Chapter 1

Introduction

Natural products are organic and inorganic compounds that occur in plants (leaves, needles, bark, roots, flowers and seeds) and microbial organisms found in highly diverse and sometimes extreme conditions. Natural products fall into several different categories: steroids from marine animal, plant and fungal sources, pyrimidines and purines from microbes and proteins, amino acids and antibiotics from microbes. Other compound classes such as polyphenols and coumarins from plants, alkaloids from plants and some bacteria, pigments from microbes and plants and terpenes, carbohydrates, fats and macromolecular products from all sources are also important natural products. A wide spectrum of natural products is created by combining different classes of compounds e.g. polyphenols and sugars to produce flavonoid glycosides. Substitutions with hydroxyl or ketones and additions with methyl or prenyl groups also increase variety. The majority of natural products are secondary metabolites, produced by microorganisms and plants for the purpose of protection, procreation and survival in general. It is thus not surprising that some natural products can have toxic effects in humans and animals.

1 The importance of natural products

The natural products that occur in food contribute to the organoleptic properties of the food products. The polyphenols and organic acids content may determine the quality of the food products. To regulate the quality of consumables, natural compounds must be analyzed in the food industry. The Quality Control must prevent off-flavors, toxins and other harmful compounds, produced by microorganisms, from reaching the market place. Many natural products have bioactivities that may be harmful to

humans. Aflatoxins produced by *Aspergillus flavus* are of the most potent liver carcinogens and phalloidins from the mushroom, *Amianita phalloides,* causes liver damage. It is important to determine sources of toxins and the presence thereof in food destined for human consumption to prevent food poisoning.

Natural products first gained prominence through the isolation of antibiotics from microbes. An example is the family of β-lactam antibiotics (cephalosporins) that are produced by the mold *Cephalosporium acremonium*. Today natural products have a variety of medicinal uses. They are used as immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigrane agents, herbicides, antiparasitic agents and bio-insecticides. The pharmaceutical industry isolates natural products as lead compounds that may potentially, after chemical modification, give rise to a new class of compounds with a certain bioactivity. The majority of the lead compounds originate from micro-organisms while the rest originate from plants (Borris, 1996). Although isolating natural products from plants is more challenging, it is important because micro-organisms and plants do not produce the same secondary metabolites. This maximizes the chemical diversity that is evaluated in pharmaceutical screening programs. Plants produce a whole range of flavonoids and flavonoid glycosides that have pharmaceutical or nutritional value. Ephedrine alkaloids from Chinese herbal preparations have medicinal value and coumarins found in several plant families have blood anti-coagulant activities. Quinoline, quinazoline and acridone alkaloids have been extracted from plants and tested as anti-malarial drugs (Michael, 1997). Similarly artemisinin drugs isolated from the plant *Artemisia annua* have been shown to have anti-malarial properties (van Agtmael, 1999). Pharmaceutical industries are exploring nature's libraries of natural products

for novel lead compounds that after combinatorial chemical modifications may result in active drugs against diseases such as cancer and AIDS.

It is important to study the metabolic pathways that synthesize these secondary metabolites, as well as their efficacy and toxicity. The separation, detection and quantification of bioactive secondary metabolites are essential in conducting these studies.

2 Natural products contributing to quality of tea

Catechins are the most abundant polyphenols in the leaves of tea (*Camellia sinensis*). They may constitute up to 30% (w/w) of the dry weight of young tea leaves. The major catechins in tea are (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg). (+)-Gallocatechin, (GC) and (+)-gallocatechin gallate (GCg) also occur in tea, but only in low concentrations (Wilson, 1992). The total catechin content is highest in young leaves and decreases significantly with leaf aging. Catechins contribute to the astringency of the tea brew. The quality of the tea correlates with its catechin content and composition.

Quercetin, kaempferol, myricetin and their glycosylated derivatives are also found in tea. Bisflavanols or theasinensis are dimers formed from the catechin monomers. These flavonoids occur at low concentrations in tea and probably do not contribute to the quality of tea. During the oxidation process required for the production of black tea, the catechins are polymerized. The major polyphenols in black tea are theaflavins (catechin dimers) and thearubigens (catechin polymers). A positive correlation has

been found between the quality of made black tea and the amount of gallated catechins in fresh leaves of Kenyan cultivars (Obanda, 1997). The gallated monomers produce gallated theaflavins that contribute more to quality than ungallated theaflavins.

Caffeine is another prominent compound of tea and may constitute 3 to 4% (w/w) of the dry weight (Wilson, 1992). It contributes to the bitterness of the tea liquor. Theophylline and theobromine are two methylxanthine precursors of caffeine also found in tea. They only occur in low concentrations and probably have very little influence on the quality.

