Effects of Medicinal Plants on Lead Induced Toxicity of Biotransformation and Redox Cycling Enzymes

Yusra Saif Khalfan Al Dhaheri

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By

Yusra Saif Khalfan Al-Dhaheri

Bachelor in Science (Biology)
College of Science, U.A.E. University (2000-2001)

A thesis Submitted to United Arab Emirates University in partial fulfilment of the requirements for the degree of M. Sc. in Environmental Science

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2006
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Dean of the Graduate Studies, Professor James E. Fletcher

United Arab Emirates University
2005/2006
FOR BEING GREAT MOTHER & FATHER

THIS WORK IS DEDICATED

TO BOTH OF YOU

WITH

MY LOVE
ACKNOWLEDGMENTS
ACKNOWLEDGMENTS

This study was carried out at the UAE University, Department of Chemistry in the Faculty of Sciences and Department of Biochemistry in the Faculty of Medicine and Health Sciences. This academic thesis is done under the ‘Environmental Sciences’ Program of the UAE Graduate studies.

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This work was conducted mainly with the support and grant of the Environmental Sciences Program Graduate studies in the UAE University. They are gratefully acknowledged.
Finally, my warmest and dearest thanks are addressed to my family for their confidence in me and for being always so supportive and interested in my work.
ABSTRACT
ABSTRACT

All organisms including humans are constantly and unavoidably exposed to foreign chemicals (xenobiotics), which include both manufactured and natural molecules such as pollutants and plant metabolites. One such alarming pollutant is lead as it is constantly produced from automobiles exhaust products and food containers. It has been documented that lead, as the major heavy metal pollutant in the air, has notable effects on nervous systems and efficiency of several enzymes.

There are some natural products such as medicinal plants that might have antagonistic effects against such pollutants. Several medicinal plants are used as traditional remedies for many ailments without knowing their detailed biochemical actions at the molecular level.

This work focused on lead toxicity that had been tested in-vitro against some enzymes that are involved in biomolecules synthesis and on enzymes involved in liver detoxication and biotransformation. Furthermore, the antagonistic (or synergestic) effects of some medicinal plants extracts was tested against lead toxicity, as an example of a commonly present heavy metal, with regards to certain metabolic and biotransformation enzymes activities. In addition, enzyme kinetic parameters (Km and Vmax) in relation to substrate binding stoichiometry and inhibition (or activation) were investigated.

In this study, three major enzymes that play important roles in the body were selected. Cytochrome P4502E1 (CYP2E1), Glutathione -S- Transfèrseres (GSTs) enzymes were chosen as representative examples of phase I and phase II of biotransformation enzymes systems respectively. Nicotinamide-adenine dinucleotide synthetase (NADS) was the third enzyme in our investigation which is involved in Nicotinamide-adenine dinucleotide (NAD+) synthesis. We have selected 4 indigenous species of plants which are commonly used as medicinal plants in the U.A.E for various ailments. These plants
are *Salvia officinalis*, *Hibiscus sabdariffa*, *Chamomilla recutita*, and *Nigella sativa*. Results of this study showed that lead solution inhibited the three sets of enzymes selected for this study *in vitro* but the extent and sensitivity of such inhibition was different among the studied enzymes systems. The study revealed that increasing the concentrations of the four selected medicinal plants have the ability to decrease the inhibitory effect of lead solution on CYP2E1 activity. GSTs activity was also affected by the medicinal plants extracts within the concentration range used. It has been shown that all the plants extracts revealed inhibitory effects on GSTs activity *in vitro*. The extent of the inhibitory effect depends on plant species and concentrations of each plant extract.

NADs activity in rat liver microsome was determined using High-performance chromatography (HPLC) and the formed NAD⁺ is separated from the substrates and the other microsome components in Retention time (Rt) of 13 minutes. It has been shown that the four plants extracts ameliorated the inhibitory effect of lead on NADs activity when low and high concentrations of each plant extract were used with lead solution. However, these plants extracts alone did not show any significant effect on NADs activity except for *Hibiscus sabdariffa* extract which caused decreased in NADs activity (11% inhibition) and such decrease in activity may be due to some active compounds in the plant that cause this decrease in activity *in vitro*.

Total phenols, flavonoids and proteins were determined and the results revealed that all the plant extracts contain phenolic compounds range from 76.4-28.6 mg GAE/ g plant extract and flavonoids content range from 7.6-28.1 mg QE/g plant extract). The four plants extracts also showed scavenging activity against DPPH free radical which may be related to the phenolic and flavonoids contents of these plants and this may-in part- contribute to the results we obtained in this study regarding lead toxicity and enzymes activities.
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% Inhibition of GST activity in rat liver cytosol caused by *Salvia officinalis* extract (1%).

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<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substances and Disease Registry</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>C_pr</td>
<td>protein concentration</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrochlorobenzene</td>
</tr>
<tr>
<td>CR</td>
<td><em>Chamomilla recutita</em></td>
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<tr>
<td>C_ST</td>
<td>standard concentration</td>
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<tr>
<td>CYP</td>
<td>cytochromes</td>
</tr>
<tr>
<td>CYP450 2E1</td>
<td>cytochrome P450 2E1</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochromes P450</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyle</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>enzyme-inhibitor</td>
</tr>
<tr>
<td>EIS</td>
<td>enzyme-inhibitor-substrate</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>f</td>
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<tr>
<td>FMO</td>
<td>flavin-containing monooxygenases</td>
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Gln: glutamine
GPx: glutathione peroxidase
GR: glutathione reductase
GSH: glutathione
GSSG: glutathione disulfide
GST's: glutathione S-transferases
hGST: human glutathione-S-transferase
HPLC: high performance liquid chromatography
HIS: *Hibiscus sabdariffa*
KD: kilo Dalton
Km: concentration of substrate at 50% of Vmax
LDL: low density lipoprotein
MFO: mixed-function oxidases reactions
mRAN: messenger RNA
MTs: methyltransferases
NAAD: nicotinic acid adenine dinucleotide
NAD: nicotinamide adenine dinucleotide
NADH: nicotinamide adenine dinucleotide, reduced
NADPH: nicotinamide adenine dinucleotide phosphate
NADPH-CYP reductase: nicotinamide adenine dinucleotide phosphate cytochrome reductase
NADS: Nicotinamide-adenine dinucleotide synthetase
NAT's: N-acetyltransferases
NDMA: N-nitrosodimethylamine
NS: *Nigella sativa*
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<td>lead-blood</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl-methylsulphonyl fluoride</td>
</tr>
<tr>
<td>QF</td>
<td>quercetine equivalent</td>
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<td>Qns1</td>
<td>glutamine dependent NAD synthetase</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>rGST</td>
<td>rat glutathione-S transferase</td>
</tr>
<tr>
<td>Rt</td>
<td>retention time</td>
</tr>
<tr>
<td>$S_4$</td>
<td>peak area of sample</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SO</td>
<td><em>Salvia officinalis</em></td>
</tr>
<tr>
<td>$S_r$</td>
<td>peak area of standard</td>
</tr>
<tr>
<td>STs</td>
<td>sulfotransfases</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyl transferases</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>ultra violet-visible</td>
</tr>
<tr>
<td>$V^\text{max}$</td>
<td>maximum velocity</td>
</tr>
<tr>
<td>$V$</td>
<td>volume of the reaction mixture</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>alpha</td>
</tr>
<tr>
<td>$\delta$-ALA</td>
<td>$\delta$-aminolevulinic acid</td>
</tr>
<tr>
<td>$\delta$-ALA-D</td>
<td>$\delta$-aminolevulinate dehydratase</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>kappa</td>
</tr>
<tr>
<td>$\mu$</td>
<td>mu</td>
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<tr>
<td>$\omega$</td>
<td>omega</td>
</tr>
<tr>
<td>$\pi$</td>
<td>pi</td>
</tr>
</tbody>
</table>

**xx**
σ  sigma
Θ  theta
ζ  zeta
CHAPTER 1

INTRODUCTION
CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Humans are exposed to various hazardous substances, which include both manufactured and natural molecules such as pollutants and plant metabolites. Lead is one of the common environmental and industrial pollutants that have been found in the environment and the biological system (Gurer and Ercal, 2000). The persistence of lead in the animals and humans is now associated with health risk (Rahman and Sultana, 2006). Lead has been found to produce wide range of biochemical and physiological dysfunctions in humans and laboratory animals (Courtois et al., 2003). The major sources of lead is from industries where lead and lead based components are used, such as lead acid battery manufacturing, leaded gasoline, cable and wire products industries, rubber and plastic industries, soldering activities, in-foundry work such as casting, forging and grinding activities. Pregnant women, infants and young children are mostly affected by lead exposure. A pregnant woman can transfer her body burden of lead to the growing fetus as there is no placental barrier for a heavy metal such as lead. Most of the environmental exposure occurs through inhalation air containing lead dust, drinking water supplied through leaded pipelines and consuming processed, preserved and stored food (Rahman and Sultana, 2006). Lead exposure has been reported to damage various cellular components including proteins, membrane lipids and nucleic acids (Hsu and Guo, 2002). It has been documented that lead, as the major heavy metal pollutant in the air, has notable effects on efficiency of several enzymes, which in turn causes the cells to be more susceptible to more deleterious effects and even death. Particularly in this study, enzymes involved in liver detoxication and biotransformation and enzymes that are involved in the
synthesis of key biomolecules such as NAD$^+$. Living organisms depend highly on vast array of enzymes to protect them from xenobiotics. Most of these xenobiotics are biotransformed into more polar metabolites that are easier to excrete (Veli'k et al., 2004). Biotransformation generally converts lipophilic pharmacologically active drug molecules and other xenobiotics into polar metabolites that are then eliminated excreted outside the body (Johnson, 2003). In mammals, the liver has long been recognized as the major site of drug/xenobiotics metabolism in the body and it has the most abundant of such enzymes (Zamek-Gliszczynski et al., 2006-in press). However, these enzymes are also present in the extrahepatic tissues (Johnson, 2003). The hepatic Cytochromes (CYP) and glutathione S-transferases (GSTs) are major enzyme systems that play critical and specific roles in the metabolism of exogenous and endogenous compounds in mammals. The CYP (phase I) enzymes are major catalysts of oxidation reaction that generate hydrophilic metabolites and also catalyze biosynthetic reactions. The GSTs (phase II), however, catalyze the conjugation of glutathione with electrophiles, some of them results of phase I oxidative metabolism followed by formation of water soluble compounds that are easily excreted (Zamek-Gliszczynski et al., 2006-in press; Veli'k et al., 2004; Ueng et al., 2002). NAD$^+$ is essential coenzyme for life in all living cells. The ratio of oxidized to reduced form (NAD$^+$/NADH) is a key regulatory control for carbohydrate and lipid metabolism. Biosynthesis of NAD$^+$ and maintenance of sufficient supply is very essential metabolic processes. Nicotinamide-adenine dinucleotide synthetase (NADS) catalyzes the final step in NAD biosynthesis in the well characterized de novo, reclaim, and import pathways (Bieganowski et al., 2004).

There are some natural products including medicinal plants and certain components of some parts of certain tree’s products that might have antagonistic effects against such pollutants. Several medicinal plants are used as traditional remedies for many ailments
without knowing their detailed biochemical actions at the molecular level. Some medicinal plants may have beneficial role in treating lead poisoning, in which can bind or remove lead from lead burdened tissues (Gurer and Ercal, 2000).

This work will focus on lead toxicity that will be tested *in-vitro* against some enzymes that are involved in biomolecules synthesis, on enzymes involved in liver detoxication and biotransformation. Furthermore, the antagonistic (or synergistic) effects of some medicinal plants extracts will be tested against lead toxicity, as an example of a commonly present heavy metal, with regards to certain metabolic and biotransformation enzymes activities.
1.2 OBJECTIVES

Specifically, the study will focus on:

1. Development of efficient and reliable assays for selected biotransformation enzymes activities & redox molecules synthesizing enzymes which can be measured quantitatively by product formation.

2. Determine the effects of lead solution, as a representative of major heavy metal pollutant in the air, on the developed enzyme activities assayed, toxicity (mainly inhibition effects) of biotransformation enzymes.

3. Determine the effects of natural products extracted from certain medicinal plants on enzyme activities.

4. Investigation of the antagonist or synergistic actions of these medicinal plants products against lead toxicity.

It is anticipated that this study will have a significant impact on better understanding of molecular mechanism of lead toxicity. This work is also expected to explore the importance of clinically valuable medicinal plants and their natural products used in U.A.E. The developed enzymatic assay will be very useful for further investigation of toxic effects of other pollutants.

1.3 APPROACH

First, enzyme assays involved in synthesis of biomolecules and in detoxification of xenobiotics will be developed and the best conditions will be optimized to measure the activity of each enzyme selected in the present study. Second, effects of aqueous extract of each medicinal plant will be assayed quantitatively on enzymatic assays developed. Third, lead toxicity will be determined and measured on the developed enzyme assays and the antagonistic action of medicinal plants on lead toxicity will be also investigated.
using cell-free system. Finally, enzyme kinetics parameters (Km and Vmax) for the inhibition effects of lead and the antagonistic or synergistic actions of medicinal plants extracts on enzymes activities will be determined.

Experimental work will be conducted in research laboratories of Chemistry Department, Faculty of Science, and the Biochemistry Department, Faculty of Medicine and Health Sciences, U.A.E University.

1.4 SCOPE OF WORK

The present investigation was conducted by selecting three major enzymes that play important roles in the body. Cytochrome P4502E1 enzyme was chosen as representative example of phase I of biotransformation enzymes systems. Glutathione-S-Transferases was chosen to be the second enzyme to be studied and it is a representative example of phase II of biotransformation enzymes systems. Nicotinamide-adenine dinucleotide synthetase (NADS) was the third enzyme in our investigation which is involved in Nicotinamide-adenine dinucleotide (NAD⁺) synthesis. We have selected 4 indigenous species of plants which are commonly used as medicinal plants in the U.A.E. for various ailments. These plants are *Salvia officinalis, Hibiscus sabdariffa Chamomilla recutita, Nigella sativa*. This study, enzyme assays for CYP2E1 and GST were developed and the best conditions were optimized to measure the activity of each enzyme selected in the present study. Next, effects of lead solution (mainly inhibition effects), as a representative of major heavy metal pollutant in the air, were determined and measured on the developed enzyme activities assayed using liver homogenate prepared in the laboratory. Rat liver microsome were used to study the effect of lead solution on CYP2E1 activity and then the antagonist or synergistic effects of the four medicinal plants aqueous extracts we selected were investigated on lead induced toxicity of CYP2E1. Also, rat liver
cytosol was used to study the effect of lead solution on GSTs activity. Furthermore, effects of the four medicinal plants aqueous extracts on cytosolic GSTs were determined. Enzyme kinetics parameters (Km and Vmax) for the inhibition effects of lead and the antagonistic or synergistic actions of medicinal plants extracts on enzymes activities were also investigated. NADS activity assay was developed and optimized using HPLC instrument to measure NADH, (the final product of the reaction catalyzed by NADS). Microsome fractions were used in NADS assays. Lead solution, four plants extracts alone, and antagonistic effects of these plant extracts were investigated on lead induced toxicity regarding NADS activity. Finally, plants contents were determined including total phenols and flavonoids contents and the capacity of these plants to scavenge DPPH free radicals were also studied.

1.5 THESIS STRUCTURE

The thesis is organized into five chapters. Chapter 1 describes a general overview of lead toxicity, enzymes of interest, and medicinal plants use and present the rational and objectives of this study. Chapter 2 reviews the literature pertaining to medicinal plants importance and use. The four medicinal plants selected for this study were explored in details in this chapter. Furthermore, Chapter 2 provide a review of the enzymes in living cells, particularly, Phase I enzymes (CYP450 2E1), phase II enzymes (GSTs) and NADS. Also, Chapter 2 explores lead toxicity. Chapter 3 represent the materials used in this study and the analytical techniques adopted for measuring enzymes activities. This chapter also highlights the details of the methods used and the way we analyze and generate data. Results of this study are presented in Chapter 4 with the figures and tables. Finally, discussion and conclusion of the obtained results are presented in Chapter 5.
CHAPTER 2

LITERATURE REVIEW
CHAPTER 2

LITERATURE REVIEW

2.1 MEDICINAL PLANTS

The vegetation on the earth is the perennial and renewable source of food and energy for the survival of living beings (Mossa and Al Yahya, 1987). Beside the aesthetic values of plants, they constitute the major natural source of food we eat, the air we breathe and considered to be the Nature's Green pharmacy, which provide drugs to maintain the good health and to cure illness (Rizk and El-Ghazaly, 1995). The relationship between man and plants has always been very close. Thus, man has experimented with many plants species. Most were innocuous, serving no purpose; a few, nourished him; some were distasteful or even toxic; occasionally, they seemed to relieve symptoms of sickness and became his "medicines" (Ayensu, 1978). From this experimentation which has been in process until complete knowledge of the properties of plants has been built up. Much of this knowledge, passed on orally from generation to generation in unlettered societies, still exists in many parts of the world (Ayensu, 1978).

From the earliest time, plants that have medicinal properties have been prized for their pain relieving and healing abilities, and people still depend on them in about 75% of our medicines (Chevallier, 1996). From the late 19th century on, medical science has been so advanced that modern medicines (drugs) include synthetic substances. Synthetic drugs are readily available for any complicated situation (Mossa et al., 1987). Thus, the use of medicinal plants neared extinction in many countries (Chevallier, 1996).

Because of dramatic revolution and advances of conventional medicine, people in many parts of the world (especially in America and Europe) became accustomed to medication that led to an almost instant short-term improvement in symptoms and the use
of medicinal plants came to be seen as outmoded and ineffective during that time (Chevallier, 1996).

Conventional medicine has brought serious infectious disease under control. However, over the years, there are worrying signs that infectious organisms are becoming resistant to synthesized drugs. Chronic illness, however, seems to be on the increase, partly because no effective conventional treatment as yet exists for many of these chronic diseases, such as asthma, arthritis, high blood pressure, and depression. Even the significant increase in the life expand in developed countries, it is starting to go into reverse. This may be as a result of environmental pollutants and toxic accumulation within the body. It is worthy to say that in the latest fifty years, humans have relied almost entirely on plants to treat all manner of illness, from minor problems to life-threatening diseases. Over the years, changes in public awareness have led to renewed interest in medicinal plants and it is coming back into prominence (Chevallier, 1996).

Herbal medicine is defined as chemically rich preparation of plant-derived material used for medicinal and health purposes (Barnes, 2002; Hodges and Kam, 2002). In some instances (e.g. use by herbalists), crude drug (e.g. dried leaf) is used. Manufactured products use extracts of plants or plant parts, formulated such as tablets, capsules, creams, tinctures. They may contain a single or multiple herbal ingredients (Barnes, 2002). It includes a wide spectrum of substances ranging from home-made teas prepared from collected herbs to medicinal products that are approved by national regulatory bodies (Hodges and Kam, 2002).

At least 122 different chemical substances derived from plants are important pharmaceutical agents in developed countries. In the pharmacopoeias of developed countries, 25% of drugs are substances first isolated from plants and further 25% are
The use of plants continues to be witnessed throughout the world, especially in developing countries where over 80% of the population depends on plants as their major health delivery system (Boulos, 1993).

Through evolution, plants have developed large numbers of chemical substances to defend themselves against insect pests and fungal and other pathogenic diseases. Some of these agents can also act within human body against microorganisms and other causes of diseases and represent an important source of natural drugs (Seters, 1997).

Plants represent a fantastic source of chemical compounds ranging in complexity from those that are simple but potentially toxic to complicated derivatives (Boulos, 1993). Each plant species is biochemically different from each other, and embraces a wide array of intraspecific biochemical variation (Duk, 1988). The ability of a medicinal plant to affect body systems depends on the chemical constituents that it contains. Scientists first
started extracting and isolating chemicals from plants in the 18th century and since that
time, we have grown accustomed to look at medicinal plants and their effects in terms of
the active constituents they contain (Chevallier, 1996).

Research into isolated plant constituents has great importance as it has given rise
to many of the world's most useful drugs. In the 1990s biomedicine still depends on
plants for at least 25% of its medicines, and many of these are among the most effective
of all conventional drugs. For example, the heart remedy digoxin (derived from Digitalis
spp.); the cough-relieving properties of ephedrine (from Ephedra sinica), and the pain-
relieving drug "aspirin" (from Spiraea ulmaria), which are all present in many
prescription remedies. These and many other conventional medicines are still derived
from plant constituents (Chevallier, 1996).

It is important to understand the actions of individual active constituents, however, herbal medicine, unlike biomedicine, is ultimately about the use and actions of
whole plants. Dividing up a medicinal plant into its constituent parts can not explain
exactly how it works in its natural form. The whole plant is worth more than the sum of
its parts and scientific research is increasingly showing that the active constituents of
many plants interact in complex ways to produce the therapeutic effect of the remedy as a
whole (Chevallier, 1996).

2.1.1 Major Plant Constituents

Plants contain hundreds, if not thousands, of different constituent chemicals that
interact in complex ways. Frequently, the details of how a particular plant works is simply
unknown even though its medicinal benefit is well established. Furthermore, although it is
very useful to know that a plant contains certain active constituents, such information can
be misleading on its own (Chevallier, 1996).
2.1.1.1 Alkaloids

Alkaloids are complex nitrogen containing compounds (-NH₂) produced by plants. They are basic (alkaline) on reaction. Most alkaloids are insoluble or slightly soluble in water, but their salts are water soluble. They are susceptible to destruction by heat, and can decompose or degrade by exposure to air or light. Many naturally derived drugs like morphine, codeine, cocaine, nicotine, reserpine, caffeine, emetine, quinine are alkaloids of plant origin (Mossa, 1987). Most of the alkaloids show pronounced pharmacological actions. Some are well known drugs and have a recognized medical use. Vincristine, for example, derived from Vinca rosea, is used to treat some types of cancer (Chevallier, 1996).

2.1.1.2 Glycosides

Glycosides are sugar-containing compounds which on hydrolysis yield one or more sugars. Glycone and aglycone are the two components contained in their molecules. The glycone is the sugar component, which can be glucose, rhamnose, xylose, arabinose or other sugars. When the glycone is glucose, the glycoside is known as a glucosid. The non-sugar component of the glycoside is aglycone which can be any type of compound such as anthraquinones, hydroquinones, saponins, aldehydes, isothiocyanates, flavonol, alcohol or phenols. They are widely present in plants and constitute major classes of drugs (Mossa, 1987).

2.1.1.3 Saponins

Saponins are glycosides generally with sterols or triterpenes as their aglycones. The aglycones portions are called sapogenins. Many saponins are hemolytic. Saponin
containing natural ingredients are sarsaparilla, alfalfa, fenugreek, licorice, etc. (Mossa, 1987).

