 29: Excision circular wound after 3 day treated with *Galium t* methanolic extract

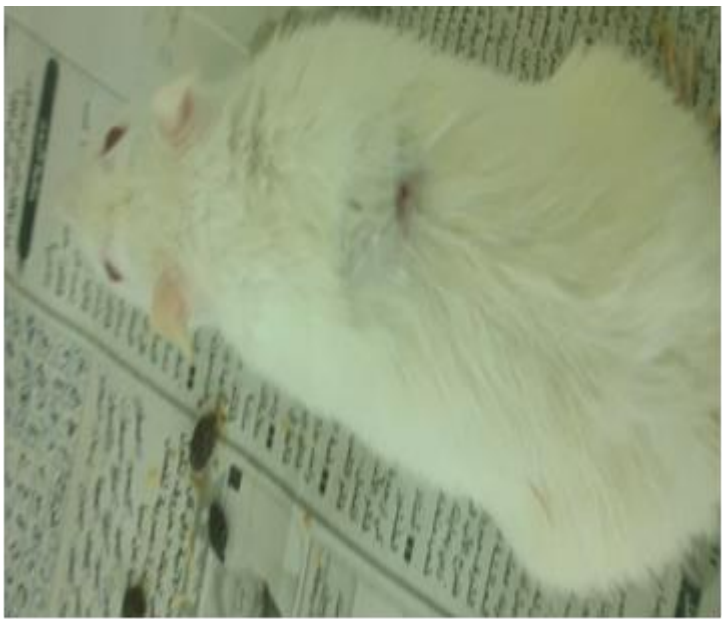


Figure 29: Excision circular wound after 12 day treated with *Galium t* methanolic extract

Many plant extracts and medicinal herbs have shown potent antioxidant activity. Tannins the main components of many plant extracts, act as free radical scavengers (Dutta and Shastry, 1959; Tran et *al*., 1996; Bekerecioglu et *al*., 1998; Ghiselli et *al*., 1998; Marja et *al*., 1999; Pulido et *al*., 2000). Research into role of antioxidants from plant extracts in wound healing has been published widely (Hwang et *al*., 2000).

Sifour et *al*. (2012), assessed 85,08± 3.8 % of wounds healing after treatment with ointment prepared with 10% of aqueous extract of *Atractylis gummifera* and 72.27± 14.07% of wound contracture treated with *MADECASSOL BAYER* (0.5g) after 14 days. Moreover, *BANEOCIN*® ointment can healing wounds at 95.69 % after 14 days of treatment (Chabane et *al*., 2013).

Therefore, secondary metabolites of *Galium t*, anti-inflammatory and antioxidant functions leads this species to possess a good wound healing properties which may be attributed to the individual or combined action of phytoconstituents. The exact component of the extract that is responsible for this effect, however, was not investigated. Further phytochemical studies are needed to isolate the active compound(s) responsible for these pharmacological activities.

Conclusion & perspectives

V – Conclusion & perspectives

To the best of our knowledge, phytochemical and therapeutic proprieties of *Galium t* presented in this investigation, has not previously been reported elsewhere. Therefore, data presented in this study could be assumed as the first report. We believe that this work will contribute to the discovery of new capacities of this plant.

The antioxidant capacities of *Galium t* were investigated to value the interaction between molecules. However, polyphenols extracted from *Galium t* can be good antioxidant agents especially flavonoids extract which present a good scavenger of ROS and a good trapper of iron ions. The tannins extract can be also better in according extraction method; they would protect DNA from oxidative damage; it is tempting to propose their use for prevention and/or experimental therapy of disorders related to iron-mediated oxidative stress, such as colorectal cancer (Nelson, 1992; Weinberg, 1994).

Moreover, saponins extract of this species offers new areas of research to demonstrate their capacities and can form a good source of effective inhibitors of free radicals. Further investigations regarding their phytoactive compounds are needed to be oriented for destruction of malignant tumors, and determine the toxic dose and cumulative effect of these molecules.

The antioxidant activity was completed with the study of essential oils, which showed a moderate antioxidant capacity but not enough to propose them as a therapeutic substances.

Considering the parameter suggested by Alves et *al.* (2000), all molecules extracted from *Galium t* were less active or inactive against strains used in antimicrobial activity. That is why, the secondary metabolites of *Galium t* are so far to be considered as antibiotics or used as antiseptic.

The *in vitro* anti-inflammatory study, inhibition of protein denaturation and membrane stabilization was studied to establish the mechanism of action of the secondary metabolites of *Galium t.* This activity, help us to determine the suitable dose for each molecules groups. All extracts demonstrate good capacities to be used against inflammatory diseases especially the flavonoids that are nontoxic and efficacy. Tannins extract can be used in a low concentrations above them, they can present a harmful effect.

The acute toxicity study, *Galium t* methanolic extract has been found to be containing many bioactive phytochemical compounds, which may be responsible for the observed clinical disorder. The use of high concentration (above 6000 µg/ kg) was necessary to determine the

effect of the over dose when the extracts of this plant were proposed as medicines. However, the LD_{50} value show that this plant may be harmful at high dose but it is not deadly, this finding was confirmed with histopathological study, hematological and biochemical blood parameters, and defines it as non-toxic up to a dose of 6600 mg/kg body weight.

The *in vivo* anti-inflammatory investigation using acute and sub-acute inflammation tests by flavonoids extract confirm that there is potential abilities to cure this kind of diseases safety at low doses.

In the analgesic activity, the hot plate method and acetic acid induced writhing method were used for detecting centrally acting analgesics. Thus, it is concluded that flavonoids extracted from *Galium t* have potent analgesic activity and demonstrate the utility of these molecules on central mechanism of pain inhibition.

The antiulcerogenic effect of tannins extracted from *Galium t*, by markedly inhibiting acid secretion and the occurrence of lesion in stomach. It appears that these phytoconstituents provides protection against gastric mucosal damage induced by ethanol/HCl and absolute ethanol, through inhibition of gastric acid, pepsin, histamine and free radical and stimulation of mucus secretion.

The wound healing effects of *Galium t* methanolic extract for excision model show a potential wound healing activity. This capacity was due to the significant content of the most important phytochemicals like total phenolic compounds, carotenoids, terpenoids…

Critical analysis was focalised on extraction methods using organic solvent which may be not specified to extract the pure molecules groups by one hand, and the preservation of the natural harmony between molecules by other hand; also the dosage of bioactive molecule groups has been shown nonspecific not only to target molecules but to any other substance . That could be oxidised by the reagent as reported by various researchers, the poor specificity of the quantification methods.

Further, acute toxicity use many animals to be sacrificed, in our case, 48 rats were used and subjected to high concentration (9 – 12 g/kg of body weight) resulting a serious disturbance in respect of animals.

Finally the agro-industrial production of the plant destined to pharmacologic manufactories may alter the genetic richness of the wild species.

As perspective, we recommend:

- The isolation of molecules responsible for each therapeutic activity and resolve the mechanism of action against diseases, to produce medicines from each extract.
- The development of a new agronomic methods able to preserve the genetic alteration and to produce in the same time this species.
- The determination of the gene map and the conservation of this endemic species in International genbank.

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VI - Bibliography

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Appendix of tables

Table 1 : Product List

Products	brand
Méthanol	Scharleau And Sigma Aldrich
Ethanol	VWR Prolabo
Acétone	Prolabo
Ether De Pétrole	Sigma – Aldrich
Ethyle Acétate	VWR Prolabo
Parafilm	Leching Plastic Packaging
2-Butanol	Prolabo
Chloroforme	Sigma – Aldrich
Papier Filtre	Selectra
Ether Di-Ethylique	VWR Prolabo
Carbonate Monsodiques	Condichim
Carbonate De Sodium En Solution	Prolabo
Acide Chlorhydrique	Riedel-De Haën
Eppendrof	Isolab
Diclofenac Sodium	Saidal
Gentamicin (10 Mg/Disc)	Pastuer Institute of Algiers
Nystatin (100 Mg/Disc)	Pastuer Institute of Algiers
Clotrimazon (50 Mg/Disc)	Pastuer Institute of Algiers
Amphotericin (100 Mg/Disc)	Pastuer Institute of Algiers
Omeprazole	Saidal
Aspirin	Sigma – Aldrich
MYCOCIDE®	Saidal
Muller Hilton	Conda pronadisa
Sabouraud	Conda pronadisa

Table 2: corporal weigh of rats in gram randomised in heterogenic groups.