Theanine (5-N-ethyl-glutamine/ γ-ethyl-glutamine) is unique to tea and the most abundant free amino acid, constituting between 1 and 2% (w/w) of the dry weight of tea leaves. Theanine is equally abundant in all types of tea (i.e. green, oolong or black). It contributes to the savory sweet taste of tea (Wilson, 1992), (Ekborg-Ott, 1997). Aspartic and glutamic acid are the other two important amino acids in tea, but their levels are usually less than 0.001% by weight. The monosodium glutamate contributes to the brothy taste of tea (Horie, 1998 (a)).

Free gallic acid is not found in fresh tea leaves. About two thirds of the catechins in fresh tea leaves are esterified at the 3-OH position with gallic acid forming complex catechins (Obanda, 1997). Some of the complex catechins are de-esterified during the manufacturing process to yield free gallic acid in the made tea. Gallic acid influences the pH and color of made black tea. Oxalic, citric, malic and quinic acids are other organic acids that are found in green tea. The organic anions chelate with aluminum,

calcium, iron and other metals, hence influencing the bioavailability of metals (Horie, 1998 (a)).

Free ascorbic acid occurs in very low concentration in tea and has no effect on the quality. Bottled ice tea is fortified with ascorbic acid for preservation and nutrition. It contributes to the quality of bottled tea in an indirect manner, making it important to quantify ascorbic acid in bottled ice teas.

3 Bioactive natural products in tea

3.1 Identification of the bioactive tea compounds

Plants have been the source of a variety of bioactive natural products. In the mid 1980's several initial studies indicated that water-extracts of green tea contained substances with antimutagenic effects (Kada, 1985), (Cheng, 1986). In several cases plant phenols have been shown to have prophylactic properties (Boone, 1990), (Das, 1987), (Mukhtar, 1988). It was therefore very likely that the prophylactic properties observed from green tea extracts were manifested through its large polyphenol contents. Green tea polyphenols (GTP) were extracted from green tea leaves with ethylacetate. The main constituents of the GTP extract were EGC, EC, EGCg, ECg and caffeine (Mukhtar, 1992). From the initial studies it could be seen that GTP's antimutagenicity was a result of at least two different mechanisms. 1) Preventing the conversion of pro-carcinogens to DNA-adduct forming carcinogens via modulation of the activity of CYP 450 systems both *in vitro* (Wang, 1989 (a)) and *in vivo* (Wang, 1989 (b)) and 2) the ability of the polyphenols to scavenge and neutralize oxygen and other genotoxic radicals (Perchellet, 1989).

3.2 Intrinsic properties of polyphenols

The polyphenolic flavonoids have the diphenylpropane ($C_6C_3C_6$) skeleton. The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to the aromatic ring structures. Various methoxylation, sulfation and glycosylation patterns also exist. This family includes monomeric subgroups such as flavanols, flavanones, anthocyanidins, flavones and flavonols, as well as isoflavonoids, neoflavonoids and flavylium salts. The differences between the sub-groups are due to variation in number and arrangement of hydroxyl groups, unsaturated and saturated bonds (Rice-Evans, 1996). The inter-relationships between the most important flavonoid sub-groups are shown in Fig. 1.1. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, singlet oxygen quenchers and metal chelators. The chemical property of polyphenols in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers predicts their antioxidant activities.

For a polyphenol to be defined as an antioxidant, it must satisfy two basic conditions: first, when present in a low concentration relative to the substrate to be oxidized, it can delay, retard or prevent the auto-oxidation, or free radical mediated oxidation (Halliwell, 1990). Second the resulting radical formed after scavenging must be stable through intramolecular hydrogen bonding after further oxidation (Shihadi, 1992). The chemistry of the flavonoids is predictive of their free radical scavenging activity, because the reduction potentials of flavonoid radicals are lower than those of alkyl peroxyl radicals and the superoxide radicals. This means that the flavonoids may inactivate these oxyl species.