2.1.1.4 Mucilage

Mucilage found in many plants, it is made up of polysaccharides that soak up water, producing a sticky jellylike mass. Mucilage also found in animal species including human. The mucous membranes of the digestive tract consist of mucilage, protecting against irritation, acidity and inflammation (Chevallier, 1996).

2.1.1.5 Phenols

Phenolic compounds contain aromatic ring(s) bearing hydroxyl group(s) and can range from simple molecules to very large oligomers. They frequently occur naturally in glycosylated forms, which make them more water-soluble although the higher molecular weight oligomers are more insoluble. Phenolic compounds are abundant in highly colored berry fruits, and due to their popularity and high consumption, these berries serve as one of the most important dietary sources of phenolics. Berry fruits are reported to contain a wide variety of phenolics including hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins) and hydrolysable tannins (Seeram et al., 2006 and Williner et al., 2003). Many plant phenolic compounds that have been studied exhibited antioxidant properties for protection against oxidation (Maisuthisakul et al., 2006-in press).

2.1.1.6 Tannins

They comprise a large group of complex substances that are usually localized in specific plant parts, such as leaves, fruits, barks, or stems. Tannins can be classified into
hydrolysable and non-hydrolysable or condensed tannins. Tannic acid, a mixture of digallic acid and glucose, falls into the hydrolysable gallotannin group, which is widely distributed in the plant kingdom and is commonly found in human diet. This compound occurs in wood, barks, leaves and roots of a large number of plants like beans, grapes, strawberries and persimmon. It is also found in beverages such as tea, coffee, cocoa and wine. Tannic acid was shown to inhibit the mutagenicity of polycyclic aromatic hydrocarbons in Salmonella typhimurium and Chinese hamster V79 cells as well as the tumorigenicity of polycyclic aromatic hydrocarbons and N-methyl-N-nitro-sourea in mouse skin, lung and forestomach (Krajka-Kuzniak and Bear-Dubowska, 2003, and Chen and Chung, 2000). Tannins have a phenolic structure capable of combining with proteins (Mossa, 1987).

2.1.1.7 Flavonoids

Flavonoids are a large group of naturally-occurring plant secondary metabolites. They are constituents of fruits, vegetables, nuts, plant-derived beverages such as tea and wine (Galati and O’Berien, 2004-in press). Flavonoids are phenolic compounds including flavones, flavonols, isoflavones, flavonones and chalcones. They are defined chemically as substances composed of a common phenylchromanone structure (C₆-C₃-C₆), with free hydroxy1 groups attached to aromatic rings (Maisuthisakul et al., 2006-in press). They have shown many biological properties that may account for cancer chemoprevention. In recent years, considerable attention has been paid to their abilities to inhibit the cell cycle, cell proliferation, and oxidative stress, inhibit lipid oxidation by scavenging radicals or by other mechanisms such as singlet oxygen quenching, metal chelation, lipoxygenase inhibition and induce detoxification enzymes, apoptosis, and the immune system (Birt et al. 2001).
2.1.1.8 Volatile oils

Volatile oils are extracted from plants to produce essentials oils. They are some of the most important plant constituents of all (Chevallier, 1996). They are usually complex mixtures of a wide variety of organic compounds like hydrocarbons, alcohols, ketones, phenols, acids, ethers, aldehydes, esters, oxides, sulphur compounds etc. They evaporate at room temperature when exposed to air. Volatile oils are generally isolated by distillation, and solvent extraction (Mossa, 1987).

2.1.1.9 Elemental contents

One important factor for the formation of active constituents in medicinal plants are the trace elements because they are known to play an important role in plant metabolism and active constituents of medicinal plants are metabolic products of plant cells. The data on major, minor, and trace elemental contents in plants is of vital importance to understand the pharmacological action of the plants. It is interesting to know that comparison of the elemental contents of the medicinal plants with that of usual plants shows that, medicinal plants are richer in elemental content than usual plants (Rajurkar and Dammale, 1997).

2.1.1.10 Vitamins

Some plants contain significant levels of vitamins like vitamin E and vitamin C. Most other medicinal plants contain at least some vitamins (Chevallier, 1996).

One of the most common ways of classifying medicinal plants is to identify their actions. For example, some plants are sedative antiseptic or diuretic, and the degree to which they affect different body systems. For example, a plant that is strongly antiseptic
in the digestive tract may be less so in the respiratory tract (Chevallier, 1996). Table 2.2 shows some examples of plants and their actions on the body.

Table 2.2: Examples of some plants and how they work on the body

<table>
<thead>
<tr>
<th>Body system</th>
<th>Plant category</th>
<th>Plant name</th>
<th>Plant action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Antiseptic</td>
<td>Tea tree</td>
<td>Disinfect the skin</td>
</tr>
<tr>
<td>Immune system</td>
<td>Immune stimulants</td>
<td>Echinacea (Echinacea spp.)</td>
<td>Encourage the immune system to ward off infection</td>
</tr>
<tr>
<td>Respiratory system</td>
<td>Antiseptic and antibiotics</td>
<td>Garlic (Allium sativum)</td>
<td>Help the lungs resist infection</td>
</tr>
<tr>
<td>Endocrine glands</td>
<td>Adaptogens</td>
<td>Ginseng (Panax ginseng)</td>
<td>Help the body adjust to external pressures and stress</td>
</tr>
<tr>
<td>Urinary system</td>
<td>Diuretics</td>
<td>Cornsilk (Zea mays)</td>
<td>Stimulate the flow of urine</td>
</tr>
<tr>
<td>Musculoskeletal system</td>
<td>Analgesics</td>
<td>Yellow gassmine (Gelsemium sempervirens)</td>
<td>Relieve joint and nerve pain</td>
</tr>
<tr>
<td>Nervous system</td>
<td>Relaxants</td>
<td>Lemon balm (Melissa officinalis)</td>
<td>Relax the nervous system</td>
</tr>
<tr>
<td>Circulation &amp; Heart</td>
<td>Diaphoretics</td>
<td>Juhua (Chrysanthemums marifolium)</td>
<td>Encourage blood flow to the surface of the body, promote sweating, and lower blood pressure</td>
</tr>
<tr>
<td>Digestive system</td>
<td>Choleretics</td>
<td>Artichoke (Cynara scolymus)</td>
<td>Stimulate secretion of bile by the liver</td>
</tr>
</tbody>
</table>

(Chevallier, 1996)

2.1.2 Supply and Uses

There are two sources of supply of medicinal plants; material collected from the wild and cultivated materials. The bulk of medicinal plants are wild harvested and only a very small number of species is cultivated.

Wild harvesting is the collection of plant material such as the herbs (plants above ground), flowers, leaves, wood and roots, from wild sources. In many tradition of medicine, wild harvested material is considered to have higher therapeutic benefits. The other main source of medicinal plants is from cultivation. Cultivated material is infinitely more appropriate for use in the production of drugs. Indeed, standardization whether for pure products, extracts or crude drugs is critical, and will become increasingly so, as
quality requirements continue to become more stringent (Kuipers, 1997). The medicines for internal use prepared in the traditional manner involve simple methods such as hot or cold water extraction, expression of juice after crushing, powdering of dried material, formulation of powder into pastes via such a vehicle as water, oil or honey, and even fermentation after adding a sugar source (Silva, 1997).

Medicinal plants are used as raw materials in the pharmaceutical industry. These raw materials are used to:

- Isolate pure active compounds for formulation into drugs (quinine, reserpine, digoxin, etc.).
- Isolate intermediates for the production of semi-synthetic drugs. For example, plant saponine can be extracted and altered chemically to produce sapogenins required to manufacture steroids.
- Prepare standardized galenicals (extracts, powders, tinctures, etc) (Silva, 1997).

Various studies have shown that plant principles, which produce medicinal effects usually, are also poisonous. In small quantities they may only stimulate and produce beneficial effects; but in large doses they begin to produce stronger physiological reactions that can prove to be quite lethal. For example, high blood pressure, which is a common fatal disorder, is currently controlled by the alkaloid reserpine found in *Rauwolfia serpentina*. This plant, if misused, can destroy stores of neurotransmitter noradrenaline in the brain and can lead to suicidal depression. It is therefore important that herbal medicine assumes new importance in health delivery systems in developing countries. Every effort should be made to ensure that avoidable catastrophes are totally eliminated. This can only be achieved when herbalists receive the confidence of plant scientists and medical doctors in their own societies (Boulos, 1983).
The plant kingdom consists of different categories of plants like monocots, dicots, lower plants, annuals, perennials, trees, shrubs and herbs. Under each plant genus there are different species, varieties, and even chemical races. So it is highly essential to follow certain procedures while obtaining the drug plants (Mossa, 1987).

2.1.3 Collection and Identification

Before proceeding to the field for plant collection, the collector should have some knowledge about the climatic conditions of the region to be explored and the flowering season of different plants. Roots, rhizomes, bark, leaves, flowers, fruits and seeds must be collected separately. The collector must ensure that the plant is free from fungal or virus diseases and he/she must not keep two different varieties of plants in the same container. Proper identification of the plants is highly important.

During collection, if a new plant is not properly identified in the field, it is safe to keep a code number in the field diary with correct description including height of plant, habits, color of flowers, aromatics properties, time and date of collection etc., and a small piece of the specimen with flowers should be pressed for the herbarium under the same number. Never use any plant, which is not correctly identified (Mossa, 1987).

2.1.3.1 Drying

The collected plants must be properly dried to remove sufficient moisture to insure good keeping qualities, to prevent fungal infection, action of enzymes and bacteria and other possible chemical changes. The plant materials may be dried either in the sun or by artificial heat. Air-drying may be done either in the sun or in the shade depending upon the material. Usually shade drying is followed to avoid any possible adverse action
of direct sunlight. The drying shed may be well ventilated and it must be without moisture or direct sunlight (Mossa, 1987).

2.1.3.2 Storage

To maintain the quality of the drug, proper storage is highly essential. The dried specimens must be stored in airtight containers. It is advisable to protect the drugs from light and to keep them in cool, dry places. Do not keep dried herbs for more than a year, because there is possibility for the loss of potency due to long time storage (Mossa, 1987).

2.1.4 Medicinal Plants in the UAE

The plant resource of a country is the most important gift from Allah (God). The United Arab Emirates is gifted with a wide range of flora, consisting of different species of trees, shrubs and herbs. A large number of these plants possess medicinal properties and known for their use in folk medicine (Mossa, 1987).

People in the country still use some plants as a remedy for many diseases. This herbal medicine may consist of one plant or a mixture of plants depending on the disease they want to treat. They may use the whole plant or herb as a medicine but usually they use some parts of the plants such as:

Leaves e.g. *Salvia officinalis, Ocimum basilicum* and *Camellia chinensis*.

Flowers: e.g. *Chamonilla recutita, Hibiscus sabdarffas* and *Carthamus tinctoris*.

Fruits: e.g. *Citrullus colocynthis*.

Seeds: e.g. seeds of *Nigella sativa* and *Citrullus colocynthis*.

Roots: e.g. *Glycyrrhize glabrata*.

Barks: e.g. *Cinnamomum cassia* (Mossa, 1987)
Medicinal plants can be prepared in many different ways. For example, people can use naturalized plants, their infusions, decoctions, powders, juices, etc. (Boulos, 1983).

In conclusion, herb is not a "magic bullet" with a single action, but a complex natural medicine composed of many active constituents that work on different body systems (Chevallier, 1996). Although the use of medicinal plants or their active principles in the prevention and/or treatment of chronic diseases is based on the experience of traditional systems of medicine from different ethnic societies, their use in modern medicine suffer from lack of scientific evidence (Iqbal et al., 2003). By combining scientific research into active constituents with clinical observation and traditional knowledge of the whole plant, we can develop a rounded picture of each herb's range of medicinal uses (Chevallier, 1996).

In this proposal, we have selected 4 indigenous species of plants which are commonly used as medicinal plants in the UAE for various ailments. These are listed below:

1. *Salvia officinalis*
2. *Hibiscus sabdariffa*
3. *Chamomilla recutita*
4. *Nigella sativa*
2.1.4.1 *Salvia officinalis*

*Salvia officinalis* (SO) is one of the oldest medicinal and aromatic plants. It is mentioned by all the medical writers in the ancient Rome. Its Latin name *Salvia* is derived from old Roman verb "salvare" meaning to heal and *officinalis* also means medicinal (Amr and Dordevic, 2000).

It is commonly known as sage. The popular names for this plant in Arab countries are meramia or marjamia (Dordevic et al., 2000). The sage belongs to the genus *Salvia* of the mint family Labiaceae which comprises about 900 plant species (Amr and Dordevic, 2000).

Sage is native to the Mediterranean; it is cultivated all round the world, thriving in sunny conditions. It is grown from seed in spring and the plants are replaced after 3-4 years. The leaves are picked in summer (Chevallier, 1996). The dried leaves of sage are used as raw material in medicine, perfumery and food industry (Santos-Gomes et al., 2002).

Sage has long enjoyed a reputation in folklore for treating many kinds of ailments (Lima et al., 2004; Lu and Foo, 2000 and Lu and Foo, 1999). It has been used as an antihyrotic, spasmylytic, antiseptic (essential oil effect), astringent, aromatic, carminative, estrogenic, tonic, in mental and nervous conditions, and it is also included as an ingredient in many phytopreparations, for mouth and throat gargling, for the treatment of inflammations and catarrhs, because it vitalizes the mucous tissue (tannin effect), antimicrobial (Lima et al., 2004; Amr and Dordevic, 2000; Chevallier, 1996). Today, the most widespread use of this herb is in flavoring foods (Iu and Foo, 1999). The sage has a great industrial significance as many Mediterranean countries where it grows have substantial gains from the production and export of sage. It is used in meat industry
(aromatization). Herbal tea and other preparations based on the sage are used for the body strengthening (Amr and Dordevic, 2000).

Experimental evidence already exists for a variety of bioactivities for different types of SO such as their antibacterial, hypoglycemic, antianoxic, antitumor (Lima et al., 2005; Liu et al., 2000), anti-oxidative (Lima et al., 2005; Bors et al., 2004; Lima et al., 2004; Wang et al., 2003; Santos-Gomes et al., 2002), anticholestatic (Oh et al., 2002), and anti-inflammatory (Lima et al., 2005) effects.

The key constituents of the sage are: volatile oil as the main bioactive ingredient (thujone- about 50%), phenolic acids, tannins (Chevallier, 1996). The antioxidant properties were found to be related to the presence of phenolic compounds such as carnosic, rosmarinic, caffeic and salvianolic acids as well as other phenolic structure-based compounds. More recent studies on sage have revealed the presence of large amount of diterpenoids, and flavonoids (Lima et al., 2004; Bors et al., 2004; Lu and Foo, 2001; Lu and Foo, 2000). Our interest in sage was promoted by its history of health properties which enhanced by recent studies.
Figure 2.1: (A) *Salvia officinalis* whole plant. (B) *Salvia officinalis* leaves. (C) *Salvia officinalis* dried leaves.
2.1.4.2 *Hibiscus sabdariffa*

*Hibiscus sabdariffa* (HS) is an attractive, tropical plant believed to be native to Africa. It is cultivated in Sudan and Eastern Taiwan that has long been recognized as a medicinal plant (Linn et al., 2005; Odigie et al., 2003). It is known commonly as "red sorrel" and "roselle" (Morton, 1987). Roselle belongs to the botanical family Malvaceae (Odigie et al., 2003).

The flower of *Hibiscus sabdariffa* calyx (local name Karkaday) is commonly consumed as cold and hot beverages. HS is an annual shrub with reddish colored stems and it is about 3.5 m tall. Leaves are dark green to red, alternate, glabrous, long-petiolate, palmately divided into 3-7 lobes, with serrate margins. Flowers are red to yellow with a dark center containing short-peduncles. Seedpods are enclosed in their red, fleshy calyces which are commonly used for making food and tea (Morton, 1987).

As a traditional medicine, it is claimed to be effective against kidney stones and urinary bladder stones, pyrexia, liver disorders. It is also used as antibacterial, antifungal, hypcholesterolemic, antispasmodic, and antihypertensive actions (Hirunpanich et al., 2005, Liu et al., 2002 and Wang et al., 2000).

It contains many chemical constituents including alkaloids, L-ascorbic acid, anisaldehyde, anthocyanin, β-carotene, β-sitosterol, citric acid, cyaniding-3-rutinoside, delphinidin, galactose, gossypetin, hibiscetin, mucopolysaccharide, pectine, protocatechuic acid, polysaccharide, quercetin, stearic acid and wax (Hirunpanich et al., 2005). *Hibiscus* anthocyanins (natural polyphenolic pigments extracted from *Hibiscus* dried flowers) had been demonstrated previously to possess antioxidative and antitumor promoting effects (Linn et al., 2005; Wang et al., 2000). It was shown in research that *Hibiscus* extract prevents the oxidation of low-density lipoproteins (LDL), or 'bad' cholesterol (Hirunpanich et al., 2005).
In human and laboratory animals, administration of *Hibiscus sabdariffa* extract caused a reduction in blood pressure; therefore, aqueous extract of *Hibiscus* significantly reduced blood pressure in essential hypertension in human (Chang et al., 2004; Odigie et al., 2003). Recently it has gained an important position in the local soft drink markets. However, its biological and pharmacological effects are still poorly defined (Wang et al., 2000).
Figure 2.2: (A) *Hibiscus sabdariffa* whole plant. (B) *Hibiscus sabdariffa* flowers. (C) *Hibiscus sabdariffa* dried flowers.
2.1.4.3 Chamomilla recutita

Chamomilla recutita (CR), commonly named German chamomile, is a daisy-like, apple-scented flower that has been used medicinally for thousands of years (O’Hara et al., 1998). It belongs to the botanical family of Asteraceae (Szöka et al., 2004). Chamomile is an annual herb, growing to 60 cm with finely cut leaves and white flower heads. It is originally from Europe, this plant has escaped to the wild and now naturalized on almost every continent (Macchioni et al., 2004).

It has been used for centuries as medicinal plants, mostly for gastrointestinal illnesses. It is also used to reduce anxiety and insomnia, to expel worms, for dropsy, jaundice; it is used externally to stimulate wound healing and to treat inflammation (Shinomiya et al., 2005; Macchioni et al., 2004; Scalia et al., 1999 and O’Hara et al., 1998).

The flower heads is strongly aromatic and has a bitter taste. The infusion is one of the most popular herbal teas and has been traditionally used as carminative, and tonic. The infusion can also be administered as a compress for skin and mucous membrane inflammation and bacterial skin disease (Kobayashi et al., 2005). The dried flower heads of chamomile have been reported to exhibit spasmolytic and sedative properties, although the active components responsible for the sedative activity have not yet been fully characterized (Avallone et al., 2000). There have been some reports on the hypnotic effects of this herb in humans (Shinomiya et al., 2005).

The most common components of chamomile reported in literature are a-bisabolol and chamazulene in the essential oils (Macchioni et al., 2004). The essential oils are of greatest importance among all effective substances (Szöka et al., 2004) but flavonoids (such as apigenin, luteolin, rutin, spiroethers), coumarins, tannins, and bitter glycosides are the main representative components in polar and medium polar extracts. Some studies
have shown that flavonoids together with some components of the essential oil of chamomile are responsible for the antibacterial activity of this plant (Macchioni et al., 2004; Chevallier, 1996). The chamomile owes its therapeutically activity to the above mentioned groups of substances that make up the complex effect of the plant (Szöka et al., 2004).
Figure 2.3: (A) Chamomilla recutita whole plant. (B) Chamomilla recutita dried flowers.
2.1.4.4 *Nigella sativa*

*Nigella sativa* (NS) is a medicinal plant that contains black seeds (Meral et al., 2004). It is commonly known as black cumin or black seed (Kanter et al., 2003). Coequal names of its seeds in Arab countries are Al-Habbah Al-Sawda, Habbet el-baraka, Kamoun Aswad (El-Dakhakhany, 2000).

NS is tall annual herb growing to 30 cm. It has an upright branching stem, fine deeply cut leaves, gray-blue flowers, and toothed seed pods (Chevallier, 1996). It belongs to the botanical family of Ranunculaceae and cultivated predominantly throughout much of Asia and the Mediterranean region for its seeds and as a garden plant (Kanter et al., 2003; Chevallier, 1996).

Seeds of NS are frequently used in folk medicine as natural remedy for variety of illnesses (Meral et al., 2004), particularly black cumin seeds were taken to treat headaches, nasal congestion, toothache, and intestinal worms, and in large quantities, as a diuretic, a promoter of menstruation, and to increase breast-milk production (Chevallier, 1996; Mossa et al., 1987).

NS contains > 30 of a fixed oil, and 0.40-0.45 % w/w of a volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone and a total of 46% of many monoterpenes such as p-cymene, and x-pinene (El-Tahir et al., 1993a). It has been shown to have bronchodilator (El-Tahir et al., 1993b), immunopotentiating activities (El-Kadi and Kandil, 1987), antibacterial (Hanafy and Hatem, 1991), hypotensive (Zaoui et al., 2000), antifungal, antelmentic and antiastmatic (El-Tahir et al., 1993a), cholericetic, and antitumoral (Galomi et al., 1992).

The multiple use of *Nigella sativa* in the folk medicine encouraged many investigators to isolate the possible active components and to conduct *in vivo* and *in vitro*
studies on laboratory animals and human beings in order to understand its pharmacological actions (El-Dakhakhany et al., 2000).
Figure 2.4: (A) *Nigella sativa* whole plant. (B) *Nigella sativa* dried black seeds.
2.2 ENZYMES IN LIVING CELLS

One of the most important functions of proteins is their role as catalysts (Mckee, T. and Mckee, J., 2003). Enzymes are extraordinarily efficient and selective biological catalysts that speed up the chemical reactions in the biological systems (Horton et al., 2002). Living processes consists almost entirely of biochemical reactions in which they would not occur fast enough at the pH and temperature of the body in the absence of enzymes (York, 2001). Enzymes are globular proteins and posses the functional characteristics of proteins in which they bind one or more substrate molecules. The substrate molecules are bound in a cleft known as the active site.

Enzymes have many remarkable properties. First, the rates of enzymatically catalyzed reactions increase by factor $10^6$ or greater. Second, enzymes are highly specific for the substrates (reactants), they act upon. Wasteful byproducts are not often formed; this is known as reaction specificity. Third, enzymes are efficient in which they save energy for living cells and prevent the buildup of potentially toxic metabolic byproducts. Finally, enzymes whose activity is regulated (regulatory enzymes) generally have complex structure than unregulated enzymes. Most regulatory enzymes have two sits, one for the substrate and the other for the modulator (regulator).

