الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالى و البحث العلمى

Université Ferhat Abbas Sétif 1
Faculté des Sciences de la
Nature et de la Vie

جامعة فرحات عباس، سطيف 1
كلية علوم الطبيعة و الحياة

DEPARTMENT OF BIOLOGY AND ANIMAL PHYSIOLOGY

N°...../SNV/**2015**

THESIS

Presented by

MERGHEM Mounira

For the fulfillment of the requirements for the degree of

DOCTORATE OF SCIENCES

BIOLOGY

Special filed: **ANIMAL PHYSIOLOGY**

TOPIC

Evaluation of toxicity in mice and rats and antioxydant activities of *Ruta montana* L. extracts

Presented publically in*:* **…/…./2015**

JURY:

President:	BAGHIANI A.	**Pr. UFA Setif 1**
Supervisor:	DAHAMNA SERAICHE S.	**Pr. UFA Setif 1**
Examiners:	LALAOUI K.	**Pr. Univ Cons 1**
	NECIB Y.	**Pr. Univ Cons 1**
	DJENIDI R.	**Dr. Univ B.B.A**

Laboratory of Phytotherapy Applied to Chronic Diseases

ACKNOWLEDGMENTS

First and foremost, praises and thanks to the God, the Almighty, for providing me the blessings throughout my research work to complete the research successfully.

I wish to express my sincere gratitude to my supervisor Pr. Dahamna Saliha, for his valuable advices and guidance, timely suggestions and whole-hearted supports. His continuous interest and great generosity helped my work to be accomplished and also supported me during a course of this study.

I would like to thank my committee members, Professor Baghiani Abderrahmane University Ferhat Abbas Setif 1, Professor Laalaoui Nacer University of Constantine, Professor Necib Youcef University of Constantine and Dr Djenidi Redha University of Borj Boariridj for critical reading of the thesis, and for their valuable comments, discussions and suggestions.

I would like to thank Pr. Bourriche Hammama, Pr. Harzallah Daoud, Pr. Khennouf Seddik, Pr. Baghiani Abderrahmane, Pr. Arrar Lakhmisi University Ferhat Abbas Sétif 1, Pr. Touabti and Dr. Krache Kaouala Samira, University Hospital of Sétif for their great help.

I would like to thank my family, especially my mother and father for always believing in me, for their continuous love and their supports in my decisions. Without whom I could not have made it here.

I also would like to thank my friend Abir Razagui for his great help, advices and consultation

Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

Abstract

The aim of this study is to evaluate the acute and chronic toxicity of *Ruta montana* aerial part aqueous extract (Fidjel) used in folk medicine in Algeria in *Albino Wistar* male and female mice and rats as well as the antioxidant activity of the aqueous extract (AqE), methanolic extract (CrE), chloroformic extract (ChE) and ethyl acetate extract (EAE). The results showed that the ethyl acetate extract contains the highest amount of total polyphenols, tannins and flavonoids with values of 257.1±0.7 EAG/mg, 251 ± 1.41 EAT/mg, 117.4± 3.457EQ/mg, 136.2±9.876ER/ mg of extract, respectively. The study of the acute toxicity of aqueous extract at doses of 2, 4, 6, 8, 10 and 12 g/kg body weight by oral route in both sexes of mice, recorded no mortality, and showed no change in the general behavior of the treated animals. The study of chronic toxicity at the doses 100, 300 and 600mg/kg body weight in male and female rats for 90 days, recorded no mortality, and showed no change in the hematological and biochemical parameters. Treated animals showed a normal weight change compared with contral. The parametres of male fertility showed a significant decrease in the weight of testis, epididymis and seminal vesicle as well as a reduction in the number and the motility of spermatozoids at treated groups by the doses 300 and 600 mg/kg body weight compared to control group. DPPH scavenging assay showed that EAE has a higher anti-radical capacity (IC_{50} = 0.044 ± 0.001 mg/ml) followed by CrE, AqE and ChE with IC_{50} of 0.067 ± 0.002 ; 0.083 ± 0.003 et 0.146 ± 0.001mg/ml, respectively. Whereas, AqE showed the best inhibitory capacity of the coupled oxidation of linoleic acid/ β-carotene (90.34 ± 0.46%) and the best chelating capacity (IC_{50} = 0.051 ± 0.004mg/ml). The antioxidant activity of aqueous extract (AqE) and methanolic extract (CrE) in vivo was estimated using the antioxidant plasma capacity (APC), DPPH and reducing power tests. All extracts did not show any significant change. The activity of catalase (CAT), the level of MDA and GSH were evaluated in rat liver and kidney homogenate. The results showed a not significant increase in CAT activity in the groups treated with AqE 300mg/kg and CrE 100mg/kg by 30.76%, 32.07% for liver tissue and 24.48%, 32.11% for kidney tissue, and also in the GSH levels by 18.57%, 22.97% for liver tissue and 12.3%, 37.36% for kidney tissue. However no significant decrease in the level of MDA in the treated groups with AqE 300mg/kg and CrE 100mg/kg.

Key words: *Ruta montana* L., acute toxicity, chronic toxicity, antioxidant acivity polyphenol

Résumé

Lobjectif de cette étude est d'évaluer la toxicité aigu et chronique de l'extrait aqueux de la partie aérienne de *Ruta montana* (Fidjel) utilisée en médecine traditionnelle en Algérie sur des souris et rats *Albino Wistar* de sexe mâle et femelle. ainsi que l'activité antioxydante de l'extrait aqueux (AqE), l'extrait methanolique (CrE), l'extrait chloroformique (ChE) et l'extrait d'acétate éthyle (EAE). Les résultats ont montré que l'extrait d'acetate d'éthyle représente la quantité la plus elevée en polyphenols, tanins et flavonoides avec des valeurs de 257.1 ± 0.7 µg EAG/mg, 251 ± 1.41 µg EAT/mg, 117.4 ± 3.451 µg EQ/mg, 139.5 ± 4.107 µg ER/ mg d'extrait respectivement. L'étude de la toxicité aiguë de l'extrait aqueux avec les doses 2,4,6,8,10 et 12 g/kg du poids corporel par voie orale chez les souris mâles et femelles, n'a enregistré aucune mortalité, et n'a montré aucun changement dans le comportement générale des animaux traités, l'étude de la toxicité chronique avec les doses 100, 300 et 600 mg/kg du poids corporel chez les rats pendant 90 jours, n'a enregistré aucune mortalité et aucun changement dans les paramètres hématologiques et biochimiques. Les animaux traités ont connu une évolution pondérale normale en comparaison avec les témoins. Concernant les paramètres de la fertilité masculine, une diminution significative des poids des testicules, épididyme, vésicules séminales ainsi que le nombre et la mobilité des spermatozoïdes chez les rats traités par les doses 300 et 600 mg/kg du poids corporel. Le test du scavenger du DPPH a montré que l'extrait EAE a un pouvoir anti-radicalaire plus élevé (IC_{50} = 0.044 ± 0.001 mg/ml) suivi par CrE, AqE et ChE avec IC_{50} de 0.067 ± 0.002 , 0.083 ± 0.003 et 0.146 ± 0.015 mg/ml, respectivement. Alors que AqE a montré une meilleur capacité inhibitrice de l'oxydation couplée de l'acide linoléique/β-carotene (90.34 ± 0.46%) et une meilleure capacité chelatrice des ions (IC_{50} = 0.005 ± 0.004 mg/ml). L'activité antioxydante *in vivo* de l'extrait aqueux (AqE) et l'extrait méthanolique (CrE) est estimée par la capacité antioxydante plasmatique (CAP), le test de DPPH et du pouvoir réducteur n'a montré aucun changement significatif. L'activité de la catalase (CAT) et les taux du MDA et du GSH ont été évalués dans l'homogénat du foie et du rein des rats. Les résultats à montré une augmentation non significative dans l'activité de la CAT dans les groupes traités par AqE 300mg/kg et CrE 100mg/kg par 30.76%, 32.07% pour le tissu hépatique et 24.48%, 32.11% pour le tissu rénal, et aussi dans les taux du GSH par 18.57%, 22.97% pour le tissu hépatique et 12.3%, 37.36% pour le tissu rénal. Cependant, une diminution non significative du taux du MDA dans les groupes traités par AqE 300mg/kg et CrE 100mg/kg.

Mots clés : *Ruta montana* L., toxicité aigüe, toxicité chronique, activité antioxidante, polyphénols

ملخص

تهدف هذه الدراسة إلى معرفة التأثير السمي الحاد و المزمن للمستخلص المائي للجزء الهوائي لنبتة *Ruta montana* (الفيجل) المستعملة في الطب التقليدي في الجزائر على فأران و جرذان بيضاء ذكور و أخرى اناث. بالاضافة إلى تقدير النشاطية المضادة للأكسدة للمستخلص المائي (AqE),المستخلص الميثانولي (CrE), مستخلص الكلوروفورم (ChE) و مستخلص ايثيل الأسيتات (EAE). بينت النتائج المتحصل عليها أن مستخلص ايثيل الأسيتات يحتوي على أكبر كمية من الفينولات، الدباغ و الفلافونويدات : 257.1±0.7 ميكروغرام مكافئ حمض الغاليك/غ مستخلص, 251±1.41 ميكروغرام مكافئ حمض التانيك/غ مستخلص, 117.4±3.451 ميكروغرام مكافئ الكرستين/غ مستخلص, 139.5± 4.107 ميكروغرام مكافئ الرتين/غ مستخلص، على الترتيب. لم تبدي دراسة التأثير السمي الحاد للمستخلص المائي على فأران ذكور و اناث بجرعات 2, 4, 6, 10 و 12غ/كغ من وزن الجسم عن طريق الفم أي تغيرات ملحوظة في السلوك العام, كما لم نسجل أي حالة وفاة. لم تسجل دراسة السمية المزمنة على الجرذان بجرعات 100, 300 و 600 مغ/كغ من وزن الجسم لمدة 90 يوم أي حالة وفاة كما لم تسجل أي تغيير في المؤشرات الدموية و البيوكيميائية. عرفت الجرذان المعالجة زيادة عادية في الوزن مقارنة بالحيوانات الشاهدة. أما فيما يخص مؤشرات الخصوبة عند الذكور فقد سجل انخفاض معنوي في وزن كل من الخصية, البربخ و الحويصلة المنوية و كذلك انخفاض في عدد و حركة الحيوانات المنوية عند الجرذان المعالجة بالجرعتين 300 و 600 مغ/كغ من وزن الجسم. بين اختبار جذر DPPH ان EAE يمتلك اعلى قدرة ازاحة (IC_{50} = 0.044 ± 0.001 مغ/مل) يليه كل من ChE, AqE و CrE ب : 0.067 ± 0.002, 0.083 ± 0.003 و 0.146 ± 0.015 مغ/مل على الترتيب. في حين بين AqE قدرة عالية على استخلاب ايونات الحديد بقيمة IC_{50} = 0.005 ± 0.004 مغ/مل كذلك قدرة عالية على تثبيط الاكسدة المزدوجة لبيتا كاروتين/حمض اللينولييك. تم تقدير النشاطية المضادة للاكسدة في الكائن الحي للمستخلص المائي و الميثانولي بواسطة النشاطية المضادة للاكسدة للبلازما. حيث لم يظهر اختبار DPPH و القدرة الارجاعية اي تغيير معنوي. كما تم تقدير نشاطية CAT و نسبة MDA و GSH في نسيج الكبد و الكلية. اظهرت النتائج ارتفاع غير معنوي في نشاطية CAT في المجموعات المعالجة بالمستخلص المائي 300 مغ/كغ و المستخلص الميثانولي 100 مغ/كغ ب 30.76 % ,32.07% بالنسبة لنسيج الكبد و 24.48%, 32.11% بالنسبة لنسيج الكلية و كذلك ارتفاع غير معنوي في GSH ب 18.57%, 22.97% بالنسبة لنسيج الكبد و 12.3%, 37.36% بالنسبة لنسيج الكلية و انخفاض غير معنوي لنسبة MDA في المجموعات المعالجة بالمستخلص المائي 300 مغ/كغ و المستخلص الميثانولي 100 مغ/كغ.

الكلمات المفتاح : *Ruta montana*, السمية الحادة, السمية المزمنة, النشاطية المضادة للاكسدة, عديدات الفنول.

ABREVIATIONS

ALT	alanine aminotransferase
ALB	albumin
ALP	alkaline phosphatase
AqE	aqueous extract
AST	aspartate aminotransferase
BHT	butylated hydroxytoluene
ChE	chloroform extract
CHOL	cholesterol
CK	creatine kinase
CREA	creatinine
CrE	crud extract
DPPH	2,2'-diphenyl-1-picrylhydrazyl
EAE	ethyl acetate extract
EDTA	ethylene diamine tetra acetic acid
GAE	gallic acid equivalents
GLU	glucose
GPx	glutathion peroxidase
GSH	gluthathion reduced
GSSG	glutathion disulfure oxidized
Hct	hematocrit
Hb	hemoglobin
HxE	hexane extract
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid

IC50 %	inhibitory concentration for 50% of activity
LOO°	lipid peroxide radical
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
VGM	mean corpuscular volume
MeOH	methanol
NO°	radical nitroxid
NOS	nitric oxidesynthase
$^1O_2$	singlet oxygen
O2°-	superoxid Radical
TG	triglycerides
OH°	hydroxyl Radical
ONOO-	peroxynitrite
MPV	platelets count and mean platelet volume
QE	quercetin equivalents
RBC	red blood cells
RNS	reactive nitrogene species
ROS	reactive oxygen species
RE	rutin equivalent
SD	standard deviation
SOD	superoxyde dismutase
AU	uric acid
WBC	white blood cell

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Summary

Introduction

Medicinal plants have important contributions in the healthcare system. Use of herbal medicines represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A number of modern drugs currently in use have been obtained through medicinal plants.

Despite the profound therapeutic advantages possessed by some of the medicinal plants, some constituents of medicinal plants have been found to be potentially toxic, mutagenic, carcinogenic and teratogenic. This raises concern about the potential toxic effects resulting from the short-term and long-term use of such medicinal plants. Therefore, evaluating the toxicity effects of any medicinal plants extracts intended to be used in humans and animals is of greatest significance.

In living systems, oxidation is a basic part of the normal metabolic process, in which reactive oxygen species (hydrogen peroxide and hypochlorous acid) and many free radicals (hydroxyl radical (OH) and superoxide anion) are generated. Rapid production of free radicals may cause alteration in the structure and function of cell constituents and membranes and can results in human neurologic and other disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular, neurodegenerative diseases, and premature aging. Therefore, the prevention of the above conditions requires the presence of antioxidants or the free radical scavenging molecules in the body (Pinton *et al*., 2012).

Medicinal plants and products made from plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng and Wang, 2001; Cai *et al*, 2003). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen *et al*, 2000; Sala *et al*, 2002).

Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals (Kahkonen *et al*, 1999). They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Proestos *et al*, 2006).

The medicinal plant *Ruta montana* L. (Family Rutaceae), is used in folk medicine as hypoglycemic, antirhematic, antihelmintic, antiepileptic, antipyretic. It is also used in treating intestinal and hepatic diseases. This plant contains various active principles able to inhibit the growth of mycobacteria. The antioxidative effect of plant extracts is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. In Algerian folk medicine, *Ruta montana* L. is also used against child fevers and as an abortive drug.

The main objectives of this study were:

- Determination of the LD_{50} of the *Ruta montana* L. aqueous extract.
- Evaluation of the acute and chronic toxicity of aqueous extract of aerial parts of *Ruta montana* L. on male and female *Albino Wistar* mice and rats.

- Evaluation of the effect of *Ruta Montana* L. aqueous extract on fertility.
- Evaluation of the effect of *Ruta montana* L. aqueous extract on organs histology.
- Evaluation of the *in vitro* and *in vivo* antioxidant activity.

1. Toxicity of medicinal plants

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially, those derived from plants. This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient, abusive and/or incorrect use of synthetic drugs results in side effects and other problems (Kiranmai and Kiran, 2014).

Traditionally, herbs have been considered to be nontoxic and have been used for treating various problems by the general public and traditional medicine doctors worldwide (Oduola *et al*, 2007). Although, the literature has documented several toxicity resulting from the use of herbs on many occasions, still the potential toxicity of herbs has not been recognized by the general public or by professional groups of traditional medicine (O'Hara *et al*, 1998). Poisonous plants are those which cause serious problems or even death occur, if a small quantity of its stem, leaves, seeds, fruits and roots are ingested. Some other plants are normally harmless but they may become toxic if preparative from them are taken in excess in strong doses or for a long period of time (Khajja *et al*, 2011). Toxicity in herbal medicine may be due to: Accidents due to mistake in botanical identification, accidental ingestion of cardiotonic plants, intoxication by popular remedies and plants that interfere with conventional pharmacological therapy (Rates, 2001).

Poisonous plants can cause superficial irritation or discomfort through contact with the skin or serious poisoning when ingested (Ndhlala et al, 2013). Toxic substances from plants can affect the entire spectrum of vital human organs while some may affect key functional body systems like the central nervous system (CNS) ,thereby interfering with the coordination of nerve functions of the body. The most dominant toxins are neurotoxins that affect the brain and CNS, followed by cytotoxins and metabolic toxins that affect

organs such as kidneys, liver, heart and lungs. The severity of a toxic effect may depend on the route of administration, growth stage or part of the plant, the amount consumed, the species and susceptibility of the victim (Botha and Penrith, 2008). Other factors that may influence the severity of toxins include the solubility of the toxin in body fluids, frequency of intoxication as well as the age of the victim.

The toxic effect of plants is based on their chemical constituents which are classified into alkaloids, glycosides, proteins, oxalates, anti-vitamins, tannins etc. They act by altering specific mechanisms involving enzymes, receptors and even genetic material at particular cells and tissues (Chandra Sekhar *et al*, 2012).

- **Alkaloids**

Alkaloids are among the most potent toxic. It is a heterogeneous group of complex organic compounds to alkaline reaction with important physiological activity. Alkaloids in general have profound toxic effects on the nervous systems of mammals due to interaction with neuronal tissue (Donald and Billie, 1978).

The pyrrolizidine alkaloids, for example, exert potent hepatotoxicity and mutagenic activity (Donald and Billie, 1978). Also, the alkaloid ephedrine contained in ephedra herb can cause serious toxic reactions ranging from liver damage to severe high blood pressure and heart problems (George, 2011).

In addition, intoxications with tropane alkaloids are characterized by dryness of the mucosa in the upper digestive and respiratory tract, constipation, pupil dilation and disturbance of vision, photophobia and changes in heart rate, hypotension, nervousness, restlessness, irritability, disorientation, ataxia and respiratory depression (Alexander *et al*, 2008).

- **Glycosides**

The diterpene glycoside, atractyloside, is present in a number of plants used as ethnomedicines throughout Africa (Obatomi and Bach 1998; Dahamna et al., 2004). These plants are known to cause acute fatal renal and liver damage in humans and domestic animals foraging on atractyloside-containing plants (Stuart *et al*, 1981; Martin *et al*, 1986). Pure atractyloside also produces a similar lesion in animals (Carpenedo *et al*, 1974; Hatch *et al*, 1982). Histologically, there was usually marked centrilobular degeneration of the liver (Caravaca-Ma- garinos *et al*, 1985; Georgiou *et al*, 1988; Hedili *et al*, 1989) and proximal tubular necrosis of the kidney (Koechel and Krejci, 1993). Atractyloside was also found to alter catabolic and anabolic functions *in vivo* (Georgiou *et al*, 1988).

A number of studies have examined the acute, chronic and carcinogenic effects of coumarin in the rat and mouse. In studies involving the rat, hepatic biochemical and morphological changes have been examined for various periods of coumarin administration (1 week to 2 years). Depending on dose administered, coumarin treatment results in an increase in relative weight and changes in various hepatic biochemical parameters. Single oral doses of coumarin have been shown to produce liver necrosis and increase plasma transaminase activities in DBA/2 strain mice (Lake, 1999).

In addition to these, saponins inhibits cellular respiration (Wittstock and Gershenzon, 2002). And are potent surfactants that can disrupt lipid-rich cellular membranes of human erythrocytes and microorganisms which explain the potent antimicrobial properties of this group of phytochemicals (Francis, 2002).

- **Proteins and peptides**

Proteins from plants are an important source in food. Amino acids are absorbed from the intestine of man and animals and are built up into adapted proteins. Nevertheless, there are also plant proteins and peptides with bioactivity. They are often not hydrolyzed in the digestive tract, but may to a certain extent be absorbed and exert their specific action in the body. Euphorbiaceae (spurge family) include plants producing such proteins, for instance ricin (lectin) in seeds of Ricinus communis (castor bean) which inhibits protein synthesis and induce systemic effects in animals and humans, with gastrointestinal symptoms dominating. Far less potent lectins are also present in seeds of several species of Fabaceae (bean family). Colic and other gastrointestinal symptoms may occur if seeds are eaten without sufficient heat treatment, which inactivates many lectins (Bernhoft, 2010).

- **Oxalates**

According to Blood and Henderson (1974), the ingestion of an excessive amount of oxalate could cause gastrointestinal irritation, blockage of the renal tubules by calcium oxalate crystals, development of urinary calculi, hypocalcaemia, muscular weakness or paralysis.

- **Minerals and heavy metals**

Another implication in the toxicity of certain herbs is the presence of toxic minerals and heavy metals like mercury, arsenic, lead and cadmium (Dwivedi and Dey, 2002). Lead and mercury can cause serious neurological impairment when an herbal medicinal product contaminated with these metals is ingested (Amster et al, 2007).

1.1. Goals of toxicity testing of herbal drugs

The primary goal of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. Two important factors which are taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed.

An equally important objective of toxicity testing is the detection of toxic plant extracts or compounds derived thereof in the early (pre-clinical) and late (clinical) stages of drug discovery and development from plant sources. This will facilitate the identification of toxicants which can be discarded or modified during the process and create an opportunity for extensive evaluation of safer, promising alternatives (Gamaniel, 2000).

1.2. Definition of Toxicity

Toxicity was defined as an aspect of pharmacology which deals with the adverse effects of bioactive substances on living organisms. Toxicological studies and experiments are very essential to establish the safety and efficiency of any new drug and to make a decision whether this drug should be adopted for clinical use or not (Anisuzzaman *et al*, 2001; Alam *et al*, 2006).

Depending on the duration of exposure of animals to drug, toxicological studies may be of three types: acute, sub-acute and chronic studies (Baki *et al*, 2007). Acute and chronic toxic effects differ principally from each other with respect to the amount of chemical compound involved and the time intervening before the effect is seen (Timbrell, 2002). Acute effects are normally observed soon after exposure and result from the uptake

of large amounts of poison, generally as a single dose. On the other hand, chronic effects are often detected over an extended period of time during which exposure may be continuous or intermittent, though obviously at levels which are too low to produce an acute effect (Loomis and Hayes, 1996; Pascoe, 1983).

1.3. Acute toxicity

Acute toxicity represents the adverse effects occurring immediately after the administration of a single dose of the tested substance, or after multiple doses given within 24 hours (Duffus *et al*, 2009).

All acute toxicity tests are performed on either rats or mice because of the low cost, the availability of the animals and the fact that toxicological reference data for many compounds in these species are available (Loomis and Hayes, 1996). In addition, these animals may have a similar metabolism manner and metabolites pharmacodynamics as well as human.

Acute toxicity studies are commonly used to determine the Lethal Dose 50 (LD_{50}) of a drug or chemical (statistically, LD_{50} is the dose of a substance that can be expected to cause the death of 50% of the tested animals). Since a great range of concentrations or doses of various chemicals may be involved in the production of harmful effects, the LD_{50} has been used by some authors to devise categories of toxicity on the basis of the amounts of the chemicals necessary to produce harm. An example of such a categorization, along with the respective lethal doses, is given in table 1.

Table 1. Classification of toxicity based on LD_{50} dose ranges Hodge and Sterner (Frank, 1992).

LD_{50} (mg/kg)	**Classification**
1 or less	Extremely toxic
1 to 50	Highly toxic
50 to 500	Moderately toxic
500 to 5000	Slightly toxic
5000 to 15000	Practically non-toxic
More than 15000	Relatively harmless

1.4. Sub-acute toxicity

In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 14 to 21 days. Sub-acute toxicity studies are used to determine effect of drug on biochemical and hematological parameters of blood as well as to determine histopathological changes (Baki *et al*, 2007; Dahamna, 1987; Dahamna *et al*., 2004; Belguet, 2010).

1.5. Chronic toxicity

Chronic toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical. In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug (Baki *et al*, 2007; Belguet, 2010). The parameters of chronic toxicity studies are same as that of sub-acute study. Multiple dose studies are necessary to assure the safety of natural products. On the other hand, clinical observations of acute assays are valuable tools to define the doses to be tested in multiple dose experiments,

along with pharmacological studies in animals and in humans (Alvarez *et al*, 2004; Hasumura *et al*, 2004, Merghem *et al*.; 2013; Boussahel *et al*., 2013).

1.6. Routes of administration

This term refers to the way in which drugs or compounds are introduced to animals or humans. To evaluate toxicity of a compound in animals, various routes may be used but two most commonly used modes of administration for animals studies are *via* intra-peritoneal injection or the oral route (Poole and Leslie, 1989).

1.6.1. Intra-peritoneal injection

This is one of the methods of dosing, which may occasionally provide information about local as well as systemic toxicity. To give drugs by intra-peritoneal injection, the animal is laid on its back and the abdomen shaved. This area is thoroughly cleansed and, using an appropriate syringe and needle, the abdominal wall is punctured. To ensure minimal danger of perforation of abdominal viscera, the injection should be made rostral and lateral to the bladder at an angle of about 15º to the abdomen. The depth of penetration should not exceed 5mm (Poole and Leslie, 1989; Waynforth, 1980).

1.6.2. Oral administration

The oral route is probably one of the most common means by which a chemical enters the body. In short, the oral administration is the form of administration involving the gastrointestinal tract, which may be viewed as a tube going through the body, starting at the mouth and ending at the anus. Although it is within the body, its contents are essentially exterior to the body fluids. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract. Oral administration of chemicals that are rapidly absorbed

from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained if other routes of administration were used (Loomis and Hayes, 1996). Furthermore, if a compound entered the entero-hepatic cycle, at least a portion of the compound would be localized in the organs involved in the cycle. Compounds that are known to be toxic to the liver would be expected to be more toxic following oral administration on repeated occasions, whereas their administration by other routes may be less hazardous (Loomis and Hayes, 1996; Waynforth, 1980).

1.7. Target tissue Toxicity

The extent to which an organ is susceptible to toxicity varies from tissue to tissue. For example the kidneys and liver are more highly vascularised making them more susceptible to toxicity than the bone tissues (Viala and Botta, 2007).

1.7.1. Hepatotoxicity

The liver is the largest internal organ in the body and is divided by the falciform ligament into two lobes: a large right lobe and a smaller left lobe. Each lobe is further divided into lobules which are the functioning units of the liver (Figure 1).There are approximately a million lobules in the liver filled with hepatocytes. The hepatocytes are responsible for bile secretion and also perform a variety of metabolic functions. Between each row of hepatocytes are small cavities called sinusoids (Berne and Levy, 1998). The main functions of the sinusoids are to destroy old or defective red blood cells, to remove bacteria and foreign particles from the blood, and to detoxify toxins and other harmful substances (Silverthorn, 2007).

