LEBANESE AMERICAN UNIVERSITY

Anticancer and Antioxidant Effects of *Tragopogon porrifolius* extract

By

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in

Molecular Biology

School of Arts and Sciences

Spring 2011

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Thesis approval Form (Annex III)

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Program : M.S. in Molecular Biology

Division/Dept : Natural Sciences Department

School : **School of Arts and Sciences**

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ACKNOWLEDGEMENTS

First, I'd like to express my most sincere gratitude to my advisor, Dr. Constantine Daher, and my committee members, Dr. Mirvat El-Sibai and Dr. Mohammad Mroueh, for their constant support, guidance and encouragement throughout my research.

I'm genuinely thankful to Dr. Mirvat who went above and beyond her role as a mentor, and became a source of inspiration and a friend.

I would also like to thank Dean Fuad Hashwa, as well as all my professors at the Lebanese American University, Dr. Roy Khalaf, Dr. Sima Tokajian, Dr. Ralph Abi-Habib, Dr. Brigitte Wex, and Dr. Pierre Zalloua for sharing their knowledge and expertise.

I'd like to express my appreciation to Ms. Maya Farah and Ms. Helena Bou Farah for their continuous presence in the labs and their technical assistance, and to Mr. Jean Karam for his immense help in the in vivo portion of my project. I couldn't have done it without you.

I would have never made it to graduation without the most incredible group of friends. I thank all my friends here at LAU for sharing the joys and miseries of being stuck in the lab, and my friends outside LAU for being the best friends anyone could ask for and for helping me maintain my sanity.

Finally, I would like to thank my amazing family for their unrelenting support of my goals and ambitions; and I dedicate this thesis to my brother who made my dream of attending graduate school a reality.

Anticancer and Antioxidant Effects of *Tragopogon porrifolius* extract

Clara A. Tenkerian

ABSTRACT

In recent years, the medicinal properties of plant-derived products have come under extensive investigation, and plants with antioxidant properties are considered as potential therapeutic tools against cancer and other diseases. This study investigates the antioxidant activity of *Tragopogon porrifolius* methanolic extract, both in vitro and in vivo; as well as assessing its effects on CCl_4-induced hepatotoxicity in rats. It also explores the antiproliferative activity of this extract against breast (MDA) and colorectal (CaCo-2) adenocarcinoma cell lines. Total phenolic and flavonoid contents were calculated using the Folin-Ciocalteu and the aluminum chloride colorimetric methods and found to be 36.96 ± 1.39 mg GAE/g and 16.56 ± 0.42 mg QE/g dry weight respectively. In vitro antioxidant activity, assessed using the FRAP assay, was determined to be 659.57 ± 13.77 μmol Fe^{2+}/g of extract. The IC_{50} using the DPPH assay was calculated to be 15.18 μg/mL. In rats subjected to CCl_4-induced hepatotoxicity, significant in vivo antioxidant activity was detected with increased levels of catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) liver antioxidant enzymes; the highest dose of the extract (250 mg/kg) recorded a fold increase of 1.68 for SOD, 2.49 for GST and 3.2 for CAT compared to the DMSO vehicle group. The extract also showed substantial hepatoprotective capacity by greatly reducing aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels with the 250 mg/kg dose recording AST, ALT and LDH levels by 56.89%, 56.45% and 64.65% respectively. Finally, measuring the effects of the extract on cell viability and proliferation by the Trypan Blue exclusion method and the WST-1 cell proliferation assay both revealed a dose-dependent inhibition of cell proliferation and increased cell death. In conclusion, the methanolic extract of *T. porrifolius* showed evidence of antioxidant activity both *in vitro* and *in vivo*, as well as an anticancer effect against MDA and Caco-2 cell lines. It also exhibited a hepatoprotective potential against liver toxicity in rats.

Keywords: *Tragopogon porrifolius*, Reactive Oxygen Species, Antioxidant, Anticancer, Hepatoprotective

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LIST OF ABBREVIATIONS

CAT: catalase

SOD: superoxide dismutase

CuZnSOD: cytosolic superoxide dismutase

MnSOD: mitochondrial superoxide dismutase

EC-SOD: extracellular superoxide dismutase

GST: glutathione-S-transferase

GPx: glutathione peroxidase

GSH: reduced glutathione

AST: aspartate aminotransferase

ALT: alanine aminotransferase

LDH: lactate dehydrogenase

GAE: Gallic Acid Equivalent

QE: Quercetin Equivalent

FRAP: Ferric Reducing/Antioxidant Power

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

ROS: Reactive Oxygen Species

$O_2^{\bullet-}$: superoxide radical

H_2O_2 : hydrogen peroxide

$^{\bullet}OH$: hydroxyl radical

DNA: deoxyribonucleic acid

BHT: Butylated hydroxytoluene

CCl_4: carbon tetrachloride

MAPK: Mitogen Activated Protein Kinase

8-OH-dG: 8-hydroxy-2'-deoxyguanosine

EAE: experimental autoimmune encephalomyelitis

MS: multiple sclerosis

CNS: Central Nervous System

VLB: vinblastine

VCR: vincristine

VRLB: vinorelbine

VDS: vindesine

DMEM : Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

DMSO: dimethyl sulfoxide

Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

ELISA: Enzyme-Linked Immunosorbent Assay

TPTZ: 2,4,6-tripyridyl-s-triazine

CDNB: 1-chloro-2,4-dinitrobencene

SEM: standard error of the means

i.p.: intraperitoneal

METP: methanolic extract of *T. porrifolius*

CHD: Coronoary Heart Disease

Chapter 1

INTRODUCTION

1.1 Cancer:

The American Cancer Society defines cancer as "a group of diseases characterized by uncontrolled growth and spread of abnormal cells". Carcinogenesis is a multistage process that often occurs over the sequence of many years (Garcia et al., 2007). Cancer is the primary cause of death in developed countries and the second in developing countries; and despite extensive research into its causes and potential new treatment strategies, cancer remains a leading cause of death globally, after cardiovascular disease (Jemal et al., 2011). Moreover, lifestyle changes and an increasingly aging population are leading to elevated cancer rates worldwide. Studies indicate that the majority of cancers are greatly influenced by environmental factors such as smoking, consumption of alcohol, and diet. They are thus attributed more to lifestyle rather than hereditary factors (Anand et al., 2008).

Oncogenesis results from mutations in several genes which can be classified into three catagories: proto-oncogenes, tumor-suppressors and stability genes. Proto-oncogenes become permanently active and thus oncogenic upon their mutation. Conversely, tumor suppressor genes lose their activity when mutated. Given that stability genes regulate functions such as DNA repair and chromosomal segregation, mutations in these genes lead to higher mutation rate in the genome (Furney, Higgins, Ouzounis, & Lopez-Bigas, 2006). Futreal et al. conducted a census from the literature to compile a list of "cancer genes" or genes in implicated in oncogenesis. At the time of the study, 291 genes had been reported in the literature, which constitutes over 1 % of all genes in the human genome. Chromosomal translocations were found to be the most common mutation class and the protein kinase to be the most common domain among the known cancer genes (Futreal et al., 2004).

1.1.1 Colorectal cancer:

Colorectal cancer is one of the most commonly occurring cancers on a global scale and is the second major cause of cancer-related deaths in the United States (Center, Jemal, & Ward, 2009a; Center, Jemal, Smith, & Ward, 2009b; El Zouhairi, Charabaty, & Pishvaian, 2011). Among the major risk factors observed are a personal or family history of colorectal cancer and/or polyps, chronic inflammatory bowel disease, obesity, heavy alcohol consumption, smoking, lack of physical activity and dietary patterns low in fruits and vegetables and high in red or processed meat (Center et al., 2009a; Center et al., 2009b; Garcia et al., 2007). It has been shown to be very responsive to diet modification (Gajula et al., 2009). Malnutrition is also common in patients suffering from colorectal cancer, effecting their recovery and performance after receiving treatment (Y. Chen et al., 2011).

Colorectal cancer is treated most commonly by surgery, which may be curative if the cancer has not yet spread. If the tumor has penetrated the bowel wall deeply or spread to lymph nodes, patients also receive chemotherapy, sometimes combined with radiation (Garcia et al., 2007).

The Caco-2 cell line, originally derived from a human colon adenocarcinoma, differentiates spontaneously in culture to form a monolayer of cells that express many of the functional and morphological characteristics of a mature enterocyte and as such can be used as an in vitro model for drug absorption and metabolism studies (Hilgers, Conradi, & Burton, 1990; Sambuy et al., 2005). Since the initial isolation of the parental cell line, several clonal cell lines which do not always express all the same characteristics have also been established and used extensively over the past few decades (Sambuy et al., 2005).

1.1.2 Breast cancer:

Breast cancer is the most commonly diagnosed cancer in women and its incidence has been increasing steadily since the 1940s; it accounts for 23% of the total cancer cases

(Jemal et al., 2011). Despite the decline in the mortality rates of breast cancer, given early detection and more advanced therapies, this malignancy remains the leading cause of cancer-related death in women, accounting for 14% of all cancer deaths (DeBruin & Josephy, 2002; Jemal et al., 2011). The risk factors associated with breast cancer can be categorized into three main groups, namely hereditary, hormonal and reproductive, and environmental (DeBruin & Josephy, 2002). Inheritance of the BRCA1 or BRCA2 genetic mutations, a family history of breast cancer, being overweight and/or the use of hormonal therapy after menopause, lack of physical activity and excess alcohol consumption are among the most important risk factors associated with breast cancer (Garcia et al., 2007). Moreover, a twin cohort study published in the New England Journal of Medicine attributed 73% of breast cancer cases to environmental causes (Lichtenstein et al. 2000). Nutrition is also hypothesized to be an important environmental determinant (Holmes & Willett, 2004).

Treatment of breast cancer depends on tumor size, stage at diagnosis and other clinical characteristics, as well as patient preference. Chemotherapy, radiation and hormone therapy are often used in combination with surgical intervention which could be in the form of either a lumpectomy (removal of the tumor with clear margins) or a mastectomy (removal of the entire breast). The stage of the tumor at diagnosis is the most important variable effecting prognosis and survival rate (Garcia et al., 2007).

The MDA cell line is a human breast adenocarcinoma cell line used as an *in vitro* model of breast carcinoma.

1.2 Reactive Oxygen Species:

1.2.1 Defining reactive oxygen species:

Any species capable of existing independently and containing one or more unpaired electrons is referred to as a free radical (Halliwell & Gutteridge, 2006). This unpaired electron is usually responsible for the high reactivity of the free radical (Valko et al., 2007; Miller, Buettner, & Aust, 1990). Oxygen free radicals or reactive oxygen species (ROS),

including the superoxide radical $O_2^{\bullet-}$, hydrogen peroxide H_2O_2, and the highly reactive hydroxyl radical $^{\bullet}OH$, derive from the metabolic reduction of molecular oxygen and are considered important signaling molecules in various pathways involved in the proliferation of cells, as well as in the induction of apoptosis (Hasan et al., 2010). Although not a free radical itself, H_2O_2 is involved in the pathway of formation of other radicals and hence considered a ROS (Vera-Ramirez et al., 2011; Waris & Ahsan, 2006). The ROS are extremely reactive and can oxidize lipids, proteins, and even DNA. Increased production of ROS and the resulting oxidative stress have thus been associated with the onset and progression of many diseases, including cancer (Hasan et al., 2010).

1.2.2 Physiological and pathological roles of free radicals:

The mitochondria are the major endogenous source of reactive oxygen species which when in equilibrium, play an important role in the regulation of physiological functions. Among these useful biological functions are their role in inflammatory responses against infectious agents and their involvement in various signaling cascades involved in maintaining cellular homeostasis. During inflammation, macrophages and neutrophils exhibit increased oxygen consumption and the resulting $O_2^{\bullet-}$ and H_2O_2 play an important role in the first line of defense against pathogens (Valko et al., 2007; Vera-Ramirez et al., 2011; Manda, Nechifor, & Neagu, 2009; Waris & Ahsan, 2006).

