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ROSMARINUS OFFICINALIS LEAVES WATER EXTRACT: A POSSIBLE HYPOGLYCEMIC, ANTI-INFLAMMATORY AND ANTI-ULCEROGENIC REMEDY

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June 2007

Rosmarinus *officinalis* leaves water extract: a possible hypoglycemic, anti-inflammatory and anti-ulcerogenic remedy

Abstract

Nowadays, herbal medicine is becoming a very popular complementary therapy based on its relative safety, effectiveness, accessibility and inexpensiveness. The present study investigates the potential role of *Rosmarinus officinalis* leaves water extract in lipemia, glycemia, ulcer and inflammation. The antibacterial activity was also assessed on several hospital isolates. After one month of water extract intake via drinking water (200, 500 and 1000 mg/kg body weight) the blood lipid profile and liver function of rats used during the experiment were not affected. However, the water extract (200 and 1000 mg/kg body weight) showed significant hypoglycemic and insulin-release inhibitory effects since both glucose and insulin concentrations were significantly reduced. The water extract of *Rosmarinus officinalis* at doses 50, 100, 250 and 500 mg/kg body weight was also studied in the rat using both acute and chronic inflammation models induced by carrageenan and formalin respectively. All dose used showed anti-inflammatory activity with significance reached at doses ≥100 mg/kg body weight in both models. The optimum dose in the acute and chronic inflammation models were 250 and 500 mg/kg body weight respectively. The anti-inflammatory effect observed was comparable to that caused by diclofenac, the control drug, in both models. Similar doses to the inflammation study were also used in the detection of anti-ulcerogenic potential of the extract. A dose dependent protection against ethanol-induced gastric ulcer was observed. However, protection became significant when rats received doses ≥ 250 mg/kg body weight. The 500 mg dose exhibited a protection that slightly exceeded that of the reference drug cimetril. The antibacterial effect of the water extract against 11 hospital bacterial isolates was investigated using disc diffusion technique (using a serial dilution: 200, 100, 50, 20 and 10μg extract) and did not show any potentials in this respect. In conclusion, the water extract of *R. officinalis* dry leaves at the doses used has no toxic signs and symptoms and it exhibits a positive effect on ulcer, inflammation and hyperglycemia, however the extract appeared to have a neutral effect upon lipemia.

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Abbreviations

•OH	Hydroxyl radical
4-AP	4-aminophenazone
ADP	Adenosine-5-diphosphate
ALT	Alanine aminotransferase
apo	Apolipoprotein
B[a]P	Procarcinogen benzo[a]pyrene
BPDE	Benzo[a]pyrene-(+)-anti-7,8-dihydrodiol-9,10-epoxide
Caco-2	Colonic adenocarcinoma cell lines
CAM	Complementary and alternative medicine
CM	Chylomicrons
DAP	Dihydroxyacetone phosphate
ELISA	Enzyme Linked Immuno-sorbant Assay
G3P	Glycerol-3-phosphate
GOD	Glucose oxidase
GPO	Glycerol dehydrogenase
GST	Glutathione S-transferase
HDL	High density lipoproteins
iNOS	Nitric oxide synthase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MDH	Malate dehydrogenase
NADH	Reduced nicotinamide adenine dinucleotide
NF-kB	nuclear factor-kappa B
OD	Optical density
PMNs	Polymorphonuclear neutrophilic leukocyte
POD	Peroxidase
QR	Quinone reductase
TAG	Triglyceride
TH	Tyrosine hydroxylase
VLDL	Very low density lipoproteins

Chapter One

Introduction

Complementary and alternative medicine (CAM) is a group of miscellaneous medical and health care systems, therapies, and products that are not presently believed to be part of conventional medicine. Many people are gradually turning to CAM therapies. This is attributed to marketing forces, availability of information on the internet and books, the curiosity of patients to be involved with medical decision making, and to the inability of conventional medicine to satisfactorily treat many chronic diseases and their symptoms such as HIV and Cancer (Barnes et al., 2004). Different types of CAM therapies such as herbal medicine and folk medicine are used by people. Herbal medicine relies on plant for healing of diseases. These plants are ingested as tea, decoctions and juice preparations; they are also made into tincture (with an alcohol base), or made into poultice to treat infected wounds and burns. Herbal medicine is not always safe to use since certain plants may be toxic or may have side effects on the human health. While certain herbs can be used to solve side effects of some conventional treatments, others can hinder conventional medicine. Therefore, it is important to seek qualified practitioners whenever they are used. In the UK the most commonly practiced type of herbal medicine is Chinese herbalism (Rojas et al., 2006; Breast Cancer Care, 2005). Some of the herbal medicine that is commonly used includes lemon balm for digestion and relaxation, and rosemary for digestion and pain (Breast Cancer Care, 2005).

1.1. Plant Distribution and Taxonomy:

Rosmarinus officinalis belongs to the Mint family *Lamiaceae* (*Labiatae*) (Table 1.1) which includes 3200 species (Katzer, 2004). Its common name is Rosemary that is derived from the Latin name Rosmarinus which is assumed to mean "dew of the sea" while officinalis designates that plant used for medicinal purposes (Katzer, 2004). Rosemary is an evergreen, perennial shrub with a fragrant needle-like leaves (Figure 1.1A). It is copious in woodland, rocky sites in the Mediterranean region like Lebanon, Syria, France, Italy and Spain (Krapp and Longe, 2001; Hui-Hui et al., 2001; Wyk and Wink, 2005). The plant is up to 1.5m long and with a spread of 4-5 feet. The flowers are pale blue arranged in clusters of 2 or 3 and appear in winter and spring (Figure 1.1B), The flower is semi-tubular with a lower lip that has three lobes and the upper lip that has two lobes (Grieve, 1900; Christman, 1999; Krapp and Longe, 2001; Wyk and Wink, 2005).

1.1.1. Cultivation and Harvest

R. officinalis can propagate from seeds, division of roots, or cutting wood of non-flowering branches in early summer and can also be grown from layers begging some lower branches under soil (Grieve, 1900). Rosemary comes off best in light and well-drained sandy soil (Christman, 1999). Leaves can be harvested anytime during the second year of growth, dried in shaded aerated room and used for water or oil infusions (Krapp and Longe, 2001).

Table 1.1: Scientific Classification of plant *Rosmarinus officinalis* *(Katzer, 2004)*

Kingdom	Plantae
Division	Spermatophyta
Subdivision	Magnoliophyta
Class	Magnoliopsida
Subclass	Lamiidae
Order	Lamiales
Family	Lamiaceae
Genus	*Rosmarinus*
Species	*officinalis*

Binomial Name: *Rosmarinus officinalis* L.

Figure 1.1: **(A)** *Rosmarinus officinalis shrub*, and **(B)** its flower (Katzer, 2004)

1.1.2. Plant History, Traditional and Medicinal Use:

Rosemary is used in various cultures for many reasons. It has been used in Europe as a symbol for remembrance, love and loyalty during interments, wedding ceremonies and war commemorations. Ancient Greek students used to put on a twig of rosemary in their hair while studying for exams to improve their memory, while lamenters threw it into tomb as a symbol of remembrance for the dead (Grieve, 1900; Krapp and Longe, 2001). Gypsy travelers used rosemary to highlight dark hairs or as reviving face wash. In the fourteenth century, the Queen of Hungary treated goat by using an alcohol extract of this herb (Krapp and Longe, 2001). Twigs of rosemary were placed under pillows at night to defend against evil spirits and bad dreams. It was used to flavor wine and in Christmas ornamentation. In French hospitals, smoke of burned Rosemary was used to purify the air and prevent infections (Grieve, 1900). Rosemary is still used in seasoning various dishes especially meat, chicken and potato (Christman, 1999).

Medicinally, *R. officinalis* has been used to stimulate hair growth and to treat poor circulation, headaches and rheumatism (Krapp and Longe, 2001; PDR, 2004; Wyk and Wink, 2005). Water infusion of Rosemary leaves and flowers is an excellent stomachic and a good therapy for cold, colic, heart disease, cataract, poor sperm motility, spasmogenic disorders, nervous depression and other nervous diseases. The smoke of rosemary leaves is used as a treatment for throat, lungs and asthma (Grieve, 1900; Slamenova et al, 2002).

1.1.3. Precautions and possible interactions

Rosemary or any herb should be taken with care and under the administration of a practitioner knowledgeable in the field of botanical therapy. Herbs contain active substances that can interact with other

medications or supplements and has side effects. It is considered safe when rosemary taken in a recommended dose, but large quantities of rosemary leaves can cause auto immune disease, vomiting, spasm, pulmonary edema and sometimes coma because of their volatile oil content (PDR, 2004). Pregnant and breast feeders should not use large quantities of *R. officinalis* since over dose can cause miscarriage and mutilation to the fetus (Barnes et al., 2002).

1.2. Plant constituents

The main constituents of *R. officinalis* are phenols, flavonoids, volatile oil and terpenoids (Lo et al., 2002). The phenols include carnosic acid, carnosol, rosmarinic acid (caffeoyl derivatives), 1,2-O-methylcarnosic acid, ursolic acid, rosmaridiphenol, rosmanol, isorosmanol, epirosmanol and rosmariquinone while the flavones are isoscutellarein 7-O-glucoside and genkwanin. During vegetative cycle, each of these constituents shows a characteristic behavior and distribution. All these compounds are found only in the leaves (Chen et al., 1992; Huang et al., 1994; Del Bano et al., 2003). The volatile constituents of rosemary are 1,8-cineole, (+)-camphor, (R)-(+)-limonene, borneol and (-)-a-pinene (Inoue et al., 2005). Other constituents are (−)-verberone (Miyazawa et al., 2003), therein and bornyl acetate (Katzer, 2004). The methanol and acetone extracts contain the greatest amount of rosmarinic acid, carnosol and carnosic acid. On the other hand, water extract contain mainly rosmarinic acid (Moreno et al., 2006).