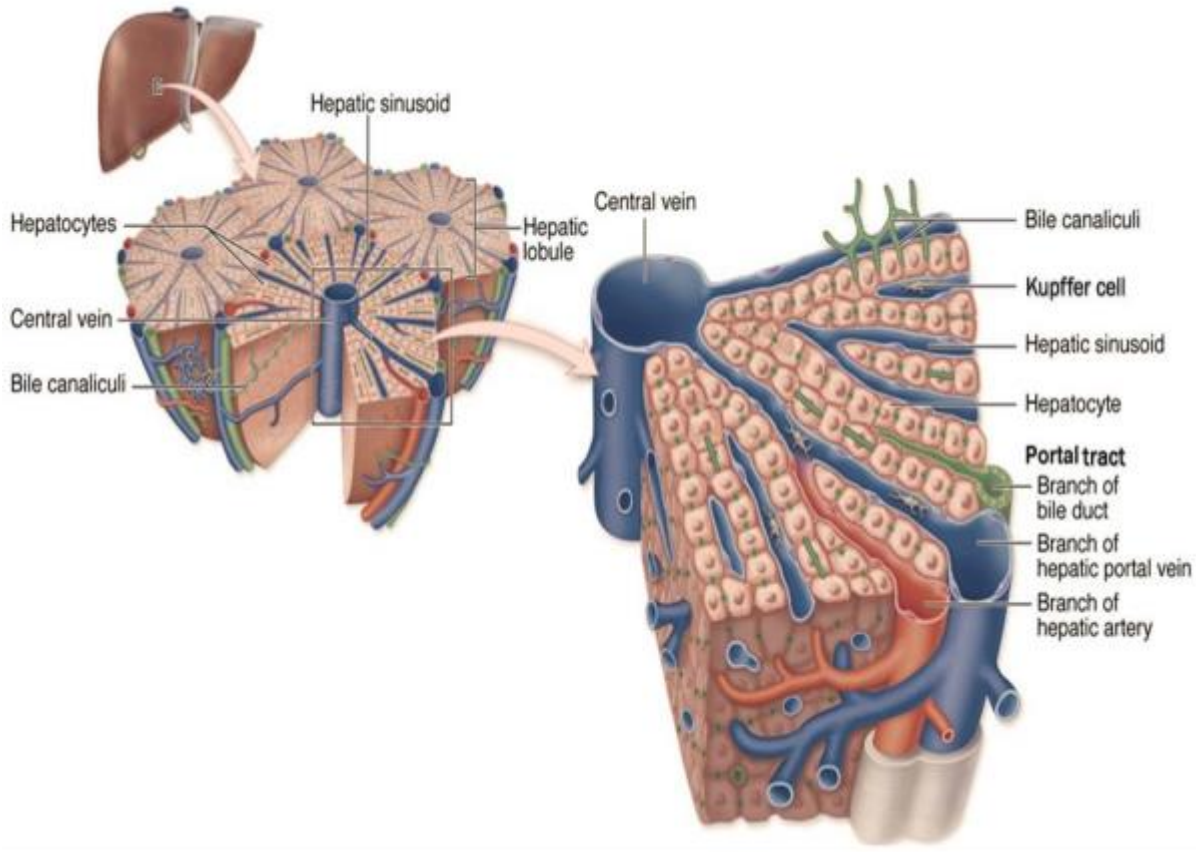


Figure 1. Structural organization of the hepatic lobule (Jacquelyn and Maher, 1997).

The liver is the target organ for chemically induced injuries. Several important factors are known to contribute to the liver's susceptibility. First, compounds absorbed in the gut tract are transported by the hepatic portal vein to the liver. Thus the liver is the first organ perfused by chemicals absorbed in the gut. A second factor is the high concentration in the liver of xenobiotic metabolizing enzymes (Deshpande, 2005; Wallace and Meyer, 2010).The liver receives 25% of the blood supply from the heart. Toxic substances absorbed from the gut are transported directly to the liver which is therefore the first target organ exposed after the gut itself. Hepatocytes are cells that make up most of the structure of the liver and are very metabolically active. Normally, they are involved in many essential biochemical processes, such as removal of nitrogen as urea, synthesis of glycogen as a glucose store, and lipid metabolism. Many toxic substances inhibit protein synthesis

because of their action in the liver. The liver also has a key role in removing external poisons (xenobiotic) (Baker, 2012).

Several plant extracts have been examined for use in a wide variety of liver disorders, Many studies have already led to the characterization of more than170 constituents isolated from 110 plants belonging to 55 families have been reported to treat liver diseases and boost liver functions (Mukazayire *et al*, 2010). While others have been reported to cause injury to the liver (Atawodi *et al*, 2011), includes elevated liver enzymes, acute or chronic hepatitis, cholestasis, hepatic necrosis or fibrosis, cirrhosis, liver failure, and hepatic veno-occlusive disease (Abdualmjid et Sergi, 2013).

1.7.2. Nephrotoxicity

The kidneys are an essential part of the urinary system responsible for producing and excreting urine into a ureter and also serve homeostatic functions such as the regulation of electrolytes. They serve the body as a natural filter of the blood, and remove wastes which are diverted to the urinary bladder. In producing urine, the kidneys excrete wastes such as urea and ammonia; the kidneys also are responsible for the reabsorption of water, glucose, and amino acids. The renal capsule is the outer skin of the kidney. The outer portion of the kidney consists of the cortex and the medulla containing millions of tiny structures called nephrons (Wheater *et al*, 1982) (Figure 2). The nephrons are responsible for filtering water out of the bloodstream. The nephron is composed of two main parts: the renal corpuscle and the renal tubule. Inside the renal corpuscle is the glomerulus, a network of blood capillaries that is surrounded, first, by a double membrane (the glomerular capsular membrane) and then is surrounded by Bowman's capsule. The renal tubule consists of the proximal tubule, the loop of Henle, and the distal tubule (which eventually leads into collecting tubules) (Silverthorn, 2007).

Frequently, exposure to large amounts of a chemical can cause kidney effects that are not observed at lesser exposures. Effects of kidney damage are frequently assessed in non specific terms such as changes in kidney weight (both increases and decreases) or increases in protein content of the urine (proteinuria) or changes in volume of urine. Acute renal failure is one of the more common responses of the kidney to toxicants. Acute renal failure is characterized by a rapid decline in glomerular filtration rate and an increase in the concentration of nitrogenous compounds in the blood. Compounds that cause renal vasoconstriction reduce the amount of blood that reaches the glomerulus (Middendorf and Williams, 2000).

In addition to the organ-level response of the kidney, many toxicants affect specific regions of the nephron. They may damage the glomerulus, the proximal tubule, or the further tubule elements such as the loop of Henle, distal tubule, or collecting duct. The most common site of injury for toxicants is the proximal tubule (Middendorf and Williams, 2000).

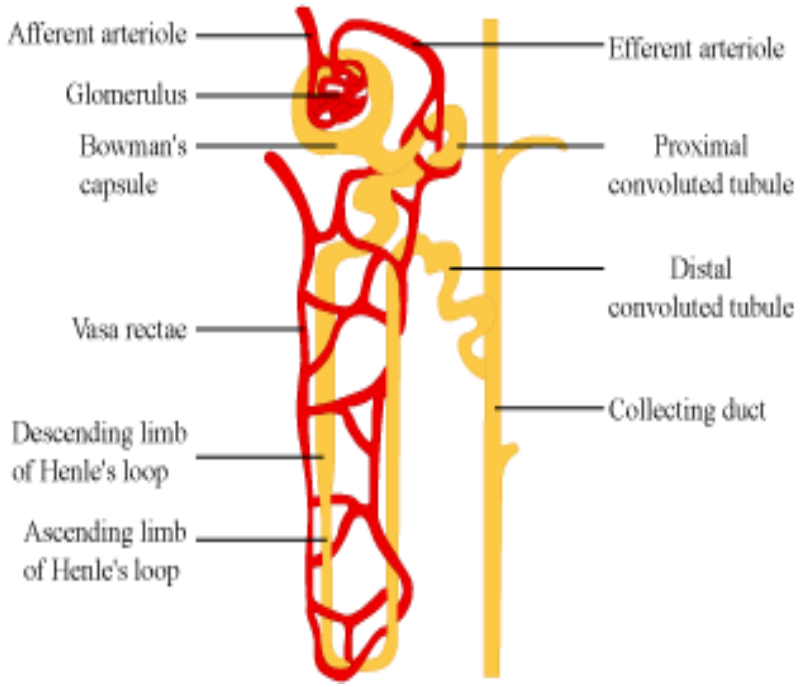


Figure 2. Structural organization of the nephron (Tarloff and Wallace, 2010).

1.7.3. Neurotoxicity

The brain is a complex array of neurons grouped to control motor, sensory, posture and higher cognitive function. The brainstem controls much of the essential physiological activity (Baker, 2012). Neurotoxicology is the study of the adverse effects of chemical, biological and certain physical agents on the nervous system and/or behavior during development and in maturity (Harry *et al*, 1998).

Like the other organ toxicities, neurotoxicity can result from different types of exposure to a substance. The major routes of exposure are oral, dermal, or inhalation. Neurotoxicity may be observed after a single (acute) dose or after repeated (chronic) dosing. (Crofton *et al*, 2011). Neurotoxicants affect the nervous system in different ways: some neurotoxicants damage the distal portions of axons without much effect on the remainder of the cell, some produce outright cell death, while others affect signaling processes in the nervous system without causing structural damage. Neurons may also be secondarily affected by neurotoxicants that target other cells in the nervous system,

disrupting normal homeostatic function and causing structural or functional damage (Blake, 2010).

1.7.4. Hematotoxicity

Blood which forms the main medium of transport in the body is a very important tissue. It serves to transport many drugs and xenobiotic. Since all foreign compounds are distributed via the bloodstream, the various components, cellular and non-cellular, are initially exposed to significant concentrations of toxic compounds (Timbrel, 2009).

Toxic injury to the blood cells and blood-forming tissues is known as hematotoxicity. In humans, the bone marrow constitutes the principal blood-forming tissue. The bonne marrow produces stem cells which are precursors of the red blood cells, white blood cells, and platelets. White cells have an essential role in inflammatory, coagulation, and immune function. Red cells primarily deliver oxygen to all cells in the body and remove carbon dioxide from these cells (Baker, 2012; Deshpande, 2005).

The bone marrow is a major target for many toxic substances. As a result of failure of generation of new cells, there may be failure of the red cell system (anemia) and failure of the white cell system, causing both overwhelming infection due to absence of granulocytes and failure of the immune system from a total reduction on the white cell count (Baker, 2012).

Some plant materials when ingested either in raw state or as extract have been reported to cause anaemia which may result from sequestration of red blood cell in the spleen, impaired red cell production or primary bone marrow dysfunction (Cheeke, 1998).

Damage and destruction of the blood cells results in a variety of consequences such as a reduction in the oxygen-carrying capacity of the blood if the cells affected are the red

blood cells. The assessment of blood is relevant to the evaluation of risks since the haematological system carries a higher predictive value for toxicity in humans (Olson *et al*, 2000).

2. Oxidative stress

Oxidative stress has been defined as a disturbance in the antioxidants/oxidants balance in favor of oxidants (Kirschvink *et al*, 2008; Lykkesfeldt and Svendsen, 2007; Abdal Dayem *et al*, 2010; Mühl *et al*, 2011). This imbalance occurs by increasing the production of oxidants, decreasing the level of antioxidants or both (Mühl *et al*, 2011; Kirschvink *et al*, 2008). Oxidative stress may cause cellular damages in macromolecules like proteins, lipids and nucleic acids, leading to cellular pathology and ultimately to cell death (zadak *et al*, 2009 ; Abdal Dayem *et al*, 2010).

Oxidative stress plays an important role in the development and progression of many human pathologies including cardiovascular and neurodegenerative disorders (Alzheimer's and Parkinson's diseases) (Pham-Huy *et al*, 2008 ; Durackova, 2010 ; Pizarro *et al*, 2009), atherosclerosis, cancer, diabetes, liver damages, rheumatoid arthritis, cataracts, inflammatory bowel disease, ulcers, pneumonia and human aging (Makker *et al*, 2009).

2.1. Free radicals

Free radicals are defined as atoms, molecules or parts of molecules containing one or more unpaired electrons in their outer orbits. They are characterized by a very short half- life and a considerable degree of reactivity (Valko *et al*, 2006).

Free radicals derivatives of oxygen like superoxide free radical anion ($O2^{\bullet -}$), hydroxyl free radical ($OH^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), lipid alkoxyl ($LOO^{\bullet}$) and lipid

peroxide (LOOH) as well as non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are collectively known as reactive oxygen species (ROS). Whereas, reactive nitrogen species (RNS) include free radicals like nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$), as well as non-radicals such as peroxynitrite ($ONOO^-$) (Kalam *et al*, 2012) (Table 2).

ROS are known to play an important role in biological systems, since they can be either harmful or beneficial to living systems. Beneficial effects of ROS involve physiological roles in defense against infections agents and the function of a number of cellular signaling systems. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids, proteins and nucleic acids. The harmful effects of ROS are balanced by the action of antioxidants (Palmieri and Sblendorio, 2007; Al-Rubae'i and Al-Musawi, 2011; Rahman, 2007).

Table 2. Classification and main effects of free radicals (Finaud *et al*, 2006).

Free Radical	Contraction	Half-life	Main effects
Reactive oxygen species	**ROS**		
Superoxide ion	$O2^{\bullet-}$	10^{-5} sec	Lipid oxidation and peroxidation
			Protein oxidation
			DNA damage
Ozone	O_3	Stable	
Singlet oxygen	$^1O_2$	1 μsec	
Hydroxyl radical	$OH^{\bullet}$	10^{-9} sec	
Hydrogen peroxide	H_2O_2	Stable	
Hypochlorous acid	HOCl	Stable	
Alkoxyl radical	$RO^{\bullet}$	10^{-6} sec	
Peroxyl radical	$ROO^{\bullet}$	7 sec	
Hydroperoxyl radical	$ROOH^{\bullet}$		
Reactive nitrogen species	**RNS**		
Nitric oxide	$NO^{\bullet}$		Lipid peroxidation
			DNA damage
			Proteins oxidation
Nitric dioxide	$NO_2^{\bullet}$	1–10 sec	
Peroxynitrite	$ONOO^{\bullet-}$	0.05–1 sec	
Reactive sulphur species	**RSS**		
Thyil radical	$RS^{\bullet}$		Proteins oxidation
			DNA damage
			ROS production

2.1.1. Production of ROS

The major biological process leading to $O2^{\bullet-}$ generation is the electron transport chain associated with the mitochondrial membrane (Bahorun *et al*, 2006). At this level, the ubiquinone – cytochrome b removes an electron from each one of the four cytochrome reduced molecules, oxidizing them and add the four electrons to the O_2 (tetravalent oxygen reduction) in order to form water (Schneider and Oliveira, 2004).

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O + \text{energy}$$

However, univalent conversion of oxygen generates the superoxide radical ($O_2^{\bullet -}$) (Gardès-Albert *et al*, 2003).

$$O_2 + e^- \longrightarrow O_2^{\bullet -}$$

Superoxide radical ($O_2^{\bullet -}$) is also formed by the NADPH oxidase of phagocytic cells in order to facilitate the elimination of pathogens. Activation of this enzyme leads to a significant increase of oxygen consumption by neutrophils and macrophages called "respiratory burst" (Bartoz, 2003).

$$NADPH + 2O_2 \longrightarrow NADP^+ H^+ + O_2^{\bullet -}$$

Superoxide radical ($O_2^{\bullet -}$) undergoes a dismutation reaction catalyzed by the Superoxide dismutase (SOD) to form H_2O_2 (Ahsan *et al*, 2003).

$$2O_2^{\bullet -} + 2H \xrightarrow{+SOD} H_2O_2$$

The hydroxyl radical ($OH^{\bullet}$) may be formed when H_2O_2 reacts with Iron or Copper ions. This reaction is known as Fenton reaction (Schneider and Oliveira, 2004).

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + Fe^{+3}$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH^{\bullet} + O^-$$

On the other hand, transition metals ions may catalyze the reaction between H_2O_2 and superoxide leading to the production of hydroxyl radical, following Haber-Weiss reaction (Schneider and Oliveira, 2004).

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe/Cu} OH^{\bullet} + OH^- + O_2$$

H_2O_2 is converted by myeloperoxidase (MPO) in neutrophils to hypochlorous acid (HOCl), a strong oxidant that acts as a bactericidal agent in phagocytic cells (Ahsan *et al*, 2003).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

Besides, the superoxide radical may react directly with the nitric oxide ($NO^{\bullet}$) and generate the peroxinitrite. This compound may lead to the formation of an oxidant agent with hydroxyl radical features (Schneider and Oliveira, 2004).

$$O_2^{\bullet -} + NO^{\bullet} \longrightarrow ONOO^{\bullet} \longrightarrow ONOO^{\bullet} + H^+ \longrightarrow OH^{\bullet}$$

The $NO^{\bullet}$ is formed by the oxidation of L-arginine using nitric oxide synthase (NOS) (figure 3) (Bahorun *et al*, 2006).

Hydroxyl radical ($OH^{\bullet}$) is the most reactive free radical *in vivo*. It has a high reactivity which making it a very dangerous radical with a very short half-life. These potentially reactive molecules are able to attack healthy cells, causing structural and functional damages (Kalam *et al*, 2012). H_2O_2 since is long-lived and membrane-permeable, may diffuse a considerable distance away from its site of generation. However, $OH^{\bullet}$ is extremely reactive having a very short half-life but with a very limited diffusion capacity. It can attack and damage almost every molecule in its vicinity at a diffusion controlled rate (Bandyopadhyay *et al*, 1999).

Nitrogen dioxide $NO_2^{\bullet}$is formed by oxidation of nitric oxide

$$2NO^{\bullet} + O_2 \longrightarrow 2NO_2^{\bullet}$$

Reaction of hypochlorous acid with hydrogen peroxide forms Singlet oxygen ($^1O_2$) (Bartoz, 2003).

$$HOCl + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O + H^+$$

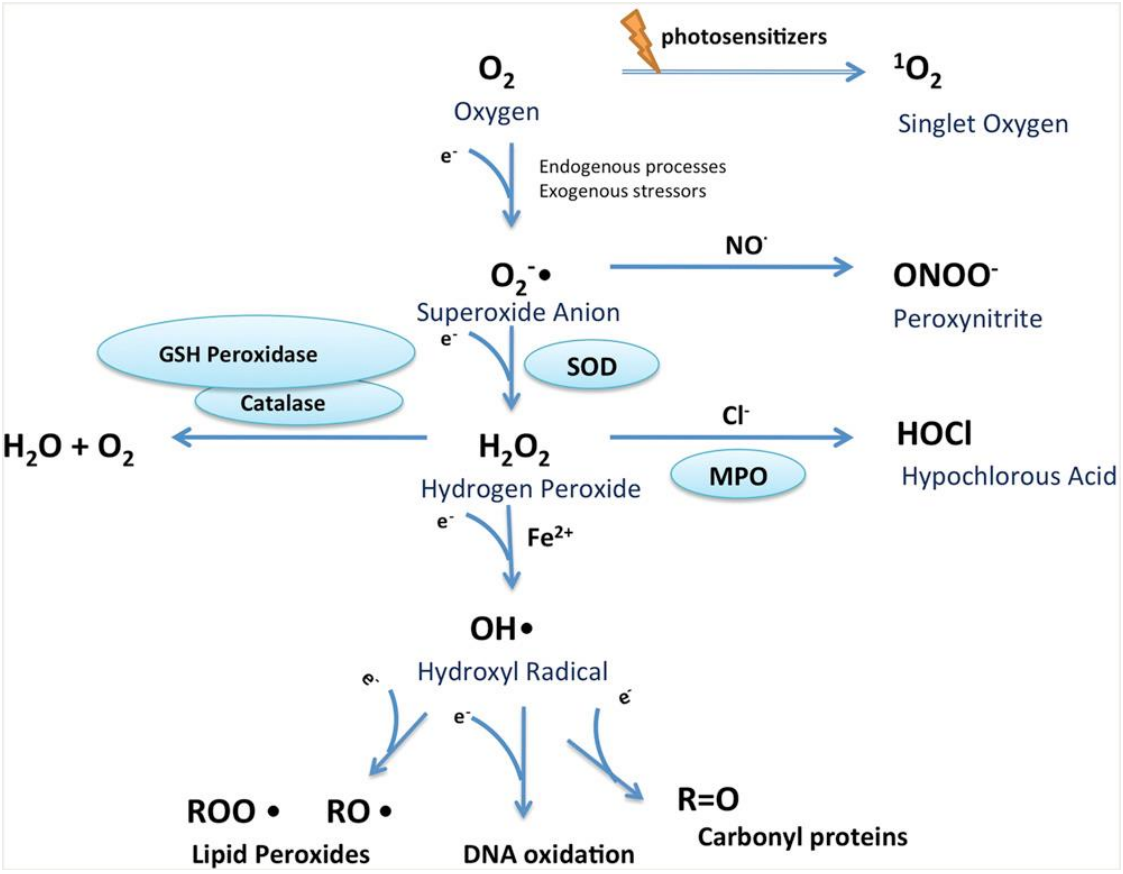


Figure 3. Generation of reactive oxygen species (ROS) (Lucy Chen *et al*, 2012).

2.1.2. Molecular damage induced by free radicals

RONS may cause oxidative damage to proteins, lipids, and DNA, which inhibits the normal functions of proteins and lipids, and facilitates DNA mutagenesis; thus, these species play a pivotal role in various clinical conditions (Rice-Evans *et al*, 1996).

2.1.2.1. Lipid peroxidation

The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are particularly sensitive to attack by ROS (Sharma *et al*, 2012). Radicals, especially hydroxyl, alkoxyl, and peroxyl, can abstract a labile H atom from the methylene group of polyunsaturated fatty acids, generating carbon-centered free radicals. Thus, the initial reaction of hydroxyl radical with fatty acids produces a lipid radical which, with the reaction of oxygen, results in the lipid peroxyl radical, which further can react with fatty acids to produce lipid hydroperoxide. This chain reaction could significantly alter the structure of membranes and other lipids, resulting in altered fluidity, permeability, transport and metabolic processes (Radak *et al*, 2011).

2.1.2.2. Protein oxidation

Proteins are also targets for free radicals. Oxidative modification of proteins by reactive oxygen species (ROS) or reactive nitrogen species (RNS) is implicated in the pathogenesis of various diseases. Oxidative damage to a specific protein, especially at the active site, can induce a progressive loss of a particular biochemical function. Several types of ROS induced protein modifications have been demonstrated (Stadtman and Le vine, 2000), including the loss of sulfhyryl (SH) groups, formation of carbonyls, disulphide crosslink, methionine sulfoxide, dityrosine cross-links, nitro tyrosine, and glyoxidation and lipid peroxidation adducts, among others. Alterations of signal transduction mechanisms, transport systems, or enzyme activities have been shown (Stadtman, 1990). Protein oxidation may be at least in part responsible for atherosclerosis, many forms of cancer, ischemia-reperfusion injury and may also be associated with aging (Stadtman, 1992).

2.1.2.3. DNA oxidation

ROS can interact with DNA and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines, damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system (Kunwar and Priyadarsini, 2011).

Free radicals such as •OH and H• react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety. The C4-C5 double bond of pyrimidine is particularly sensitive to attack by •OH, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Similarly, interaction of •OH with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products (Devasagayam *et al*, 2004). The consequence of DNA damage is the modification of genetic material resulting in to cell death, mutagenesis, carcinogenesis and ageing (Kunwar and Priyadarsini, 2011).

2.2. Antioxidants

Uncontrolled generation of ROS can lead to their accumulation in the cells, causing oxidative stress. Therefore, cells have evolved antioxidant mechanisms for the protection against ROS mediated oxidative damage. An antioxidant is defined as a molecule capable of slowing or preventing the oxidation of other molecules, or as any substance that when present at low concentrations compared to those of an oxidized substrate significantly delays or prevents oxidation of that substrate (Aher *et al*, 2011). Antioxidant systems are

classified into two major groups, enzymatic antioxidants and non-enzymatic antioxidants (Kunwar and Priyadarsini, 2011).

2.2.1. Enzymatic antioxidants

The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) that act as body's first line of defense against ROS by catalyzing their conversion to less reactive or stable species (Kunwar and Priyadarsini, 2011).

Superoxide dismutase (SOD) is the first line of defense against free radicals, and catalyzes the dismutation of superoxide anion radical ($O2^{\bullet -}$) into hydrogen peroxide (H_2O_2) (Pham-Huy, 2008). SOD exists in several isoforms, which differ in the nature of the coenzyme (metal), the amino acid composition, the co-factors and other features. Three forms of SOD are present in humans: cytosolic (Cu/Zn-SOD), mitochondrial (Mn-SOD) and extracellular SOD (Rahman, 2007).

$$\mathbf{2O_2^{\bullet -} + 2H \xrightarrow{+SOD} H_2O_2 + O_2}$$

The hydrogen peroxide H_2O_2 is then transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx).

$$\mathbf{2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2}$$

The selenoenzyme GPx removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoenzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (Pham-Huy, 2008). Besides, hydrogen peroxide, GPx also reduces the lipid peroxides (ROOH) formed

by the oxidation of polyunsaturated fatty acids (PUFA) to a stable, non-toxic molecule-hydroxyl fatty acid (ROH) (Kumar, 2011).

$$2H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG$$

2.2.2. Non-enzymatic antioxidants

The non-enzymatic antioxidants are divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants (endogenous antioxidants) are produced by the body metabolism such as glutathione, lipoid acid, L-arginine, etc. Whereas, nutrient antioxidants (exogenous antioxidants) cannot be produced in the body and must be provided through foods or supplements such as vitamin E, vitamin C, carotenoids, Polyphenols and other antioxidants *(*Pham-huy *et al*, 2008).

- **Vitamin E**

This is a fat-soluble vitamin existing in eight different forms. In humans, α-tocopherol is the most active form and is the major powerful membrane bond antioxidant employed by the cell. The main function of vitamin E is to protect against lipid peroxidation, and there is also evidence to suggest that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction α-tocopherol is converted to α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical, and the α-tocopherol radical can therefore be reduced to the original α-tocopherol form by ascorbic acid (Rahman, 2007).

- **Vitamin C**

Also known as ascorbic acid and it is the major essential water-soluble antioxidant in human serum. It is able to neutralize ROS in the aqueous phase before that the lipid

peroxidation is initiated (Percival, 1998). Vitamin C is an electron donor; and this property accounts for all its known functions. It can scavenge the $O_2^{\bullet -}$, $^1O_2$, $OH^{\bullet}$ and neutralize the hypochlorous acid (HOCl) (Kelly and Tetley, 1997). Vitamin C works synergistically with vitamin E to quench free radicals and regenerate the reduced form of vitamin E (Pham-Huy, 2008).

- **Glutathione**

An important water-soluble antioxidant is synthesized from glycine, glutamate and cysteine (Fang *et al*, 2002). The main protective roles of glutathione against oxidative stress are:

1- Incorporation as cofactor in several antioxidant enzymes against oxidative stress.
2- Scavenger effect against hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase.
3- Regeneration of the most important antioxidant, vitamin C and E (Valko *et al*, 2007).

- **Carotenoids**

Carotenoids are the mainly colored pigments present in plants and microorganisms. These molecules contain conjugated double bonds and their antioxidant activity arises due to the ability of these bonds to delocalize unpaired electrons (Rahman, 2007). The β-carotene or pro-vitamin A is a fat soluble member of the carotenoids. It is considered as the strongest antioxidant and the best scavenger of singlet oxygen (Pham-Huy, 2008).

- **Polyphenols**

Polyphenols represent one of the largest groups of plant metabolites. More than 8000 polyphenolic compounds have been identified in various plant species, having several important functions (Pandey and Rizvi, 2009).

Polyphenols are regular components of the human diet and possess several beneficial health effect and biological activities such as antioxidant, antibacterial, antiviral, immuno-modulatory, anti-inflammatory, anti-allergic, anti-mutagenic, antiviral, antineoplastic, antithrombotic, and vasodilation activities (Celep and Rastmanesh, 2013).

The most prominent activity of polyphenols is expressed to be their antioxidant activity against oxidative stress by scavenging hydroxyl radicals, superoxide anions and lipid peroxy radicals (Celep and Rastmanesh, 2013). Polyphenols have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. Polyphenols suppress the generation of free radicals, thus reducing the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals. More frequently, they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers). In addition to radical scavenging, polyphenols are also known as metal chelators. Chelation of transition metals such as Fe^{2+} can directly reduce the rate of Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals (Tsao, 2010).