The Mitogen Activated Protein Kinase (MAPK) family is one of the protein families regulating ROS activated signal transduction pathways. The MAPK pathways respond to a wide range of stimuli and are involved in the regulation of gene expression, proliferation, mitosis, metabolism, mitosis, and apoptosis. Therefore, ROS may lead to both proliferation and death of cancer cells, depending on the exogenous and endogenous environment (Manda et al., 2009; Waris & Ahsan, 2006).

However, when the tight regulation maintaining equilibrium between free radicals and the antioxidant defense system is compromised, either due to the excess production of ROS or the down-regulation of antioxidants, it results in oxidative stress which has been

shown to be involved in the initiation and progression of various human diseases, including cancer (Evans, Dizdaroglu, & Cooke, 2004; Waris & Ahsan, 2006).

1.2.3 Free radicals and cancer:

DNA damage induced by free radicals is well documented and widely accepted as a major cause of genomic instability and cancer. Human studies have shown that oxidative DNA damage is an important carcinogenic and mutagenic factor in the sense that it favors the acquisition of mutations and contributes to cellular transformation and cancer cell survival (Waris & Ahsan, 2006).

Oxidative lesions are induced in the genetic material by both exogenous and endogenous free radicals; if not efficiently repaired, these will lead to mutations and the subsequent deleterious effects. For instance, the hydroxyl radical tends to interact with double bonds of DNA bases generating modified bases, DNA strand breaks and aberrant DNA-protein interactions (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004; Vera-Ramirez et al., 2011).

The formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) adducts, which induce errors in the reading frame during replication, is one of the well-known consequences of DNA exposure to $OH^{\bullet}$. The damage in the deoxyribose molecules also interferes in the activity of DNA polymerase and DNA ligase enzymes. Such modifications have been detected in oncogenes and tumor suppressor genes, and it is assumed that reactive oxygen species are involved in both the initiation and the progression of cancer (Vera-Ramirez et al., 2011; Waris & Ahsan, 2006).

Elevated levels of 8-OH-dG adducts are reported to play a role both in breast cancer (Malins & Haimanot, 1991) and in hepatocellular carcinoma (Ichiba et al., 2003). Lungs from cigarette smokers also have a two to three fold elevation in 8-OH-dG (Olinski et al., 1992).

In addition, levels of the antioxidant enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) were reported to be elevated in

colorectal cancer tissues (Kanbagli et al., 2000). SOD and GPx levels have been found to be elevated in breast cancer tissue as well (Punnonen et al., 1994).

1.3 The Oxidative balance:

1.3.1 The antioxidant defense system:

Exposure to higher than normal concentrations of oxygen has deleterious consequences in all aerobes and although ROS exist in all aerobic cells, they are normally in a balance with antioxidants which help maintain lower levels of free radicals, thus allowing them to perform their beneficial physiological roles (Halliwell & Gutteridge, 2006; Waris & Ahsan, 2006).

Antioxidants are a class of plant secondary metabolites that improve the body's cellular defense system against oxidative damage. Although synthetic antioxidants such as butylated hydroxytoluene (BHT) are available, most lead to toxic side effects like liver damage and mutagenesis. Consequently, there has been an increased interest in the search for natural antioxidants in recent years. Numerous studies have reported that the intake of fruits and vegetables, which have been shown to be rich in antioxidants, plays an important role in the prohibition of free radical-associated diseases and decreases the incidence of heart disease and cancer in humans. These beneficial effects of plants have been ascribed to the polyphenolic compounds they contain (El-Far & Taie, 2009; Sahgal et al., 2009; Winkler, Wirleitner, Schroecksnadel, Schennach, & Fuchs, 2005).

The antioxidant defense system of the body can be in the form of low molecular weight antioxidants such as Vitamins E and C which block free radicals, or in the form of enzymes such as superoxide dismutase, catalase, and the glutathione system (glutathione, glutathione reductase, peroxidase and transferase) that constrain the levels of reactive oxygen species (Evans et al., 2004; Manda et al., 2009). However, even though this system attenuates the harmful effects caused by free radicals, the damage accumulates over the years and is implicated in the development of age-related disorders (Vera-Ramirez et al., 2011).

1.3.2 The major antioxidant enzymes:

As shown in Figure 1.1, maintaining the oxidative balance and preventing oxidative stress requires the cooperation of several antioxidant enzymes (Halliwell, 2006). Overproduction of the superoxide radical $O_2^{\bullet-}$ is believed by many scientists to be one of the major causes of oxygen toxicity (Fridovich, 1995). Superoxide dismutase enzymes (SODs) catalyze its dismutation (reaction 1), whereby one is oxidized to form O_2 and another reduced into H_2O_2 (Valko et al., 2004).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \textbf{(1)}$$

SODs must thus work simultaneously with other enzymes to remove H_2O_2, which would otherwise undergo the "Fenton reaction" (reaction 2) and produce the very reactive hydroxyl radical (Halliwell, 2006).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \quad \textbf{(2)}$$

Catalases and glutathione peroxidases are two enzymes responsible for this function. Catalases break down hydrogen peroxide into water and oxygen (reaction 3), whereas glutathione peroxidases reduce it to water by coupling it to the oxidation of reduced glutathione (GSH) which is a thiol-containing tripeptide (reaction 4). Glutathione reductase enzymes eventually convert back the oxidized glutathione (GSSG) formed by this reaction to GSH (Rhee, Chae, & Kim, 2005; Halliwell, 2006).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad \textbf{(3)}$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad \textbf{(4)}$$

It is important to note however that catalases are not abundant in the mitochondria where substantial amounts of the superoxide radical are generated; rather they are mostly present in peroxisomes where they catalyze the breakdown of H_2O_2 produced by oxidative enzymes (Halliwell & Gutteridge, 2006; Schrader & Fahimi, 2004).

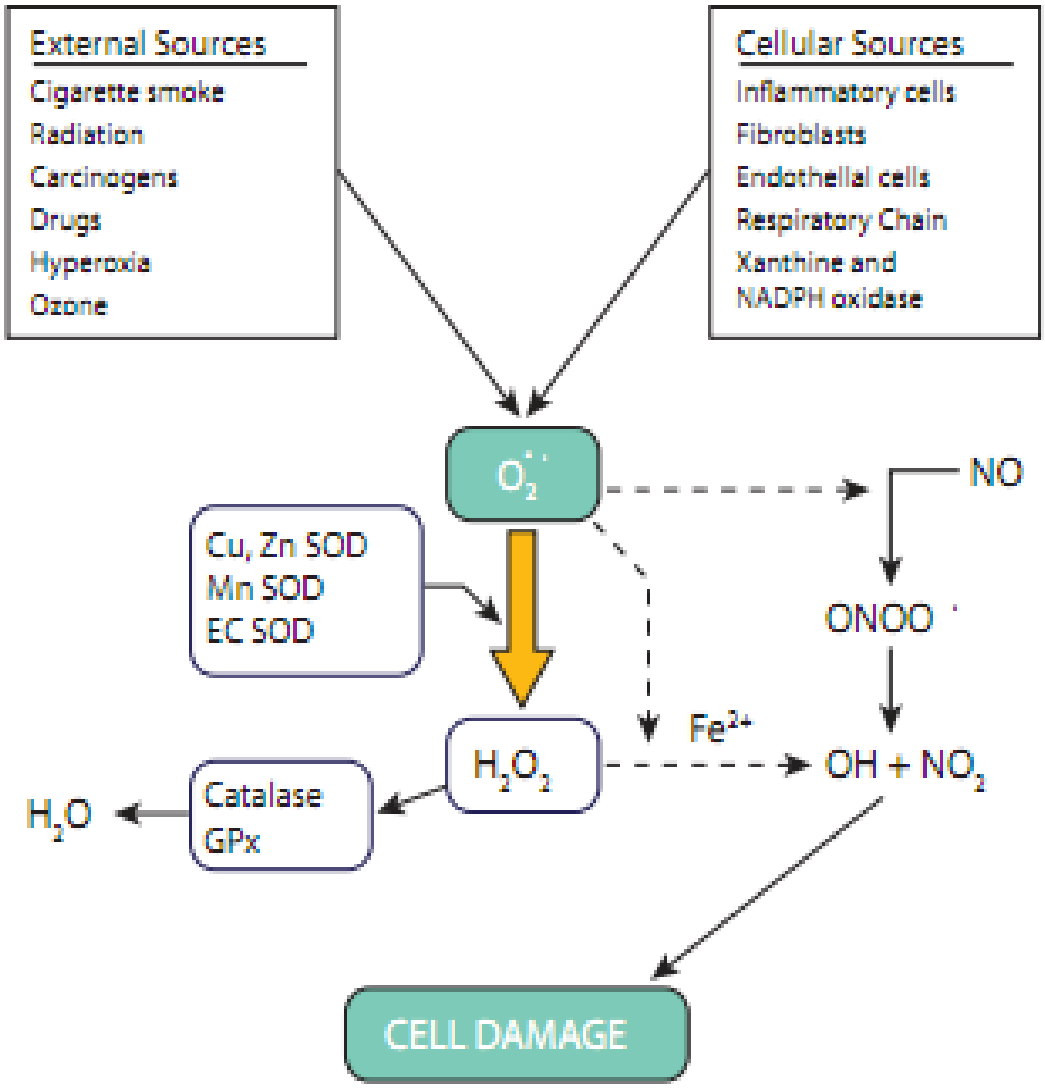

Figure 1.1. Exogenous and endogenous sources of reactive oxygen species and the antioxidant defense system. ("Literature review of a cantaloupe melon", 2007)

1.3.3 The importance of superoxide dismutase:

SOD is the primary enzyme defending an organism against the superoxide anion, and it exists in three forms: cytosolic CuZnSOD (coded by the *Sod1* gene) which is the predominant form responsible for 70-80% of SOD activity, MnSOD (coded by the *Sod2* gene) expressed in the mitochondrial matrix of cells and responsible for 10-20% of SOD activity, and extracellular EC-SOD (coded by the *Sod3* gene) which is expressed selectively in a limited number of tissues. Due to its location, MnSOD is the major contributor against reactive oxygen species resulting from mitochondrial respiration (Fukui & Zhu, 2010; Van Remmen et al., 2003).

The importance of this antioxidant enzyme has been reported in many transgenic studies over the years. One group reported that mice lacking SOD suffered from extensive mitochondrial damage, cardiac abnormalities, and accumulation of fat in the liver and skeletal muscles leading to death of the animals within 10 days of their birth. These mice also had significant reductions in size and growth rate, unlike the heterozygous mice which had similar sizes and growth rates as the wild-type group (Lebovitz et al., 1996). Another study showed that although they appear normal at birth, with age SOD heterozygous mice exhibited elevated levels of mitochondrial oxidative damage and oxidative DNA damage and they developed cancer at an accelerated rate (Van Remmen et al., 2003). A third group determined that SOD-knockout mice are less resistant to ROS-generating toxins and similar to the previous study, they suffer from an increased risk of cancer, liver cancer in particular; as well as neurological damage and hearing loss (Elchuri et al., 2005).

1.4 Medicinal usage of plants:

1.4.1 Transition from traditional to modern medicine:

Ethnobotanic medicine is broadly defined as the utilization of plants for medicinal uses (Fabricant & Farnswort, 2001). Throughout history, plants have always been used as medicinal products. Having served as the primary sources of treatment for numerous

human diseases for centuries, they continue to be utilized as therapeutic and preventive agents in traditional, alternative and even modern medicine, where over 25% of prescribed drugs used today include plant-derived constituents (Ma et al., 2005; X.H. Chen et al., 2011). Moreover, according to the World Health Organization, 80 % of the population of developing countries in Asia and Africa rely on traditional medicine for primary health care (World Health Organization [WHO], 2009).

Currently, the term traditional medicine is used to describe any non-Western medical practice. Resorting to traditional medicine in order to discover plants with therapeutic properties has proven valuable in the search for new bioactive compounds. Ethnopharmacology refers to the observation, description, experimental investigation, and determination of biological activities of indigenous drugs as an approach to drug discovery (Fabricant & Farnswort, 2001).