Plants like *R. officinalis* belonging to the Lamiaceae family are very rich in phenolic compounds and owing to these compounds they exhibit antioxidant activity (Kaliora and Andrikopoulos, 2005). Carnosol, carnosic acid, rosmarinic acid, ursolic acid and rosmaridiphenol are such compounds that show antioxidant activity (Chen et al., 1992; Debra et al., 1997; Moreno et al., 2006). Oxidation, the foremost reason of food

deterioration arises spontaneously in lipid containing foods and lipids. It causes unfavorable flavor and color, destruction of vitamins and nutritional loss (Wei and Ho, 2005). LDL is prone to oxidation which results from metabolic reactions in the presence of oxygen. Oxidized LDL is implicated in fatty streak accumulation and instigation of arthrosclerosis (Kaliora and Andrikopoulos, 2005). It has been reported (Wei and Ho, 2005) that *R. officinalis* extract is effectual for impeding the rancidity development in potato chips which have been fried in a deep-fat conditions and its antioxidant activity is as good as that of synthetic antioxidants. Carnosic acid and carnosol are responsible for 90 % of antioxidant activity of rosemary. The antioxidant activities of acetone and hexane extract of rosemary that contains mainly carnosic acid and carnosol showed a stronger antioxidant activity than the synthetic antioxidants butylated hydroxyanisole and butylated hydoxytoluene (Chen., et al, 1992).

Pearson et al. (1997) reported that 5 μM of carnosic acid and carnosol inhibited oxidation of liver rat microsomes by 92 and 95% respectively and LDL oxidation by approximately 91 %. Hui-Hui et al. (2001) investigated the inhibition capacity of *R. officinalis* phenolic compounds carnosol, rosmanol and epirosmanol to oxidize LDL in human blood and detected their scavenging activities to free lipid radicals. They showed that carnosol, rosmanol and epirosmanol have the capacity to inhibit LDL oxidation or cell membrane oxidation mediated by copper. Also these phenolic compounds inhibited the interaction of lipid oxidation products and apolipoprotein at low concentration and inhibited the process of oxidative modification of apo B in LDL. Epidemiological surveys showed that the ingestion of flavonols and flavones was inversely correlated with coronary heart disease in an aged person and in a cross cultural population (Pearson et al., 1997). Currently, there is a product of the *R. officinalis* in the U.S. market (Hui-Hui et al., 2001) and publicized on the internet (www.earthturns.com/index.asp?PageAction=VIEWPROD&ProdID=226)

to have a protective effect against cardiovascular disease mainly atherosclerosis.

Rosemary and its constituents (carnosic acid, carnosol, rosmarinic acid andursolic acid) have been extensively studied against cancer during the last decade (Slammenova et al., 2002). Sharabani et al (2006) have shown that the major *R. officinalis* polyphenol, carnosic acid, enhances the differentiating and antiproliferative effects of low concentration of vitamin D_3 in human myeloid leukemia cell lines. Combining carsonic acid with a synthetic vitamin D3 was found to reduce the risk of vitamin D3-induced hypercalcemia.

One study found that 10 μM of carsonic acid prevented cell proliferation in HL60 cells and caused transient G_0/G_1 phase cell cycle arrest (Steiner et al., 2001). In another study, the effect of carsonic acid and carnosol on growth of Caco-2 (colonic adenocarcinoma cell lines) was determined by the uptake of [^{3}H]thymidine. It was shown that after 21 hours of treatment with carnosic acid and carnosol, [^{3}H]thymidine incorporation is inhibited in a concentration-dependent way, where a 23μM concentration for both carnosol and carnosic acid exhibited a 50% inhibitory effect. This study demonstrated that carnosic acid induces cell cycle arrest at G_2/M phase before prometaphase or late S phase by reducing the level of cyclin A that would decrease the activity of CDK2 (cyclin-dependent kinase 2) and cdc2 (cell division cycle 2) kinases, activities essential for progression from S to G_2/M phase. Carnosol induces cell cycle arrest at G_2/M phase after prometaphase by increasing the level of cyclin B1. Degradation of cyclin B1 occurs in anaphase that is required for completion of mitosis; the retention of cyclin B1 arrests the cells at, or before anaphase prevents the cells from exiting mitosis (Visanji et al., 2006). Offord et al. (1995) studied the mechanism by which *R. officinalis* constituents block the initiation of carcinogenesis by the carcinogenic

DNA-binding anti-BPDE (benzo[a]pyrene-(+)-anti-7,8-dihydrodiol-9,10-epoxide), the activated carcinogenic metabolite of procarcinogen benzo[a]pyrene (B[a]P), in human bronchial cells. It was shown that 6µg/ml of *R.officinalis* extract or an equivalent concentration of its antioxidant components, carnosic acid or carnosol, inhibited DNA adduct formation by 80% after 6 hours of incubation with 1.5 µM of anti-BPDE. Offord et al.(1995) study is in good correlation with that conducted by Singletary and Guieterrez (1993), who showed that DMBA-DNA adduct formation was inhibited by 0.25-1.0% of *R. officinalis* extract added to rats' diet, and GST and QR detoxification activity increased 3-4 folds in the liver.

Recently, the antimutagenic effect of different *R. officinalis* constituents, carnosic acid, carnosol and rosmarinic acid, against γ-rays-induced chromosomal damage were determined pre and post γ-ray irradiation (Del Bano et al., 2006). Results showed a significant antimutagenic effect of carnosol and carsonic acid before and after γ-ray irradiation. This antimutagenic activity is explained by the capacity of phenolic carsonol and carnosic acid in scavenging reactive oxygen species, especially, hydroxyl radical ($^{\bullet}OH$) that is produced massively during γ-radiation. The capacity to inhibit hydroxyl radicals is built on the presence of catechol group of the phenolic diterpene skeleton and the presence of carboxylic groups on this skeleton (Del Bano et al., 2006). *R. officinalis* extract also showed antimutagenic activity in bacteria (Santamaria et al., 1987; Minnuni et al., 1992).

Kim et al., (2006) studied the role of carnosol, a phenolic diterpene, against rotenone-mediated dopaminergic neuron death. They showed that pretreatment of carnosol (10µM) significantly increased cell survival 90% and 81% after 24 and 48 hours respectively. This protective effect of carnosol is mediated by down regulation of caspase-3. Also carnosol

treatment showed a two-fold increase in tyrosine hydroxylase (TH) protein, a specific marker to dopaminergic neurons, and retained a moderate level of TH after rotenone treatment (Kim et al., 2006). Another study done by Kosaka and Yokoi (2003) showed that carnosol and carnosic acid induced the synthesis of nerve growth factor in glial cells. Ono et. al. (2003) reported that rosmarinic acid could be a therapeutic agent used in treatment of Alzheimer's disease.

Recently, it was reported (Moreno et al., 2006) that the methanol and ethanol extracts of *R. officinalis* leaves, in correlation to its carnosol and carnosic acid contents, showed a high antimicrobial activity against gram positive bacteria, gram negative bacteria and yeast. On the contrary, water extract, contanning rosmarinic acid, didn't show antimicrobial activity except against *Staphylococcus aureus* bacteria. In another study, methanolic extract of R. officinalis leaves showed a good activity against *Helicobacter pylori*, that is an etiological agent in numerous gastrointestinal disorders (Mahady et al., 2005). Also methanolic extract of concentration 2mg/ml inhibited completely the movement of cultured epimastigotes of *Trypanosoma cruzi* or killed all epimastigotes (protozoan parasite that cause Chagas' disease) after 2 hours of incubation (Abe et al., 2002).

Volatile oil is one of the major constituents of *R. officinalis* (Lo et al., 2002). A study by Aqel (1990) shows that the volatile oil of *R. officinalis* leaves inhibited the contraction of tracheal smooth muscles *in vitro* of guinea pig and rabbit induced by histamine and acetylcholine stimulation in a dose dependent manner. Another study (Inoue et al., 2005) showed that volatile oil of *R. officinalis* inhibited the allergic airway inflammation in mice *in vivo* after being induced by house dust mite allergen. This study showed that volatile oil prevented the increase in the number of pulmonary granulocytes and mononuclear cells accompanied by significant

suppression in the expression of IL-13 after intratracheal instillation of house dust mite in mice.

1.3. Lipid overview

Lipid plays a diversity of cellular roles. They are the major constituents of cell, as well as the principal form of stored energy. Special forms of lipid serve as cofactors (vitamin K), hormones (sex hormones), pigments (carotene), and detergents (bile salts and transporters (eicosanoids). A major class of biological important lipids is fatty acids, the main components of both triacyglecyrols (triglycerides) and phospholipids (Nelson and Cox, 2004). A second class of lipids that plays a major role in biological system is cholesterol and its derivatives (Ger, 2004). Most lipids in human diets are triglycerides that are broken in the small intestine by the action of lipase (Horton et al., 2002). The level of cholesterol and triglycerides in blood are significantly correlated to cardiovascular diseases (Grundy and Denke, 1990; Nelson and Cox, 2004).

1.3.1. Cholesterol

Cholesterol is an important lipid constituent of biological membranes, specially influencing membrane fluidity also as a precursor for bile formations and steroid hormones, but it is not required in the mammalian diet since all cells can synthesize it from simple precursors (Nelson and Cox, 2004).

1.3.2. Lipoproteins

Cholesterol, triglycerides and phospholipids are insoluble in water. These lipids carried in the blood plasma from one tissue to another as plasma lipoproteins, macromolecular complexes of specific carrier proteins called apolipoproteins with different combinations of phospholipids, cholesterol, triglycerides and cholesteryl esters (Nelson and Cox, 2004).

The major lipoproteins are classified according to density. Since lipids have a lower boyant force than proteins, lipoproteins that have a high ratio of lipid to protein have a lower density than a lower ratio of lipid to protein. Chylomicrons (CM) are synthesized in the intestine for transport of dietary triacylglycerols to various tissues. Very low density lipoproteins (VLDL) are synthesized in the liver for the export of endogenous triacylglycerols, while low density lipoproteins (LDL) arise from the metabolic transformation of VLDL in circulation. The function of LDL is to deliver cholesteryl ester to peripheral tissues and liver. High density lipoproteins (HDL) are synthesized and assembled in the liver and intestine or are formed from metabolic transformations of other lipoproteins in circulation, and from cellular lipids at the cell membranes. HDL removes excess cholesterol from cells and transports it to liver and steroidogenic tissue for metabolism and excretion (Vance, 2004).

A high level of HDL is correlated with decreased risk of coronary artery disease since HDLs prevent accumulation of cholesterol in the blood (Tortora and Grabawski, 2003). LDLs contain apoprotein, apoB-100. LDLs transport cholesterol to tissues that have specific plasma membrane receptors that recognize apoB-100. These receptors mediate the endocytosis of LDL into the cell within an endosome (Nelson and Cox, 2004). Excessive numbers of LDL form fatty plaques that increase the risk of coronary heart disease (Tortora and Grabowski, 2003).