- **Flavonoids**

Flavonoids are the most common group of plant polyphenols. More than 4000 varieties of flavonoids have been identified, many of them are responsible for the attractive

colors of flowers, fruits and leaves. Flavonoids can be found in vegetables, fruits, nuts, seeds, stem, flowers, tea, wine etc (Pandey and Rizvi, 2009).

Flavonoids contain C15 atoms which form two aromatic rings linked through a heterocyclic pyrane ring. All flavonoids share the basic C6-C3-C6 structural skeleton, consisting of two aromatic C6 rings (A and B) and a heterocyclic ring (C) that contains one oxygen atom (Figure 4). According to their molecular structures they can be subdivided into six sub classes: flavonols, flavones, flavanones, isoflavone, anthocyanidin and chalcones (Saxena *et al*, 2012).

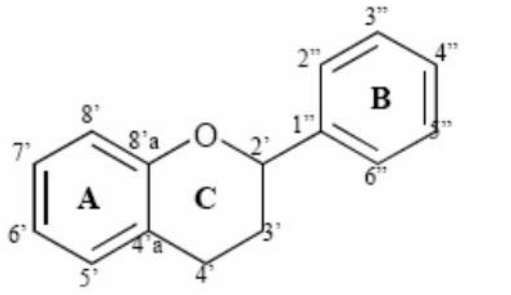


Figure 4. Basic structure of flavonoids (Sandhar *et al*, 2011).

In addition, flavonoids are known to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, cyclooxygenase and lipoxygenase activities. These activities are attributed to the powerful antioxidant effects (radical scavenging and chelation of divalent cation) and the hydrogen-donating ability exerted by flavonoids (Sandhar *et al*, 2011).

The free radical scavenging capacity is primarily attributed to the high reactivity of hydroxyl substituents that participate in the following reaction:

$$\mathbf{F\text{-}OH + R \longrightarrow F\text{-}O + RH}$$

Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate the chain radical reaction by donating hydrogen atom to a peroxy radical and forming flavonoids radical which, further, react with free radicals thus terminating propagating chain (Sandhar *et al*, 2011).

- **Tannins**

Plant tannins are one of the major groups of antioxidant polyphenols found in food and beverages. They have attracted a lot of attention in recent years because of their multifunctional beneficial properties in human health. The tannins name is given to polymeric phenolic substances which are able to tanning leather or precipitate proteins (Fritos *et al*, 2004). Tannins can be classified into two general groups: hydrolysable tannins and condensed tannins. Hydrolysable tannins are usually present in low amounts in plants. They are molecules with a polyol (D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid or ellagic acid. Whereas, condensed tannins are a group of naturally occurring polyphenolic bioflavonoids, specifically taking the form of oligomers or polymers of poly-hydroxy flavan-3-ol units such as catechin and epicatechin, flavan-3,4-diols such as eucoanthocyanidins or a mixture of the two (Kumari and Jain, 2012).

3. Plant description

3.1. Rutaceae

Rutaceae is a large family of trees and shrubs predominantly tropical and subtropical family. It is consisting of 150–162 genera and 1500–2096 species with three main centers of diversity: Tropical America, southern Africa, and Australia (Groppo *et al*,

2012). This family has long been economically important for edible fruits (especially Citrus, with many varieties of oranges, lemons, tangerines, etc.), aromatic oils (Boronia and Ruta), drugs (e.g., Pilocarpus, source of pilocarpine, used against glaucoma), and bitter beverages used to treat fevers (Angostura, Galipea). Species of Flindersia, Zanthoxylum, Balfourodendron, and Euxylophora are sources of timbers.

More recently, the antimicrobial and antifungal properties of rutaceous compounds are being exploited as natural pesticides, herbicides, and antimicrobials, while others are medically useful (Oliva *et al*, 2000).

Ruta is the richest genus of Rutaceae family. It features mainly shrubby plants, native to the Mediterranean region and present in traditional medicine of this region since Antiquity. But now, genus *Ruta* is cultivated in many parts of the world (Parray *et al*, 2012). The three most diffused species *Ruta chalepensis* L., *Ruta graveolens* L., and *Ruta montana* L. are morphologically poorly differentiated and were probably interchangeably used during Antiquity (Pollio *et al*, 2008).

3.2. *Ruta montana* L.

The medicinal plant *Ruta montana* L. (*Rutaceae*) is known in Algeria as "mountain rue" or "Fidjel" (Bellakhdar, 1977). This plant grows in the arid regions in the Mediterranean zone, distributed in Spain, Portugal, Italy, Greece, Turkey, Algeria and Morocco (Delahaye *et al*, 2008). *Ruta montana* L. has several communally names (Fidjla el-djebeli in Arabic; rue des montagnes in French and mountain rue in English).

3.2.1. Taxonomy is as the following (Wiart, 2006; Takhtajan, 2009).

Kingdom: *Plantae*

Subkingdom: *Tracheobionta*

Super division: *Spermatophyta*

Division: *Magnoliophyta*

Subdivision: *Angiospermae*

Class: *Magnoliopsida*

Subclass: *Rosidae*

Super order: *Rutanae*

Order: *Sapindales*

Family: *Rutaceae*

Genus: *Ruta*

Specie: *Ruta montana* L.

3.2.2. Botanical description

Ruta montana L. is a perennial aromatic herb with 20-40 cm of height: the leaves (Figure 5a) are small oblong deeply divided, the flowers (Figure 5b)are small yellow and in clusters and the fruits are round, small and lobulated (Delahaye *et al*, 2008).

(a)

(b)

(c)

Figure 5. *Ruta montana L.* plant (a): leaves, (b): flowers: (c): arial part (http:// luirig.altervista. org/flora/taxsa/index l.php?scientific-name.

3.2.3. Traditional uses and side effects

Ruta montana L. is used in folk medicine as hypoglycemic, anti-rheumatic, anti-helminthic, antiepileptic and antipyretic. It is also used in the treatment of intestinal and

hepatic diseases (Bnouham *et al*, 2002). *Ruta montana* L. contains many active principles able to inhibit the growth of mycobacteria (Sqalli *et al*, 2007). In Algerian folk medicine, *Ruta montana* is used against child fevers and as an abortive drug (Bellakhdar *et al*, 1991).

However, the uncontrolled use of this plant can produce several toxic effects like gastro-enteritis, hypothermia and coma (Bellakhdar *et al*, 1991). These effects can be referring to the presence of methyluonylcetone (Paris and Moyse, 1981). Moreover, it is also described that furo-coumarins of *Ruta montana* L. can induce skin eruptions (Charnot and Faure, 1945).

3.2.4. Chemical composition

Ruta species are sources of diverse classes of natural products such as flavonoids, alkaloids, essential oils, coumarins, phenols, saponins lignans, and triterpenes, with biological activities including antifungal, antioxidant, phytotoxic, abortive, depressant, antidotal and anti-inflammatory (Shahidi *et al*, 1992; Kabouche *et al*, 2003; Raghav *et al*, 2006; Kuzovkina *et al*, 2009; Mejrib *et al*, 2010;Zellagui *et al*, 2012).

In previous studies with *Ruta montana* L. the presence of alkaloids : 1-methyl-4-methoxy-2-quinolone, evolitrine, 2-(nonan-8-one)-(1H)-4-quinolone, 2-(nonan- 8-one)-4-methoxy-quinoline, 2-(nonan-8-one)-N- methyl-4-quinolone, 2-(decan-9-one)-N-methyl-4-quinolone and coumarins were shown (Touati*et al*, 2000). Kabouche *et al* (2003) were isolated two furocoumarins, heraclenol and isopimpinellin from *Ruta montana*.

The study of Belkassam *et al* (2011) revealed that the major essential oil components of the aerial parts of *Ruta montana* were found to be: 2-Undecanone, 2-Nonanone, Monoethylhexyl phthalate, Decanone, 2-Acetoxytridecane, and 2-Tridecanol.

3.2.5. Pharmacological properties

Pharmacological studies carried out with different extracts of various *Ruta* species have reported many effects which include anti-epileptic, sedative, and hypnotic actions (Di Stasi *et al*, 2002, Gonzalez-Trujano *et al*, 2006), analgesic, antipyretic, anti-inflammatory effects (Al-Said *et al*, 1990; Atta and Alkofahi, 1998; Ciganda and Laborde, 2003; Lauk et al., 2004), antispasmodic, emmenagogue, antihelminthic and antibacterial actions (Di Stasi *et al*, 1994). In the cardiovascular system *Ruta* has been shown to have positive chronotropic and inotropic effects on isolated right atria and to prolong the AV-node refractoriness in isolated rat hearts, suggesting cardiotonic and antiarrhythmic activities (Chiu and Fung 1997; Khori *et al*., 2008).

Ruta montana essential oils and extracts have antibacterial properties against pathogenic bacteria and fungi (Belkassam *et al*, 2011 ; Bouzidi *et al*, 2012; Djarri *et al*, 2013; Hammami *et al*, 2015; Sqalli *et al*, 2007).

Materials and methods

1. Materials

1.1. Biological materials

1.1.1. Plant

Ruta montana L. was collected in October 2011, from Beniaziz region, Wilaya of Sétif in North-East of Algeria, and it was identified by Pr. Laouar H. (Department of Ecology & Plant Biology, Faculty of Nature and Life Sciences, university Ferhat Abbas, Sétif 1).

1.1.2. Animals

Males and females of *Albino Wistar* mice were used for acute toxicity and *Albino Wistar* rats for chronic toxicity and *in vivo* antioxidant activity. All experimental animals were purchased from Pasteur Institute (Algiers, Algeria). These animals were kept in the animal house, at a temperature of 20°C and a natural photoperiod cycle. The animals were housed in plastic cages (5 rats or mice per cage) and had free access to standard commercial diet and tap water.

1.2. Chemicals

Folin-Ciocalteu, aluminum chloride ($AlCl_3$), gallic acid, quercetin, rutin, 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH), ethylenediamine tetraacetic acid (EDTA), gallic acid and tween 40 were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic acid, β-carotene and butylated hydroxytoluene (BHT) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

2. Methods

2.1. Preparation of plant extracts

The aerial parts of the plant material were cleaned with tap water, dried in shade at room temperature for 2 weeks and ground into fine powder using an electric grinder.

2.1.1. Aqueous extract

The aqueous extract was prepared according to the method described by Mbiantcha *et al*., (2011), with some modifications. Briefly, 100g of *Ruta montana* L. powder was mixed with 1L of boiled distilled water (100 °C) at room temperature during 72h, the mixture was filtered using Wattman filter paper n°3 and then evaporated in rotary vacuum evaporator .

2.1.2. Methanolic extract

The methanolic extract was obtained by maceration in methanol/water mixture (25:75, V/V) for 72 h. The resultant extract was filtered through Wattman filter paper n°3 and the solvent was removed by rotary evaporator under reduced pressure at 45°C. The resulting crude extract was then stored until further analysis.

2.1.3. Fractionation of the crude extract

Fractionation of the crude extract is performed according to the method of (Markham, 1982) with slight modifications, using a series of solvents of increasing polarity (figure 6). The crude extract was initially mixed with the hexane (V/V) to eliminate lipids and pigments, and after separation the upper organic phase was recovered. This step is repeated several times with renewal of the solvent until it becomes transparent. The lower

aqueous phase was subjected to another fractionation with chloroform to give the chloroform extract (ChE), and finally by ethyl acetate to give the fraction of ethyl acetate (EAE) following the same steps as for the first fractionation by hexane.

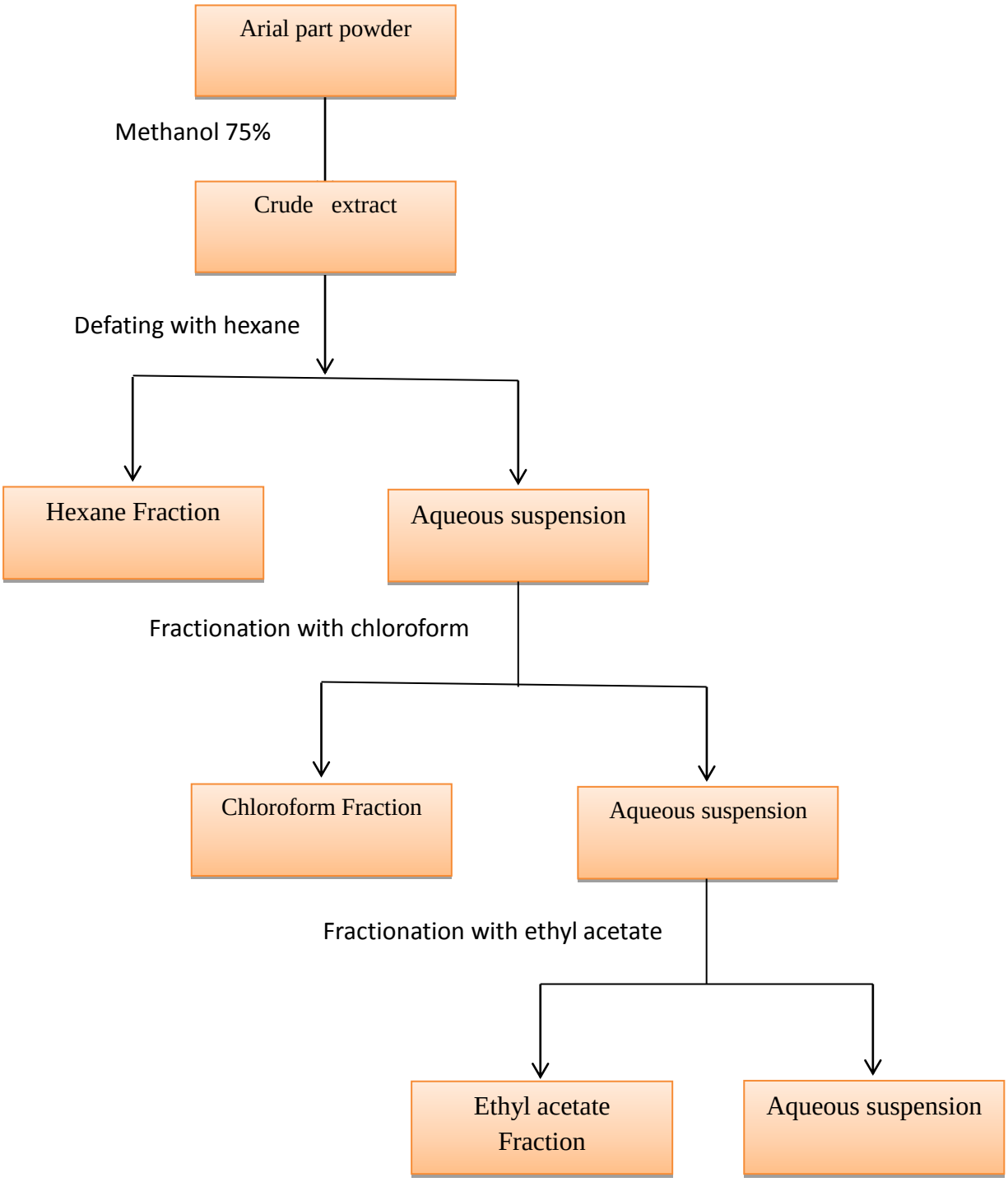


Figure 6. Fractionation steps of the crude extract of *Ruta montana* L. (Markham, 1982) with slight modifications

2.2. Determination of total polyphenols, flavonoids and tannins content in plant extracts

2.2.1. Determination of total polyphenols content in plant extracts

Total polyphenols content was determined spectrophotometrically, using the Folin–Ciocalteau reagent according to the method described by Li *et al*., (2007). Folin-Ciocalteu reagent is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungsten (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurring under alkaline conditions is carried out with sodium carbonate. Blue coloration is mesured at 765 nm and reflects the quantity of polyphenols usually expressed as gallic acid equivalent (GAE). Briefly, a volume of 100 µl of the extract was mixed with 500 µl of Folin–Ciocalteau reagent (diluted 10% in distilled water). After 4 min, 400 µl of sodium carbonate solution Na_2CO_3 (75 g/l) was added to the mixture, the reaction mixture was incubated at room temperature for 1h 30 min and the absorbance of the mixture was read at 765 nm, Gallic acid (0-160 µg/ml) was used as standard for the calibration curve. The total polyphenols content was expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (µg GAE/mg of extract). All samples were analyzed in three replications.

2.2.2. Determination of total flavonoids content in plants extracts

The flavonoids content were determined by the aluminum chloride solution according to Bahorun *et al*., (1996). Briefly, 1 mL of 2% $AlCl_3$ in methanol was mixed

with 1 mL plant extract. After incubation in dark at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm. Quercetin and rutin (1-40

µg/mL) were used as standards for calibration curve and the total flavonoids content was expressed as µg quercetin and rutin equivalent (QE and RE, respectively)/mg of extract.

2.2.3. Determination of tannins content in plant extracts

Tannins content of plant extracts were determined by the precipitation test for hemoglobin using fresh bovine blood according to the method described by Bate-Smithe (1973). Briefly, a volume of each plant extract was mixed with an equal volume of hemolysed-blood (absorbance = 1.6). After 20 minutes of incubation, this mixture was centrifuged for 10 minutes at 4°C and the absorbance of the supernatant was measured at 576 nm. Different concentrations of tannic acid were also mixed with an equal volume of hemolysed-blood and the absorbance was measured in the same manner. The tannins content was expressed as µg tannic acid equivalent/mg extract.

2.3. Evaluation of toxic effect of *Ruta montana* L. aqueous extract

2.3.1. Acute toxicity of *Ruta montana* L. aqueous extract

Albino Wistar mice of both sexes weighing between 18 and 22 g were divided into seven groups of 10 mice (5 males and 5 females). The animals were fasted overnight (12 h) with free access to water prior to the orale administration of single doses of the extract (0, 2, 4, 6, 8, 10 and 12 g/kg of body weight), the extract dissolved in 1 mL of distilled water per 100 g of body weight. The general behavior of the mice was continuously monitored after dosing, periodically during the first 24 h (with special attention given during the first 4 hours), and then daily thereafter, for a total of 14 days.

2.3.2. Chronic toxicity of *Ruta montana* L. aqueous extract

Albino Wistar rats of both sexes weighing between 149 and 200 g were divided into four groups of 10 rats each (5 females and 5 males). The aqueous extract dissolved in distilled water, was administered daily by gavage for 90 days to groups I to IV (doses of 0, 100, 300 and 600 mg/kg, respectively). The animals were observed for signs of toxicity and mortality throughout the experimental period. At the end of the treatment, animals were fasted overnight, but allowed access to water ad libitum. They were subsequently anesthetized with diethyl ether and blood samples were obtained by retro-orbital puncture (Waynforth, 1980) and collected in two tubes. The first one containing EDTA and it was processed immediately for hematological parameters analysis. The second containing heparin and it was centrifuged at 4000g at 4°C for 15 min to obtain serum (stored at −20°C until analysis). The organs (kidneys, liver, heart, lungs, spleen, brain, stomach, tests, epididymis and ovaries) were weighed and fixed in 10 % formalin for histopathological examination.

2.3.2.1. Effect of *Ruta montana* L. aqueous extract on heamatological and biochemical parameters

Hematological analysis was performed using an automatic hematological analyzer Medonic (Coulter Beckman -USA-) at the Central Laboratory of the University Hospital of Sétif. Parameters included: Red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (VGM), platelets count and mean platelet volume (MPV). For biochemical analysis, the serum

analysis for cholesterol (CHOL), glucose (GLU), creatinine (CREA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides (TG), uric acid (AU), alkaline phosphatase (ALP), creatine kinase (CK), albumin (ALB), were made using automatic analyzer (Beckman).

2.3.2.2. Effect of *Ruta montana* L. aqueous extract on organs weight

After the sacrifice of all animals, the kidneys, liver, heart, lungs, spleen, brain, stomach, tests, epididymis, seminal vesicle and ovaries were carefully removed and weighed individually (absolute organ weight).

2.3.2.3. Histopathological examination

The treated and control rats organs were taken out. They were weighed and examined for the evidence of gross lesions. Similar samples were fixed in 10% formalin solution, dehydrated ingraded (70-90%) alcohol, cleared in xylene, and placed and embedded in paraffin wax.

To perform histology of tissues, 5-6 μm sections were prepared using Microtome (Leica, RM 2145). These sections then deparaffinated in xylene, passed through 70% to 90% alcohol, and stained with hematoxylin and eosin (H&E). The slides prepared by this process were observed under light microscopy (Bensalem-Bendjelloul, 1998).

2.3.2.4. Effect of *Ruta montana* L. aqueous extract Fertility

a. Sperm suspension

The epididymis of each rat was placed in 1 mL of ringer buffer, the spermatozoa were obtained by making small cuts in caudal epididymis and diluted by adding 9 mL of ringer buffer (1/10) and then incubated at 37°C for 10 minutes. The sperm suspension was used for analysis of motility and counts.

b. Sperm count and motility

A sample of sperm suspension was taken and the number of sperm counted using a haemocytometer under the light microscope at a magnification of x40. Four squares were counted in triplicate. The count was expressed as 2500/mm of suspension. Sperm motility (%) was also assessed immediately by counting both motile and immotile spermatozoa at the magnification of x40 and calculated by the formula:

Number of motile sperm x 100/total number of motile and immotile sperm.

2.4. Evaluation of antioxidant activity of plant extracts

2.4.1. *In vitro* antioxidant activity

2.4.1.1. DPPH free radical-scavenging activity of plant extracts

This assay is based on the measurement of the reducing ability of antioxidants towards the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The free radical DPPH, which bears a deep purple color, is reduced to the yellow-colored diphenyl picryl-hydrazine when it reacts with hydrogen donors. This ability is evaluated by measuring the loss of DPPH color at 517 nm after reaction with extracts, that color intensity is inversely related to the test antioxidant concentration. After dissolving the plant extracts, the solution of DPPH in methanol (0.04 mg/mL) was prepared and 1250µL of this solution was added to 50µL of extracts solution at different concentration. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature. Then, the absorbance was measured at 517 nm in a spectrophotometer. All tests were performed in triplicate (Burits and Bucar, 2000). Radical-scavenging activity was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = \{(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}\} \times 100.$$

A_{blank}: Absorbance of the control.

A_{sample}: Absorbance of the reagent with extract.

2.4.1.2. Determination of antioxidant activity of plant extracts using β-carotene/linoleate model system

In this test, the antioxidant capacity of the extracts was determined by measuring the inhibition of the oxidative degradation of β-carotene (discoloration or bleaching) by the oxidation products of the acid linoleic according to the method described by Kartal *et al*., (2007). The β-carotene solution was prepared by dissolving 0.5 mg β-carotene in 1 mL of chloroform. One milliliter of this solution was pipetted to a flask covered with aluminum foil. Then 25 µL of linoleic acid and 200 mg of tween 40 were added. The chloroform was evaporated using evaporator at 45°C. Then 100 mL of distilled water saturated with oxygen was added. 2.5 mL of this prepared β-carotene solution were transferred to test tubes, and 350 µL of the extracts (2mg/mL methanol) were added before incubation for 48h at room temperature. The same procedure was repeated with butylated hydroxyl toluene (BHT) as a positive control and with distilled water and methanol as a negative control. The absorbance was reading at 490 nm after 1h, 2h, 6h, 24h and 48h. The antioxidant activity of extracts was calculated using the following equation:

$$(AA\ \%) = A_{sample} / A_{BHT} x\ 100.$$

2.4.1.3. Chelation of ferrous iron by plant extracts

The chelating ability of the extracts is determined according to the method of Le *et al*., (2007), which is based on the inhibition of the formation of Fe^{2+}-ferrosine complex after treatment of samples with Fe^{2+} ions. Briefly, 250 µL of extract solutions at different concentrations were added to 50 µL of $FeCl_2$ (0.6 mM in distilled water) and 450 µL of

methanol. After 5 min of incubation, 50 µL of ferrosine (5 mM in methanol) was added, and the mixture was stirred and allowed to react for 10 min to allow the complexation of residual iron. The absorbance of the Fe^{2+}-ferrosine complex was measured at 562 nm against a blank contained all the reaction reagents except ferrosine. Moreover, the negative control containing all reagents except that the test sample is replaced by the same volume of methanol. EDTA was used as reference chelators. The chelating activity is expressed as a percentage using the following equation:

$$\text{Chelating activity (\%)} = [(Ac - At)/ Ac] \times 100$$

Ac: absorbance of the control.

At: absorbance of the test.

2.4.2. *In vivo* antioxidant activity of plant extracts

Thirty male rats were randomly divided into five groups, each consisting of 6 animals, which included:

- Group 1: Untreated control rats which received distilled water for 21 days.
- Group 2: Positive control received 50mg/kg bw of vitamin C for a period of 21 days.
- Group 3: Received 100 mg/kg bw of *Ruta montana* L. aqueous extract for 21 days.
- Group 4: Received 300 mg/kg bw of *Ruta montana* L. aqueous extract for 21 days.
- Group 5: Received 100 mg/kg bw of *Ruta montana* L. methanolic extract for 21days.

Administrations were done orally. 24 h after the last dose all animals were anesthetized with diethyl ether and blood samples were obtained by retro-orbital puncture

and collected into tubes containing heparin and it was centrifuged at 3000g for 15 min at 4°C to obtain serum stored at −20°C until used for DPPH and reducing power test. After the sacrifice the liver and kidney from each animal were dissected out, washed in ice-cold saline, patted dry and weighed and homogenized in Tris HCL buffer (pH 7.4), to prepare 10% (w/v) homogenate. Then, the homogenate was centrifuged at 4000 rpm at 4 °C for 15 min and the supernatant was collected and used for the estimation of MDA, reduced glutathione (GSH),catalase(CAT) and total proteins.

2.4.2.1. Effect of extracts on plasma antioxidant capacity using DPPH radical

The ability of plasma to scavenge DPPH radical was evaluated by the method of Hasani *et al*., (2007) with some modifications, based on the same principle as the DPPH test previously conducted *in vitro*. Briefly, a volume of 50 µL of plasma was added to 1250 µL of methanolic DPPH solution (2.4 mg / 100 mL methanol). After 30 min of incubation in the dark, followed by centrifugation the absorbance is measured at 517. The plasma antioxidant capacity is then calculated according to the equation below:

$$\text{Radical scavenging activity (\%)} = \{(A_{blank} - A_{sample}) / A_{blank}\} \times 100.$$

2.4.2.2. Effect of extracts on plasma reducing power

Reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, TCA and $FeCl_3$ and it was measured by the method reported by Jayprakash *et al*., (2001). 1mL of plasma was mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1% w/v). The mixture was incubated at 50℃ for 20 min. After terminating the reaction by adding trichloroacetic acid (10% w/v), the mixture was centrifuged at 3000 rpm for 10 min. The supernatant of solution 0.5 mL was mixed with 0.5 mL distilled water

and 0.1 mL $FeCl_3$ (0.1% w/v). After 5 min later, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

2.4.2.3. Estimation of lipid peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formation, using the method of Ohkawa *et al*., (1979). Malondialdehyde (MDA), the end product of lipid peroxidation, is a good marker of free radical–mediated damage and oxidative stress.

The principle of this method consists in the reaction of MDA with thiobarbituric acid (TBA) in acidic conditions and at a higher temperature (90-100°C) to form a pink MDA-$(TBA)_2$ complex (Figure7), which can be quantified spectrophotometrically at 530 nm.