Enzymes are compounds metabolizing in the living cells. Both membrane-bound and soluble enzymes are involved in maintaining metabolite homeostasis. In addition to metabolizing nutrients, enzymes are also involved in the metabolism of foreign compounds (xenobiotics) such as drugs, chemicals, and other environmental and dietary contaminants.
2.2.1 Biotransformation

Living organisms depend highly on vast array of enzymes to protect them from xenobiotics (foreign molecules to the body). In the modern world, organisms have been exposed to hundreds of thousands of new compounds. These compounds become new threats for all living organisms since the beginning of the Industrial Revolution (Mckee, T. and Mckee, J., 2003). As any compound enter the body, it maybe harmful because the high concentration of any substance in any organism will lead to damage. Substances can enter the body either with the diet or inspired air and may (depending on their physiochemical properties) accumulate in the body. Particularly, substances which are easily soluble in fat (lipophilic compounds) tend to accumulate in the body (Bloauboer, 1996). In the organism, most of these xenobiatics are biotransformed into more polar metabolites that are easier to excrete (Veli'k et al., 2004). Biotransformation generally converts lipophilic pharmacologically active drug molecules and other xenobiotics into polar metabolites that are then eliminated by the kidneys or other organs (Johnson, 2003).

Biotransformation process can be defined as" a series of enzyme-catalyzed reactions in which toxic substances are converted into less toxic metabolites". Biotransformation reactions occur in several locations within the cell (e.g., the cytoplasm and mitochondria), but mostly occur within the Endoplasmic reticulum (ER). Also, biotransforming potential differ between different cell types. Generally, cells located near the major points of xenobiotic entry into the body have more biotransforming potential than other cell types (Mckee, T. and Mckee, J., 2003).

In mammals, the liver has long been recognized as the primary site of drug metabolism in the body and it has the most abundant drug-metabolizing enzymes (Zamek-Gliszczynski et al., 2006-in press). However, these enzymes are also present in the extrahepatic tissues including kidney, intestine, and skin. Tissues specific expression
of these enzymes plays an important role in the detoxication and bioactivation of xenobiotics (Johnson, 2003; Ueng et al., 2002).

Biotransformation can be considered to occur in three phases. Oxidations, reductions, or hydrolyses of the substrate represent phase I metabolism. Among the most important enzyme systems involved in phase I reactions are cytochromes P450 (CYP) and flavin-containing monooxygenases (FMO) (Zamek-Gliszczynski et al., 2006-in press; Jokanovic', 2001). In phase II, drugs (or xenobiotics) or their metabolites can undergo conjugation reactions with endogenous compounds. UDP-glucuronosyl transferases (UGT) and glutathione-S-transferases represent the main conjugation enzymes. Transport of substrates, metabolites or conjugates through membranes mediated by special protein transporters is currently considered to be the third phase of biotransformation (Vel'k et al., 2004; Ueng et al., 2002).

In phase I reactions, a polar group such as hydroxyl (-OH), carboxyl (-COOH), thiol (-SH) and amino (-NH₂), is introduced into the molecule through the reactions of oxidation, reductions, and hydrolysis. The alteration of the molecule may also lead to the unmasking of such groups. Metabolites formed can be more toxic than parent compounds, but some other nontoxic metabolites can be formed as well (Jokanovic', 2001). Phase I metabolism usually does not result in a large change in molecular weight or water solubility of the substrate, but it is of great importance because oxidative reactions add or expose sites where phase II metabolism can subsequently occur (Zamek-Gliszczynski et al., 2006-in press; Lyer and Sinz, 1999). In contrast, phase II conjugation typically results in an appreciable increase in molecular weight and water solubility.

Microsomal CYP-catalyzed oxidations require nicotinamide adenine dinucleotide phosphate cytochrome (NADPH-CYP) reductase for electron transferring from NADPH to the hemoprotein CYP. CYP enzymes comprise a family of isoforms with diverse
functions. For some CYPs such as CYP2E1, cytochrome bs is essential for optimal catalytic activity (Ueng et al., 2002). Inhibition, induction, and polymorphisms of cytochrome P450 enzymes are an interest area of research, because this class of metabolic enzymes is responsible for the biotransformation of the majority of drugs, thus their modulation has important therapeutic and toxic implications (Evans and Johnson, 2001). Most compounds undergo phase I oxidation prior to phase II conjugation (e.g. phenobarbital), but molecules with sites amenable to conjugation may undergo phase II reactions directly (e.g. acetaminophen). Molecules that undergo direct phase II conjugation may also undergo competing (e.g. acetaminophen) or additional (e.g. troglitazone) phase I oxidation (Zamek-Gliszczynski et al., 2006- in press).

In phase II reactions, polar metabolites are conjugated with endogenous substrates such as glucuronides, sulfates, glutathione, acetates and amino acids, which form hydrosoluble products that can be readily excreted in urine (Jokanović, 2001). Phase II enzymes are generally transferases and are responsible for conjugating a xenobiotic or its metabolite, thus, resulting in a further enhancement of polarity (Iyer and Sinz, 1999).

Phase II reactions involve glucuronosyltransferases (UGTs), sulfotransferases (STs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs), and O-, S- and N-methyltransferases (MTs) (Jokanović, 2001; Iyer and Sinz, 1999). These enzymes with sufficient levels of cellular reduced glutathione (GSH) play a critical role in the detoxification of toxic and carcinogenic environmental chemicals by catalyzing their conjugation or reduction. The phase II reaction is the true detoxification pathway resulting in products that are generally water soluble and easily excreted (Hughes and Gallagher, 2004).

Unbound compounds in sinusoidal blood are taken up into hepatocytes typically by diffusion across the basolateral membrane, depending on molecular lipophilicity.
charge, size, and three-dimensional structure. Substrates of phase II metabolism are typically lipophilic (e.g., acetaminophen, 4-methylumbelliferone, dehydroepiandrosterone) and access the intracellular space by diffusion (Mayer, 1995). In contrast, phase II conjugates formed inside the hepatocyte are typically too hydrophilic to passively diffuse across the canaliculal membrane into bile or across the hepatic basolateral membrane into sinusoidal blood, and necessitate carrier-mediated transport to cross this diffusional barrier. Distinct transport proteins are present in the apical (canaliculal) and basolateral (sinusoidal) domains of the hepatocyte’s plasma membrane, where they efficiently pump substrates out of the cell (Zamek-Gliszczynski et al., 2006-in press).

Generally, the parent substance and the metabolite or conjugate can differ in both physico-chemical properties and pharmacodynamic effect, as well as in toxicity and pharmacokinetics behavior. The occurrence and activity of biotransformation enzymes determines the way and extent in which the chemical drug or other xenobiotic is metabolized. Therefore, the biotransformation enzymes fundamentally influence the biological system (both desirable and undesirable) of a drug administered (Veli’k et al., 2004). Thus, detoxification is governed by biotransformation enzymes that facilitate the efficient excretion of harmful compounds (Hughes and Gallagher, 2004).

2.2.1.1 Phase I enzymes

Cytochrome P450 (CYP) enzyme system is a major catalyst of oxidation reactions of many endogenous and exogenous chemicals including therapeutic drugs and carcinogens (Gyamfi et al., 2004; Wang et al., 2002). The cytochrome P450 isoenzymes are a super family of haemoproteins which contains ferroprotoporphyrin IX group of heme and a polypeptide chain (apoprotein) having molecular weight in the range between 45 and 55 KDa. Iron from prosthetic group of heme is placed in the center of
protoporphyrin ring and its four coordinative bonds are bound to porphyrin skeleton. The fifth ligand is thiolate anion from cysteine in apoprotein, and the sixth ligand is probably hydroxyl group from an amino acid or water. They are the terminal oxidases of the mixed function oxidases system (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1) (Jokanović, 2001; Chang and Kam, 1999). P450s are located predominantly in the smooth endoplasmic reticulum of the mammalian cells and are entirely distinct from the cytochrome proteins that comprise the mitochondrial electron-transport function (Piao et al., 2003). All organisms examined so far, microorganisms, such as bacteria, as well as higher organisms, have been shown to possess one or more isoenzymes of cytochrome P450 (Bloauboer, 1996).

The abbreviation "cytochrome P450" (P denotes pigment) describes hemoprotein in which complex of iron and carbon monoxide shows a spectrophotometric absorption maximum at or near 450 nm and which functions as electron transporter. In humans, the cytochrome enzymes are involved in the metabolism of exogenous substances (drugs, alcohols, organic solvents, anaesthetic agents, dyes, environmental pollutants and chemicals) producing metabolites which may be toxic or carcinogenic. They are also important in the oxidative, peroxidative and reductive metabolism of endogenous physiological compounds such as steroids, fatty acids, liposoluble vitamins, prostaglandins, leukotriens and thromboxanes (Chang and Kam, 1999).

A. CYP 450 classification

Researchers initially assigned names to the isoenzymes according to their spectral properties, electrophoretic mobility or their substrates. As a result of rapid advances in knowledge of the amino acid sequences, currently, more than 700 P450s have been
characterized and standard nomenclature has been adopted that categorizes the individual P450s into respective families and subfamilies based on the presence of common amino acid sequence in which the prefix CYP is used to donate both human and rat cytochrome P450 isoenzymes (Nebert and McKinnon, 1994).

This system, CYP, is followed by an Arabic numeral denoting the family (e.g. CYP2) in which cytochrome P450 proteins from all sources having more than 40% identity in amino acids is placed in the same family. A subfamily consists of enzymes in which the amino acid sequence is more than 55% identical and this is designated by a capital letter (e.g. CYP2C) and then another Arabic numeral after the letter represent the individual gene or enzyme (e.g. CYP2C9). The individual enzyme is denoted by a numeral, as in CYP2C9, and the gene associated with the enzyme is denoted in italics such as CYP2C9. Each family of cytochrome P450 has several subfamilies, for example, the CYP2 family has several subfamilies such as CYP2C, CYP2D and CYP2E (Johnson, 2003; Piao et al., 2003). The important human isoforms of the enzyme include CYPs 1A1 and 2, 2B6, 2C8-10, 2C19, 2D6, 2E1 and 3A4 and 5. Genetic polymorphisms exist for a number of CYPs including 2D6, 2C9, 2C19 and 3A5. The CYP3A subfamily are the most abundant enzymes in human liver and small intestine and are involved in the metabolism of around 50% of drugs (Johnson, 2003).

The advantage of this nomenclature is that structurally identical or highly similar cytochrome P450s are easily identified regardless of their sources or their catalytic activities. Of the 74 gene families described, 14 families have been reported in humans and 20 subfamilies have been mapped in the human genome (Chang and Kam, 1999). Table 2.3 shows major cytochrome P450 forms found in human.
Table 2.3: Major Human Cytochrome P450 Forms.

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(Okita and Masters, 2002)

Most carcinogens and xenobiotics which are primarily metabolized by microsomal cytochrome P450 system is localized in the liver more abundantly than other organs (Sheweita et al., 2002). Chang and Kam (1999) reported that extra hepatic cytochrome P450 has been identified in a wide range of tissues which include the small intestine, pancreas, brain, lung, adrenal gland, kidney, bone marrow, mast cells, skin, ovary and testis. CYP1, 2 and 3 families account for most drug metabolism. Beside on their expression in the liver, it appears that CYP3A accounts for about 30% of the total hepatic P450; CYP2, about 20%; CYP1A2, 13%; CYP2E1, 7%; CYP2A6, 4%; and CYP2D6, 2% (Chang and Kam, 1999).

CYP-dependent monooxygenase activity requires an electron transporting enzyme. NADPH-CYP reductase and phospholipids (Gyamfi et al., 2004a). In the mixed-function oxidases reactions (MFO), cytochrome P450 is the site where both the
substrate and the oxygen bind. The most characteristic feature of the reaction cycle catalyzed by cytochrome P450 is the ability of the heme iron to undergo cyclic oxidation-reduction reactions in relation to substrate binding and oxygen activity (Bloauboer, 1996).

Multiple forms of CYP exist and the composition of these isozymes in tissues as well as relative concentrations is influenced by xenobiotics including natural products (Gyamfi et al., 2004a). The typical reaction catalysed by cytochrome P-450, a monoxygenation, can be summarized as following: First, a substrate (RH) such as a steroid, fatty acid or compound with an alkene, alkane, aromatic ring or heterocyclic ring substituent that serves as a site for oxygenation binds to the oxidized (Fe³⁺) form of cytochrome P450. Second, an electron (donated by NADPH) can be transferred to the resulting enzyme-substrate complex via a flavoprotein called NADPH cytochrome P-450 reductase. Third, molecular oxygen is then incorporated into the reduced (Fe²⁺) enzyme substrate complex. Finally, the substrate-complex then accepts a second electron, donated by NADH and transferred via a second flavoprotein or NADH cytochrome b₅ reductase. The second electron may, however, also be donated via NADPH cytochrome P-450 reductase. Thus, the system can also function using NADPH alone (Chang and Kam, 1999; Bloauboer, 1996). The typical reaction catalysed by cytochrome P450 is shown in Figure 2.5.
Figure 2.5: General scheme for P450-catalyzed oxidation reactions. RH, substrate; ROH, product. The reversibility of some of the latter steps is unknown. Outlets for the uncoupled reduced oxygen products $O_2^-$, $H_2O_2$, and $H_2O$ are shown (Bell and Guengerich, 1999).
Drug metabolising cytochromes P450s have distinct but often overlapping substrate specificity. Another characteristic of cytochrome P450s is the large intra- and interspecies variability in catalytic activity and in regulation. More than one cytochrome P-450 isoenzyme can be involved in the metabolism of a drug (Chang and Kam, 1999).

Since CYPs are involved in metabolism of drugs and in the process of chemical carcinogenesis, potential consequences in the interaction of xenobiotics with CYPs may include reduced therapeutic efficacy, enhanced systemic toxicity or chemoprevention. Inhibition of drug metabolizing enzymes especially CYPs is implicated in the chemoprotective action of natural products such as resveratrol (Gyamfi et al., 2004a).

Change and Kam (1999) believe that changes in the activity of the cytochrome P450s can result from genetic polymorphism, enzyme inhibition, enzyme induction and physiological factors. They also mentioned that these changes would have several clinical implications as they may result in changes in the pharmacokinetics of the drugs leading to altered efficacy of the drugs, increased toxicity due to reduced metabolism or increased production of toxic metabolites, and drug interactions.

A.1 CYP 2 family

This is a large family and includes 2A, 2B, 2C, 2D and 2E subfamilies. The CYP2E subfamily contains only one gene and is toxicologically and clinically important enzyme. It is constitutively expressed in the liver and many other tissues (Tanaka et al., 2000; Bell and Guengerich, 1997).

A.1.1 CYP450 2E1

The CYP2E1 metabolizes many substrates (e.g. dimethylnitrosamine N-demethylase). It is the classical ethanol-inducible CYP (Wang et al., 2002) and highly
significant as it is responsible for the metabolism of a wide variety of chemicals with different structures, in particular small (molecular weight < 100) and hydrophobic compounds, including potentials cytotoxic and carcinogenic agents (Tanaka et al., 2000; Bell and Guengerich, 1997). These compounds include many volatile anaesthetic agents (such as sevoflurane, enfurane, isoflurane, methoxyflurane, diethyl ether, trichloroethylene and chloroform) (Tanaka et al., 2000), and ethanol. Also it has been shown to catalyse the bioactivation of several procarcinogens and protoxins including N-nitrosodimethylamine, benzene and N-alkylformamides (Piao et al., 2003; Wang et al., 2002).

Hepatic cytochrome CYP2E1 is one of the best conserved xenobiotic-metabolizing P450s because rodent and human CYP2E1 enzymes catalyze similar reactions, the rat and mouse are good models for screening for substrates of this enzyme. Over 70 substrates have been shown to be metabolized by this enzyme (Tanaka et al., 2000).

The enzyme can be induced by acetone, isoniazid, ethanol and other compounds which are substrates for the enzyme (Tanaka et al., 2000; Chang and Kam, 1999) and it is inhibited by disulfiram. Chronic ethanol consumption increases the rate of metabolism of pentobarbital, tolbutamide, propanolol and rifampicin as a result of induction of CYP2E1. In contrast, acute ethanol consumption may competitively inhibit 2E1 and decrease the metabolism of benzodiazepines, phenothiazines, barbiturates, morphine and warfarin (Chang and Kam, 1999). In addition, the activity of CYP2E1 is affected by some pathophysiological conditions such as diabetes, starvation, and obesity (Tanaka et al., 2000). Woodcroft and Novak (1999) reported that pathophysiological alterations such as diabetes, fasting, and high-fat diet increase cytochrome CYP2E1 expression by about 3 to 8 fold at both the mRNA and protein levels.
2.2.1.2 Phase II enzymes

These enzymes conjugate contaminants to endogenous polar compounds forming a more excretable product (Hughes and Gallagher, 2004).

Glutathione (γ-glutamyl-cysteinyl-glycine) is a water-soluble tripeptide, thiol, and low molecular weight compound that present at high concentrations (generally in the millimolar range) in most mammalian cells mainly in the form of reduced glutathione (GSH) with minor fractions being glutathione disulfide (GSSG) (Townsen et al., 2003; Mitty et al., 2003). GSH acts both as a nucleophilic ‘scavenger’ of numerous compounds and their metabolites converting electrophilic centers to thioether bonds, and as a substrate in the GSH peroxidase-mediated destruction of hydroperoxides. Conjugation with intracellular glutathione results in the detoxification of electrophilic compounds, which could otherwise covalently bind intracellular macromolecules. A broad spectrum of diverse electrophiles can undergo glutathione conjugation. Molecular moieties amenable to glutathione conjugation include electrophilic carbon atoms as well as electrophilic nitrogen, oxygen, and sulfur atoms. Substrates of glutathione conjugation can be parent compound electrophiles, as well as electrophilic phase I metabolites, and even certain phase II conjugates (Parkinson, 2001). GSH is synthesized in vivo in the liver from γ-glutamyl-cysteine and glycine via GSH synthetase in an adenosine triphosphate (ATP)-dependent (Jokanović, 2001). High cellular GSH: GSSG ratios, which are maintained by the action of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase (GR), make an important contribution to the redox state of the cell. Hence, maintaining optimal GSH: GSSG ratios in the cell are critical to survival. As an important antioxidant, GSH protects against a range of peroxides, xenobiotics and heavy metals via catalysis by glutathione -S-transferases (GSTs) and glutathione peroxidases (GPx) (Townsen et al., 2003). A deficiency of GSH
puts the cell at risk for oxidative damage. GSH can be depleted directly by conjugation with electrophiles and indirectly by the addition of inhibitors of GSH synthesis and regeneration. GSH depletion to about 20-30% of total glutathione levels can impair cellular defense capacity against the toxic action of such compounds and may lead to cell damage and death (Mittova et al., 2003; Jokanović, 2001).

Environmental factors alter both the size and the redox state of the glutathione pool. For example, salt stress (in plants), heavy metals, and pathogens all lead to GSH accumulation (Mittova et al., 2003 and Townsen et al., 2003).

A. Glutathione-S-transferases (GSTs, EC 2.5.1.18)

The glutathione S-transferases (GSTs) are a polymorphic super family of multifunctional enzymes that known to catalyze the conjugation of sulphydryl group of reduced glutathione (GSH) with a wide variety of endogenous and exogenous electrophilic compounds (Moon et al., 2006; Seo et al., 2000). GSTs are an important part of the cellular detoxification system and, perhaps, evolved to protect cells against reactive oxygen species (ROS), have a main function in the binding and transport of a wide variety of harmful compounds, and they have a considerable important role in the detoxification of carcinogens (Moon et al., 2006). These proteins are found in all eukaryotic and prokaryotic systems and present in both membrane and cytosolic fractions and they are classified by their cellular localization. Cytosolic (soluble) GSTs are responsible predominantly for conjugation of xenobiotics with glutathione. Mammalian GSTs are encoded by at least nine different gene families, eight of them encode the cytosolic GSTs and the ninth encodes a membrane-bound form of the enzymes. Approximately 20 cytosolic GSTs have been identified in vertebrates, and have been categorized into at least eight distinct classes thus far: alpha (α), mu (μ), pi (π), sigma (σ),
theta (θ), kappa (κ), omega (ω), and zeta (ζ). This classification is in accordance with the
substrate specificity, chemical affinity, structure, amino acid sequence and kinetic
behavior of the enzyme (Zamek-Gliszczynski et al., 2006-in press; G Yamfi et al., 2004b;
Landi, 2000). The soluble GSTs exist as dimeric proteins of approximately 25 KDa, and
the sequences and the known three-dimensional (3D) structures suggest that these
proteins share a common ancestry, though the precise details of their evolution remain
obscure. They are expressed at high levels in mammalian liver constituting up to 4% of
the total soluble proteins (Townsend et al., 2003; Habdous et al., 2002). The membrane
bound isoforms include the mitochondria and the microsomal GSTs; some membrane-
bound GST isoforms have been implicated in the metabolism and biotransformation of
drugs in mammals (Zhang et al., 2005, 2004) and they play a key role in endogenous
metabolism of leukotrienes and prostaglandins. Cytosolic GSTs have very little structural
similarity to their membrane-bound counterparts, and exist as hetero- (only alpha and mu)
or homo-dimers in the cytoplasm. Dimerization is necessary for GSTs to carry out their
function in catalyzing glutathione conjugation as well as some reduction reactions
(Parkinson, 2001)

A.1 The GSTs nomenclature

Several different nomenclatures have been adopted during the years as new GSTs
were discovered. The nomenclature is based on primary structure similarities and the
division of GSTs into families of more closely related sequences. Briefly, the more recent
and commonly used classification for the mammalian subunit GSTs designated by the
names of the Greek letters: Alpha, Mu, Pi, etc., abbreviated in Roman capitals: A, M, P, T
and so on, denoting the family (e.g. T for theta.). A lower case letter preceding 'GST' is
used to name the species (e.g. for human theta subunits written as hGST, and for rat GST
subunits written as \( \text{rGST} \). An Arabic number denotes the subfamily such as the human theta subunits are named "\( \text{hGST}_{\text{TT1}} \)". When enzymes, instead of the subunit, are named, they are named with the repeated number of their subunits (e.g. \( \text{hGST}_{\text{TT1-1}, \text{rGST}_{\text{TT1-1}}} \)) according to the homodimeric structures. Finally, the gene coding for each subunit adopt the names of the respective subunit in italic (e.g. \( \text{hGST}_{\text{TT1}} \) for \( \text{hGST}_{\text{TT1}} \)) (Mannervik et al., 2005; Landi, 2000).