	1	2	3	4	5	6	7	8
Group 1	115	155	156	172	127	140	124	156
Group 2	130	205	162	155	157	159	144	177
Group 3	162	158	107	112	134	150	126	197
Group 4	110	194	128	127	126	102	142	159
Group 5	188	172	158	136	146	159	120	126
Group 6	144	117	148	154	133	133	190	200

Appendix of figures

Appendix of figures:

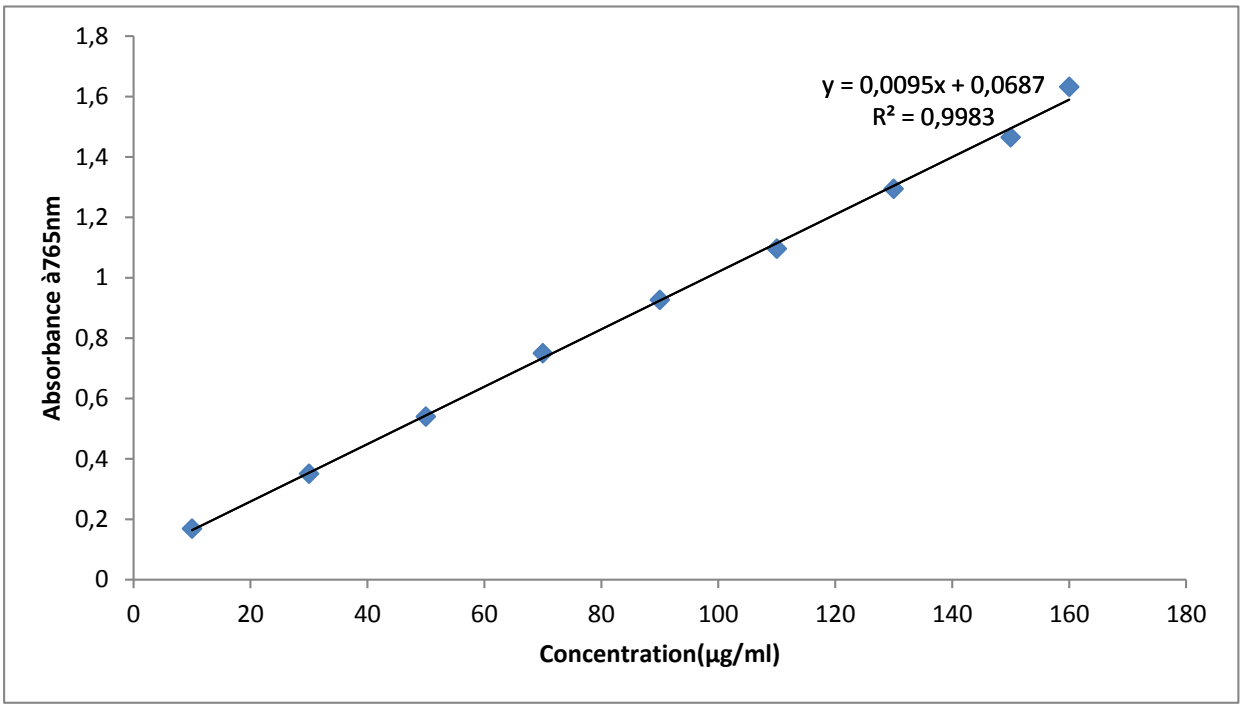

Figure 1: calibration curve of polyphenols (gallic acid: means ± sd)

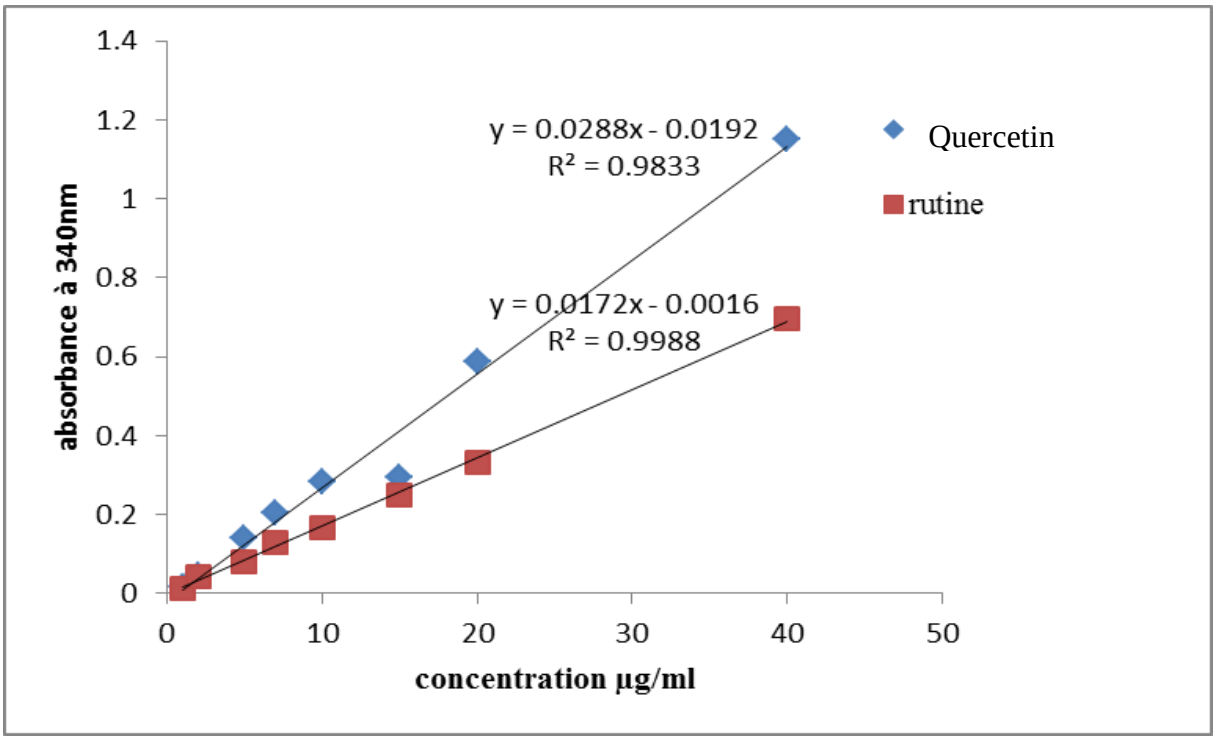

Figure 2: calibration curve of flavonoids (quercetin and rutin: means ± sd)