1.3.3. Triglycerides

Most triglycerides are carried in the VLDL fraction, in the fasting state. Two factors determine serum levels of triglycerides; rates of hepatic secretion of VLDL triglyceride and the capability for hydrolyzing circulating triglyceride. Hepatic overproduction of VLDL triglycerides or defective lipolysis of triglyceride-rich lipoproteins cause hypertriglyceridemia (Grundy and Denke, 1990)

1.4. Inflammation

Inflammation is an essential and common pathologic process; it indicates that the body is struggling to deal with some infectious agent or damaged tissues. Its symptoms are redness, pain, heat and swelling of the inflamed or damaged tissue (Tortora and Grabowski, 2003). The process of inflammation is divided into acute and chronic forms. Acute inflammation is characterized by vasodialation, vasopermeability and infiltration of polymorphonuclear neutrophilic leukocyte (PMNs) into injured area while chronic inflammation is characterized by infiltration of lymphocytes and macrophages into injured area, and restoration of normal structure or scarring. Chronic inflammation follows acute inflammation if the latter is not enough to clear the tissue of necrotic debris produced by acute necrosis. Chronic inflammation is more defensive against persistent infections and completes the healing process (Sell and Max, 2001).

1.5. Gastric Damage

Gastric and duodenal ulcers affect a great number of the world population and induced by many factors, including genetic susceptibility, alcohol, drugs, stress, smoking, nutritional deficiencies, bile salts, and an excessive secretion of acid and pepsin. However, the major factor is the presence of *Helicobacter pylori* that is present in the stomachs of most patients with peptic ulcer (Widmaier et al., 2004; Belaiche et al., 2002).

Ulcer treatment is based on the inhibition of acid secretion. Two drug classes are effective inhibitors of acid secretion. One class act by blocking specific histamine receptors (H_2) found on parietal cells, which stimulate acid secretion, example cimetidine. The second class of drugs inhibits the H^+/K^+-ATPase pump in parietal cells, which pumps proton into gastric lumen (Widmaier et al., 2004). Several plants and herbs have been used to treat gastrointestinal disorders in traditional medicine (Toma et al., 2003), and have shown promising results in the treatment of gastric ulcer as being sources of new drugs (Borelli and Izzo, 2000). Methanolic extract of *R. officinalis* leaves showed a good activity against *Helicobacter pylori* (Mahady et al., 2005).

1.6. Glycemia

Insulin secreted by β-cells of the islets of Langerhans decreases the blood glucose level by enhancing the uptake of glucose into the cells where it is stored as glycogen or used as an energy substrate in the synthesis of proteins or fats (Hadley, 2000). The destruction of β-cells by auto immune disease leads to Type-I Diabetes Mellitus, whereas Type II Diabetes Mellitus is a metabolic disease caused by decreased insulin secretion and action (Cavaghan et al., 2000). Many studies were conducted on plants to monitor their hypoglycemic and insulin release stimulatory effect (Ivorra et al., 1989). It has been previously reported that rare number of plants have hyperglycemic and insulin release inhibitory effect (Morrison et al., 1987). A study by Al-Hader et al (1994) suggested that volatile oil of R. officinalis has insulin release inhibitory and hyperglycemic effects. Administration of 25mg/kg of volatile oil extracted from *R. officinalis* leaves to normal rabbits increased the plasma glucose 20%, 27% and 55%, above those of control group, at the 60, 90 and 120 min intervals respectively, and decreased the insulin serum level 20% in comparison with control group at 30 min interval (AL-Hader et al., 1994).

1.7. Liver assessment

Biotransformation of all drugs and xenobiotics (foreign substances) is central to the liver along with synthesizing an array of body proteins (Lee, 1993). Analysis of some enzyme activities in blood serum helps in diagnosis of a number of disease conditions. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are two important enzymes in the diagnosis of liver damage caused by drug toxicity (Nelson and Cox, 2004). AST is found in high concentration in heart muscle, liver cells and skeletal muscles. The ALT is found in high concentration in liver and kidney (Pratt and Kaplan, 2001). The use of herbal medicine is very popular and common. Medicinal plants are being sold almost everywhere in a random manner and reasonable prices. It was proven that plants consumed can be detoxified by the liver and its second metabolites issued might cause liver damage and elevate liver enzymes (Grunhage et al., 2003; Pak et al., 2004). Therefore, it is extremely important to test all herbs and herbal supplements for their possible liver toxicity before being consumed.

1.8. Antimicrobial activity

The antimicrobial effect of plants is an interesting field of research especially when it comes to pathogenic microbial species (Avani and Neeta, 2005). Plant antimicrobial compounds may inhibit bacterial growth by several mechanisms different from the presently used antimicrobials and can be effective in treating resistant microbial strains (Eloff, 1988). Patients that were using plants for their antimicrobial activity have a reduced risk to get infectious diseases from resistant pathogens than people from urban areas using traditional antibiotics (Rojas et al., 2006). Many plants' antimicrobial compounds were identified against different bacterial strains. They belong to polyphenols, alkaloids and monosaccharide classes

and include latex, tannins, essential oils, salicylic acid and many others. The mechanism of action of phenols is through inhibiting certain enzyme activities in bacteria, while flavonoids are known to bind bacterial cell walls leading to their destruction (Cowan, 1999).

1.9. Purpose of the Project:

The aim of the present project is to investigate the potential medicinal effect of aqueous extract of *R. officinalis* leaves in lipemia, glycemia, inflammation, gastric ulcer and as an antimicrobial agent. Also, the present study evaluates the safety of plant extract intake by assessing liver toxicity. The study included the following:

❖ Assessment of blood lipid and glycemic profiles and liver toxicity in rats after 30 days of intake of *R. officinalis* leaves water extract:

- ➢ Plasma total cholesterol.
- ➢ Plasma HDL- cholesterol.
- ➢ Plasma LDL- cholesterol.
- ➢ Plasma Triglycerides
- ➢ serum glucose
- ➢ Serum insulin
- ➢ Serum Enzymes ALT and AST

❖ Assessment of *in-vivo* anti-inflammatory activity of *R. officinalis* leaves water extract using acute and chronic inflammation models.

❖ Assessment of the anti-ulcerogenic effects of aqueous extract of *R.officinalis* leaves in Ethanol induced gastric ulcer.

❖ Assessment of antimicrobial effects of aqueous extract of *R.officinalis* leaves on several hospital bacterial pathogenic species.

Chapter 2

Materials and Methods

2.1. Plant Collection and water extract preparation:

The leaves of *Rosmarinus officinalis* were collected from West Bekaa valley especially from Jib Jannine. The plant was identified according to the characteristics described in botanic and plant taxonomy book (Duke et al., 2002). All collected leaves were left in shade until they became dry before being used in the different experiments. The dry weight of water extract of the leaves per g dry leaves was determined by soaking 20 g of dry leaves in pre-boiled water for 30 minutes, filtering the solution using Whattman filter paper and evaporating the filtrate first on a mild flame until a small volume is left and then in an oven at 45^0C overnight to assure total evaporation of water. Data have shown that every 1g of plant leaves yields 0.20 g of pure water extract, and this was taken into consideration in all the experimental protocols conducted.

2.2. Animals on lipid diet:

Male Sprague rats (n=40) (Lebanese American University stock) were divided into four groups (10 rats/group) with an average weight of 235 g. Animals of all groups were maintained at an ambient temperature of 20-25^0C and received rat chow diet (Hawa chicken stock) to which 5 % (w/w) coconut oil was added. The first group was considered as the control group while the other three groups were considered as experimental groups and received the plant water extract in drinking water at doses 200 (group I), 500 (group II) and 1000 (group III) mg/kg body weight. After one month of extract intake, fasted animals (12hr) were sacrificed using diethyl ether

and approximately 8 ml of blood were collected from the inferior vena cava.

2.2.1. Serum Analysis:

2.2.1.1. Samples preparation:

The collected venous blood (8 ml) was put into a glass tubes and allowed to clot for 30 minutes at room temperature. Blood was then subjected to centrifugation for 15 minutes at 2000g and 4^0C. The serum (supernatant) samples were aliquoted into different eppendorf tube. Liver enzyme (ALT and AST) activities were directly measured using fresh samples while the remaining samples were stored at -20^0C for later use in the assessment of lipid (TAG, total cholesterol, HDL cholesterol, LDL cholesterol) and glycemic (glucose and insulin) profiles.

2.2.1.2. Determination of Triglyceride:

Triglycerides (TAG) concentration was determined using SPINREACT kit. The lipoprotein lipase (LPL) is an enzyme that rapidly hydrolyzes TAG to glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. G3P is then converted by Glycerol dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Finally H_2O_2 reacts with 4-aminophenazone (4-AP) and chlrophenol in presence of peroxidase (POD) enzyme to give a red color solution.

The color intensity is proportional to the concentration of TAG of the sample which can be measured by determining the optical density (OD) by the spectrophotometer at wavelength of λ=505 nm

Calculation of TAG:

Concentration of TAG in the unknown sample (mg/dl) =

$$\frac{\text{Abs of unknown} \times \text{Conc.of Standard}}{\text{Abs of Standard}}$$

The concentration of the standard is 200mg/dl according to SPINREACT kit

2.2.1.3. Determination of the Total Cholesterol:

Cholesterol concentration was determined following the SPINREACT kit protocol. Cholesterol esterase hydrolyzes the cholesterol ester form and releases free cholesterol and fatty acids. Then H_2O_2 is formed in the subsequent enzymatic oxidation of cholesterol by cholesterol-oxidase. The hydrogen peroxide produced reacts with 4-aminophenazone (4-AP) and phenol in the presence of peroxidase (POD) and forms a red dye quinonimine

The color intensity is proportional to the concentration of cholesterol of the sample which can be measured by determining the absorbance (Abs.) by the spectrophotometer at wavelength of λ=505 nm

Calculation of Cholesterol:

$$\text{Concentration in the unknown sample (mg/dl)} = \frac{\text{Abs. of unknown} \times \text{Conc. of Standard}}{\text{Abs. of Standard}}$$

The concentration of the standard is 200mg/dl according to SPINREACT kit

2.2.1.4. Determination of HDL-Cholesterol:

Low density lipoproteins (LDL) and very low density proteins (VLDL) are specifically precipitated by phosphotungstic acid and magnesium ions and then removed by centrifugation while HDL remains in the supernatant.

