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Lebanese Plants and Plant-Derived Compounds Against Colon Cancer

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ACADEMIC DISSERTATION

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To My Family

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LIST OF ORIGINAL PUBLICATIONS

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The publications are referred to in the text by their roman numerals.

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ABBREVIATIONS

ACF	Aberrant crypt foci
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, excretion
AGP	Alpha-1-acid glycoprotein
BSA	Bovine serum albumin
DCC	Deleted in colon cancer
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMH	1, 2-Dimethylhydrazine
DMSO	Dimethyl sulfoxide
DiOC6	3,3' - Dihexyloxacarbocyanine iodide
ESI	Electrospray ionisation
CRC	Colorectal cancer
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
HNPCC	Hereditary non-polyposis colon cancer
HPLC	High performance liquid chromatography
HSA	Human serum albumin
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LLE	Liquid-liquid extraction
MAPK	Mitogen-activated protein kinases
MS	Mass Spectrometry
MTT	Method of transcriptional and translational assay
NAC	N-acetyl-cysteine
NQO	NAD(P)H:quinone oxido-reductases
PBS	Phosphate buffer saline
PD	Pharmacodynamic
PK	Pharmacokinetic
PI	Propidium iodide
ROS	Reactive oxygen species
SPE	Solid phase extraction
TQ	Thymoquinone
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
i.p.	Intraperitoneal
s.c.	Subcutaneous

ABSTRACT

Colorectal cancer (CRC) is a major health concern and demands long-term efforts in developing strategies for screening and prevention. Over the past two decades, CRC has become a preventable disease. This remarkable revolution is a consequence of a better understanding of colorectal carcinogenesis, in particular, pre-symptomatic transitional lesions (i.e. adenomas) and their association with CRC occurrence and mortality. However, current therapy is unsatisfactory and necessitates the exploration of other approaches for the prevention and treatment of cancer. Plant based products have been recognized as preventive with regard to the development of colon cancer. Therefore, the potential chemopreventive use and mechanism of action of Lebanese natural product were evaluated.

Towards this aim the antitumor activity of *Onopordum cynarocephalum* and *Centaurea ainetensis* has been studied using *in vitro* and *in vivo* models. *In vitro*, both crude extracts were non cytotoxic to normal intestinal cells and inhibited the proliferation of a host of colon cancer cells in a dose-dependent manner. Flow cytometric analysis showed that both extracts induced apoptosis. Extract induced apoptosis has been associated with the ability to induce the expression of p53 and p21 proteins and cause a significant increase in Bax/bcl-2 ratio. *In vivo*, both crude extracts injected intraperitoneally (i.p) prior to the subcutaneous (s.c) injection of the carcinogen 1, 2-dimethylhydrazine (DMH), reduced the number of tumors by an average of 65% at weeks 20 and 30.

The activity of the *C. ainetensis* extract was attributed to Salograviolide A, a guaianolide-type sesquiterpene lactone, which was isolated and identified through bio-guided fractionation. Salograviolide A was found to induce apoptotic cell death in colon cancer cells at non-cytotoxic concentrations to normal human intestinal cells.

The mechanism of action of thymoquinone (TQ), the active component of *Nigella sativa*, was established in colon cancer cells using *in vitro* models. By the use of N-acetyl cysteine (NAC), a radical scavenger, the direct involvement of reactive oxygen species (ROS) in TQ-induced apoptotic cells was established. TQ-increased phosphorylation states of the mitogen-activated protein kinases (MAPK) was linked to ROS generation. By using specific MAPK inhibitors, the two kinases JNK and ERK were found to possess pro-survival activities in TQ-induced apoptotic cell death.

The analytical detection of TQ from spiked serum and its protein binding were evaluated. TQ was extracted from spiked serum by several extraction procedures prior to HPLC analysis. The average recovery from all the methods used was 2.5% proving the inability of conventional methods to analyze TQ from serum. Ultracentrifugation with 3K cut off filter and HPLC analysis were used to determine the percentage of binding of TQ to protein serum. More than 98% of protein binding was observed as of 0 min of TQ incubation with fetal bovine serum (FBS). Extensive binding was observed between TQ and two major plasma proteins, bovine serum albumin (BSA) and alpha-1-acid

glycoprotein (AGP). Incubation of TQ with BSA and AGP resulted in 94.5 ± 1.7 and 99.1 ± 0.1, percentage of binding, respectively. BSA and AGP's binding showed a differential effect on TQ's antiproliferative activity. While TQ bound to AGP retained its inhibitory effect as compared to TQ alone, its inhibitory effect was altered when bound to high concentration of BSA. Using mass spectrometry analysis, TQ was confirmed to bind covalently to the free cysteine in position 34 and 147 of the amino acid sequence of BSA and AGP, respectively.

The results of this work put at the disposal for future development new plants with anti-cancer activities and enhance the understanding of the pharmaceutical properties of TQ, a prerequisite for its future clinical development.

1. INTRODUCTION

Cancer is classified as the second leading cause of death after cardiovascular diseases (Heron *et al.* 2009). Worldwide more than 20 million people are living with cancer, a fatal disease estimated to kill 9 million people by 2015 (Darwiche *et al.* 2007).
The most frequent cancers are lung, colorectal (CRC), stomach, liver, and breast. CRC is the most common gastrointestinal cancer and a leading cause of death in the world. Although surgical excision is the best option for treatment, many patients who undergo therapeutic resection will develop tumor recurrences. Therefore, other approaches for the prevention and treatment of cancer are necessary. The fact that CRC is a stepwise process that takes 5-20 years from the time of initiation to adenoma formation, and another 5-15 years until the beginning of the invasive stage offers a great opportunity for its prevention.

Medicinal plants have been used by diverse cultures around the world and recognized as preventive with regard to the development of cancer (Lee *et al.* 2006). In addition compelling evidence from epidemiological and experimental studies emphasizes the importance of compounds derived from plants to reduce the risk of cancer and inhibit the development and spread of tumors in experimental animals. Actually, more than 50% of drugs used during the last 20 years are directly or chemically altered natural products (Newman *et al.* 2007). For instance, vincristine and vinblastine are examples of plant-derived compounds used against hematologic cancers such as multiple myeloma, acute lymphoblastic leukemia, Hodgkin's and non-Hodgkin's lymphoma and Wilms' tumor (Advani *et al.* 2006; Cragg *et al.* 2005; Kumar *et al.* 2006; Darwiche *et al.* 2007). The advantage of plant derived compounds for cancer treatment is due to their health benefits and long use in folk medicine when substantial information have been collected on the safe use of plants, contrasted by the limitations of chemotherapeutic agents (Manson *et al.* 2005).

The utilization of plants is greatly affected by geography, climate and other major influencing factors; therefore, the higher the diversity of a certain region, the higher the possibilities of finding plants with medicinal properties. The Mediterranean coast has varied microclimates with a wide diversity of plant species. Lebanon, a Mediterranean country, comprises more than a hundred plant species that are reported to have medicinal properties (Rouwayha 1983), have been used traditionally for hundreds of years (Rouwayha 1981), and are still commonly used. Some of the reported plants are *Achilea falcate*, *Calendula officinalis*, *Centaurea ainetensis*, *Onopordum cynarocephalum*, *Ranunculus constantinopolitanus* and *Nigella sativa.* Most of the aforementioned plants belong to the Astarecae/Compositae or Ranunculucae families known to have beneficial effects including anti-inflammatory, anti-diabetic, anti-rheumatic properties amongst others (www.ibsar.org). The scientific investigations on these plants are at different stages of development. While the reported data for most plants used in folk medicine is limited, the beneficial effects of *N. sativa* have been extensively studied and are already attributed to its quinone contents and specifically to thymoquinone (TQ).

Therefore, as part of the ongoing search for natural products against colon cancer from traditionally used plants, two approaches have been followed: 1) unbiased approach to identify potential plant extracts against colon cancer based on screening studies, and 2) biased approach in which further mechanistic and analytical-investigations are carried on the well established anticancer compound TQ, derived from *N. sativa*, to gather more information that is needed before TQ can be further developed towards clinical applications.

2. REVIEW OF THE LITERATURE

2.1. Colorectal Cancer

2.1.1. Colorectal Cancer: Types and Molecular Genetics

CRC is the most common gastrointestinal cancer and a leading cause of cancer deaths worldwide. Environmental as well as hereditary factors play a role in CRC development. For instance, sporadic cancer, those cases that occur in individuals over age 50 without any identifiable predisposing factors, account for more than 75% of the various causes of CRC while the remaining are accounted for by familial incidence and inflammatory bowel diseases.

Familial adenomatous polyposis (FAP) is an autosomal dominant colon cancer predisposition syndrome that appears upon the inheritance of a single copy of a mutant gene (Farinella *et al.* 2010; Kucherlapati *et al.* 2001). FAP syndrome is characterized by the appearance of hundreds to thousands of colonic polyps during the second and the third decade of life and is considered to be the least common type of colon cancer (Farinella *et al.* 2010; Kucherlapati *et al.* 2001). The gene responsible for FAP has been identified in 1991 by Groden et al., (Groden *et al.* 1991) and termed the adenomatous polyposis coli (APC) gene.

Hereditary non-polyposis colon cancer (HNPCC), also known as lynch syndrome, is another autosomal dominant disorder that accounts for 2-4% of the cancer cases (Jasperson *et al.* 2010; Kucherlapati *et al.* 2001). Individuals with HNPCC are also at increased risk of other cancers, such as stomach, ovarian, small bowel, biliary, and kidney (Jasperson *et al.* 2010). Germline mutations in any of the following mismatch repair genes (mut l homolog 1 [MLH1], mut s homolog 2 [MSH2], mut s homolog 6 [MSH6], post-meiotic segregation increased 1 [PMS1], post-meiotic segregation increased 2 [PMS2], and mut s homolog 3 [MSH3]) results in HNPCC (Neibergs *et al.* 2002). Mutations in MLH1 and MSH2 are the most commonly observed mutations in HNPCC patients (Markowitz *et al.* 2009).

Human sporadic colon cancer is thought to arise from spontaneous or induced somatic mutations in critical tumor suppressor genes or DNA mismatch repair genes (Nambiar *et al.* 2003). It has been proved that chromosomal instability or loss of heterozygosity (LOH) in tumor suppressor gene such as p53, APC, or deleted in colon cancer (DCC), or proto-oncogenes such as *Kras, c-erb,* or *c-myc* are implicated in 80% of sporadic CRC (Neibergs *et al.* 2002; Rajamanickam *et al.* 2008; Zhou *et al.* 2004). Microsatellite instability (MSI) associated with mutations in DNA repair genes, on the other hand, has been identified in 15-20 % of the cases (Mutch *et al.* 2007; Neibergs *et al.* 2002).

2.1.2. Morphological Changes in Colorectal Cancer

The occurrence of cancer is associated with the incidence of pre-morphological alterations in apparently normal mucosa prior to the appearance of adenomatous polyps. The development of these morphological lesions is preceded and accompanied by a disorder of cell proliferation and differentiation. These lesions known as aberrant crypt foci (ACF) have been identified in the colonic mucosa of rodents exposed to colorectal carcinogens and on the colonic mucosa of patients operated on for cancer or benign diseases of the large bowel (Bird 1987; Bird *et al.* 1989; Dias *et al.* 2010; Janakiram *et al.* 2010; Pretlow *et al.* 1991). ACF are distinguished from normal crypts by their darker staining, larger size, elliptical shape, and thicker epithelial lining. Although not all ACF have the potential to be transformed into adenomas and then carcinomas, these lesions by their morphological and molecular features are known to be precursors of colorectal cancer, extending hence the notion of adenoma-carcinoma sequence to ACF-adenoma-carcinoma (Cappell 2007). In fact, histological examination of ACF shows that these foci have variable features ranging from mild hyperplasia to dysplasia (Siu *et al.* 1997; Siu *et al.* 1999). Only dysplastic ACF, a hallmark of malignant transformation, have the potential to develop into tumors. The progression of ACF to carcinoma results from the succession and accumulation of series of genetic alterations in colonic mucosa (Humphries and Wright 2008).

Vogelstein *et al*. in 1988 have been the first to describe the stepwise process of colorectal carcinogenesis through which a series of genetic and epigenetic alterations lead to the development of carcinoma from the normal mucosa (Vogelstein *et al.* 1988). Substantial data suggest that carcinomas (malignant tumors) originate from adenomas (benign tumors) (Cunningham *et al.* 2010; Sugarbaker *et al.* 1985). The evolution of colorectal cancer through the adenoma-carcinoma sequence can be simplified by highlighting the intervention of some of the most important markers: normal epithelium, upon loss or mutation of the APC gene, is transformed into hyper-proliferative epithelium. DNA methylation is responsible for the transformation of the hyper-proliferative epithelium into early adenoma. Due to the mutation of the K-ras gene and loss of DCC, the early adenoma is transformed into dysplastic adenoma, upon which the loss of p53 function results in the appearance of carcinoma. Though the adenoma-carcinoma sequence is a well-established concept for the development of colorectal cancer, this, however, does not imply that all adenomas will transform into carcinomas since many of them may regress (Sillars-Hardebol *et al.* 2010). In fact, the total accumulation of the genetic alterations, rather than their order according to a preferred sequence, is responsible for the determination of the tumor's properties (Vogelstein *et al.* 1988).

2.1.3. Treatment of Colon Cancer by Natural Anticancer Compounds

Cancer chemoprevention is defined as the use of natural or synthetic compounds capable of inducing biological mechanisms necessary to preserve genomic fidelity (Hauser and

Jung 2008; Pan and Ho 2008b; Umar *et al.* 2002). Such mechanisms include protection against mutagens/carcinogens through the inhibition of uptake, activation or via enhanced DNA repair/replication or apoptosis (Hauser *et al.* 2008; Sporn 1976). A potential chemopreventive agent has to be able to prevent, inhibit, or reverse carcinogenesis prior to the development of the invasive disease (Krzystyniak 2002; Pan and Ho 2008b). An ideal agent is one that 1) kills cancer cells while sparing normal ones, 2) has a defined mechanism of action, 3) is effective in multiple sites, 4) can be given orally, and 5) has low cost and high human acceptance.