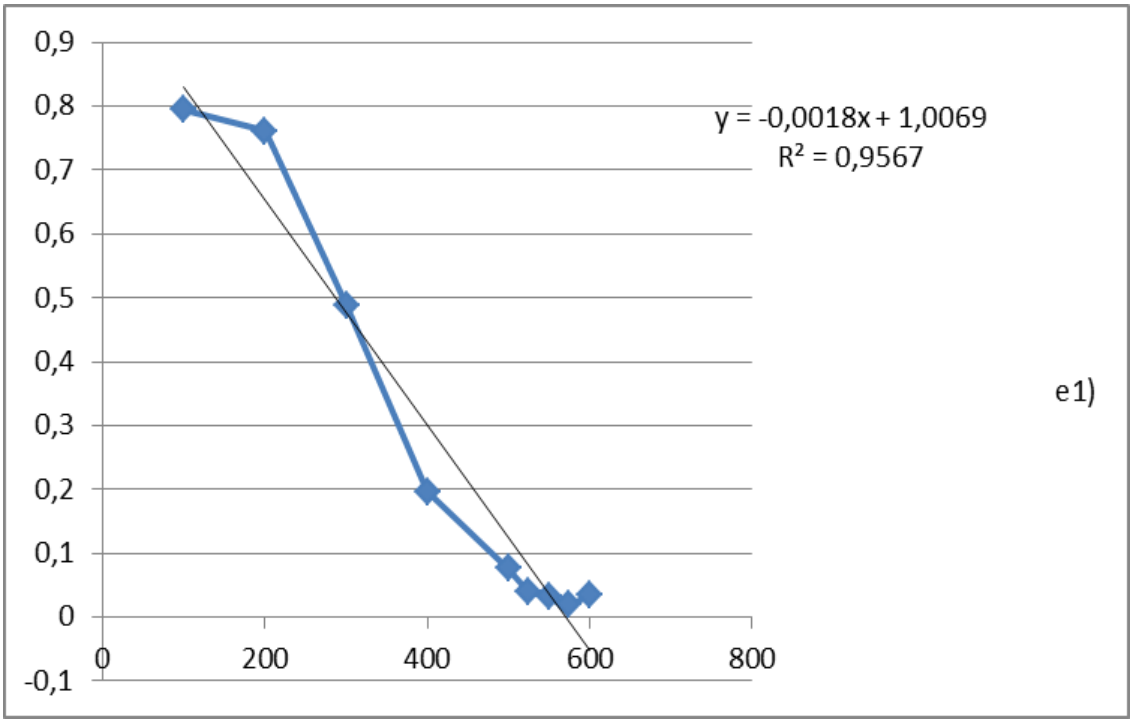

Figure 3: calibration curve of tannins (means ± sd)

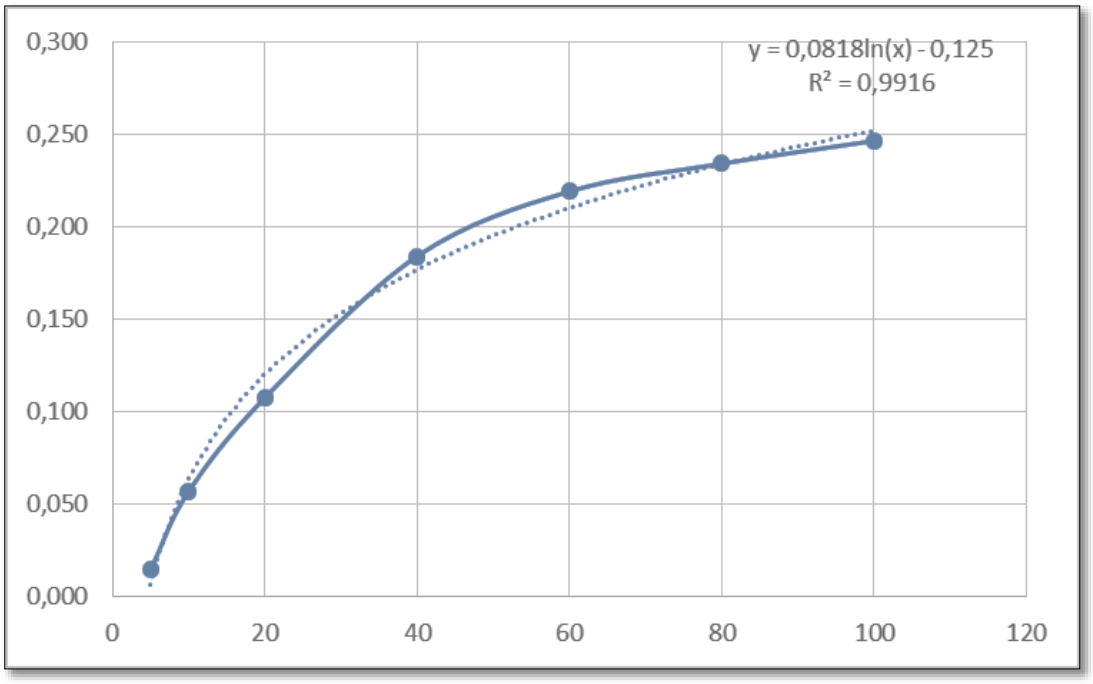

Figure 4: calibration curve of alkaloids (atropine: means ± sd)

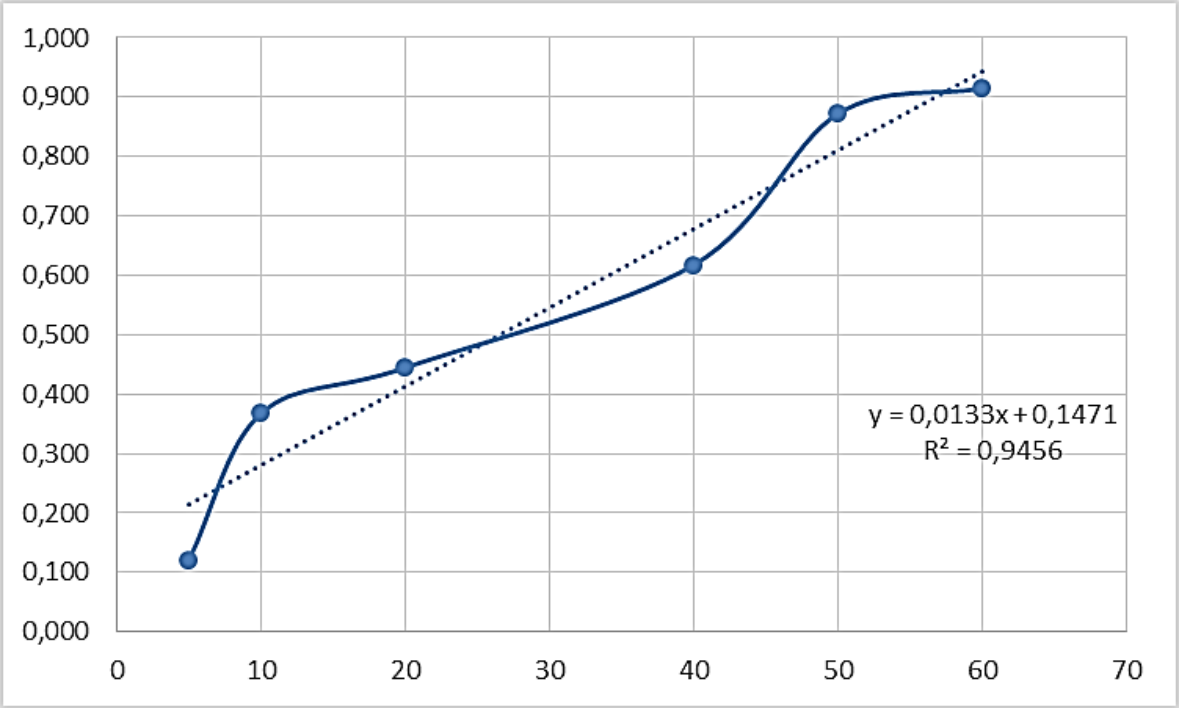

Figure 5: calibration curve of pepsin (tyrosine: means ± sd)