Multiple isoenzymes have been identified in the hepatic tissue of many mammals, including human. In the liver, alpha-GST is located in the hepatocytes whereas pi-GST is restricted to the intrahepatic bile duct cells. GSTs comprise 1-4% of the soluble cytosolic protein in human tissues and up to 10% of the total cytosolic protein in other mammalian tissues. More than 5% of soluble protein from human liver consists of alpha- and pi-GST. In human liver, the alpha-class of GST constitutes more than 80% of the total GST activity while the pi-class of GST is the major isoenzyme in mouse liver and in the extrahepatic tissues of other mammals (Hayes et al., 2005; Raza et al., 1997; Hayes and Pulford, 1995). The expression of certain GST isoenzymes has been proposed to be tissue-specific which may enable individual tissues to meet their specific detoxification needs (Gadagbui and James, 2000). Regulation of GSTs expression both by induction and inhibition is a crucial factor in determining the sensitivity or resistance of cells to a wide variety of foreign chemicals, carcinogen, drugs and metabolites under normal and pathophysiological conditions. Many of GSTs inducers and inhibitors occur naturally in vegetables and fruits (Hayes and Pulford, 1995). Biological mechanisms responsible for controlling the selective expression of GSTs are complex due to the multiplicity of these enzymes and their tissue specific and subcellular distribution. In addition, GSTs also exhibit differential response to a wide variety of chemicals and oxidative stress in the normal and pathophysiological conditions. It appears that GSTs are also regulated both in
*vivo* and *in vitro* by ROS such as superoxides, \( \text{H}_2\text{O}_2 \) and by the products of membrane lipid peroxidation. The controlled expression of the different GST isoenzymes has presumably evolved as an adaptive response to chemical stress within cells. It has also reported that there is a difference in the subcellular distribution and targeted translocation of GST isoenzymes in the different compartment of the cells by chemical and oxidative stress (Raza et al., 2004).

The GSTs are also known to be involved in the bioactivation of dichloromethane, haloalkenes, vicinal dihaloalkenes and haloacids to toxic metabolites and organ damage (Gyamfi et al., 2004b). GSTs are readily released in the event of hepatocellular injury making them the only sensitive and specific biomarkers of acute hepatic injury. Among them, pi-and alpha-GSTs have attracted most of the interest mainly because they are highly expressed in different pathologies such as cancer, liver and kidney diseases (Habdous et al., 2002). These enzymes are potentially essential in regulating susceptibility to cancer as they are able to metabolize reactive metabolites of carcinogens. They are present in relatively large amounts in the epithelial tissues of human gastrointestinal tract. A significant negative correlation was demonstrated between GST enzymes activity and tumor incidence in the mucosa along the human gastrointestinal tract, suggesting the importance of GSTs in cancer prevention (Moon et al., 2006).

GSTs can be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis including, phenobarbital, planar aromatic compounds, ethoxyquin, and trans-stilbene oxide. Some of the compounds known to induce GSTs are themselves substrates of the enzymes, suggesting that induction is an adaptive response mechanism. Many clinical useful drugs are also potential substrates for GSTs and development of drug resistance can frequently be a key element in treatment failure (Townsend et al., 2003).
CYPs and GSTs are responsive to the inductive and inhibitory effects of many exogenous factors including herbal medicines (Ueng et al., 2002). Frequent administration of pharmacologically active substances of naturally occurring dietary or herbal components can have serious consequences for the induction or inhibition of biotransformation enzymes. Induction causes an increase in activity of these enzymes in response to the presence of a xenobiotic substance. This increase of activity is mainly a result of an increase in respective gene expression and/or lowering of protein degradation. Inhibition, (e.g. decrease the activity of biotransformation enzymes due to a xenobiotic), can be caused by interaction of the xenobiotic, both with protein and at all stages of genetic information transfer. In the case of drug/herbal interactions, many drugs along with herb components are capable of interacting with biotransformation enzymes and such modulation of these enzymes activity changes both the intensity and half time of simultaneously or successively administered drugs. This can result in increase of plasma levels of the effective substance with associated risk of toxic action or, alternatively, in lowering of plasma concentration of effective substance below the limit of therapeutic effect. The modulation of biotransformation enzymes activity also increases the risk of undesirable side effects as well as increasing sensitivity to environmental contaminants and the risk of pathogenesis (Pelkonen et al.; 1998; Nebbia, 2001; Velik et al. 2004; Pal and Mitra, 2005, in press).
2.2.3 Redox Molecules Synthesizing Enzymes

Under the normal physiological conditions, biotransformation is highly dependent on cellular oxidative stress and redox molecules. Both CYPs and GSTs are known to require substrates and co-enzymes involved in redox cycling in cells. The cellular levels of oxygen, glutathione and Nicotinamide-adenine dinucleotide, phosphorylated, reduced forms (NAD(P)H) are playing key roles in providing appropriate environment for biotransformation and CYP/GST activities. NAD(P)H is an absolute requirement for CYP activity. NAD(P)H also maintains homeostasis of GSH (glutathione) by regenerating reduced GSH from oxidized GSSG by the enzyme glutathione reductase:

\[
\text{G Reductase} \\
\text{GSSG + NADPH} \rightarrow \text{GSH + NADP}
\]

Therefore, in this study we have also studied the rate of NAD(H) synthesis in cellular fractions using rat liver cytosols and microsome after treatment with plant extracts and/or lead compounds.

Coenzymes are small organic molecules, often are derivatives of vitamins that function with the enzyme in the catalytic process. Often the coenzyme has an affinity for the enzyme that is similar to that of the substrate; consequently the coenzyme can be considered to be a second substrate (York, 2002).

2.2.3.1 Nicotinamide-Adenine Dinucleotide Synthetase (NADS)

NAD⁺ and its phosphorylated from NADP are essential coenzyme for redox reactions (oxidizing reactions) in the cell, whereas reduced forms of these co-enzymes, NADH and NADPH, are essential for supplying reducing equivalents (Bieganowski and Brenner, 2004). NAD(H) is a relatively large and complex molecule (Figure 2.6). It is vitamin B₃ (niacinamide) combined with a ribose (5 carbon sugar), a phosphate group and
an adenine nucleotide (a DNA component). NAD(H) can be made in the liver and other cells from vitamin B₃. NAD⁺ represents the oxidized (burned) coenzyme form of vitamin B₃ and NADH represents the reduced (electron-energy rich) form of vitamin B₃. It functions as intermediates in transfer of two electrons between an electron donor and an acceptor. The donor and acceptor need not be involved in the same metabolic pathway. Therefore the reduced form of these nucleotides acts as a common “pool” of electrons that arise from many oxidative reactions and can be used for various reductive reactions. The adenine, ribose, and pyrophosphate components of NAD⁺ are involved in binding of NAD⁺ to the enzyme (York, 2002).

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**Figure 2.6:** (a) NAD(H) structure and (b) NADP(H) structure.

NAD⁺ and NADH are converted into each other in numerous different metabolic activities. In some metabolic reactions it is NAD⁺ which is the needed catalyst, with NADH a useful by product; in other reactions the situation is reversed. NAD⁺ and NADH also activate various enzymes, for example, NAD⁺ activates alcohol dehydrogenase and acetaldehyde dehydrogenase that are the two enzymes needed to detoxify alcohol in the
body into carbon dioxide and water. NADH is the first of five enzyme complexes of the electron transport chain.

The chemistry of NAD(H) is one of the most complex in the human body. NAD(H) is necessary to oxidized (burn) all food stuffs (fats, sugar, amino acids) into ATP bioenergy. There are three interlinked energy production cycles (glycolytic, citric acid, and electron transport side chain cycles) (Sakai et al., 1997; Bieganowski and Brenner, 2004).

Nicotinamide-adenine dinucleotide synthetase (NADS) catalyzes the final step in NAD biosynthesis in the well characterized de novo, reclaim, and import pathways. (Preiss-Handler pathway for NAD⁺ biosynthesis) that originate from tryptophan (or aspartic acid), nicotinic acid, and nicotinamide, respectively, and meet on nicotinic acid mononucleotide (Bieganowski and Brenner, 2004). It has been long known that eukaryotic NAD⁺ synthetase uses glutamine to amidate nicotinic acid adenine dinucleotide. NAD⁺ synthetase transfers an amino group from glutamine to nicotinic acid adenine dinucleotide (NAAD) to form NAD⁺ via an adenylated intermediate (Morita et al., 1997) (Figure 2.7). All of the known eukaryotic NAD⁺ synthetases are glutamine-dependent, hydrolyzing glutamine to glutamic acid to provide the attacking ammonia. In the prokaryotic world, some NAD⁺ synthetases are glutamine-dependent, whereas others are ammonia-dependent (Bieganowski and Brenner, 2004).
Figure 2.7: Reaction scheme of glutamine-dependent NAD$^+$ synthetase. Glutamine-dependent NAD synthetase (Qns1) catalyzes ATP-Mg$^{2+}$-dependent adenylation of NAAD to form NAAD-AMP. In the second step, glutamine is consumed to glutamate and the released ammonia attacks the adenylated carbon, releasing AMP and forming NAD$^+$. 

The World Health Organization (WHO) referred to the decrease in NADS activity as one of the important effects of lead on humans (WHO, 1995). It was demonstrated by Morita et al. that the dose-effect relationship of NAD$^+$ synthetase activity versus blood lead concentration (Pb-B), indicating that the activity could be useful as a biological effect index (Morita et al., 1997).
2.3 LEAD TOXICITY

Lead (Pb\textsuperscript{12}) is a non-essential toxic heavy metal and environmental pollutant (Struzyn'ska and Sulkowski, 2004) with no biological function (Suwalsky et al., 2003). It is present throughout the ecosystem and is detectable in practically all phases of the environment and in all biological systems. (Colombo et al., 2004; Amdour, 1991). Lead (Pb\textsuperscript{2+}) is both an acute and chronic toxin (Burris and Ashwood, 2001). Routes of lead exposure include ingestion of contaminated drinking water, food or soil, and via inhalation of lead-contaminated dust. For children, intake of lead-containing paint is still an important font of intoxication (Garber and Heiman, 2002).

Lead (Pb) as one of the heavy metal pollutants of the environment originates from various sources like mining and smelting of lead-ores, burning of coal, effluents from storage battery industries, automobile exhausts, metal plating and finishing operations, fertilizers, pesticides and from additives in pigments and gasoline (Verma and Dubey, 2003).

2.3.1 Biological Fate and Toxicokinetics

Absorption and biological fate of lead (Pb) depend on a number of factors. First, physiological characteristics of exposed person. For example, children and pregnant women can absorb up to 70% of ingested lead, while adults absorb up to 20% only. Second, the chemical form of lead (Pb). Organic lead compounds are metabolized in the liver, whereas inorganic lead (the most common form of lead) does not undergo such transformation. Third, absorption of lead will vary directly with solubility and inversely with particle size (ATSDR, 1999).

Following exposure, lead is taken up in the bloodstream. In human blood, ~99% lead is associated with erythrocytes, leaving about 1% in the plasma (Suwalsky et al.,
Approximately 90% of the total body lead is contained within bones. Blood accounts for 4% and the remaining is transported via the blood to soft tissues (resides mainly in the liver and kidneys) (Sivaprasad et al., 2004).

Environmental or occupational exposure to this metal results in toxicity which has been well documented to virtually all body systems in every sector of the population (El-Safty, 2004). Lead has been found to induce a wide range of physiological, biochemical, and behavioral dysfunction in man and in experimental animals. In line with this, many studies have investigated the mechanisms and symptoms of this toxicity through the years (Sivaprasad et al., 2004).

Even though there appears to be no threshold below which lead has no effect, many studies reveal a deficit in cognitive and behavioral function in children, as well as experimental animals, at blood lead levels (Pb-B) as low as 10-15 μg/dl (Garber, 2002). Low levels of human exposure to lead have a number of negative consequences such as impairment of the function of renal tubular cells (El-Safty, 2004), inhibition of sperm formation, slow of motor nerve velocity, cardiovascular diseases (Suwalsky et al., 2003), dysfunction of the central nervous system (Dabrowska-Bouta et al., 2004; Strużyńska and Sulkowski, 2004; Garber and Heiman 2002) Extensive experimental investigation has shown that lead alters several immune system parameters, cause increased susceptibility to infections, autoimmune diseases, and allergic responses (Colombo et al., 2004). On the other hand, at high levels there is damage to almost all organs, and most importantly to the central nervous system, kidneys and blood, culminating in death at excessive levels (Suwalsky et al., 2003).
2.3.2 Biochemical Effects

Lead has been found to produce wide range of toxic-biochemical effects. Liver, kidneys and brain have been considered as the target organs for the toxic effects of lead (Patra et al., 2001). Lead interferes with several aspects of cellular metabolism in tissues throughout the body.

First, lead can disturb calcium metabolism in low dose by substituting this essential cation as an intracellular second messenger, altering the distribution of calcium in subcellular compartments, activating protein kinase C (calcium-dependent enzyme involved in the phosphorylation of regulatory proteins involved in multiple cellular processes), binding more avidly than calcium to calmodulin (a key calcium-binding regulatory protein) (Verity, 1990). Lead also inhibits Na+, K+-ATPase, which may in turn contribute to lead associated increases in intracellular calcium. These and other biochemical effects of lead on cellular calcium have substantial impacts, which may in turn, contribute to lead-related health effects such as neurotoxicity and hypertension (Staessen, 1995).

Second, impairment of heme biosynthesis (Amdour et al., 1991). The heme biochemical alterations caused by lead exposure are of particular interest because of the significant increase in intermediate metabolites, which are easily detected and are widely used as biomarkers of damage and exposure (Hernández et al., 1998). Zn$^{2+}$-containing enzymes, particularly those containing vicinal groups, such as δ-aminolevulinate dehydratase (δ- ALA-D) are considered classical target molecules as it has been reported as the most sensitive enzymes to be affected by lead exposure. δ- ALA-D activity is inhibited by lead because this metal interacts with protein sulfhydryl groups and this inhibition may be detectable at blood concentrations as low as 10 μg/dl (Amdour et al., 1991; Ford et al., 2001 and Perottoni et al., 2004).
Morita et al. (1997) study had shown that δ-ALA-D activity decreased logarithmically with increasing Pb-B and reached plateau at Pb-B level between 40-60 μg/dl. This study also had shown that Pb-B levels inducing 50% inhibition of the δ-ALA-D activity was calculated to be 20 μg/dl (Morita et al., 1997). Accumulation of δ-aminolevulinic acid (δ-ALA), which results from δ-ALA-D inhibition by Pb²⁺ or other agents, can have hepatic and neurotoxic effects (Perottoni et al., 2004).

Lead (Pb) also had an inhibition effect on many enzymes that are involved in heme synthesis. For example it is avidly inhibits prohobilinogen synthase, reductase which is a heme synthesis enzyme necessary to produce Fe⁺²; inhibition of reductase causes accumulation of protoporphyrine in erythrocytes. It had been shown that lead decreased coproporphrinogen oxidase activity (Amdour et al., 1991). Ferrochelatase is also affected by exposure to high lead levels (Hernandez et al., 1998). This enzyme catalyzes the incorporation of iron into the protoporphrin ring. Any depression of the above mentioned enzymes will lead to depressed heme formation (Amdour et al., 1991). Thus, urinary δ-aminolevulinic acid, urinary coproporphyrin and zinc protoporphyrin in red blood cells are useful as lead exposure biomarkers (Hernandez et al., 1998).

Third, lead (Pb) binds and affects proteins/enzymes structures (Buritis and Ashwood, 2001). The molecular targets that have been accounted for the symptoms associated with lead poisoning fall in two primary categories: proteins that naturally bind calcium and zinc in which lead metal competes with these two essential metals in the body (Perottoni et al., 2004; Hernandez, 1998). Due to moderate-high affinity of lead for sulfhydryl groups of cysteines in proteins, it is commonly found in cells and tissues attached to thiol-containing proteins and small molecular weight thiols (Perottoni et al., 2004; Buritis and Ashwood, 2001; Hernandez, 1998).
Some proteins become labile as lead binds with them because lead causes the tertiary structure of the protein to change. Cells of the nervous system are particularly susceptible to this effect. In some cases, lead-bound proteins change their tertiary configurations sufficiently so that they become antigenic (Buritis, and Ashwood, 2001). Renal tubular cells are particularly susceptible to this effect because they are exposed to relatively high lead concentrations during clearance (El-Safy et al., 2004; Buritis, and Ashwood, 2001).

Finally, recent studies demonstrate that relatively low levels of lead (Pb) trigger an increased production level of reactive oxygen species (ROS). These ROS cause damage to the biomolecules such as proteins, enzymes, membrane lipids, pigments, nucleic acids, etc. It had been suggested that ROS may lead to oxidative destruction of thiol groups of amino acids and proteins through the process of peroxide formation ((Rahman and Sultana, 2006; Sivaprasad et al., 2004; Verma, 2003;).

Persistence of lead (Pb) in the humans and animals and the associated health risk is a topic of current debate and concern. Therefore, more studies and investigations need to be done to explore mechanisms underlie lead toxicity especially on the molecular and cellular levels.
CHAPTER 3

MATERIALS AND METHODS
3.1 CHEMICALS

Lead nitrate was obtained from BDH Laboratory Supplies (England). β-nicotinamide adenine dinucleotide, oxidized form (NAD⁺) was purchased from ICN Biomedicals, β-nicotinamide adenine dinucleotide phosphate, tetrasodium salt reduced form (β-NADPH), N-nitrosodimethylamine (NDMA), adenosine 5'-triphosphate magnesium salt (ATP), Nicotin acid adenine dinucleotide sodium salt (NAAD), 2,4-chlorodinitrobenzene (CDNB), L-Glutathione reduced form (GSH), glutamine (Gln), gallic acid, Folin-Ciocalteau's reagent, quercetine, 1,1-diphenyl-2-picrilhydrazyle (DPPH), Butylated hydroxytoluene (BHT) from Sigma Chemical Co. (St Louis, MO, USA). Standard laboratory chemicals used in all experiments and other laboratory techniques were of highest purity commercially available. The water used in all experiments was deionized and distilled with a Milli-Q Gradiant distiller.

3.2 PREPARATION OF MEDICINAL PLANTS EXTRACTS

Medicinal plants (Salvia officinalis, Hibiscus sabdariffa, Nigella sativa, and Chamomilla recutita) were used. Each plant (dried leaves of Salvia officinalis, dried flowers of Hibiscus sabdariffa and Chamomilla recutita, and dried seeds of Nigella sativa) were powdered. Aqueous extraction was applied to each plant powder (10g/200 ml, 5% plant extraction), (5g/200 ml, and 2.5% of Chamomilla recutita powder). The extraction time was 24 hours at room temperature and was periodically sonicated to enhance recovery. The supernatant of each plant extract was filtered using 0.2μm
Millipore filters (Sigma Chemical Co., St Louis, MO, USA) and then stored at 4°C until use. Each plant extract was prewarmed to room temperature before use.

3.3 PREPARATION OF HOMOGENATE, CYTOSOLIC, AND MICROSONAL FRACTIONS

A liver from adult male Wistar (265 g) rat was obtained from the UAE University animal house facility. Liver specimen was quickly excised from the rat, weighed (15 g), then rinsed in ice-cold saline (0.9% NaCl) to clear them from blood, mixed, finely minced, and homogenized (20% w/v) in 100 mM potassium phosphate, buffer pH 7.4 containing 0.15 M KCl, 0.1 mM ethylenediamine tetra acetic acid (EDTA), and 1.0 mM phenyl-methylsulphonyl fluoride (PMSF) using homogenizer (Glas-Cd, Terre Haut, U.S.A). Microsonal and post-microsonal (cytosolic) fractions were prepared by ultracentrifugation as described by Bailey et al. (1998). Briefly, the homogenate was centrifuged for 10 minutes at 1000 g to separate nuclei from cellular debris. Mitochondria were isolated by centrifugation of resulting supernatant for 15 minutes at 10,000 g. Post-mitochondria supernatant was centrifuged for 60 minutes at 100,000 g using Beckman Ultracentrifuge. The resulting pellet contained the microsonal fraction with supernatant containing the cytosolic fraction. The microsonal fraction was suspended in the homogenizing buffer containing 20% glycerol. Homogenate, microsonal, and cytosolic fractions were quickly aliquoted, frozen in dry ice, and stored at -80 °C until assayed.

3.4 PROTEIN DETERMINATION

Protein concentration was measured in homogenate, microsome, and cytosol fractions by the method of Bradford (1976) using bovine serum albumin as standard.
3.5 CYTOCHROME P450 2E1 ACTIVITY

3.5.1 Cytochrome P450 2E1 (CYP2E1) Assay

CYP2E1 activity was measured as described by Raza et al. (2004). Briefly, the reaction mixture (total volume: 1.0 ml) components consisted of 4.0 mM NDMA, 1.0 mM NADPH, assay buffer (35 mM potassium phosphate, pH 7.4, 63 mM MgCl₂) and rat liver microsome (50 µL, 11.5 mg/ml protein). Reactions were started by the addition of 10 mM NADPH and terminated after 30 minutes at 37°C with 25% ZnSO₄ and saturated Ba(OH)₂. After removal of precipitated protein by centrifugation, 0.75 ml of the supernatant was mixed with equal volume of freshly prepared Nash reagent (9.0 g ammonium acetate, 0.12 ml acetyl acetone, 0.18 glacial acetic acid, complete volume to 30 ml with distilled water) and then incubated for 30 minutes at 60°C. The amount of formaldehyde (HCHO) produced was measured spectrophotometrically (CARY 50, VARIAN) at 415 nm.

3.5.2 In Vitro Effect of Lead Nitrate on CYP2E1 Activity

For studying in vitro effect of lead nitrate Pb(NO₃)₂ on CYP2E1, various concentrations (0.006, 0.05, 0.1, 0.15, 0.2, 0.24, and 0.3 mM) of lead nitrate were added to the reaction mixture, before reaction is started by NADPH and then the reaction mixture is incubated and the CYP2E1 activity was measured as described above.

3.5.3 In Vitro Effect of Four Medicinal Plants Extracts on CYP2E1 Activity

Inhibited with Lead Nitrate

To test in vitro effect of medicinal plants extracts on CYP2E1 activity treated with fixed concentration (0.24 mM) of lead nitrate Pb(NO₃)₂, various concentrations of each plant extract (Hibiscus sabdariffa, Nigella sativa, Salvia officinalis, Chamomilla

61
were added to the reaction mixture before addition of lead nitrate. After addition of lead nitrate, the CYP2E1 activity was measured as described above.