Several natural anticancer compounds are already on the market to treat different types of cancer. These agents belong to four different classes; the vinca alkaloids, the epipodophyllotoxins, the taxanes, and the camptothecin derivatives (Darwiche *et al.* 2007). For instance, vincristine and vinblastine (vinca alkaloids) isolated from the plant *Catharanthus roseus* have been used for the treatment of hematologic cancers (Advani *et al.* 2006; Cragg and Newman 2005; Kumar *et al.* 2006). Vindesine and vinorelbine are semisynthetic analogues of vinca alkaloids that show less toxicity and a wider range of antitumor properties as compared to vincristine and vinblastine (Drawiche *et al.* 2007). The anti-cancer properties of podophyllotoxin, from the plant extract of *Podophyllum peltatum*, were discovered in 1942, but due to its high toxicity it has been discarded (You 2005). In the mid 1960, semisynthetic analogues of podophyllotoxin, etoposide and teniposide, were discovered and are used for the treatment of lymphomas, non-Hodgkin's lymphoma, Hodgkin's lymphoma, acute leukemia, prostate, lung, testicular, bronchial and ovarian cancers (Bhutani *et al.* 2006; Kelland 2005; You 2005). Paclitaxel, discovered from the Pacific Yew tree *Taxus brevifolia*, and its semisynthetic analogue docetaxel are the most commonly used taxanes, a class of alkaloids that possess potent anticancer activities. While paclitaxel is used for the treatment of ovarian, breast, prostate, urological and bladder cancers; docetaxel is used for the treatment of metastatic breast, ovarian, urothelial, lung, head and neck, gastric and prostate cancers (Kelland 2005; Lyseng-Williamson and Fenton 2005; Yusuf *et al.* 2003). 20(S)-camptothecin (CPT), isolated in 1966 from the bark of the Chinese tree *Camptotheca acuminate*, was discarded in the 1970s due to its severe toxicity despite its strong antitumor properties against gastrointestinal and urinary bladder tumors (Li *et al.* 2006; Wall *et al.* 1966). Other plant-derived compounds such as flavopiridol, combretastatin A4, roscovitine, among others, are still under clinical trials and show promising anticancer results (Darwiche *et al.* 2007; Desai *et al.* 2008; Cragg and Newman 2005).

Due to the fact that plants are home to many secondary metabolites that may provide chemoprotection against cancer, they potentially represent an inexhaustible source for the discovery of new drugs.

2.2. Lebanese Plants in Drug Discovery

Lebanon comprises more than 83 plant families with more than 2500 species distributed along its relatively small geographical area. More than hundred plant species used

traditionally are still commonly used to promote health (Rouwayha 1981; Rouwayha 1983). However, the scientific studies that evaluate the activities of the Lebanese plants used in folk medicine are limited. In an effort to study the folk use of these plants, the Nature Conservation Center for Sustainable Futures (IBSAR) at the American University of Beirut has set forth to conduct a thorough investigation to prove scientifically their use against diseases such as cancer. Towards this end, a screening program has been initiated since 2002 and a total of 110 crude extracts have been tested for their antiproliferative potential and cytotoxicity effects in a panel of normal and cancer cells using two bioassays, namely cell proliferation and cytotoxicity. The selection criteria have been pre-defined as the potential extract has to inhibit the proliferation of cancer cells by more than 50% without being toxic to normal cells. Based on the selection criteria cited previously, several plant extracts with potent and selective bioactivities against cancer cells versus normal cells have been identified among which are the crude extracts from *O. cynarocephalum* and *C. ainetensis.* On the other hand, *N. sativa*, used in Lebanese folk medicine, has been extensively studied. In fact, several studies have shown that *N. sativa* plant and its active constituent TQ have chemopreventive effect against colon cancer using both *in vitro* and *in vivo* models.

2.2.1. *O. cynarocephalum* Boiss. & Blanche

O. cynarocephalum (Fig. 1a), a species of *Onopordum*, belongs to the Compositae family, which comprise about 40 species distributed throughout the Mediterranean and semiarid areas of Eurasia and North Africa (Rees *et al.* 1999).

Onopordum is an early Latin name given to a group of thistles. The name is derived from the Greek ‘’onos’’ which means ass and ‘’porde’’ which means flatulence, due to the belief that the plant induces flatulence in donkeys. *Cynarocephalum* also known as kynaros akantha, kynara or kinara derived from the Greek describing a spiny plant; Latin cinara has been attributed for a kind of artichoke and for a native of the island of Cinara, in the Aegean Sea, now Zinara;-cephalum means headed; cynara headed.

O. cynarocephalum, an endemic plant to Lebanon known in Arabic as Shawk, grows wild to 0.5-3 meters tall on the mountain Hermon. The plant has an alternate leaf pattern and purple flowers. The flowering period of the plant is between May and July and its optimal growth necessitates a nutrient rich soil (http://www.ibsar.org/Research/Traditional Knowledge and Biotechnology/Plants/Onopordum cynarocephalum.html). Although *O. cynarocephalum* has been traditionally used in Lebanon for its antibacterial, haemostatic, and hypotensive properties as well as for the treatment of skin cancer, the reported scientific studies proving the folk use for this species are limited. However, other species from *Onopordum* genera have been found to exert several biological activities such as the inhibition of TNF-α and nitric oxide in murine endothelial cells (Strzelecka *et al.* 2005), the augmentation of the NK cell activity (Abuharfeil *et al.* 2000; Abuharfeil *et al.* 2001), and the ability to change the rhythmic system by adjusting heart rate activity (Cysarz *et al.* 2000). The beneficial effects of *Onopordum* genera are due to the presence of many secondary metabolites with flavonoids, lignans, and sesquiterpene lactones being the most

common constituents (Braca *et al.* 1999; El-Moghazy *et al.* 2002; Cardonas *et al.* 1992; Lazari *et al.* 1998).

2.2.2. *C. ainetensis* Boiss.

C. ainetensis (Fig. 1b) is one of more than 500 species of the genus *Centaurea* (Asteraceae family) that are distributed around the Mediterranean area and West Asia (Mabberlay 1997).

C. ainetensis, an endemic plant to Lebanon known in Arabic as shawkil-dardar, grows wild in specific areas such as sterile, stony, or bushy places. The plant, however, does not grow in rocky habitats. The plant has a short and erect stem and its flowering period falls between May and June (http://www.ibsar.org/Research/Traditional Knowledge and Biotechnology/Plants/Centaurea ainetensis.html). *Centaurea* species have been traditionally used for their therapeutic and curative properties specifically by acting as stimulants, diuretics, anti-rheumatics, antimicrobial, anti-inflammatory, anti-tumor and anti-diabetics. The wide range of activities observed in the use of *Centaurea* in folk medicine along with the availability of many species has attracted the attention of scientist to test their efficacy. *C. ainetensis* and other *Centaurea* species have been reported to induce biological activities such as antioxidant (Karamenderes *et al.* 2007), antimicrobial (Buruk *et al.* 2006; Skliar *et al.* 2005), wound healing (Csupor *et al.* 2010), anti-tumor (Ghantous *et al.* 2008; Koukoulitsa *et al.* 2002), and anti-inflammatory properties (Al-Saghir *et al.* 2009).

2.2.3. *N. sativa* L.

N. sativa (Fig. 1c) (Ranunculaceae) is an annual flowering plant widely grown in different parts of the world specifically in countries bordering the Mediterranean area, in Pakistan and in India.

In English, *N. sativa* seeds are known as Black Seed, Black Caraway, Black Cumin, and Roman Coriander. It is referred to as Melanthion by Hippocrates and Dioscorides. In old Latin it is called Panacea meaning cure all, while in India and China it is called Kalonji and Hak Jung Chou, respectively (Aggarwal *et al.* 2008). In Arabic the seeds are known as Habbatul-Barakah, which translates directly to the seeds of blessing. This latter description comes from the fact that the seeds are mentioned in religious texts such as the Quran, Bible and Torah.

The plant grows to 20-30 cm tall. The plant has finely divided leaves with usually pale blue and white coloured flowers. The fruit is a capsule made of several united follicles containing each numerous seeds.

This plant has been used for centuries in many Middle Eastern and Indian countries for culinary and medicinal purposes (Ali and Blunden 2003; Marsik *et al.* 2005; Norwood *et*

al. 2006). The folk uses of this plant include treatment of headache, coughs, abdominal pain, diarrhaea, asthma, rheumatism and others. For the last three decades, this plant has been the focus of scientific research to study its chemical composition as well as its biological activities *in vitro* and *in vivo*. Numerous studies show a broad spectrum of the plant's biological activities including anti-inflammatory (Ali and Blunden 2003; Landa *et al.* 2009), anti-diabetic (Benhaddou-Andaloussi *et al.* 2010), anti-hypertensive (Dehkordi *et al.* 2008), anti-histaminic (Boskabady *et al.* 2007), along with significant anti-neoplastic properties (Ait Mbarek *et al.* 2007; Worthen *et al.* 1998). In addition, oral use of the seed in moderate amount in food has been found to be safe (Der Marderosian *et al.* 2005). Therefore, based on the above reported activities, development of black seed-derived compounds could be useful in modern medicine.

2.3. Salograviolide A and Thymoquinone

Salograviolide A. The reported activities of *Centaurea* species have been attributed to the presence of various types of sesquiterpene lactones (Al-Saghir *et al.* 2009; Koukoulitsa *et al.* 2002; Ghantous *et al.* 2008; Giordano *et al.* 1992). For instance, Solstitialin A and acetyl solstitialin, sesquiterpene lactones isolated from *C. solstitialis* and *C. depressa*, have been found to be the components responsible for the anti-nociceptive and anti-pyretic activities of these plants (Akkol *et al.* 2009).

Sesquiterpene lactones isolated from *C. pullata* and *C. deusta*, have been found to exert antibacterial and antifungal activities (Djeddi *et al.* 2008; Karioti *et al.* 2002). Salograviolide A (Fig. 1d), a sesquiterpene lactone guaianolide, isolated from *C. nicoli* has been found to possess antifungal properties (Vajs *et al.* 1999). In addition, anti-inflammatory and anti-skin cancer properties have been attributed to Salograviolide A isolated from *C. ainetensis* (Al-Saghir *et al.* 2009; Ghantous *et al.* 2008).

Thymoquinone. The activities exerted by *N. sativa* have been attributed to its quinone content, specifically to TQ ($C_{10}H_{12}O_2$) (Fig. 1e) (Ali and Blunden 2003). The therapeutic potential of TQ has been confirmed in cancer research. *In vivo*, TQ has been found to reduce tumor growth using DMH and xenograft models of human colon cancer (Gali-Muhtasib *et al.* 2008a)**,** prevents tumor angiogenesis in a xenograft model of human prostate cancer (Richards *et al.* 2008), inhibits the incidence and multiplicity of benzo(a)pyrene (BP)-induced fore-stomach tumors (Badary *et al.* 1999) and 20-methylcholanthrene (MCT)-induced fibrosarcoma tumors (Badary *et al.* 2001). *In vitro*, the inhibitory effect of TQ against colon cancer cells (El-Mahdy *et al.* 2005; El-Najjar *et al.* 2010; Gali-Muhtasib *et al.* 2008b), lymphoblastic leukemia cells (Alhosin *et al.* 2010), laryngeal carcinoma cells (Richards *et al.* 2006; Womack *et al.* 2006), pancreatic cells (Chehl *et al.* 2009), and prostate cancer cells (Richards *et al.* 2008; Richards *et al.* 2006) is well established.

Generally, in the process of drug discovery a potential lead compound has to undergo preclinical evaluation prior to clinical trials. This includes lead optimization, definition of

mechanism of action, animal toxicity, and determination of its absorption, distribution, metabolism and excretion (ADME). Once the ADME is defined, the compound enters the phase of drug development, production/formulation prior to clinical trials (Lee 2010). Even though a review of the literature has shown that TQ induces apoptotic cell death via p53-dependent (Gali-Muhtasib *et al.* 2004), p53-independent (El-Mahdy *et al.* 2005; Roepke *et al.* 2007), and p73-dependent pathways (Alhosin *et al.* 2010); its exact mechanism of action is not yet fully elucidated. In addition, there are no reports on TQ's analytical detection or ADME.

To better delineate TQ's pharmaceutical properties it is important to clarify the chemical and analytical properties of quinones, the family to which TQ belongs.