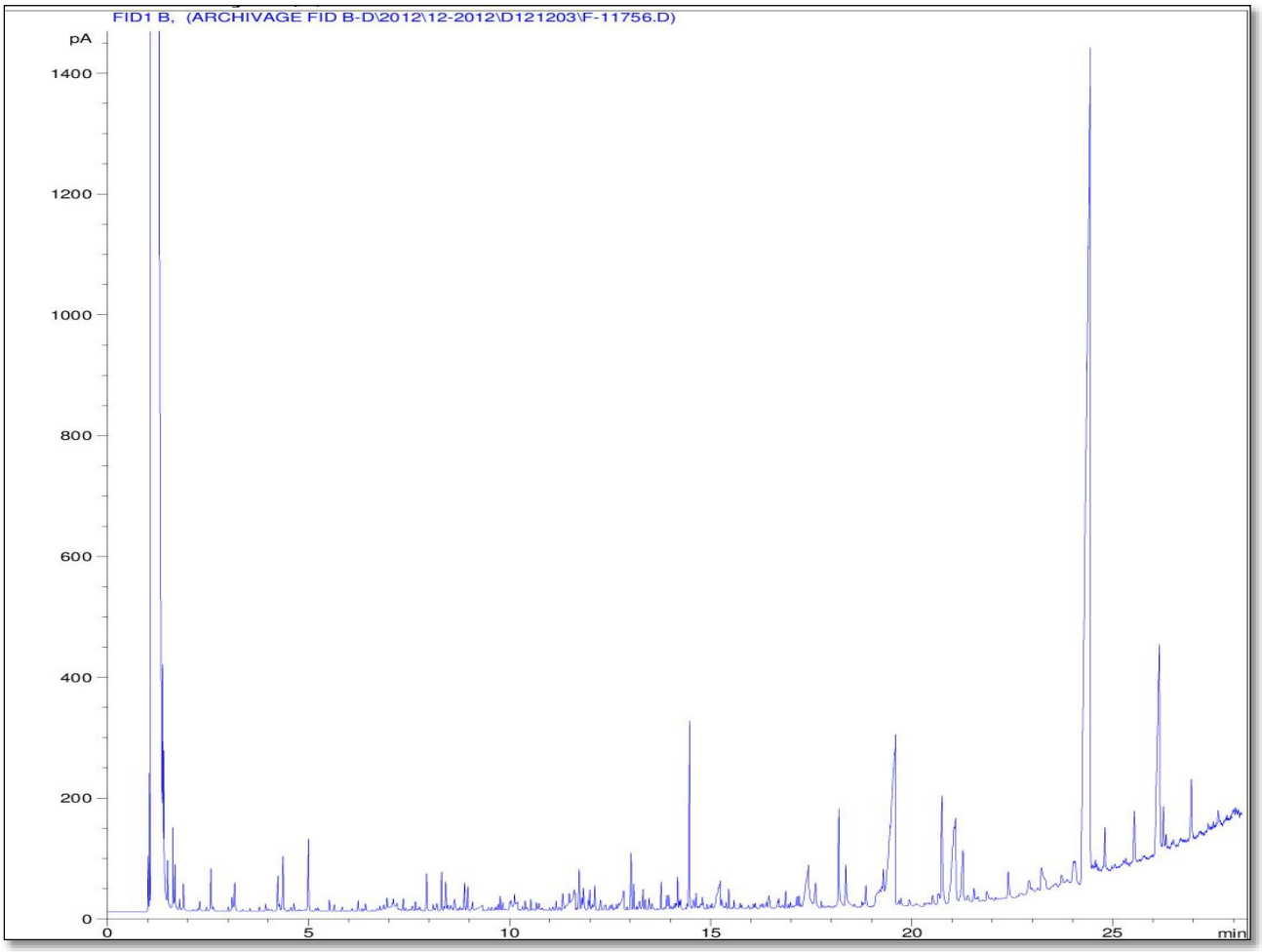

Figure 6: chromatogram of essential oil of *Galium tunetanum* Poiret

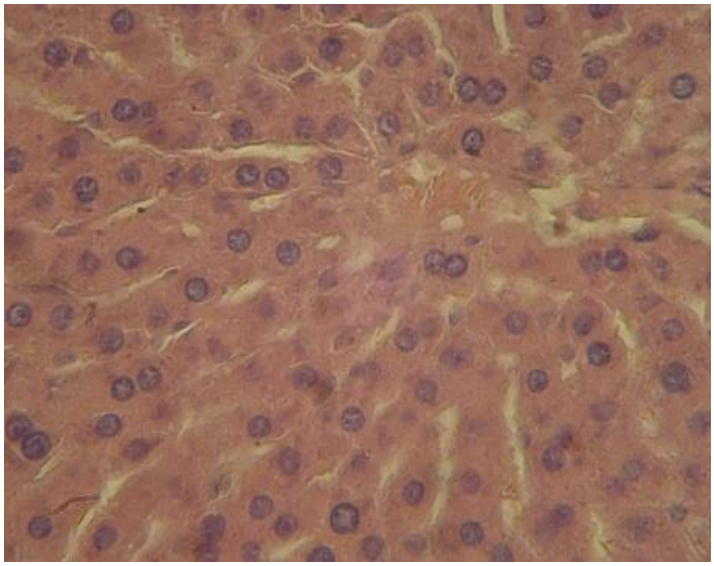

Figure 7: liver control tissue

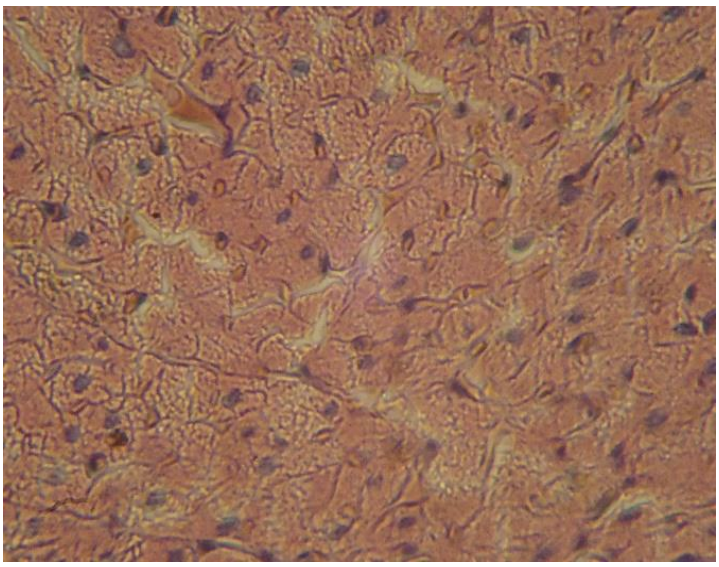

Figure 8: heart control tissue

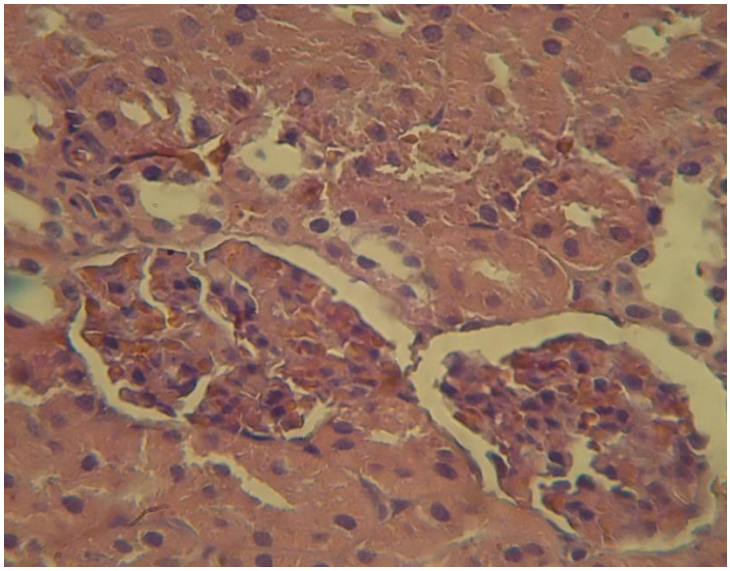

Figure 9: kidney control tissue

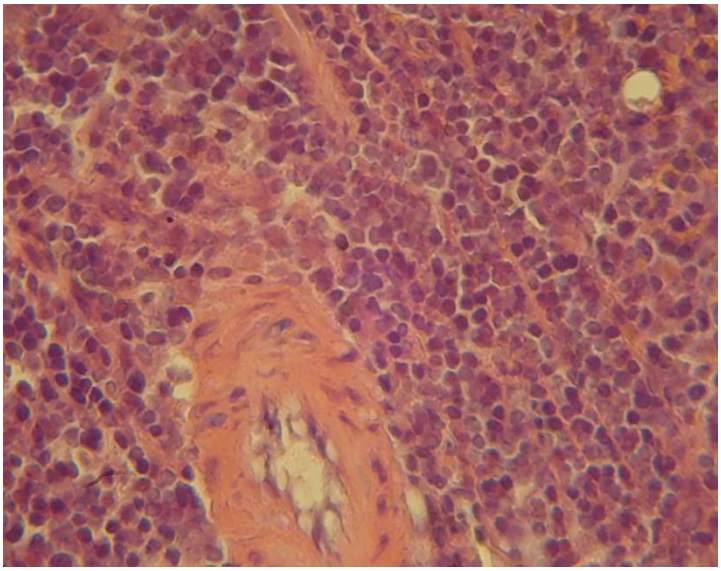

Figure 10: spleen control tissue

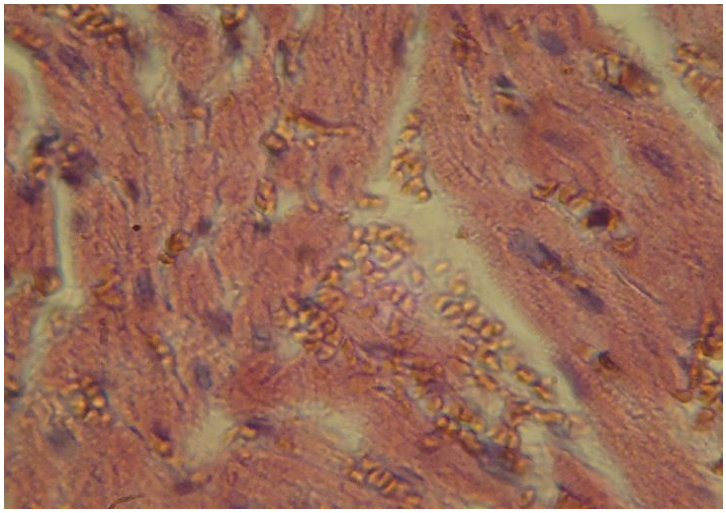

Figure 11: heart tissue of 1000 mg/kg treated group

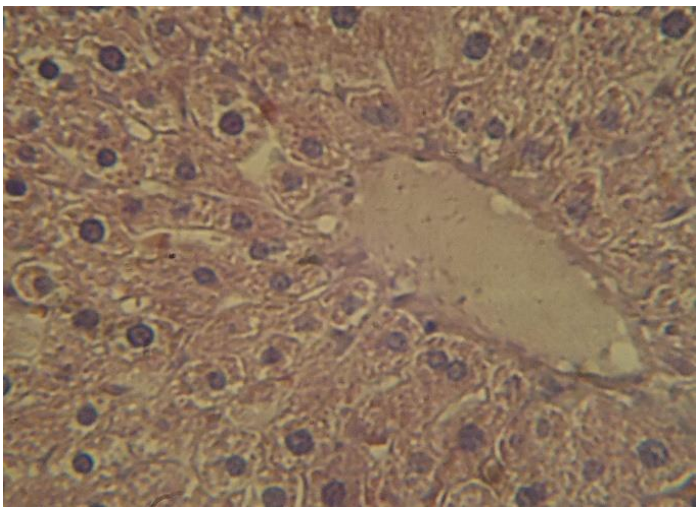

Figure 12: liver tissue of 1000g/kg treated group

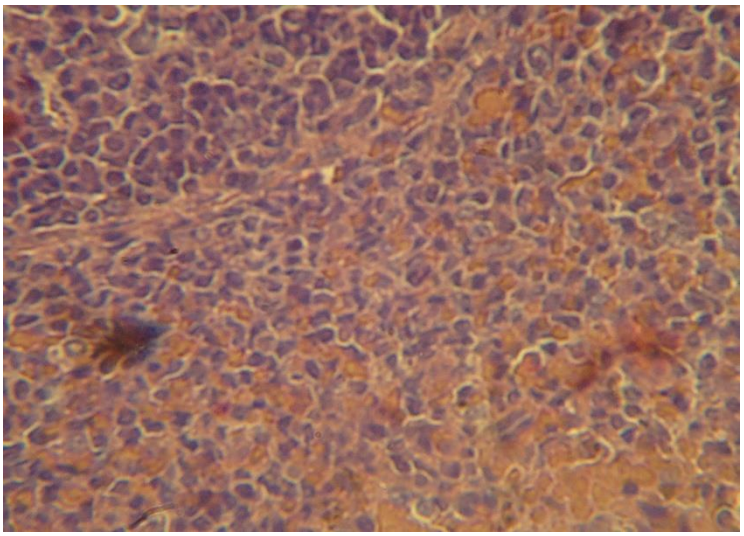

Figure 13: spleen tissue of 1000 mg/kg treated group

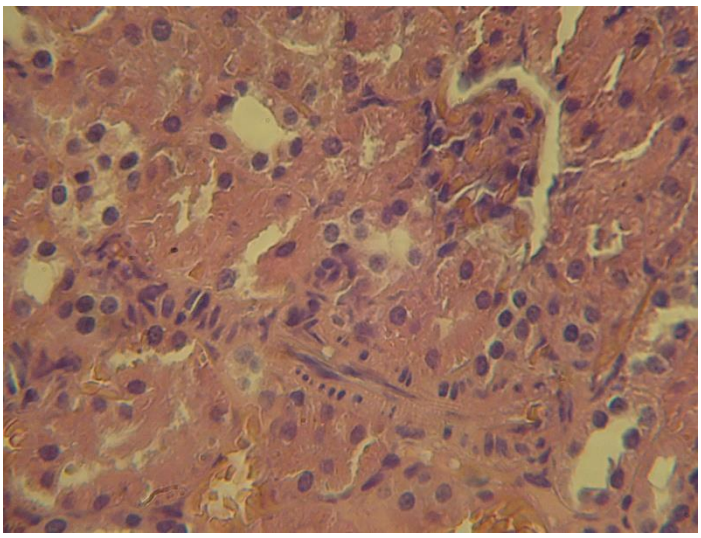

Figure 14: kidney tissue of 1000g/kg treated group

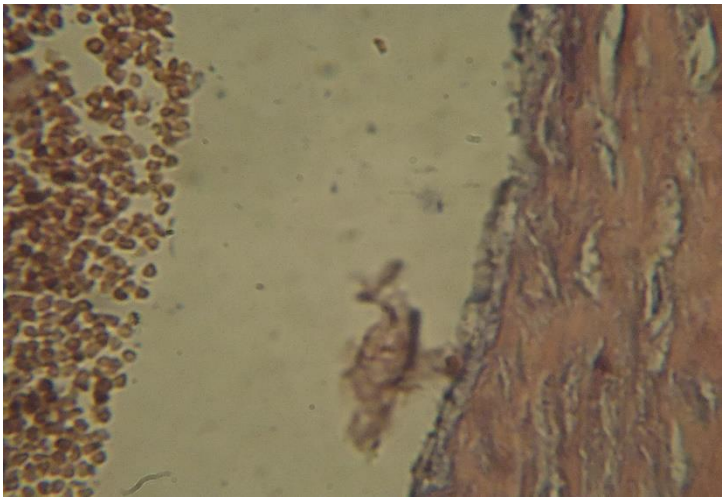

Figure 15: heart tissue of 3000 mg/kg treated group

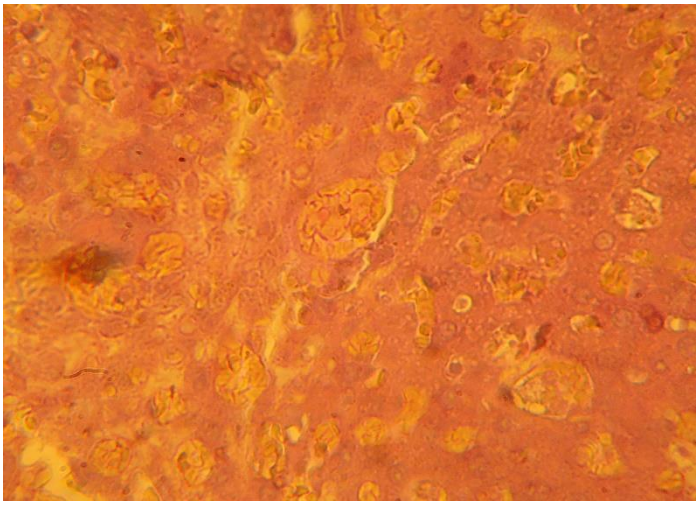

Figure 16: liver tissue of 3000g/kg treated group

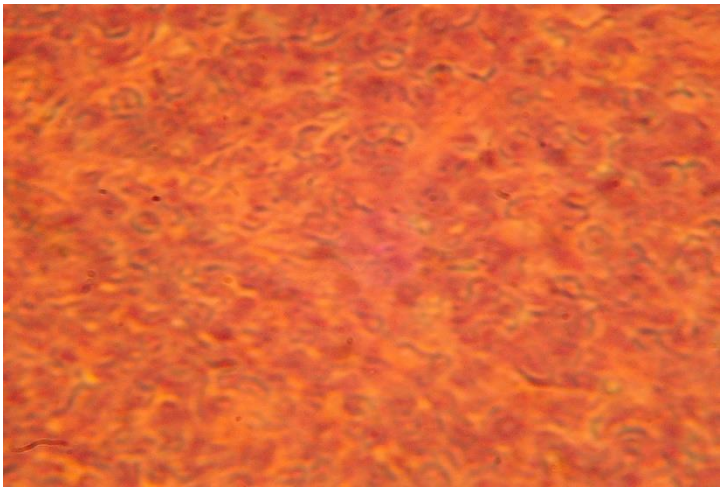

Figure 17: spleen tissue of 3000 mg/kg treated group

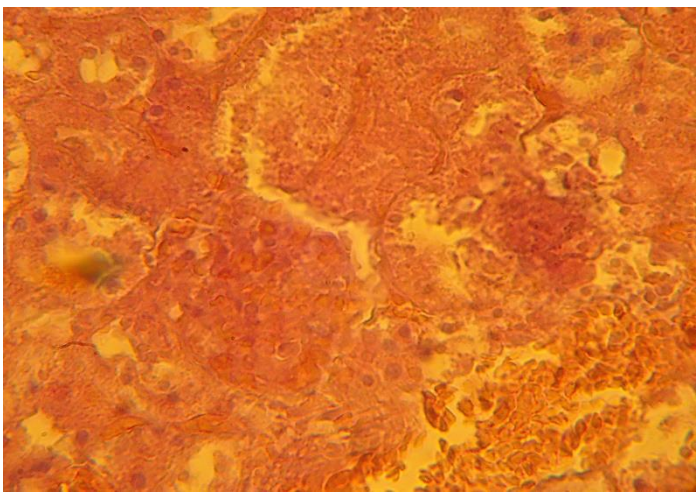

Figure 18: kidney tissue of 3000g/kg treated group

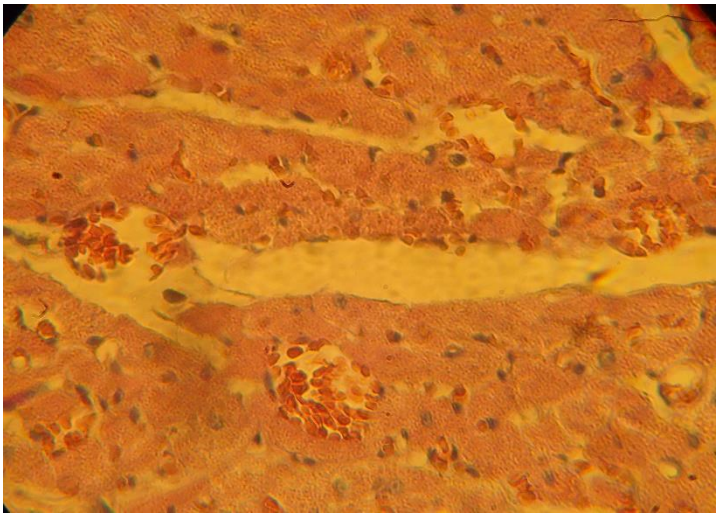

Figure 19: heart tissue of 6000 mg/kg treated group