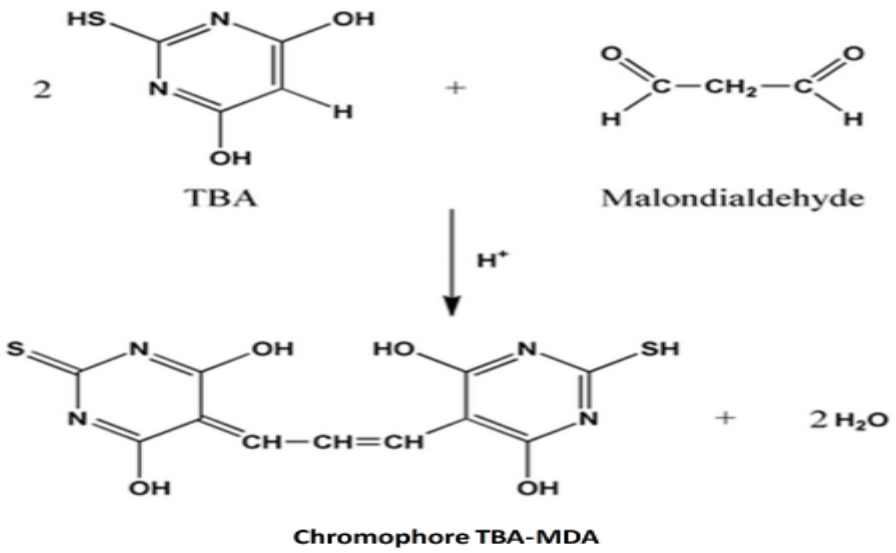


Figure 7. Malondialdehyde assay (Santos-Fandila *et al.*, 2014)

In the procedure, 0.5mL of 20% TCA was added to 0.5mL of the tissue homogenate, then there was an addition of 1 mL of 0.67% TBA. The mixture was incubated at 100^{o}C for 15 min in a water bath, cooled and then added with 4 mL of *n*-butanol and centrifuged at 3000rpm for 15min. The absorbance of the clear pink

supernatant was then read against a blank at 532nm spectrophotometrically. The concentration of MDA is expressed in nmol / g of the tissue.

2.4.2.4. Determination of reduced glutathione (GSH)

Reduced glutathione was determined by the method of Ellman (1959).The assay is based on the oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid (TNB) which has yellow color. Therefore, GSH concentration can be determined by measuring absorbance at 412 nm. According to the following reaction (figure 8):

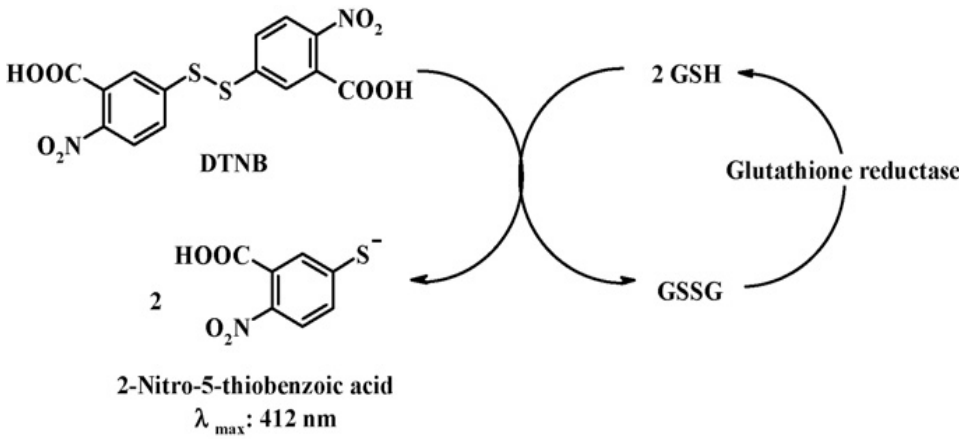


Figure 8. Ellman reaction (Araujo *et al*, 2008)

For this assay, 50 µL of the tissue homogenate was diluted in 10 mL of phosphate buffer (0.1 M, pH 8). To 3 mL of the mixture of dilution, 20 µL of DTNB (0.01 M) was added. Absorbance is read at 412 nm against a blank prepared under the same conditions.

The concentrations of GSH are expressed in µmol / g of the tissue were obtained from calibration curve of GSH realized in the same condition (figure 9).

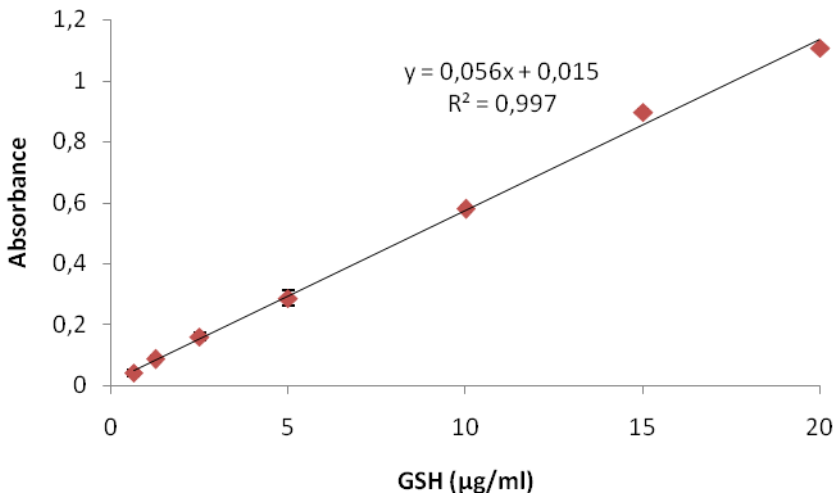


Figure 9. Standard curve of reduced glutathione (GSH). Each value represents the mean ± SD (n = 3).

2.4.2.5. Determination of catalase activity

The enzymatic activity of catalase was determined by the method of Claiborne (1985). The principle is based on the hydrogen peroxide H_2O_2 degradation in the presence of the enzyme according to the following reaction:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

The reaction mixture contained 50 mM potassium phosphate buffer pH 7.4, 19 mM H_2O_2 and 16.5 uL tissue homogenate. The consumption of H_2O_2 was monitored spectrophotometrically at 240 nm for 1 min. and the enzymatic activity is calculated according to the formula:

$$\mathbf{K = 2.303/T \times \log (A1/A2)}$$

Where:

K: Rate of reaction

T : Time interval (minutes)

A1 : Absorbance at time zero

A2 : Absorbance at 60 seconds interval

The enzymatic activity of CAT is calculated by K/n in terms of units per milligram of protein (U /mg protein); where n is protein content.

2.4.2.6. Estimation of total proteins

Total proteins were determined by the Biuret method using the kit total protein reagent (REF: 1001290, 2x250 ml). Proteins give a blue-violet color with copper sulfate in alkaline medium. The intensity of the color formed, measured at 540 nm is directly proportional with the concentration of total protein. 1ml of kit total protein was added to 25µl of the tissue homogenate, then the mixture was incubated for 5 min. The absorbance was then read at 540 nm. The amount of protein in the sample is expressed in mg / dl.

Statistical analysis

The results are expressed as the mean value ± standard deviation. One-way analysis of variance (ANOVA) followed by the Tukey test was performed to assess differences between groups. Differences were considered significant at $p < 0.05$. Statistical analyses were performed with the software GraphPad Prism 5®.

Results

1. Extraction yield

The maceration of 100g of the aerial part powder of *Ruta montana* L. in 1L of distilled water was given 13.53% of aqueous extract. Moreover, *Ruta montana* L. powder was extracted according to Sunday and Uguru (2010) and was performed in two steps. The first one is the extraction with hydroalcoholic mixture methanol/water (75/25 v/v) to give the crude extract containing the total flavonoids. The second step is the fractionation of crude extract with increasing polarity solvents (hexane, chloroform, ethyl acetate) to separate the crude extract compounds according to their solubility in solvents and their glycosylation degree (a glycone flavonoids, mono, di and tri-glycosylated). Therefore, five different extracts were obtained: aqueous extract (AqE), crude extract (CrE), hexane extract (HxE) (it is eliminated because it contains only lipids, chlorophylls and impurities), chloroform extract (ChE) and ethyl acetate (EAE). The yield of each extract are shown in table 3.

Table 3. Yield of different extracts from *Ruta montana* L.

Extract	Yield %
Aqueous extract	13.53
Crude extract	15.04
Hexane	1.21
Chloroform	0.76
Ethyl acetate	1.48

The highest yield was obtained for crude extract (15.04%) followed by aqueous extract (13.53%), ethyl acetate (1.48%), hexane (1.21%) and chloroform (0.76%), respectively.

2. Total phenolics, flavonoids and tannins content in *Ruta montana* L. extracts

Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many phenolic compounds including flavonoids, tannins and phenolic acid, exhibit a strong antioxidant activity (Rohman *et al*, 2010). Therefore, the amounts of total phenolics, flavonoids and tannins in *Ruta montana* L. extracts were measured in this study.

The total phenolics in extracts was determined according to the Folin-Ciocalteau method using gallic acid as a standard and expressed as micrograms of gallic acid equivalents per milligram of extract (µg GAE/mg extract). A linear calibration curve of Gallic acid, in the range of 0-160 µg/ml, was obtained (figure10).

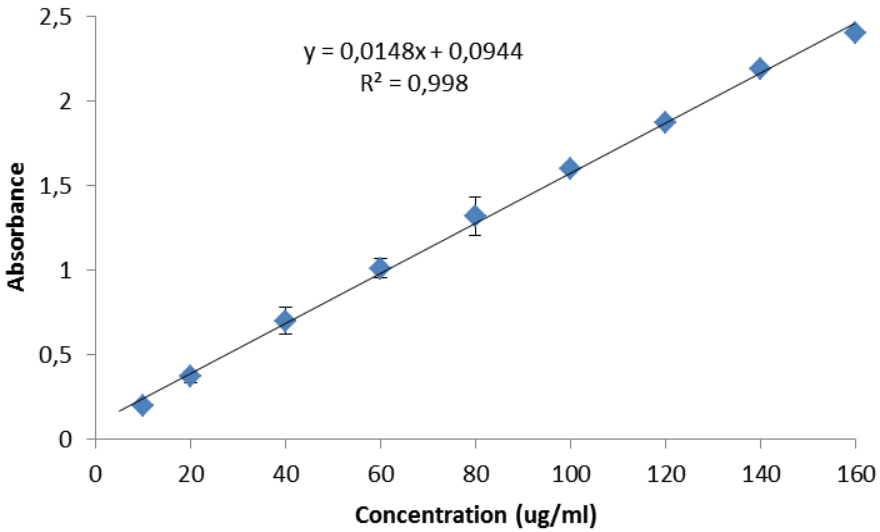


Figure 10. Standard curve of gallic acid for determination of total polyphenols in *Ruta montana* L. extracts. Each value represent mean ± SD (n = 3).

Whereas, the flavonoids content was estimated by the $AlCl_3$ method (Bahorun *et al*, 2003) and expressed as microgram of rutin and qurcetin equivalents per milligram of extracts. The calibration curve of rutin and qurcetin was shown in figure11.

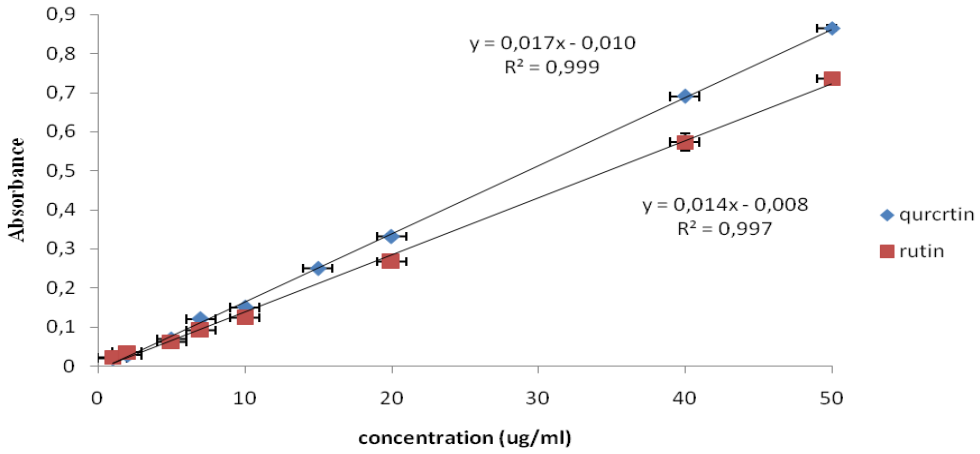


Figure 11. Standard curve of Rutin and Quercetine for determination of total flavonoids in *Ruta montana* L. extracts. Each value represent mean ± SD (n=3).

Total phenolics, flavonoids and tannins content in extracts was compiled in table 4.

Table 4. Total polyphenols, flavonoids and tannins content of *Ruta montana* L. extracts.

Extract	Polyphenols	Flavonoids		Tannins
	µg GAE/mg extract	µg QE/mg extract	µg RE/mg extract	µg TAE/mg extract
CrE	81,56± 0,766	50,80± 1,24	60,10± 1,484	80.33 ± 0.70
ChE	245,6± 6,005	35,81± 1,11	42,23± 1,330	83 ± 0.98
EAE	257,1± 0,703	117,4± 3,451	139,5± 4,107	251 ± 1.41
AqE	76,90± 0,681	22,54± 0,220	26,54± 0,3	33.6 ± 0.84

Each value represents the mean ± SD (n = 3). **GAE**: gallic acid equivalent. **QE**: quercetin equivalent. **RE**: rutin equivalent. **TAE**: tannic acid equivalent.

The results showed that EAE contain the highest amount of phenolic compounds (257.1 ± 0.703 µg GAE/mg E), followed by ChE (245.6 ± 6.005 µg GAE/mg E), CrE (81.56 ± 0.766 µg GAE/mg E) and AqE (76.9 ± 0.681 µg GAE/mg E). On the other hand, the highest value of flavonoids found in EAE was 117.4 ± 3.451 µg QE/mg E,139.5 ± 4.107 µg RE/mg E and the lowest was 22.54 ± 0.22µg QE/mg E, 26.54± 0.3 µg RE/mgE for AqE with the following decreasing order: EAE > CrE > ChE > AqE. EAE contained the highest amount of tannins (251 ± 1.41µg TAE/mg E), followed by ChE (83 ± 0.98µg TAE/mg E), CrE (80.33 ± 0.70 µg TAE/mg E) and the AqE (33.6 ± 0.84 µg TAE/mg E).

3. Toxicity

3.1. Acute toxicity

No mortality was observed during the 14 days of the observation period after administration of aqueous extract of *Ruta montana* L. at the doses of 2, 4, 6, 8, 10 and 12g/kg of body weight. According to Hodge and Sterner (Frank, 1992), substances with LD_{50} higher than 5 g/kg by oral route are regarded as being safe or practically nontoxic. The behavioral signs of toxicity such as diarrhea, vomiting, convulsion and difficulty in movement were not observed.

3.2. Chronic toxicity

In chronic toxicity study, rats were divided into 4 groups of 10 animals (5 female and 5 male). The rats were treated with the aqueous extract of *Ruta montana* L. at three doses (100, 300 and 600 mg/kg of body weight/day) for 90 days, while the control group was given only the distilled water.

No toxicity signs or death were recorded during the 90 consecutive days of treatment *via* oral route with *Ruta montana* L. aqueous extract at doses of 100, 300 or 600 mg/kg of body weight.

3.2.1. Effect of *Ruta montana* L. aqueous extract on rats body weight

Changes in body weight are an indicator of adverse effects of drugs and chemicals and it will be significant if the body weight loss is more than 10% from the initial body weight occurred (Raza *et al*, 2002; Teo *et al*, 2002).

The results of body weight of control and treated rats are presented in figure 12 and figure 13. Statistically, no significant difference was noted in the body weight between the control and any of treated groups (1-4) at any time period.

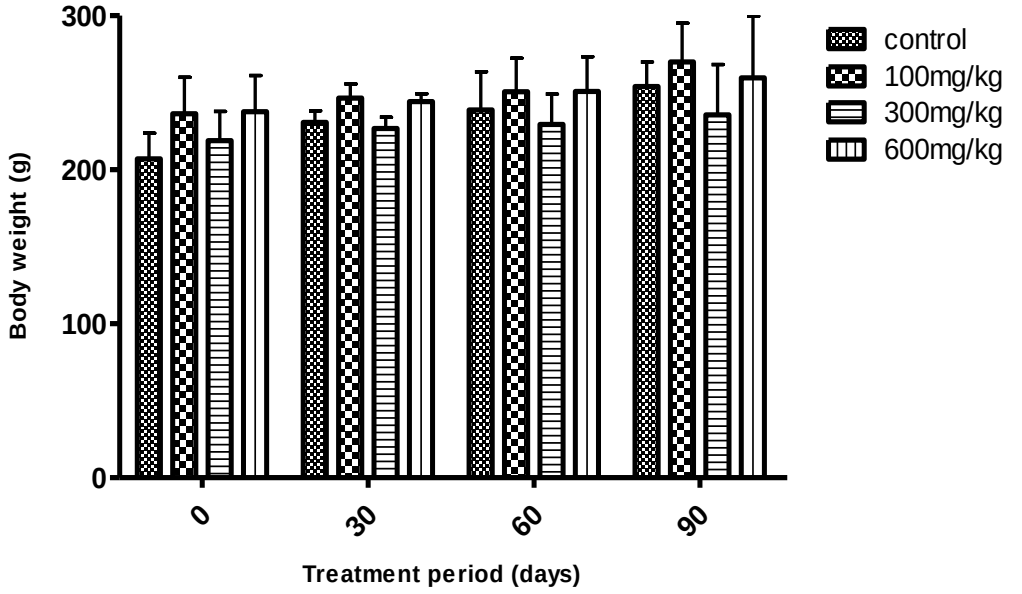


Figure 12. Changes in body weight of male rats after chronic oral treatment with aqueous extract of *Ruta montana* L. The values are expressed as mean ± SD (n = 5).

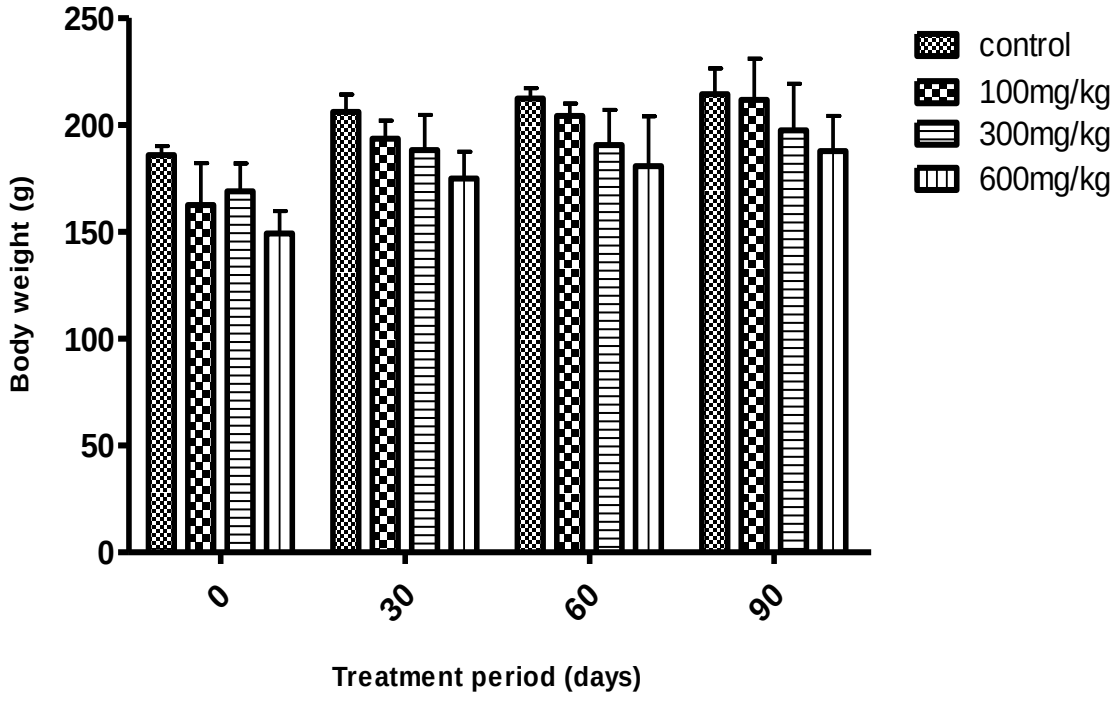


Figure 13. Changes in body weight of female rats after chronic oral treatment with aqueous extract of *Ruta montana* L. The values are expressed as mean ± SD (n = 5).

3.2.2. Effect of *Ruta montana* L. aqueous extract on hematological parameters

The hematological parameters of the treated and control groups are presented in table 5 and table 6. In female rats, no significant differences were recorded in any of the parameters examined at 30 and 90 days. In the male groups, there was no significant difference observed in any of the parameters examined at 90 days. However, a significant decrease was noticed at 30 days in the WBC, Hct, Hb and platelets at the dose 600mg/kg and a significant increase in the MPV at the dose 300 and 600mg/kg when compared to the control group.

Table 5. Effect of aqueous extract of *Ruta montana* L. (100, 300 and 600mg/kg) on hematological parameters of male and female *Wistar* rats treated for 30 days.

Parameters	Control	100mg/kg	300mg/kg	600mg/kg
Male				
RBC(10^6/mm^3)	9.34 ± 0.58	9.77 ± 0.51	8.94 ± 0.00	7.05 ± 2.2
WBC (10^3/mm^3)	16.63 ± 3.84	14.57 ± 3.23	14 ± 0.00	8.56 ± 2.4*
Hematocrit (%)	46.73 ± 2.4	48.27 ± 0.25	42.7 ± 0.00	31.97 ± 5.65**
Platelets (10^3/mm^3)	842.3 ± 145.9	971.7 ± 28.5	485 ± 0.00**	529.7.7 ± 53.13*
Hemoglobin (g/dL)	15.9 ± 0.79	16.5 ± 0.6	14.5 ± 0.00	12.53 ± 0.51**
VGM (fL)	50.07 ± 1.87	49.5 ± 2.76	47.8 ± 0.00	47.53 ± 9.0
MPV (fL)	6.1 ± 0.36	6.53 ± 0.2	8.2 ± 0.00***	8.53 ± 0.15***
MCH (pg)	17.03 ± 0.89	16.9 ± 0.75	16.2 ± 0.00	19.2 ± 6.35
MCHC (g/dL)	34.03 ± 0.56	34.23 ± 1.45	33.9 ± 0.00	39.87 ± 5.55
Female				
RBC(10^6/mm^3)	9.1 ± 0.11	8.31 ± 0.3	8.86 ± 0.48	8.88 ± 0.05
WBC (10^3/mm^3)	12.3 ± 0.51	8.6 ± 0.7	9.16 ± 2.08	12.7 ± 1.83
Hematocrit (%)	45.17 ± 3.3	39.35 ± 0.91	45.47 ± 0.55	44.95 ± 0.91
Platelets(10^3/mm^3)	1009 ± 257.9	1480 ± 113.1	1443± 75.79	1460 ± 84.15
Hemoglobin (g/dL)	15.47± 0.75	13.85 ± 0.49	15.73 ± 0.68	15.65 ± 0.35
VGM (fL)	49.67 ± 3.98	49.7 ± 0.42	52.77 ± 2.19	50.43 ± 0.57
MPV (fL)	6.16 ± 0.75	5.6 ± 0.14	5.86 ± 0.25	5.85 ± 0.05
MCH (pg)	17 ± 0.88	17.7 ± 0.28	17.77 ± 0.4	17.53 ± 0.23
MCHC (g/dL)	34.3 ± 1.01	35.65 ± 0.21	34.3 ± 0.65	34.8 ± 0.00

Values represent the mean ± SD. (n =10/group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ **RBC** : Red Blood Cell, **WBC** : White Blood Cell, **VGM** : Mean Corpuscular Volume, **MPV** : mean platelet volume, **MCH** : Mean Corpuscular Hemoglobin, **MCHC** : Mean Corpuscular Hemoglobin Concentration.

Table 6. Effect of aqueous extract of *Ruta montana* L. (100, 300 and 600mg/kg) on hematological parameters in male and female *Wistar* rats treated for 90 days.

Parameters	Control	100mg/kg	300mg/kg	600mg/kg
Male				
RBC(10^6/mm^3)	6.3 ± 0.94	7.99 ± 0.56	7.76 ± 1.3	8.21 ± 0.21
WBC (10^3/mm^3)	9.7 ± 3.39	9.03 ± 1.51	9.65 ± 5.43	13.77 ± 4.25
Hematocrit (%)	32.05 ± 4.03	38.77 ± 2.41	38.4 ± 4.99	38.9 ± 1.49
Platelets (10^3/mm^3)	814 ± 207.9	997.7 ± 233.4	927.3 ± 115	988 ± 170.2
Hemoglobin (g/dL)	11.05 ± 1.76	13.7 ± 0.8	13.5 ± 1.64	13.43 ± 0.56
VGM (fL)	50.33 ± 1.36	56.8 ± 16.63	49.5 ± 3.12	47.33 ± 0.55
MPV (fL)	5 ± 0.55	5.5 ± 0.42	5.25 ± 0.23	5.63 ± 0.25
MCH (pg)	17.87 ± 0.55	17.08 ± 0.51	17.55 ± 0.98	16.55 ± 0.07
MCHC (g/dL)	35.53 ± 2.08	38.08 ± 5.56	35.53 ± 0.77	34.75 ± 0.21
Female				
RBC(10^6/mm^3)	8.99 ± 0.83	6.63 ± 0.58	7.89 ± 1.3	8.86 ± 0.98
WBC (10^3/mm^3)	6 ± 2.26	5.45 ± 1.06	6.33 ± 0.41	7.85 ± 0.91
Hematocrit (%)	49.35 ± 0.77	36.45 ± 6.57	40.73 ± 7.89	45.9 ± 4.95
Platelets(10^3/mm^3)	848 ± 128.7	596 ± 55.15	787.5± 174.1	697 ± 76.37
Hemoglobin (g/dL)	16.2± 0.7	12.5 ± 1.41	14.58 ± 2	15.8 ± 0.7
VGM (fL)	53.3 ± 4.23	55.6 ± 3.84	51.43 ± 2.87	51.67 ± 2.87
MPV (fL)	6.46 ± 0.6	6.36 ± 0.46	6.42 ± 0.22	6.13 ± 0.5
MCH (pg)	17.9 ± 0.7	18.6 ± 0.45	18.63 ± 0.86	18.53 ± 1.35
MCHC (g/dL)	33.7 ± 1.6	34.7 ± 1.7	36.47 ± 3.25	35.93 ± 3.05

Values represent the mean ± SD. (n =10/group). **RBC** : Red Blood Cell, **WBC** : White Blood Cell, **VGM** : Mean Corpuscular Volume, **MPV** : mean platelet volume, **MCH** : Mean Corpuscular Hemoglobin, **MCHC** : Mean Corpuscular Hemoglobin Concentration.

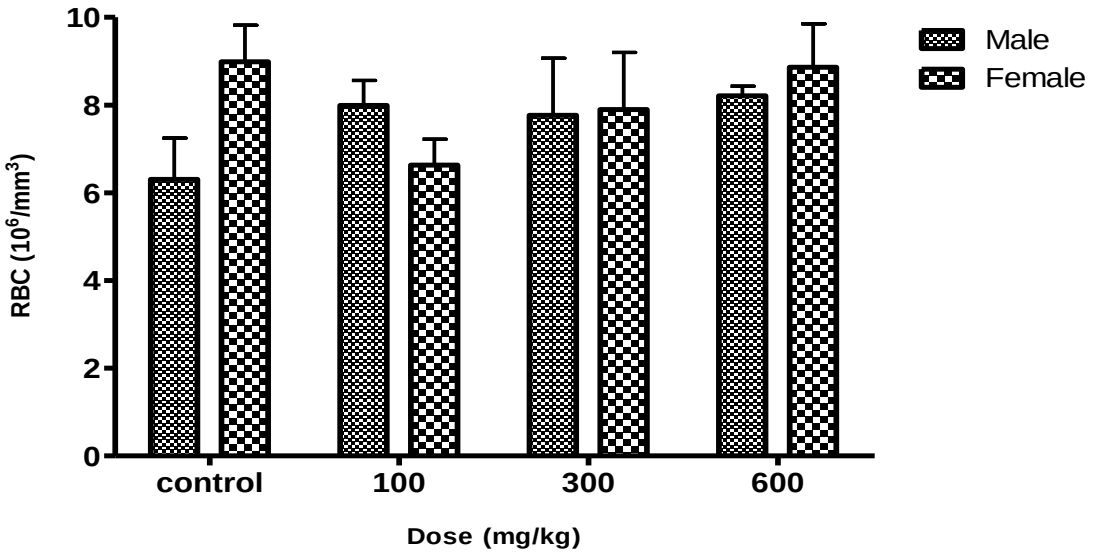


Figure 14. Effect of chronic administration of aqueous extract of *Ruta montana* L. on plasma red blood cells. The values are expressed as mean ± SD (n = 10).