For the past few decades, the advances made in the fields of cell and molecular biology have allowed shorter drug discovery timelines by simultaneous testing of a larger number of molecular targets. On the other hand, the fact that some natural compounds are available only in small quantities, combined with the prolonged lag between the initiation of research on a potential therapeutic agent and its actual launch into the market have reduced the emphasis of the pharmaceutical companies on natural products (Koehn & Carter, 2005; Paterson & Anderson, 2005). In recent years however, concerns over harmful side-effects of synthetic compounds have shifted the focus back to natural plant resources which represent an abundant source of biologically active molecules (Ma et al., 2005; Palombo, 2011).

1.4.2 Medicinal plants in the literature:

A vast number of reports have been published dealing with the potential use of plants against microbial and fungal infections, several disease conditions and even cancer (Cragg & Newman, 2005; Kalemba & Kunicka, 2003; Willcox, Bodeker, & Rasoanaivo, 2004). During the last few decades, numerous plant extracts and their essential oils have been

investigated for their antimicrobial effects and dozens of herbs and spices have been found to which various species of bacteria and fungi are susceptible (Kalemba & Kunicka, 2003). In addition, over a thousand plants, either as crude extracts, isolated active components or as a combination of several plants, were found to be used in traditional medicine for the treatment of malaria, a parasitic disease (Willcox et al., 2004).

Plant-derived compounds have shown effectiveness in non-infectious diseases as well. For instance, Kizelsztein, Komanytsky, and Raskin (2009) reported that treatment of experimental autoimmune encephalomyelitis (EAE) mice with a molecule isolated from *Tripterygium wilfordii*, a twining vine, resulted in a delay of multiple sclerosis (MS) onset, a reduction in relapse rate, and a decrease in demyelination of CNS tissue.

1.4.3 Plant-derived anticancer agents in clinical use:

Active constituents derived from plants have been used to treat cancer for years and the search for chemotherapeutic agents that are efficient against malignant cells, but non-toxic in non-tumor cells is a major line of research (Jimenez-Medina et al., 2006).

There are currently four classes of plant-derived anticancer agents in clinical use around the world: the vinca alkaloids, the podophyllotoxins, the taxanes, and the camptothecin derivatives (Cragg & Newman, 2005).

The vinca alkaloids, vinblastine (VLB) and vincristine (VCR), were the first plant-derived anti-cancer agents discovered and developed in the 1950s. They were obtained from the Madagascar periwinkle, *Catharanthus roseus*, which was traditionally used by many cultures for the treatment of diabetes. These compounds, as well as two other active alkaloids namely vinleurosine and vinrosidine, were being studied as potential hypoglycemic agents when investigators observed reductions in the levels of white blood cells and suppression in the bone marrows of rats. Further investigations led to the discovery that the extracts were active against lymphocyte leukemia in mice. VLB and VCR were isolated as the active compounds and after selective modifications two semi-synthetic agents, vinorelbine (VRLB) and vindesine (VDS), were developed and are currently used for the

treatment of different types of cancer, such as Kaposi's sarcoma, breast, lung and advanced testicular cancers, leukemias and lymphomas (Cragg & Newman, 2005; Newman, Cragg, & Snader, 2000). The vinca alkaloids function by binding to β-tubulin near the GTP-binding site and inhibiting microtubule dynamics. And despite the fact that the structures of the various vinca alkaloids vary only slightly, they have distinct niches as chemotherapeutic agents and are therefore used in the treatment of different types of cancers (Amin, Gali-Muhtasib, Ocker, & Schneider-Stock, 2009).

Podophyllum peltatum, commonly referred to as the American mandrake or Mayapple, and *Podophyllum emodii* have been used historically to treat skin cancers and warts (Cragg & Newman, 2005). They were also used hundreds of years ago by natives of the Himalayan mountain area and by American Indians as a cathartic and an anthelmintic (Wang & Lee, 1997). Although podophyllotoxin, the major bioactive agent, was first isolated as early as in 1880, it was only in the 1950s that scientists identified its correct structure. Other similar podophyllotoxin-like molecules were also isolated and used in clinical trials; however, due to insufficient efficacy and high toxicity reports they were discarded. After further studies, etoposide and teniposide, two semi-synthetic derivatives of epipodophyllotoxin (an isomer of podophyllotoxin), were found to be clinically active and are now used to treat lymphomas and testicular cancer. Etoposide is also extremely active against small cell lung carcinoma (Cragg & Newman, 2005; Mans, Da Rocha, & Schwartsmann, 2000). Etiposide and teniposide act as inhibitors of topoisomerase II (Wang & Lee, 1997).

The taxanes are a more recent group of chemotherapeutic drugs. Isolated from the bark of the Pacific yew, *Taxus brevifolia*, paclitaxel (Taxol®) is used as an anti-cancer drug against ovarian cancer, breast cancer and non-small cell lung cancer. It is also used in the treatment of some forms of Kaposi's sarcoma. *T. brevifolia* and other *Taxus* species were traditionally used by Native American tribes to treat several conditions excluding cancer. There was even one reported use of *Taxus baccata* leaves for cancer treatment in traditional Asiatic Indian medicine. Additionally, numerous other taxanes are currently in preclinical trials for the development of novel chemotherapeutic drugs. Docetaxel

(Taxotere®), an active semi-synthetic analog of paclitaxel, provides a major, renewable source of this important class of drugs (Cragg & Newman, 2005). Along with the vinca alkaloids, the taxanes represent another major class of antimitotic drugs. Taxanes are microtubule-targeting agents that bind to polymeric tubulin, stabilizing it and inhibiting its disassembly, and thus inhibit cell replication by disrupting normal mitiotic spindle formation and ultimately lead to cell death by apoptosis (Amin et al., 2009; Wang & Lee, 1997).

The latest additions to the classes of plant-derived anti-cancer drugs are the clinically active compounds obtained from camptothecin which was isolated from *Camptotheca acuminate*, a Chinese ornamental tree. Camptothecin first reached the clinical trial stage in the 1970s, but due to severe issues of bladder toxicity it was dropped. It eventually came back in modified forms called topotecan and irinotecan, gaining approval in the USA in 1996. These are used for the treatment of ovarian and small cell lung cancers, and for colorectal cancers respectively (Cragg & Newman, 2005; Newman et al., 2000). Camptothecin and its analogues function as inhibitors of DNA Topoisomerase I (Wang & Lee, 1997). Their potency as cytotoxic agents has been verified by various studies to be related directly to their ability to inhibit topoisomerase I catalytic activity (Hertzberg et al., 1989; Jaxel, Kohn, Wani, Wall, & Pommier, 1989; Kingsbury et al., 1991).

1.5 *Tragopogon porrifolius*:

Taking into consideration that of the estimated 500,000 plant species on Earth, only about 1% have been investigated for their medicinal qualities, the potential for the discovery of novel bioactive compounds is substantial (Palombo, 2011).

Tragopogon porrifolius, which belongs to the family Asteraceae, is subdivided into three subspecies, namely *T.porrifolius* subsp. *australis*, *T.porrifolius* subsp. *cupani* and *T.porrifolius* subsp *porrifolius*, only the latter of which is edible (Zidorn et al., 2005).

T. porrifolius subspecies *porrifolius*, more commonly referred to as white salsify, is an annual or biennial herb indigenous to Central and Eastern Mediterranean, as well as Asia Minor. It has also gained the alternate name "goat's beard" due to the appearance of its foliage and that of "vegetable oyster" due to the taste of its roots. Ranging in height from 30 to 125 cm, it has lilac to reddish-purple ligules and its long, grass-like narrow leaves form a daisy-like rosette (Figure 1.2).

Figure 1.2. *Tragopogon porrifolius* (Spooner, 1997).

Although its usage as a vegetable has decreased in Southern and Central Europe in recent years, it is still popular in the United Kingdom. It is grown for its long, tapering and frequently branched roots which are used for flavoring. Its roots, leafy shoots and open

flowers are used both cooked or as a raw salad (Gupta, Talwar, V. Jain, Dhawan, & S. Jain, 2003).

As to the use of *T. porrifolius* as a potential source of bioactive compounds against cancer or other free radical associated diseases, studies are thus far very limited. One group reported the isolation of phenolic acids and flavonoids which are plant secondary metabolites associated with antioxidant activity (Sareedenchai, Ganzera, Ellmerer, Lohwasser, & Zidorn, 2009). In another study, it was reported that two out of six compounds isolated from *T. porrifolius* had moderate radical scavenging activity measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Zidorn et al., 2005). *T. porrifolius* is also used to treat cancer in Lebanese folk medicine.

The present study investigates the methanolic extract of *Tragopogon porrifoilus*, a biennial wildflower native to Mediterranean regions, as a potential chemotherapeutic agent. Total phenolic and flavonoid contents, higher contents of which are correlated with increased antioxidant activity and potentially protective roles for human health, are assessed. Antioxidant activity is assessed both in vitro through the Ferric Reducing Antioxidant Power (FRAP) and the DPPH radical scavenging assays, and in vivo through the calculation of catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) hepatic antioxidant enzymes levels. In addition, the in vivo experimentation examines the hepatoprotective effects of the *T. porrifolius* methanolic extract by calculating the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) marker enzymes. Finally, the cytotoxicity, the anti-proliferative activity, and the pro-apoptotic effects of this extract against colon (Caco-2) and breast (MDA) cell lines are also evaluated.

Chapter 2

MATERIALS & METHODS

2.1 Plant material and extraction in methanol:

The *Tragopogon porrifolius* plant material was collected from Lebanon and air dried in shade. The dried plants were then cut to small pieces and 300 grams were soaked for 72 hours in methanol and then filtered. This process was repeated twice to ensure maximal extraction. Finally, the solvent was distilled off under reduced pressure using a rotary evaporator and the residue was refrigerated for further use.

2.2 Determination of total phenolic and flavonoid contents:

2.2.1 Determination of total phenolic content:

Total phenolic content was estimated by the Folin–Ciocalteu colorimetric method, using gallic acid (100-1000 mg/L in 80% methanol) as a standard (Tawaha, Alali, Gharaibeh, Mohammad, & El-Elimat, 2007). This assay is based on the chemical reduction of the Folin–Ciocalteu reagent, a metal oxide mixture of tungsten and molybdenum. Upon reduction, a blue color with maximum absorption at 765 nm is produced, whereby the intensity of light at this wavelength is proportional to the concentration of phenols in the tested sample (Waterhouse, 2002).

Briefly, 50 μL of the 1:5 diluted and filtered extract (at an original concentration of 100 mg/mL in methanol) was mixed with 450 μL of distilled water and 2.5 mL of 0.2 N Folin–Ciocalteu reagent. After 5 min, 2 ml of saturated sodium carbonate (75 g/L) were added and the mixture was incubated at 30 °C for 90 minutes with intermittent shaking. The absorbance of the resulting blue-colored solution was measured at 765 nm and the total phenolic content was expressed as gallic acid equivalents mg(GAE)/g of dry weight.

2.2.2 Determination of total flavonoid content:

Total flavonoid content was determined by the aluminum chloride colorimetric method, using quercetin (10-100 mg/L) as a standard (Lin & Tang, 2007). The principle of this technique is the formation of complexes between $AlCl_3$ and the keto or hydroxyl groups of flavones, flavonols, and flavonoids (Chang, Yang, Wen, & Chern, 2002).

Briefly, 500 µL of the 1:20 diluted and filtered extract (at an original concentration of 100 mg/mL in methanol) was mixed with 1.5 ml of 95% methanol, 100 µl of 10% aluminum chloride ($AlCl_3$), 100 µl of 1 M potassium acetate (CH_3COOK), and 2.8 ml of deionized water. The mixture was incubated at room temperature for 40 minutes and the absorbance was measured at 415 nm. The data were expressed as milligram quercetin equivalents (QE)/g lyophilized powder. The total flavonoid content was expressed as quercetin equivalents mg (QE)/g dry weight.

2.3 Cell lines and cell culture:

MDA (human breast adenocarcinoma) and Caco-2 (human colorectal adenocarcinoma) cell lines were used in the in vitro experiments of cytotoxicity and proliferation.

Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.4 Cytotoxicity assay:

Cytotoxicity of the *T. porrifolius* methanolic extract on the cell lines was assayed at 24 and 48 hours using the Trypan Blue exclusion method, a technique measuring cell viability.