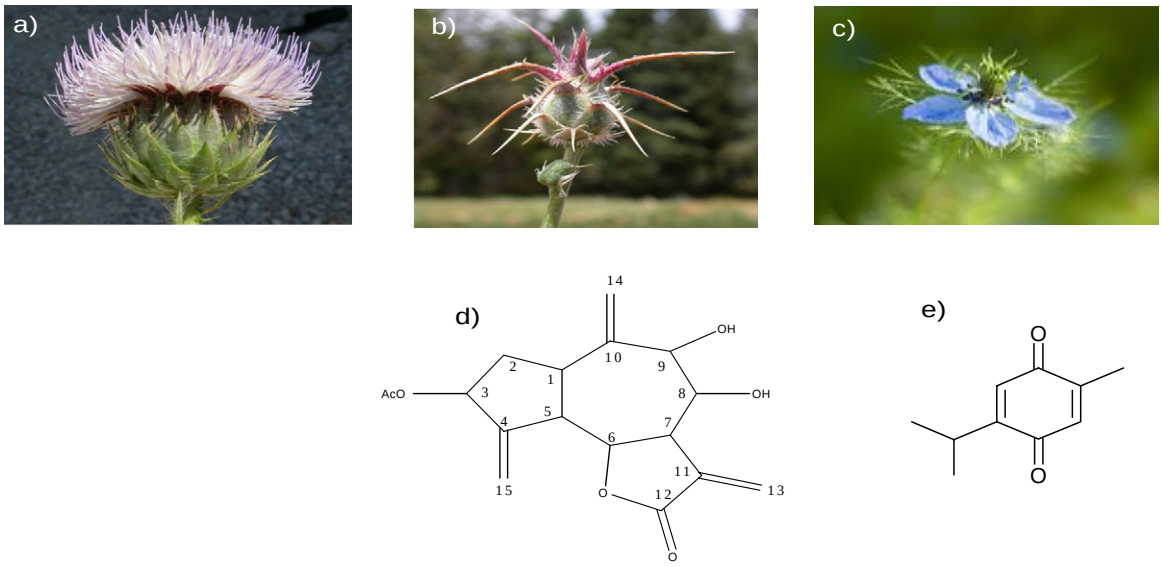


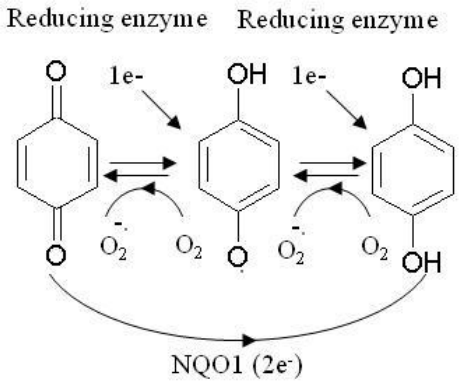
Figure 1. a) *O. cynarocephalum*, b) *C. ainetensis*, c) *N. sativa*, d) Salograviolide A, e) Thymoquinone.

2.4. Chemistry of Quinones

Quinones are an important class of molecules harbouring physiological and therapeutic effects. They have two properties that define their biological activities. The first is their ability to undergo one or two electron reduction and the second is their ability to undergo nucleophilic attack. The knowledge of the inherent chemical reactivity of quinones is relevant to understand their physiological and toxicological properties.

2.4.1. Quinones Redox Cycle

The mechanism of quinones cytotoxicity is attributed mainly to their ease of reduction and therefore their ability to act as oxidizing or dehydrogenating agents. In biological systems quinones can undergo one or two electron reduction by cellular reductases leading to the corresponding semiquinones or hydroquinones, respectively (Scheme 1).



Scheme 1. Illustration, using benzoquinone as an example, of one and two electron reduction yielding semiquinone and hydroquinone respectively.

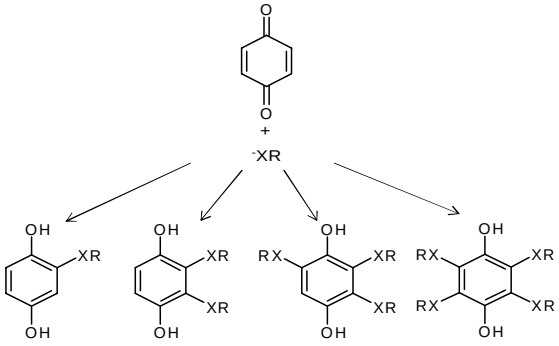
The one-electron reduction of quinones can be catalyzed by a number of enzymes, including microsomal NADPH cytochrome P450 reductase (P450R), microsomal NADH cytochrome b5 reductase (b5R), and mitochondrial NADH ubiquinone oxidoreductase (Holtz *et al.* 2003; Monks and Jones 2002; Yan *et al.* 2008). The semiquinone radical, formed by one electron reduction, gets oxidized under aerobic conditions to the initial quinone with the generation of superoxide anion radicals. In aqueous solutions the former radicals interact with molecular oxygen to give rise to hydrogen peroxide, which in the presence of iron forms toxic hydroxyl radicals to which the toxicity of quinones is attributed (Asche 2005; Kappus *et al.* 1986). Ample evidence proves that ROS production, by numerous anticancer agents, is responsible for apoptosis induction in cervical (Lin *et al.* 2008), pancreatic (Zhang *et al.* 2008), gastric (Qian *et al.* 2008b), breast (Xiao *et al.* 2008), colon (Pan *et al.* 2008a), and hematologic cancers (Feng *et al.* 2007). For instance one electron reduction of doxorubicin (Bartoszek and wolf 1992), tirapazamine (Chinje *et al.* 1999; Patterson *et al.* 1995; Patterson *et al.* 1997), indoloquinone (Bailey *et al.* 2001), and mitomycin (Belcourt *et al.* 1998; Cowen *et al.* 2003; Joseph *et al.* 1996; Wang *et al.* 2007) leads to significant increase in their cytotoxicity against cancer.

In addition to the one-electron reduction, quinones can undergo two-electron reduction catalysed by the cytosolic flavoenzymes NAD(P)H:quinone acceptor oxidoreductases (NQO). NQO1, also known as DT-diaphorase, is a well-studied NQO and is considered a distinctive flavoenzyme for three reasons. First, it displays a nonspecific reactivity towards NADH and NADPH and shows broad electron acceptor specificity, catalyzing the reduction of quinones and structurally related compounds. Second, it is strongly inhibited by the NAD(P)H competitive inhibitor dicumarol and other oral anticoagulants. Third, the most striking feature is its ability to catalyze the so-called “obligatory” two-electron transfers (Bianchet *et al.* 2004; Cadenas 1995). This obligatory 2-electron reduction competes with the one-electron reduction of quinones by enzymes such as P450R and protects cells against oxidative stress (Guo *et al.* 2008). This protection is the result of conversion of quinones to hydroquinones rather than semiquinones and ROS, which is generated by redox cycling of semiquinones in the presence of molecular oxygen (Bianchet *et al.* 2004; Kappus and Sies 1981; Tampo and Yonaha 1996). Three types of hydroquinones are formed by DT-diaphorase action, 1) redox-stable hydroquinones, 2) redox-labile hydroquinones that subsequently auto-oxidize with formation of ROS and 3) hydroquinones that readily rearrange to potent electrophiles participating in bio-alkylation

reactions (Cadenas 1995). The properties of the hydroquinone generated by DT-diaphorase determine whether this reduction leads to the activation or deactivation of quinones.

2.4.2. Quinones Nucleophilic Addition

Quinones's electrophilic character enables them to undergo nucleophilic attack, which may lead to either detoxification or enhanced toxicity (Scheme 2). In a biological system, such nucleophiles may be found as reactive side-groups of lysine, serine and cysteine (Magee 2000). However, the thiol group of glutathione (GSH) represents the first to be involved in the nucleophilic addition reaction with quinones. In fact, the first line of cellular defense is controlled by GSH, an active ROS scavenger and the most abundant non-protein antioxidant in the cell. Many quinones can be conjugated to the sulfhydryl group of GSH and this reductive addition represents their major route of elimination. Quinone-GSH conjugation is a detoxification reaction because of the more hydrophilic character of the formed adduct as compared to the parent quinone. This conjugation can occur either spontaneously via a reductive addition or is catalyzed by glutathione-*S*-transferase leading to hydroquinone-glutathionyl conjugates (Buffinton *et al.* 1989; Jakoby and Ziegler 1990). Yet, nucleophilic addition might lead to their enhanced toxicity. For instance, quinone-GSH conjugation can also contribute to compound toxicity. This is caused in some cases by the faster redox cycling of the glutathionyl conjugates compared to that of the parent quinone (Buffinton *et al.* 1989; Jakoby and Ziegler 1990; van Ommen *et al.* 1992). Another mechanism of toxicity stems from the significant depletion of the reduced thiol form of glutathione by alkylation in the presence of high concentrations of quinones. Once the detoxification system is saturated by GSH depletion, cellular SH-dependent proteins can be alkylated thereby causing irreversible changes and cell death (Buffinton *et al.* 1989; Jakoby and Ziegler 1990). The propensity of quinones to bind to nucleophilic functional groups commonly found on many cellular components, represents the most popular mechanistic theory underlying their toxicity. Mutation and/or protein dysfunction can result from the conjugation of quinones to proteins or DNA (Buffinton *et al.* 1989; Jakoby and Ziegler 1990).



Scheme 2. Illustration, using benzoquinone as an example, of the nucleophilic addition with formation of mono-,di-,tri-, and tetra-susbtitution.

2.4.3. Analytical Detection of Quinones

A wide range of analytical methods has been reported for the determination of quinones in plants, pharmaceutical preparations, as well as in biological samples. Gas chromatography (GC) (Zuo *et al.* 2008), Raman microscopy (Beattie *et al.* 2007), high-performance liquid chromatography (HPLC) (Xue *et al.* 2008), and mass spectrometry (MS) (Kang *et al.* 2007) have been used for the identification and quantification of quinones. HPLC and HPLC/MS are the most frequently used methods.

Sample cleanup procedures for quinones are usually performed using solid phase extraction (SPE), liquid-liquid extraction (LLE) or protein precipitation. Protein precipitation using methanol (MeOH), ethanol (EtOH), and acetonitrile (ACN) has been usually used to disrupt protein binding and remove interferences from biological samples. SPE, in addition to its use as a cleanup method, is performed to concentrate the samples.

Detection methods such as UV (Fahmy *et al.* 2004; Qian *et al.* 2008a; Xue *et al.* 2008), chemiluminescence (Ahmed *et al.* 2007) and fluorescence (Azharuddin *et al.* 2007), have been combined to HPLC methods. Several quinones can be detected by chemiluminescence due to their ability to generate hydrogen peroxide and a fluorophore when subjected to UV irradiation, a property that allows their determination through a peroxyoxalate chemiluminescence reaction by mixing with aryloxalate (Ahmed *et al.* 2007). Also post column chemical reduction for the detection of the reduced form of the quinone using a catalyst reduced column and a MeOH-EtOH mobile phase as reductant have been used (Azharuddin *et al.* 2007).

Mass spectrometry, in negative or positive ionisation mode, is often coupled to HPLC for the identification of the quinones. Different mass analysers are used, depending on the structures of the studied compounds, especially electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) instruments such as triple-quadrupole and ion trap instruments, which enable tandem mass spectrometry (MS/MS) measurements.

Despite the fact that many methods have been used, the identification and quantification of quinones is still challenging due to their high reactivity as fast redox cycling molecules as well as their potential of binding to hydroxyl, thiol, and amine groups. Therefore, efforts to establish efficient, accurate and precise procedures for their quantification are ongoing.

Wherever the conventional analytical methods for studying quinones in biological samples have failed in their detection, other approaches such as the use of radiolabeled or isotopically labeled compounds have been adopted.

Tracer compounds whether isotopic or radioactive are useful tools for measuring and understanding the metabolism and disposition of both endogenous molecules and drugs. This is true in the case of compounds that are unstable or require to be detected at low

concentrations. Several studies using labeled quinones have been conducted so far and have been instrumental in clarifying their metabolic fate and/or mode of action. This is true in the case of studying vitamin K_1 whereby specific challenges for its analysis in plasma result from its low concentration, interference of plasma lipid components, and the sensitivity of the molecule to degradation by light and strong alkaline conditions. Between 1972 and 1979, three attempts to measure vitamin K_1 turnover in human subjects have been made by using [1', 2'-$^3H_2$] vitamin K_1. However, none of these studies has allowed the calculation of the body pool of vitamin K_1 due to the absence of a suitable method for measuring vitamin K_1 in plasma (Bjornsson *et al.* 1979; Shearer *et al.* 1972; Shearer *et al.* 1974). Nearly 20 years later, Olson *et al.*, succeeded to determine the total body vitamin K_1 and its turnover in human subjects at two levels of vitamin K intake using tritiated vitamin K_1 (Olson *et al.* 2002). Another example involves the study done by Miao *et al.* (Miao *et al.* 2008) on β-lapachone, a promising anticancer compound. While studying its *in vitro* metabolism in plasma and whole blood, the compound could not be detected with conventional LC-MS. The use of ^{14}C β-lapachone has allowed studying the metabolic profiling and determining the reason for the failure of its detection in blood using conventional analytical methods. Using LC-MS coupled to a radioisotope counting system it has been shown that β-lapachone is extensively metabolized in whole blood under *in vitro* conditions and the enzymatic activity is located in red blood cells. By determining the percent of radioactivity present in protein pellet prepared from whole blood spiked with ^{14}C β-lapachone, it has been proved that covalent protein binding of β-lapachone and/or its metabolites is a minor contributor in the failure of its detection in blood (Miao *et al.* 2008).

2.5. Methods in Preclinical Cancer Research

To assess the efficacy of chemopreventive agents, it is not feasible to use cancer incidence or large polyp prevention as a primary method for evaluation; however, surrogate markers of carcinogenesis, of biological or morphologic events, instead of the actual cancer can be more useful (Kelloff *et al.* 1994; Liou *et al.* 2010; Sillars-Hardebol *et al.* 2010).

These markers have to 1) display variability of expression through the different stages of cancer progression, 2) be detectable early in the carcinogenesis process, and 3) be coupled to the occurrence of pre-cancer or cancer. The efficacy of the chemopreventive agents will be reflected by the degree of modulation exerted by the agent on this marker. Many promising surrogate endpoint biomarkers for CRC chemoprevention have been evaluated (Einspahr *et al.* 1997; Liou *et al.* 2010). These biomarkers are classified into different categories (cellular, molecular, biochemical, genetic, and pathologic markers).