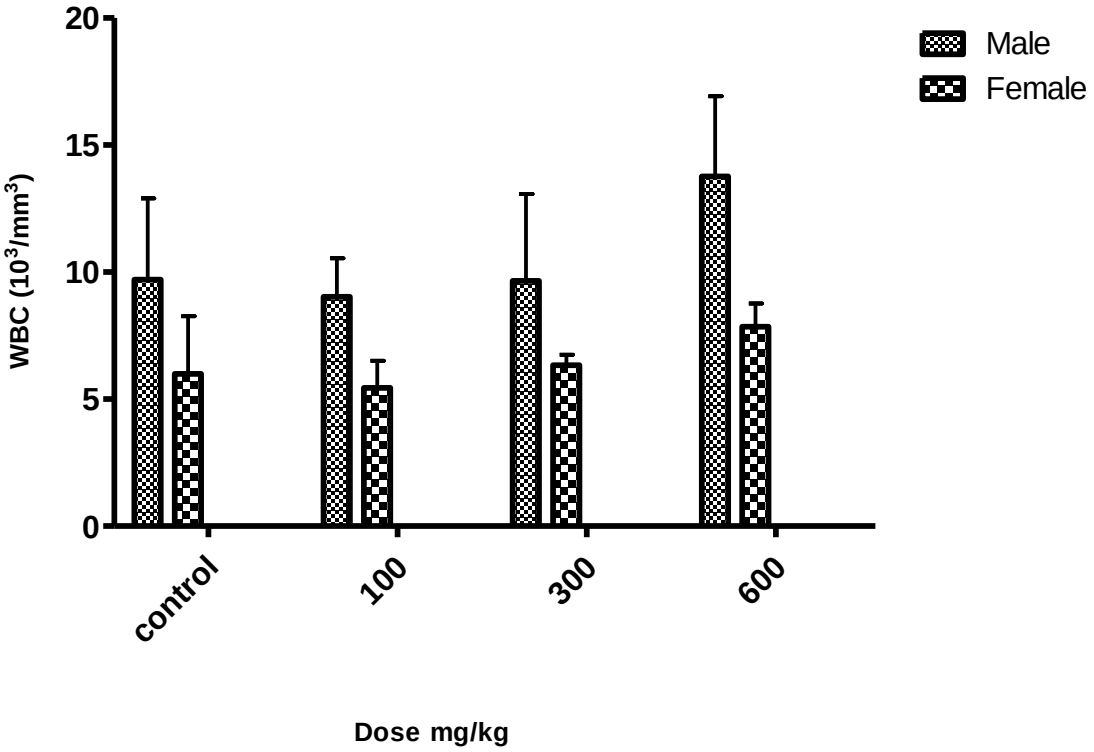


Figure 15. Effect of chronic administration of aqueous extract of *Ruta montana* L. on plasma white blood cells. The values are expressed as mean ± SD (n = 10).

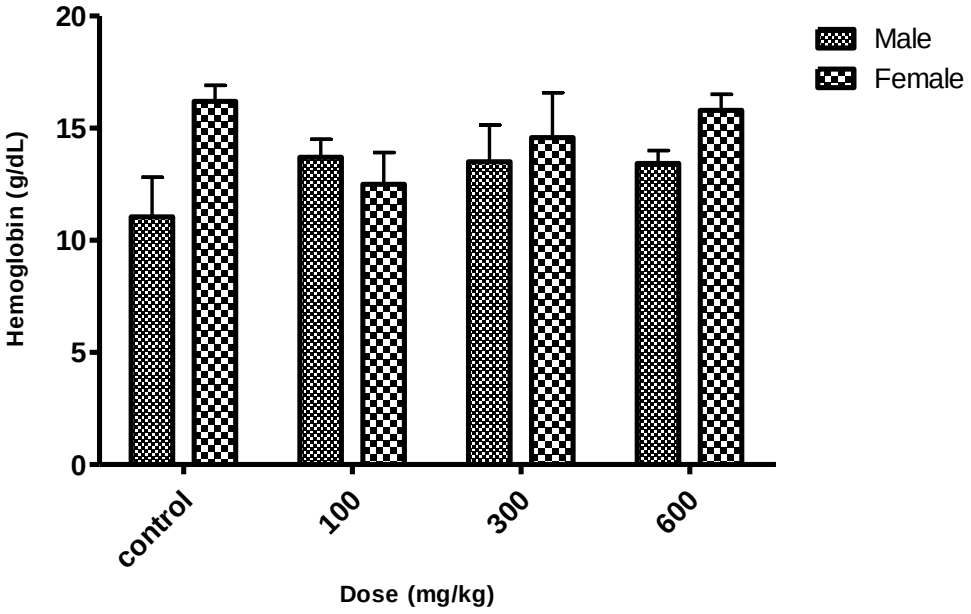


Figure 16. Effect of chronic administration of aqueous extract of *Ruta montana* L. on plasma hemoglobin.The values are expressed as mean ± SD (n = 10).

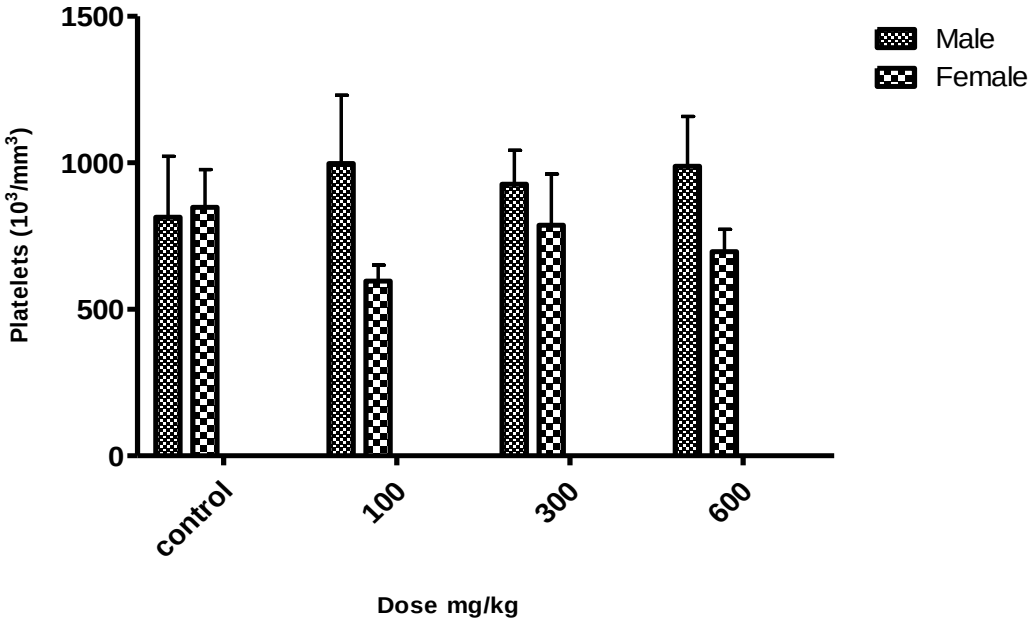


Figure 17. Effect of chronic administration of aqueous extract of *Ruta montana* L. on platelets. The values are expressed as mean ± SD (n = 10).

3.2.3. Effect of *Ruta montana* L. aqueous extract on biochemical parameters

The values for the biochemical parameters in treated and control rats are presented in table 7 and table 8. Oral administration of aqueous extract did not cause any significant differences in the biochemical parameters examined of the male and female rats.

Table 7. Effect of aqueous extract of *Ruta montana* L. (100, 300 and 600mg/kg) on bio-chemical parameters in male and female *Wistar* rats treated for 30 days.

Parameters	Control	100mg/kg	300mg/kg	600mg/kg
Male				
AU (mg/L)	8.33 ± 5.57	11.97 ± 2.3	12.33 ± 2.08	10.67 ±5.03
ALT (UI/L)	78.67 ± 7.5	80.67 ± 41.43	40.67 ± 2.3	48 ± 1.73
ALB (g/L)	30.67± 2.08	29 ± 13	36.67 ± 1.52	36.33 ± 5.03
ALP (UI/L)	194.8 ± 27.71	115.9 ± 6.5	118.4 ± 35.08	124.4 ± 29.9
AST (UI/L)	119.3 ± 6.02	102 ± 7.07	117.3 ± 43.89	105 ± 13
CHOL (g/L)	0.37 ± 0.08	0.73 ± 0.43	0.6 ± 0.13	0.52 ± 0.01
CK (UI/L)	171.3 ± 61.58	178.3 ± 70.06	107 ± 4.24	172 ± 48.5
CREA (mg/L)	5 ± 0.00	5± 1	5 ± 0.00	4 ± 0.00
GLU (g/L)	1.17 ± 0.07	1.25 ± 0.52	1.74 ± 0.41	1.48 ± 0.25
TG (g/L)	0.86 ± 0.12	0.64± 0.06	0.89± 0.16	0.88 ± 0.1
Female				
AU (mg/L)	6 ± 2.82	7.33 ± 2.51	6.66 ± 0.57	8.5 ±2.12
ALT (UI/L)	46.5 ± 2.12	36 ± 4.35	34.5 ± 4.95	34 ± 2.82
ALB (g/L)	34± 9.89	38 ± 2.0	36.67 ± 4.04	34.67 ± 4.04
ALP (UI/L)	103.7 ± 5.23	81.1 ± 3.55	69.07 ± 2.34	86.47± 20.76
AST (UI/L)	96.5 ± 3.53	81.67 ± 19.35	71 ± 10	82.33 ± 5.5
CHOL (g/L)	0.76 ± 0.28	0.52 ± 0.08	0.36 ± 0.03	0.49 ± 0.13
CK (UI/L)	123.5 ± 20.51	134.3 ± 12.7	130.7 ± 51.07	169.7 ± 21.94
CREA (mg/L)	5.5 ± 0.7	5.33± 0.57	5.33 ± 1.15	5 ± 0.00
GLU (g/L)	1.01 ± 0.4	1.44 ± 0.01	1.15 ± 0.19	1.19 ± 0.15
TG (g/L)	0.69 ± 0.28	0.59 ± 0.03	0.45± 0.25	0.3 ± 0.04

Values represent the mean ± SD (n =10/group). **AU**: uric acid, **ALT** : alanine aminotransferase, **ALB** : albumin, **ALP** : alkaline phosphatase, **AST** : aspartate aminotransferase, **CHOL** : cholesterol, **CK** : creatine kinase, **CREA** : creatinine, **GLU** : glucose, **TG** : triglycerides.

Table 8. Effect of aqueous extract of *Ruta montana* L. (100, 300 and 600mg/kg) on bio-chemical parameters in male and female *Wistar* rats treated for 90 days.

Parameters	Control	100mg/kg	300mg/kg	600mg/kg
Male				
AU (mg/L)	16.73 ± 0.48	18.88 ± 1.49	14.9 ± 0.00	16.27 ±7.8
ALT (UI/L)	47.75 ± 8.61	50± 2.58	39 ± 5.56	41.33 ± 13.2
ALB (g/L)	42.25± 8.53	35 ± 4.89	36 ± 6	30.33 ± 17.01
ALP (UI/L)	389.3 ± 192.9	320.3 ± 238.9	261.3 ± 56.3	357.7 ± 243.8
AST (UI/L)	103.3± 13	104.5 ± 45.51	91.67 ± 20.6	108 ± 32.79
CHOL (g/L)	0.9 ± 0.18	0.78 ± 0.31	0.86 ± 0.14	0.79 ± 0.27
CK (UI/L)	85.25 ± 70.03	69.25 ± 59.42	22.67 ± 5.68	32.67 ± 2.88
CREA (mg/L)	7.5 ± 1.91	8± 1.82	9.66 ± 0.57	9 ± 1
GLU (g/L)	0.41 ± 0.14	0.35 ± 0.09	0.36± 0.0.09	0.38 ± 0.09
TG (g/L)	0.61 ± 0.25	0.43 ± 0.05	0.6± 0.34	0.82 ± 0.31
Female				
AU (mg/L)	13.95 ± 3.91	19.77 ± 10.07	16.63 ± 3.13	21.27 ±9.29
ALT (UI/L)	32.5 ± 3.53	56.67 ± 17.21	60 ± 11.58	66.75 ± 23.36
ALB (g/L)	32.5± 3.53	31.5 ±4.95	34.5 ± 5.74	39 ± 6.92
ALP (UI/L)	152.5 ± 74.25	142.7 ± 92.68	273.3 ±80.66	236± 31.58
AST (UI/L)	102.5 ± 3.53	132.3 ± 49.92	101.3 ± 42.03	110.7± 41.67
CHOL (g/L)	0.87 ± 0.23	1 ± 0.00	0.69± 0.16	0.62 ± 0.05
CK (UI/L)	18.5 ± 9.19	108.3 ± 46.65	94 ± 24.04	175.8 ± 176.9
CREA (mg/L)	7.75 ± 3.59	8.33± 2.08	8.25 ± 1.5	6.66 ± 0.57
GLU (g/L)	0.36 ± 0.21	0.41 ± 0.07	0.54 ± 0.21	0.45 ± 0.34
TG (g/L)	0.55 ± 0.15	0.49 ± 0.08	0.55± 0.5	0.51 ± 0.14

Values represent the mean ± SD (n =10/group). **AU**: uric acid, **ALT** : alanine aminotransferase, **ALB** : albumin, **ALP** : alkaline phosphatase, **AST** : aspartate aminotransferase, **CHOL** : cholesterol, **CK** : creatine kinase, **CREA** : creatinine, **GLU** : glucose, **TG** : triglycerides.

3.2.4. Effect of *Ruta montana* L. aqueous extract on rat's organs weight

Organ weight also is an important index of physiological and pathological status in man and animals. Absolute and relative organ weights of 90 days treated rats are shown in Table 9. *Ruta montana* L. aqueous extract administration did not cause statistical difference in organ weight of male and female rat except a significant decrease was observed in testis, epididymis and seminal vesicle in the doses 300 and 600mg/kg, when compared to the control group. Moreover, gross examination of internal organs of all the rats revealed no detectable abnormalities.

Table 9. Effect of chronic oral administration of aqueous extract of *Ruta montana* L. on rats organs weight.

Organ	Relative organ weight			
	Control (0mg/kg)	Group I (100mg/kg)	Group II (300mg/kg)	Group III (600mg/kg)
Male				
Kidneys	0.31±0.06	0.33±0.08	0.29±0.00	0.3±0.37
Liver	3.68±0.41	3.41±0.37	3.07±0.58	3.05±1.15
Heart	0.4±0.09	0.4±0.14	0.32±0.02	0.36±0.09
Lungs	0.81±0.2	0.69±0.08	0.77±0.11	0.77±0.19
Spleen	0.37±0.08	0.41±0.16	0.36±0.10	0.4±0.14
Brain	0.73±0.1	0.68±0.04	0.66±0.13	0.66±0.18
Stomach	0.65±0.08	0.51±0.07	0.59±0.12	0.71±0.1
Testis	0,58±0,07	0,48±0,13	0,23±0,15*	0,19±0,10**
Epididymis	0,22±0,00	0,19±0,04	0,11±0,02**	0,07±0,00**
Seminal vesicle	0,90±0,43	0,55±0,26	0,13±0,05*	0,16±0,11*
Female				
Kidneys	0.31±0.04	0.43±0.12	0.32±0.02	0.35±0.02
Liver	3.81±0.39	4.09±0.77	4.14±0.13	3.65±0.39
Heart	0.33±0.02	0.37±0.08	0.43±0.04	0.39±0.00
Lungs	0.64±0.08	0.6±0.11	0.6±0.05	0.74±0.04
Spleen	0.32±0.19	0.38±0.04	0.36±0.1	0.38±0.07
Brain	0.73±0.16	0.85±0.03	0.79±0.09	0.87±0.09
Stomach	0.69±0.09	0.63±0.09	0.78±0.13	0.68±0.02
Ovaries	0,04±0,01	0,04±0,00	0,03±0,00	0,04±0,007

Values represent the mean ± SD (n =10/group). * $p<0.05$; ** $p<0.01$

3.2.5. Effect of *Ruta montana* L. aqueous extract on sperm count and motility

The sperm count in control group was 13.21 ± 1.99 x 2500 sperm/mm^3, in group I 9.66 ± 2.22 x 2500 sperm/mm^3, in group II 5.4 ± 0.04 x 2500 sperm/mm^3 and in group III 1 ± 0.00 x 2500 sperm/mm^3. Whereas the motility in control group was 63.73 ± 2.25 (%), in group I 60.05 ± 2.08 (%), in group II 19.42 ± 4.30 (%) and in group III 0.00 ± 0.00 (%) (Table 10). In *Ruta montana* L. aqueous extract treated rats the epididymidal sperm parameters showed evidence of dose dependent toxicity. The sperm count and motility were significantly decreased in group II and group III.

Table 10. Effect of *Ruta montana* L. aqueous extract on sperm count and motility

	Control (0 mg/kg)	Group I (100mg/kg)	Group II (300mg/kg)	Group III (600mg/kg)
Count (2500/mm^3)	13.21±1.99	9.66±2.22	5.4±0.04**	1±0.00***
Motility (%)	63.73±2.25	60.05±2.08	19.42±4.30***	0.00±0.00***

Values are presented as means ± SD; ** P < 0.01, *** P < 0.001 compared with control

3.2.6. Effect of *Ruta montana* L. aqueous extract on rats organs histology

Microscopic observations in control and treated groups showed a normal liver histomorphology consisting of central vein with hepatocytes separated by sinusoids (Figure 18). However, the doses at 100, 300 and 600 mg/kg revealed a vascular congestion. The kidneys of control and treated rats showed normal glomeruli and renal tubules (Figure 19), with presence a vascular congestion in treated rats. In addition, there are no histological changes observed in brain and ovary in all treated groups compared with control group (Figure 20 and Figure 21).

Histological examination of the testes of control group showed normal histological structure of the seminiferous tubules associated with complete spermatogenic series as demonstrated in figure (22 C). The testes of rats given 100 mg/kg of body weight of *Ruta montana* L. aqueous extract had similar histological appearance as the control rats (Figure 22 D1)**,** and showed active spermatogenesis, however, the testes of the rat treated with 300 and 600 mg/kg of body weight of *Ruta montana* L. revealed degeneration of seminiferous tubules with absence of sperm in tubular lumen as shown in figure (22 D2) and figure (22 D3). For the epididymis of control group and the group treated by 100 mg/kg of body weight showed normal tubules that contained spermatozoa in the lumen (Figure 23 C and D1). Whereas, the treatment with the doses 300 and 600 mg/kg of body weight caused damage in epididymis tubules and absence of sperm (Figure 23 D2 and D3).

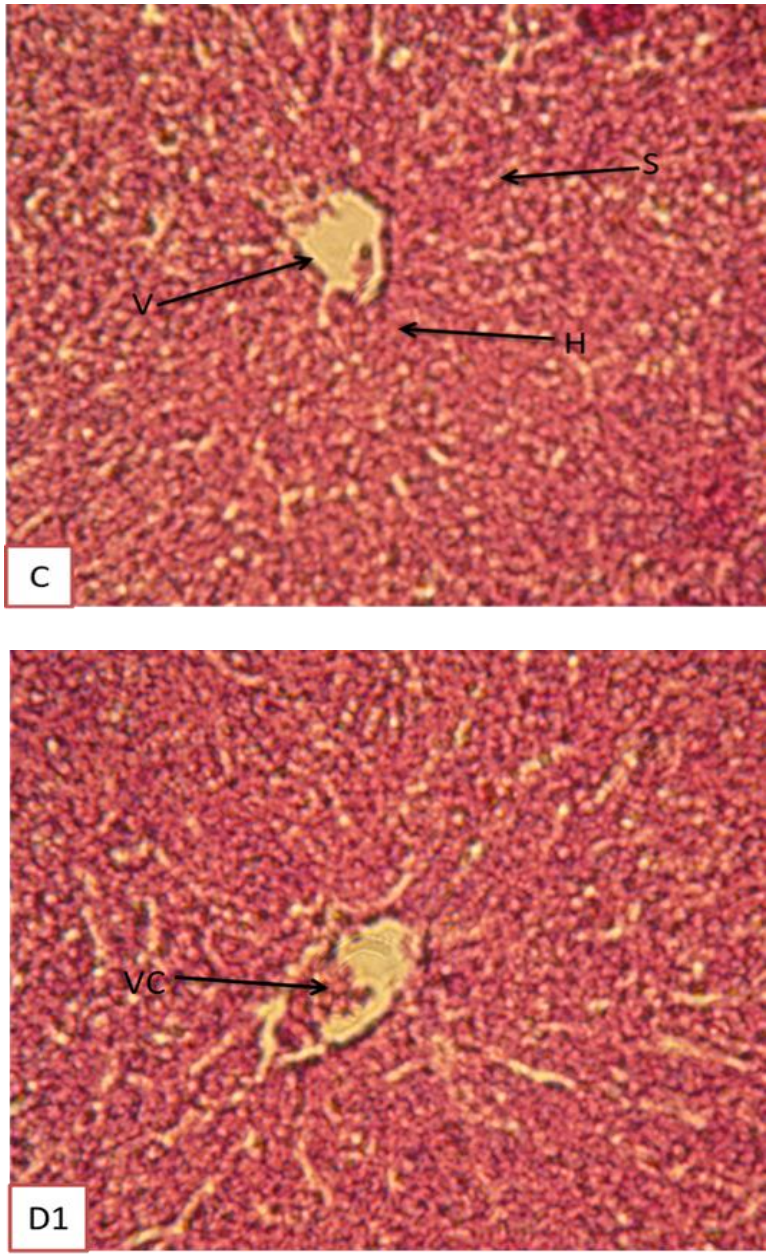


Figure 18: Histological sections of liver of control group (C) and group 1 treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal histomorphology. V: vein ; H: hepatocytes ; S: sinusoids ; VC: vascular congestion. (HE X 200)

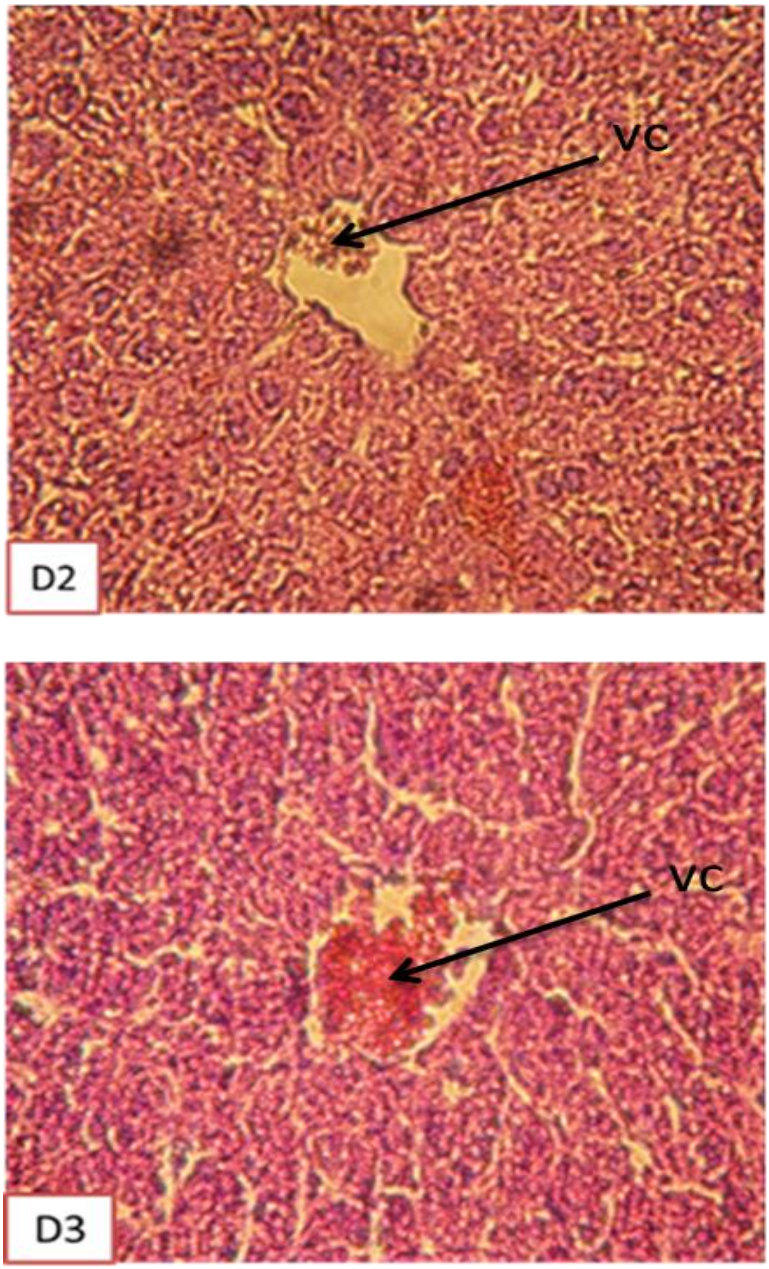


Figure 18: Histological sections of liver of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg bw of *Ruta montana* L. aqueous extract respectively after 90 days of treatment showing normal histomorphology with a vascular congestion. VC: vascular congestion. (HE X 200).