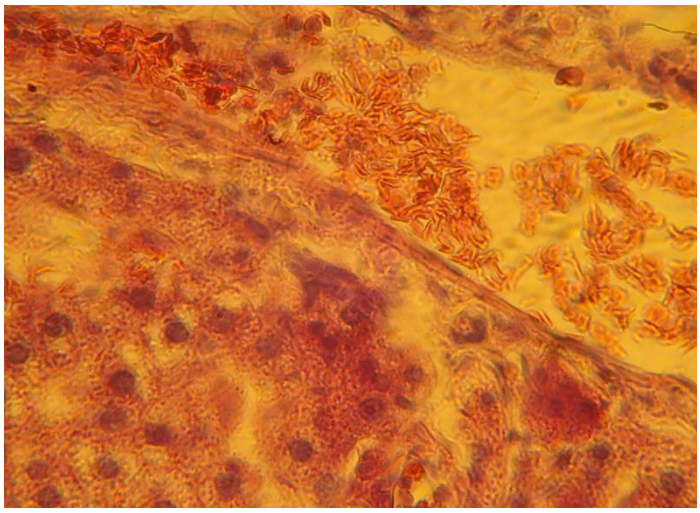

Figure 20: liver tissue of 6000g/kg treated group

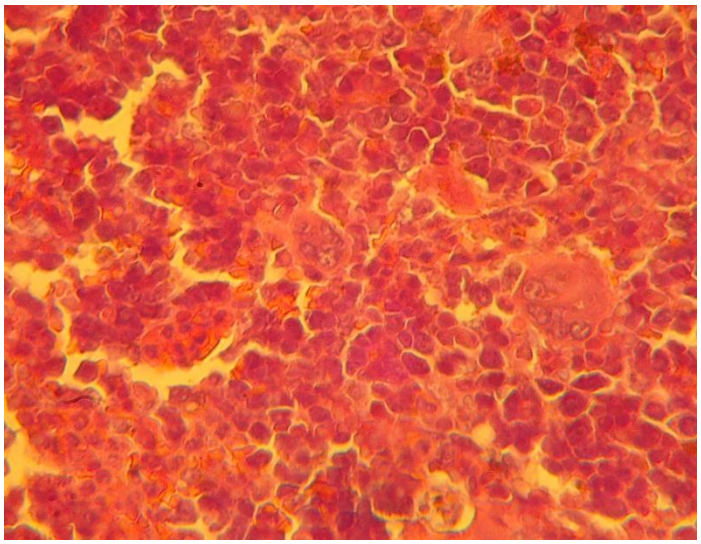

Figure 21: spleen tissue of 6000 mg/kg treated group

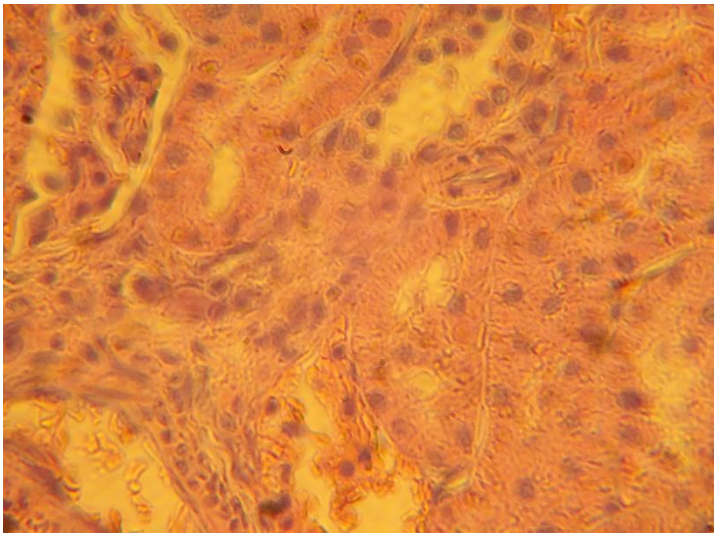

Figure 22: kidney tissue of 6000g/kg treated group

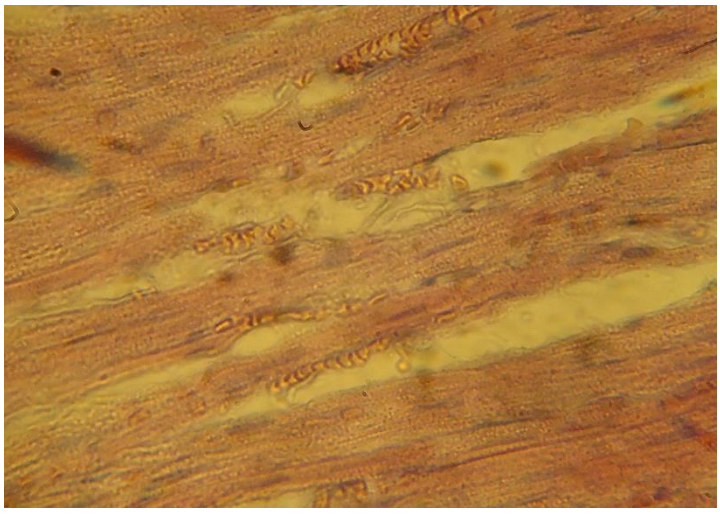

Figure 23: heart tissue of 9000 mg/kg treated group

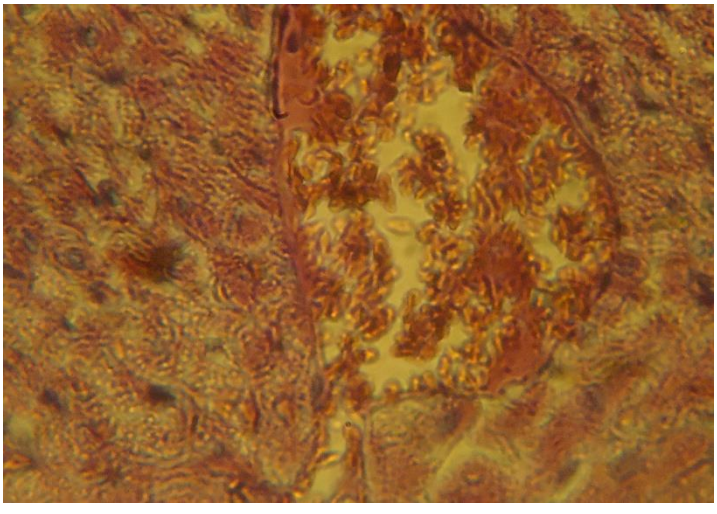

Figure 24: liver tissue of 9000g/kg treated group

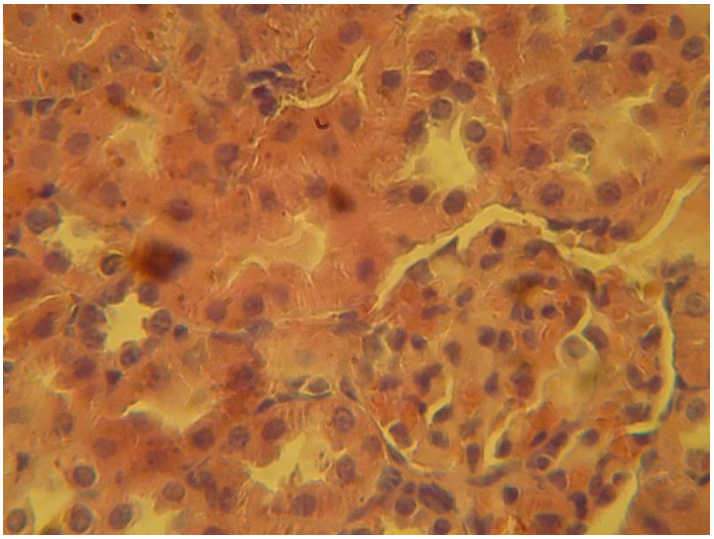

Figure 25: kidney tissue of 9000 mg/kg treated group

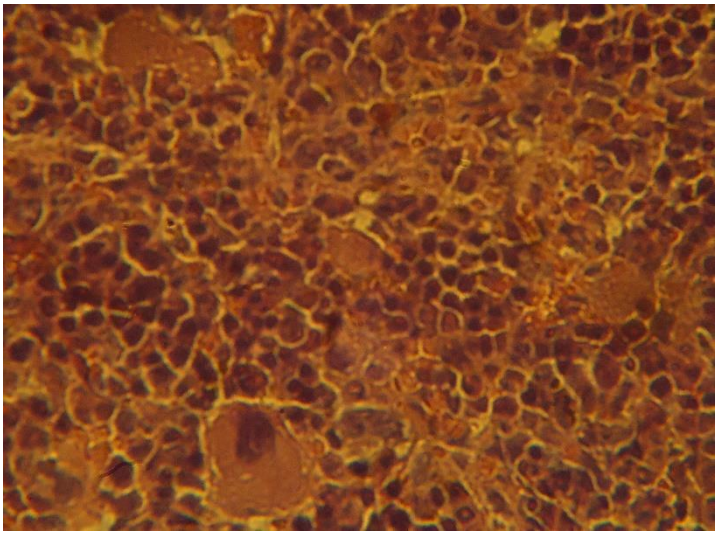

Figure 26: spleen tissue of 9000g/kg treated group

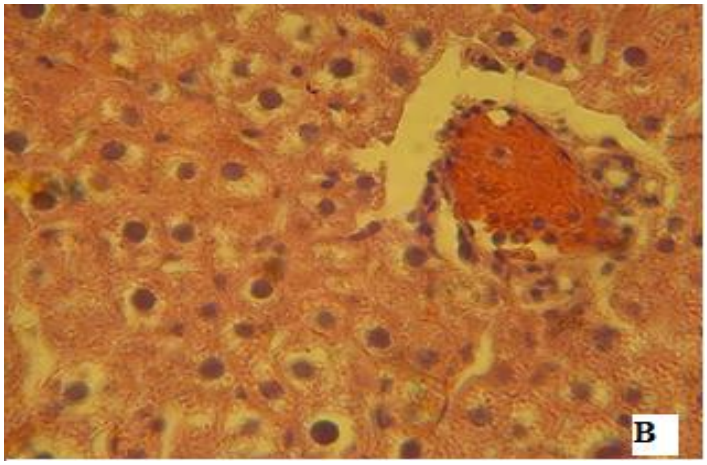

Figure 27: liver tissue of 12000 mg/kg treated group

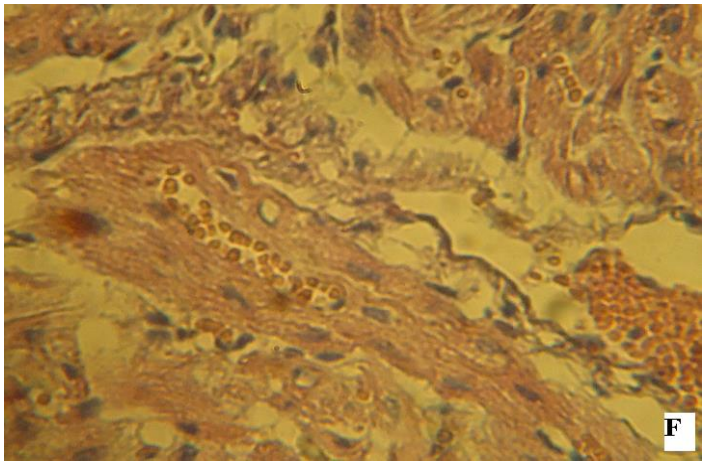

Figure 28: heart tissue of 12000g/kg treated group

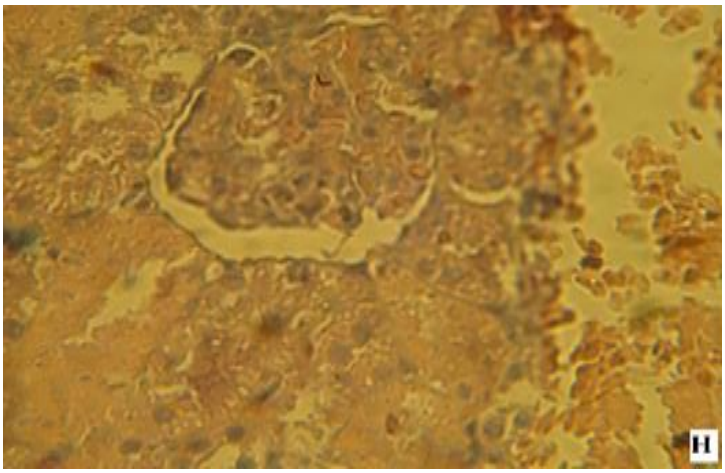

Figure 29: kidney tissue of 12000 mg/kg treated group

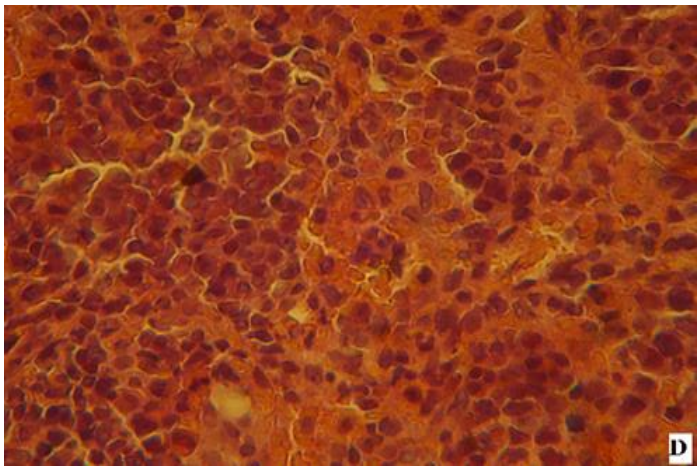

Figure 30: spleen tissue of 12000g/kg treated group

ملخص

تعتبر نبتة الجويسئة (*Galium tunetanum Poiret*) احدى النباتات المتبقية من أثار الغابات الكثيفة التي وجدت في المنطقة حيث تنتمي إلى الفصيلة الفَويّة المستوطنة شمال أفريقيا وتشكل هذه النبتة لغزا من حيث