3.6 GLUTATHIONE-S-TRANSFERASE ACTIVITY

3.6.1 Glutathione-S-Transferases (GSTs) Assay

GST activity in cytosol was determined according to Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The enzyme assay was carried out in a final volume of 3.0 ml containing 100 mM phosphate buffer (pH 6.5), 1 mM CDNB, 1.5 mM GSH and 10 μL cytosol (~100 μg/assay). The reference cuvette contained similar reaction mixture except the CDNB substrate (an equal amount of buffer was added). The reaction started by the addition of the substrate. The increase in absorbance at 343 nm due to the formation of CDNB-GS conjugates was recorded for 3 minutes at room temperature using UV-VIS spectrophotometer (CARY 50, VARIAN). The enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 mM⁻¹ cm⁻¹. The GST-catalyzed formation of CDNB-GSH produces a dinitrophenyl thioether as shown below in the reaction:

\[
gSH + CH-\text{NO}_2 \rightarrow \text{GST} \rightarrow GS-\text{NO}_2 + CH + H^+\]

3.6.2 In Vitro Effect of Lead Nitrate on GSTs Activity

For studying the in vitro effect of lead nitrate Pb(NO₃)₂ on GSTs activity, rat liver cytosol, 10 μL cytosol (~100 μg/assay), 100 mM phosphate buffer (pH 6.5), were incubated at 37°C for 20 minutes with various concentrations of (Pb(NO₃)₂) (0.02, 0.04,
0.16, 0.32, 0.48, 0.8, 1.13, 1.6, 2.4 mM). Immediately afterwards GSH and CDNB were sequentially added and the enzyme activity measured as described above.

3.6.3 In Vitro Effect of Four Medicinal Plants Extracts on GSTs Activity

To test the in vitro effect of the four plant extracts (Hibiscus sabdariffa, Nigella sativa, Salvia officinalis and Chamomilla recutita) on GSTs activity, rat liver cytosol, 10μL (~100 μg/assay), 100 mM phosphate buffer (pH 6.5) were incubated at 37°C for 20 minutes with 10, 20, 30, 50, 100, 150 and 200μl of each plant extract (from original concentration 10 mg/ml). Immediately afterwards GSH and CDNB were sequentially added and the enzyme activity measured as described above.

3.6.4 Kinetics Parameters Studies

Enzyme kinetic studies were carried out using various concentrations of GSH and CDNB and one concentration (50 μL from original 10 mg/ml) of the plant extract Salvia officinalis; in another set of experiments, one concentration (0.16 mM) of lead nitrate (Pb(NO₃)₂). When CDNB was varied the GSH concentration was kept at 1.5 mM and when GSH was varied the CDNB concentration was kept at 1 mM.

3.7 NAD SYNTHETASE (NADS) ACTIVITY

3.7.1 NAD Assay by HPLC

Fifty μL of the microsome were used for the NADS reaction. The total volume of the reaction mixture was 400 μL placed in a microtube (1.5 ml) with cap, and consisted of 30 mM Tris-HCl buffer (pH 7.4), 60 mM KCl, 2 mM MgCl₂, 0.5 mM ATP, 15 mM Glutamine, 0.5 mM NAAD and 50 μL of the microsome (11.5 mg protein /ml). The reaction was carried out at 37°C for 60 minutes and stopped by
cooling the microtubes in ice cold water. After centrifugation at 12,000 g for 10 minutes at 4°C (using Biofuge Centrifuge, Brimo R, Heraeus), 20 μL of the supernatant was used for NAD determination by HPLC (Sakai et al., 1997)

3.7.2 HPLC Analysis

A liquid chromatograph (Agilent HPLC Liquid Chromatography system) located at Chemistry Department in the UAE University Campus was used. The HPLC instrument was used with a pump, an automatic sample injector, a detector and a data processor. The column (Symmetry® C18, 5 μm) packed with a C18 reversed-phase silica was used for the separation. The mobile phase was 3% methanol containing 10 mM KH₂PO₄. The flow-rate and detector wavelength were set at 1 ml/min and 260 nm, respectively. 20 μL of the supernatant was injected at 20 minutes intervals. An aqueous solution of NAD⁺ (200 μM) was injected into HPLC system as the standard. Figure 3.1 shows chromatograms of an aqueous solution of NAD⁺ (200 μM), 0.5 mM NAAD, reaction mixture contains microsome, substrate and the cofactors to form end product of NAD under the analytical conditions stated above.
Figure 3.1 Examples of the obtained chromatograms for (a) 200 µM NAD, (b) 0.5 mM NAAD, and (c) NADS reaction mixture in the presence of 0.5 mM NAAD using rat liver microsome as source of the enzyme.
3.7.3 **Calibration of NADS Activity**

NADS activity was calculated by the amounts of formed NAD. The following equation was used for the calibration of the NADS activity.

\[
\text{NADS activity (\(\mu\text{mol/h/g protein}\))} = \frac{S_A}{S_T} \cdot C_{ST} \cdot V \cdot f / C_{\text{prot}}
\]

Where \(S_A\) and \(S_T\) are peak area of sample NAD and that of standard NAD used (200 \(\mu\text{M}\)). \(V\) is the volume of the reaction mixture (0.0004 l). \(f\) (2000) is the factor for volume of microsome used (50 \(\mu\text{l}\)) to convert the microsome volume of 100 ml and \(C_{\text{prot}}\) is the protein concentration of microsome used (g/100ml). \(C_{ST}\) is the standard concentration. The peak area is automatically calculated by the software which is originally provided with the data processor.

3.7.4 **In Vitro Effect of Lead Nitrate on NADS Activity**

For studying the *in vitro* effect of lead nitrate \(\text{Pb(NO}_3\text{)}_2\) on NADS activity. Various concentrations of lead nitrate, (0.036, 0.06, 0.08, 0.12 mM) were added to 50 \(\mu\text{l}\). microsome (11.56 mg protein/ml), and then immediately afterward all the reaction chemicals were added to the reaction mixture. The reaction was carried out at 37 \(^\circ\text{C}\) for 60 minutes and stopped by cooling the microtubes in ice cold water. After centrifugation at 12,000 g for 10 minutes at 4\(^\circ\text{C}\) (using Biofuge Centrifuge, Brimo R. Heraeus), 20 \(\mu\text{l}\). of the supernatant was used for NAD determination by HPLC as described above.
3.7.5 In Vitro Effect of Four Medicinal Plants Extracts on NADS Activity

To test the in vitro effect of the four plant extracts (Hibiscus sabdariffa, Nigella sativa, Salvia officinalis and Chamomilla recutita) on NADS activity, two different concentrations of each plant extract (50 mg/ml from original extraction; 25 mg/ml in case of using Chamomilla recutita) 20 µl of each plant extract were added to 50µl of microsome (11.56 mg/ml) and then immediately afterward all the reaction chemicals were added to the reaction mixture. The reaction was carried out at 37°C for 60 minutes and stopped by cooling the microtubes in ice cold water. After centrifugation at 12,000 g for 10 minutes at 4°C, 20 µL of the supernatant was used for NAD determination by HPLC as described above.

3.7.6 In Vitro Effect of Four Medicinal Plants Extracts on NADS Activity Inhibited with Lead Nitrate

To test the in vitro effect of the four plant extracts (Hibiscus sabdariffa, Nigella sativa, Salvia officinalis and Chamomilla recutita) with fixed concentration of lead nitrate on NADS activity, two different concentrations of each plant extract (50 mg/ml from original extraction, 25 mg/ml in case of using Chamomilla recutita) 20, 100 µl of each plant extract was added to 50µl of microsome (11.56 mg/ml) prior of lead nitrate addition (0.06 mM) and then immediately afterward all the reaction chemicals were added to the reaction mixture. The reaction was carried out at 37°C for 60 minutes and stopped by cooling the microtubes in ice cold water. After centrifugation at 12,000 g for 10 minutes at 4°C, 20 µL of the supernatant was used for NAD determination by HPLC as described above.
3.8 DETERMINATION OF TOTAL PHENOLS CONTENT

Total soluble phenolics derivatives were determined according to the Folin-Ciocalteau method (Singleton and Rossi, 1965) using a reaction mixture containing 50 μl aliquot of each plant extract, blank or gallic acid standards, respectively, 50 μl of 7% (v/v) acetic acid, 50 μl of Folin-Ciocalteau’s reagent, 50 μl of 35% (w/v) sodium carbonate and 800 μl of ultra-pure distilled water. After mixing, the reaction was incubated for 90 minutes, at room temperature, in the dark. Light absorbance was measured at 725 nm using UV-VIS spectrophotometer (CARY 50 Conc., VARIAN). All determinations were carried out in duplicates. The total phenolic content was expressed as gallic acid equivalents (GAE) per g of extract (mg gallic acid/ g dry weight of plant extract). The amount of phenols derivatives in each plant extract, in gallic acid equivalents, was calculated using a standard curve obtained from various concentration of gallic acid:

\[ X = \frac{(A_o - 0.0289)}{10.98}/m. \]

where \( X \) is the phenols content, mg/g plant extract solution, \( A_o \) is the absorbance of plant sample, and \( m \) is the weight of plant extract in g.

3.9 DETERMINATION OF TOTAL FLAVONOIDS CONTENT

Total flavonoids present in the crude extract were determined by the method described by Costa (1972) using quercetine as a reference compound. The reaction mixture containing 200 μL of sample, blank or a quercetine standards (dissolved in 0.05 N NaOH solution), 60 μL of glacial acetic acid, 1 ml of pyridine: water: 12% aluminum chloride solution (17: 80: 3), 1.24 ml of DMSO:H₂O (1:1). The reaction product was spectrophotometrically determined at 420 nm. All determinations were carried out in duplicates. The total flavonoids content was expressed as quercetine equivalents (QE) per
g of extract (mg quercetine/ g dry weight of plant extract). The amount of flavonoids in each plant extract, in quercetine equivalents, was calculated using a standard curve obtained from various concentration of quercetine:

\[ X = \frac{(A_0 - 0.0395)}{0.002} / m, \]

where \( X \) is the flavonoid content, mg/g dry weight of plant extract solution, \( A_0 \) is the absorbance of plant sample, and \( m \) is the weight of plant extract in g.

### 3.10 MEASUREMENT of DPPH FREE RADICAL SCAVENGING ACTIVITY

The free radical-scavenging activity of plants extracts, based on the scavenging activity of the stable radical 1,1-diphenyl-2-picrylhydrazyle (DPPH), was measured spectrophotometrically by the method described by Parejo et al. (2002) with some modifications. A 0.5 ml aliquot of each plant extract was mixed with 1.5 ml of 0.1 mM DPPH-methanol solution and 1.0 ml Tris-HCl buffer (50 mM, pH 7.4). After 30 min incubation at 37°C in the dark, the resultant absorbance was recorded spectrophotometrically at 517 nm. Butylated hydroxytoluene (BHT, 1.5 mg/L) was used as a positive control. All determinations were performed in duplicate. The percentage inhibition of the DPPH radical by the samples was calculated using Eq (1):

DPPH radical scavenging activity (%) 

\[ \% \text{ inhibition} = \left\{ \frac{(A_0 - A_s)}{A_0} \right\} \times 100, \quad \text{Eq(1)} \]

where \( A_0 \) is the absorbance of the control solution (contain only DPPH), \( A_s \) is the absorbance in the of the plant extract in DPPH solution.
3.11 STATISTICAL ANALYSIS

The data was analyzed and plotted using SigmaPlot Version 9.0 (Systat Software, Inc., USA) and Excel software (2003). The statistical significance of differences between data means was determined by using t-test. A value of $P$-value<0.05 was considered as statistically significant. Values in tables and graphs are reported as means ± standard deviation (SD) of at least two independent determinations.
CHAPTER 4

RESULTS
CHAPTER 4

RESULTS

4.1 EFFECTS ON CYTOCHROME P450 2E1 (CYP2E1) ACTIVITY

4.1.1 Effect of Lead on CYP2E1 Activity

When rat liver microsome fractions were incubated with increasing concentrations of lead nitrate, the CYP2E1 activity decreased gradually (Figure 4.1). From Figure 4.2, it can be seen that with (0.006, 0.05, 0.1, 0.15, 0.2, 0.24, and 0.3 mM) of lead nitrate, CYP2E1 activity inhibited is inhibited by 10%, 24%, 21%, 25%, 63%, 72%, and 73% respectively (Table 4.1).

4.1.2 Effect of Salvia Officinalis, Hibiscus Sabdariffa, Chamomilla Recutita, and Nigella Sativa Extracts on CYP2E1 Activity Inhibited by Lead

As presented previously, CYP2E1 activity was strongly inhibited when rat liver microsome was incubated with 0.24 mM lead nitrate. This concentration of lead is chosen to test the effect of increasing concentrations of the four medicinal plants on lead toxicity with regard to CYP2E1 activity.

Table 4.2 illustrates the effect of Salvia officinalis on inhibition of CYP2E1 activity caused by lead. A significant recovery in enzyme activity was noted when increasing concentrations of Salvia officinalis extract were incubated with rat liver microsome prior to lead nitrate addition (protective effect)(Figure 4.3). Incubation of 20, 40, 60, 80, 100, and 150 μL of Salvia officinalis extract (5% extract, 50 mg/ml) gradually increased CYP2E1 activity by 12.5%, 33.5%, 48.0%, 63.3%, 132.8%, and 206.0%
respectively with regard to microsome incubated with lead nitrate only. It is clear that 150.0 µL of *Salvia officinalis* extract returned CYP2E1 activity almost back to normal.

The effect of tested *Hibiscus sabdariffa* extract on inhibited CYP2E1 activity of rat liver microsome is shown in Figure 4.4. It is clear from this figure that incubation of increasing concentrations of *Hibiscus sabdariffa* extract prior to lead nitrate addition gradually recovered the inhibited CYP2E1 activity caused by lead. Pre-treatment of rat liver microsome with 20, 40, 60, 80, 100, and 150, and 200 µL of this extract (5% extraction, 50 mg/ml) increased CYP2E1 activity by 5.3%, 36.6%, 53.4%, 81.6%, 116.0%, 193.8%, and 287.6% respectively (Table 4.3).

Plant extract of *Chamomilla recutita* showed the same effect on inhibited CYP2E1 caused by lead nitrate. This effect is presented in Figure 4.5 which clearly shows that the inhibitory effect on CYP2E1 activity caused by lead was ameliorated with this plant extract. Pre-incubation of rat liver microsome with 20, 40, 60, 80, 100, and 150, and 200 µL of the *Chamomilla recutita* extract (2.5% extraction, 25 mg/ml) caused 14.0%, 33.0%, 48.0%, 64.0%, 75.5.0%, 120.0%, and 155.0% increase in CYP2E1 activity respectively (Table 4.4).

Figure 4.6 shows the effect of *Nigella sativa* extract on inhibited CYP2E1 activity in rat liver microsome caused by lead nitrate. Compared to microsome incubated with lead nitrate only, sharp increase in CYP2E1 enzyme activity (110.6%) occurred when treated with the lower concentration of *Nigella sativa* extract (20 µL). With 40 µL of extract, enzyme activity increased to 98.5% compared to microsome incubated with lead nitrate only but the percentage of increase declined compared to the lower concentration of the extract used which is 20 µL. 113.7% increase occurred with 60 µL of the plant
extract. Enzyme activity continued to increase with 80, 100, 150, and 200 μL of the
Nigella sativa extract by 154.2.0%, 192.4%, 205.0%, and 205.0% respectively. However,
the percent increases observed in CYP2E1 activity with 150 and 200 μL of this extract
are similar (205.0%) (Table 4.5).
Table 4.1 *In vivo* effect of various concentrations of lead nitrate on rat liver microsomal CYP2E1 activity using NDMA as substrate.

<table>
<thead>
<tr>
<th>Lead nitrate (mM)</th>
<th>0.006</th>
<th>0.05</th>
<th>0.1</th>
<th>0.15</th>
<th>0.2</th>
<th>0.24</th>
<th>0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1 (^a)</td>
<td>4.9**</td>
<td>4.13*</td>
<td>4.31***</td>
<td>4.07**</td>
<td>2.01*</td>
<td>1.54***</td>
<td>1.47**</td>
</tr>
<tr>
<td>S.D. (^b)</td>
<td>0.06</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>10.0</td>
<td>24.0</td>
<td>21.0</td>
<td>25.0</td>
<td>63.0</td>
<td>72.0</td>
<td>73.0</td>
</tr>
</tbody>
</table>

Control value: 5.45 nmol of HCHO formed/min/mg protein.

\(^a\) CYP2E1 activity in nmol of HCHO formed/min/mg protein.

\(^b\) Standard deviation.

Values are statistically significant at \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \) versus control.

\( (P \text{ values} = \text{statistical comparison with the control group}). \)
Figure 4.1: *In vitro* effect of lead nitrate on CYP2E1 activity in rat liver microsome. Microsomal fractions were incubated with different concentrations of lead nitrate at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without lead. Each point represents the mean of duplicate determinations.
Figure 4.2: % inhibition of CYP2E1 activity in rat liver microsome caused by lead nitrate. Microsomal fractions were incubated with different concentrations of lead nitrate at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without lead. Each point represents the mean of duplicate determinations.
Table 4.2 *In vitro* effects of various concentrations of *Salvia officinalis* extract on rat liver microsomal CYP2E1 activity using NDMA as substrate.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lead</th>
<th><em>Salvia officinalis</em> Extract (5%) (μL) + Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.06</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>CYP2E1 a</td>
<td>1.44* (12.5%) b</td>
<td>1.71* (33.5%) b</td>
<td>1.90** (48.0%) b</td>
</tr>
<tr>
<td>S.D. c</td>
<td>0.00</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

* a CYP2E1 activity in nmol of HCHO formed/mg protein.

b % increase in activity versus microsome incubated with lead only.

* c Standard deviation.

d 0.24 mM lead nitrate (fixed concentration throughout experiment).

Values are statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001 versus control.

(P values = statistical comparison with the control group.)
Figure 4.3: *In vitro* effect of *Salvia officinalis* extract on CYP2E1 activity in rat liver microsome treated with 0.24 mM lead nitrate. Microsomal fractions were incubated with different concentrations of plant extract prior to lead addition and then incubated at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without either lead or plant extract. Each column represents the mean of duplicate ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001 versus microsome incubated with 0.24 mM lead nitrate only.
Table 4.3 *In vitro* effect of various concentrations of *Hibiscus sabdariffa* extract on rat liver microsomal CYP2E1 activity using NDMA as substrate.

<table>
<thead>
<tr>
<th>Control</th>
<th>Lead</th>
<th><em>Hibiscus sabdariffa</em> Extract (5%) (µL) + Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>CYP2E1</td>
<td></td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>0.02</td>
</tr>
</tbody>
</table>

* CYP2E1 activity in nmol of HCHO formed/min/mg protein.

* % increase in activity versus microsome incubated with lead only.

* Standard deviation.

* 0.24 mM lead nitrate (fixed concentration through experiment).

Values are statistically significant at * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

(P values = statistical comparison with the control group.)
Control (No lead, No plant extract)
0.24 mM Lead nitrate
Plant extract *Hibiscus sabdariffa* (5%) volume in µL

Figure 4.4: *In vitro* effect of *Hibiscus sabdariffa* extract on CYP2E1 activity in rat liver microsome treated with 0.24 mM lead nitrate. Microsomal fractions were incubated with different concentrations of plant extract prior to lead addition and then incubated at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without either lead or plant extract. Each column represents the mean of duplicates ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001 versus microsome incubated with 0.24 mM lead nitrate only.
Table 4.4 *In vitro* effect of various concentrations of *Chamomilla recutita* extract on rat liver microsomal CYP2E1 activity using NDMA as substrate

<table>
<thead>
<tr>
<th></th>
<th><em>Control</em></th>
<th><em>Lead</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Chamomilla recutita Extract (2.5%) (µL) + Lead</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>CYP2E1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.06</td>
<td>1.31</td>
<td>1.49*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(14.0%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S.D.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15</td>
<td>0.00</td>
<td>0.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> CYP2E1 activity in nmol of HCHO formed/min/mg protein.
<sup>b</sup> % increase in activity versus microsome incubated with lead only.
<sup>c</sup> Standard deviation.
<sup>d</sup> 0.24 lead nitrate (fixed concentration through experiment).

Values are statistically significant at * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

(*P* values = statistical comparison with the control group).
Figure 4.5: In vitro effect of Chamomilla recutita extract on CYP2E1 activity in rat liver microsome treated with 0.24 mM lead nitrate. Microsomal fractions were incubated with different concentrations of plant extract prior to lead addition and then incubated at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without either lead or plant extract. Each column represents the mean of duplicates ± S.D. *p < 0.05, **p < 0.01, ***p < 0.001 versus microsome incubated with 0.24 mM lead nitrate only.
Table 4.5 *In vitro* effect of various concentrations of *Nigella sativa* extract on rat liver microsomal CYP2E1 activity using NDMA as substrate.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lead d</th>
<th>Nigella sativa Extract (5%) (μL) + Lead</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>CYP2E1 a</td>
<td>4.06</td>
<td>1.31</td>
<td>2.76* (110.6%) b</td>
<td>2.60** (98.5%) b</td>
<td>2.80*** (113.7%) b</td>
<td>3.33** (154.2%) b</td>
<td>3.83*** (192.4%) b</td>
<td>4.00*** (205.0%) b</td>
<td>4.00*** (205.0%) b</td>
</tr>
<tr>
<td>S.D. c</td>
<td>0.03</td>
<td>0.04</td>
<td>0.43</td>
<td>0.07</td>
<td>0.00</td>
<td>0.09</td>
<td>0.10</td>
<td>0.04</td>
<td>0.00</td>
</tr>
</tbody>
</table>

a CYP2E1 activity in nmol of HCHO formed/min/mg protein.
b % increase in activity versus microsome incubated with lead only.
c Standard deviation.
d 0.24 mM lead nitrate (fixed concentration through experiment).

Values are statistically significant at * P <0.05, ** P <0.01, *** P <0.001 versus control.

(P values = statistical comparison with the control group).

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Figure 4.6: *In vitro* effect of *Nigella sativa* extract on CYP2E1 activity in rat liver microsome treated with 0.24 mM lead nitrate. Microsomal fractions were incubated with different concentrations of plant extract prior to lead addition and then incubated at 37°C for 30 minutes before activity measurements. Control microsome was also used similarly without either lead or plant extract. Each column represents the mean of duplicates ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001 versus microsome incubated with 0.24 mM lead nitrate only.
4.1 EFFECTS ON GLUTATHIONE-S-TRANSFERASES (GSTs)

ACTIVITY

4.2.1 Effect of Lead on GSTs Activity

In rat liver cytosol the total cytosolic GSTs activity was altered when incubated with increasing concentrations of lead nitrate (Figure 4.7 (a, b)). From Figure 4.8, it is seen that increasing lead nitrate concentrations strongly inhibited GSTs activity with 50% inhibition at 0.16 mM (I50) of lead nitrate. At 2.4 mM lead nitrate, almost 96% reduction in enzyme activity was found. From Figure 4.8, it is clear that a sharp decrease in GSTs activity occurred from 63% inhibition at 0.8 mM to 94% inhibition at 1.13 mM. By increasing lead concentration from 1.13 mM to 2.4 mM, GSTs activity reduced by 2% only. Table 4.6 shows decrease values in GSTs activity with increasing lead nitrate concentrations.