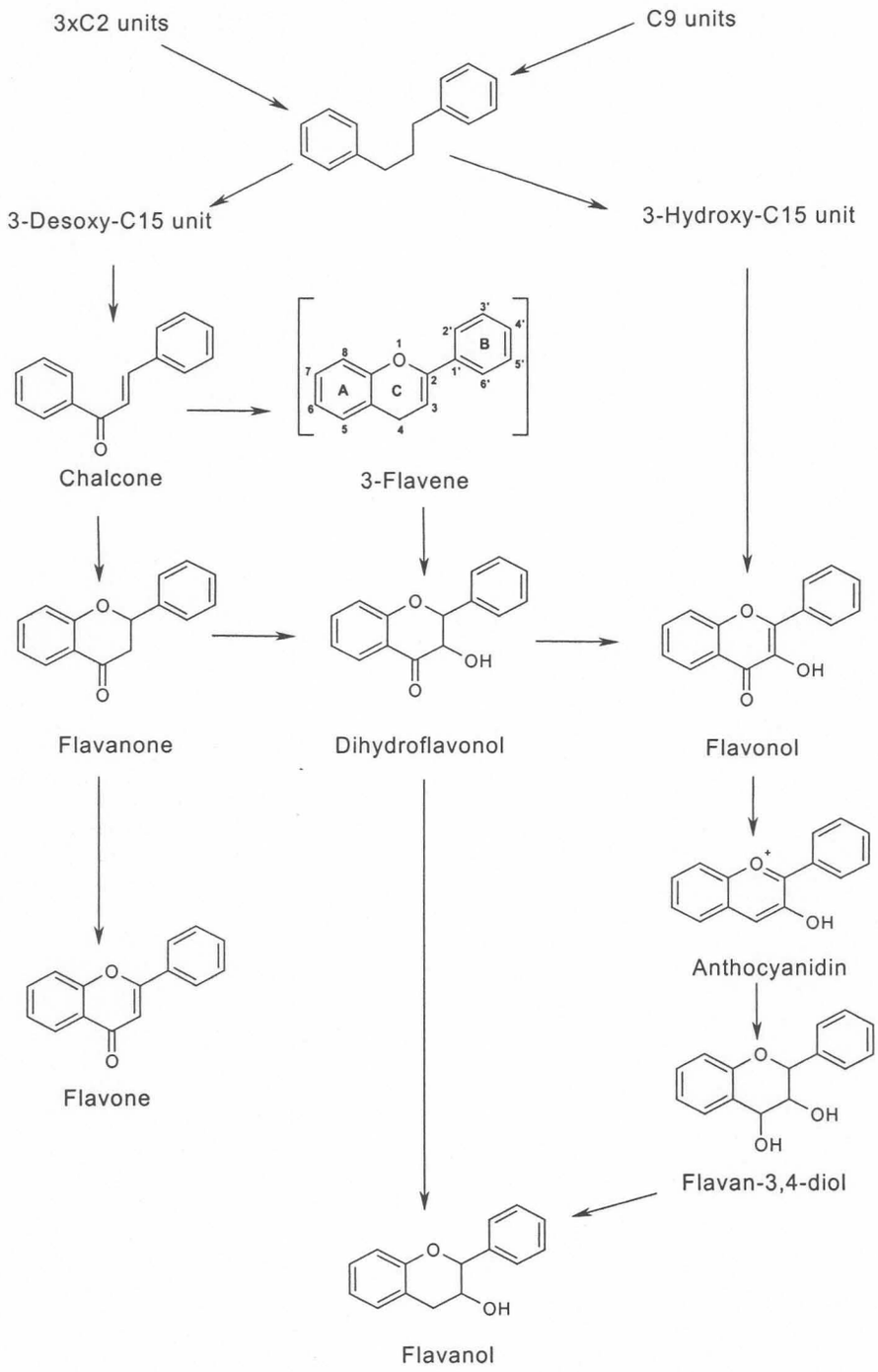
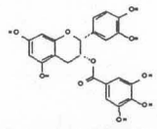
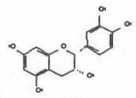
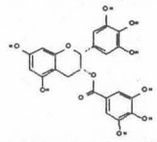
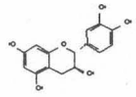
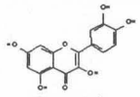
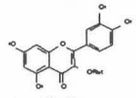
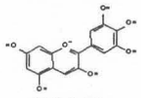
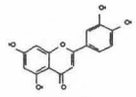
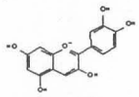
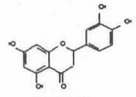
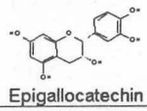
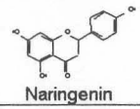
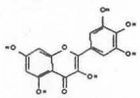
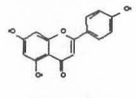
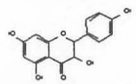
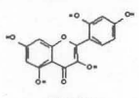
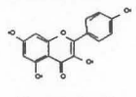


Figure 1.1 A schematic representation of the inter-relationship between the most important flavonoid sub-groups (Adapted from (Rice-Evans, 1996)).