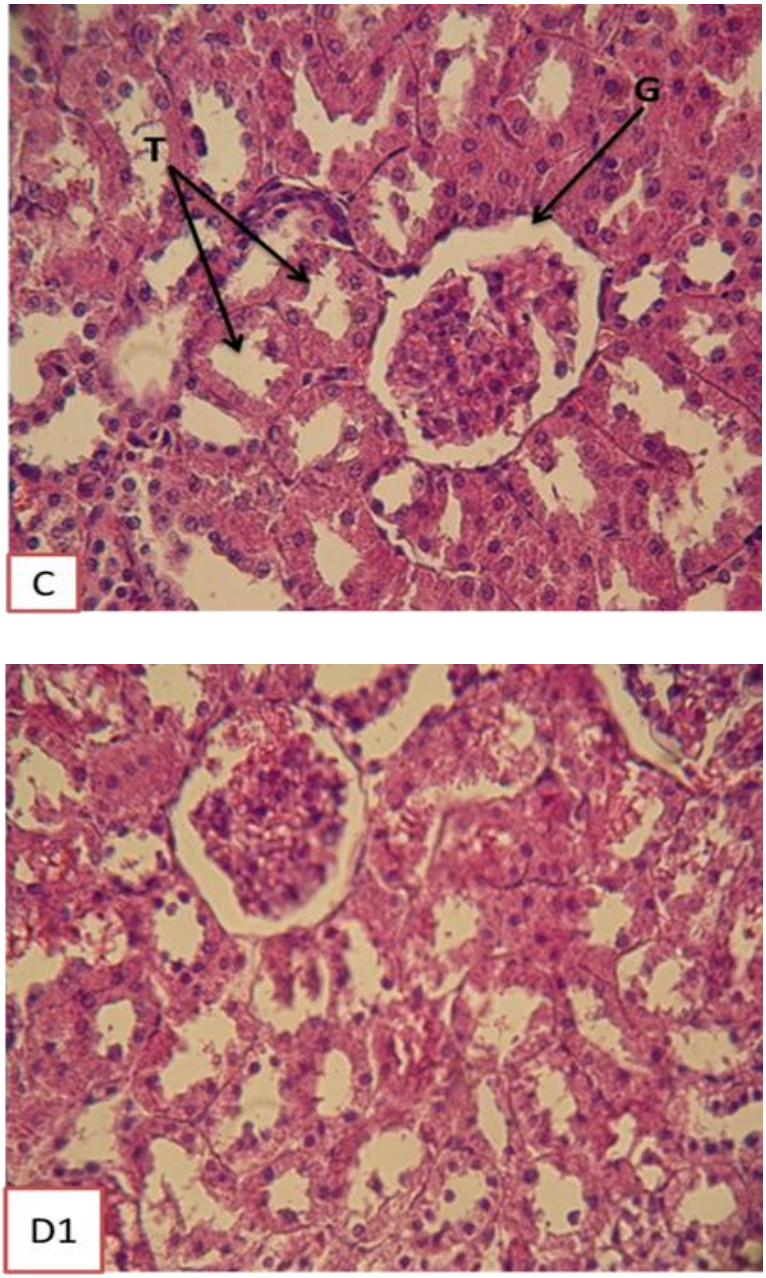


Figure 19: Histological sections of kidneys of control group (C) and group 1 (D1) treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal glomeruli and renal tubules. G: glomeruli ; T: tubules. (HE X 200)

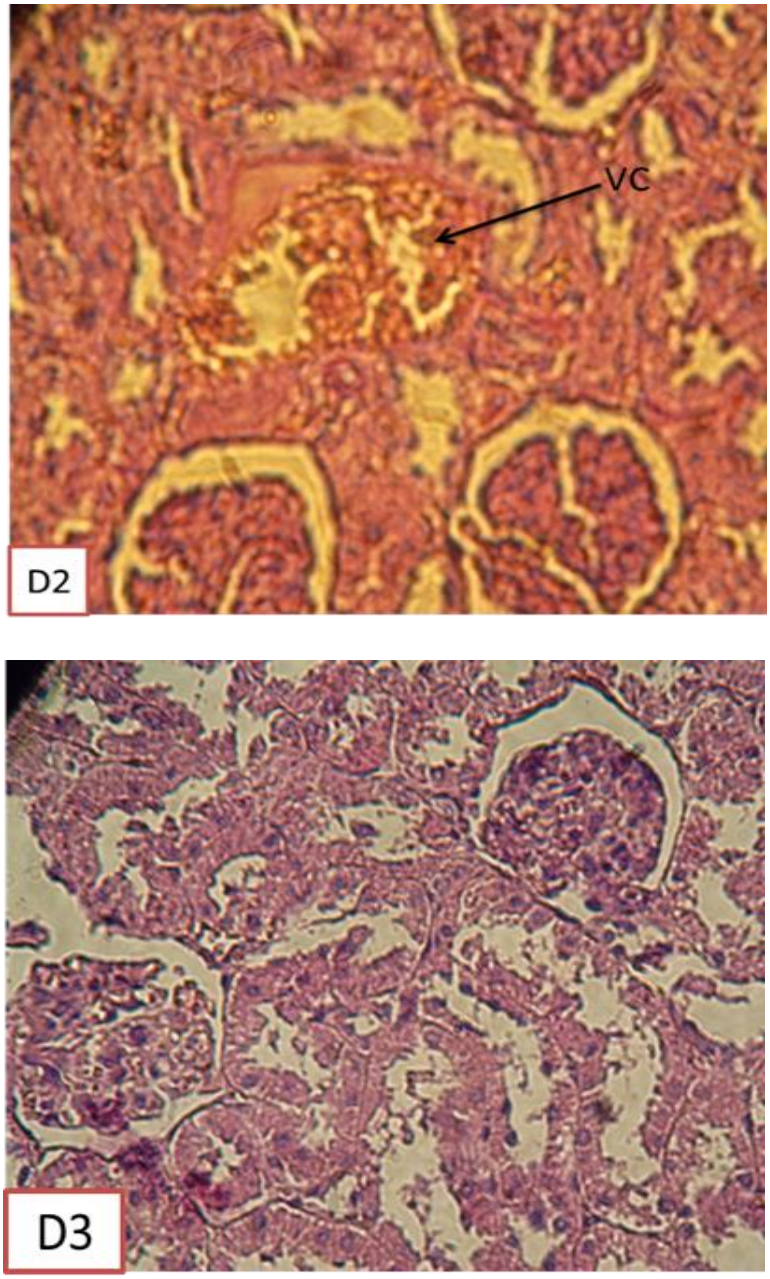


Figure 19: Histological sections of kidneys of group 2 (D2) and group 3 (D3) treated with 200 mg/kg and 600 mg/kg bw respectively of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal glomeruli and renal tubules with presence a vascular congestion. VC: vascular congestion. (HE X 200)

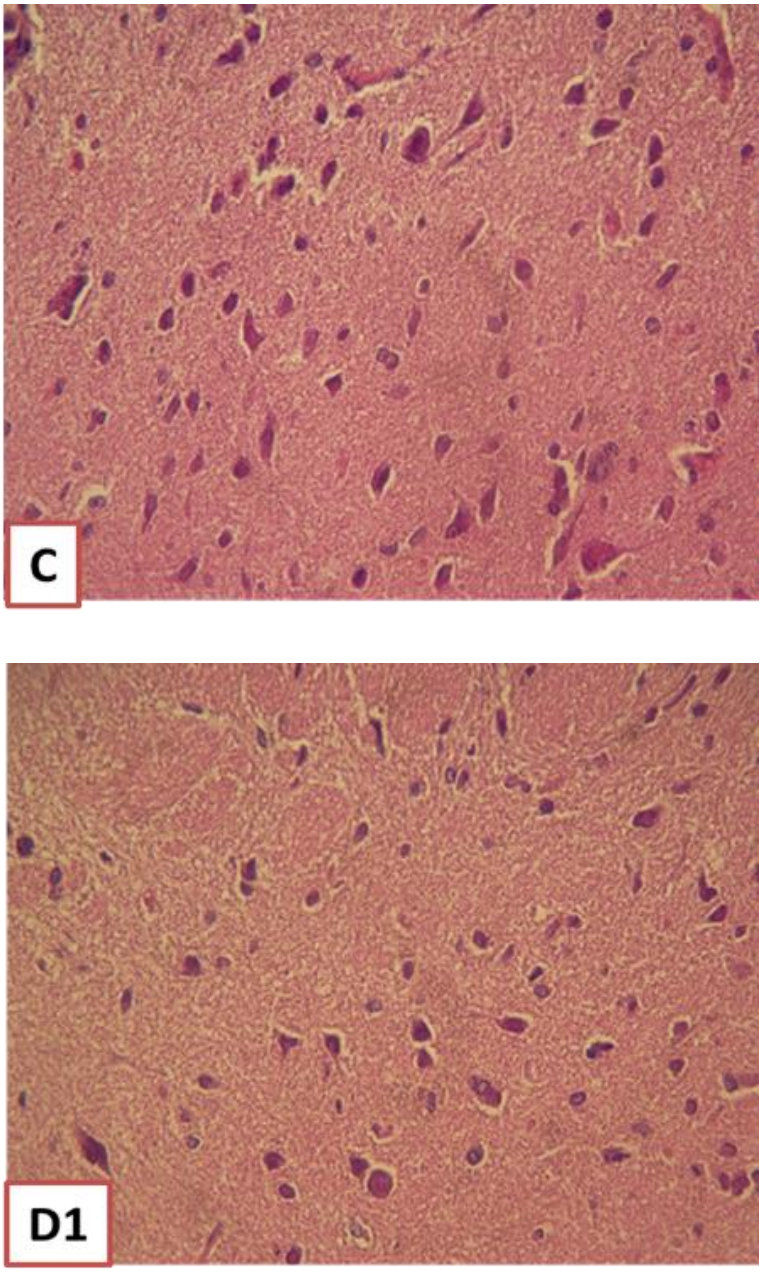


Figure 20: Histological sections of Brain of control group (C) and group 1 (D1) treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal structure. (HE X 200)

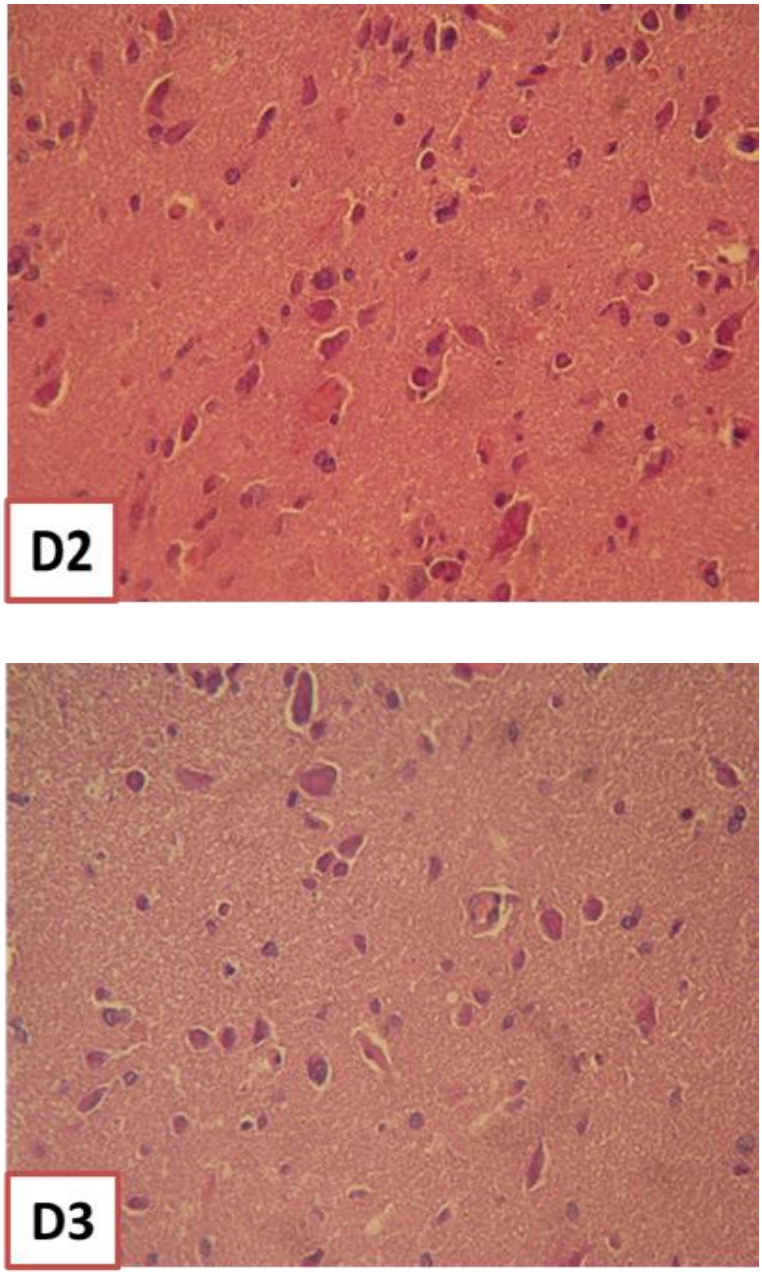


Figure 20: Histological sections of Brain of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg bw respectively of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal structure. (HE X 200)

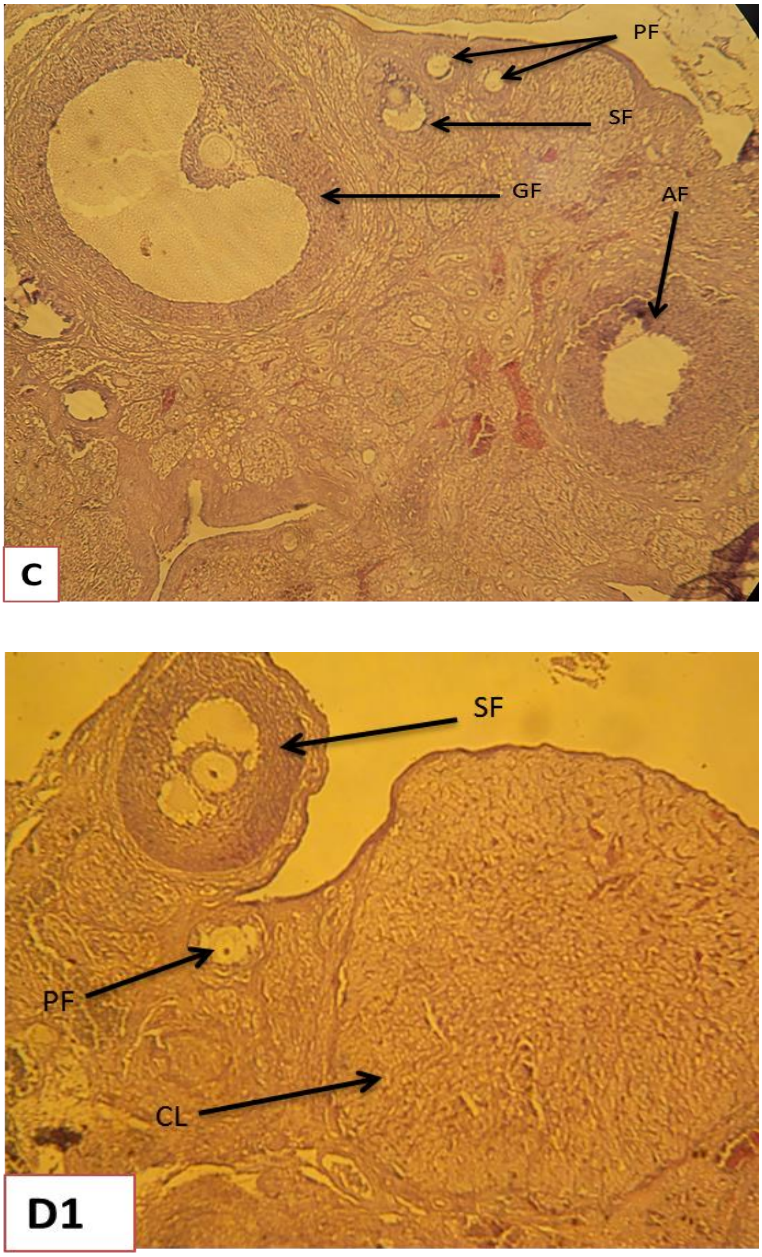


Figure 21: Histological sections of Ovary of control group (C) and group 1 (D1) treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal structure. PF: primary follicle ; SF: secondary follicle ; GF: grafian follicle ; atresia follicle ; CL : corpus luteum. (HE X 200)

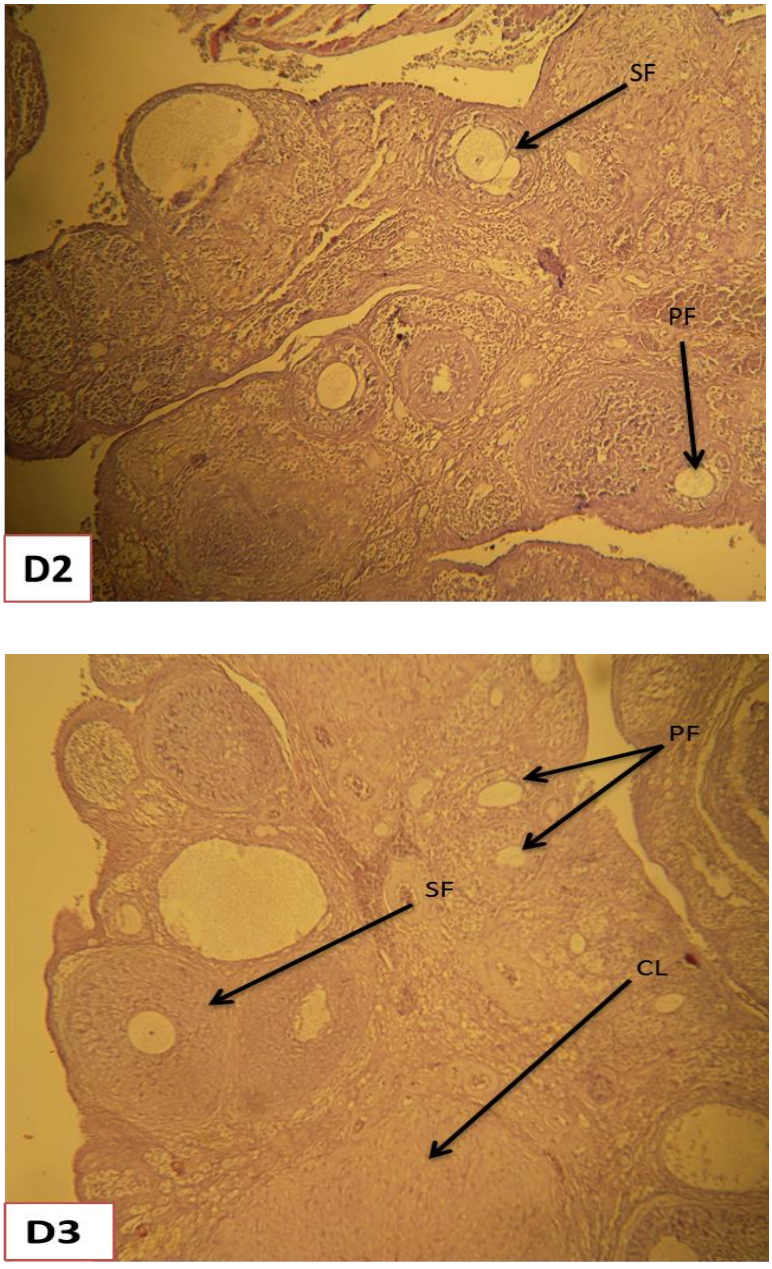
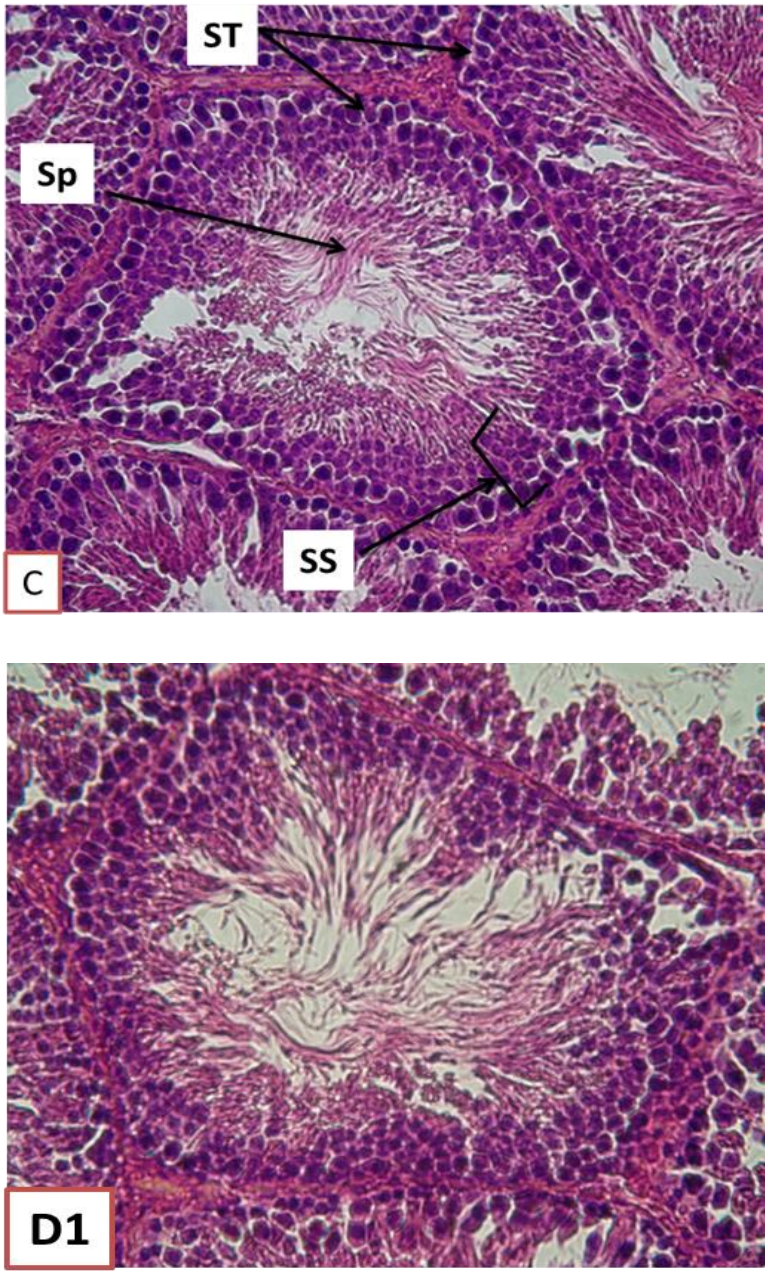


Figure 21: Histological sections of Ovary of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg bw respectively of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal structure. PF: primary follicle ; SF: secondary follicle ; CL : corpus luteum. (HE X 200)



Fifure 22: Histological sections of testes of control group (C) and group 1 (D1) treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal histological structure of the semiferous tubules associated with complete spermatogenic series and spermatozoa in the lumen. ST: seminiferous tubules; Sp: sperm; SS: stages of spermatogenesis. (HE X 200)

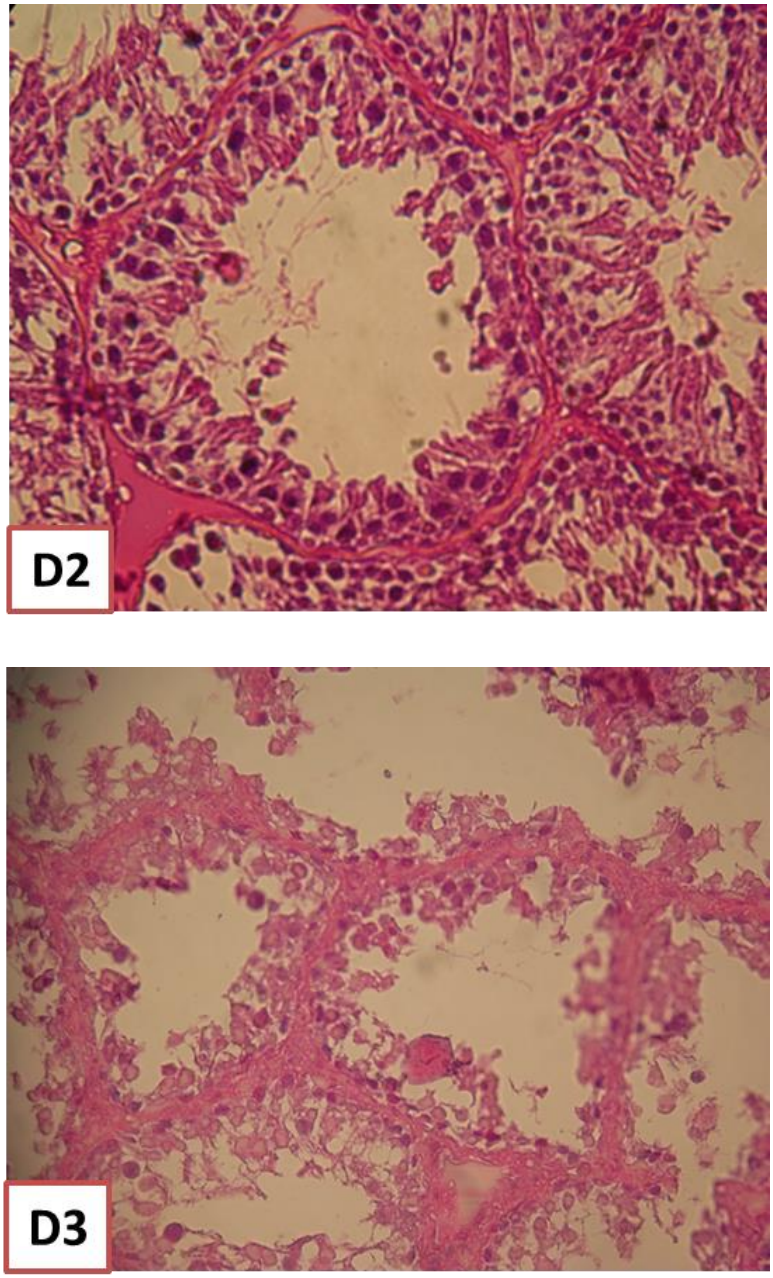


Figure 22: Histological sections of testes of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg bw respectively of *Ruta montana* L. aqueous extract after 90 days of treatment showing degeneration of seminiferous tubules with absence of sperm in tubular lumen. (HE X 200)

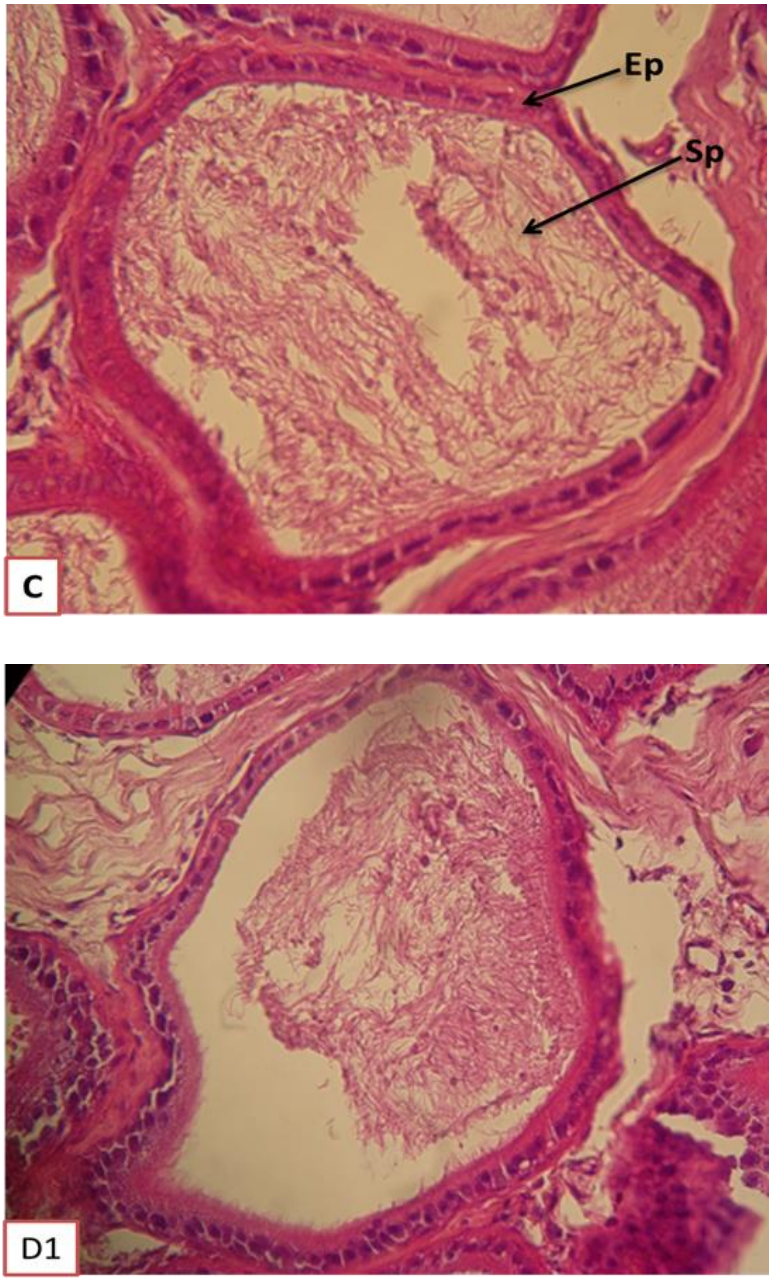


Figure 23: Histological sections of the epididymis of control group (C) and group 1 (D1) treated with 100 mg/kg bw of *Ruta montana* L. aqueous extract after 90 days of treatment showing normal tubules that contained spermatozoa in the lumen. Ep: Epethelial cells; Sp: Sperm. (HE X 200)

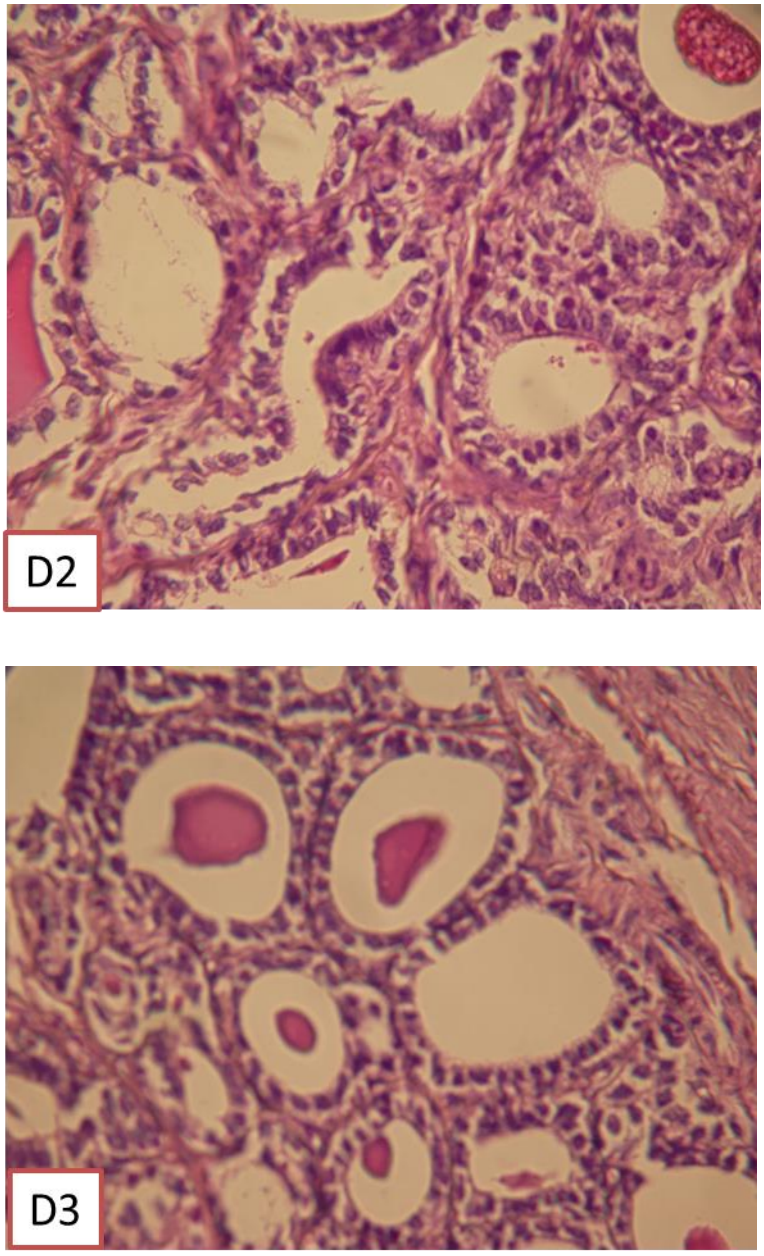


Figure 23: Histological sections of the epididymis of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg bw respectively of *Ruta montana* L. aqueous extract after 90 days of treatment showing damage in epididymal tubules and absence of sperm. (HE X 200).