To obtain information on the nature of inhibition of the cytosolic GSTs caused by lead nitrate, GSTs activity was measured with variable concentrations of either CDNB or GSH with fixed concentration of lead (0.16 mM). Table 4.7 & Figure 4.9 shows GSTs activity with varying concentrations of CDNB as a substrate. The Lineweaver-Burk plot of CDNB as variable substrate and the type of inhibition produced by lead is presented in Figure 4.10. From this figure it is clear that lead nitrate is non-competitive inhibitor of GSTs with respect to CDNB as substrate. Moreover, the Km and Vmax for cytosolic rat liver GSTs were calculated to be 0.3 mM and 1362.39 nmol/min/mg protein, respectively. Vmax is changed (lowered) while Km value is unchanged with respect to CDNB (Table 4.10).
GSTs activity with varying concentrations of GSH as a substrate is shown in Table 4.8 & Figure 4.11. Double reciprocal plot with varying concentrations of GSH is presented in Figure 4.12. This figure indicates that lead nitrate inhibits GSTs activity non-competitively. $V_{\text{max}}$ was changed (lowered) with respect to GSH while $K_m$ value unchanged. $V_{\text{max}}$ for cytosolic rat liver GST and $K_m$ values were calculated to be 0.8 mM and 1440.9 nmol/min/mg protein, respectively (Table 4.11).

4.2.2 Effect of *Salvia Officinalis, Hibiscus Sabdariffa, Chamomilla Recutita*, and *Nigella Sativa* Extracts on GSTs Activity

Effect of plant extract *Salvia officinalis* on hepatic cytosolic GST activity using CDNB as substrate is presented in Figure 4.13. *Salvia officinalis* inhibited GSTs activity dose-dependently with ~50% inhibition of the enzyme activity (Figure 4.14) when we use 50 μL of the plant extract (1% extraction, 10 mg/ml) (Table 4.9). Enzyme inhibitory kinetic studies were carried out using various concentrations of GSH and CDNB and one (50 μL) concentration of the plant extract (Figure 4.9 & Figure 4.11). The Lineweaver-Burk plot for CDNB as variable substrate and the type of inhibition produced by *Salvia officinalis* is presented in Figure 4.15. *Salvia officinalis* lowered the $V_{\text{max}}$ and increased $K_m$ value for CDNB suggesting uncompetitive inhibition towards CDNB. However, *Salvia officinalis* exerted a non-competitive inhibition towards GSH ($V_{\text{max}}$ decreased while $K_m$ is unchanged, Table 4.10) and these values were calculated to be 1021.4 nmol/min/mg proteins, 0.8 mM respectively (Figure 4.16).

The effect of *Hibiscus sabdariffa* on GSTs activity is shown in Table 4.12 and Figure 4.17 which show that *Hibiscus sabdariffa* decreased cytosolic GSTs activity in rat liver. In the range of 10 μL and up to 50 μL of the plant extract (10 mg/ml) used in the
experiment, an average of about 15% inhibition of GST's activity occurred. The maximum reduction (25%) in enzyme activity occurred when hepatic cytosol was incubated with 200 μL of *Hibiscus sabdariffa* extract (10 mg/ml) (Figure 4.18).

From Figure 4.19 we can see that incubation of 10, 20, 30, 40, 50, 100, and 200 μL of *Chamomilla recutita* extract (1% extraction, 10 mg/ml) with rat liver cytosol for 20 minutes at 37°C GSTs activity decreased by 28%, 31%, 32%, 35%, 45%, 54%, and 64% respectively compared to control (Table 4.13 & Figure 4.20).

There were significant changes in the hepatic cytosolic GSTs activity when incubated 20 minutes at 37°C with increasing concentrations of the *Nigella sativa* extract (Figure 4.21). Table 4.14 shows that with CDNB as substrate, 10, 20, 30, 40, 50, 100, and 200 μL of *Nigella sativa* extract (1% extraction, 10 mg/ml) inhibited cytosolic GSTs activity in rat liver by 14%, 18%, 32%, 38%, 45%, 50% and 73% respectively (Table 4.22).
Table 4.6 *In vitro* effect of various concentrations of lead nitrate on rat liver cytosolic GST activity using CDNB as substrate.

<table>
<thead>
<tr>
<th>Lead (mM)</th>
<th>0.0</th>
<th>0.02</th>
<th>0.04</th>
<th>0.16</th>
<th>0.32</th>
<th>0.48</th>
<th>0.8</th>
<th>1.13</th>
<th>1.6</th>
<th>2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST activity <em>a</em></td>
<td>1234.25</td>
<td>1025.17*</td>
<td>902.52**</td>
<td>620.28**</td>
<td>583.31***</td>
<td>513.58***</td>
<td>457.52***</td>
<td>77.0***</td>
<td>56.93***</td>
<td>48.31***</td>
</tr>
<tr>
<td>S.D. <em>b</em></td>
<td>68.4</td>
<td>59.6</td>
<td>41.2</td>
<td>8.8</td>
<td>25.9</td>
<td>31.1</td>
<td>71.7</td>
<td>6.58</td>
<td>15.3</td>
<td>11.3</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.0</td>
<td>17.0</td>
<td>27.0</td>
<td>50.0</td>
<td>53.0</td>
<td>58.0</td>
<td>63.0</td>
<td>94.0</td>
<td>95.0</td>
<td>96.0</td>
</tr>
</tbody>
</table>

* GST activity in nmol/min/mg protein, values are mean ± S.D. of triplicate determinations.

* Standard deviation.

Values are statistically significant at * * *P* < 0.05; ** * *P* < 0.01; *** *P* < 0.001 versus control.

(*P* values = statistical comparison with the control group).
Figure 4.7(a) *In vivo* effect of lead nitrate on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of lead nitrate at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without lead. Each point shows mean for triplicate determinations.
Figure 4.7(b): *In vitro* effect of lead nitrate on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of lead nitrate at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without lead. Each point shows mean for triplicate determinations.
Figure 4.8. % inhibition of GST activity in rat liver cytosol caused by lead nitrate. Cytosolic fractions were incubated with different concentrations of lead nitrate at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without lead. Each point shows mean for triplicate determinations.
Table 4.7: GST activity in rat liver cytosol with varying CDNB concentrations and 1.5 mM GSH.

<table>
<thead>
<tr>
<th></th>
<th>CDNB concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1368.17±226.68</td>
</tr>
<tr>
<td><strong>Lead nitrate</strong></td>
<td>653.34±23.66</td>
</tr>
<tr>
<td><strong>Salvia officinalis</strong></td>
<td>243.5±5.4</td>
</tr>
</tbody>
</table>

* GST activity measured in nmol/min/mg protein.
** GST activity was measured in the absence of lead nitrate and *Salvia officinalis* (control), in the presence of lead nitrate (0.16 mM) and in the presence of 50 µl. of 1% *Salvia officinalis* extract. Each point shows mean ± S.D. for triplicate determinations.
Figure 4.9  GST activity in rat liver cytosol with varying CDNB concentrations. 1.5 mM GSH GST activity was measured in the absence of lead nitrate and *Salvia officinalis* (●), in the presence of lead nitrate (0.16 mM) (▲), and in the presence of 50 μL of 1% *Salvia officinalis* extract (▼) at 37°C for 20 minutes. Each point shows mean for triplicate determinations.
Control
△ Lead nitrate

Figure 4.10 Lineweaver-Burk plot of GST activity in rat liver cytosol with varying CDNB concentration with 1.5 mM GSH. GST activity was measured in the absence (■) and presence (△) of lead nitrate. Samples were incubated at 37°C for 20 minutes. Each point shows mean for triplicate determinations.
Table 4.8: GST activity in rat liver cytosol with varying GSH concentrations, 1.0 mM CDNB.

<table>
<thead>
<tr>
<th></th>
<th>GSH concentration (mM)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3</td>
<td>3.5</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>1051.6±47.6</td>
<td>1186.69±29.88</td>
<td>1395.52±26.11</td>
<td>1500.79±52.22</td>
<td>1420.38±38.86</td>
<td>1544.68±59.58</td>
<td>1319.82±104.66</td>
<td>1635.84±190.75</td>
</tr>
<tr>
<td>Lead nitrate</td>
<td>564.33±61.24</td>
<td>793.0±103.71</td>
<td>823.71±92.77</td>
<td>978.68±87.06</td>
<td>1190.83±117.2</td>
<td>1234.75±86.92</td>
<td>1196.63±32.45</td>
<td>1129.51±158.45</td>
</tr>
<tr>
<td>Salvia officinalis</td>
<td>436.97±7.52</td>
<td>631.46±26.37</td>
<td>696.93±17.64</td>
<td>651.33±25.84</td>
<td>811.28±13.72</td>
<td>836.14±75.66</td>
<td>907.41±52.91</td>
<td>962.11±97.82</td>
</tr>
</tbody>
</table>

* GST activity measured in nmol/min/mg protein.

** GST activity was measured in the absence of lead nitrate and Salvia officinalis (control), in the presence of lead nitrate (0.16 mM), and in the presence of 50 µL of 1% Salvia officinalis extract. Each point shows mean ± S.D., for triplicate determinations.
Figure 4.11: GST activity in rat liver cytosol with varying GSH concentrations. 1.0 mM CDNB. GST activity was measured in the absence of lead nitrate and *Salvia officinalis* (•), in the presence of lead nitrate (0.16 mM) (○), and in the presence of 50 μL of 1% *Salvia officinalis* extract (▼) at 37 °C for 20 minutes. Each point shows mean for triplicate determinations.
Figure 4.12: Lineweaver-Burk plot of GST activity in rat liver cytosol with varying concentrations of GSH with 1.0 mM CDNB. GST activity was measured in the absence (■) and presence of lead nitrate (▲) at 37°C for 20 minutes. Each point shows mean for triplicate determinations.
Table 4.9 In vitro effect of various concentrations of *Salvia officinalis* extract on rat liver cytosolic GST activity using CDNB as substrate.

<table>
<thead>
<tr>
<th>Salvia officinalis Extract (1%) (µL)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST activity a</td>
<td>1319.65</td>
<td>1112.52**</td>
<td>879.48**</td>
<td>811.37***</td>
<td>739.9***</td>
<td>684.45***</td>
<td>495.22***</td>
<td>347.96***</td>
</tr>
<tr>
<td>S.D. b</td>
<td>60.81</td>
<td>40.9</td>
<td>103.17</td>
<td>25.62</td>
<td>77.85</td>
<td>5.55</td>
<td>12.17</td>
<td>50.39</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.0</td>
<td>16.0</td>
<td>33.0</td>
<td>39.0</td>
<td>44.0</td>
<td>48.0</td>
<td>62.0</td>
<td>74.0</td>
</tr>
</tbody>
</table>

*a* GST activity in mmol/min/mg protein, values are mean ± S.D. of triplicate determinations.

*b* Standard deviation.

Values are statistically significant at * P <0.05; ** P < 0.01; *** P <0.001 versus control.

(P values = statistical comparison with the control group).
Figure 4.13: *In vitro* effect of *Salvia officinalis* extract (1%) on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Figure 4.14: % Inhibition of GST activity in rat liver cytosol caused by Salvia officinalis extract (1%). Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Figure 4.15 Lineweaver-Burk plot of GST activity in rat liver cytosol with varying CDNB concentration with 1.5 mM GSH. GST activity was measured in the absence (●) and presence of Salvia officinalis extract (●). Samples were incubated at 37°C for 20 minutes. Each point shows mean for triplicate determinations.
Figure 4.16: Lineweaver-Burk plot of GST activity in rat liver cytosol with varying concentrations of GSH with 1.0 mM CDNB. GST activity was measured in the absence (■) and presence of *Salvia officinalis* extract (○) at 37°C for 20 minutes. Each point shows mean for triplicate determinations.
Table 4.10 $V_{\text{max}}$ and $K_m$ values with increasing CDNB concentration.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3225.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Lead nitrate</td>
<td>1362.4</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>918.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 4.11 $V_{\text{max}}$ and $K_m$ values with increasing GSH concentration.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2016.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Lead nitrate</td>
<td>1440.9</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>1021.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 4.12 In vitro effect of various concentrations of *Hibiscus sabdariffa* extract on rat liver cytosolic GST activity using CDNB as substrate.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hibiscus sabdariffa Extract (1%) (μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST activity <em>a</em></td>
<td>1234.25</td>
<td>1064.57*</td>
<td>1093.2</td>
<td>1068.18</td>
<td>1023.43</td>
<td>1056.58*</td>
<td>999.4**</td>
<td>924.82**</td>
</tr>
<tr>
<td>S.D. <em>b</em></td>
<td>68.39</td>
<td>2.65</td>
<td>65.96</td>
<td>81.19</td>
<td>191.31</td>
<td>67.31</td>
<td>47.95</td>
<td>30.25</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.0</td>
<td>14.0</td>
<td>11.0</td>
<td>13.0</td>
<td>17.0</td>
<td>14.0</td>
<td>19.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

*a* GST activity in nmol/min/mg protein, values are mean ± S.D. of triplicate determinations.

*b* Standard deviation.

Values are statistically significant at *P* < 0.05; **P** < 0.01; ***P*** < 0.001 versus control.

(*P* values = statistical comparison with the control group).
Figure 4.17: *In vitro* effect of *Hibiscus sabdariffa* extract (1%) on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Figure 4.18. % Inhibition of GST activity in rat liver cytosol caused by *Hibiscus sabdariffa* extract (1%). Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Table 4.13 In vitro effect of various concentrations of *Chamomilla recutita* extract on rat liver cytosolic GST activity using CDNB as substrate.

<table>
<thead>
<tr>
<th>GST activity a</th>
<th>Chamomilla recutita Extract (1%) (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1123.453</td>
<td>979.09</td>
</tr>
<tr>
<td>S.D.</td>
<td>93.97</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* GST activity in nmol/min/mg protein, values are mean ± S.D. of triplicate determinations.

b Standard deviation.

Values are statistically significant at * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

(P values = statistical comparison with the control group).
Figure 4.19. *In vitro* effect of *Chamomilla recutita* extract (1%) on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Figure 4.20: % Inhibition of GST activity in rat liver cytosol caused by *Chamomilla recutita* extract (1%) Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Table 4.14 In vitro effect of various concentrations of *Nigella sativa* extract on rat liver cytosolic GST activity using CDNB as substrate.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST activity$^a$</td>
<td>1358.22</td>
<td>1167.58</td>
<td>1119.06*</td>
<td>929.46**</td>
<td>841.6**</td>
<td>752.16**</td>
<td>672.81***</td>
<td>362.3***</td>
</tr>
<tr>
<td>S.D.$^b$</td>
<td>90.37</td>
<td>160.63</td>
<td>65.21</td>
<td>27.77</td>
<td>97.3</td>
<td>108.11</td>
<td>24.64</td>
<td>31.63</td>
</tr>
</tbody>
</table>

$^a$ GST activity in nmol/min/mg protein, values are mean ± S.D. of triplicate determinations.

$^b$ Standard deviation.

Values are statistically significant at $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$ versus control.

(P values = statistical comparison with the control group).
Figure 4.21. *In vitro* effect of *Nigella sativa* extract (1%) on GST activity in rat liver cytosol. Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
Figure 4.22. % Inhibition of GST activity in rat liver cytosol caused by *Nigella sativa* extract (1%). Cytosolic fractions were incubated with different concentrations of the plant extract at 37°C for 20 minutes before activity measurements. Control cytosol was also used similarly without a plant extract. Each point shows mean for triplicate determinations.
4.1 EFFECTS ON NAD SYNTHEASE (NADS) ACTIVITY

4.3.1 Effect of Lead on NADS Activity

Figure 4.23 shows lead dose dependent decrease in NADS activity in rat liver microsome when the fractions were incubated with increasing concentrations of lead. It was clear that with 0.036 mM lead nitrate, a 13% inhibition of the enzyme activity was observed. However, a profound decrease in enzyme activity up to 64% inhibition was observed when incubated with 0.06 mM lead nitrate and when 0.08, 0.12, and 0.6 mM of lead nitrate were used, NADS activity was inhibited by 82.5% and 90%, 100% respectively (Table 4.15).

4.3.2 In Vitro Effect of Four Medicinal Plants Extracts on NADS Activity

When 20 µL from each plant extract (5% extraction, 50 mg/ml; 2.5% extraction, 25 mg/ml in case of *Chamomilla recutita*) was incubated with the microsome fraction from rat liver, no significant change was observed in NADS activity in all plants extracts used except for *Hibiscus sabdariffa* extract which showed significant decrease in NADS activity (*P*-value < 0.05) by 11% compared to control value (Table 4.16 & Figure 4.24). Also, we tried increasing concentrations of plants extracts; some plants did not show significant results using higher concentrations and some plants extracts showed increase in NADS activity when used at higher concentrations. Therefore, middle concentration (20 µL, 50 mg/ml; 25 mg/ml in case of *Chamomilla recutita*) was used in this study.

4.3.3 In Vitro Effect of Four Medicinal Plants Extracts on NADS Activity Inhibited with Lead Nitrate

As presented previously, NADS activity was strongly inhibited (64% inhibition) when rat liver microsome was incubated with 0.06 mM lead nitrate. This concentration of
lead is chosen to test the effect of each plant extract on lead toxicity with regard to NADS activity. Two concentrations (20 and 100 μL from 5% extraction, 50 mg/ml; 2.5% extraction, 25 mg/ml in case of *Chamomilla recutita* ) of each plant extract were chosen to test if these extracts have the capacity to ameliorate the decrease in the enzyme activity caused by lead nitrate.

Table 4.16 illustrates effects of the four plants extracts on inhibition of NADS activity caused by lead. A significant elevation in enzyme activity was noted when microsomes fraction were incubated with 20 μL (50 mg/ml; 25 mg/ml in case of *Chamomilla recutita*) of each plant extract. When microsome fractions were incubated with 20 μL of *Salvia officinalis*, *Hibiscus sabdariffa*, *Chamomilla recutita*, and *Nigella sativa* prior to lead nitrate addition (protective effect) a profound recovery in NADS activity was observed by 80%, 75%, 90%, and 95% respectively. With 100 μL (50 mg/ml; 25 mg/ml in case of *Chamomilla recutita*) of each plant extract, 90%, 95% increases in enzyme activity were observed for *Salvia* sp. and *Chamomilla* sp. respectively. It should be noticed that using half concentration of *Chamomilla* sp. showed 95% recovery and therefore can be considered the highest recovery percentage among the other studied plants. However, 68.5% decrease in NADS activity was observed when microsome incubated with 100 μL of *Hibiscus* sp. Incubation of microsome with 100 μL of *Nigella* sp. caused 160% increase in NADS activity compared to microsome incubated with 0.06 mM lead nitrate. This increase percentage nearly brought NADS activity close to control value (Figure 4.25).
Table 4.15 Effect of lead nitrate on NADS activity in rat liver microsome. Microsomal fractions were incubated with varying concentrations of lead nitrate at 37°C for 60 minutes. Samples were analyzed by HPLC for NAD determination.

<table>
<thead>
<tr>
<th>Lead concentration (mM)</th>
<th>0.0</th>
<th>0.036</th>
<th>0.06</th>
<th>0.08</th>
<th>0.12</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADS activity (µmol/min/g protein)</td>
<td>0.55</td>
<td>0.48</td>
<td>0.2</td>
<td>0.096</td>
<td>0.057</td>
<td>0</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.0</td>
<td>13.0</td>
<td>64.0</td>
<td>82.5</td>
<td>90.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Figure 4.23. *In vitro* effect of lead nitrate on NADS activity in rat liver microsome. Microsomal fractions were incubated with varying concentrations of lead nitrate at 37 °C for 60 minutes. Samples were analyzed by HPLC for NAD determination.
Table 4.16 NAD activity (μmol/min/g protein) in rat liver microsome. Microsomal fractions were incubated with (20 μL) of each plant extract and with (20 μL, 100 μL) of each plant extract prior of lead nitrate addition. Samples were incubated at 37 °C for 60 minutes then analyzed by HPLC for NAD determination.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Control</th>
<th>lead nitrate a</th>
<th>Plant extract + lead nitrate</th>
<th>Plant extract only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td><em>Salvia officinalis</em> (5%)</td>
<td>0.55</td>
<td>0.2</td>
<td>0.36* (+80%)</td>
<td>0.38** (+90%)</td>
</tr>
<tr>
<td><em>Hibiscus sabdariffa</em> (5%)</td>
<td>0.55</td>
<td>0.2</td>
<td>0.35* (+75%)</td>
<td>0.063*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-68.5%)</td>
<td></td>
</tr>
<tr>
<td><em>Chamomilla recutita</em> (2.5%)</td>
<td>0.55</td>
<td>0.2</td>
<td>0.38**(+90%)</td>
<td>0.39** (+95%)</td>
</tr>
<tr>
<td><em>Nigella sativa</em> (5%)</td>
<td>0.55</td>
<td>0.2</td>
<td>0.39** (+95%)</td>
<td>0.52 ** (+160%)</td>
</tr>
</tbody>
</table>

a Lead nitrate 0.06 mM.
b +% increase in activity of NADs in relation to microsome incubated with lead only.
c -% decrease in activity of NADs in relation to microsome incubated with lead only.

^P value < 0.05, compared to control.
Plants Extracts

Figure 4.24: *In vitro* effects of the four plants extracts selected on NADS activity in rat liver microsome. Microsomal fractions were incubated with 20 μL of each plant extract. Samples were incubated at 37 °C for 60 minutes. Samples were analyzed by HPLC for NAD determination.
Figure 4.25: *In vitro* effects of the four plants extracts selected on NADS activity in rat liver microsome. Microsomal fractions were incubated with 20 and 100 µL of each plant prior to lead nitrate addition (0.06 mM) to the reaction mixture. Samples were incubated at 37 °C for 60 minutes. Samples were analyzed by HPLC for NAD determination.
4.4 TOTAL PHENOLS CONTENT

Content of total phenolics in the extracts of *Salvia officinalis*, *Hibiscus sabdariffa*, *Chamomilla recutita* and *Nigella sativa* is determined using the Folin-Ciocalteu assay, calculated from regression equation of calibration curve \( y = 10.981x + 0.0289, \ r^2=0.9993 \) and expressed as gallic acid equivalents (GAE). The total phenolic content of the four plants extracts used in our study ranged from 28.6 to 76.4 mg (GAE)/g dry weight of plant extract (Table 4.17). It was observed that content of phenolics in extracts is in the following order: *Salvia officinalis > Hibiscus sabdariffa > Nigella sativa > Chamomilla recutita* (Figure 4.26).