3.3 Structural Principles for Radical Scavenging by Flavonoids

The glycosylation of flavonoids reduce their antioxidant activity when compared to the corresponding aglycones. The antioxidant values of polyphenols are expressed as Trolox-Equivalent Antioxidant Capacity values (TEAC). This value indicates the concentration of a Trolox solution in mM units with equivalent antioxidant potential as a 1 mM solution of the polyphenol of interest. A high TEAC value for a polyphenol indicates that it has good radical scavenging properties. Different flavonoids, their hydroxylation patterns and TEAC values are shown in Table 1.1. Blocking the C3-hydroxyl group in the C ring of quercetin with rutinose forming rutin (quercetin rutinoside) results in a decrease the compound antioxidant activity. Removal of the C3-hydroxyl group as in luteolin also results in decreased antioxidant activity. Maximal effectiveness for radical scavenging apparently requires the C3-hydroxyl group attached to the C2,C3-double bond and adjacent to the C4-carbonyl in the C ring. Removal of the C2,C3 double bond in the C ring, eliminates the means of delocalization of radicals from the aryloxyl radical on the B ring to the A ring as in taxifolin. The comparison of quercetin with luteolin and rutin demonstrates the influence of the C3-hydroxyl in combination with the adjacent double bond in the C ring. If one is dispensed with, the other apparently loses its impact on the antioxidant activity. The reduction of the C2,C3 unsaturated bond in the C ring of kaempferol forming dihydrokaempferol has no influence on the total antioxidant activity. This substantiates the notion of the lack of effect of the specific structural activities in the C ring on the total antioxidant activity in the absence of the orthodiphenolic structure in the B ring. The single hydroxyl group in the B ring makes little contribution to the antioxidant potential, despite the double bond conjugation and the C3-hydroxyl group.

Table 1.1 A list of selected polyphenols with their free hydroxyl group distribution and antioxidant potential (Adapted from Rice-Evans, 1996)

Compound	Free OH-Substituents	TEAC (mM)	Family	Compound	Free OH-Substituents	Glycosylation position	TEAC (mM)	Family
 Epicatechin gallate	3,5,7,3',4',3'',4'',5''	4.9 ± 0.02	Flavanol	 Epicatechin	3,5,7,3',4'		2.5 ± 0.02	Flavanol
 Epigallocatechin gallate	3,5,7,3',4',5',3'',4'',5''	4.8 ± 0.06	Flavanol	 Catechin	3,5,7,3',4'		2.4 ± 0.05	Flavanol
 Quercetin	3,5,7,3',4'	4.7 ± 0.10	Flavonol	 Rutin	5,7,3',4'	3-rutinose	2.4 ± 0.06	Flavonol
 Delpinidin	3,5,7,3',4',5'	4.44 ± 0.11	Anthocyanidin	 Luteolin	5,7,3',4'		2.1 ± 0.05	Flavone
 Cyanidin	3,5,7,3',4'	4.4 ± 0.12	Anthocyanidin	 Taxifolin	3,5,7,3',4'		1.9 ± 0.03	Flavanone
 Epigallocatechin	3,5,7,3',4',5'	3.8 ± 0.06	Flavanol	 Naringenin	5,7,4'		1.53 ± 0.05	Flavone
 Myricetin	3,5,7,3',4',5'	3.1 ± 0.30	Flavonol	 Apigenin	5,7,4'		1.45 ± 0.08	Flavone
 Gallic acid	3,4,5	3.01 ± 0.05	Hydroxybenzoate	 Dihydrokaempferol	3,5,7,4'		1.39 ± 0.02	Flavonol
 Morin	3,5,7,2',4'	2.55 ± 0.02	Flavonol	 Kaempferol	3,5,7,4'		1.34 ± 0.08	Dihydroxyflavonol

The C2,C3 double bond apparently contributes very little to the hydrogen-donating ability without the diphenolic structure in the B ring, for naringenin with a saturated heterocyclic ring and no C3'-hydroxyl has approximately the same antioxidant potential as apigenin.

Quercetin with two hydroxyl groups in the *o*-dihydroxy arrangement in the B ring has a higher antioxidant activity than morin with the two hydroxyl groups in the meta arrangement. Kaempferol with a lone C4' hydroxyl group in the B ring has 27% less antioxidant activity than quercetin with the additional C3' hydroxyl group. Presumably the C2,C3 double bond is not so relevant when the B ring lacks the *o*-hydroxyl arrangement because the monophenolic ring is not as an effective hydrogen donor. The presence of a third hydroxyl group in the B ring does not enhance the effectiveness against aqueous phase radicals as in myricetin compared with quercetin and anthocyanidin delphinidin compared with cyanidin.

It can be concluded that there are three structural groups that are important in determining the radical scavenging and/or antioxidative potential. a) The *o*-dihydroxy (catechol) structure in the B ring, which is the obvious radical target site for all flavonoids with a C2,C3-saturated bond (flavan-3-ols, flavanones and cyanidine chloride). The catechol moiety does not confer stability to aroxyl radicals and participates in electron delocalization. b) The C2,C3-double bond in conjugation with a C4-oxo function that is responsible for electron delocalization from the B ring to the A ring. c) The additional presence of both the C3- and C5- hydroxyl groups for maximal radical-scavenging potential and stronger radical absorption.

3.4 Antioxidant activity of the catechins and catechin gallate esters

Catechins are the most important antioxidant compounds in tea. Catechins include epicatechins, gallocatechins (with the three hydroxyl groups in the B ring) and catechin gallates with a gallic acid esterified to the C3-OH group in the C ring.