Cells were seeded into 24 well plates and a dose/response curve was generated using five different concentrations of the crude extract dissolved in DMSO (5, 10, 25, 50, and 100 μg/mL). After the specified time periods following treatment, the cells were trypsinized, diluted in 0.4% Trypan Blue and counted in a hemocytometer chamber. While viable cells exclude the dye, the dead cells are stained blue. Cell viability was determined and used to plot a dose-response curve and derive the cytotoxic concentration IC_{50}, the concentration causing a 50 % decrease in cell viability. All experiments were plated in triplicate wells and were performed three times.

2.5 Proliferation assay:

The effect of the *T. porrifolius* methanolic extract on proliferation of the cell lines was measured at 24 and 48 hours using the cell proliferation reagent WST-1, a tetrazolium salt which is cleaved by mitochondrial dehydrogenases in metabolically active cells.

Cells were seeded into 96 well microplates at a density of 1 x 10^5 cells/well and treated with different concentrations of the extract (5, 10, 25, 50, and 100 μg/mL). At the end of the treatment period, 10 μL of the cell proliferation reagent WST-1 was added to the cells already cultured in 100 μL/well (1:10 final dilution). After 2 hours of incubation in a humidified atmosphere (37 °C, 5% CO_2), the absorbance of the samples was measured in an ELISA microplate reader at 450 nm. All experiments were plated in triplicate wells and were performed three times.

2.6 Determination of *in vitro* antioxidant activity:

The *in vitro* antioxidant activity of the *T. porrifolius* methanolic extract was measured using the Ferric Reducing/ Antioxidant Power (FRAP) and the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assays.

2.6.1 The FRAP assay:

The FRAP assay measures the change in absorbance due to the reduction of the colorless Fe^{3+} by the action of electron donating antioxidants and the formation of Fe^{2+}-tripyridyltriazine compound which is blue in color. The FRAP value was calculated based on the method of Benzie and Strain (1996), using ferrous sulphate (10-100 mM $FeSO_4$.7H2O) as a standard (Griffin, Bhagooli, & Weil, 2006).

The working FRAP reagent consists of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) solution in a 10:1:1 ratio. 150 µL of the freshly prepared and prewarmed (at 37 °C) FRAP reagent was added to each well in a 96 well microplate and after a blank reading, 20 µL of the sample was added. The absorbance was measured after 8 minutes in an ELISA microplate reader at 600 nm. The initial blank reading for each well was subtracted from the final reading to determine the FRAP value (micromol ferric ions reduced to ferrous form per gram) of each sample.

2.6.2. The DPPH assay:

The DPPH assay is based on the ability of this stable radical to react with hydrogen donors (Chu, Y.W. Lim, Radhakrishnan, & P.E. Lim, 2010). A solution of DPPH, originally deep violet, is decolorized to light yellow after reduction with an antioxidant. The degree of reduction in absorbance reflects the radical scavenging power of the tested compound (Agbafor & Nwachukwu, 2011).

Briefly, 50 µL of the extract and 50 µL of 0.5 mM DPPH in ethanol were added to each well in a 96 well microplate. After incubation in the dark for 40 minutes, the absorbance was read at 492 nm. Ascorbic acid and Trolox were used as reference compounds and the radical scavenging activity was calculated as percentage inhibition of absorbance.

IC_{50}, the concentration needed to reduce the initial absorbance of DPPH radical by 50%, was calculated graphically using a calibration curve in the linear range by plotting extract concentration versus scavenging effect.

2.7 Hepatotoxicity bioassay:

2.7.1 Animals:

Male Wistar rats weighing 180-220 g were supplied by the Lebanese American University animal unit. The animals were housed under stable conditions of temperature (20 ± 2 °C) and humidity (50 ±5 %), and an alternating cycle of light and dark. The animals were supplied with standard laboratory rat chow diet and water and were maintained in accordance to the appropriate ethical and legal requirements.

2.7.2 Experimental design:

Liver damage was induced with CCl_4 in a 1:1 (v/v) mixture with olive oil at a dose of 1.5 mL/kg administered intraperitoneally (Coballase-Urrutia et al., 2011).

Animals were divided into 9 groups of 6 animals each:

Group I – normal control (no treatment)

Groups II and III – extract-treated groups without CCl_4 injections (*T. porrifolius* methanolic extract in DMSO, 50 and 250 mg/kg, i.p.)

Group IV – positive control (olive oil 2 mL/kg + CCl_4/olive oil 1.5 mL/kg, i.p)

Group V – vehicle group (DMSO 2 mL/kg + CCl_4/olive oil 1.5 mL/kg, i.p)

Groups VI, VII and VIII – extract-treated groups (*T. porrifolius* methanolic extract in DMSO, 50, 100 and 250 mg/kg + CCl_4/olive oil 1.5 mL/kg, i.p)

Group IX – only CCl_4/olive oil 1.5 mL/kg

All the groups were treated for 6 consecutive days, in combination with the CCl_4 injections for the last 3 days of treatment. 48 hours after the administration of the last dose of CCl_4 and the respective treatment of each group (day 8), the animals were anaesthetized with diethyl ether and sacrificed. Blood samples were withdrawn from each rat and the liver was excised for the determination of antioxidant enzyme levels.

2.7.3 Measurement of biochemical parameters:

The blood collected from each animal was left to clot at room temperature for 45 minutes. The serum was then separated by centrifugation at 600g for 15 minutes and used for the determination of marker enzymes AST, ALT and LDH. The enzyme levels were assayed using commercially available kits (SPINREACT) according to the manufacturer's instructions.

Hepatic tissue was homogenized in 0.1 M phosphate buffer (pH 7.0) containing 0.1% Triton X-100 with buffer volume being four times the weight of the tissue. The homogenate was centrifuged at 19,000g for 10 minutes and the supernatant was used for the determination of total protein and the activity of the antioxidant enzymes CAT, SOD and GST.

Total protein concentration was determined using the Bio-Rad Protein Assay Kit II, according to the manufacturer's instructions and using bovine serum albumin (0.1-1 mg/mL) to generate the standard curve.

2.7.3.1 CAT assay:

Catalase activity was assayed on the basis of H_2O_2 disappearance (Pedraza-Chaverri et al., 1999, 2001, 2005). A 5 μL aliquot of the 1:40 diluted supernatant was added to 720 μL of 30 mM H_2O_2 in 10 mM potassium phosphate solution. The decomposition of H_2O_2 by CAT contained in the samples was measured at 240 nm for a period of 15 seconds. The reaction follows a first-order kinetics given by the equation $k = 2.3/t \log A_0/A$ where k is the first-order reaction rate constant, t is the time over which the decrease of H_2O_2 was measured, and A_0 and A are the optical densities at times 0 and 15 s respectively. The results were expressed in k/mg protein.

2.7.3.2 SOD assay:

SOD activity was assayed according to the method of Marklund and Marklund (1974) as modified by Naskar et al. (2010). 50 μL of the homogenized liver supernatant was added to 2.8 mL Tris-EDTA (49.78 mM Tris, 1.2 mM EDTA; pH= 8.5) and 100 μL of 2mM pyrogallol. The mixture was scanned for 3 minutes at 420 nm and compared to the control which consists only of Tris-EDTA and pyrogallol.

One unit of SOD is the amount of enzyme that inhibits the rate of auto-oxidation of pyrogallol by 50%.

The results were calculated according to the following equations:

Rate (R) = $(A_3 – A_0)/t$ where t = 3

% inhibition= $[(R_{control} – R)/R_{control}]$ x 100

Enzyme unit (U) = (%inhibition/50) x dilution factor

2.7.3.3 GST assay:

GST activity was assayed based on the method of Habig, Pabst and Jakoby (1974) as modified by Al-Saffar, Ganabadi, Fakurazi and Yaakub (2011). 600 μL of the supernatant was added to 2.2 mL of 0.1 M potassium phosphate buffer (pH 6.5), 100 μL of 30 mM CDNB (1-chloro-2,4-dinitrobencene) and 100 μL of 30 mM GSH. The absorbance was read at 340 nm after 1 minute and the activity of GST was expressed in nmol of GSH-CDNB conjugates formed/min/mg protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Chapter 3

RESULTS

3.1 Yield of plant material:

The extraction of *Tragopogon porrifollius* plant material, using methanol as a solvent, generated 27.7 grams of a semi-solid crude extract. The percent yield was 9.23 %.

3.2 Total phenolic and flavonoid contents:

Total phenolic content of the methanolic extract of *T. porrifolius* was estimated by the Folin–Ciocalteu colorimetric method using gallic acid to generate the standard curve and was determined to be 36.95 ± 1.39 mg GAE/g dry weight.

Total flavonoid content of the methanolic extract of *T. porrifolius* was estimated by the aluminum chloride colorimetric method using quercetin to generate the standard curve and was determined to be 16.56 ± 0.42 mg QE/g dry weight.

3.3 Cytotoxicity of *T. porrifolius* methanolic extract:

The cytotoxic effects of *T. porrifolius* methanolic extract on the MDA and Caco-2 cell lines, determined by the Trypan Blue exclusion method, are shown in Figure 3.1 and Figure 3.2 respectively and demonstrate a decrease in cell viability that is both dose-dependent and time dependent. IC_{50} after 48 hours of exposure to the extract was determined to be 74.29 μg/mL for the MDA cell line, and 93.00 μg/mL for the Caco-2 cell line.

Two tailed *t*-tests, performed to validate the reductions in cell viability, demonstrated that results for the MDA cell line were statistically significant for all concentrations of the

extract and for both time periods ($P < 0.02$), except for the 5 μg/mL concentration at 24 hours for which $P > 0.05$. For the Caco-2 cell line however, none were determined to be statistically significant ($P > 0.05$) except for the highest concentration 100 μg/mL concentration at 24 hour time period, and the 50 μg/mL and 100 μg/mL concentrations at the 48 hour time period ($P < 0.05$).

3.4 Effect of *T. porrifoilius* methanolic extract on proliferation:

The effect of the *T. porrifolius* methanolic extract on the proliferation of the MDA and Caco-2 cell lines was measured using the WST-1 proliferation assay which is based on the enzymatic cleavage of the tetrazolium salt WST-1 into formazan by mitochondrial dehydrogenases in viable cells. The results, shown in Figure 3.3, demonstrate a dose and time dependent decrease in proliferation. Although the IC_{50} wasn't reached, the highest concentration used (100 μg/mL) reduced proliferation to 51.86% and 56.26% viability in the MDA and Caco-2 cell lines respectively.

The results were shown to be statistically significant for all of the concentrations tested at both 24 and 48 hours when compared to the group that received only DMSO ($P < 0.04$ for the Caco-2 cell line and $P < 0.05$ for the MDA cell line). The only exceptions were the 5 μg/mL and 25 μg/mL concentrations in the MDA cell line for which $P > 0.05$.