4.5 TOTAL FLAVONOID CONTENT

The total flavonoid content of the four plants extracts used in our study is shown in Figure 4.26. Total flavonoid is calculated from regression equation of calibration curve \( y = 0.002x + 0.0395, \ r^2=0.975 \) and expressed as quercetin equivalents (QE). The total flavonoid content of the *Salvia officinalis* was the highest (28.1 mg QE/g dry weight of plant extract) among the four plants extracts screened for their flavonoid content in this study. *Chamomilla recutita*, *Hibiscus sabdariffa* and *Nigella sativa* show flavonoid content of 21.5, 14.2 and 7.6 mg QE/g plant extract respectively (Table 4.17).
Table 4.17  Total amount of plant phenolic compounds, flavonoids, and DPPH scavenging activity and protein content of the four plants extracts used in this study.

<table>
<thead>
<tr>
<th>Plant Extract</th>
<th>Total phenols mg/g dry weight of plant extract (in GAE*)</th>
<th>Total flavonoids mg/g dry weight plant extract (in QE**)</th>
<th>DPPH scavenging activity (% Inhibition)</th>
<th>Protein content mg/g dry weight plant extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salvia officinalis</em></td>
<td>76.4±0.21</td>
<td>28.1±2.3</td>
<td>53.25±3.9</td>
<td>16.2±0.01</td>
</tr>
<tr>
<td><em>Hibiscus sabdariffa</em></td>
<td>68.7±0.08</td>
<td>14.2±0.7</td>
<td>36.32±1.8</td>
<td>6.10±0.01</td>
</tr>
<tr>
<td><em>Chamomilla recutita</em></td>
<td>28.6±0.19</td>
<td>21.5±2.0</td>
<td>63.1±0.0</td>
<td>18.0±0.0</td>
</tr>
<tr>
<td><em>Nigella sativa</em></td>
<td>40.7±0.01</td>
<td>7.6±1.2</td>
<td>64.52±0.4</td>
<td>9.90.01</td>
</tr>
</tbody>
</table>

*GAE: Gallic acid equivalent
**QE: Quercetin equivalent
* Chamomilla recutita (2.5% extraction, 25 mg/ml) was used in DPPH scavenging activity assay
Figure 4.26: Total amount of plant phenolic compounds and flavonoids of the four plants extracts used in this study. (GAB: Gallic acid equivalent, QE: Quercetin equivalent). Values are mean ± S.D. of triplicates determinations.
4.6 DPPH FREE RADICAL SCAVENGING ACTIVITY

The DPPH radical is a stable free radical and the DPPH radical-scavenging activity was determined by the decrease in absorbance at 517 nm, due to reduction by the antioxidant (AH) or reaction with a radical species, as shown (Gordon, 2001):

\[ \text{DPPH}^\cdot + \text{AH} \rightarrow \text{DPPH-H} + \text{AH}^\cdot \]
\[ \text{DPPH}^\cdot + R^\cdot \rightarrow \text{DPPH-R}. \]

The free radical scavenging effect of aqueous extract of *Chamomilla recutita* was the highest since we used only half concentration which showed 63.1% scavenging activity. The free radical scavenging effect of aqueous extracts of the other three plants extracts relative to the standard with the DPPH radical is in the following order: *Nigella sativa* (64.5%) > BHT (54%) > *Salvia officinalis* (53.3%) > *Hibiscus sabdariffa* (36.3%) at the dose of 0.5 ml (50 mg /ml) for each plant extract and 1.5 mg /L of BHT. The experimental data we obtained reveal that all studied plants extracts used here are likely to have the effect of scavenging free radicals (Figure 4.27).
Figure 4.27: Scavenging activity of free radicals (DPPH) by the aqueous extracts of *Salvia officinalis*, *Hibiscus sabdariffa*, *Chamomilla recutita*, and *Nigella sativa*. Data are expressed as percentage of scavenging of DPPH• radicals. All used plants extract were compared with BHT as known pure antioxidant. Values are mean ± S.D. of duplicate determinations. In case of *Chamomilla recutita* we used only half concentration compared to other tested plants.
CHAPTER 5

DISCUSSION & CONCLUSION
CHAPTER 5

5.1 DISCUSSION

Lead is a persistent environmental pollutant with no beneficial role and its toxicity continues to be a major health problem due to its interference with natural environment. The quantity of lead used in the 20th century far exceeds the total consumed in all previous eras. This heavy use has caused local and global contamination of air, dust, and soil (Rahman and Sultana, 2006). It has been known to damage vital organs and suppresses cellular processes. One of the best known toxic effects of lead is its interference with the enzymes activities. Many studies have explored the mechanisms of lead-induced toxicities and explained this toxicity in different ways through the years. Besides its competition with essential metals like calcium and zinc, and its high affinity to thiol groups in proteins; these known mechanisms have not been successful in explaining some of the symptoms of lead poisoning (Othman et al., 2004). Thus, alternative mechanisms are now being investigated. Recent studies have reported lead's potential for induction oxidative stress through production of reactive oxygen species (ROS) as well as depressing endogenous antioxidants and enhancing lipid peroxidation and evidence is accumulating in support of the role for oxidative stress in pathophysiology of lead poisoning (Rahman and Sultana, 2006; Farmand et al., 2005; Othman et al., 2004; Hsu and Guo, 2002; Gurer and Erkal, 2000).

The assessment of enzyme activity impairments caused by toxic substances is hard to evaluate in vivo models due mainly to homeostatic responses. Thus these evaluations are easier in vitro models (Hernaández et al., 1998). The hepatic CYP and GSTs are major enzyme systems that play critical and specific roles in the metabolism of exogenous and endogenous compounds in mammals (Gyamfi et al., 2000).
This work focused on lead toxicity, as an example of a commonly present heavy metal, tested in vitro against CYP2E1 as representative example of phase I biotransformation enzymes and GSTs as representative example of phase II enzymes in the liver. NADS was chosen as a representative example of enzymes involved in biomolecules synthesis. In addition, we have also studied the activation/or inhibition of GSTs enzymes by some medicinal plants extracts. Furthermore, the antagonistic (or synergistic) effects of these plants extracts against lead toxicity, with regard to CYP2E1 and NADS activities were also studied.

5.1.1 Cytochrome P450 2E1 (CYP2E1) Activity

The objectives of the first part of the present study were in two-folds: firstly, to examine the effects of increasing concentrations of lead nitrate on rat microsomal CYP2E1 activity, and secondly, to determine the possible antagonist or synergistic effects of aqueous extracts of the medicinal plants we chose on CYP2E1 inactivation induced by lead nitrate. Yet, no biochemical in vitro studies have been done on antagonist effects of plants extracts against lead induced enzyme inactivation.

Cytochrome P450 proteins are cysteine thiolate-bound heme. The liver is the major site where xenobiotic metabolism takes place. Hepatic cytochrome P450 2E1 is one of the best conserved xenobiotic-metabolizing P450s. Because rodent and human CYP2E1 enzyme catalyzes similar reactions, the rat and mouse are good models for studying this enzyme activity (Tanaka, 2000). Our results showed that lead nitrate decreased rat microsomal CYP2E1 activity, with maximum inhibition 73% obtained when 0.3 mM of lead was used. The inhibition of CYP2E1 activity may be related-in part to the high affinity of lead to the sulphydryl groups of cysteine in CYP2E1 protein and hence enzyme inactivation as discussed previously. Oxidative stress has been
suggested to be one of the important mechanism(s) of toxic effects of lead (Flora et al., 2003). It has been demonstrated by Verma (2003) that relatively low levels of lead trigger an increased production level of reactive oxygen species (ROS). These ROS cause damage to the biomolecules such as proteins, enzymes, membrane lipids, pigments, nucleic acids, etc. It had been suggested by Sivaprasad et al. (2004) that ROS may lead to oxidative destruction of thiol groups of amino acids and proteins through the process of peroxide formation. A number of recent studies also confirmed the possible involvement of ROS in lead-induced toxicity (Flora et al., 2003). It is therefore speculated that the toxic action produced by lead might be attributed to its ability to generate ROS which induce the CYP2E1 inactivation effect observed in our study.

As shown in the results, all the plants selected in this study reversed the decreased in CYP2E1 activity caused by lead nitrate. The results of this study showed that different concentrations of Salvia officinalis (50 mg/ml) can evoke significant prevention for rat liver microsome CYP2E1 activity from damage or inactivation caused by lead nitrate. It is obvious from the data we obtained here that incubation of microsome with increasing concentrations of Salvia officinalis prior to lead nitrate addition prevent the enzyme inactivation caused by lead metal and recovery percentages depend on the concentration of the plant we used and as had been shown in the results that highest concentration of SO brought the enzyme activity nearly back to normal.

Nigella sativa, Hibiscus sabdariffa, Chamomilla recutita aqueous extracts showed the same preventive effects on lead-induced CYP2E1 inactivation. However, in case of using NS, lower concentrations caused higher increase in the enzyme activity compared to SO effects. However, with higher concentrations, NS and SO showed almost the same percentage increase in the enzyme activity. Concentrations higher than 150 μL (50 mg/ml) did not show further increase in activity. Both 150 μL and 200 μL (50 mg/ml)
resulted in 205.0% increase in enzyme activity. HS caused gradual increase in enzyme activity depending on the amount of plant added and with the highest concentration used, the enzyme activity exceeded the control enzyme activity. We do not have explanations for such an increase in enzyme activity and will require further experimental research.

*Chamomilla recutita* showed also significant increase in enzyme activity with increasing plant extract concentrations as we used only half extraction percentage (2.5% extraction, 25 mg/ml) compared with the other plants used in this study (5% extraction, 50 mg/ml). The highest concentration of this plant (200 µL, 25 mg/ml) did not bring enzyme activity back to normal compared to control; therefore, using higher concentrations may bring enzyme activity back to normal.

The protective and improved effects of the plants extracts on lead-induced CYP2E1 inactivation may be due to the antioxidant properties of the four plants extracts (*Salvia officinalis, Nigella sativa, Hibiscus sabdariffa, Chamomilla recutita*), which may contribute to the inhibition of the reactive free radicals produced by lead metal. Hence, decreased enzyme inactivation with increasing concentrations of each plant extract and the magnitude of percentage increase depends on the plant species used. In addition, some important naturally occurring vitamins have been found to be effective in reducing the toxic manifestation of lead. These vitamins are low molecular mass antioxidants that interact directly with the oxidizing radicals and decrease the effects of ROS (Flora et al., 2003). The four plants studied here may contain some important naturally occurring compounds that have the capability to scavenge the ROS by very rapid electron transfer that inhibit lipid peroxidation (Halliwell, 1994, Buettner, 1993, and Halliwell et al., 1987). Also, the protective and improved effects of these plants extracts on lead-induced CYP2E1 inactivation could be due to the ability of the plants extracts to chelate lead metal as they form an insoluble complex with lead and therefore protect the enzymes.
from damage. For example, ascorbic acid has been shown to have protective effects against lead intoxication and its beneficial effects could be attributed to its ability to complex with lead (Flora and Tandon, 1986). It is likely that the reversion of CYP2E1 enzyme activity by plants extracts could be attributed either to a direct effect of plants on enzyme or to antagonistic effect of some compounds present in these plants to CYP2E1 inhibition. In the present study, we suggest a pronounced beneficial role of *Salvia officinalis*, *Nigella sativa*, *Hibiscus sabdariffa*, *Chamomilla recutita* aqueous extracts in the recovery of lead-induced CYP2E1 inactivation. We suggested that presence of antioxidants and ROS scavenging by plants extracts might have played a role in the modulation of CYP2E1 activity. Although several mechanisms have been proposed to explain the lead-induced toxicity, no mechanism has been defined clearly. Further detailed study will be needed in this regard to clarify the mechanisms of the protective and improved effects of the plants extracts used on lead-induced CYP2E1 inactivation.

5.1.2 Glutathione-S-transferases Activity

The glutathione-S-transferases (GSTs) catalyze the conjugation of glutathione to a variety of electrophilic compounds and thus play an important role in detoxification of many environmental chemicals (Hayes et al., 2005 and Sheweita, 1998). Many chemicals, including glutathione analogues phenolic and carcinogenic compounds have been shown to be inhibitors of glutathione-S-transferases (GSTs) activity (Sheweita and Mostafa, 1996). Previous studies demonstrated that glutathione (GSH) and GST reduce the covalent binding of epoxides with DNA and other macromolecules. This reduction was found to be effective in decreasing the toxicity and hepatocarcinogeninity caused by these compounds. Therefore, the inhibition of GSTs activity might potentiate the deleterious effects of many environmental toxicants (Sheweita, 1998). It has been reported that the
subcellular distribution of total GSTs activity in rat liver is principally associated with the
cytosol (Casalino et al., 2004). They found that using CDNB as substrate more than 90% of
total GSTs activity is found in cytosol while only 5.9% and 3.5% is present in
mitochondria and microsomes, respectively. Therefore, we examined the effects of lead
on GSTs activity in rat liver cytosol.

As we have shown in the results of this study, the presence of lead caused
decrease in cytosolic GSTs activity of rat liver. The extent of this inhibition was highly
dependent on lead concentration. It is speculated that decreased GSTs activity, evoked by
lead metal, may increase the chance of this metal ions to associate with other intracellular
ligands, and may therefore enhance the deleterious effects of this toxicant. The inhibition
mechanism of the cytosolic GSTs by lead nitrate was investigated in some details with
respect to substrates GSH and CDNB. Lead metal is found to be non-competitive
inhibitor (lower Vmax and unchanged Km) in case of both CDNB and GSH suggesting
that the inhibitory binding site of lead is distant from the catalytic sites leading to
conformational changes and hence enzyme inactivation. In another words, lead does not
compete with CDNB for the CDNB binding site (H-site) neither with GSH for GSH
binding site (G-site) on the enzyme (York, 2002).

Glutathione is one of the most abundant thiol-containing molecules in animal cells
and its role in the protection of the tissues from the toxic effects of xenobiotics and
endogenous electrophilic toxicants has been extensively studied (Sheweita, 1998). The
interaction of metals (such as lead) with glutathione is an integral part of the toxic
response to many metals (Sheweita and Mostafa, 1996). Lead may bind to glutathione
and other thiols and thus decreased cytosolic GSTs activity (Garber, 2002). The
sulfhydryl-reactive lead metal is particularly insidious and can affect a vast array of
biochemical and nutritional processes. It is known that lead and other metals such as Hg
and Cd have the potential to disrupt the metabolism and biological activities of many proteins due to their moderate-high affinity for free sulphydryl (SH) groups of cysteine in proteins and mercaptides are formed with the SH group of cysteine, and less stable complexes with other amino acid side chains (Hsu and Guo, 2002; Gurer and Ercal, 2000) and it is commonly found attached to thiol-containing proteins and small molecular weight thiols. Therefore, inhibition of enzyme activity may be due to lead binding to thiol groups of glutathione, decreasing the amount of glutathione needed to enzyme-activity and hence decreasing enzyme activity (Quig, 1998).

In addition, some proteins become labile as lead binds to them because lead might cause the tertiary structure of the proteins and enzymes to change, thereby altering its functional activity. Alternatively, many authors have suggested that free radical-mediated inactivation of enzymes due to enzyme protein fragmentation (Casalino et al., 2002). In summary, these speculations mentioned above offer an explanation for the results we obtained regarding decreased rat liver cytosolic GSTs activity by addition of lead nitrate.

Previous study done by Sivaprasad et al. (2004) showed that administration of 0.2% lead acetate in drinking water for five weeks to rats decreased cytosolic rat GSTs (-42%) and decreased glutathione (-43%). Also, Sheweita (1998) reported that lead acetate found to inhibit GSTs activity in mice after 1 and 24 hours of administration at a dose of 15 mg/Kg. In contrast, in the same study, he found that GSTs activity increased after repeated doses of lead for 3 consecutive days and he stated that such increase in activity might be an adaptive mechanism of the liver to enhance the GSH conjugation capacity with the accumulated lead ions in order to reduce its toxicity. He concluded from his study that the alteration in GSTs activities is dependent mainly on the period of treatment. Nakahama et al. (2001) found in their study that lead chloride inhibited
cytosolic GSTs activity of rabbit lung and the extent of inhibition depend on the concentration of the metal.

In our study we also examined the in vitro effects of some medicinal plants, which are used as traditional remedies for many ailments in the U.A.E. on rat liver cytosolic GSTs activity in view of studying the biological and pharmacological properties of these plants extracts. Our results revealed that all the plants aqueous extracts used in this study are found to be potent inhibitors of rat liver cytosolic GSTs. However, the potentials of individual plant species are variable.

*Salvia officinalis* (SO) was generally the most potent in the inhibition of tested enzyme activity. This plant is commonly used as a medicinal supplement in various ailments. The results revealed that *Salvia officinalis* inhibited GSTs activity in rat liver cytosol dose-dependent.

The inhibition mechanism of the cytosolic GSTs by SO was investigated in some detail with respect to GSH and CDNB. SO was found to be un-competitive inhibitor with respect to CDNB suggesting that the inhibitory binding site of SO is remote from CDNB binding sites (H-site). Therefore, SO may bind only to enzyme-substrate (ES) form (York, 2002). With regards to GSH, SO was found to be non-competitive inhibitor (lower Vmax and unchanged Km) suggesting that in case of GSH the inhibitory binding site of SO is distant from the catalytic sites leading to conformational changes and hence enzyme inactivation. In other words, SO does not compete with GSH for the GSH binding site (G-site) on the enzyme. This type of inhibition kinetics suggest that components in SO might bind not only to the substrate binding site but also to an additional site that causes loss of enzymes activity. It is also possible for components in SO to interact with other cofactors or substrates in the reaction which are necessary for enzyme activity.
The modulation (inhibitory effects) of GSTs activity as one of xenobiotic metabolizing enzymes might be attributed to one or several compounds present in the plants extracts we used in our study (Debersac et al., 2001). It has been reported previously that SO contains phenolic acids (carnosic, rosmarinic, caffeic and salvianolic acids), tannins, as well as other phenolic structure-based compounds (Chevallier, 1996) in which all possesses hydroxyl groups and this might be important for GSTs inhibition. The mechanism of inhibition of GSTs by plant polyphenols has not been fully explained yet, however it has been suggested that the presence of polyhydroxylations in plant polyphenols is important for GSTs inhibition (Gyampfi et al., 2004b). More recent studies on SO have revealed the presence of a large content of diterpenoids, flavonoids (Lima et al., 2004; Bors et al., 2004; Lu and Foo, 2001; Lu and Foo, 2000).

Another plant tested, the flower heads of *Chamomilla recutita* (CR) used in this study is strongly aromatic and has a bitter taste. The infusion is one of the most popular herbal teas and has been traditionally used as carminative, sedative and tonic (Kobayashi et al., 2005). Our study showed that CR decreased GSTs activity with maximum inhibition of 64% when 200 μL (10 mg/ml) of this plant extract was used. Flavonoids (such as apigenin, luteolin, rutin, spiroethers), coumarins, tannins, and bitter glycosides are the main representative components in polar and medium polar extracts (Macchioni et al., 2004; Chevallier, 1996).

Seeds of *Nigella sativa* (NS) also showed inhibitory effect on GSTs activity when cytosolic fractions were incubated with increasing concentrations of plant's seeds extraction. Seeds of NS are commonly used in folk medicine as natural remedy for variety of illnesses (Meral et al., 2004), particularly to treat headaches, nasal congestion, toothache, and intestinal worms, and in large quantities, as a diuretic, a promoter of menstruation, and to increase breast-milk production (Chevallier, 1996 and Mossa et al.,
1987). NS contains > 30 of a fixed oil, and 0.40-0.45 % w/w of a volatile oil (El-Tahir et al., 1993a). Whether these compounds present in NS contribute to the effect of NS on GSTs we obtained in this study or not, further investigations should be done.

Our study also showed that aqueous extract from the flowers of *Hibiscus sabdariffa* (HS) showed inhibitory effect on GSTs activity. However, this plant extract showed the lowest inhibitory effect on GSTs among all the other tested plants in our studies with maximum inhibition of 25% occurred with 200 μL (10 mg/ml) of the flower extract used. The flower of HS calyx used in this study is commonly consumed as cold and hot beverages (Morton, 1987). As a traditional medicine, it is claimed to be effective against kidney stones and urinary bladder stones, pyrexia, liver disorders. It is reported that HS contains many chemical constituents but one interesting compound in HS that may contribute to the effect we obtained in this study is the anthocyanins (natural polyphenolic pigments extracted from *Hibiscus* dried flowers) which had been demonstrated previously to possess antioxidative and antitumor promoting effects (Linn et al., 2005; Wang et al., 2000). Falade et al (2005) has reported in his study that the tannin content was low in HS clays. The low tannin content in HS flowers compared to tannin content in the other plants extracts tested on GSTs activity could explain in part the low inhibitory effect of this plant compared to the inhibitory effects we obtained from other plants extracts used in this study. These findings presumably suggest that tannin content in the plants could likely play significant role in GSTs activity we tested *in vitro*.

In the present study, it was revealed that exposure of cytosolic GSTs from rat liver to plant aqueous extracts of SO, CR, NS, and HS decreased the activity of GSTs when using the electrophilic substrate CDNB. Although the different classes of GSTs show different active site structures at the GSH binding site, it is known that all GSTs share similar catalytic mechanism where the thiol group of glutathione is activated to thiolate. It
has been reported that the ability of GSTs to catalyze the nucleophilic conjugation of
GSH with electrophiles is attributed to a highly conserved invariant tyrosine residue
located at the GSH binding site which in its undissociated form appears to stabilize the
nucleophilic thiolate anion of GSH (Hubatsch and Mannervik, 2001; Armstrong, 1997).
Moreover, it has been reported by Vega et al. (1998) that the specific sensitivity of the pi-
class GST to inactivation is linked to the oxidation of a reactive thiol, at position 47 in the
amino acids sequence with a concomitant formation of a disulfide. While inactivation of
mouse liver GST-μ by reactive nitrogen species was reported to involve nitration of
individual tyrosine residues, the inhibition of GSTP1-1 by HOCl and calvatic acids were
thought to involve oxidation of cysteine residues in the enzymes (Haalften-van et al.,
2001; Wong et al., 2001). Modification of amino acids residues such as cysteine,
tryptophan and methionine have also been found to be sensitive to oxidation besides
tyrosine and hence inactivation of enzymes such as GSTs (Alvrez et al., 1999). Whether
such modifications contribute to inactivation of cytosolic rat liver GSTs remains to be
examined. A study done by Gyamfi et al. (2004b) showed that Th A (thonningianin A, is
a polyphenolic compound with potent antioxidant action recently isolated from medicinal
plant Thonningian sanguinea) was found to be a non-competitive inhibitor with respect to
CDNB and GSH. Additionally, it was found by Haslam (1996) that SO and CR contain
many tannins. Tannic compounds have the potential to bind proteins, including the GST's
enzyme through hydrogen bond formation causing steric hindrance and hence enzyme
inactivation. It was suggested in previous studies with tannins that the O-dihydroxy
groups in the natural tannin molecule were sites of complex formation (Gyamfi et al.,
2004b; Haslam, 1974).