There is no electron delocalization between the A and the B rings due to the saturation of the hetrocyclic C ring. The antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity. Quercetin has an identical number of hydroxyl groups in the same positions as catechin, but also contains the C2,C3-double bond in the C ring and the C4-oxo group. The catechin structure can be modified to enhance its antioxidant potential by incorporating the C2,C3-double bond and C4-oxo function or by forming epigallocatechin gallate. Both the modifications result in approximately the same antioxidant potential. The antioxidant potential of the tea catechins on a molar basis, against the radicals generated, in the aqueous phase are, in order of decreasing effectiveness: ECg ≈ EGCg > EGC > Gallic acid > epicatechin > catechin.

A green tea preparation at 1000 ppm (0.1% w/v) gave a TEAC value of 3.78. A black tea preparation of the same concentration gave a similar TEAC value of 3.49. A total green tea polyphenol extract (44% w/w of the dry weight of the green tea preparation) shows a total antioxidant activity of 3.36. The total polyphenol content of a black tea extract is similar to that of green tea (44.94% w/w), but only 6.9% by weight is comprised of catechin and catechin gallate components. The rest of the polyphenols includes theaflavins, thearubigens and undefined polymeric polyphenols formed during the oxidation process. The antioxidant activity of black tea extract is 3.49

which is similar to that of green tea (Salah, 1995). The above results suggest that theaflavins and thearubigens have similar antioxidant capacity as their catechin precursors.

3.5 Properties of methylxanthines

Theophylline, theobromine and caffeine are three closely related alkaloids from the family of methylated xanthines. Beverages containing methylxanthines have been popular since antiquity. One such beverage is tea. The basic belief is that beverages containing caffeine have stimulatory effects that elevate mood, decrease fatigue and increase capacity for work (Gilman, 1985). The solubility of the methylxanthines is low and is enhanced by the formation of complexes with a wide variety of more soluble compounds. In black tea the methylxanthines complex with gallated theaflavins to increase their solubility. At low temperatures these complexes aggregate to form "cream". This is a problem with bottled iced tea. "Cream" formation must be prevented to be able to produce and deliver a product with freshly made and appetizing appearances. To prevent the formation of "cream" the tea must be decaffeinated or the gallated theaflavins must be hydrolyzed to simple theaflavins with tannin acylhydrolase enzymes [E.C. 3.1.1.20].

4 Bioactivities documented for tea components

4.1 Tea polyphenols and cancer

The antimutagenic and anticarcinogenic properties of tea polyphenols are poorly understood and are being investigated extensively. From the evidence currently available it is certain that there are several mechanisms of action by which polyphenols manifest their prophylactic properties.

Several studies have shown that tea polyphenols prevent tumor initiation. Tumor initiation is usually the result of permanent covalent DNA modification. Oxygen radicals oxidize the nucleotide bases thus causing alterations of the genetic code. This may lead to tumorigenesis. The antioxidant activities of polyphenols reduce tumor initiation occurring in this manner (Steele, 1985), (Nagabhushan, 1988), (Bu-Abbas, 1994 (a)).

Activated chemical compounds, known as carcinogens, can form adducts with DNA resulting in erroneous transcription or replication of DNA also altering the genetic code. Polyphenols may act as alternative targets for the activated chemical compounds, thus sparing the DNA. Many carcinogens enter the body as inactive pro-carcinogens that do not have the ability to bind to DNA. Cytochrome P-450's (P450), phase I detoxification enzymes, convert pro-carcinogens to electrophillic carcinogens through oxidation. The P450 is a superfamily of isoenzymes with different substrate specificities and reactivities. Different isoenzymes may convert the same xenobiotic to different products of which one may be carcinogenic and the other may be harmless. It was shown that EGCg inhibited the activities of P450 1A, 2B1 and 2E1 from liver microsomes, preventing the activation of NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) a tobacco-specific carcinogen (Shi, 1994). Quercetin, kaempferol and other polyphenols are known to be selective inhibitors of P450 isoenzymes (Yang, 1994). Tea polyphenols are able to selectively induce P450 1A2 and 4A1 (Bu-Abbas, 1994 (b)), (Sohn, 1994). This is an indication that it may not only prevent pro-carcinogens from being converted to carcinogens, but may also be

able to induce alternative options for converting pro-carcinogens to harmless compounds.

From the literature it is evident that phase II detoxification enzymes are modulated in a similar manner by tea polyphenols as with the phase I enzymes. Tea treatment enhanced the UDP-glucuronosyl transferase activity, resulting that tobacco-specific carcinogens were conjugated more readily with glucuronic acid (Bu-Abbas, 1995). This renders the carcinogen inactive and increases the excretion of the carcinogens. It was found that tea polyphenols inhibited the glucuronidation of estradiol and estrone (endogenous compounds) which shows that there is some sort of selectivity in the induction/inhibition process (Zhu, 1998). Glutathione-S-transferase, another phase II enzyme, is also inhibited by several plant polyphenols, including morin, quercetin and apigenin (Zhang, 1994).