HDL-Cholesterol concentration was determined following the SPINREACT kit protocol

Calculation of HDL- Cholesterol:

Concentration in the unknown sample (mg/dl) =

$$\frac{\text{Abs of unknown} \times \text{Conc of Standard}}{\text{Abs of Standard}}$$

2.2.1.5. Determination of LDL-Cholesterol:

In order to determine the LDL concentration, no kit was used. LDL concentration was calculated using Friedewald Formula (SPINREACT Kit):

$$\text{Concentration in the unknown sample (mg/dl)} = \text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL Cholesterol}$$

2.2.1.6. Liver enzymes:

2.2.1.6.1. Aspartate aminotransferase (AST) :

Oxaloacetate and glutamate are formed by the reversible transfer of an amino group from aspartate to α-ketoglutarate catalyzed by AST formerly called glutamate oxaloacetate (GOT). Oxaloacetate produced is reduced by malate dehydrogenase (MDH) and NADH to malate. The rate of decrease in concentration of NADH, measured photometrically (340nm) is proportional to the catalytic concentration of AST present in the sample (SPINREACT Kit)

Calculation of AST:

$\Delta A/min \times 1750 = U/L\ AST$

2.2.1.6.2. Alanine aminotransferase (ALT) :

Pyruvate and glutamate are formed by the reversible transfer of an amino group from alanine to α-ketoglutarate catalyzed by ALT formerly called glutamate pyruvate transaminase (GPT). Pyruvate produced is

reduced by lactate dehydrogenase (LDH) and NADH to lactate. The rate of decrease in concentration of NADH, measured photometrically (340nm) is proportional to the catalytic concentration of AST present in the sample (SPINREACT Kit)

Calculation of ALT:

$\Delta A/min \times 1750 = U/L\ ALT$

2.2.1.7. Glycemia:

2.2.1.7.1. Glucose:

The oxidation of glucose to gluconic acid is catalyzed by glucose oxidase (GOD). The formed H_2O_2 is detected by phenol-aminophenazone, a chromogenic oxygen acceptor, in the presence of peroxidase (POD). The intensity of the color formed is proportional to the glucose concentration in the sample measured by spectrophotometer at 505 nm (SPINREACT)

Calculation of glucose concentration:

$$\text{Concentration in the unknown sample (mg/dl)} = \frac{\text{Abs. of unknown} \times \text{Conc.of Standard}}{\text{Abs. of Standard}}$$

The concentration of the standard is 100 mg/dl according to the SPINREACT

2.2.1.7.2. Insulin:

Insulin concentration is determined using the Rat/Mouse insulin ELISA (Enzyme Linked Immuno-sorbant Assay) kit (LINCO Research, USA). It is used for non-radioactive quantification of insulin in rat. This technique is based on Sandwich ELISA which a microtiter plate coated with pre-titered amount of monoclonal anti-rat serum antibodies is used. After adding of the samples to the wells, insulin molecules bind to the monoconal anti-insulin antibodies. Then bioltinylated anti-insulin antibodies were added and binded to the captured insulin. After washing the unbound materials from the sample, horseradish peroxidase were added

and binds to immobilized biotinylated antibodies. A simple washing step removes unbound enzyme labeled antibodies. The immobilized antibody-enzyme conjugates quantified by monitoring the activity of horseradish peroxidase enzyme in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The reaction is stopped by adding acid and the enzyme activity is measured using Spectra Max Plus ELISA reader by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

2.2.2. Water, TAG and Cholesterol content in Stool:

Stool samples were collected from each animal in every group, weighed immediately and then placed overnight in an oven at 70^0C. The next day, the dried stools were weighed again and the percentage water content was calculated. Stools were then crushed into powder using mortar and pestle. 0.200g of the powdered stools was weighed, placed in a glass tube and 2 ml of n-hexane was added. The sample mixtures were put in a water bath at 40^0C for 2 hours with frequent stirring using a glass rod. Samples were subjected to centrifugation (3500 g) for 10-15 minutes and aliquots (300µl) of the supernatant were taken for triglyceride and cholesterol assays. Before assessment of triglyceride and cholesterol the aliquot samples were put in a water bath at 72^0C in order to evaporate the n-hexane. The left-over lipids in the tubes were then tested for TAG and cholesterol using the "SPINREACT" kit.

2.2.3. Body weight change:

In order to study the effect of the water extract of *Rosmarinus officinalis* leaves on bodyweight changes, all animals were subjected to body weight measurement at day 1 and day 30 of the experiment. During the study

period, rats were given 6.5g of food per 100 g body weight and their eating behavior was monitored. The percentage increase in body weight was calculated according to the formula below:

$$\% \text{ of increase in weight} = \frac{\text{increase in body weight}}{\text{initial body weight}} \times 100$$

2.3. Inflammation:

Sprague-Dawley male rats (Lebanese American University) weighing between 250 and 300 g were used. Acute and chronic anti-inflammatory effects were appraised using carrageenan and formalin induced inflammation respectively in rat hind paw (Jose et al., 2004). All rats were fed regular chow diet and had open access to water. The water extract of *Rosmarinus officinalis* was sterilized using syringe filtration before intraperitoneal injection.

2.3.1. Acute Inflammation:

The thickness of right hind paw of all animals (n=42) was measured with a vernier caliper at the beginning of the experiment. Rats were then randomly divided into six groups each of 7 rats. Four groups I, II, III and IV received *Rosmarinus officinalis* water extract through intraperitoneal injection at dose of 50, 100, 250, and 500 mg/kg body weight respectively. The remaining two groups served as control groups where one group was injected with Diclofenac (10mg/kg) intraperitoneally and considered as positive control while the other group was left untreated and served as negative control. After 30 minutes of water extract and Diclofinac injection, rats of all groups were subjected to subplanter injection with 0.02 ml of 1% carrageenan in the right hind paw to induce edema. Paw thickness was remeasured after 3 hours post-edema induction (Jose et al., 2004).

2.3.2. Chronic inflammation:

The thickness of right hind paw of all animals (n=42) was measured with a vernier caliper at the beginning of the experiment. Rats were then randomly divided into six groups each of 7 rats. Four groups I, II, III and IV received *Rosmarinus officinalis* water extract through intraperitoneal injection at dose of 50, 100, 250, and 500 mg/kg body weight respectively. The remaining two groups served as control groups where one group was injected with Diclofenac (10mg/kg) intraperitoneally and considered as positive control while the other group was left untreated and served as negative control. After 30 minutes of water extract and Diclofinac injection, rats of all groups were subjected to subplanter injection with 0.02 ml of 2% formalin in the right hind paw to induce edema. The administration of the extracts and reference drug was continued once daily for 6 consecutive days. The paw thickness was measured using vernier calipers the 6th day after 3 hours of injection (Jose et al., 2004).

2.3.3. Calculations:

the anti-inflammatory effect of *Rosmarinus officinalis* in acute and chronic inflammation was callculated as follows:

P_0: the paw thickness at time zero (pre-inflammation induction)

P_t: the paw thickness at time t (post-inflammation induction)

$C = P_t - P_0$: Increase in paw thickness of the control

$T = P_t - P_0$: Increase in paw thickness of the treatment

$$\text{percentage of inhibition} = \frac{(C - T)}{C} \times 100$$

2.4. Gastric damage:

Sprague-Dawley male rats (Lebanese American University) weighing between 250 and 300 g were used to assess the anti-ulcer activity of *Rosmarinus officinalis.* Animals (n=42) were divided into 6 groups of 7 rats each. Animal groups were fasted for 48 hours to ensure empty stomach and kept in cages with raised floors of wide wired mesh to prevent coprophagy (eating excrement). During the fasting period all groups were provided with a solution containing 8% sucrose and 0.2% NaCl in order to prevent excessive dehydration. This solution was removed 1 hour before ulcer induction (Alkofahi and Atta, 1999; Gharzouli et al., 1999).

Also during the fasting period, the six groups received the treatment shown in table 2.1:

Table 2.1: The treatment solutions that were received by the experimental groups in assesment of anti-ulcer activity of *R. officinalis.*

Group I	Group II	Group III	Group IV	Group V	Group VI
Drinking water	Cimetril	Water extract	Water extract	Water extract	Water extract
	10mg/kg body weight	50 mg/kg body weight	100 mg/kg body weight	250 mg/kg body weight	500 mg/kg body weight

Either cimetril or plant extract were dissolved in drinking water, and all gastric instillation volumes were equivalent to 10 ml/kg body weight.

The drinking water, reference drug and plant extract doses were administered orally using an intubation needle. On the first day, 2 doses were given orally at 9:00 h and 16:00 h and a third dose was given on the next day at 8:00h, 1.5 hour before gastric ulcer induction. The six groups were subjected to gastric ulcer induction by administration of ethanol 50% (w/v) (in drinking water) orally in a dose of 10ml/kg body weight. All rats were sacrificed by an overdose of diethyl ether after 1 hour of ethanol administration. The stomachs were removed and washed under running tap water and opend along their great curvature (Alkofahi and Atta, 1999).

2.4.1. Calculations:

Using an illuminated magnifying microscope, long lesions were measured with a ruler along their length while petechial lesions were counted, and every 5 petechial lesion were considered as 1 mm of measurment. The sum of total length of ulcer and petechial lesions in each group of rats was divided by its number to calculate the ulcer index (mm) (Alkofahi and Atta, 1999).

The curative ratio was determined using the formula:

$$\text{Curative Ratio} = \frac{(\text{Control Ulcer index} - \text{Test Ulcer index})}{\text{Control Ulcer index}} \times 100$$

2.5. Antimicrobial Activity of Water Extract of *Rosmarinus officinalis:*

The antibacterial activity of the *Rosmarinus officinalis* water extract was tested. Ten hospital isolates of different bacterial strains from different patients were used in this experiment using the disc diffusion assay. The bacterial strains used were *Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Serratia marcesens, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphilococcus aureus methicillin resistant (MRSA), Staphilococcus aureus methicillin sensitive (MSSA), Ewingella americana* (table 2.2). These strains were provided from the bacteriology laboratory of Beirut Govermental Hospital.