2.5.1. Cellular and Molecular Markers *in vitro*

Proliferation and Toxicity Assays. The potential inhibitory effect of chemopreventive agents on the proliferation of cancer cells can be determined using the MTT (method of

transcriptional and translational) assay (Lawnicka *et al.* 2010; Zhou *et al.* 2010). In this method the ability of metabolically active cells to convert a tetrazolium salt, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole), into a blue formazan product by dehydrogenases and reductases is measured. The conversion takes place only when the enzymes are active. The effectiveness of any agent to cause death or induce metabolic dysfunction is therefore deduced by comparing the amount of purple formazan produced by treated cells to untreated cells (Mosmann 1983). A decrease of the color in treated cells as compared to control is an indication of metabolic dysfunction and inhibitory effect due to treatments. To measure treatment induced-cell cytotoxicity, however, the cytotoxicity assay that assesses cell membrane integrity is used (Chen *et al.* 2010; Momeny *et al.* 2008). Toxic compounds often compromise cell membrane integrity and result in the passage, to the outside of the cells, of substances that are normally sequestered inside the cells. Lactate dehydrogenase (LDH) is one of the most commonly measured molecules for the assessment of drug toxicity. In this assay the extent of LDH release is proportional to the red formazan product obtained via a coupled enzymatic assay of LDH and tetrazolium salt, the absorbance of which is recorded at 490nm (Decker and Lohmann-Matthes 1988).

Cell Cycle Distribution, Apoptosis, and Molecular Markers. To determine the effect of treatment on the distribution of cells in the different phases (preG_1, G_0/G_1, S, G_2/M) of the cell cycle, flow cytometry analysis of DNA content stained with propidium iodide (PI) is used (Moore *et al.* 1998). PI is a DNA intercalating agent that emits fluorescence upon excitation. This method has been used to determine if the inhibitory activity observed with the proliferation assay is associated with specific changes in the different phases of the cell cycle such as cell cycle arrest and/ or apoptosis. By comparing data from treated and non-treated cells, it is possible to define the mechanism by which the drug might be acting. While an increase in the G_0/G_1, S, or G_2/M (Schonn *et al.* 2010; Xiong *et al.* 2010) phase implies that the drug inhibits cell proliferation by inducing cell cycle arrest, an increase in the preG_1 phase implies apoptosis/necrotic induction (Qin *et al.* 2010; Tabata *et al.* 2009). Whereas apoptotic cell death is characterized by well-defined cytological and molecular events leading to DNA fragmentation; necrotic cell death results in rapid loss of membrane integrity and release of cell content such as LDH to the environment (Riss and Moravec 2004).

In treatments where an increase in preG_1 has been observed with PI by flow cytometry analysis, other methods such as TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and M30 cytodeath assays can be applied to differentiate apoptotic from necrotic cell death. TUNEL enzyme catalyzes the addition of dUTPs that are secondarily labeled with a marker to the terminal end of the nucleic acid. Using flow cytometry, apoptotic-induced fragmented DNA is visualized by the increase of fluorescence in treated cells as compared to control untreated cells (Gavrieli *et al.* 1992; Negoescu *et al.* 1996). M30 cytodeath antibody allows the determination of early apoptotic events in single cells by its ability to bind specifically to a caspase cleavage site, apoptosis marker, within cytokeratin 18 that is not detected in native normal cells (Dionne *et al.* 2010).

To characterize the molecular basis of drug-induced cellular growth inhibition, western blot analysis is used to study changes in the expression of key cell cycle mediators known to accompany cell cycle arrest and apoptosis such as cyclin D_1, cyclin B_1, p53, p21, Bax and Bcl-2 (Hedstrom *et al.* 2009; Kim *et al.* 2006; Sherr and Roberts 1995; Stewart *et al.* 1995; Taylor and Stark 2001; Yasuda *et al.* 2010).

2.5.2. Pathologic Markers *In Vivo*

Effectiveness of Animal Models for Colon Cancer. The stepwise progression of human CRC may take from one to three decades from the time of initiation until the beginning of the invasive stage. Therefore, to study human CRC, animal models have to be used. The ideal animal model should have exactly the same histology, morphology and anatomy of human colonic neoplasms. However, based on a review of the available CRC models such an ideal animal model does not exist. On the other hand, some of the models approximate many of the characteristics of human colonic carcinogenesis; therefore, they can be used for clarification of the mechanisms of colorectal carcinogenesis and prevention of the occurrence of colorectal cancer (Femia *et al.* 2010; Kobaek-Larsen *et al.* 2000; Mori *et al.* 2004).

DMH and its metabolites are frequently used to study CRC in animal models for they induce colonic tumors with a similar histology, morphology and anatomy to human colonic neoplasms (Femia *et al.* 2010; Ma *et al.* 2002; Ma *et al.* 2004). Human colon cancer, however, differs from DMH-induced colon cancer by the metastatic potential of human adenocarcinomas. While half of the patients with colon cancer end up with lymphatic or liver metastasis, DMH-induced adenocarcinomas have little potential for metastasis (Dionne *et al.* 1965; Haase *et al.* 1973).

Description of the DMH Model. In order to become a carcinogen, DMH must be activated by a series of metabolic transformations leading to a number of intermediates (Fiala 1977). In the first step DMH is oxidized to azomethane, which after another oxidation forms azoxymethane (AOM). Hydroxylation of the latter molecule forms methylazoxymethanol (MAM), which in the final step is converted to formalin and methyldiazonium ion, alkylating agents of DNA, RNA, and protein principally in the colon, liver, and kidneys (Kobaek-Larsen *et al.* 2000).

Activation of AOM and MAM has been attributed to the effect of the alcohol-inducible cytochrome P-450 isoform, CYP2E1 (Sohn *et al.* 2001). The specificity of DMH to colonic mucosa is probably the consequence of the relative stability of its metabolite MAM (Nagasawa *et al.* 1972). With a half-life of approximately 12h, MAM has sufficient time to distribute to the colon (Feinberg and Zedeck 1980).

The fact that CRC is a stepwise process where dysplastic ACF transform to adenomas, the majority of which transform to adenocarcinomas, makes the use of these intermediates

potential pathologic biomarkers for CRC (Cai *et al.* 2010; O'Brien *et al.* 1990; Velmurugan *et al.* 2008).

The DMH model has been widely used to assess the potency of chemopreventive agents on ACF (initiation), adenomas (promotion), and adenocarcinomas (progression) of CRC development. For instance, the DMH model has been used to study the chemopreventive effect of etoricoxib (a cyclooxygenase inhibitor), astaxanthin (a carotenoid), Yogurt, and refecoxib. While the reported chemopreventive effects of etoricoxib and astaxanthin have been due to their significant inhibition of DMH-induced ACF (Prabhu *et al.* 2009; Sharma *et al.* 2010), Yogurt and rofecoxib, however, have been found to exert their effects on adenomas and adenocarcinomas formation (progression and promotion stage of CRC) (De Moreno de Leblanc and Perdigon 2004; Noguera Aguilar *et al.* 2005).

2.6. Protein-Drug Binding in Plasma

Protein binding is generally referred to as the reversible binding of drugs to plasma components. Once a drug reaches the blood circulation after intravenous injection or absorption, it interacts with plasma proteins via two different ways: either adsorption to the protein surface or more rarely by covalent binding (Barnaby and Bottacini 2004). Human plasma contains more than 100 proteins among which six are able to bind drugs: human serum albumin (HSA), AGP, lipoproteins (VLDL, HDL and LDL, and immunoglobulin G (Barnaby and Bottacini 2004).

HSA is the most abundant protein in plasma whereby it represents more than 60% of all plasma proteins. HSA has 17 disulfide bridges and one free cysteine (Cys-34). The free thiol of the cysteine residue is redox active capable of thiolation and nitrosylation, and accounts for more than 80% of the thiols in plasma. HSA binds to many endogenous and exogenous compounds and participates in different processes such as drug delivery, detoxification, and antioxidant properties (Quinlan *et al.* 2005).

AGP, the second major protein in plasma, has very high carbohydrate content of 45%. AGP is considered as a major member of the positive acute phase protein family; while its concentration remains stable in physiological conditions, it increases several-fold during acute-phase reactions. AGP is encoded by two genes that differ by 22 substitutions resulting in two different polypeptides (Fournier *et al.* 2000). The two isoforms differ mainly by the presence of an additional cysteine in position 147 of the amino acid sequence in one of the isoforms (Schmid *et al.* 1973). AGP binds to basic drugs and small molecules such as steroid hormones. Although AGP plays a key role in binding basic drugs, it binds to neutral and acidic drugs but to a lesser extent (Banker *et al.* 2008; Israili and Dayton 2001). In addition, AGP can induce various immunomodulating effects (Fournier *et al.* 2000). In plasma the concentration of AGP is 100 fold lower than that of albumin (Barnaby and Bottacini 2004). While HSA is considered as a high capacity low

affinity protein, AGP on the other hand is a low capacity high affinity protein (Barnaby and Bottacini 2004).

In addition to plasma proteins, drugs can bind to tissue proteins. The reversible binding of the drug to plasma components results in an equilibrium between the concentration of bound and unbound drug (Longmei *et al.* 2005). Binding of drugs to proteins has serious implications since it can affect the therapeutic, pharmacodynamic, as well as the toxicologic actions of the drug. This can be explained by the fact that only unbound drug reaches the site of action, and tightly bound drugs tend to distribute in a smaller body volume and cause a delay of their elimination etc., therefore, the determination of the unbound concentrations of the drug is important for the determination of its pharmacokinetic (PK) and pharmacodynamic (PD) studies (Vallner 1977). The desire to predict ADME parameters to assist in early PK/PD modeling in the drug discovery process places an increased demand for protein and tissue binding studies (De Buck *et al.* 2007; McGinnity *et al.* 2007).

3. AIMS OF THE STUDY

The aim of this thesis is to discover bioactive natural products from Lebanese plants used in folk medicine as candidates for future clinical development against colon cancer. Molecular and cellular biology as well as analytical chromatographic studies have been performed to achieve the goal.

Specific aims of the study:

1. Evaluate the anticancer activities of *O. cynarocephalum* and *C. ainetensis* selected in screening studies, using *in vitro* and *in vivo* models for colon cancer (I, II).

2. Investigate the *in vitro* mechanism of action of the anticancer molecule TQ, the active constituent in *Nigella sativa*, for further preclinical evaluation (III).

3. Investigate the analytical detection of TQ in biological matrices as well as its binding to plasma proteins, necessary for future kinetic studies (IV).

4. MATERIALS AND METHODS

A thorough description of the materials and methods is presented in the original publications (I-IV).

4.1. Reagents

Cell culture reagents such as media, serum, etc. were obtained from Gibco BRL Life Technologies (Gaithersburg, Maryland, US). Other kits and reagents were purchased from Promega Corp (Madison, Wisconsin, US), Molecular Probes (Eugene, Oregon, US), Roche Diagnostics Corporation (Mannheim, Germany), Bio-Rad (Hercules, California, US) and Amersham Pharmacia Biotech (Amersham, England). Antibodies and inhibitors were from Santa-Cruz (Santa-Cruz, California, US), Biogenesis (Poole, UK), Cell Signaling Technology (Beverly, US), Acros Organics (New Jersey, US) and Sigma Chemical Company (St. Louis, Missouri, US). TQ was purchased from MP Biomedical (Strasbourg, France). LC solvents were HPLC grade from Rathburn (Walkerburn, UK). C_{18} PrepSep solid phase extraction (SPE) cartridges were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, US). Amicon Ultra centrifugal filters with 3K cut off were obtained from Millipore (Carrigtwohill, Co. Cork, Ireland).

4.2. Animal Cells and Plant Material

Normal mouse intestinal ModeK cells were kindly provided by Prof. F. Homaidan (Department of Human Morphology, American University of Beirut, Lebanon). FHs74Int normal human intestinal epithelial cells, HCT-116 and HT-29 human colon cancer cells were kindly provided by Prof. R. Schneider-Stock (Experimental Tumor pathology, Institute for Pathology, University Erlangen-Nuremberg, Erlangen, Germany). DLD-1 cells were kindly provided by Prof. M. Ocker (Institute for Surgical Research, Philipps University Marburg, Marburg, Germany). *O. cynarocephalum* and *C. ainetensis* were collected from Lebanon and voucher specimens were deposited in the George Post Herbarium at the American University of Beirut (Beirut, Lebanon).

4.3. Animal Experiments

In studies I and II, 6-9 weeks old, female Balb/c mice were randomly distributed to treatment and control groups. During the toxicity study, crude extracts (I, II) were given i.p. to the mice for 10 consecutive days and toxicity signs such as death and body weight loss were monitored. For the long term experiment (30 weeks), control groups were injected (i.p.) with 0.9% sodium chloride, crude water extracts (I, II), or (s.c.) with 20 mg/kg mouse body weight of DMH. Treatment groups were injected i.p. with the crude extract 15 min prior to DMH injection. The injections were done once a week. At week 10, mice were sacrificed and colon tissues were processed and stained with Schiff's reagent. ACF were examined and their size and location recorded using fluorescent

microscopy. At weeks 20 and 30, colon tissues were processed and visible tumors were counted using an optical microscope.
For TQ analysis in spiked serum (IV), intact male Sprague-Dawley rats, average weight of 250 g were used. The blood was collected by heart puncture into EDTA containing tubes, centrifuged (3000 rpm, 4 °C, 5 min), and serum aliquots were stored in plastic tubes at -20 °C until used. Handling the animals was conducted using a protocol approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

4.4. Methods

4.4.1. Crude Extract Preparation, Fractionation, Isolation and Molecular Identification Procedures

Water extraction. Twenty grams of air-dried ground material (I, II) was placed in 160 ml of boiled distilled water [material/solvent concentration (w/v) of 1:8] and shook for 20 min using an incubator shaker. The resulting solution was then filtered through Whatman No. 2 filter paper and stored at -20 ºC until used in well-sealed 150-ml bottles covered with aluminium foil for the bioassays. Crude extracts were filter sterilized using 0.2µm non-pyrogenic sterile-R filters before treatment.