Polyphenols have been shown to be able to prevent or reduce tumor promotion (Mukhtar, 1992). Recent research presents evidence that tea polyphenols are modulators of various cell growth regulators. Tea polyphenols have been shown to inhibit lipoxygenase from soybeans (Wang, 1990). It has been shown that lipoxygenase plays an important role in tumor promotion, since the metabolic products (HETE) of this pathway via the arachidonic acid cascade are involved in the process of tumor promotion (Nakadate, 1989).

Tea polyphenols inhibit one or several signal transduction pathways. It was shown that tea polyphenols prevent tumor promotion induced by either epidermal growth factor (EGF) or phorbol esters (Mukhtar, 1992), (Conney, 1992). Phorbol esters

mimic 1,2-diacylglycerol activation of protein kinase C (PKC) and EGF activate PKC via a receptor mediated pathway. It was shown that EGCg and theaflavin inhibits EGF and phorbol esters induced phosphorylation of the protein c-jun (Dong, 1997). The c-jun NH_2 terminal kinase is downstream of PKC in the signal transduction pathway, and is required to form an active AP-1 complex. AP-1 is a transcription factor that is important in tumor promoter-induced neoplastic transformation (Angel, 1991).

Recently it was also shown that EGCg inhibits UVB-induced AP-1 activity (Barthelman, 1998). UVB-induced signal transduction pathways are normally PKC independent, indicating that EGCg also modulates other signal transduction enzymes. Evidence was presented showing that EGCg inhibits the phosphorylation of p38 MAPK resulting in the reduced expression of the c-fos gene (Chen, 1999). The c-fos protein is required to form the active AP-1 heterodimer complex. GTP was shown to inhibit UVB-induced ornithine decarboxylase and cyclooxygenase and restore UVB-induced catalase, GSH and glutathione peroxidase activity in epidermal cells. Additional evidence shows the inhibition of UVB-induced TNFα, known to be released by keratinocytes subsequent to UVB irradiation. EGCg, EGC and theaflavin-3,3'-digallate inhibited cell growth in Ha-ras transformed cells. The Ha-ras activated AP-1 pathway is the major growth stimulant in Ha-ras transformed cells (Yang, 1999).

Green tea polyphenols have an inhibitory effect on the growth of transformed cell lines and tumors transplanted into mice, indicating it may play a role in regulating cell cycle progression (Conney, 1999). Tea polyphenols are able to inhibit the phosphorylation of retinoblastoma protein (pBR). pBR binds transcription factors

such as the E2F family required for the progress of the cell cycle from G1 to the S phase (Khafif, 1998). It has been demonstrated that EGCg can induce apoptosis and cell cycle arrest in human epidermoid carcinoma cells A431. The apoptotic response was specific to cancer cells.

It was shown that green tea inhibits *in vivo* metastasis and *in vitro* invasion of mouse lung carcinoma cells (Sazuka, 1995). It was shown that EGCg, ECg, theaflavin and theaflavin digallate inhibited the type IV collagenase activity of MMP-2 and MMP-9 matrix-metallo proteinases *in vitro* (Sazuka, 1997) and stabilizes collagen against collagenase activity (Wauters, 1986). With computer modeling it was suggested that EGCg might well serve as an inhibitor of urokinase, one of the enzymes that are most frequently expressed in human cancers (Jankum, 1997).

4.2 Inhibition of other enzymes

In vitro studies with tea extracts and pure compounds have shown inhibition of salivary α-amylase (Hara, 1990), small intestine sucrase (Welsch, 1989), liver NADH-cytochrome c reductase (Wanmg, 1988), glucosyltransferase from *Streptococcus mutans* (Otake, 1991) as well as HIV reverse transcriptase and cellular DNA and RNA polymerases (Nakane, 1990). The tea polyphenols do not only display selective inhibition with the P450 isoenzymes. Specific inhibition of type 1 but not type 2 steroid 5-reductase is seen (Liao 1995) and strong inhibition of angiotensin converting enzyme (ACE) is found, while only weak inhibition is found for mechanistically similar carboxypeptidase A (Hara, 1989).

4.3 Prophylactic properties of tea methylxanthines

Some of the beneficial health properties in animal studies can be produced with caffeine in the drinking water (Chung, 1998). Caffeine induces hepatic CYP 1A2 in rats just as polyphenols, although the induction is somewhat weaker (Ayalogu, 1995). Consumed carcinogens are probably converted to innocuous compounds by the induced P450 isoenzymes, or the increased biotransformation leads to increased excretion of the toxin. Theophylline and theobromine and caffeine share common pharmacological actions. They stimulate the central nervous system, act on the kidney to produce diuresis, stimulate cardiac muscle, and relax smooth muscle. Theophylline is used as therapy for bronchial asthma. High doses of caffeine or theophylline may have adverse effects on the central nervous system (Gilman, 1985).