4. Antioxidant activity of *Ruta montana* L. extracts

4.1. *In Vitro* Antioxidant Activity

4.1.1. DPPH-scavenging assay

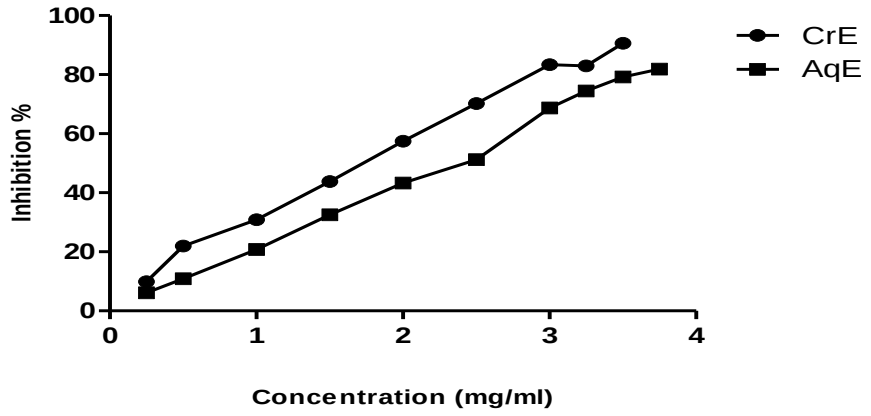
The scavenging ability of the extracts was expressed as IC_{50} value, which is the concentration of substrate that causes 50% loss of DPPH activity (color) (Molyneux, 2004). Low IC_{50} value indicates strong ability of the extract to act as DPPH scavenger. The DPPH scavenging activity of the extracts is represented in table 11.

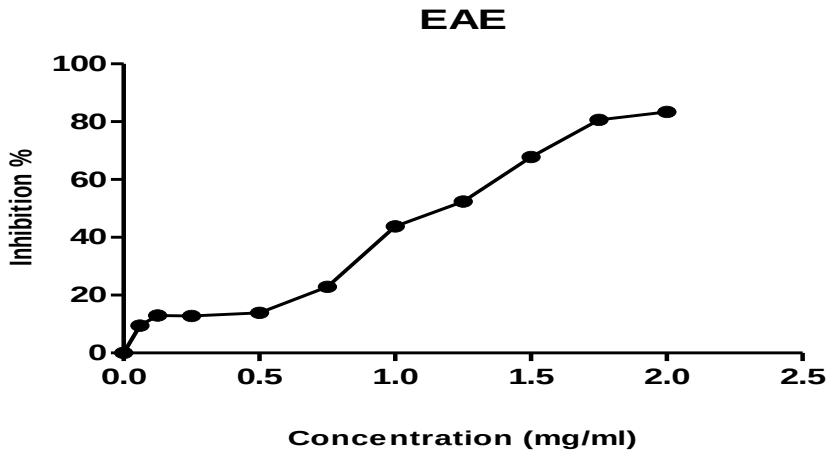
Table 11: DPPH scavenging activity of *Ruta montana* L. extracts and phenolic standards.

Standards	IC_{50}(mg/ml)	Extracts	IC_{50}(mg/ml)
BHT	0.032 ± 0.000	CrE	$0.067 \pm 0.002^{**}$
Gallic acid	$0.056 \pm 0.001^{\#}$	ChE	$0.146 \pm 0.015^{***}$
Quercetin	$3.491 \pm 0.001^{\#}$	EAE	0.044 ± 0.001^{ns}
Rutin	$4.179 \pm 0.000^{\#}$	AqE	$0.083 \pm 0.003^{***}$

#: µg/ml. Comparison was realized against BHT, ns: not significant, **: $p \leq 0.01$, ***: $p \leq 0.001$. Each value represents the mean ± SD (n = 3).

Results showed that the extracts exhibited a dose-dependent activity in scavenging DPPH radicals (Figure 37). The EAE exhibited the highest radical scavenging activity with IC_{50} of (0.044± 0.001mg/ml), which was significantly near to that of BHT (0.032± 0.000mg/ml), followed by CrE with 0.067± 0.002mg/ml, AqE with 0.083±0.003mg/ml and ChE with 0.146±0.015mg/ml. Moreover, CrE, AqE and ChE had a radical scavenging activity lower by 2-folds, 2.5-folds and 4.5-folds than BHT, respectively.





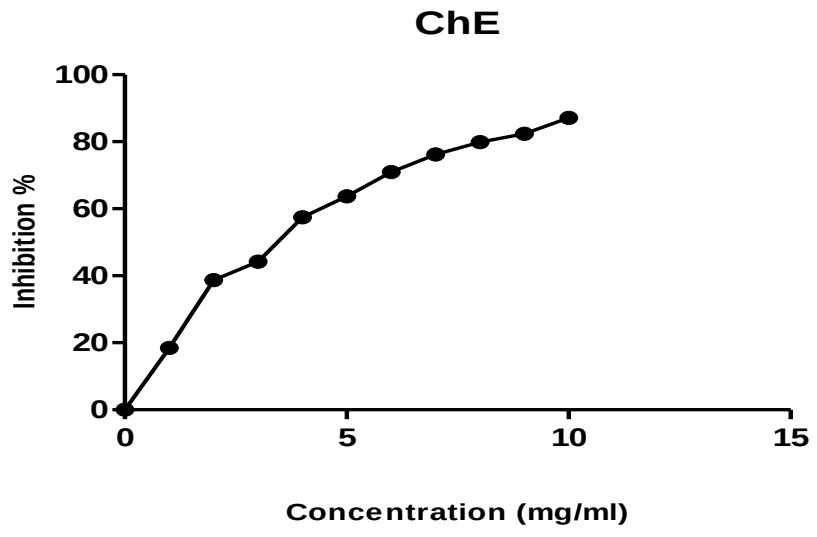


Figure 24. The percentage inhibition of free DPPH radical in the presence of different concentrations of *Ruta montana* L. extracts. Each value represents the mean ± SD (n =3).

4.1.2. β-carotene/linoleic acid bleaching assay

Figure 38 and Figure 39 show the changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of *Ruta montana* L. extracts compared to that of synthetic antioxidant BHT as a positive control during 48 h under the same condition. AqE and ChE exhibited the highest antioxidant activity (90.34 ± 0.46% and 89.92 ± 0.29%, respectively), which was near to that of BHT (100 ± 0.52%), following by EAE and CrE with an antioxidant activity (73.56 ± 0.63 % and 63.27 ± 2.27 %, respectively).

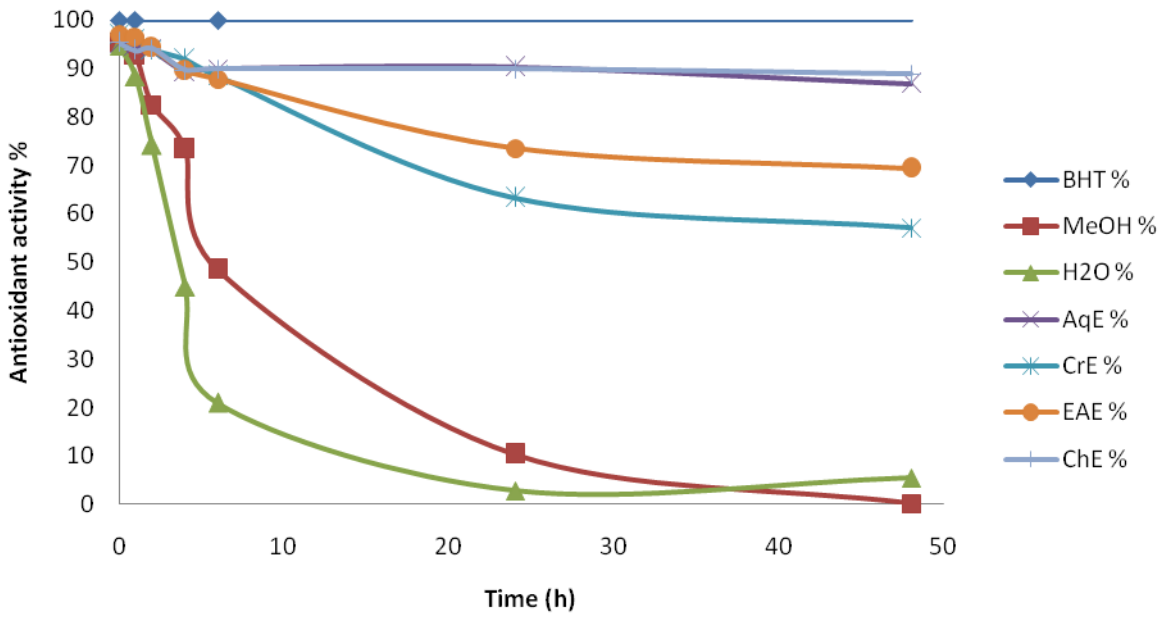


Figure 25. The changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of *Ruta montana* L. extracts (2mg/ml), compared to BHT as a positive control during 48 h. **AqE:** aqueous extract, **CrE:** crude extract, **ChE:** chloroform extract, **EAE:** ethyl acetate extract.

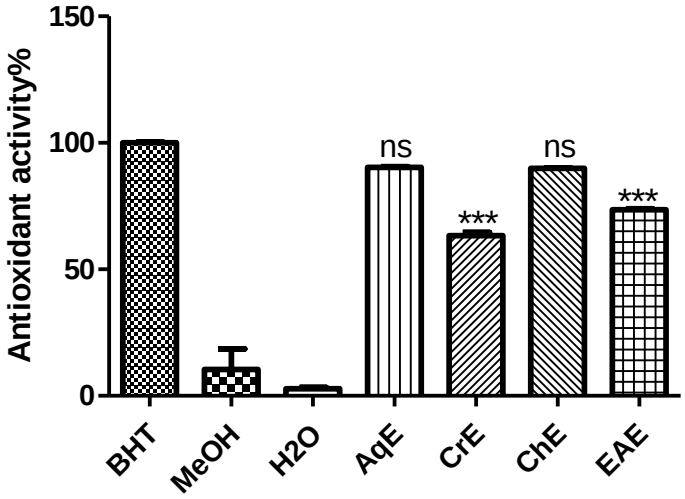


Figure 26. Inhibition percentage of extracts of *Ruta montana* L. in β-carotene/linoleic acid assay after 48 h compared with BHT, methanol and water as standards. Results represent the means ±SD (n = 3). **ns:** not significant, *******: $p \leq 0.001$. **AqE:** aqueous extract, **CrE:** crude extract, **ChE:** chloroform extract, **EAE:** ethyl acetate extract.

4.1.3. Chelation of ferrous iron by plant extracts

Table 12 list IC_{50} values of *Ruta montana* L. extracts obtained from measurement of metal chelating activity. The smaller IC_{50} value represents the higher metal chelating activity. The chelating effects of the extracts on ferrous increased as a function of concentration (Figure 40).The AqE and CrE appear to have the most important chelating activity with IC_{50} values of 0.005 ± 0.004 mg / ml and 0.021 ± 0.005 mg/ml, respectively. The chelating effect of ChE and EAE is very low (IC_{50} = 0.146 ± 0.015 and 0.771 ± 0.021 mg / ml, respectively).

Table 12. Metal chelating activity of *Ruta montana* L. extracts and EDTA.

Extracts	IC_{50} (mg/ml)
CrE	0.021 ± 0.005^{ns}
ChE	$0.146 \pm 0.015^{***}$
EAE	$0.771 \pm 0.021^{***}$
AqE	0.005 ± 0.004^{ns}
EDTA	$5.32 \pm 0.03^{\#}$

#: µg/ml. Comparison was realized against EDTA; ns: not significant, ***: $p \leq 0.001$. Each value represents the mean ± SD (n = 3).

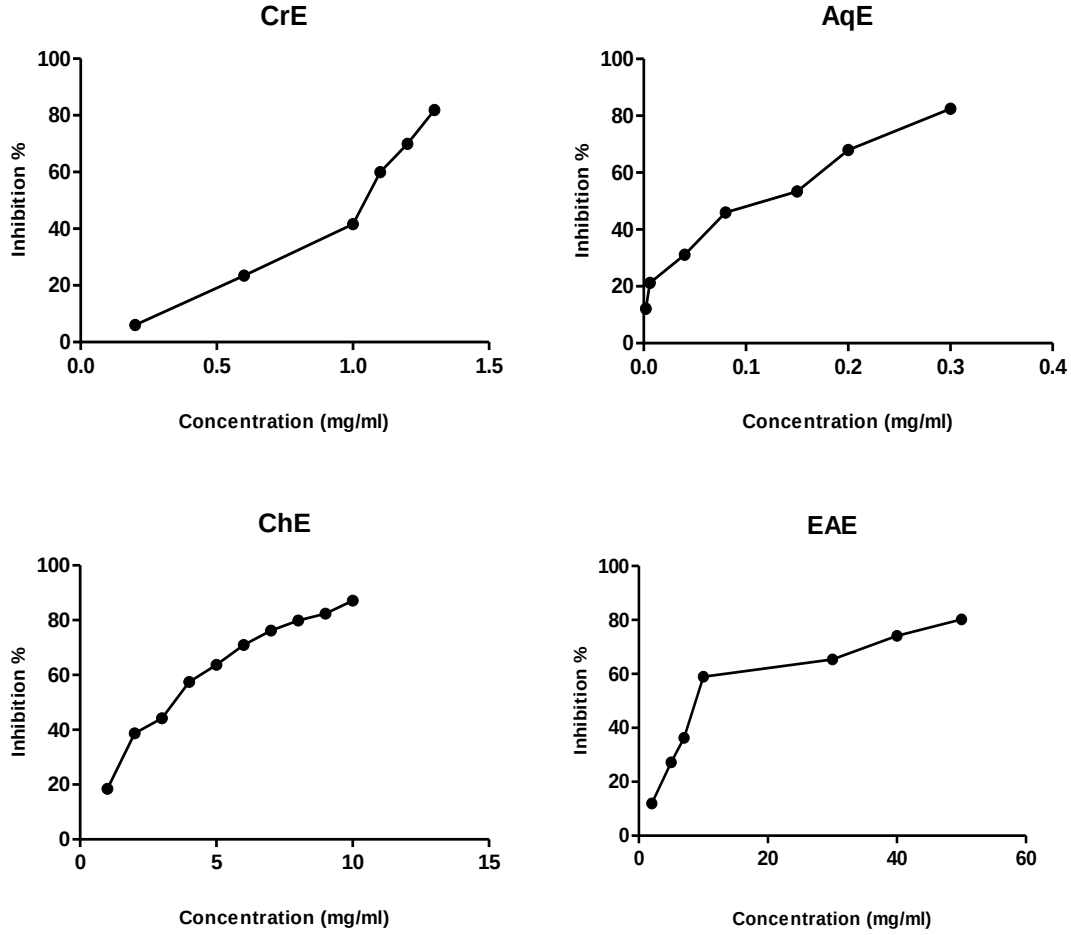


Figure 27. Metal chelating activity of different *Ruta montana* L. extracts. Data were presented as mean ± SD (n= 3)

4.2. *In vivo* antioxidant activity

4.2.1. Effect of extracts on plasma antioxidant capacity using DPPH radical

The obtained results (Figure 41) showed that oral administration of AqE (300 mg / ml) and CrE (100 mg / ml) leads to increased plasma antioxidant capacity (18,13±5,366% and 17,57±5,869%, respectively). This increase is not significant statistically compared with the control group13,65±1,46%. However, the administration of AqE at the dose 100 mg/kg did not cause any change 13,28 ± 3,253% compared with the control group.

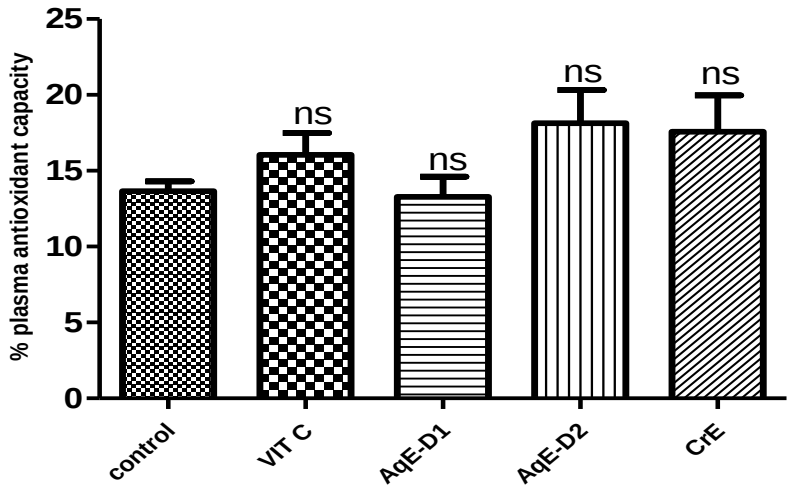


Figure 28. Plasma antioxidant capacity toward DPPH radical for different groups. Values are means ± SD (n = 6). Comparisons are made relative to the control group, ns: not significant.

4.2.2. Effect of *Ruta montana* L. extracts on plasma reducing power

The obtained results (Figure 42) showed that oral administration of AqE (100 and 300 mg / ml) and CrE (100 mg / mL) did not show any significant changes compared with the control group.

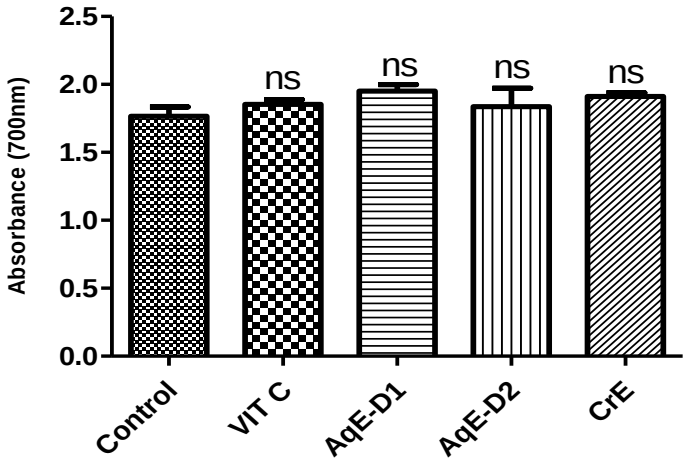


Figure 29. Reducing power of plasma for different groups. Values are means ± SD (n = 6). Comparisons are made relative to the control group, ns**:** not significant.

4.2.3. Effect of *Ruta montana* L. extracts on lipid peroxidation in liver and kidney

The results of lipid peroxidation (Table 13) revealed a not significant decrease in liver and kidney tissues for the group treated with AqE 300mg/kg and CrE 100mg/kg with the value of 11,20 ± 2,56, 14,99 ± 3,70 nmol/g tissue and 11,85±3,59, 11,15±3,30 nmol/g tissue respectively, compared to group treated with distilled water 18,50±3,31 and 16,54±3,45 nmol/g tissue.

Table 13: Effect of *Ruta montana* L. extracts on lipid peroxidation in liver and kidney

	MDA concentration (nmol/g tissue).	
Group	Liver	Kidney
Group (distilled water)	18,50±3,31	16,54±3,45
Group (Vit C 50mg/kg)	12,90±5,18^{ns}	14,59±4,95^{ns}
Group (AQE 100mg/kg)	18,90±5,49^{ns}	17,88±3,22^{ns}
Group (AQE300mg/kg)	11,20±2,56^{ns}	11,85±3,59^{ns}
Group (CrE 100mg/kg)	14,99±3,70^{ns}	11,15±3,30^{ns}

Each value represents the mean ± SD (n = 6). ns: not significant

4.2.4. Effect of *Ruta montana* L. extracts on reduced Glutathione (GSH)

The obtained results (Table 14) indicated that AqE at the dose of 300mg/kg and CrE at the dose of 100mg/kg presented a not significant increase in GSH in liver tissue 0,7±0,16 and 0,74±0,15 µmol / g tissue, respectively compared with the control group 0,57±0,15µmol / g tissue.

In the kidney tissue AqE at the dose of 300mg/kg and CrE at the dose of 100 mg/kg showed an non significant increase with 0.65±0.06 and 0,91±0,09 µmol /g tissue, respectively, compared to the control group 0,57±0,06 µmol / g tissue.

Table 14: Effect of *Ruta montana* L. extracts on reduced Glutathione (GSH)

	GSH concentration (µmol / g tissue)	
Group	Liver	Kidney
Group(distilled water)	0,57±0,15	0,57±0,06
Group (Vit C 50mg/kg)	$0,79±0,11^{ns}$	$1,02±0,11^{ns}$
Group (AqE 100mg/kg)	$0,44±0,13^{ns}$	$0,58±0,09^{ns}$
Group (AqE 300mg/kg)	$0,7±0,16^{ns}$	$0,65±0,06^{ns}$
Group (CrE 100mg/kg)	$0,74±0,15^{ns}$	$0,91±0,09^{ns}$

Each value represents the mean ± SD (n = 6). ns: not significant.

4.2.5. Effect of *Ruta montana* L. extracts on catalase activity

The results of catalase (Table15) revealed not significant increase in liver and kidney tissue for the groups treated with AqE 300 mg/kg and CrE 100 mg/kg with the value of 0,52 ± 0,038 , 0,53±0,13 and 0,98 ± 0,17, 1,09±0,02 μmol/g tissue, respectively compared to group treated with distilled water 0,36 ± 0,02 and 0,74 ± 0,14 μmol/g tissue.

Figure 15. Effect of *Ruta montana* L. extracts on catalase (CAT) activity

	Catalase activity (μmol/g tissue)	
Group	Liver	Kidney
Group(distilled water)	0,36±0,02	0,74±0,14
Group (Vit C 50mg/kg)	$0,53±0,11^{ns}$	$0,99±0,12^{ns}$
Group (AqE 100mg/kg)	$0,31±0,031^{ns}$	$0,75±0,10^{ns}$
Group (AqE 300mg/kg)	$0,52±0,038^{ns}$	$0,98±0,17^{ns}$
Group (CrE 100mg/kg)	$0,53±0,13^{ns}$	$1,09±0,02^{ns}$

Each value represents the mean ± SD (n = 6). ns: not significant.

DISCUSSION

Medicinal plants have been in use all over the world to treat various diseases including inflammation, heart diseases, cancer, etc. Today the large numbers of drugs in use are derived from plants, which are rich in secondary metabolites and essential oils of therapeutic importance. The important advantages claimed for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective and their easy availability (Verma and Kumar, 2011).

Plant phenolics, tannins and flavonoids represent major groups of plant constituents that work predominantly as powerful antioxidants or scavenger of free radicals. They play beneficial role in human health and cure or prevent ailments such as inflammatory disorders, cardiovascular diseases, cancer and diabetes which occur due to the deregulation of free radicals generation in the cells (Chouhan and Singh, 2011).

In the present study, total phenolics, flavonoids and tannins contents among the different extracts are evaluated. The total polyphenols content varied in the different extracts and ranged from 76,90 ± 0,681 to 257,1 ± 0,703 µg GAE /mg extract, and their contents were in the following order: EAE > ChE > CrE >AqE.

Flavonoid contents were in the order: EAE > CrE > ChE > AqE. While the tannins contents were in the order: EAE > ChE > CrE > AqE. Based on these data, it can be concluded that the EAE extract of *Ruta montana L.* contains the highest amount of phenolics (257,1 ± 0,703 µg GAE/mg extract), flavonoids compounds (117,4 ± 3,451 µg QE/mg extract, 139,5 ± 4,107 µg RE/mg extract) and tannins (251 ± 1.41µg TAE/mg extract), indicating that ethyl acetate was to be the best solvent to concentrate phenolic substances of intermediate polarity.

Phytotheraputic products are many times, mistakenly regarded as safe because they are natural (Gesler, 1992). Nevertheless, these products contain bioactive principles with potential to cause adverse effect (Bent and Ko, 2004).Therefore, it is important to evaluate the adverse effects of all the natural products used in therapeutics.

This study tested the acute and chronic toxicity of the aqueous extract of *Ruta montana* L. In the acute toxicity study, the aqueous extract at doses of 2, 4, 6, 8, 10 and 12 g/kg body weight was administered orally to both sexes of mice during 14 days. Signs of toxicity are the most important indicator of drug or clinical related toxicity or morbidity in all types of toxicity studies. Clinical signs including behavior, movements were observed. The results of this study indicated that *Ruta montana* L. aqueous extract *via* oral route with the doses from 2 to12 g/kg body weight did not produce any sign of toxicity or death in both female and male mice after 14 days of observation.

According to the classification of Hodge and Sterner (Frank, 1992), Chemical substances with a LD_{50} between 5000 and 15000 mg/kg body weight determined after a single oral doses in rats is considered as practically non-toxic in humans. Therefore, the aqueous extract of *Ruta montana* L. could be considered practically non-toxic.

In the chronic toxicity study, both female and male rats were given with three different doses of the aqueous extract from *Ruta montana* L. (100, 300 and 600 mg/kg body weight) for 90 days. In the aspect of general behaviors, both male and female rats treated with these three doses presented no signs of behavior changes and toxicity.

In rats receiving the *Ruta Montana* L. aqueous extract orally at doses of 100, 300 or 600 mg/kg during 90 consecutive days, there was no significant change in the body weight in the treated groups compared to the control group. Changes in body weight have been used as an indicator of adverse effects of drugs and chemicals (El Hilaly *et al*., 2004).

Since, no significant changes were observed in the body weight, in the treated groups compared to that of the control group, it suggested that at the oral doses administered, *Ruta montana* L. aqueous extract had no effect on the growth of rats.