Methanolic extraction and fractionation. Plant material was soaked in MeOH at a 1:10 ratio (w/v) and incubated on a shaker at 20 °C for 16 h. After extraction the extract was filtered and separated into residue labeled as (R-I) and a filtrate labeled as “I” which was named as "MeOH crude extract". The residue labeled as R-I was then soaked in EtOAc at 10 to 1 ratio (10:1 w/v) and filtered to give “I.1”.
Concentrated sulfuric acid solution was added to the crude extract “I” until the pH reached 2 after which a mixture of chloroform ($CHCl_3$): water (2:1 v/v) was added.
The chloroform phase was collected and labeled as “I.2”. The pH of the aqueous layer was adjusted to 10 by adding conc. ammonia solution; the obtained mixture was then suspended in a $CHCl_3$:MeOH mixture (3:1 v/v), separated into organic and aqueous layers and labeled “I.3” and “I.4”, respectively. All fractions were dried *in vacuo* at 40 °C, the residues were dissolved in minimal amounts (0.1-1ml) of EtOH.
Only fraction I.2, which exhibited anticancer activity was concentrated *in vacuo* at 40 °C. The residue (0.48 g) was applied to a chromatographic column consisting of 150 g of silica gel (0.035-0.07 mm and 6 nm pore diameter). A gradient elution was performed using petroleum ether:$CHCl_3$:EtOAc (2:2:1), followed by petroleum ether:$CHCl_3$:EtOAc (1:3:1) (500 ml), $CHCl_3$:EtOAc (3:2) (250 ml), $CHCl_3$:EtOAc:MeOH (3:3:1) (525 ml), $CHCl_3$:EtOAC:MeOH (3:3:2) (900 ml), $CHCL_3$:MeOH (3:2) (750 ml) and MeOH successively. The anticancer activity was found in sub-fraction I.2.2, a fraction eluted with $CHCl_3$:EtOAC:MeOH (3:3:1). Normal phase SPE cartridges (Alltech, silica, 200 mg/4.0 ml, 50/P; particle size 50 µm and average pore size 60 °A) were used to purify the active subfraction (I.2.2). Gradient elution consisting of CH_2Cl_2 (20 ml), $CHCl_3$ (20 ml), and MeOH (10 ml) was used and the pure compound was eluted with $CHCl_3$ (20 ml).

Spectroscopic Measurements. 1D and 2D NMR spectra were recorded using deuterated chloroform ($CDCl_3$) (Acros Organics, New Jersey, US) by a Bruker 300 MHz spectrometer (Bruker, Bremen, Germany). Chemical shifts were reported in δ (ppm) values relative to TMS (Acros Organics, New Jersey, US). For GC analysis, a Hewlett-Packard 6890 gas chromatograph equipped with HP-5 capillary column was used (Fisher Scientific, Pittsburgh, Pennsylvania, US). The carrier gas was helium and the flow rate was 1ml/min. The column was heated from 35ºC to 290ºC and the maximum temperature was 350°C. Results were recorded as percent of total peak areas. The mass spectrometer employed in the GC-MS analysis was a Hewlett Packard 7972 series mass selective detector in the electron impact (EI) ionization mode (70 eV) (Fisher Scientific, Pittsburgh, Pennsylvania, US). FTIR spectra were recorded on a Nicolet AVATAR 360 FTIR spectrometer. UV-Vis spectra were measured in MeOH using a JASCO V-570 UV/VIS/NIR spectrophotometer (JASCO Inc., US).

4.4.2. Cell Culture

Colon cancer and normal intestinal cells, listed in table 1, were maintained at 37 ºC in a humidified atmosphere of 5 % CO_2, 95 % air, supplemented with 1 % penicillin–streptomycin (100 U/ml), and 10 % fetal bovine serum (FBS). In all experiments (I, II, III, IV) except ELISA assays (III), cells were seeded at 10^5 cells/ml and exposed at 40–50 % confluency to different concentrations of crude extracts of *O. cynarocephalum* (I), *C. ainetensis* and its fractions { I.1-I.4, I.2.1-I.2.6 } (II), Salograviolide A (II), and TQ (III, IV). For experiments involving inhibitors (III), cells were pre-treated with 5 mM NAC for 2 h, 50 µM PD98059 for 2 h, 20 µM SP600125 for 1.5 h or with 100 µM dicoumarol for 1 h prior to TQ. For experiments involving protein binding (IV), 6.6 µg/ml TQ was prepared in different concentrations of FBS {5, 10, 20, and 40 %}, BSA {30, 60,120, and 240 µM}, AGP {1.25, 2.5, 5, and 10 µM} and added to the cells after incubation for 30 min at 37 °C. TQ stock was prepared in MeOH (III)/DMSO (IV) and the final concentration of either vehicle on cells was less than 0.5 %.

Table 1: list of all cancer and normal cells used in this study

Colon cancer cells	Publications
HCT-116 p53+/+	I, II, III
HCT-116 p53-/-	II
HT-29	I, III
DLD-1	II, III, IV
Lovo and Caco-2	III
Normal intestinal cells	Publications
FHs74Int	I, II, III
Modek	I

4.4.3. Cell Proliferation and Viability Assays

Inhibition of cell proliferation was studied using the Cell Titer 96 non-radioactive cell proliferation kit, MTT (Promega Corporation, Madison, Wisconsin, US) in (I, II, III) and using a commercial colorimetric WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate) cell proliferation assay (Roche Diagnostics GmbH, Mannheim, Germany) in (IV), according to the manufacturer's protocol.
Briefly, colon cancer cells were plated in 96-well plates and treated with different concentrations of crude extracts (I, II), fractions and Salograviolide A (II), TQ in the presence or absence of NAC, PD98059, SP600125, or dicoumarol for 24 h and 48 h (III), and TQ in the presence or absence of FBS, BSA or AGP for 6 h (IV).
The CytoTox 96 (viability) assay was done 6 h (II) and 24 h (I, III) post-treatment using the CytoTox 96 non-radioactive cytotoxicity kit (Promega Corporation, Madison, Wisconsin, US). The absorbance was recorded at 550, 440 and 490 nm for MTT, WST-1 and cytotox assay, respectively.

4.4.4. Cell Cycle Analysis Using Flow Cytometry

PI staining was used to follow the distribution of cells in the different phases (preG_1, G_0/G_1, S, G_2/M) of cell cycle upon treatment. Cells were seeded, treated, and collected 24 h (I, II, III) and 48 h (III) post-treatment. Collected cells were washed with phosphate buffer saline (PBS) and fixed in 70% EtOH at least for 2 h at -20 ºC. After fixation washed cells were stained with PI and analyzed by fluorescence activated cell sorting FACScan flow cytometer (Becton Dickinson, US). The percentage of cells in the different phases was then determined using the Cell Quest Histogram analysis program. Cells that were less intensely stained than G_1 cells in flow cytometric histograms were considered as apoptotic cells and marked as preG_1.

4.4.5. Evaluation of Apoptosis

Apoptotic cell death was measured in treated cells 24 h post treatment by TUNEL assay (I, II) and 24 h/48 h post-treatment by M30 cytodeath assay (III), mitochondrial membrane potential analysis DiOC6 (III) and caspase-3 activity (III). The TUNEL assay measures the extent of DNA fragmentation by the Terminal deoxy-transferase (tdT)-mediated dUTP nick end-labeling technique. The M30 cytodeath antibody recognizes a specific caspase cleavage site with cytokeratine 18 that is not detected in native cells. The dihesiloxalocarbocyanine Iodide (DiOC6 (3)) measures the loss in mitochondrial potential due to apoptosis induction. Collected and stained cells were analyzed using FACScan flow cytometer (I, II, III) or by fluorescent microscope Leica DM6000B using 20-fold magnification (III). Caspase-3/7 activity assay, which measures caspase 3/7 activation-associated apoptosis, was determined in cell lysates and the luminescence was measured by a microplate reader (TECAN GENious, Switzerland).

4.4.6. Intracellular ROS Generation by DCFH

Oxidative stress was examined in study III by measuring the level of ROS using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a molecule that passively diffuses into the cells and is cleaved and oxidized in the intracellular environment to the green fluorescence emitting compound, 2',7'-dichlorofluorescein (DCF). Cells were treated at 50% confluency with TQ in the presence and absence of NAC then incubated with DCFH-DA for 30 min in the dark. ROS generation was determined by flow cytometric analysis.

4.4.7. Western Blot

In studies I, II and III, colon cancer cells were treated as described earlier and cellular protein extracts were prepared in 2X SDS-lysis buffer. Protein quantification was done using the DC Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, California, US) according to the manufacturer's protocol. Equal amount of protein lysates, prepared in 10 % β-mercaptoethanol and 2X sample buffer containing bromophenol blue, were placed on 12% SDS–PAGE for 2 h at 90 V then transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham, Arlington, Illinois, US) in transfer buffer under 30 volts overnight at 4 ºC. Each membrane was immunoblotted with appropriate primary and secondary antibodies, reacted with enhanced chemiluminescence reagent, and exposed to X-ray films for different time periods. Membranes were re-probed with GAPDH or actin to ensure equal loading. Band quantification was performed using the Labworks software (Ultraviolet Products, Upland, Canada).

4.4.8. Cellular Activation of MAPK Signaling

In study III cells were plated at 1.5 x 10^5 cells/ml in 96-well plates and after overnight starvation in serum-free medium treated with TQ. Cells were fixed and after 30 min the relative extent of target protein phosphorylation (p-ERK, p-JNK, p-p38) was determined using CASE kits according to the manufacturer's procedures (SuperArray Bioscience Corporation, Frederick, Maryland, US).

4.4.9. High Performance Liquid Chromatography (HPLC)

In all the experiments (IV) rat serum was diluted (1:1) with PBS and spiked with TQ to yield a final concentration of 10 µg/ml. Spiked samples were subjected to several extraction methods (a-c) prior to HPLC analysis as follows: a) C_{18} PrepSep SPE followed by drying under a stream of nitrogen, b) LLE step with different ratios of EtOAc (1:1, 1:1.5, 1:2, and 1:3, v/v) followed by centrifugation (14000 g, 4 °C, 10 min), c) protein precipitation with different ratios (1:1, 1:2, 1:4, v/v) of MeOH and ACN followed by centrifugation at 14000 g for 10 min. For quantification, stock solutions of TQ were prepared in ACN and standard calibration curves of the stock were generated by serial dilution using ACN or PBS (IV). The analysis was conducted on an Agilent Technologies 1100 series instrument (Waldbronn, Germany), comprising a vacuum degaser, an

autosampler, a binary pump, and a diode array detector. Chromatographic separation was performed on a Phenomenex (Torrance, California, US) C_{18} column (25 × 4.6 mm I.D.) with 5µm packing material. Samples were eluted using an isocratic mobile phase of water: ACN (45:55% v/v) at a flow rate of 1 ml/min. Diode array detector signal was recorded at 254 nm and the injection volume was 20 µl. The chromatographic data were acquired and analyzed using Agilent Chemstation software package.

4.4.10. Protein-Binding Studies

To determine the percentage of protein binding (IV), FBS and 10 % FBS (diluted with DMEM high glucose as used in cell culture experiments) were spiked with TQ to yield a final concentration of 3.3 µg/ml, 6.6 µg/ml and 13.2 µg/ml. Samples were incubated at 37 °C and 400 µl were collected at 0, 5, 10, 15, 30, 60, 120, 240, and 360 min. Further binding experiments were performed using BSA and AGP. The concentrations of BSA and AGP in serum are approximately 40 mg/ml (600 µM) and 0.97 mg/ml (25 µM), respectively. Therefore, BSA and AGP at concentrations corresponding to 10 % FBS were incubated at 37 °C with TQ 6.6 µg/ml for 30 min prior to analysis. Samples were filtered using Amicon Ultra with 3K cut off by centrifugation at 14000 g for 30 min. The filtrates were then analyzed by HPLC and the percentage of binding was calculated using the following equation: [% binding= (Total-free)/Total *100]. TQ recovery from standard subjected to the same procedure was 91.3 ± 0.6 %, therefore no correction was made while calculating the percentage of binding.

4.4.11. Amino Acid Sequence Synthesis, Purification, and Binding Assay

TQ binding to free thiol from BSA and AGP was tested using synthesized sequences corresponding to the region containing the free thiol. While one sequence YLQQCPFED was synthesized for BSA, two variants - CLCIP and CLAIP - were synthesized for AGP. The major difference between the two AGP isoforms is the presence of an additional free cysteine in one isoform while the other isoform had an arginine residue instead. The first cysteine from each AGP sequence was alkylated and consequently not available for binding. The position of free cysteine was determined by using the protein data bank.
The selected peptides were synthesized on an ACT-396 peptide synthesizer at a 0.2 mmol scale, using Rink amide resin, double coupling standard fluorenylmethyloxycarbonyl FMOC-chemistry protocols (6 fold excesses of protected amino acids), TBTU/HOBT as coupling reagent, 20 % piperidine in NMP for FMOC de-protection and mixture of phenol:H_2O:EDT:thioanisol:TFA = 1.5:1:1:1:10 for 2 h at room temperature for final cleavage/de-protection. After cleavage/de-protection, crude peptides were precipitated with ice-cold diethylether, filtered, dried and analyzed by RP-HPLC (HP 1050 series chromatography, Supelco Discovery C18 column (15 cm x 4.6 mm, 5 µm), λ = 220 nm, linear gradient from 0 to 70% B in 40 min; A – 0.1% TFA, B – 0.1% TFA in 80% ACN). TQ was then incubated with the sequence for 30 min at 37 °C after which covalent binding was detected by mass spectrometry.

4.4.12. Mass Spectrometry Analysis

Waters (Micromass) QTOF micro mass spectrometer (Manchester, UK) with electrospray ion source was used in positive ion mode to study TQ binding to the synthesized sequences. Samples were dissolved in 50/50 H_2O/MeOH containing 0.1% formic acid. Samples were injected at a flow rate of 10 µl/min. The desolvation temperature was 75 °C and source temperature was set to 150 °C. Capillary voltage was 3 kV, sample cone was 15.0 V, and extraction cone was 2.0 V. Cone gas and desolvation gas flow was set to 200 l/hr.