2.5.1. Disc Diffusion Test:

Rosmarinus officinalis dry leaves (5 g) were soaked in pre-boiled distilled water (100 ml) to obtain a concentration of 200µg/20µl. This solution was filtrated using a Whatman filter paper and sterilized by passing it through a 0.45µm membrane filter. Different concentrations (200, 100, 50, 20 and 10 µg/20µl) of the extract were prepared by serial

dilutions. Inoculum from each of the bacterial cultures were inoculated in 5 ml of 0.85% NaCl then the optical density was measured and amended to 0.125 at 550 nm (150×10^6 bacteria/ml of suspension). Bacterial strains each of 0.1 ml were spread on a Muller-Hinton agar plates and four blank sterile discs were placed on the surface. One disk served as a control by adding 20µl of sterile water and the other discs were soaked with 20µl of the different concentrations (200, 100, 50, 20 and 10 µg/20µl) of the extract and in duplicate. A reference antibiotic was used as a positive control and placed at the center of the plates according to each bacterial strains (table 3.5). Plates were incubated 24 hours at 37°C then the antimicrobial activity was determined by measuring the zone of inhibition and compared to the Reference antibiotic (Romero et al., 2005; Tadeg et al., 2005; Barbour et al., 2005)

Table 2.2. List of bacterial strains used in antibacterial activity of *Rosmarinus officinalis* and their identification tests used in the hospital.

Bacterial strains		Identification test
1.	*Citrobacter freundii*	API 20E
2.	*Enterobacter cloacae*	API 20E
3.	*Escherichia coli*	Urea: Negative/ Indole: Positive
4.	*Serratia marcesens*	Phoenix
5.	*Klebsiella pneumoniae*	API 20E
6.	*Proteus mirabilis*	Urea: Positive/ Indole: negative
7.	*Pseudomonas aeruginosa*	Oxidase positive/ Isolation at 42 °C
8.	*Salmonella typhi*	API 20E
9.	*(MRSA)**	Oxacillin resistant
10.	*(MSSA)**	Oxacillin sensitive
11.	*Ewingella americana*	Phoenix

*Glucose positive, Catalase positive, Coagulase positive and DNase positive

2.6. Stastical analysis:

Values are presented as ± S.E.M. Student *t* test was used to determine the significant difference between the treated groups and the control. All values were considered significant when $p < 0.05$.

Chapter 3

Results

3.1. Serum parameters

3.1.1. Total Cholesterol, HDL, LDL, and TAG

All animal groups that received the water extract of *Rosmarinus officinalis* leaves (200, 500 and 1000mg/kg body weight) showed no significant changes in the serum concentration of total cholesterol, HDL cholesterol, LDL cholesterol, and TAG with respect to the control. Results are shown in table 3.1.

Table 3.1: Serum total Cholesterol, HDL cholesterol, LDL cholesterol and TAG concentrations in control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight respectively.

Parameters	Control	Treatment groups		
		Group I	Group II	Group III
Total Cholesterol	60.03 ± 2.58	60.72 ±2.44	67.29± 3.41	61.37 ± 3.52
HDL-Cholesterol	26.86 ± 1.89	31.95±2.55	28.94 ±1.81	24.24 ± 2.67
LDL-Cholesterol	21.23 ± 1.72	17.49 ± 1.33	25.41 ± 2.52	24.98 ± 2.63
Triglycerides	61.12 ± 3.17	56.37 ±2.79	64.58± 3.43	60.75± 3.83

Values are expressed as mean ± S.E.M. (n=10/group)

3.1.2. Liver functions tests

The activities of liver enzymes ALT and AST were determined in Control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration (200, 500, and 1000 mg/kg body weight respectively). Results are shown in table 3.2. Data analysis did not reveal any significant difference between the control and experimental groups with both enzymes.

Table 3.2: Determination of serum hepatic enzymes ALT and AST levels (U/L) in Control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight respectively.

Parameters	Control	Treatment groups		
		Group I	Group II	Group III
ALT	44.43 ±4.57	42.88 ±6.25	64.88 ±11.57	46.99 ±10.13
AST	53.38 ± 2.89	59.50 ± 5.30	52.50 ± 4.45	63.97 ± 5.35

Values are expressed as mean ± S.E.M. (n=10/group)

3.1.3. Glycemia

Glucose and insulin concentrations were measured in the serum of control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight respectively.

3.1.3.1. Glucose:

The serum concentrations of glucose in the different experimental groups were lower than that of the control. However, significance was only reached in groups I and III (figure 3.1).

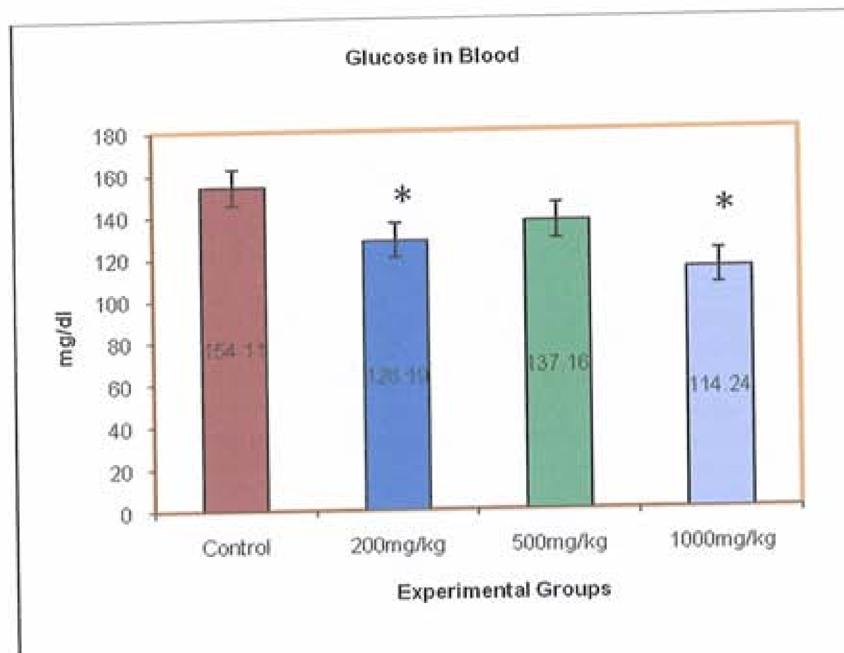

Figure 3.1: Serum glucose concentration (mg/dl) after 1 month of intake of *Rosmarinus officinalis* water extract in the control and experimental groups * Significant difference with respect to the control ($p < 0.05$). Bars represent means ± S.E.M. (n=10/group)

3.1.3.2. Insulin:

Data have shown a significant decrease in serum insulin concentration in group I and III with respect to the control. However, group II showed similar serum concentration of insulin as the control. Data are presented in figure 3.2

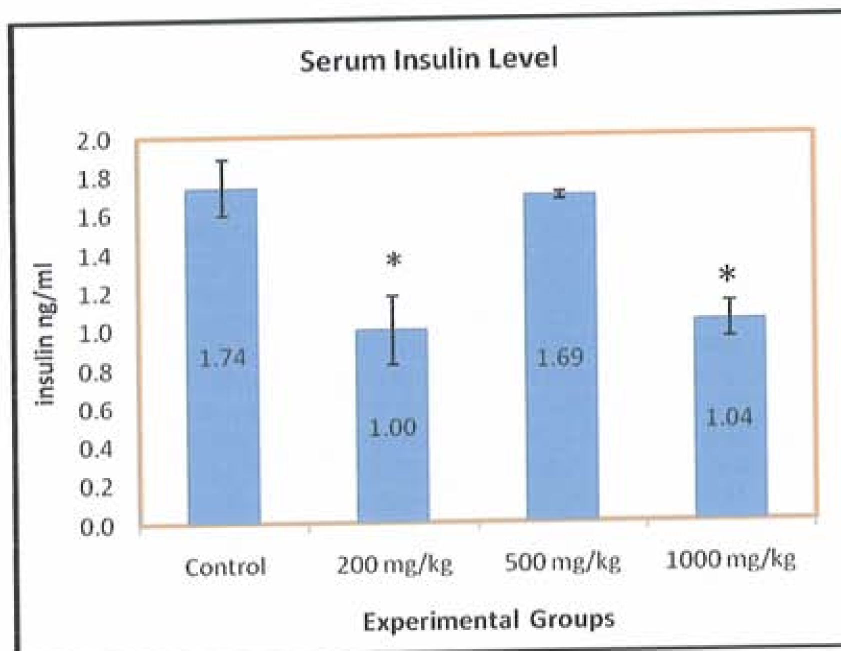

Figure 3.2: Serum insulin concentration (ng/ml) after 1 month of intake of *Rosmarinus officinalis* water extract in the control and treated groups. *Significant difference with respect to the control ($p < 0.05$), Bars represent means ± S.E.M. (n=10/group)

3.2. Effect on body weight

The *Rosmarinus officinalis* water extract (200, 500 and 1000 mg/kg) didn't show any significant effect on body weight of rats after one month of intake of the extract (Figure 3.3).

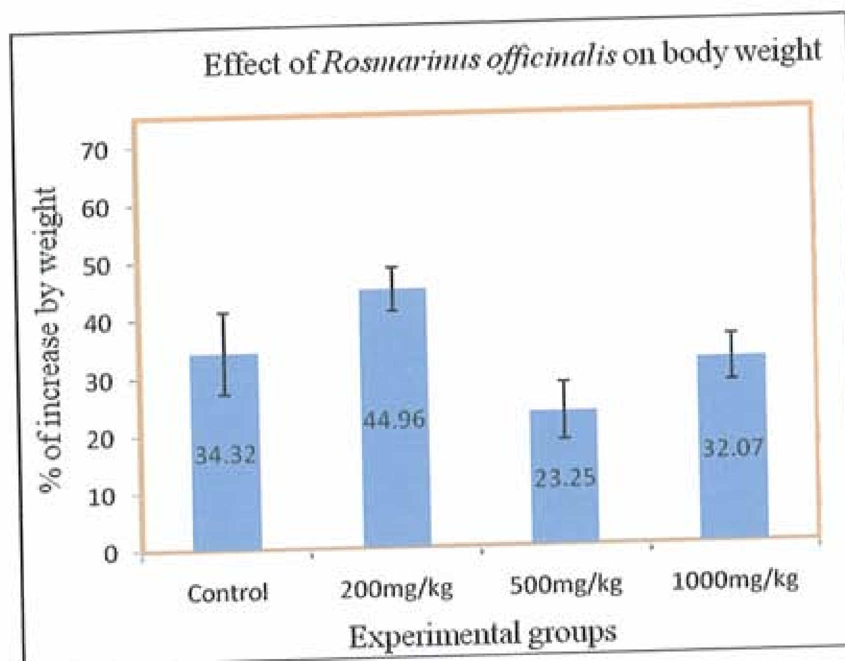

Figure 3.3: The increase in body weight of animals after 1 month of intake of *Rosmarinus officinalis* water extract in the control and experimental groups (200, 500 and 1000 mg/kg of water)

3.3. Stool Analysis

3.3.1. Water, cholesterol and triglyceride content in Stools

The effect of water extract of *Rosmarinus officinalis* leaves on water, cholesterol and triglyceride content in stools was evaluated. Water extract intake appeared not to have any significant effect on the water content of stools (figure 3.4). Similarly, there were no significant changes in the concentration of cholesterol (figure 3.5) and triglyceride (figure 3.6) in the stools of experimental groups with respect to control.