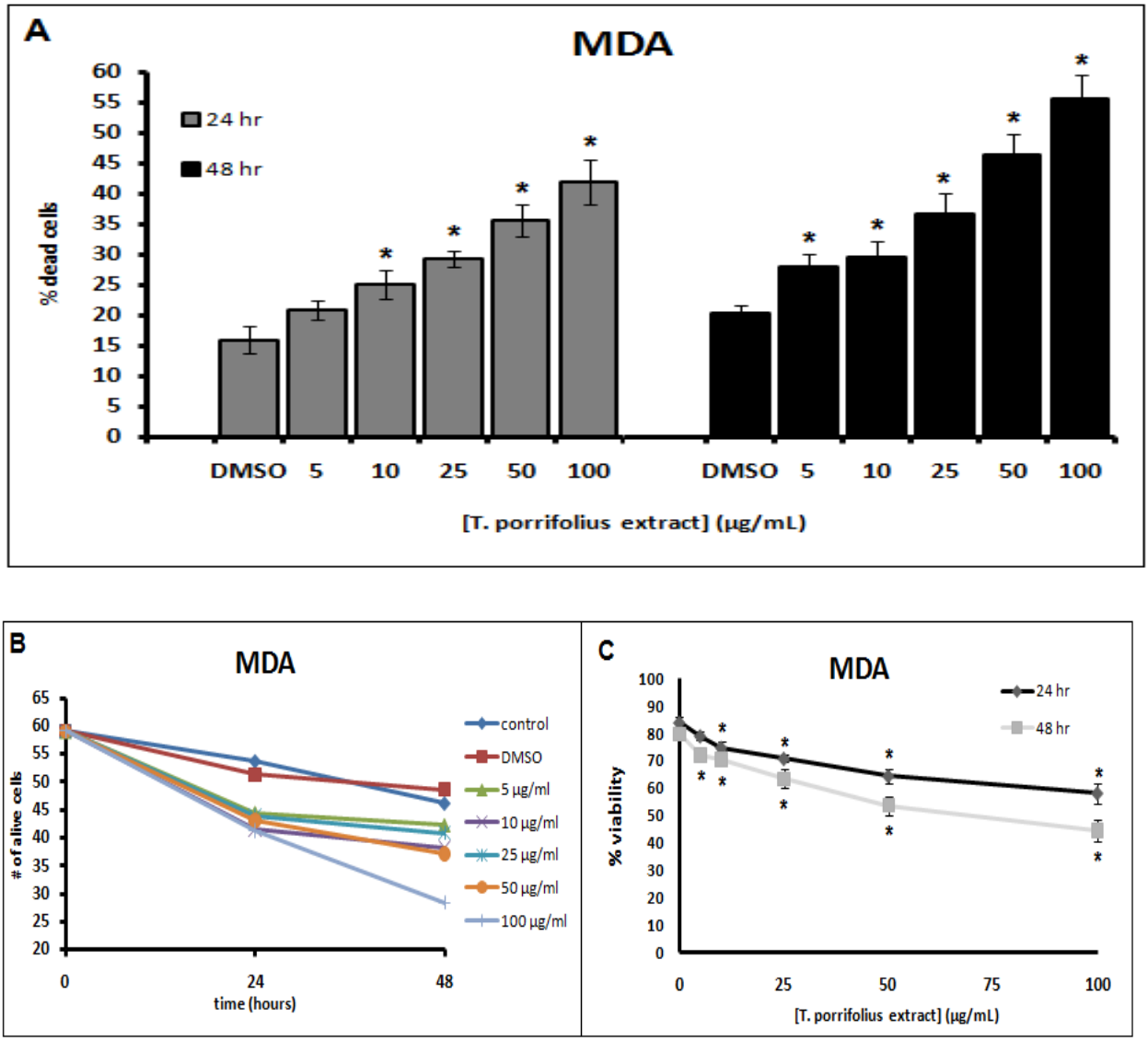

Figure 3.1. Cytotoxicity of *T. porrifoilius* methanolic extract on MDA cells at 24 and 48 hours of treatment. **(A** and **B)** Effect of the extract on cell viability. **(C)** Dose response curve of the extract.

The error bars represent the standard error of the means for three independent experiments (SEM).

* $P < 0.02$ with respect to the group that received only DMSO.

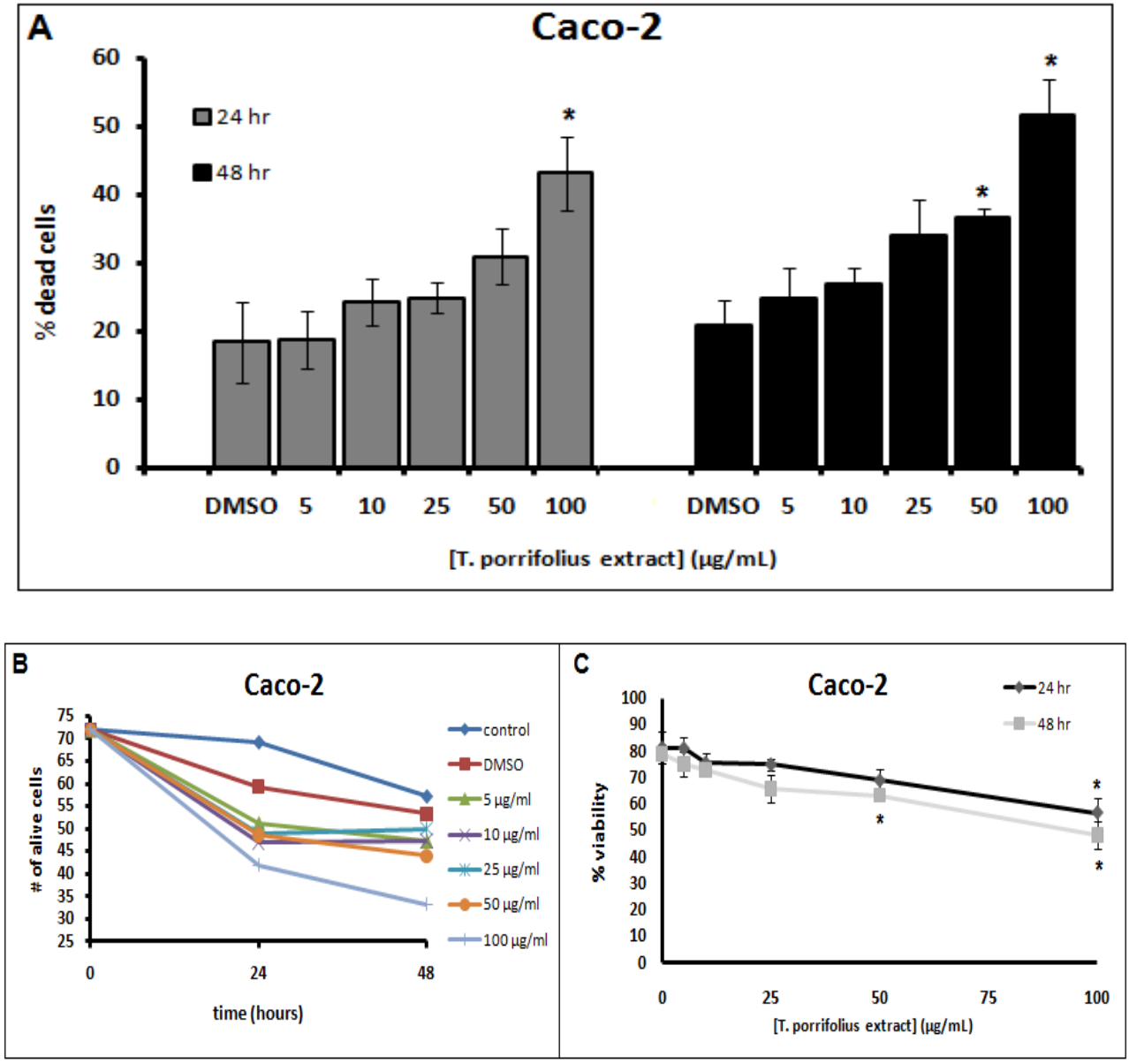

Figure 3.2. Cytotoxicity of *T. porrifoilius* methanolic extract on Caco-2 cells at 24 and 48 hours of treatment. **(A** and **B)** Effect of the extract on cell viability. **(C)** Dose response curve of the extract.

The error bars represent the standard error of the means for three independent experiments (SEM).

* $P < 0.05$ with respect to the group that received only DMSO.

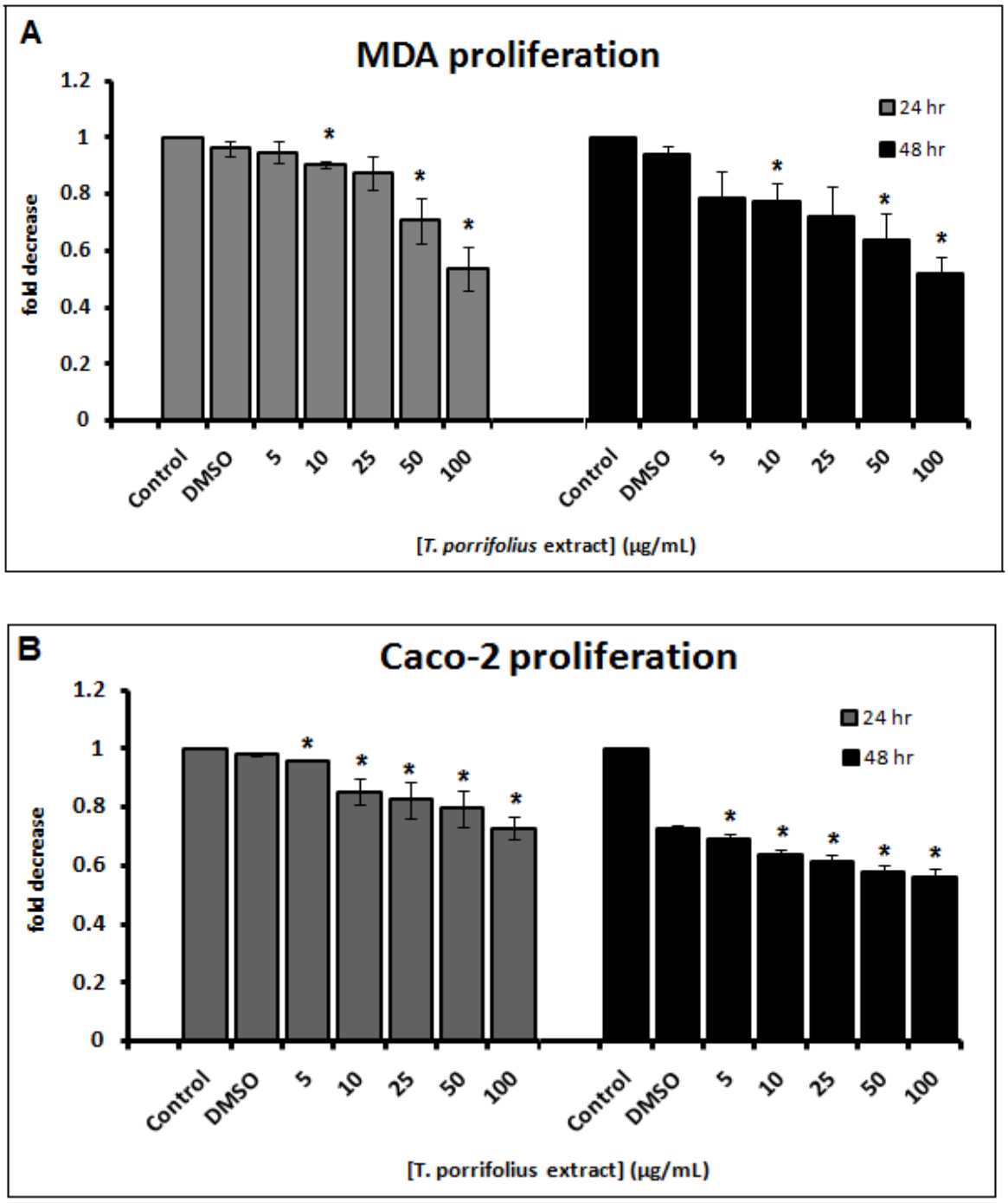

Figure 3.3. The effect of *T. porrifolius* on proliferation of MDA **(A)** and Caco-2 **(B)** cell lines in the presence of increasing concentration of the methanolic extract at 24 and 48 hours of treatment.

* $P < 0.05$ with respect to the group that received only DMSO.

3.5 *In vitro* antioxidant activity:

The *in vitro* antioxidant activity of the T. porrifolius methanolic extract was determined by two methods: the ferric reducing/antioxidant power (FRAP) assay and the DPPH free radical scavenging assay.

3.5.1. The FRAP assay:

In the FRAP assay, antioxidant activity is measured as the capacity of the tested compound to reduce Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ; a higher reduction corresponds to a higher antioxidant activity. The FRAP value of the methanolic extract was calculated using ferrous sulphate to generate a standard curve and was determined to be 659.57 ± 13.77 μmol Fe^{2+}/g. Ascorbic acid and Trolox were used as reference compounds and their FRAP values were calculated to be 889.27 ± 17.13 and 1349.86 ± 53.41 μmol Fe^{2+}/g respectively. The results are shown in Figure 3.4.