Generally, the alterations of body weight gain and internal organ weights of mice would reflect the toxicity after exposure to the toxic substances (Carol, 1995). Organ weight 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 (Dybing et al, 2002). In this study, no significant changes were observed in the internal organ weight after the 90 days period, except a significant decrease in testis, epididymis and seminal vesicle in groups treated by the doses 300 and 600mg/kg when compared to the control group. In addition, there is no changes were observed in gross observation of organs of both control and treated groups.

The haematopoietic system is one of the most sensitive targets for toxic compounds (Mukinda and Syce, 2007 ; Li *et al*, 2010), and an important index of physiological and pathological status in human and animals (Olson *et al*, 2000 ; Wallace and Meyer, 2010). The changes in the hematological system have a higher predictive value for human toxicity when data are translated from animal studies (Rahman *et al*, 2001 ; Jodynis-Liebert *et al,* 2010).

Research has shown that some plants materials when ingested, either raw or extract have deleterious effect on the blood parameters as well as the bone marrow (Omodamiro and Nwankwo, 2013), The principal effects, in order of frequency, are neutropenia , thrombocytopenia, hemolytic anemia, aplastic anemia and macrocytic anemia (Lubran, 1989). On the other hand some plants also have stimulatory effects on the blood parameters and the bone marrow (Omodamiro and Nwankwo, 2013).

There were no significant differences in the hematocrits, red blood cell and white cell counts of the control versus the treated rats after 90 days of administration. These results therefore indicated that 90 days administration of *Ruta montana* L. aqueous extract had no effects on the circulating cells nor interfered with their production. The hemoglobin and the red blood cell levels were not affected suggesting that hemolytic anemia and polycythemia, (that are characterized by decreases and increases in RBC count, hematocrits and hemoglobin, respectively) (Mishra and Tandon, 2012), were not likely to be induced by *Ruta montana* L.

The platelet counts were also not adversely affected indicating that the plant extract also did not affect the production of platelets nor induced thrombocytopenia, the latter may result from a direct toxic effect of the drug on platelet precursors in the bone marrow or on the circulating platelets (Lubran, 1989).

The levels of white blood cells, (which serve as scavengers that destroy microorganisms at infection sites, remove foreign substances and debris that results from dead or injured cells (Miller and Harley, 1996; Guilhermino, 1998), were also not changed suggesting that the aqueous extract of *Ruta montana* L. was also not toxic to the immune system. Collectively, all the results suggest that the chronic ingestion of the aqueous extract of *Ruta montana* L. did not alter the hematological parameters of the rats.

While the study of Shama *et al.,* (2014) showed that the administration of ethanolic and aqueous seed extract of *Ruta graveolens* L. at a dose of 200 mg/kg body weight for 4 weeks by oral route induces a significant decrease ($p<0.05$) in WBC, RBC and PCV parameters when compared with control and no significant changes in the other hematological parameters.

In addition, biochemical examination was performed in order to evaluate any toxic effects on liver, kidney, and glucose metabolism. The parameters such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, albumin, creatinine, uric acid, creatine kinase, glucose, triglycerides and cholesterol were examined. It is known that the liver and kidneys play significant roles in various metabolic processes. The liver plays an important role in xenobiotic function; and the kidneys are the main organs involved in drugs elimination and therefore, particularly exposed to the toxic effects of exogenous compounds (Bidhe and Ghosh, 2004).

Serum enzymes like AST, ALT and ALP are well-known enzymes used as good indicators of liver function (Rahman et al, 2001) and as biomarkers predicting possible toxicity (Mdhluli, 2003). The transaminases AST and ALT leak into the circulation when hepatocytes or their cell membranes are damaged. ALT is more specific marker of hepatocellular injury because it occurs exclusively in the liver, whereas AST occurs to some extent also in heart, skeletal muscle, kidney, brain, pancreas and blood cells (Kew, 2000; Belguet, 2010).Moreover, serum ALP activity increases in case of damage to the hepatic cells and obstruction of the bile ducts (Akhtar *et al*., 2012; Dahamna, 1987).Therefore, no changes in ALT, AST and ALP activities suggest that the chronic administration of *Ruta montana* L. extract did not alter the hepatocytes function.

The liver is the site of cholesterol disposal or degradation and its major site of synthesis. In the same perspective, the liver also controls glucose synthesis and generates free glucose from hepatic glycogen stores (Li *et al*., 2010). As no significant changes were observed in glucose and cholesterol levels in this study, this suggests that *Ruta montana* L. aqueous extract had no effects on the lipid and carbohydrate metabolism of the rats.

Equally, there was also no significant change in uric acid and creatinine, between the treated groups and the control group. Indeed, uric acid and creatinine are considered as important markers for kidney dysfunction (Mukinda and Eagles, 2010; Gnanamani *et al*, 2008). creatinine is nitrogenous end products of metabolism removed from the blood by the kidneys. It is the most commonly used clinical serum biomarkers of renal damage. (Hayes, 2008). Any rise in creatinine levels is only observed if there is marked damage to functional nephrons (Lameire *et al*, 2005). Thus, the results recorded in this study suggest that *Ruta montana* L. aqueous extract did not alter the renal function.

The other biochemical parameters such as ALB, Ck and TG showed no significant changes between treated groups and control group. Albumin is the protein of the highest concentration in plasma. Albumin transports many small molecules in the blood, for example, bilirubin, calcium, progesterone, and drugs. Because albumin is made by the liver, decreased serum albumin may result from liver disease. It can also result from kidney disease, which allows albumin to escape into the urine. Decreased albumin may also be explained by malnutrition or a low protein diet (Young and Holland, 1995; Sacher and Mcpherson, 1991; Russo et al, 2007).

However, Shama et al (2014) showed that the value of AST, ALT and urea concentrations in groups treated with ethanolic and aqueous seed extract of *Ruta graveolens* L. at a dose of 200 mg/kg body weight for 4 weeks by oral route were significantly increased compared to control. And no significant changes occurred among all groups regarding total protein, bilirubin and cholesterol concentrations.

Several lines have suggested that some herbal drugs can be used as anti-fertility agents in mice, rats, rabbits and even humans (Koneri *et al*, 2007). Although many reports have showed that the use of plant extract can affect reproductive physiology of the animal.

In the present study, a significant weight reduction were seen in the testes, epididymis and seminal vesicle in the doses 300 and 600 mg/kg when compared to the control group. The weight reduction was dose dependent.These results are similar to those obtained by Khouri and EL-Akawi (2005), which suggest that the administration of aqueous extract of *Ruta graveolens* L. at a dose of 500 mg/kg body weight for 60 days induces a significant decrease in the weight of reproductive organs ($P<0.01$) when compared to control. But, do not agree with the results obtained by Al Qarawi (2005) which suggest that the oral administration of an aqueous extract of the leaves of *Ruta chalepensis* in daily oral doses of 0.5 g , 1.0 g and 2.0 g for 30 days caused an increase of the testicular and epididymis weights.

Generally, the reductions in internal organ weight are simple and sensitive indices of toxicity after exposure to toxic substances (Teo *et al*, 2002).The significant reduction of testis weight is known to be mostly related to number of spermatids and spermatozoa present in the tissue (Gupta *et al*., 2005). The significant reduction in the weight of reproductive organs indirectly supports the reduced availability of androgen (Zeherea *et al*., 1998). It is known that the accessory sex organs are androgen dependent target organs and manifest differential sensibility to androgens for maintenance of their structure and function. It is also known that, any change in circulating androgens would affect the internal microenvironment of epididymis and thereby lead to alteration in sperm motility and metabolism (Khan and Awasthy, 2003).

Androgen deprivation not only suppresses spermatogenesis, leading to low sperm concentration, but also alters the epididymal milieu which renders it hostile for maturation and survival of the spermatozoa (Setty, 1979 ; Rao, 1988). Testosterone, an important androgen, plays a pivotal role in maturation, spermatogenesis and the maintenance of

accessory sex organs (Keel and Abney, 1980). The structural and functional integrity of reproductive tissues depends on the circulating androgen (Chinoy *et al*., 1982) and therefore, any small change in testosterone content may result in reductions in the weights of the reproductive organs.

Sperm characteristics are important reproductive indices as they account for male fecundity. The aqueous extract at the doses of 300 and 600 mg/kg body weight produced a significant reduction in the sperm count and motility. Two possible hypotheses may be proposed to explain this reduction. One hypothesis is that the principles active of the extract may alter the pituitary gonadotropins hormones : luteinizing hormone(LH) and follicle stimulating hormone (FSH) (Kusemuji *et al*., 2010). It is well known that the weight, size and the Secretary function of testes, epididymis and seminal vesicles are closely regulated by androgens hormones (Almasad *et al*, 2007).The production of the sperm cells (spermatozoa) and testosterone in the testis are mainly regulated by the follicle stimulating hormone and Luteinizing hormone, which are released from the interior pituitary (Steinberger, 1971). FSH stimulates spermatogenesis in the sertoli cells, while LH stimulates the production of testosterone in leydig cells of the testis (Wannang *et al*, 2008). Low levels of these hormones decrease endogenous testosterone secretion from the testis depriving developing sperm of the signal required for normal maturation and also it suppress testicular steroidogenesis and spermatogenesis (Kusemuji et al., 2010) since the pituitary-testicular axis is a central regulatory conduit for testicular function that culminates in the production of spermatozoa (Cheng *et al.,* 2010). Besides hormonal alteration, the alternative hypothesis is that the principles active may induce oxidative stress in testicular tissue and stored germ cells leading to generation of free radical products, as they exert a detrimental effect on spermatogenesis (Gosh *et al*, 2002).

The histological examination is the golden standard for evaluating treatment related pathological changes in tissues and organs (Subramanion *et al*, 2011). In the present study, histopathological evaluation of chronic oral administration of *Ruta montana* L. indicated that the aqueous extract did not cause toxicity towards the organs as there was no structural damage to the organs of liver, kidney, brain and ovary of the rats. This agrees with the results of biochemical analysis.

The liver is the main target organ of toxicity where exposed to the foreign substances being absorbed in intestines and metabolized to other compounds which may or may not be hepatotoxic to the mice (Rhiouania et al, 2008). In this study, the liver histology revealed normal. Hepatocytes did not show any alteration in the structure in treated animals compared to control. Except slight congestion. Also there was no necrosis, inflammatory reaction, fibrosis or local fatty degeneration observed in liver and the arrangement of cell structure almost similar to the organs of rats in control groups. In contrast, the histological examination study conducted by Shama *et al* (2014) showed that After 4 weeks of treatment with the daily oral doses of *Ruta graveolens* seeds extracts, there were lesions in the liver and kidney of rats given ethanolic extract at 200 mg/kg/day and in aqueous extract at 200 mg/kg/day, there is fatty cytoplasmic vacuolation of the centirlobular hepatocytes, cell necrosis and hemorrhage in the liver of rats given 200 mg/kg/day aqueous extract and glomerular alteration, dilatation and fatty change in the kidney at dose of 200 mg/kg ethanolic extract.

A great number of medicinal plants contain compounds exhibiting antioxidant properties as phenolic compounds, which possess strong natural antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals. They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors,

and singlet oxygen quenchers. Antioxidants from plant materials terminate the action of free radicals thereby protecting the body from various diseases (Narayanaswamy and Balakrishnan, 2011).

According to Chu *et al*. (2000), total antioxidant activities of the plant extracts cannot be evaluated with a single method due to the complex nature of phytochemicals. Two or more methods should always be employed in order to evaluate the total antioxidative effects of plant material (Nuutila *et al*., 2003). Thus, in the present study we applied three methods *in vitro,* DPPH scavenging activity, β-carotene bleaching and ferrous ion chelating to evaluate the antioxidant capacities of *Ruta montana* L extracts.

DPPH radical is a stable free radical that shows a maximum absorption at 517 nm, and is widely used to evaluate the free radical scavenging ability of natural compounds. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH (purple color) to the yellow coloured diphenylpicrylhydrazine. Therefore, the antioxidant activities of a sample can be expressed as its ability in scavenging the DPPH radical. In DPPH assay, the antioxidant effect was likely to be due to the hydrogen donating ability of the extract (Conforti *et al*, 2005).

EAE exhibited the highest activity toward DPPH scavenging (IC_{50} = 0.044±0.001 mg/ml) followed by CrE with 0.067±0.002 mg/ml, AqE with 0.083± 0.003 mg/ml and ChE with 0.146±0.015 mg/ml. The anti-radical activity may be related to the presence of flavonoids and polyphenols in the extracts. The antioxidant effect of plant extract is likely related to the amount of polyphenols present (Jayaprakasha and Patil, 2007; Hodzic *et al*, 2009), and may also differ depending on the quality of polyphenols and flavonoids (Wang and Mazza, 2002). The mechanism of the reaction between the antioxidants and DPPH depends on the structural conformation of the antioxidant. Some compounds react rapidly

with the DPPH, with the reducing the number of DPPH equal to that of the hydroxyl groups present in the antioxidant compound (Dapkevicius *et al*, 1998).

Similar research conducted in other plants and fruit have shown that high radical scavenging activities are usually associated with high phenolic contents. For instance, Lim *et al* (2006) described that high radical scavenging activity was contributed by the presence of high phenolic content in guava extracts. Surveswaran *et al* (2007) found positive correlations between antioxidant activities and total phenolic contents of 133 medicinal plants. The high radical scavenging activity of polyphenols can be attributed to their high degree of hydroxylation of aromatic rings, the arrangement of the hydroxyl group, as well as the number of galloyl group and ortho-hydroxyl groups, on benzene nucleus structure (Cai *et al*, 2006).

Differently, other research found no such relationship, since other compounds are responsible for the antioxidant activity (Heinonen *et al*., 1998; Kahkonen *et al*., 1999), which is the case in our study. The results showed that there is no accordance between the order of decreasing scavenging activity among the *Ruta montana* L. extracts and the amount of phenolic compounds present in these extracts.

The bleaching of β-carotene is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radicals, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As a result, β-carotene will be oxidized and broken down in part; subsequently, the system loses its chromophore and characteristic orange color, which can be monitored

spectrophotometrically. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleic acid-free radical and other free radicals formed in the system (Jayaprakasha *et al*, 2001). AqE and ChE exhibited the highest antioxidant activity (90.34 ± 0.46% and 89.92 ± 0.29%, respectively), which was near to that of BHT (100 ± 0.52%), following by EAE and CrE with an antioxidant activity (73.56 ± 0.63 % and 63.27 ± 2.27%, respectively).

Finally, the antioxidant potentials of the *Ruta montana* L. extracts were evaluated by determining their ferrous ion chelating ability. ferrous ion chelating ability measures the ability of secondary antioxidants to chelate metal ions. Primary antioxidants prevent oxidative damage by directly scavenging free radicals, while secondary antioxidants act indirectly by preventing the formation of free radicals through Fenton's reaction (Chan *et al*. 2010). Fe^{2+} has been known to accelerate formation of hydroxyl radicals via the Fenton reaction, leading to occurrence of many diseases (Halliwell, 1996). It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Lai and Lim, 2011). In the Ferrous ion chelating assay, ferrozine acts as a chelating agent and forms purple complex ion with Fe^{2+} giving maximum absorbance at 562 nm. The complex formation can be disrupted by the presence of other complexing agents which cause a decrease in the purple color intensity of complexes. Substances or samples that can reduce its color intensity can be considered as antioxidant through the mechanism of inhibition of heavy metal (Rohman *et al*, 2010).

The AqE and CrE appear to have the most important chelating activity with IC_{50} values of 0.005 ± 0.004 mg / ml and 0.021 ± 0.005 mg/ml, respectively. The chelating effect of ChE and EAE is very low (IC_{50} = 0.146 ± 0.015 and 0.771 ± 0.021 mg / ml, respectively).

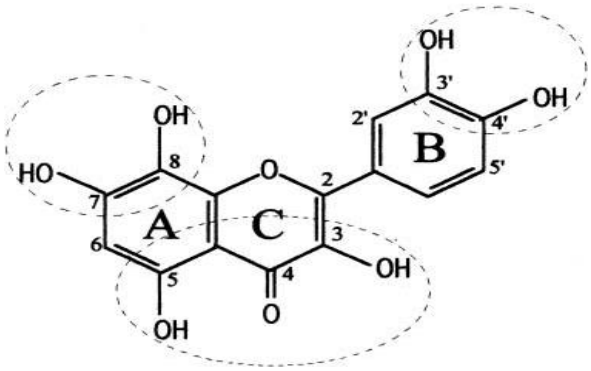
There are contradictory reports in the literature regarding metal chelating capacities of polyphenols. Some studies have shown that polyphenols derived from different plants are potent ferrous ion chelators (Chew *et al*, 2008 ; Seneviralhne *et al.*, 2006) and metal chelating ability are dependent upon their unique phenolic structure and the number and location of the hydroxyl groups (Santoso *et al*, 2004). In contrast, other authors have reported that metal chelation played a minor role in the overall antioxidant activities of several plant-derived phenolic compounds (Rice-Evans *et al*, 1996). In agreement with this finding, our study showed that ferrous ion-chelating was not correlated with total phenolic compounds, aqueous extract was more effective in chelating ferrous ion than EAE. Saiga *et al*. (2003) and Wang *et al*. (2009) explained that other components such as polysaccharides, proteins or peptides in the extracts were more effective chelators of ferrous ions than phenolic compounds.

There have been two opinions on the correlation between phenolics and total antioxidant activity. Some reports demonstrated positive correlation between them (Wangensteen *et al*, 2004 ; Zielinski and Kozlowska, 2000) and the others showed no correlation (Sun and Ho, 2005). Our results agree with the latter, it seems that no correlation exists either between the percentage inhibition (% scavenging effect) assayed by DPPH and total phenolics, between the antioxidant activity (AAC) assayed by β-carotene blenching method and total phenolics, or between the antioxidant activity assayed by ferrous ion chelating (FIC) ability and total phenolics. Kaur and Kapoor (2002) thought

this phenomenon can be explained on the basis of high antioxidant activity of some individual phenolic units, which may act as efficient antioxidants rather than contributing to high total phenolics, while Kähkönen *et al* (2001) thought that total phenolics content did not include all the antioxidants, such as ascorbic acid, carotenoid and tocopherol. Vinson *et al*. (2001) held the idea that the synergism among the antioxidants in the mixture made the antioxidant activity not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants. This probably is the reason why samples with similar concentrations of total phenolics may vary remarkably in their antioxidant activity.

In general the antioxidant activity of phenolic compounds reportedly varies with the structure and degree of hydroxylation of the aromatic ring (Burda and Oleszek, 2001; Aruoma, 2002). It is associated with the number of hydroxyl groups and the most active possess from 3 to 6 hydroxyl groups. Hydroxylation in the C3 position seems to be detrimental for their antioxidant potency (Huguet *et al*., 1990). Fukumoto and Mazza (2000) reported that for benzoic and cinnamic acid derivatives, flavonols and anthocyanidins, an increase in the number of hydroxyl groups on the aromatic ring lead to higher antioxidant activity *in vitro*. Compounds with three hydroxyl groups on the phenyl ring of phenolic acids or the B-ring of flavonoids had high antioxidant activity. The loss of one hydroxyl group decreased activity slightly.

Amic *et al* (2003) studied the relationship between structural characteristic of flavonoids with their antiradical activity. Results of this study revealed that free radical quenching activity of polyphenolic compounds strongly depends on the specific substitution pattern of free hydroxyl moieties on flavonoids structure. B ring with 3', 4'dihydroxy groups and 3-OH group at ring C are essential features for strong antioxidant activity.



The structure and position of hydroxyl groups are crucial for antioxidant activity. 3' 4' ortho dihydroxy configuration (catechol) in B ring and 3-OH group in C ring is very important for antiradical efficacy. Catechol ring and C2 – C3 double bond conjugated with 4- keto moiety are essential for electron delocalization from the ring B, and it increases the antiradical activity (Sharififar *et al*., 2009). Many studies showed that when these structural features were removed from flavonoids structure their antioxidant activity was decreased (Amic *et al*., 2003; Silva *et al*., 2002, Furusawa *et al*., 2005).

Plasma antioxidant capacity (PAC) is one of the most commonly used biomarkers to assess the effectiveness of dietary supplementation or antioxidant treatment. In fact, the plasma contains a network of endogenous antioxidants such as (albumin, bilirubin, reduced glutathione and uric acid) as well as exogenous antioxidants derived from food. These antioxidants may act in a complementary and synergistic manner to provide a better protection against ROS. Due to the large number of antioxidants present in plasma, several methods have been developed: the ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), total radical absorption potential (TRAP) and the DPPH radical scavenging activity (Janaszewska and Bartosz, 2002; Huang and Prior, 2005). In our study, tow complimentary tests were used to evaluate the antioxidant activity of *Ruta montana* L. extracts in plasma: DPPH scavenging activity and reducing power.

The first method is widely used to evaluate the antioxidant activity of plant extracts and food, it is also used to assess the plasmatic antioxidant capacity which is expressed as a percentage inhibition of discoloration of reaction mixture in the presence of the plasma compared to the methanolic solution containing only DPPH.

In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe^{3+}–Fe^{2+} by donating an electron. An amount of Fe^{2+}complex can then be monitored by measuring the formation of Perl's Prussian blue ($Fe_4[Fe(CN)_6]_3$) at 700 nm (Fakhfakh *et al.*, 2012). Increasing absorbance at 700 nm indicates an increase in reductive ability.

The results obtained in present study showed that the oral administration of AqE (300 mg / ml) and CrE (100 mg / ml) increased plasma antioxidant capacity. This increase is statistically not significant compared with the control group. The scavenging activity of aqueous extract increased with the increase of their concentration. While in reducing power assay, the results showed that oral administration of AqE (100 and 300 mg / ml) and CrE (100 mg / ml) did not show any significant changes compared with the control group.

Liver and kidney are most vulnerable to injury by free radicals resulting in serious health problems (Sreenivasamurthy *et al*, 2012). This is due to their involvement in metabolism, detoxification, storage and excretion of xenobiotics and there metabolites, making them important target organs for xenobiotic induced injuries (Tayeb et al, 2010).

ROS and RNS deteriorate many biological molecules like fatty acid, lipids, proteins and DNA, and become a major cause of heart diseases, diabetes, cancer, inflammations and weak immune system (Cakir *et al*., 2006; Fei *et al*., 2007; Gulcin *et al*., 2007; Adedapo *et al.*, 2009; Jayakumar *et al*., 2009; Sharififar *et al*., 2007).

The body has evolved a complex defence strategy to minimize the damaging effects of various oxidants. Central to this defence are the non-enzymatic and enzymatic antioxidants. These include reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) respectively, which act in concert to protect the organism from oxidative damage (Magdi *et al*, 2011). Thus, In present work, we evaluated the effect of extracts on oxidative stress parameters MDA, GSH and CAT at certain organs (liver and kidney) which oxidative stress appears to play a crucial role.

Lipid peroxidation is an auto-catalytic, free-radical mediated, destructive process, where by polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Linden *et al*, 2008). Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde (Pryor and Stanley, 1975). This compound is a reactive aldehyde, and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del Rio *et al.* 2005).

The non-enzymic antioxidant, glutathione is one of the most abundant tripeptides present in the liver. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST (Prakash *et al*, 2001).

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues. It decomposes hydrogen peroxideand protects the tissue from highly reactive hydroxyl radicals (Chance *et al*, 1952). Catalase activity varies greatly from tissue to tissue, the highest activity is found in liver and kidney, whereas the lowest activity is seen in the

connective tissue (Deisseroth and Dounce, 1970). Inhibition of this enzyme may enhance sensitivity to free radical-induced cellular damage. Therefore reduction in the activity of CAT may leads to deleterious effects as a result of superoxide and hydrogen peroxide assimilation.

The results revealed no significant decrease in lipid peroxidation and no significant increase in GSH and CAT in liver and kidney tissue for the group treated with AqE (300 mg/kg) and CrE (100mg/kg) compared to control group. This is mainly due to their high content in phenolic compounds, which are able to donate atoms of hydrogen to inhibit lipid peroxidation.

Among the rue constituents (Asgarpanah et Khoshkam, 2012), phenolics, flavonoids and cumarines are the most probable ones which could be able to have inhibitory activity on DPPH radicals. The pharmacological investigations clearly indicated that plant flavonoids have various properties such as antioxidant, anticancer, and anti-inflammatory activities (Raghav *et al*, 2006) and inhibit lipid peroxidation in biological membranes (Mariddnneau-Parini et al, 1986).

Similar research indicated that in *in vivo* model, *Ruta gravoelens* L. has been found to reduce oxidative stress by decreasing TBARS level and increasing antioxidant enzymes activities such as SOD, catalase and GPX in liver and heart in hypercholesteromic rats (Halliwell et al, 1999). (Preethi *et al*, 2006) also indicated in *in vitro* experiments that *Ruta gravoelens* L. extract was found to scavenge hydroxyl radical and inhibit lipid peroxidation.

In addition, Ashour *et al*, (2011) showed that treatment with the ethanolic extract of *Ruta chalepensis* L. could reduce oxidative stress as well as inflammation in hypercholesteromic rats. Flavonoids widely distributed in plants have the ability to inhibit

oxidative damage. Indeed, these flavonoids have the potential to function as in vitro antioxidants by scavenging superoxide anion (Razali *et al*, 2008), singlet oxygen (Almeida *et al*, 2008) lipid peroxy-radicals (Alejandro *et al*, 2000; Hsu, 2006), and/or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Shahidi *et al*, 1992). Flavonoids existing in *Ruta chalepensis* L., in addition to their free radical scavenging properties also enhance the expression of intracellular endogenous antioxidants such as superoxide dismutase (SOD), catalase, and GPX (Kacem *et al*, 2014).

According to Toker *et al* (1998), the aerial part of *Ruta montana* L. plant contains rutin. Recently, a study by Mahmoud (2012) demonstrated that rutin, the major active constituent of *Ruta graveolens*, markedly decreased lipid peroxidation, increased glutathione concentration and ameliorated the antioxidant enzyme activities in liver and brain of AC-induced hyperammonemic rats. Other studies carried out by (Shen *et al*, 2002) have reported that rutin decreases nitric oxide and reduces inducible nitric oxide synthase (iNOS) protein in BALB/c mice pretreated with lipopolysaccharide.

Conclusion

The present study was carried out to evaluate the acute and chronic toxic effects of aqueous extract prepared from the aerial parts of *Ruta montana* L. Antioxidant activity of aqueous, methanolic, chloroformic and ethyl acetate extracts of the plant was studied *in vitro* by the main of different methods. Furthermore, antioxidant potential of aqueous and methanolic extracts was also tested *in vivo* using *Albino Wistar* rats.

This study showed that extracts from *Ruta montana* L. contained high levels of total phenolic compounds. The ethyl acetate extract had the highest amount of polyphenols, flavonoids and tannins.

The toxicity study revealed that aqueous extract from *Ruta montana* L. did not cause any mortality or other toxicity signs. Acute toxicity study suggested that aqueous extract of the plant is safe up to the dose of 12 g/kg of mice body weight when consumed by oral route. Therefore, according to the classification of Hodge and Sterner, aqueous extract could be considered practically non-toxic. In chronic toxicity study, aqueous extract did not affect hematological or biochemical parameters. In contrast, the extract affected male fertility by causing a decrease in the weight of testis, epididymis and seminal vesicle, as well as a reduction in the number and the motility of spermatozoa.

Studied extracts from *Ruta montana* L. showed an important antioxidant activity *in vitro*. The ethyl acetate extract exhibited the highest scavenging activity, whereas aqueous extract showed the best chelating capacity and the best inhibitory capacity of the coupled oxidation system of linoleic acid/ β-carotene.

However, aqueous and methanolic extracts caused an amelioration in the liver and kidney antioxidant status when administred orally to rats, by decreasing the MDA

concentration and increasing the rate of reduced glutathione (GSH) and catalase activity (CAT).

Perspectives

Further research needs to be carried out to:

- Identify the active molecules which may be responsible for antioxidant activity or toxic effects caused by the studied plant.
- Evaluate the bioactive molecules in therapeutic significance in the prevention of diseases induced by oxidative stress.

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