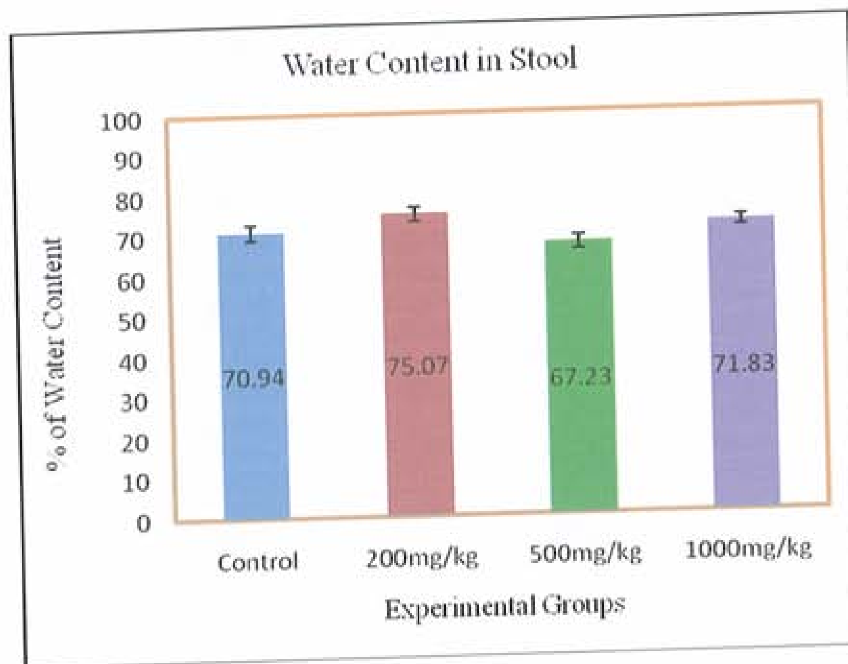

Figure 3.4: The % of water content of stools in control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight.

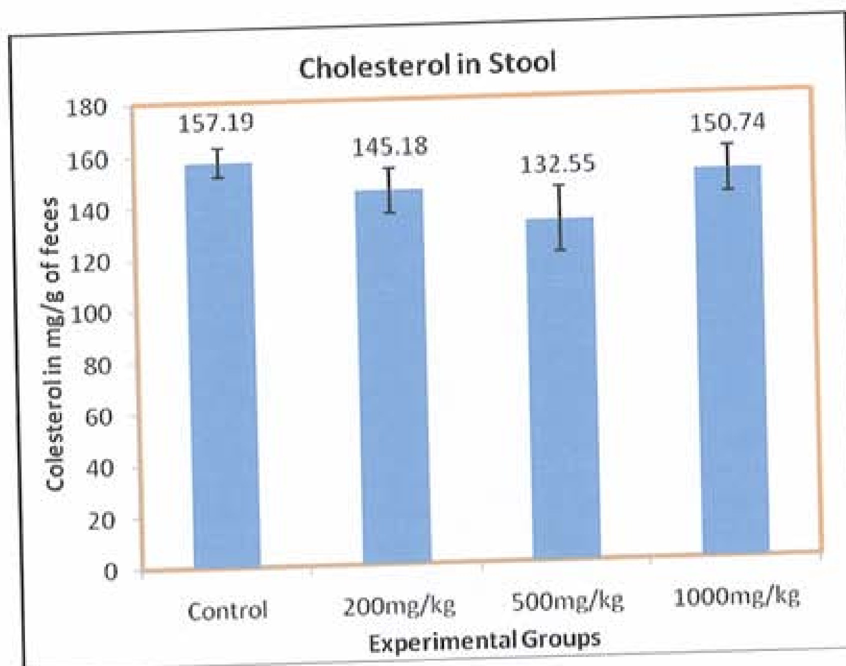

Figure 3.5: Cholesterol content of stools in control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight.

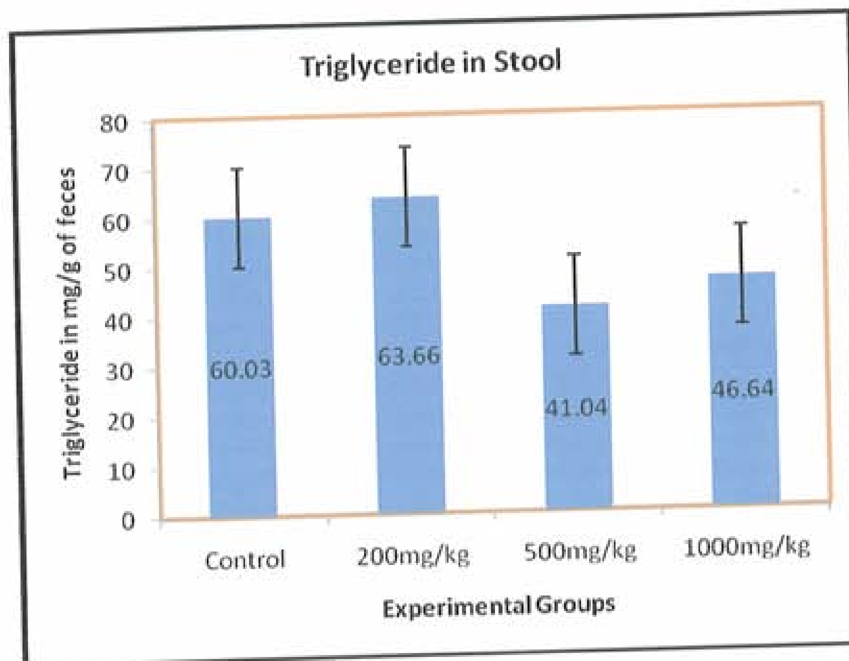

Figure 3.6: Triglyceride content of stools in control and experimental groups I, II, and III after one month of *Rosmarinus officinalis* administration of 200, 500, and 1000 mg/kg body weight.

3.4. Gastro protective effect against ethanol-induced gastric ulcer

The extended and petechial lesions were established in the control and treated groups I, II III and IV after ethanol-induced gastric ulcer. Animals that received the *Rosmarinus officinalis* water extract showed a dose dependent protection against ulcer with respect to the negative control group. Protection was significant in group III and IV (table 3.3; Figure 3.7; 3.8).

Table 3.3: Effect of water extract of *Rosmarinus officinalis* dry leaves on ethanol-induced gastric ulcer.

Treatment groups	Ulcer Index
Negative Control	35.17 ± 4.29
Cimetril (10 mg/kg)	14.09 ± 2.46 *
Group I (50 mg/kg)	25.60 ± 6.29
Group II (100 mg/kg)	23.80 ± 6.87
Group III (250 mg/kg)	17.11 ± 1.48 *
Group IV (500 mg/kg)	10.27 ± 1.8 *

* Significant difference with respect to the negative control ($p < 0.05$).
Ulcer index values are represented as mean± S.E.M.

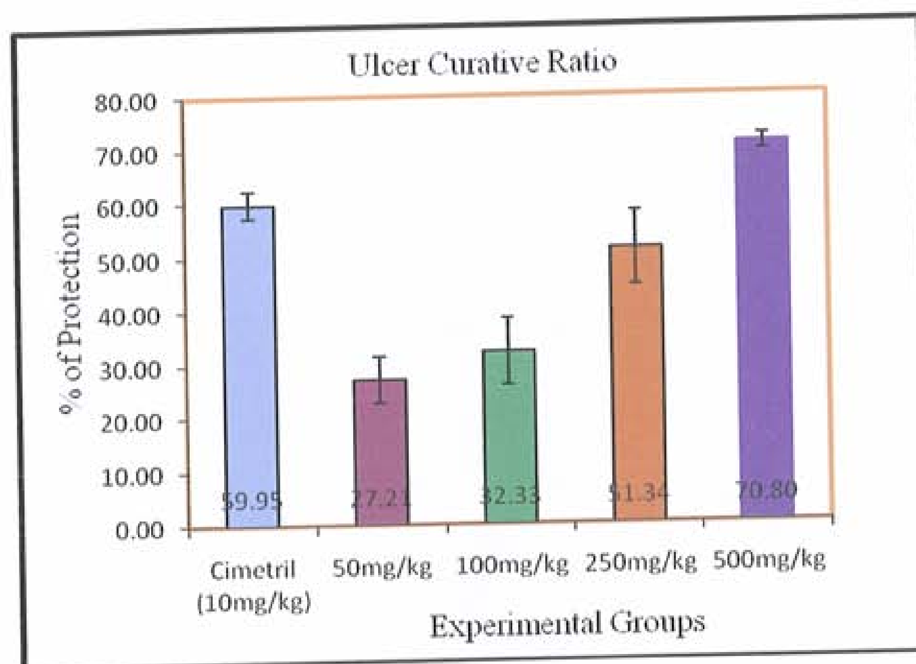

Figure 3.7: The Ulcer Curative ratio in Cimetril (10 mg/kg) treated group and the 50, 100, 250 500 mg/kg of *Rosmarinus officinalis* water extract treated groups. Bars represent the % protection against ethanol-induced gastric ulcer.