3.5.2 The DPPH assay:

In the DPPH free radical scavenging assay, antioxidant activity is measured as the capacity of the tested compound to reduce the stable DPPH radical. The IC_{50} value represents the concentration needed to cause a 50% inhibition of $DPPH^{\bullet}$. Different concentrations of the extract, as well as ascorbic acid and Trolox, were used and the IC_{50} values were determined to be 15.18 μg/mL for the extract, 9.13 μg/mL for ascorbic acid and 6.82 μg/mL for Trolox. The results are shown in Figure 3.5.

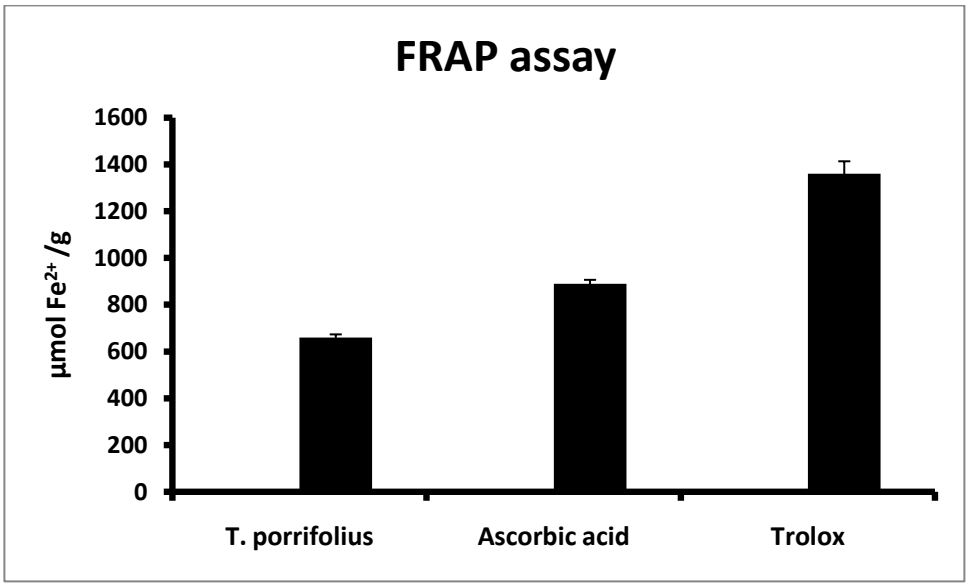

Figure 3.4. Ferric reducing abilities of *T. porrifolius* methanolic extract, ascorbic acid, and Trolox. Each column represents the mean of 3 experiments and the error bars represent the standard error of the means (SEM).

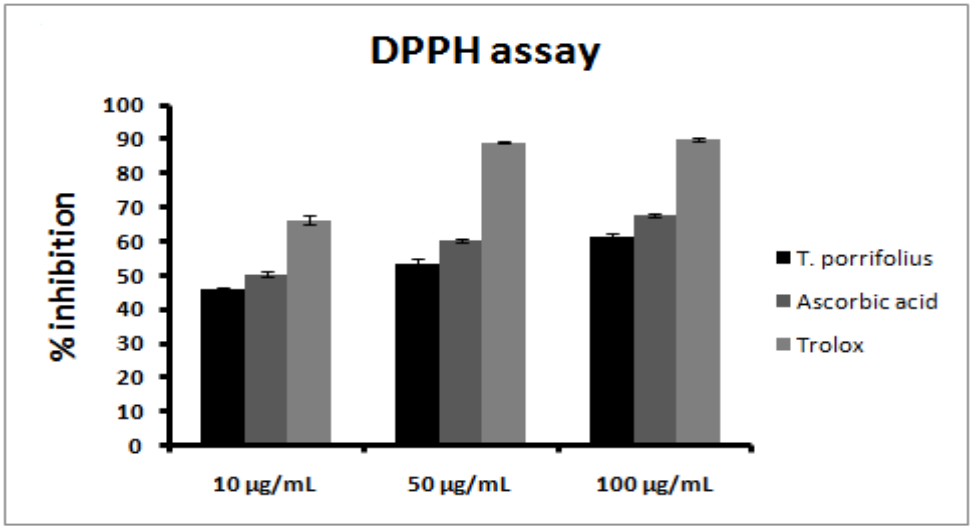

Figure 3.5. DPPH radical scavenging activities of *T. porrifolius* methanolic extract, ascorbic acid, and Trolox. Each column represents the mean of 3 experiments and the error bars represent the standard error of the means (SEM).

3.6 *In vivo* antioxidant activity:

The *in vivo* antioxidant activity of the *T. porrifolius* methanolic extract was determined by the evaluation of catalase, glutathione-S-transferase and superoxide dismutase antioxidant enzymes in the livers of both normal and CCl_4-treated rats. The increases in the level of these enzymes are shown in figures 3.6, 3.7 and 3.8 respectively.

Enzyme activity observed in the hepatic tissue of CCl_4-treated rats was significantly lower compared to the normal group. However, in the groups pretreated with the *T. porrifolius* methanolic extract, there was a significant dose-dependent increase in activity when compared to the group that received CCl_4 alone, and the highest dose (250 mg/kg body weight) restored the activity of all three enzymes to normal levels. On the other hand, the groups that received only the extract without the CCl_4 injections had higher enzyme activity compared to the normal group.

The results of all the groups were shown to be statistically significant ($P < 0.02$) by a two-tailed *t*-test when compared to the normal group, except groups II (50 mg/kg *T. porrifolius* extract only) and VIII (250 mg/kg *T. porrifolius* extract + CCl_4) for which $P > 0.05$. However, the results of group VIII were highly significant ($P < 0.001$) when compared to the vehicle group which received DMSO and CCl_4.

3.7 Hepatoprotective activity:

The animals treated only with *T. porrifolius* methanolic extract at doses of 50 and 250 mg/kg body weight exhibited no significant changes in AST, ALT and LDH marker enzymes. The slight elevations at the level of these enzymes were found to be statistically insignificant ($P > 0.05$). Furthermore, in the groups subjected to CCl_4-induced hepatotoxicity, those pretreated with the extract exhibited substantial and significant ($P < 0.05$; with the exception of the 50 mg/kg dose for ALT for which $P > 0.05$) hepatoprotective capacity. The three doses (50, 100 and 250 mg/kg) used respectively reduced AST levels by 26.90, 40.19 and 51.74 percent, ALT levels by 17.91, 31.84 and 50.74 percent, and LDH levels by 30.50, 48.81 and 61.18 percent compared to the vehicle group that received DMSO and CCl_4. The results are shown in figures 3.9, 3.10 and 3.11.

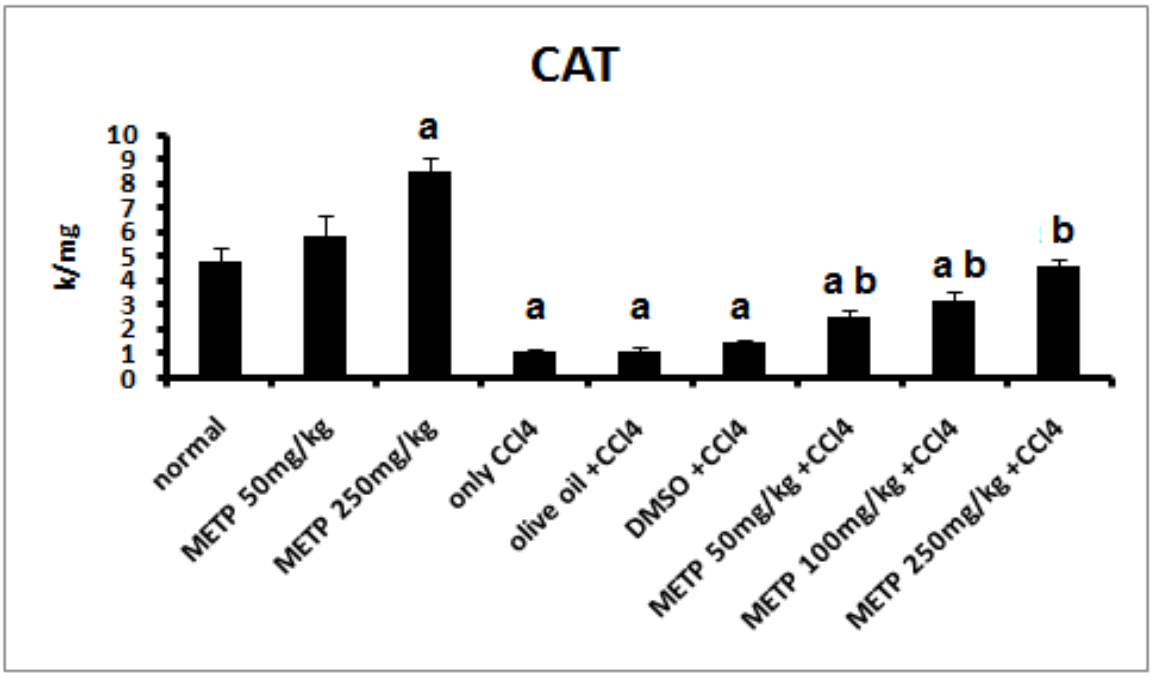

Figure 3.6. Effect of the methanolic extract of *T. porrifolius* (METP) on the activity of CAT antioxidant enzyme in vivo. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).

[a] $P < 0.05$ with respect to the normal group

[b] $P < 0.001$ with respect to the vehicle group (DMSO + CCl_4)

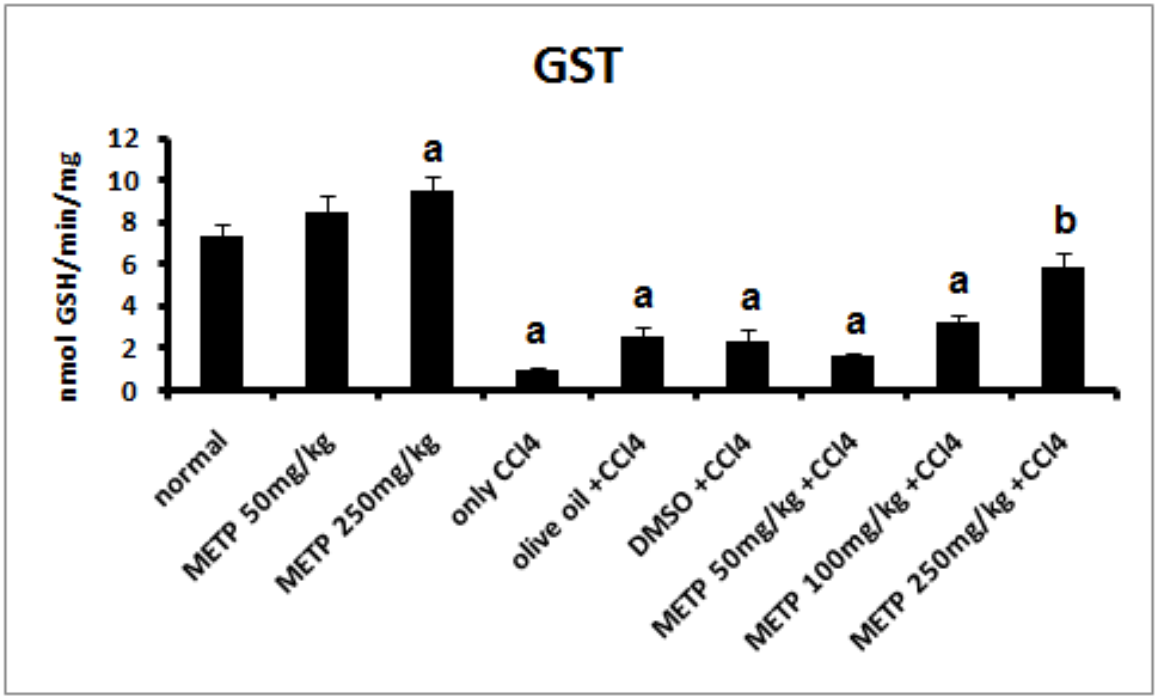

Figure 3.7. Effect of the methanolic extract of *T. porrifolius* (METP) on the activity of GST antioxidant enzyme in vivo. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).