خصائصها الكيميائية و العلاجية. تم اخذ عينات المادة النباتية من جبل مقرس بسطيف -الجزائر حيث جففت في الظل، سحقت ثم تم استخلاص المركبات الكيمائية الثانوية، تم تثمين وتقييم الأنشطة المضادات للأكسدة بواسطة عدة اختبارات منها اختبارDPPH، اختبار بيتا-كاروتين واختبار إرجاع القوة. أما فيما يخص دراسة النشاطية المضادة للميكروبات، فقد تم تقديرها باستعمال ستة سلالات بكتيرية وثلاث فطريات من بينها خميرة وتم التحقق أيضا من النشاطية المضادة للالتهابات باستعمال عدة اختبارات منها اختبار ثبات اغشية خلايا الدم الحمراء (HRBC)، اختبار فقدان طبيعة البروتين في المختبر، اختبار الزيلين المسبب لذمة الأذن لدى الفئران واختبار القطن المسبب للورم. وقد درس أيضا التأثير المسكن للفلافونويدات المستخلصة من *Galium tunetanum* Poiret عن طريق اختبار الصفيحة الساخنة واختبار حمض الخليك. أما فيما يخص دراسة القرحة المعدية فتم استخدام جزيئات الدباغة كاختبار وقائي وعلاجي من خلال دراسة معايير مختلفة، إضافة الى دراسة مدى قدرة المستخلص المثيلي في علاج الجراح وفي النهاية تم دراسة السمية الحادة للنبتة. تعتبر جزيئات البوليفينول والصابونين المستخرجة من *Galium tunetanum* Poiret من المضادات للأكسدة الجيدة في حين كانت جميع الجزيئات المستخلصة أقل نشاطا أو غير نشطة ضد سلالات الميكروبات المستخدمة. وقد أظهرت مركبات الفينولية قدرات جيدة في علاج الأمراض الالتهابية وبالأخص مركبات الفلافونويد بجرعات