4.4.13. Statistical Analysis

Results are expressed as mean ± standard error (SE) (I, II, IV) and mean ± standard deviation (SD) (III). Statistical analysis was performed using SPSS Student Version 11.0 Software Package (I, II, III) and SYSTAT VERSION 12 Software Package (IV). Comparisons between the different groups were evaluated using ANOVA followed by Dunnett test (I, II), and one-tailed Student's *t*-test (III, IV). The level of significance was set at 0.05. For all tests, $p < 0.05$ was considered as significant.

5. RESULTS

The main findings are described in the section below and more detailed results can be found in the original publications (I-IV).

5.1. Anticancer Properties of *O. cynarocephalum* and *C. ainetensis* (I, II)

The inhibitory effect of the crude extract of *O. cynarocephalum* and *C. ainetensis* has been tested *in vitro* against a panel of colon cancer and normal intestinal cells using MTT-Cytotox assays. Both extracts inhibited the proliferation of colon cancer cells in a concentration-dependent manner (Fig. 2a) and were more cytotoxic to cancer cells than to normal cells. PI staining of DNA content showed that the inhibitory effect observed with both extracts was due to apoptosis induction as evident by the increase in the percentage of fragmented DNA in the $preG_1$ phase of the cell cycle (Fig. 2b). The TUNEL assay, which measures the extent of DNA fragmentation by the Terminal deoxy-transferase (tdT)-mediated dUTP nick end-labeling technique, was used to confirm the nature of cell death. Extract-treated cells underwent a significant amount of apoptosis as compared to untreated control cells. The percentage of TUNEL positive cells correlated well with the increase in $preG_1$ phase observed by flow cytometry analysis, thus confirming apoptosis induction.

To further investigate the mechanism of extract-induced apoptosis, changes in the expression of key cell cycle and apoptotic mediators such as cyclin B_1, p21, p53, Bax, and Bcl-2 were monitored in HCT-116 treated cells by western blot.
An increase in the expression of p53, p21 and Bax/bcl-2 ratio along with a decrease in cyclin B_1 expression was observed upon treatment with both extracts (Fig. 2c).
The tumor suppressor gene p53 is known to protect mammals from neoplasia by inducing cell cycle arrest, DNA repair, and apoptosis through activation of its downstream effectors p21, Gadd45 and Bax. This activation occurs in a response to a variety of stresses such as DNA damage, hypoxia and chemotherapeutic drugs (Hedstrom *et al.* 2009; Kim *et al.* 2006; Taylor and Stark 2001). Up-regulation of Bax and down-regulation of Bcl-2 favors the pro-apoptotic over the anti-apoptotic response in cells, leading to programmed cell death. Cyclin B_1 rises during the G_2/M phase of the cell cycle and peaks into mitosis and its destruction is fundamental for mitotic exit and cytokinesis (Takizawa and Morgan 2000; Yasuda *et al.* 2010). Extract-mediated cell cycle arrest and apoptosis correlated well with their ability to differentially modulate the expression of components of the signaling pathways involved in cell cycle arrest and apoptosis.

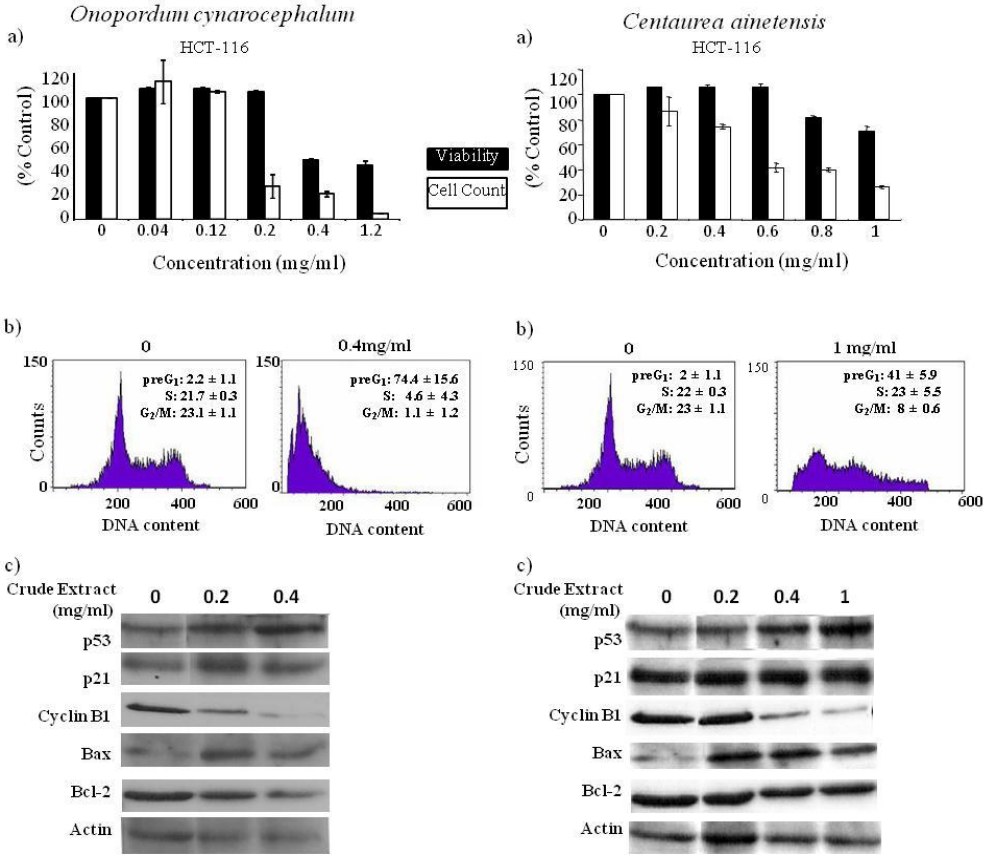


Figure 2. Effect of crude extracts of *O. cynarocephalum* and *C. ainetensis* on HCT-116 colon cancer cells: a) cell proliferation, b) cell cycle, and c) protein expression.

The *in vivo* toxicity of *O. cynarocephalum* and *C. ainetensis* extracts was tested in Balb/c mice. Each extract was injected (i.p.) daily for 10 consecutive days and toxic signs such as weight loss and death were monitored. Both extracts were not toxic to the animals as evident by the normal weight gain and absence of death in all the groups (data not shown). Based on the toxicity data, the chemopreventive effect of the extract was studied in the well-established DMH model of colon cancer. This model induces colonic tumors of epithelial origin with similar histology, morphology, and anatomy to human colonic neoplasms (Maskens 1976). In addition, it offers multiple opportunities for assessment and intervention by the stepwise development of colorectal carcinogenesis from dysplastic aberrant crypt foci (ACF) (week 10) to adenomas (week 20) and adenocarcinomas (week 30). ACF are potential markers of early tumor growth and are considered as useful intermediate biomarkers for the modifying effect of certain natural or synthetic compounds on chemically induced carcinogenesis (Sengupta *et al.* 2004).

Balb/c mice were treated as described in the materials and methods. At week 10, animals were selected randomly from each group, sacrificed and monitored for the presence of ACF. Following staining with Schiff's reagent, no ACF were observed in control treated groups (saline, plant extract) whereas in the DMH-treated group, all animals developed

ACF (Fig. 3a,b). Although treatment with the extracts prior to DMH did not affect the average number of ACF, their average sizes were significantly inhibited ($p < 0.05$) as compared to DMH-treated group. At 20 and 30 weeks of treatment and using an optical microscope, significant inhibition was observed in DMH-extract treated mice. While at week 20, *O. cynarocephalum* and *C. ainetensis* induced 65% and 79% ($p < 0.001$) inhibition in extract-treated mice as compared to DMH-treated group, 63% and 73 % ($p < 0.0001$) inhibition were observed at week 30, respectively (Fig. 3a,b).

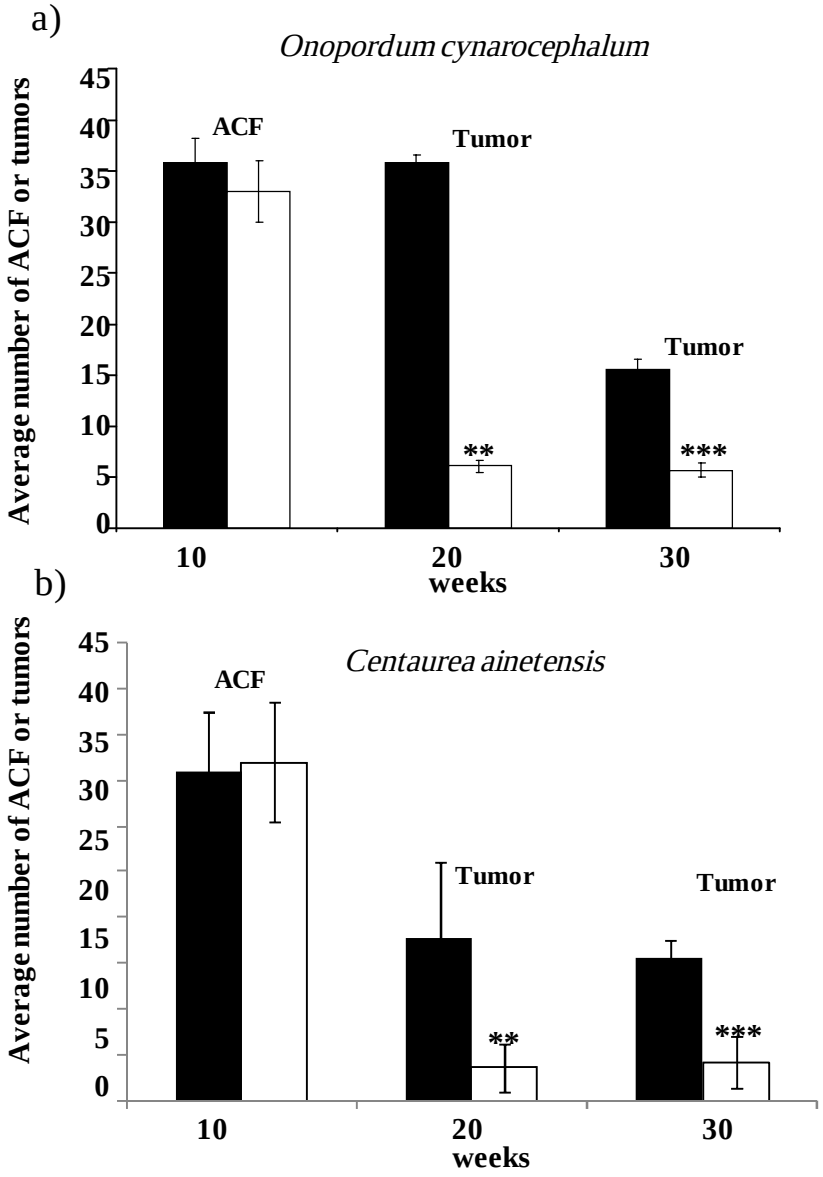


Figure 3. Effect of crude extract of a) *O. cynarocephalum* and b) *C. ainetensis*, *in vivo*, on aberrant crypt foci (ACF, week 10), adenomas (week 20) and adenocarcinomas (week 30).

5.2. Bio-guided Fractionation *of C. ainetensis* and Isolation of Salograviolide A (II)

Based on the availability of sufficient plant material, only *C. ainetensis* was selected for the characterization of the active ingredient through bio-guided fractionation using the proliferation assay as an end point.

The extract was fractionated into 4 fractions (I.1, I.2, I.3, and I.4) and their antiproliferative effect was investigated on HCT-116 cells. Only fraction I.2 significantly inhibited the growth of these cells. Therefore, further fractionation was performed on the active fraction and a total of six subfractions (I.2.2-I.2.6) were obtained and consequently tested. The activity was retained in the I.2.2 subfraction, which induced significant growth inhibitory effects against colon cancer cells. The purification and identification of the I.2.2 subfraction by solid phase extraction and spectroscopic techniques led to the isolation of Salograviolide A, which belongs to the guaianolide sesquiterpene lactones (Fig. 4a). The mechanism of action of Salograviolide A was further defined and compared to the effect observed with total extract using *in vitro* model. Salograviolide A inhibited the growth of a panel colon cancer cells in a concentration-dependent manner, and induced apoptotic cell death as evidenced by the TUNEL assay (Fig. 4b,c). Consequently, Salograviolide A, isolated from *C. ainetensis* was responsible, at least in part, for the activity of the crude biological extract.

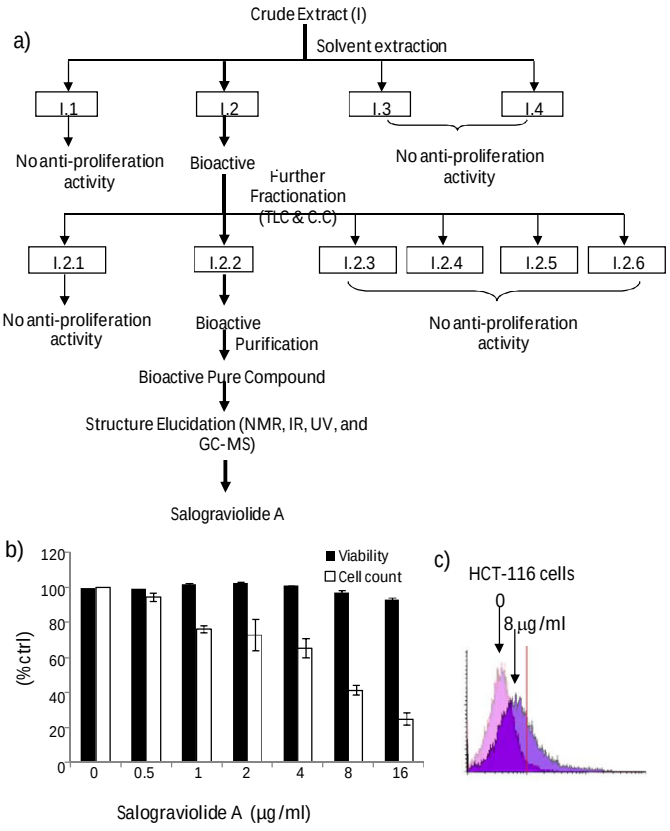


Figure 4. a) Bio-guided fractionation of *C. ainetensis.* Effect of Salograviolide A on colon cancer cell proliferation (b) and apoptosis induction (c).