[a] $P < 0.05$ with respect to the normal group

[b] $P < 0.001$ with respect to the vehicle group (DMSO + CCl_4)

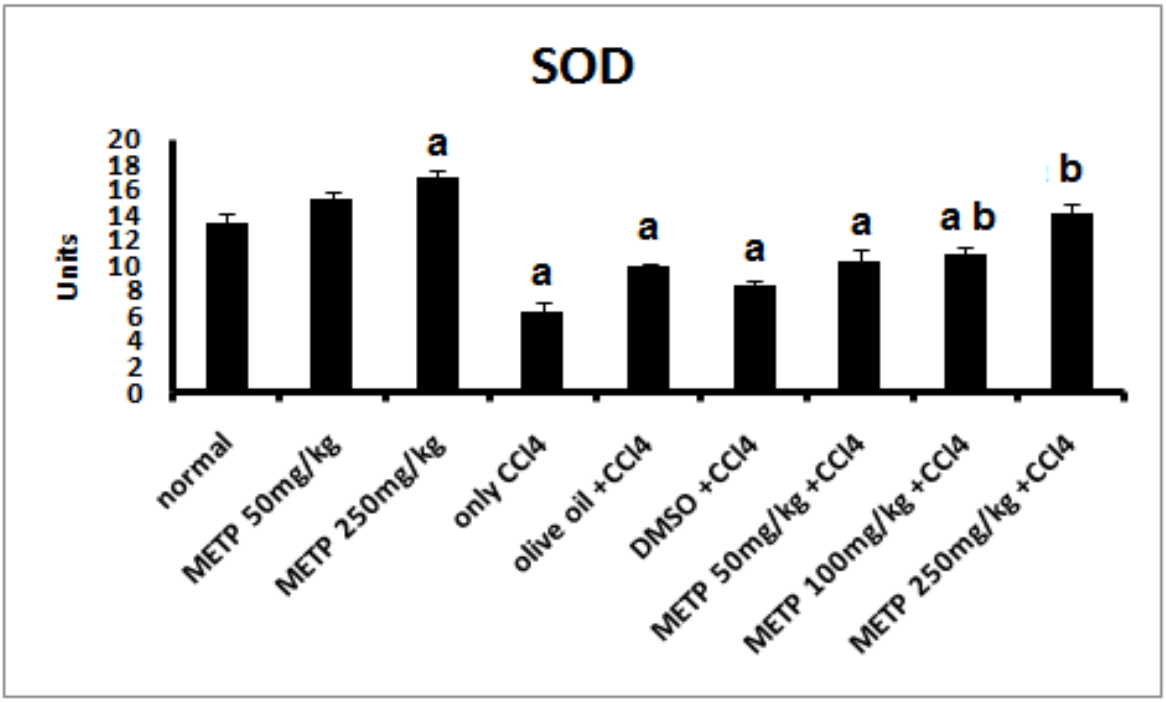

Figure 3.8. Effect of the methanolic extract of *T. porrifolius* (METP) on the activity of SOD antioxidant enzyme in vivo. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).

[a] $P < 0.05$ with respect to the normal group

[b] $P < 0.001$ with respect to the vehicle group (DMSO + CCl_4)

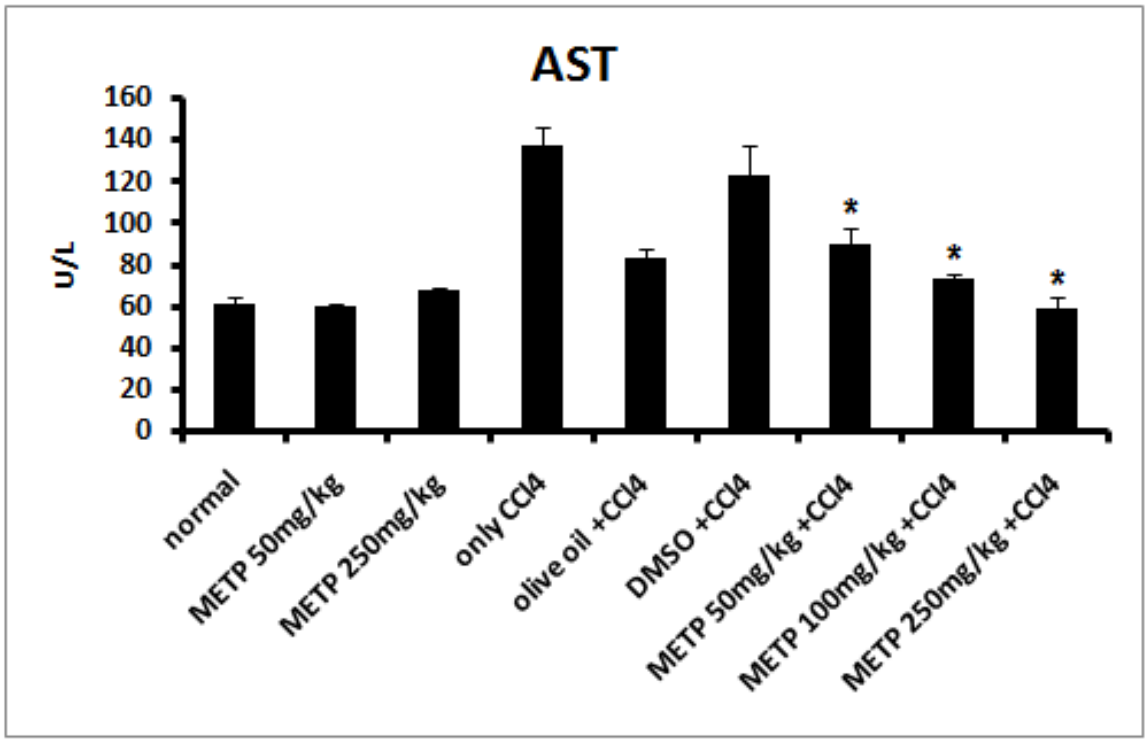

Figure 3.9. Effect of the methanolic extract of *T. porrifolius* (METP) on AST enzyme levels in serum. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).
* $P < 0.05$ with respect to the group that received DMSO and CCl_4.

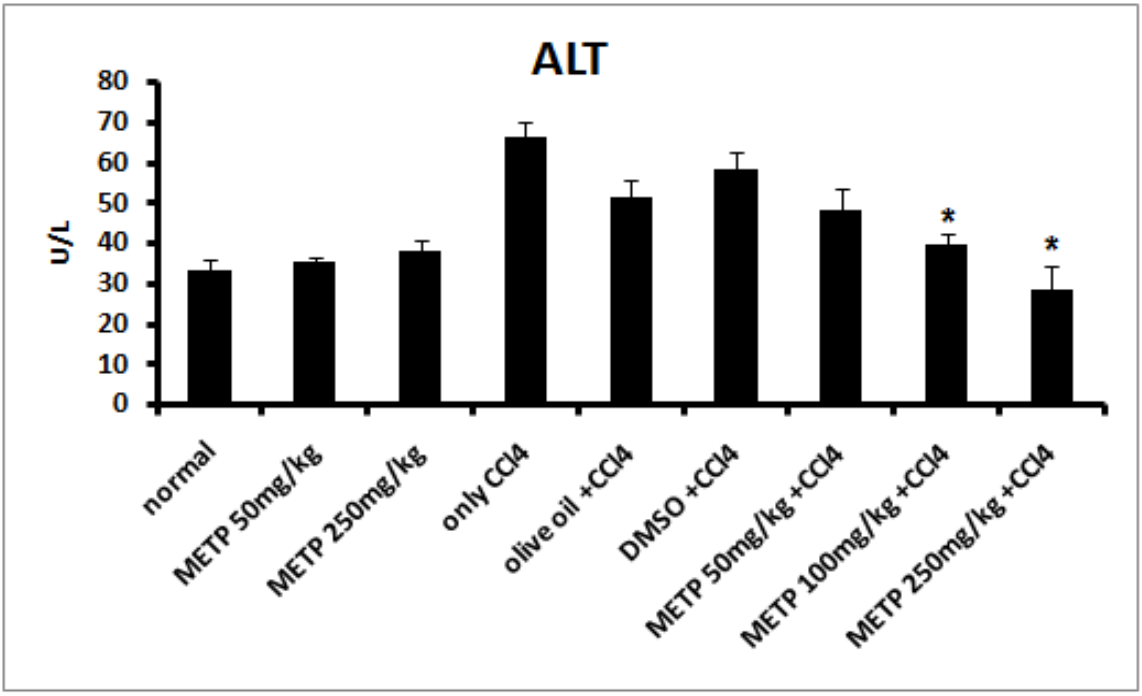

Figure 3.10. Effect of the methanolic extract of *T. porrifolius* (METP) on ALT enzyme levels in serum. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).

* $P < 0.05$ with respect to the group that received DMSO and CCl_4.

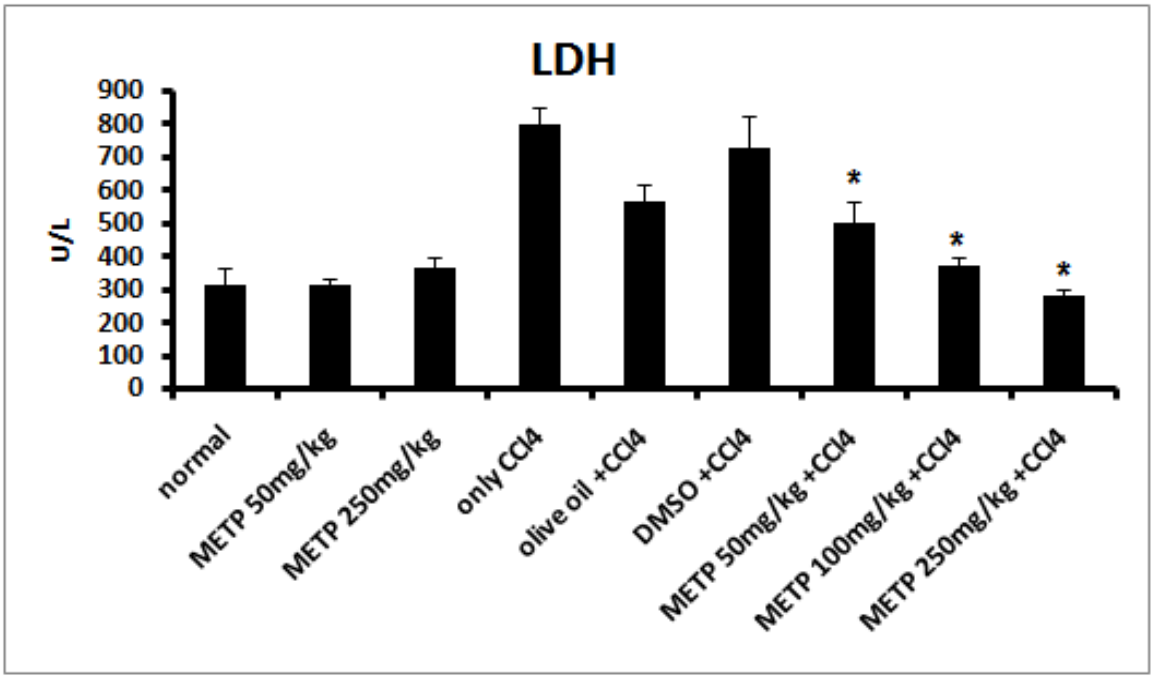

Figure 3.11. Effect of the methanolic extract of *T. porrifolius* (METP) on ALT enzyme levels in serum. Each column represents the mean of 6 animals and the error bars represent the standard error of the means (SEM).

* $P < 0.05$ with respect to the group that received DMSO and CCl_4.

Chapter 4

DISCUSSION & CONCLUSIONS

Reactive oxygen species (ROS) have been associated with many degenerative diseases and are considered potential carcinogens. These oxygen-derived free radicals can induce injury to nuclear DNA, facilitating mutagenesis and leading to cancer initiation and progression. When regulated, ROS act as signal transduction molecules involved in various signaling pathways (Ferreira et al., 2010; Manda et al., 2009). Antioxidants, which can be of endogenous and exogenous origin, provide protection to cellular systems from the harmful effects of excessive oxidations and consequently contribute to the inhibition of many diseases such as CHD and cancer (G.G. Duthie, S.J. Duthie, & Kyle, 2000).