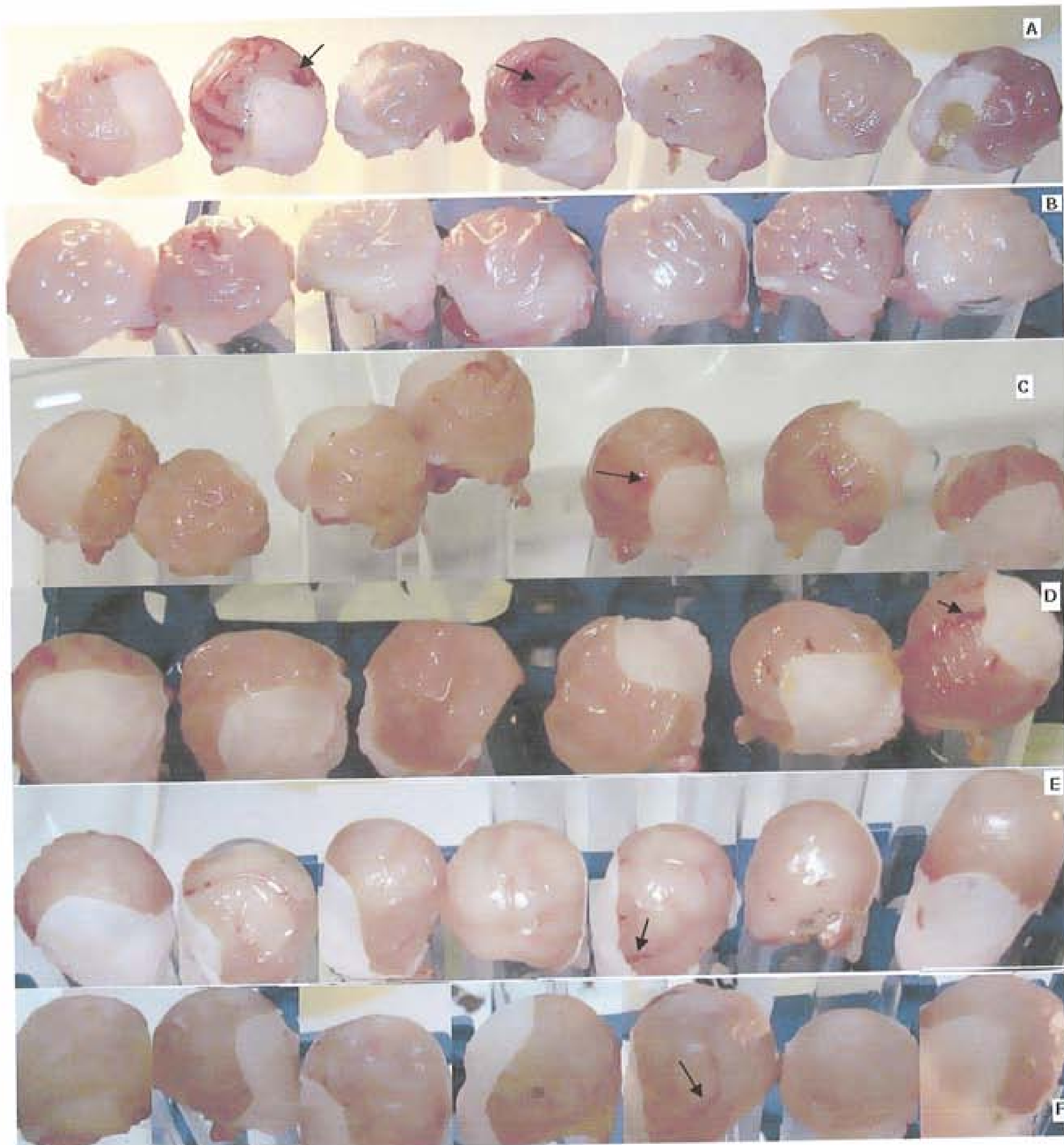

Figure 3.8: Photo illustration showing the stomachs of rat groups after ethanol–induced gastric ulcer. **(A)** Untreated group. **(B)** Cimitrel (10 mg/kg) treated group. **(C)** 50 mg/kg water extract treated group. **(D)** 100 mg/kg water extract treated group. **(E)** 250 mg/kg water extract treated group. **(F)** 500 mg/kg water extract treated group.

⟶: Ulcer formation

3.5. Anti-inflammatory activity

Water extract of *Rosmarinus officinalis* leaves showed substantial anti-inflammatory activity in acute and chronic inflammation models. Doses of 100, 250, and 500 mg/kg body weight showed a significant anti-inflammatory activity in the acute and chronic inflammation models. The optimum dose was 500 mg/kg in the chronic inflammation; its anti-inflammatory effect surpassed that of Diclofenac. However, two optimum doses of 100 and 250 mg/kg were observed in acute inflammation. Data are shown in table 3.4.

3.6. Anti-microbial Activity:

The anti-bacterial consequences of water extract *of Rosmarinus officinalis* leaves were investigated against 11 bacterial strains (table 3.5). The five doses (200, 100, 50, 20 and 10 μg/20μl) that were used didn't show any antibacterial activity against any of the tested strains. The zones of inhibition produced by the antibiotics, specific to each bacterial strain, were measured and shown in table 3.5

Table 3.4: The effect of *Rosmarinus officinalis* water extract dry leaves on Carrageenan induced acute and formalin induced chronic inflammation

		Carrageenan		Formalin	
Treatment	Dose (mg/kg)	Increase in paw thickness after 3 h	Inhibition (%)	Increase in paw thickness after 6 days	Inhibition (%)
Control		0.9 ± 0.13[a]	–	1.57 ± 0.12[a]	–
***R. officinalis* Extract**	50	0.58 ± 0.14[a]	36	1.25 ± 0.13	21
	100	0.36 ± 0.12*	61	1.09 ± 0.15*	31
	250	0.36 ± 0.10*	61	1.10 ± 0.06*	30
	500	0.42 ± 0.11*	54	0.87 ± 0.13*	45
Diclofenac	10	0.24 ± 0.03*	73	0.95 ± 0.17*	40

* Significant difference with respect to the control ($p < 0.05$)

[a] Significant difference with respect to the diclofenac ($p < 0.05$)

Values are represented as mean ± S.E.M. (n=7)

Table 3.5. List of bacterial strains used in antibacterial activity of *Rosmarinus officinalis* leaves, and their reference antibiotic drug.

Bacterial strains	Inhibition zone of extract	Reference Drug	Inhibition zone (mm)
Citrobacter freundii	_	Cefepime/ Imipenim (30/10µg/disc)	26/25
Enterobacter cloacae	_	Cefepime/ Imipenim(30/10µg/disc)	37/25
Escherichia coli	_	Cefepime/ Imipenim (30/10µg/disc)	14/30
Serratia marcesens	_	Cefepime/ Imipenim (30/10µg/disc)	32/26
Klebsiella pneumoniae	_	Cefepime/ Imipenim (30/10µg/disc)	35/26
Proteus mirabilis	_	Cefepime/ Imipenim (30/10µg/disc)	15/23
Pseudomonas aeruginosa	_	Cefepime/ Imipenim (30/10µg/disc)	32/25
Salmonella typhi	_	Bactrim* (25µg/disc)	29
(MRSA)	_	Vancomycin (30µg/disc)	22
(MSSA)	_	Vancomycin (30µg/disc)	14
Ewingella americana	_	Cefepime/ Imipenim (30/10µg/disc)	35/30

*Bactrim consisted of sulfamethoxazol (23.75) + Trimethoprim (1.25)

Chapter 4

Discussion and Conclusions

Rosmarinus officinalis leaves and flower are traditionally used as flavoring agent in food and in treatment of headache, sciatica, intercostal neuralgia, flatulence, and other gastrointestinal complaints (Barnes et al., 2002). A search of the literature on *Rosmarinius officinalis* revealed that most studies dealt with the pharmacological actions of its methanolic and ethanolic extracts (Chen et al., 1992; Debra et al., 1997; Pearson et al., 1997; Moreno et al., 2006). The present study investigates the medicinal role of *R. officinalis* water extract in lipemia, glycemia, inflammation and ulcer protection in rats. This animal model was chosen in order to avoid any possible toxic effect on humans that may arise during the course of the study and to the fact that animals are better controlled in term of food, activity and other parameters that may affect the outcome of the study. Also, the present study covers the anti-microbial activity of the plant water extract against certain hospital pathogenic bacterial isolates.

The drying of the plant may cause conformational transformations in some of its dried preparations than in fresh plant material (Romero et al, 2005). In order to reduce variations due to geographic factors, the plant should be harvested from the same area (Ellof, 1999). Condition of leaves and time of harvest have also been reported to have a main role in the overall quality of the extract (Tewari and Virmani, 1987). In the present study, we used dried leaves that were collected only from Jib-Jannine area (Lebanon, West Bekaa Valey) during the month of June in order to avoid both geographic and harvest time effect on the plant constituent variation.

Lipid and lipoprotein abnormalities are correlated with development of coronary heart disease (CHD) (Skoumas et al., 2003). The intake of diet

that is high in cholesterol and lipid can produce hyperlipidemia and thus the researchers have centered their interest on the protecting effect of some plant extracts (Blazovics., 1993). In order to understand the effect of water extract of *Rosmarinus officinalis* dry leaves on blood lipid profile, serum total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol were assessed after one month of extract intake in drinking water at three different doses. To increase atherogenecity of the diet, animals were fed a diet rich in coconut oil that contains the atherogenic saturated fatty acids lauric, myristic and palmitic acids. Data have shown that the extract did not show any significant effect on total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides. Consequently, the plant water extract seems to have a neutral effect on lipemia. *R. officinalis* leaves are known to be rich in phenols and flavonoids (Barnes et al., 2002). Previous investigations revealed the importance of flavonoids in improving blood lipid profile (Grglewski et al., 1987, Hertog et al., 1993). The no effect observed in the present study may be attributed to the fact that different species of plants may contain different types and concentrations of flavonoids. Also, not all flavonoids and other plant constituents are water soluble to be extracted. Flavonoids and other polyphenol constituents of *R. officinalis* leaves are aromatic or saturated organic compounds; they are most often extracted through ethanol, chloroform or methanol extraction (Hufford et al., 1993; Nakahara et al., 1993; Perrett et al., 1995 Vijaya et al., 1995).Therefore, the concentration of flavonoids in the water extract used may be too low to exert the positive effect upon lipemia. On the other hand, the oxidative modification of LDL is viewed as a crucial step in the pathogenesis of artherosclerosis (Esterbauer et al., 1992). Rosemary phenolics (carnosol > carnosic acid □ rosmarinic acid) are known to prevent oxidation of LDL and may contribute to the prevention of atherosclerosis (Pearson et al., 1997). Although not investigated, the presence of such phenols in the water extract may have a potential role in reducing LDL oxidation, a parameter that needs further investigations.