5.3. TQ mechanism of action (III)

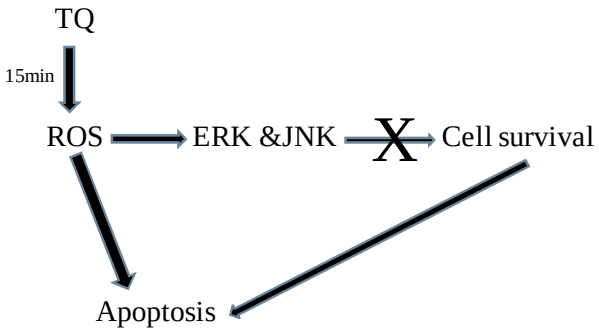
The present study (III) delineates the mechanism by which TQ induces its effect in colon cancer cells. The same *in vitro* techniques described previously for the evaluation of the anticancer activities used in the first two studies (I, II) were used in this study (III). Using the MTT-cytotox assay, TQ was found to inhibit the proliferation of a panel of colon cancer cells in a time and concentration-dependent manner (Fig. 5a) without inducing any toxicity to human normal intestinal cells (III). Investigations relating to the mechanism of action were carried out using DLD-1, a cell line that showed moderate sensitivity to TQ. A 24h post-treatment significantly increased the percentage of cells observed in the $preG_1$ phase of the cell cycle (from 2.5 to 18.8%) in DLD-1 treated cells (Fig. 5b). The increase in the $preG_1$ population was confirmed to be due to apoptosis by the M30 cytodeath antibody. The M30 cytodeath antibody recognizes a specific caspase cleavage site with cytokeratine 18 that is not detected in native cells. Using fluorescent microscopy the M30 immunofluorescent images showed clear cytoplasmic signals for M30 antibody after TQ treatment, which confirmed apoptosis induction by TQ (Fig. 5c).
Many chemotherapeutic agents induce their effect by ROS generation. As a quinone, TQ can undergo redox cycling in the presence of oxygen to produce ROS. Therefore, to confirm ROS generation in TQ-treated cells and to determine their involvement in TQ-induced cell death, the antiproliferative effect of TQ was tested in DLD-1 cells in the presence and absence of the strong antioxidant NAC. NAC pretreatment completely reversed the inhibitory effects of TQ and cell viability was restored to 100 % (Fig. 5d). These findings correlated well with the ability of TQ to induce ROS, as measured by the extent of DCF fluorescence, an effect that was inhibited in the presence of NAC by 40%.

TQ can also be a substrate for DT-diaphorase, an enzyme that catalyzes the two-electron reduction of quinones (oxidized form) to hydroquinones (reduced form) (Cullen *et al.* 2003). HT-29 cells, known to express high levels of DT-diaphorase (Karczewski *et al.* 1999), were the least sensitive to TQ's antiproliferative effect and did not elicit any oxidant shift as compared to the controls. Therefore, to study the involvement of DT-diaphorase in HT-29 cells resistance to TQ, the enzyme was inhibited by dicoumarol prior to TQ-treatment. As seen in Figure 4e, DT-diaphorase appeared to be partly responsible for the resistance of HT-29 cells whereby its inhibition sensitized the cells to TQ and reduced the IC_{50} from 95 to 63 µM ($p < 0.05$). These findings confirm that the mechanism of TQ-induced apoptotic cell death is via ROS production.

ROS are known to activate members of the mitogen activated protein kinases (MAPK), so a thorough investigation was conducted to elucidate the link between ROS production by TQ and the subsequent activation of MAPK. The modulation of expression of ERK, JNK, and p38 (MAPK family) by TQ was studied in the presence and absence of NAC by western blot. As early as 15 min, a 12 and 14-fold induction was observed for p-ERK and p-JNK, respectively, in DLD-1 treated cells without any significant increase in total ERK and JNK. No changes in p-p38 and total p38 protein expression were observed in response to TQ. MAPK induction was confirmed to be due to ROS generation by TQ by the fact

that the induction observed in TQ-treated cells was completely abrogated in the presence of NAC. The role played by the MAPK in TQ-induced DLD-1 cell death was characterized by pre-treating the cells with the specific ERK 1/2 inhibitor PD98059 or with the specific JNK inhibitor SP600125. Data compiled from the proliferation assay, M30 cytodeath assay, caspase activation and DiOC6 assay proved a protective role for MAPK in TQ-induced cell death. Inhibition of both ERK and JNK pathways sensitized DLD-1 cells to TQ's antiproliferative effect and potentiated TQ-induced apoptotic cell death (Fig. 5f).

The proposed mechanism of TQ anti-tumor effect in colon cancer cells is as follows:



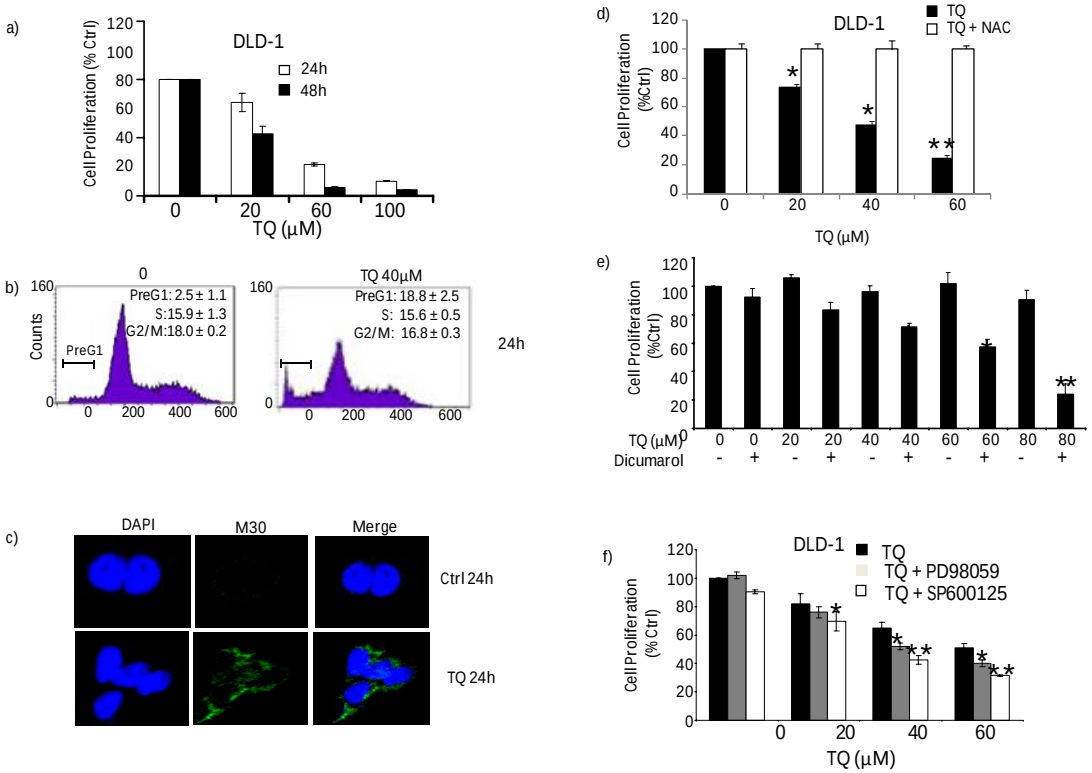


Figure 5. TQ's effect on DLD-1 cell proliferation (a), cell cycle (b), apoptosis (c), and ROS generation (d). Sensitization of HT-29 cells to TQ's effect by inhibiting DT-diaphorase (e), and potentiation of TQ's effect by MAPK inhibitors (f).

5.4. Impact of Protein Binding on TQ'S Analytical Detection and Anticancer Activity (IV)

The recovery of TQ (10 µg/ml) from spiked serum following SPE, LLE, and protein precipitation prior to HPLC analysis was less than 5%. The use of different ratios of EtOAc and MeOH/ACN for extraction and protein precipitation did not improve TQ recovery. However, the recovery from serum spiked with 100 µg/ml TQ improved to 65-80 % with all the methods used. The ability to detect TQ at high concentration (100 µg/ml) and not at a 10 times lower concentration implies that fresh serum has a capacity above which TQ can be detected. In addition, the fact that none of the organic solvents used during LLE or protein precipitation was effective in extracting TQ from serum implies that TQ might be covalently bound to serum components and that the non-covalently bound TQ is below the detection limit. To investigate this assumption TQ binding to serum proteins was studied. Towards that end, 10 % and 100 % FBS were spiked with different concentrations of TQ (3.3 µg/ml, 6.6 µg/ml and 13.2 µg/ml) and the percentage of protein binding was determined, over 6 h, at different time intervals using the equation [% binding= (Total-free)/Total *100]. At all concentrations used, TQ binding in 10 % FBS was 86-93 % at 0 min and >96% after 30 min of incubation. In 100% FBS, however, 0 min of incubation resulted in more than 98% of binding. TQ binding to BSA and AGP, the most abundant protein in plasma, was determined and found to be 94.5% and 99.1%, respectively.

The effect of protein binding on TQ's anticancer activities was further investigated in DLD-1 human colon cancer cells. Pre-incubation of TQ with different FBS concentrations for 30 min prior to addition to the cells resulted in a significant decrease ($p < 0.05$) in TQ's anticancer activities. Since TQ was incubated with FBS prior to treatment, we suggest that the observed loss of TQ's activity comes from FBS component (s) on TQ rather than from FBS action on the cells.

The protective effect exerted by FBS components on TQ-induced cell death was further explored by studying the effect of TQ binding to BSA and AGP. While incubation of TQ with increasing concentrations of BSA resulted in a concentration-dependent loss of its activity, pre-incubation with AGP prior to treatment did not alter its inhibitory effect at almost all the concentrations used. The loss of TQ activity was more pronounced in cells treated with TQ + 240 µM BSA than with those treated with TQ + 40 % FBS. These results imply that TQ binds to proteins in serum where they may play a protective role and allow TQ to exert its effect while other(s) inactivate TQ.

Further experiments were conducted in which TQ was incubated for 30 min with 60 µM BSA and for an additional 30 min with 2.5 µM AGP and vice versa. Interestingly, when TQ was pre-incubated with AGP prior to the addition of BSA, AGP protected TQ from binding to BSA and TQ's effect was retained. The contrary was observed when the incubation order was reversed, whereby pre-incubation of TQ with BSA prior to AGP resulted in a significant loss of TQ's activity as compared to TQ alone.

TQ as a quinone can undergo facile adduction with electron-rich nucleophilic species such as activated amino, hydroxyl and thiol groups (Land *et al.* 2004; Li *et al.* 2005). BSA has a free cysteine on the position 34 of its amino acid sequence. AGP, on the other hand, has 2 isoforms. Each has 2 disulfides bridges but the major difference is the presence of a free cysteine in position 147 in one isoform while the other has an arginine residue instead (Schmid *et al.* 1973). TQ binding to the free thiol group in BSA and AGP was further studied using sequences that were synthesized from regions surrounding the free thiol in BSA and AGP. Mass spectrometric analysis confirmed a covalent binding between TQ and sequences having a free thiol (Fig. 6). However, no binding was observed when TQ was incubated with the AGP isoform that has no free cysteine.

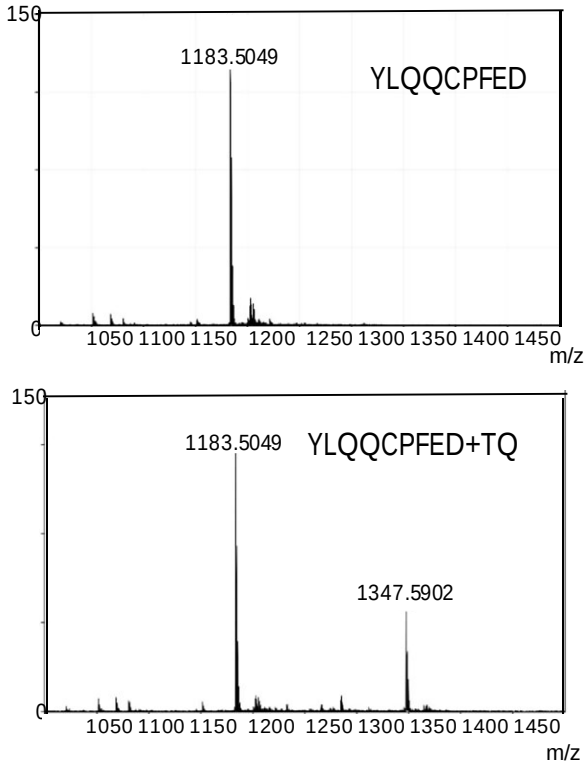


Figure 6. TQ covalent binding to BSA sequence on free thiol of Cyst-34.

6. DISCUSSION

Medicinal plants have been used by diverse cultures around the world to promote health conditions and fight diseases. Due to the health benefits associated with the use of plants in folk medicine, the plants became an essential source for new chemical entities and the basis of many clinically approved drugs.

Colorectal cancer (CRC) is one of the most devastating malignancies in the world. Yet the prolonged series of neoplastic events required for clonal expansion from the time of initiation until the beginning of invasive stage provides time and targets for preventive interventions. Many clinically used anticancer drugs that have provided hope for people with cancers resistant to conventional treatment are based on plant products. Therefore, finding new plants/plant-derived compounds with medicinal properties holds great potential to prevent and cure this invasive disease.