5 Analytical methods in natural product chemistry

In the search and identification of novel natural products several analytical techniques are employed. These include high performance liquid chromatography (HPLC), high performance centrifugal counter current chromatography (HPCCC), capillary electrophoresis (CE) and high resolution mass spectrometry (HRMS). Analytical HPLC has always been the favorite and most popular analytical tool in natural product and clinical chemistry. Since the development of capillary electrophoresis many papers on the topic of CE have been catalogued. The pioneer days of trial and error method development have passed. This work has been integrated forming trends and concepts that enable the design of systematic strategies for the development of new analytical methods. The technique has matured to such an extent that similar CE methods exist for almost all HPLC methods, particularly in the fields of natural product and clinical chemistry.

CE has become the preferred technique because it has several advantages over HPLC. The separation efficiency of CE is much higher than that of HPLC and comparable to that of GC. The electro-osmotic flow causes less increase in the peak width than conventional laminar flow, which reduce the width of analyte peaks and increase the plate numbers. Plate numbers for CE are typically in the region of 10^5 to 10^6 m^{-1}, while platenumbers for HPLC are generally in the region of 10^4 m^{-1}. Uncoated CE columns are generally rinsed with acid or base for regeneration taking between 2 and five minutes while HPLC columns require long rinse times for column equilibration. This simplifies CE methods considerably, making automation a lot easier and increase the sample throughput. The amounts of solvents and in particular organic solvents are reduced to milliliters compared to the 1-2 liter organic solvents per day that require disposal with HPLC. Since uncoated capillaries can be regenerated with harsh acid or base conditions, extensive sample pretreatment is not as important as with HPLC where regeneration conditions are much milder (Issaq, 1997). The different modes of CE enable it to be a very powerful and versatile technique. CE has been used to analyze proteins, peptides, amino acids and a whole variety of drugs in serum. CE is ideal for therapeutic drug monitoring and testing for intoxication and drug abuse since small volumes of sample is required and the method automation is possible. The analysis of the low volumes of serum and urine collected for time dependent therapeutic monitoring and the huge sample throughput required can be achieved with CE.

All specialized techniques have limitations and CE is no exception. Analytes should not be highly volatile and have to be soluble in polar solvents to some extent. Analyte

concentrations in the order of 1mg/l (1ppm) is a necessity to avoid sample pre-concentration steps. On-column detection restricts the limit of detection (LOD) to between 0.1 and 1.0 mg/l, which is not as good as obtained with HPLC (0.02–0.1 mg/l) but still comparable. Flow-cell designs, different detection window geometries, stacking procedures as well as electrochemical and fluorescence detection can improve the LOD (Albin, 1993). The maximum peak capacity with CE is 10 peaks/min and more complex samples should undergo sample pretreatment. The low volume of buffer and sample used in CE restricts it to analytical tasks while preparative work should preferably be done with LC.

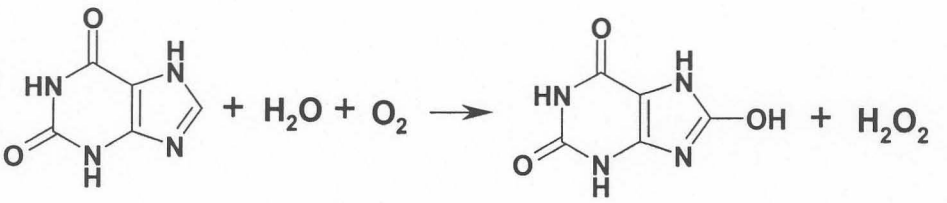
6 Objectives of the study

6.1 The detection and quantification of important constituents in tea leaf extracts

Plant extracts normally contain a large range of closely related secondary metabolites such as flavonoids, flavonoid glycosides, alkaloids or coumarins. Tea leaf extracts have an array of closely related catechins. CE with its high resolution and column efficiency is ideal for analysis of complex plant extracts without performing any sample clean-up procedures. Differences of secondary metabolite types and distribution patterns between cultivars or species can be detected easily with CE. The ability of MEKC to separate both charged and neutral analytes in one run makes this mode of CE an ideal analytical method to separate and quantify all the pharmacologically and organoleptically important tea constituents in one run.