The effect of water extract of *R. officinalis* leaves on fecal cholesterol and triglyceride have shown that the water extract had no impact on these parameters. These findings reveal that the water extracts of rosemary do not interfere with gastrointestinal lipid absorption/or digestion. Although the fecal concentration of TAG were somehow reduced (without reaching significance) in rats that received 500mg/kg and 1000mg/kg body weight of rosemary leaves water extract, no similar trend was observed in the serum TAG concentration. Also, the intake of *R. officinalis* leaves water extract for a period of four weeks appeared not to have an effect on the water content of stools and accordingly it causes neither constipation nor diarrhea. Similarly, the *Rosmarinus officinalis* water extract didn't show any significant effect on body weight changes over the period of the study.

Drugs or herbal medicine can be harmful to liver since it is responsible for metabolism of exogenous toxins (Pak et al., 2004). The level of liver enzymes, ALT and AST, provide a sign on the extent of this hepatocellular damage (Mason, 2004). To test the hepatotoxicity or the hepatoprotective activity of the water extracts of *R. officinalis* leaves, the activity of alanine transaminase (ALT) and aspartate transaminase (AST) in serum of rats were measured after one month of plant extract intake. The increase in the transaminases' concentrations indicates a cellular damage and to the structural integrity of the liver (Sallie et al., 1991). The water extract of *R. officinalis* leaves had no significant effect on both enzymes studied and consequently on the integrity of the liver. In addition, calculations of the ratios of AST/ALT in the experimental groups showed that all groups had values of less than 2 (data not shown) meaning that there is no worry of liver disease and that extract is safe to be consumed (Cohen and Kaplan, 2001). All these data indicate the harmless effects of the doses used when taken for a period of 30 days.

Early studies on the hypoglycemic effect of *R. officinalis* volatile oil were done by Al-Hader et al (1994). This study showed that volatile oil of *R. officinalis* has a hyperglycemic and insulin release inhibitory consequences in normal rabbits and in alloxin diabetic rabbits. The administration of volatile oil 25mg/kg to a fasting diabetic rabbit increases the glucose plasma level 20%, 27% and 55% compared with control animals at the 60, 90, and 120 minutes time intervals respectively. Also the same treatment decreases the insulin level by 30 % in fasting diabetic rabbit after 30 minutes in comparison with the control animal (Al-Hader et al., 1994). In the present study, the administration of water extract of *R. officinalis* leaves at concentrations 200mg/kg (group I) and 1000mg/kg (Group III) to rats produced 17% and 26% significant decrease in glucose level respectively. Also the same treatment resulted in a 42.5% and 40% decrease in serum insulin level respectively. A mild decrease in insulin and glucose levels was observed in rats that received the 500 mg/kg body weight (Group II) dose. The discrepancy in result between the present study and that of Al-Hader et al. (1994) may be explained by the fact that different extracts were used (water extract versus volatile oil). The volatile constituents of rosemary are 1,8-cineole, (+)-camphor, (R)-(+)-limonene, borneol and (-)-a-pinene (Inoue et al., 2005) while the water extract contains mainly rosmarinic acid (Moreno et al., 2006). The hypoglycemic effect of *R. officinalis* water extract is shown to be a non-insulin mediated mechanisms since the two groups that showed hypoglycemia also showed a reduced level of serum insulin. Possible mechanisms behind the observed hypoglycemia may include decreasing glycogenolysis and gluconeogenesis by the liver, increased uptake of glucose by the adipocytes and muscle cells and inhibited secretion of regulatory hormones (glucagon, cortisol, growth hormones and epinephrine). Also, another possibility may be an increase in tissue sensitivity to insulin since the decrease in serum glucose concentration was accompanied with a decrease in serum insulin

concentration. Further investigations are necessary to elucidate the mechanism involved.

The anti-inflammatory effect of *R. officinalis* leaves water extract was investigated using *in vivo* model of chronic and acute inflammation induced by formalin and carrageenan respectively. The inhibition of inflammation was computed from the reduction in the edema produced in the hind-paw of male Sprague-Dawley rats. In the acute inflammation model, the extracts of doses 100, 250 and 500 mg/kg showed high percentages of anti-inflammatory effects (61, 61 and 54% respectively) that were comparable to that of the reference drug (73%). For the chronic inflammation, the extracts of doses 100, 250 and 500 mg/kg showed substantial inhibition of the inflammation (21, 31 and 45% respectively) and values observed were not significantly different from of the reference drug Diclofenac (40%). In acute inflammation, the 100 and 250 mg/kg doses appeared to be optimum doses that can be used. By increasing the dose beyond 250 mg/kg, the potential anti-inflammatory effect reduced drastically and became no more useful. The decrease in the anti-inflammatory activity observed in the 500mg/kg dose with respect to 250 mg/kg dose may be attributed to the presence of a compounds in the water extract that if present in high concentrations, makes a negative feedback effect on the anti-inflammatory effect of the active constituents. Further studies using dose extract between 100 and 250 mg/kg must be performed in order to locate better the appropriate dose. In chronic inflammation, the 500 mg/kg dose appeared to be the optimum dose. The difference in the optimum concentrations between the acute and chronic inflammation can be attributed to the fact that the acute and chronic inflammations have different sequence of events. A study on the anti-inflammatory effect of *R. officinalis* constituents, carnosol and ursolic acid, on TPA-induced mouse ear edema showed that both compounds have a strong anti-inflammatory activity (Huang et al 1994). Carnosol inhibits the production of nitric oxide

(NO) and inducible nitric oxide synthase (iNOS) gene expression in macrophage by preventing nuclear factor-kappa B (NF-kB) activation (Lo et al., 2002). Flavonoids are known to modify eicosanoid biosynthesis and in turn anti-inflammatory responses (Kuo et al., 1995). Thus the presence of certain flavonoids, carnosol and ursolic acid in the leaves can explain the anti-inflammatory activity of the water extract of *R. officinalis* leaves. Further studies are required in order to specify the ingredient of *R. officinalis* water extract behind the anti-inflammatory activity. Also since the water extract of *R. officinalis* leaves contains mainly rosmarinic acid (Moreno et al., 2006), a study is required to find if rosmarinic acid has an anti-inflammatory activity or not.

The anti-ulcer effect of *R. officinalis* leaves water extract was investigated using *in vivo* model of ethanol induced gastric ulcer on rats. A pre-treatment by oral administration of rosemary leaves water extract have resulted in a dose-dependent protection against ethanol induced gastric ulcer. The protection was by 27.21% (dose of 50mg/kg), 32.33% (dose of 100mg/kg), 51.34% (dose of 250mg/kg), and 70.8% (dose of 500mg/kg) compared with the positive control group that has received anti-ulcer drug Cimetril (reference drug) which showed 60% protection. The dose 500 mg/kg, the highest dose, showed a better protection than Cimetril. The protection mechanism against ulcer by the drug Cimetril is known to be mediated through blocking histamine receptors on parietal cells and in turn prevents acid secretion. But the protection mechanism of the rosemary water extract is not known. The gastro protective effect of *R. officinalis* can be explained most probably by the presence of active compounds in the water extracts including flavonoids, rosmarinic acid and terpenoids. Previous studies done on these compounds have shown that flavonoids have anti-ulcer activity by shielding and protecting the mucosa (Izzo et al., 1944) while terpenoids have prevented ulcer formation in chemically

induced ulcer in rats (De Pasquale et al., 1995). Also a study by Sereiti et al. (1999) showed that rosmarinic acid has therapeutic potential to treat peptic ulcer. Mahady et al. (2005) reported that *R. officinalis* has antimicrobial activity against *H. pylori*, the most important causing agent in gastric ulcers, which may explain the anti-ulcer effect of *R. officinalis* leaves water extract. Further studies are required in order to elucidate clearly the mechanism of action. Moreover it is important to purify the extract into its active elements and isolate the ones that are responsible for anti-ulcer activity.

The effect of *R. officinalis* leaves water extract upon bacterial inhibition was also investigated. The study was screened against 11 hospital isolates of Gram negative and Gram positive bacteria. The extract didn't show any noticeable antibacterial effect against the different bacterial strains studied. In a recent study, water extract of *R. officinalis* at concentrations 50 and 25µg/ml has shown antibacterial activity only against *Staphylococcus aureus.* The same study showed that methanolic and acetone extract of *R. officinalis* at concentrations 60-125µg/ml were active against *E. coli, K. pneumonia* and *P. mirabilis* (Moreno et al., 2006). The water extract were shown less active against bacteria than hydrocarbon extract of *R. officinalis* dry leaves and this can be attributed to the difference in the constituents between water and methanolic extract where they are more soluble in methanol. Although flavonoids, active constituents of *R. officinalis*, are synthesized by plants in response to microbial infection (Dixon et al., 1983) and terpenoids are also active against bacteria (Scortichini and Rossi, 1991; Mendoza et al., 1997), flavonoids and terpenoids are most often extracted through ethanol, chloroform or methanol extraction and not through water extraction (Hufford et al., 1993; Nakahara et al., 1993; Perrett et al., 1995 Vijaya et al., 1995).

In conclusion, after one month period of chronic consumption of *R. officinalis* water extracts in the rat model, the extract appeared not to have any significant effect upon blood lipemia. Similarly the *R. officinalis* water extract at the doses used didn't affect the absorptive property of the intestine and the integrity of the liver cells. The water extract of doses 200 mg/kg and 1000 mg/kg exhibited hypoglycemic and insulin release inhibitory effect. The hypoglycemic effect seemed to be insulin independent since the glycemic level was proportional to insulin level. The water extract of *R. officinalis* had a substantial anti-inflammatory activity in both acute and chronic types of inflammation. Also it showed a potent protective effect against gastric ulcer. The water extract didn't show any anti-bacterial activity against different hospital isolates. An over all conclusion can be drawn, that water extract of *R. officinalis* dry leaves at the doses used has no toxic signs and symptoms and it exhibits a positive effect on ulcer, inflammation and hyperglycemia. The present study demands further studies to explore the exact mechanisms and clinical applicability of *R. officinalis* as an anti-ulcerogenic, anti-inflammatory and hypoglycemic plant. Also, it is worth investigating the potentials of the water extract as mean for LDL oxidation protection since the plant is very rich in antioxidant compound. On the hand, because of the anomalous behavior of the water extract in reducing both glucose and insulin levels in the blood further studies are necessary where doses of 400 and 600 mg/kg body weight can be used in order to have a solid conclusion in this respect.

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