منخفضة وغير سامة إضافة ان لها نشاط مسكن قوي في تثبيط الآلية المركزية للألم. اما فيما يخص جزيئات الدباغة فهي توفر حماية ضد تضرر الغشاء المخاطي للمعدة المحدث باستعمال الايثانول / حمض الهيدروكلوريد أو الايثانول المطلق. كما أظهرت النتائج أن للمستخلص المثيلي قدرات معتبرة في التئام الجراح.

الكلمات الدالة :جبل مقرس، جويسئة، مركبات الايض الثانوية، المضادة للأكسدة، المضادة للالتهاب، المسكنة للألم، المضادة للقرحة المعدية، المعالجة للجراح.

Summary:

Galium tunetanum Poiret is one of dense pre-existent forest vestige belong to Rubiaceae is a North African endemic species constitute an enigma toward their phytochemicals and therapeutic proprieties. Areal part of the plant material is harvested from Megriss – Setif - Algeria, dried in shade, and reduced to a fine powder; then the major groups of secondary metabolites were extracted and quantified. The evaluation of antioxidant activities of species were carried out with DPPH, β-Carotene bleaching test and reducing power assay. Whereas, the antimicrobial capacity was tested against six bacterial strains, three fungi and one yeast. The anti-inflammatory activity was investigated *in vitro* and *in vivo* by human red blood cell (HRBC) membrane stabilization method, the denaturation of protein *in vitro*, Xylene-induced ear oedema in mice and cotton pellet-induced granuloma. The analgesic effect of the flavonoids extracted from *Galium tunetanum* Poiret was studied using hot plate and acetic acid induced writhing tests. The antiulcer activity of tannins were carry out by the preventive and curative test by studying various parameters. All of these therapeutic activities were completed by the acute toxicity study of methanolic extract. Polyphenols and saponins can be good antioxidant agents. However, they were less active or inactive against strains used in antimicrobial activity. Phenolic compounds demonstrate good capacities to be used against inflammatory diseases, especially the flavonoids that are nontoxic and efficacy against this kind of diseases at low doses. Those flavonoids have also potent analgesic activity and demonstrate the utility of these molecules on central mechanism of pain inhibition. Moreover, tannins provide protection against gastric mucosal damage induced by either ethanol/HCl or absolute ethanol, and the methanolic extract was found to possess significant wound healing activity.

Key words: Megriss, *Galium tunetanum*; secondary metabolites; antioxidant; anti-inflammatory; analgesic; anti-ulcer; healing wounds