Cellular and molecular evidence obtained from the first two studies (I, II) indicate that crude plant extracts of *O. cynarocephalum* and *C. ainetensis,* plants indigenous to the Mediterranean region, inhibit the growth of colon cancer cells at concentrations that showed no apparent toxicity effects against normal cells. *In vitro* both extracts induced cell cycle arrest and apoptosis through modulation of the expression of specific components of the cell signaling pathways (p53, p21, cyclinB_1, Bax and Bcl-2). The tumor suppressor protein p53 is known to play a key role in cell cycle arrest and apoptosis. Upon DNA damage, hypoxia or other stress stimuli, p53 induces the expression of p21, gadd45, and Bax, resulting in cell cycle arrest, DNA repair and apoptosis, respectively (Hedstrom *et al.* 2009; Kim *et al.* 2006; Taylor and Stark 2001). Furthermore, p53 by inhibiting the cyclin-dependent kinase (Cdc2) required for entering mitosis and by the repression of cyclin B_1 gene plays a role in the regulation of the G_2/M checkpoint (Sherr *et al.* 1995; Stewart *et al.* 1995; Taylor *et al.* 2001). The cyclin dependent kinase inhibitor p21 on the other hand, leads to cell cycle arrest at the G_1 phase. Other mediators playing a fundamental role in apoptosis signaling are Bax and bcl-2. While the latter is an anti-apoptotic protein that prevents the release of apoptotic factors such as cytochrome c and inhibits the activation of caspases, the former is a pro-apoptotic protein. When p53 induces Bax, the balance between pro-apoptotic and anti-apoptotic proteins favors the former leading to the release of cytochrome c and promotes cell death. Correlation between cell cycle deregulation and carcinogenesis has been extensively studied. Therefore, inhibition of the cell cycle has been considered as a target for cancer treatment (Weinstein *et al.* 1997).

The increase in p53 protein levels correlated well with the observed increase in the expression levels of p21 and Bax/bcl-2 ratio. In addition, apoptotic induction was further confirmed by the extract-induced inhibition of cyclin B_1, the destruction of which is fundamental for mitotic exit and cytokinesis (Takizawa and Morgan 2000).

The tumor inhibitory effect of *O. cynarocephalum* and *C. ainetensis* extracts were further investigated *in vivo*. Since cancer intervention can take many decades and harmful effects must be minimal, toxicity or adverse side effects must be evaluated prior to long-term treatment. The results show that administration of the extracts for 10 consecutive days have no effect on mice mean body weight nor have resulted in any death. Based on the toxicity data, the tumor inhibitory effect of the extracts was carried on using the DMH model of colon cancer.

Tumor development is divided into stages of initiation, promotion, and progression.
While in the initiation phase irreversible genetic changes, such as mutations take place in normal cells, promotion of initiated cells to pre-neoplastic lesions is governed by epigenetic and potentially reversible events. During the progression phase additional DNA mutations and epigenetic changes play a major role in driving mitogenesis and apoptosis resistance to eventual neoplasia (Trosko 2001).

Depending on the timing of anticarcinogenic actions, chemopreventive agents can be classified into those that inhibit tumor initiation, and those that block tumor promotion and progression (Wali *et al.* 2002). The results in study I and II show that both extracts did not decrease the average numbers of ACF (week 10), potential markers of tumor growth, however they significantly ($p < 0.001$) inhibited tumor formation at weeks 20 and 30.

The inhibitory effects of the extracts on tumor formation and not on ACF development suggest that the two extracts act during the promotion and progression stages and not during the initiation stage of colorectal carcinogenesis. A similar effect was observed with rofecoxib, a highly selective inhibitor of cyclooxygenase-2 shown to inhibit the later stages of DMH-induced colon carcinogenesis in Wistar rats (Perse *et al.* 2005).

In studies II and III, the inhibitory effects of two plant-based products, Salograviolide A and TQ, have been investigated *in vitro*. Salograviolide A, isolated through bio-guided fractionation of *C. ainetensis* crude extract, has been found to induce apoptosis in colon cancer cells as the mother crude extract (II). While Salograviolide A, isolated from other *Centaurea* plants, was shown to possess antifungal activities (Vajs *et al.* 1999), no other activities have been reported so far. Therefore, the anti colon-tumor activities of Salograviolide A reported in this study (II) are new activities identified for this molecule.

TQ, the active constituent of *N. sativa*, has been found to induce apoptotic cell death as well (III). The fact that the further studies have been conducted on TQ is based on three facts: 1) TQ has already potent *in vitro*/*in vivo* activities and lacks toxicity, 2) in many countries the plant is incorporated into diets and everyday lifestyles to improve health conditions, and 3) TQ is readily obtained from several companies, bypassing hence the difficulties of plant supply and extraction procedures.

Although *in vitro* and *in vivo* studies show that TQ is a potent anticancer agent, its mechanism of action is not yet fully defined. Therefore, to decipher the exact mechanism

of TQ action, a wide variety of molecular and cellular biology experiments have been performed. TQ, as a quinone, is known to undergo one or two electrons reduction by cellular reductases. While one electron reduction results in the formation of semiquinones, two electron reductions produce hydroquinones. ROS are the by-product of the reaction of semiquinones with molecular oxygen. Hydroquinones formed by DT-diaphorase action are of three types, 1) redox-stable hydroquinones, 2) redox-labile hydroquinones that subsequently auto-oxidize with formation of ROS and 3) hydroquinones that readily rearrange to potent electrophiles participating in bioalkylation reactions (Cadenas 1995). The properties of the hydroquinone generated by DT-diaphorase determine whether this reduction leads to the activation or deactivation of quinones. Several reports attribute the potency of TQ to its antioxidant properties (Badary *et al.* 2003; Brewer *et al.* 2006; Nagi and Mansour 2000; Richards *et al.* 2006; Tan *et al.* 2006). In study III, however, TQ-induced apoptotic cell death has been confirmed to be mediated by ROS resulting from its one electron reduction and that its two electrons reduction by DT-diaphorase leads to its detoxification.

The pro-oxidant effects of TQ are in accordance with studies showing that TQ is involved in mitochondrial ROS generation in human osteosarcoma and leukemia Jurkat cells (Alhosin *et al.* 2010; Roepke *et al.* 2007). The pro-oxidant/antioxidant activities of TQ depend on the milieu where it is present.

Oxidative stress is known to activate the MAPK family and therefore might contribute to influencing survival (Matsuzawa and Ichijo 2005; Navarro *et al.* 2006). MAPK family consists of three subfamilies: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAP kinase that play a key role in the regulation of many cellular processes, such as cell growth and proliferation, differentiation, and apoptosis (Kyosseva 2004). Many studies have been made to link MAPK activation and apoptosis induction during stress conditions. While the ERK pathway usually confers a survival signal, JNK and p38 activation are associated with pro-apoptotic signals (Brenner *et al.* 1997; Guyton *et al.* 1996). JNK and ERK, but not p38 kinases, were activated significantly in the presence of TQ (III). The activation of ERK and JNK has been shown to play a survival role whereby inhibition of the ERK pathway by PD98059 and JNK pathway by SP600125 potentiated apoptosis induction by TQ (III). Therefore to further improve the anti-tumor activities of TQ, combinatorial treatment with a kinase inhibitor such as sorafenib could be used. In fact, sorafenib has successfully improved the anticancer activity of doxorubicin and irinotecan.

Despite the considerable data reported for the biological activities of TQ against cancer and inflammation, no studies have dealt with its pharmacokinetic profile. This is probably due to the absence of analytical methods for the isolation and quantification of TQ from blood/plasma, a prerequisite for the determination of its pharmacologically achievable doses and clearance from blood. However, only two HPLC methods have been reported for its quantification from black seed oil (Aboul-Enein and Abou-Basha 1995; Ghosheh *et al.* 1999). The absence of analytical methods for TQ detection from serum might be the

reason for the absence of data describing its pharmacokinetic profile, a prerequisite for its further clinical evaluation. As TQ belongs to the quinone family, known to have electrophilic property, it has the ability to undergo nucleophilic attack as activated amino, hydroxyl and thiol groups (Land *et al.* 2004; Li *et al.* 2005), which may lead to detoxification or enhanced toxicity. In biological systems, such nucleophiles may be found as reactive side-groups of lysine, serine and cysteine (Magee 2000). The ability of TQ to bind covalently to proteins might influence its analytical detection for irreversible binding might result in a decrease in its free concentration to a level that is below the detection limit. In study IV, spiked serum subjected to several sample cleanup procedures (SPE, LLE, protein precipitation) prior to HPLC analysis, resulted in low recovery < 5% when 10 µg/ml of TQ was used and increased to 72 % when the extremely high concentration of 100 µg/ml was used. The results show that TQ extraction and quantification from spiked serum is not possible using conventional analytical methods since high recovery is observed with a concentration that is not reflective of the real pharmacological achievable dose.

The proposed explanation is that TQ may undergo reversible and irreversible binding to serum components. When serum is spiked with low concentration of TQ, many irreversible binding sites are available and the free and reversibly bound TQ is below the detection limit thus explaining the low recovery. On the other hand, when higher concentrations of TQ were used, all the irreversible binding sites were occupied and the free TQ was easily extracted and detected by the proposed method. To prove this hypothesis, a thorough investigation was performed. Our findings show that the extensive binding of TQ to serum proteins (> 90%) may play a major role in the observed low recovery and provide evidence for the absence of conventional analytical methods for TQ.

The study of the binding effect on TQ-induced cell death in colon cancer shows that TQ binding to BSA resulted in its inactivation while its binding to AGP maintains its activity. The fact that TQ extensive binding to AGP did not alter its inhibitory effect suggests that in serum there is a competition in the binding of TQ to different plasma components. Mass spectrometric analysis shows that TQ binds covalently to the free thiol on the 34^{th} position of the amino acid sequence of BSA. Serum contains many components that have free thiol groups to which TQ can be bind covalently confirming again the difficulties in detecting TQ in serum. Our data on TQ covalent binding contradicts a recent study published by Lupidi G *et al.*, where they showed that TQ binds to the site I of human serum albumin (HSA) by a hydrophobic interaction (Lupidi *et al.* 2010). The discrepancy between our results using BSA and the results reported by Lupidi et al. on TQ binding to HSA could be due to differences in species and analytical techniques used. The effect of serum components on TQ's activity resembles that observed with ET-743, a novel marine antitumor compound against soft tissue sarcomas and ovarian cancer, which is in phase I and II clinical studies (Tognon *et al.* 2004). ET-742's antitumor activity was lost when the percentage of FBS was increased; however, the activity was restored when the compound was first prepared in HSA then diluted with FBS, confirming thus the protective effect of HSA on the drug against the inactivation exerted by FBS components (Tognon *et al.*

2004). The protection offered by HSA is similar to the effect offered by AGP prior to the addition of BSA. Our result on the loss of TQ activity when TQ is bound to serum components correlate well with a new study showing that encapsulation of TQ in nanoparticles improves the antitumor effect of TQ against colon, breast, prostate, and myeloma cancer *in vitro* (Ravindran *et al.* 2010). The nanoparticles protected TQ from such an inhibitory effect and increased TQ potency against cancer cells. TQ non-covalent and covalent binding to plasma proteins can cause problems in its detection thus limiting the use of conventional methods for determining its bioavailability. In addition to the effect on detection, the binding of drugs to plasma and tissue proteins can affect the drug's ADME and can result in adverse side effects. The ability of TQ to bind covalently to proteins with free thiol raises concerns about its long term use. While the covalent binding and thus the decrease in the available free drug might result in instant absence of toxicity, consecutive administration of the drug could be responsible in drug-induced toxic side effects. This can be explained by the fact that once, in the presence of quinones, the plasma free thiols and cellular GSH are depleted, cellular SH-dependent proteins can be alkylated thereby causing irreversible changes and cell death (Buffinton *et al.* 1989; Jakoby and Ziegler 1990). Eventhough short term *in vivo* toxicity studies has shown that TQ is not toxic (Gali-Muhtaseb *et al.* 2008a), long term toxicity studies should be performed to confirm its safe use.

7. CONCLUSIONS

The increase in the incidence of CRC along with the undesirable side effects observed with the available chemopreventive drugs urges the discovery of new agents from natural sources. The goal of prevention is to decrease morbidity and mortality from CRC. To achieve this goal, it is important to delay the progression of early neoplasia or reverse/ inhibit the development of invasive cancer.

Strong side effects are usually associated with most of the known chemotherapeutic drugs. Interestingly, in studies I, II, and III the plants (*O. cynarocephalum, C. ainetensis)* and plant-based products (Salograviolide A and TQ) show high selectivity against cancer cells with no apparent toxic effects on normal cells. This differential effect makes the aforementioned natural products potentially harmless chemopreventive agents against colon cancer development (I, II, III).

The results of study III and IV cover the lack of useful information for the better understanding of the PK/PD profile of TQ, a well-established anticancer plant derived-compound. In study III, early events of TQ's induced apoptotic cell death are defined. Using a radical scavenger and several inhibitors the mechanism is identified as follows: TQ, absorbed by the cells as early as 15 min, undergoes redox-cycling and generates ROS. ROS mediate apoptosis and result in the activation of p-ERK and p-JNK, which are known to play a protective role against TQ-induced cell death (III). In study (IV) evidences prove that the absence of analytical methods for TQ detection from blood/serum and therefore pharmacokinetic studies are due to extensive protein binding, especially covalent binding. Even though many reports show that TQ is active *in vitro* and *in vivo*, the actual behavior of TQ in plasma remains an unanswered question that merits further investigation.

This thesis confirms the usefulness of two endemic Lebenase plants used in folk medicine, identifies a new compound with anticancer activity and enhances the understanding of the pharmaceutical properties of the promising anticancer compound TQ.

Based on the reported data in this study there is a need to 1) further fractionate *O. cynarocephalum* to isolate the active constituent (s) responsible for the plant's *in vitro* and *in vivo* activity, 2) develop a method for Salograviolide A extraction and quantification from blood/serum for future pharmacokinetic studies, and 3) synthesize radiolabeled or isotopically labeled TQ for clarifying its pharmacokinetic properties, required for its future clinical development.

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