6.2 To study the inhibition of Xanthine Oxidase by tea polyphenols

Xanthine oxidase (XO) belongs to the oxomolybdenum group of enzymes. This group contains both oxidase and reductase enzymes. Oxomolybdenum enzymes differ from other oxidase enzymes by utilizing water instead of dioxygen as the source of the oxygen molecule destined to be incorporated into the products. They also produce reducing equivalents instead of consuming them (Fig. 1.2). Xanthine oxidase catalyzes the oxidation of a broad spectrum of aromatic heterocycles and simple aliphatic aldehydes.



Xanthine **Uric acid**

Figure 1.2 Xanthine oxidase catalyzes an oxidation-reduction reaction. It obtains its reducing equivalents from water. In the process xanthine is oxidized to uric acid and the reducing equivalents are donated to molecular oxygen to produce hydrogen peroxide.

Physiologically XO catalyzes the irreversible formation of uric acid from xanthine and therefore it is one of the enzymes that regulate the levels of uric acid in body fluids. Uric acid levels of more than 7 mg/dl usually result in a condition known as hyperuricemia. This may lead to the formation of monosodium urate crystals in the kidneys (kidney stones) and joints (gout). Inhibition of XO alleviates hyperuricemia, reducing the occurrence of kidney stones and gout. Oxygen radicals are the byproduct in the reaction which may result in increased oxidative stress that lead to diseases such as cancer and atherosclerosis. The inhibition of radical formation may reduce oxidative stress and increase the antioxidant potential in body fluids. Inhibition of uric acid formation may compromise the antioxidant potential of the serum, since urate

also serves as antioxidant that contributes to the total antioxidant status of the serum (Rice-Evans, 1994).

Allopurinol is the most widely used drug for the treatment of hyperuricemia. It is a substrate analogue of hypoxanthine. It causes hypersensitivity reactions as well as debilitating side-effects such as nausea, headaches and drowsiness. Many other synthetic inhibitors of XO have been identified. These inhibitors include several anti-allergy drugs with aromatic heterocycles (White, 1981) and several derivatives of allopurinol (Okamoto, 1995), (Springer, 1976).

Different natural compounds also inhibit XO. These include coumarins (esculetin and 7-hydroxycoumarin) (Chang, 1995 (a)), phenolic carboxylic acids (2,3,4 hydroxybenzoic acid and β-resorcylic acid) (Chang, 1995 (b)), caffeic acid and its analogues such as *m*-coumaric acid (Chang, 1995 (c)) and flavonoids such as quercetin, baicalein and hesperidin (Chang, 1993). Polyphenol (anthocyanidins) extracts from berries are capable of inhibiting XO and scavenge the superoxide radicals that have formed (Costantino, 1992). Polyphenols are the most potent of all the natural product inhibitors. Several polyphenols have K_i values that are similar to that of allopurinol. Many of the allopurinol analogues have been tested *in vivo* to determine their potency and selectivity and evaluate the possibilities of replacing allopurinol as drug of choice. No such studies have been conducted for the polyphenols (*in vivo*).

The different tea polyphenols are likely to inhibit XO *in vitro*. This needs confirmation before *in vivo* studies can be conducted. Most studies on the anti-

carcinogenic and anti-mutagenic properties of tea polyphenols were conducted in rat models. The inhibition of XO *in vivo* may reduce the amount of superoxide radicals, making it a significant mechanism whereby tea polyphenols may prevent tumorigenesis. A rat model was selected for the *in vivo* inhibition studies even though little data is available on the pharmacokinetic behavior and bioavailability of tea polyphenols. Evidence available suggested that this model would not be inappropriate for this type of study. In male Wistar rats that were orally fed, catechins were absorbed from the intestinal tract and were carried to the liver by the rat portal vein (Okushio, 1996). According to (Chen, 1997) the absorption and elimination of EGCg fits a one compartment model for the intragastrically (i.g.) route and a two compartment model for the intravenously route in male Sprague-Dawley rats. Glucuronide and glucuronide-sulfate conjugates of catechins are found in serum (Da Silva, 1998). This indicates that the catechins do reach the liver, which is the target organ for the inhibition of XO. EGCg reaches levels of 0.1-0.5 μg/ml in rat and 2-4 μg/ml in human plasma. CE will be used to quantify the amounts of xanthine and uric acid in the serum and urine of the rats. The analyte levels will be an indication of the amount of inhibition obtained with the polyphenols.

7 Aims of study

a) Develop a MEKC method that is suitable for the analysis of the important constituents in tea.

b) Determine whether tea polyphenols inhibit XO.

c) Identify an enzyme inhibition model and predict structure-activity relationships.

d) Identify a suitable method for analyzing rat urine and serum for XO substrates and products and implement it.

e) Determine the ability of the EGCg and a polyphenol extract as *in vivo* inhibitors of XO in a rat model and compare them with a positive control (allopurinol).

8 Hypotheses

a) MEKC will be a useful method for the separation and quantification of the quality contributing components in tea.

b) Tea polyphenols are a potential novel class of XO inhibitors.