In this study, the antioxidant and the anticancer effects of the methanolic extract of *Tragopogon porrifolius* were investigated through *in vitro* studies on breast and colorectal adenocarcinoma cell lines, as well as by testing its effects on the levels of hepatic antioxidant enzymes in a rat model.

The results obtained from the *in vitro* studies on the MDA and Caco-2 cell lines indicate that the *T. porrifolius* methanolic extract exhibits an antiproliferative effect on both cell lines in a time and dose-dependent manner. In the WST-1 proliferation assay, the highest dose of the extract (100 μg/mL) significantly ($P < 0.001$) reduced the proliferation of both cell lines by approximately 50% after 48 hours of treatment. However, given that this dose is higher than the IC_{50} values calculated by the Trypan Blue exclusion method, which were 74.29 μg/mL for the MDA cell line and 93.00 μg/mL for Caco-2 cell line, this reduction in proliferation might be due to cytotoxicity of the extract at this dose. Nevertheless, the 50 μg/mL concentration of the extract, which is lower than the IC_{50} calculated for both cell lines, reduced proliferation by 36% and 42% for the MDA and Caco-2 cell lines respectively.

Studies have shown that cancer cells are usually under increased oxidative stress in comparison to normal cells. This is exhibited by the detection of accumulated ROS-mediated reaction products in urine or plasma, by elevated ROS production, or by overexpression of antioxidant enzymes such as superoxide dismutase, catalase and glutathione-S-transferase as a result of the oxidative stress. Given that cancer cells are metabolically more active than normal cells, with functions associated with proliferation and uncontrolled cell growth, they demand a higher supply of ATP and consequently burden the mitochondrial respiration chain with additional stress and lead to greater ROS production. In response, the cell adapts by upregulating the expression of antioxidant enzymes in order to protect itself against the oxidative stress. Nevertheless, such adaptation mechanisms are limited because cancer cells tend to exhaust ROS-buffering capacity (Pelicano, Carney, & Huang, 2004).

CCl_4 is a potent toxin that is used extensively to study ROS-induced liver damage. This model is analogous to hepatotoxicity in humans (Ferreira et al., 2010). The results of the *in vivo* studies show a substantial and significant ($P < 0.001$) decrease in the levels of liver antioxidant enzymes in group IX, which received only CCl_4, relative to group I, which was the untreated control group. This result is consistent with many reports in the literature observing the hepatotoxicity of CCl_4 (Agbafor & Nwachukwu, 2011; Ganie, Haq, Masood, & Zargar, 2010; Lima, Suja, Sathyanarayanan, & Remya, 2010). However, the levels of these enzymes were reversed in the groups pretreated with the *T. porrifolius* methanolic extract. In addition, when the extract alone was given, without inducing toxicity with CCl_4, enzyme levels were higher than the normal untreated group (Table 1). These observations reflect an antioxidant activity by the extract.

The aforementioned antioxidant enzymes constitute the first-line defense against oxidative stress and damage caused by free radicals (Agbafor & Nwachukwu, 2011). The most important effects of *T. porrifolius* methanolic extract were observed on the levels of catalase. The groups that received the extract alone at concentrations of 50 and 250 μg/mL (groups II and III) increased the level of this enzyme by 22.56% and 78.15% respectively. In addition, in the CCl_4- treated groups, the three doses (50, 100 and 250 μg/mL) raised CAT levels by 72, 124, and 222 percent respectively compared to the vehicle group that received DMSO and

CCl_4. The extract had a less effectual influence on the levels of superoxide dismutase and glutathione-S-transferase enzymes whereby group II increased SOD and GST levels by only 13 and 16 percent, and group III by 27 and 29 percent. Similarly, in the CCl_4- treated groups, the three doses (50, 100 and 250 μg/mL) increased SOD levels by 23, 37 and 68 percent respectively. As for GST, the lowest dose exhibited no effect on this enzyme and the higher two doses lead to a 36 and 149 percent increase compared to the vehicle group.

Furthermore, the antioxidant capacity of the *T. porrifolius* extract was also tested by *in vitro* assays. In the Ferric Reducing/Antioxidant Power assay, the extract had a FRAP value of 659 μmol Fe^{2+} formed per gram of dry weight which was fairly comparable to that of ascorbic acid, 889 Fe^{2+}/g. In an article by Song et al. (2010) reporting the antioxidant capacities of 56 Chinese medicinal plants, the FRAP values ranged from as low as 0.15 μmol Fe2+/g to 856.92 μmol Fe2+/g. Based on this assay, *T. porrifolius* methanolic extract can be considered to have a relatively high antioxidant capacity. The second *in vitro* assay, the DPPH radical scavenging assay, also revealed good antioxidant activity comparable to ascorbic acid. The IC_{50} of ascorbic acid was determined to be 9.13 μg/mL, which was consistent with Kano, Takayanagi, Harada, Makino and Ishikawa (2005). The IC_{50} of the extract was calculated to be 15.18 μg/mL.

Numerous phenolic compounds are produced by plants as secondary metabolites. Among their diverse functions, including pollination, pigmentation, and resistance against pathogens and predators, polyphenols are also capable of scavenging free radicals by donating a hydrogen atom and are thus considered efficient antioxidants in a wide range of oxidation systems (Duthie et al., 2000). Total phenolic content of the *T. porrifolius* methanolic extract was calculated to be 36.95 ± 1.39 mg GAE/g dry weight. In the article by Song et al. (2010), the total phenolic content of the 56 Chinese medicinal plants ranged from 0.12 to 59.43 mg GAE/g. In another study of 51 Jordanian plant species, total phenolic content of the methanolic extracts ranged from 2.6 to 59.6 mg GAE/g dry weight, with any value greater than 20 mg GAE/g considered to be "remarkably high" (Tawaha et al., 2007). The total content of flavonoids, which are considered to have beneficial effects on a variety of diseases, including cardiovascular diseases and cancer (Hou & Kumamoto, 2010), was determined to be

16.56 ± 0.42 mg QE/g dry weight. In one study of the methanolic extracts of six species of edible Irish seaweeds, the total flavonoid content ranged from 7.41 to 42.50 mg QE/g (Cox, Abu-Ghannam, & Gupta, 2010). Consequently, the antioxidant activity of the *T. porrifolius* methanolic extract can be attributed to the relatively high levels of phenols and flavonoids.

Finally, given that any new potential therapeutic agent should not have harmful side-effects on health, the effects of the *T. porrifolius* methanolic extract on liver damage were also assessed. Levels of ALT, AST and LDH, marker enzymes released into the bloodstream in the event of tissue damage (Lima, Suja, Sathyanarayanan, & Remya, 2010), were used to evaluate hepatic damage. The extract itself didn't cause significant hepatotoxicity, with only slight elevations in AST, ALT and LDH levels. However, in the CCl_4-treated groups, all three concentrations of the extract (50, 100 and 250 mg/kg) were able to protect the liver from the CCl_4-induced toxicity by reducing the levels of these enzymes in serum. In a similar study by Jadhav, Thakare, Suralkar, Deshpande, and Naik (2010), a concentration of 200 mg/kg of silimarin, which is a standard reference drug, reduced AST, ALT and LDH levels at rates very similar to the 250 mg/kg dose of *T. porrifolius* methanolic extract when compared to the group that received only CCl_4 injections (75.84, 57.33 and 57.57 percent respectively in the case of silymarin, compared to 64.63, 56.89 and 56.45 percent in the case of the extract). These observations indicate that the methanolic extract of *T. porrifolius* has a hepatoprotective activity. The 100 mg/kg concentration of the extract also showed substantial and significant ($P < 0.01$) antihepatotoxic effects by reducing AST, ALT and LDH levels by 46.57, 39.73 and 53.36 percent respectively compared to group IX (received only CCl_4).

In conclusion, the *Tragopogon porrifolius* methanolic extract exhibited anticancer, antioxidant and hepatoprotective effects. Its anticancer activity was revealed through *in vitro* assays on the MDA and Caco-2 cell lines, which showed a time and dose-dependent inhibition of cell proliferation and increased cell death. Antioxidant activity was demonstrated both *in vivo* by increasing the activities of SOD, CAT and GST antioxidant enzymes in extract-treated groups in a rat model, and *in vitro* by exhibiting high reducing capacity in the FRAP and DPPH assays. This antioxidant activity may be attributed to the relatively high contents of total phenols and flavonoids determined to be contained in the extract. Lastly, reducing the levels of AST, ALT

and LDH marker enzymes in CCl_4-treated rats indicated that not only is *T. porrifolius* non-toxic to the liver, but it also provides a protective role against liver toxicity in rats.

Given that the crude extract was used in these experiments, further studies are needed to determine the active component in *T. porrifolius* and establish its mechanism of action.

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APPENDIX A

Group		**CAT** (k/mg)	**GST** (nmol/min/mg)	**SOD** (units)
I	Normal (no treatment)	4.77 ± 0.54	7.37 ± 0.52	13.44 ± 0.60
II	*T. porrifolius* 50 mg/kg	5.85 ± 0.81	8.51 ± 0.73	15.25 ± 0.68
III	*T. porrifolius* 250 mg/kg	8.51 ± 0.54 [a]	9.54 ± 0.65 [a]	17.00 ± 0.56 [a]
IV	olive oil + CCl_4	1.07 ± 0.15 [a]	2.58 ± 0.43 [a]	9.88 ± 0.35 [a]
V	DMSO + CCl_4	1.43 ± 0.11 [a]	2.35 ± 0.51 [a]	8.38 ± 0.52 [a]
VI	*T. porrifolius* 50 mg/kg + CCl_4	2.46 ± 0.34 [a b]	1.64 ± 0.16 [a]	10.32 ± 0.91 [a]
VII	*T. porrifolius* 100 mg/kg + CCl_4	3.19 ± 0.35 [a b]	3.19 ± 0.41 [a]	10.91 ± 0.63 [a b]
VIII	*T. porrifolius* 250 mg/kg + CCl_4	4.61 ± 0.27 [b]	5.86 ± 0.65 [b]	14.11 ± 0.73 [b]
IX	only CCl_4	1.05 ± 0.12 [a]	0.92 ± 0.13 [a]	6.33 ± 0.74 [a]

Table 1. Effect of *T. porrifolius* methanolic extract on the activities of liver antioxidant enzymes. Values are mean ± SEM of 6 animals in each group and evaluated by two-tailed *t*-test. $P < 0.02$ compared to the normal control group (group I) except for groups II and VIII for which $P > 0.05$.

[a] $P < 0.05$ with respect to the normal group

[b] $P < 0.001$ with respect to the vehicle group (DMSO + CCl_4)

Group		**AST** (U/L)	**ALT** (U/L)	**LDH** (U/L)
I	Normal (no treatment)	61.83 ± 2.44	33.44 ± 2.63	312.74 ± 53.27
II	*T. porrifolius* 50 mg/kg	60.67 ± 0.86	35.58 ± 1.20	315.2 ± 16.74
III	*T. porrifolius* 250 mg/kg	67.81 ± 1.54	38.35 ± 2.45	369.37 ± 27.17
IV	olive oil + CCl_4	83.32 ± 4.91	51.72 ± 3.83	565.83 ± 52.77
V	DMSO + CCl_4	122.89 ± 14.22	58.62 ± 4.24	727.53 ± 96.12
VI	*T. porrifolius* 50 mg/kg + CCl_4	89.83 ± 8.20 *	48.12 ± 5.71	505.63 ± 62.30 *
VII	*T. porrifolius* 100 mg/kg + CCl_4	73.5 ± 1.86 *	39.96 ± 2.63 *	372.38 ± 28.12 *
VIII	*T. porrifolius* 250 mg/kg + CCl_4	59.30 ± 5.82 *	28.87 ± 5.65 *	282.37 ± 17.97 *
IX	only CCl_4	137.57 ± 9.03	66.30 ± 4.10	798.40 ± 53.57

Table 2. Effect of *T. porrifolius* methanolic extract on the activities of liver function enzymes in serum. Values are mean ± SEM of 6 animals in each group and evaluated by two-tailed *t*-test.

* $P < 0.05$ with respect to the group that received DMSO and CCl_4.