Previous studies showed in vivo and in vitro effects of other plant species on the
same tested enzyme here. For example, it was shown that dietary administration of water
soluble extract of *Rosmarinus officinalis* (rosemary, an aromatic evergreen herb highly distributed in the Mediterranean region and well known and greatly valued medicinal plant used in pharmaceutical products and folk medicine as a digestive, tonic, astringent, diuretic and useful for urinary ailments) to rats at 0.5% for two weeks enhanced GSTs activity (Mahmoud et al., 2005; Debersac et al., 2001). The work of Yang et al (2003) showed that treatment of male rats for 10 days with 100 mg/kg and 200 mg/kg of *Ginkgo biloba* extract (widely used in traditional Chinese medicinal herb for treatment of cardiovascular diseases) did not affect GSTs activity in liver microsomes but significantly decreased the activity in kidney microsomes. Sasaki and his colleagues (2002) found that *Ginkgo biloba* leaves extract elevated activity of cytosolic GSTs in mouse liver. Another study revealed that the medicinal herb, *Thonningia sanguinea* did not have any significant effect on microsomal GSTs. Gyamfi et al. (2004b) also found that aqueous extract of *Thonningia sanguinea* is a potent inhibitor of liver cytosolic GSTs *in vitro* and it was reported in their study that certain plant phenols, such as ellagic acid, ferrulic acid, caffeic acid, quercetine and chlorogenic acid are *in vitro* inhibitors of GSTs activity from rat liver. Also they reported that ellagic acid and curcumin are *in vitro* inhibitors but *in vivo* inducers of GSTs (Gyamfi et al., 2000). However, Gyamfi et al. (2004a) showed that *in vivo* administration of the same medicinal plant extraction did not have any significant effect on the enzyme except after 6 hours of the plant extract injection (5ml/kg), cytosolic GSTs activity was increased by 30% and authors suggested the possibility that some components of this plant have the potential to increase activity of GSTs. Based on the previous mentioned works and studies, we can conclude that *in vivo* effects of a plant extract might be different from their *in vitro* potentials.
5.1.3 NAD Synthetase (NADS) Activity

NADS catalyzes the final step in NAD⁺ biosynthesis in the cell. NAD⁺ is essential for life in all living cells (Bieganowsk and Brenner, 2004). Impaired rate of NAD⁺ synthesis in erythrocytes was reported in some disorders (e.g. pyruvate kinase deficiency and thalassemia) (Sakai et al., 1997). Our experimental data showed clearly that the formed NAD⁺ is completely separated (at 13 minutes) from the substrates (NAAD and ATP) and microsome components in the present HPLC system (Figure 3.1). Our results showed that lead nitrate decreased NADS activity (dose-dependent) in microsomal fractions from rat liver. When microsome fractions were incubated with 0.036 mM lead nitrate, NADS activity was inhibited by 13%. During experimental work, we started with lead nitrate concentrations less that 0.036 mM but no significant differences in inhibition percentage were observed compared to 13% inhibition. With 0.06 mM of lead, a sharp increase in enzyme inhibition occurred. Then with increasing lead concentrations, the inhibitory effect increased. When we used 0.6 mM of lead, no peak was formed at the corresponding retention time of NAD⁺ (at 13 minutes) in HPLC chromatogram profile (as the end product of the reaction catalyzed by NADS). This indicates that lead nitrate caused 100% enzyme inhibition. Zerez et al. (1990) were the first to report that NADS activity was markedly decreased in three workers exposed to lead. Also it was reported that NADS activity was reduced in erythrocyte from lead exposed subjects and dose-effect relationship of NADS activity versus blood lead concentration (Pb-B) was demonstrated in this study (Morita et al., 1997).

It is known that lead metal has the potential to disrupt the metabolism and biological activities of many proteins due to their moderate to high affinity for free sulphydryl groups of cysteines in proteins. The inhibition of NADS activity may be related- in part- to the high affinity of lead to the sulphydryl groups of cysteines in NADS
enzyme and hence enzyme inactivation as discussed previously. A number of recent studies also confirmed the possible involvement of ROS in lead-induced toxicity (Flora et al., 2003). It is therefore speculated that the toxic action produced by lead might be attributed to its ability to generate ROS which induce the NADS inactivation effect observed in our study. These ROS cause damage to the biomolecules such as proteins, enzymes, membrane lipids, pigments, nucleic acids, etc. As it has been suggested by Casalino et al. (2002) that free radical-mediated inactivation of enzymes may be due to enzyme protein fragmentation. In summary, these speculations offer explanations for the results we obtained regarding decreased microsomal NADS, CYP2E1, and cytosolic GSTs activities in rat liver caused by lead nitrate.

In this study, we tested increasing concentrations of plants extracts on NADS activity; some plants did not show significant results using higher concentrations and some plants extract showed increase in NADS activity when used at higher concentrations. Therefore, middle concentration (20 μL) was used and presented here in the study. No significant changes observed in NADS activity when we used 20 μL (50 mg/ml, 25 mg/ml in case of Chamomilla recutita) of each plant extract except for Hibiscus sabdariffa which caused decrease in NADS activity (11% inhibition, P-value <0.05). Such decrease in enzyme activity may be due to some compound in the plant that can inhibit enzyme activity in vitro. More experimental works needed to be done to confirm such observation. When two concentrations (20 and 100 μL from 5% extraction, 50 mg/ml; 2.5% extraction, 25 mg/ml in case of Chamomilla recutita) of each plant extract were chosen to test if these extracts have the capacity to ameliorate the decrease (antagonistic effect) in the enzyme activity caused by lead nitrate, a significant recovery occurred in enzyme activity and the four plant extracts can evoke significant prevention - at least partly- for rat liver microsome NADS activity from damage or inactivation caused
by lead. It is obvious from the data we obtained here that incubation of microsome with
the four plants (20 and 100 µL from 5% extraction, 50 mg/ml; 2.5% extraction, 25 mg/ml
in case of Chamomilla recutita) prior to lead nitrate addition prevented the enzyme
inactivation caused by lead metal and this effect was concentration dependent of the plant
extract we used and as was shown in the results that 100 µL (50 mg/ml) of Nigella sativa
used brought the enzyme activity nearly back to normal (+160%). However, using 100 µL
(50 mg/ml) of Hibiscus subdariffa extract decreased enzyme activity (-67.5%) and this
result may be related to the decrease of NADS activity when we used only 20 µL of the
same plant as shown previously. Chamomilla recutita and Nigella sativa have the highest
protection activity among the other studied plants against lead-induced NADS
inactivation. It should be noticed that in case of Chamomilla recutita we used only half
the concentration (25mg/ml) compared to the other studied plants, so we can considered
this plant to have the highest protection activity. The protective and improved effects of
the plants extracts on lead-induced NADS inactivation may be due- in part- to the
antioxidant properties of the four plants extracts (Salvia officinalis, Nigella sativa,
Hibiscus subdariffa, Chamomilla recutita) and the presence of antioxidants and ROS
scavenging properties by plants extracts might have played a role in the modulation of
NADS activity and may contribute to the inhibition of the suggested reactive free radicals
produced by lead metal, hence, reversing enzyme inactivation (Flora et al., 2003). Also,
the protective and improved effects of these plants extracts on lead-induced NADS
inactivation could be due to the ability of the plants extracts to chelate lead metal as they
form an insoluble complex with lead and therefore protect the enzymes damage. For
instance, it has been shown that ascorbic acid has protective effects against lead
intoxication and its beneficial effects could be attributed to its ability to complex with
lead (Flora and Tandon, 1986). It is likely that the reversion of NADS enzyme activity by
plants extracts could be attributed either to a direct effect of plants on enzyme or to antagonistic effect of some compounds present in these plants to NADS inhibition.

5.1.4 Total Phenolic and Flavonoid Content in Plants Extracts

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Maisuthisakul et al., 2006-in press). Flavonoids are phenolic compounds known to be very effective antioxidants (Yanishlieva-Maslarova, 2001). The Folin-Ciocalteu method is a rapid and widely used assay, to investigate the total phenolic content but it is known that different phenolic compounds have different responses in the Folin-Ciocalteu method (Maisuthisakul et al., 2006- in press). Therefore, in this work, we measured the total phenolic contents in units of mg gallic acid equivalent (GAE) of phenolic compounds. The total phenolic content of the four selected plants extracts ranged from 28.6 to 76.4 mg GAE/g dry weight of plant extract. Based on total phenolic content in the extracts, the four plants extracts can be divided into three ranges of GAE values. The lower, middle and higher ranges of total phenolic compounds were below 10, 10-20 and higher than 40 mg GAE/g dry weight of plant extract, respectively (Maisuthisakul et al., 2006- in press). Plants with higher total phenolic content include Salvia officinalis, Hibiscus sabdariffa and Nigella sativa. The total flavonoid content of Salvia officinalis was remarkably high and was the highest in the group of plants in our study compared to those obtained from other plants extracts (7.6-21.5 mg QE/g dry weight of plant extract). Each plant extract contained lower total flavonoid content than the total phenolic content, since other compounds besides flavonoids are phenolic substances present in plants as well. It can be noticed that plants extracts with a higher phenolic content also contained a higher flavonoid content as was apparent for Salvia.
officinalis (28.1 mg QE/g dry weight of plant extract). However, extracts with higher phenolic content did not always have a higher flavonoid content, as was evident for the seeds of Nigella sativa which had the lowest total flavonoid content (7.6 mg QE/g dry weight of plant extract) among the four studied plants, although the total phenolic content was high (40.7 mg GAE/g dry weight of plant extract). The results suggest that different plants extracts contain different levels of total flavonoids as a proportion of the total phenolic compounds.

5.1.5 DPPH Free Radicals Scavenging Activity

Since the main mechanism of antioxidant action in food is radical scavenging, many methods have been developed in which the antioxidant activity is evaluated by the scavenging of synthetic radicals in polar organic solvents such as methanol at room temperature (Gordon, 2001). The radical used in this study was DPPH and this method was selected in this study since it is one of the most effective methods for evaluating the radical-scavenging materials acting by a chain-breaking mechanism (Maisuthisakul et al., 2006- in press).

DPPH is known to abstract labile hydrogen and the ability to scavenge the DPPH is related to the inhibition of lipid peroxidation (Gyamfi et al., 1999). In this study, we tested the DPPH radical scavenging action of the plants extracts. The experimental data we obtained in our study revealed that the selected plants, which are used for treating various ailments, have the capacity to scavenge DPPH free radicals. The results of scavenging activity of the extracts were compared with reference compound BHT. BHT showed 54% of scavenging activity against DPPH. Chamomilla recutita showed scavenging activity of 63.1% scavenging activity and considered to be the highest in scavenging activity among other studied plants since using only half plant concentration
(2.5% extraction, 25 mg/ml) gave 63.1% scavenging activity. Since phenolic compounds are reported in many studies to be responsible for antioxidant activity and as they related the measurements of phenols in plants extracts to their antioxidant properties (Katalinic et al., 2006) Chamomilla recutita, however, had the lowest phenolic contents among all the plants extracts studied with good flavonoid content. Therefore, the antioxidant capability of this medicinal plant could be due to factors other than phenolic compounds. Nigella sativa extract (0.5 ml, 50 mg/ml) demonstrated to have the highest scavenging activity (64.5%) among the other three selected plants (5% extraction, 50 mg/ml) and even higher than scavenging activity of BHT (1.5 mg/L). As presented previously, it has been shown that Nigella sativa extract contained the lowest content of flavonoid compared to those from other studied plants and it contains high content of phenolic compounds and this may be related to suggestion that phenolic compounds other than flavonoid may contribute to free radicals scavenging activity of this plant. Earlier study done by Burits and Bucar (2000) showed that Nigella sativa essential oil and its four constituents (thymoquinone, carvacrol, 1-anethol and 4-terpineol) had antioxidant effect in different chemical assays, such as DPPH assay for non-specific hydrogen atom or electron donating activity. It should noted that Salvia officinalis extract had both the highest phenolic content and flavonoid content among all the plants investigated, and showed 53.25% scavenging activity of free radicals. Hibiscus sabdariffa showed the lowest scavenging activity with high phenolic content and low flavonoid content compared to other plant extracts studied. It has been reported that flavonoids, tannins, ascorbic acid, tocopherol, and aromatic amines reduce and decolorize DPPH by their hydrogen donating ability (Kumaran, and Karunakaran, 2005-in press). Beside the previously studies and reports which indicate that phenolic compounds in the plants are probably involved in their scavenging activity against DPPH free radical, from our experimental data in this
regard we can conclude that other factors (or compounds) may play a role in the antioxidant capability of the selected plants in this study. We have found that both \textit{Chamomilla recutita} and \textit{Nigella sativa} have the highest protection activity and this could be explained by their high free radical scavenging activity.
5.2 RECOMMENDATIONS & FUTURE RESEARCH

Taking together the results we obtained from this study, we recommend that additional studies regarding the effects of these medicinal plants on tested enzymes activities to be done in vivo to confirm and extend these results to include more additional information on the possible mechanisms actions of these plants. Furthermore, molecular separation and the effects of individual bioactive constituents of these medicinal plants aqueous extracts are of great value to characterize detoxification natural compounds.

The elevation of cellular GSH and the increased expression and activity of GSTs have been implicated in the development of resistance of cells and organisms towards drugs, pesticides, herbicides and antibiotics (Gymfli et al., 2004b). In particular the over expression of GSTs in tumor cells to clinically important anticancer drugs such chlorambucil, melphalan, nitrogen mustard and nitrosourea has served as a stimulus to the search for non-toxic GSTs inhibitors to improve cytotoxic drugs in the treatment of tumors (Morrow et al., 1998; Hayes and Pulford, 1995).

Our results in this study revealed that Salvia officinalis, Chamomilla recutita, Nigella sativa, and Hibiscus subdariffla are potent in vitro inhibitors of rat liver cytosolic GSTs activity. It is suggested that these plants have the potential as GSTs inhibitors and could be used in cancer drug efficacy studies where multi-drug resistance is related to over expression of GSTs and as a chemoprotective agent against GST-induced toxicities. Therefore these plants and their active constituents may be good candidates for investigation for use in cancer chemo-modulation. Besides GSTs, the glutathione-conjuate efflux pump, P-glycoprotein has been implicated in the resistance in cancer chemotherapy (Marrow et al., 1998). So, the effects of SO, CR, NS, and HS on this multi-drug resistance protein would be also interested to examine. This is a major area for future research to clarify the paradoxical role of GSTs in protection against degenerative
diseases such as cancer on the one hand and in the development of transitional cell carcinoma and its role in cellular resistance to chemo-therapy on the other.

It would be of great interest to explore whether plants extracts used in this study can protect against carcinogenesis. Obtaining more detailed information about plants chemical compositions could perhaps allow us to identify the compound(s) that account for the effects of plants extracts we observed on GSTs activity. But whatever beneficial effects the plants bring in rats, the efficacy as well as the safety of plants use with promising chemo-preventive potential should be always further confirmed.

In the present study also we suggest that aqueous extracts of the selected medicinal plants are a potential source of natural antioxidants and may even be better antioxidants than known synthetic antioxidant such as BHT. The results obtained are promising and therefore further studies are necessary for the isolation and identification of individual active compounds and also in vivo studies are needed for better understanding of their mechanisms of action as an antioxidant.
5.3 CONCLUSION

In conclusion, the present study achieved the purpose for which it was conducted. Our results revealed that lead, as a representative example of common heavy metal, is found to be a potent inhibitor for all the enzymes studied here and the extent of such inhibitory effect depends on the lead concentrations used and enzyme sensitivity to such pollutant. All the medicinal plants selected in this study reversed the decreased in CYP2E1 activity caused by lead nitrate. Our results showed that increasing concentrations of each plant extract can evoke significant prevention for rat liver microsome CYP2E1 activity from damage or inactivation caused by lead nitrate. Also our study revealed that SO, CR, NS, and HS are potent in vitro inhibitors of rat liver cytosolic GSTs activity. With regard to NADS activity, we found that aqueous extracts of selected plants ameliorated the decrease in enzyme activity caused by lead nitrate.

In addition, we found that the four plants contain different levels of phenolic and flavonoids compounds and we suggested that such compound may play the role in the results we obtained regarding reversing the decrease in enzymes activities of CYP2E1 and NADS and GSTs inhibition caused by lead. Finally, the results revealed that all the plants are found to have free radical DPPH scavenging activity which can be a potential source of natural antioxidants.
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نوع من النباتات المستخدمة كان له القدرة على تقليل من تأثير الأنزيم السلبي (تتبيط نشاطها) على إنزيم CYP2E1.

مجموعة الأنزيمات GST أظهرت تأثيرها بالنباتات الطبية عندما تم اختيار زيادة تركز كل نبات على حدة على نشاط هذا الأنزيم حيث أظهرت نشاطًا مثبطًا لهذا الأنزيم وكانت نسبة تثبيط هذا الأنزيم مختلفة باختلاف نوع النبات المستخدم وتركيز النبات المستخدم.

أما نشاط إنزيم NAD في التفاعل قد انفصلت من مادة الركيزة والمواد الأخرى الموجودة في التفاعل عند NADS تنتج عن نشاط NADS في الانتهاج (Rt. 13 دقائق) وقت الانتهاج 13 دقيقة. أثبتت الدراسة أن النباتات المستخدمة كان لها القدرة على تقليل الأثر السلبي (المنع أو الكبح) للرصاص على هذه الأنزيمات عند استخدام تركيز قليل وتركيز عالي من كل نبات. ولكن لم تظهر هذه النباتات أي تأثير على نشاط الإنزيم عند اختيارها بدون مادة الرصاص بعده عند استخدام ثلاث نبات الكركديه الذي قل من نشاط الأنزيم (11%) وهذا التأثير يمكن أن يكون بسبب بعض المركبات الأساسية الموجودة في النبات وله القدرة على تقليل نشاط الأنزيم.

مجموع مركبات الفلانولات والفلافونولات والبروتين تم تحديدها في جميع النباتات المستخدمة وأظهرت النتائج أن جميع النباتات تحتوي مركبات الفلانولات (28.6-76.4 ملغم/جرام من وزن النبات المستخدم) وتحوي أيضاً الفلانولات (1.2-7.6 ملغم/جرام من وزن النبات المستخدم).

أظهرت الدراسة أيضاً قدرة النباتات المستخدمة على التخلص والقضاء على الجذور الحرة من مادة DPPH ويمكن أن يرجع هذا الأثر (جزئياً) لوجود مواد الفلانولات والفلافونولات في هذه النباتات و التي تم تحديدها في هذه الدراسة وهذا يمكن أن يكون له علاقة بنتائج هذه الدراسة المتعلقة بسمية الرصاص ونشاط الأنزيمات.
الملخص العربي

تعرض جميع الكائنات الحية ومن ضمنها الإنسان لمواد كيميائية وأجسام غريبة بشكل مستمر. وهذـاء المواد تتضمن مواد مصنعة مثل الملوثات الكيميائية ومـواد طبية مثل نواتج بعض النباتات. يعتبر الرصاص واحد من هذه الملوثات التي تتنـذ بالخطر لأنه ينتج باستمرار من عوامل المحركات وموجود في معلـبات الطعام المحفوظ و من أهم المعادن القليلة الملوثة الموجودة في الهواء الذي تم توثيق أثره السلبي في كثير من الدراسات السابقة على الجهاز العصبي وعلى كفاءة عمل كثير من الإنزيمات في الجسم. يوجد بعض المصادر الطبيعية مثل النباتات الطبية التي يمكن أن يكون لها تأثير مضاد لـنسبة هذه الملوثات ومن ضمنها مادة الرصاص. كثير من النباتات الطبية تستخدم كعلاج شعبي لـ كثاـر من الأمراض بدون معرفة تفاعـلها البيوكيميائي على المستوى الجزيئي.

إن الهدف من هذه الدراسة هو اختيار سمية مادة الرصاص باستخدام فصل الأنظمة اللاخلوية على نشاط الإنزيمات معينة وهي الإنزيمات التي تعمل على بناء المركبات الحيوية (إنزيمات دورة الأكسدة و الانتقال) و الإنزيمات التي تعمل على التخلص من المواد السامة والملوثات في الكبد (إنزيمات التحلل الحيوي). بالإضافة لذلك، اختيار إمكانية وجود تأثير مضاد لبعض النباتات الطبية المعروـوف استخدامها محليا على سمية مادة الرصاص على هذه الإنزيمات. كـ ذلك قـيم الإنزيمات الحركية (Vmax، Km) المتعلقة بـ ارتباط المادة (الركيزة) الكيميائي واختيار تثبيط (منع) أو تنشيط عمل هذه الإنزيمات.

تم في هذه الدراسة اختيار ثلاثة مجموعات من إنزيمات لـها دور فعال ومهم في الجسم لأجراء التجارب. تم اختيار إنزيم (CYP2E1) لـ تمثيل مرحلة المرور 1 و مجموعة إنزيمات (GSTs) لـ تمثـيل مرحلة المرور 2 من إنزيمات التحلل البيولوجي في الكبد. أما إنزيم (NADS) في تم اختياره لـ تمثيل الإنزيمات التي تعمل على بناء المواد البيوكيميائية (إنزيمات دورة الأكسدة و الانتقال). تم في هذه الدراسة اختبار 4 نباتات طبية التي لها استخدامات شائعة كعلاج شعبي في دولة الإمارات العربية المتحدة. وهي أوراق نبات المرومية، نبات الكركم، نبات البابونج، ونباتات الحبة السوداء.

أظهرت نتائج هذه الدراسة أن مادة الرصاص نبتت عمل ونشاط الإنزيمات الثلاثة التي تم اختيارها وأجزاء التجارب عليها (باستخدام جسم الـأنظمة اللاخلوية) و كانت نسبة تثبيط نشاط كل نوع من الإنزيمات مختلفة بينهم معتمدة على حساسية الإنزيم وتركيز الرصاص المستخدم. كما أثبتت الدراسة أن زيادة تركيز كل
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