2. Review of Literature

2.1. Plants as therapeutic agents

Plant-derived medicines are the most widely used medicines in the world today. The use of herbs and plants as first medication is practiced universally. Every culture on earth, through written or oral tradition, has relied on the vast variety of natural chemistry found in healing plants for their therapeutic properties (Serrentino 1991). Nearly 5.1 billion people worldwide employ natural plant-based remedies as their primary medicines for both acute and chronic health problems (Stockwell, 1988). Plants with therapeutic potential may be defined as any plant that can be put to culinary and/or medicinal use. Recent researches have found that food and their constituents act in a manner similar to modern drugs without dreaded side effects (Serrentino 1991). Sometimes plant medicine is viewed as a complementary medicine, working closely with allopathic drugs.

Most of the drugs have substances with a particular therapeutic property extracted from plants. Some of the medicines such as Taxol (anti-cancer drug) and Quinine (anti-malarial drug) are manufactured from Taxus brevifolia and Cinchona pubescens. Other medicinal agents such as pseudoephedrine originally derived from ephedra species and methylsalicylate, derived from Gaultheria procumbens are now chemically synthesized. Plant medicines remain indispensable to modern pharmacology and clinical practices. Much of the current drug discovery and development process are plant-based and new medicines derived from plants are inevitable.

2.2. Functional foods

A food can be regarded as a “functional food” if it demonstrates an ability to affect one or more target functions in the body beyond adequate nutrition and improves health or reduces the risk of diseases (Tsao and Akhtar, 2005). On this basis, a functional food can be a natural food, a food to which a positive component has been added, or a food from which a deleterious component has been removed or a food where nature of one or more components has been modified (Tsao and Akhtar, 2005). While searching for new sources of functional foods, attention has been paid to vegetables from Cruciferae family, which is often used in human diets. Recent investigations have shown that
Cruciferous vegetables contain appropriate amounts of bioactive compounds such as GLs, ITCs, tocopherols, L-ascorbic acid, vitamin B, reduced glutathione, inositol phosphates and polyphenolic compounds [Nakamura et al, 2001; Zielinski and Kozłowska, 2003; Zielinski et al, 2005; Takaya et al, 2003].

2.3. Cruciferous plants

The family Cruciferae (Brassicaceae) is an economically important family with about 350 genera and 3000 species that includes several edible plants. Despite great diversity among crucifers, members of only few genera are generally consumed. The most commonly consumed cruciferous vegetables belong to genus Brassica that includes broccoli, cabbage, cauliflower, kale and Brussels sprouts. Other cruciferous vegetables commonly used in human diet such as radish, water cress, wasabi, horseradish, garden cress, Italian cress, Swiss chard and crambe belong to another genera of family such as Raphanus, Nasturtium, Wasabia, Armoracia, Lepidium, Eruca, Beta and Crambe respectively.

Cruciferous vegetables are important dietary constituents in many parts of the world and account for about 10 – 15% of total vegetable intake, reaching as high as 25% in some countries (Bosetti et al, 2002; Chiu et al, 2003). However, regional pattern of crucifer consumption varies substantially in different parts of the world. The highest intake of cruciferous vegetable is reported to be in China with an average consumption of more than 100 g per day, representing about one-fourth of their total vegetable intake (Chiu et al, 2003). Other Asian countries such as Japan, Singapore and Thailand and Middle Eastern country such as Kuwait also have a relatively high intake of cruciferous vegetables, ranging from 40 – 80 g per day (Bosetti et al, 2002; Seow et al, 2002; Shannon et al, 2002; Memon et al, 2002). In North America, daily estimated consumption is in the range of 16 – 40 g per day (Lin et al, 1998) and in South America, it is about 3 – 15 g per day (Atalah et al, 2001). The daily intake of cruciferous vegetables is reported to be about 5 – 30 g per day in Europe (Bosetti et al, 2002), 50 g per day in Australia (Nagle et al, 2003) and 15 g per day in South Africa (Steyn et al, 2003) respectively. The only study carried out in India by Rajkumar et al, (2003) has shown an average consumption of only 17 g per day of cruciferous vegetables.
2.3.1. *Raphanus sativus*

*R. sativus* is believed to have originated in southern Asia and cultivated in Egypt. The first cultivated *R. sativus* was black variety and white and red *R. sativus* have been developed later. It was highly esteemed in ancient Greece, and the Greek physician Androcydes ordered his patients to eat *R. sativus* as a preservative against intoxication. The Japanese white *R. sativus*, also named daikon, is the vegetable for which literature reports the highest per capita consumption, quoted at 55 g per day in Japan (Talalay and Fahey, 2001). In addition to this, Japanese also consume *R. sativus* sprouts under the name of “Kaiware Daikon”.

2.3.2. Botanical classification of *R. sativus*

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th><em>Raphanus sativus</em> Linnaeus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Plantae</td>
</tr>
<tr>
<td>Subkingdom</td>
<td>Viridaeplantae</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Class</td>
<td>Dilleniidae</td>
</tr>
<tr>
<td>Order</td>
<td>Capparales</td>
</tr>
<tr>
<td>Family</td>
<td>Brassicaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Raphanus</em> Linnaeus</td>
</tr>
<tr>
<td>Species</td>
<td><em>Sativus</em></td>
</tr>
</tbody>
</table>

2.3.3. Varieties of *R. sativus*

There are six main varieties of *R. sativus* including White, Red Globe, White Globe, Black, White Icicles and California Mammoth White (Figure 2.1).

a) **White** (*R. sativus* Linn)

This variety is indigenous to Asia. They are large and carrot-shaped, have white flesh that is juicy and hotter than red radish, but milder than black variety.

b) **Red Globe** (*R. sativus* var. red)

This variety is most popular in the United States. They are small, round or oval shaped and referred to as "button" red radish and have solid crisp flesh.
c) **White Globe** (*R. sativus var. white*)

This variety is small and oval shaped and referred to as “hailstone” or “white button”. They have white flesh and milder than red variety.

d) **Black** (*R. sativus var. niger*)

This variety is thought to be native of Egypt and Asia. They are turnip-like in size and shape. They are quite pungent and drier than other varieties of radishes.

e) **White Icicles** (*R. sativus L var. thin*)

This variety is long and tapered. They have white flesh that is milder than red variety.

f) **California Mammoth White** (*R. sativus L var. large*)

This variety is larger than white icicle. They have oblong-shaped roots and their flesh is slightly pungent.

### 2.3.4. Nutritive value of *R. sativus*

*R. sativus* root and its leafy part provide an excellent source of vitamin C. Leafy part contains almost six times the vitamin C content of its root and also a good source of calcium and iron. *R. sativus* is also a good source of potassium and folic acid. It is very low in fats. Approximately, 100 g of raw vegetable provides roughly 20 Kcal, largely from carbohydrates (Table 2.1). Thus *R. sativus* is a dietary food that is relatively filling for its caloric value. Some sources list *R. sativus* as being rich in dietary fibers, whereas other sources differ in respect of its roughage content (USDA Nutrient Database, 1999; Duke and Ayensu, 1985).

#### Table 2.1

Nutrient values of root and leafy part of *R. sativus*

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Root (Per 100g FW)</th>
<th>Leafy part (Per 100g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>94.84 g</td>
<td>0 g</td>
</tr>
<tr>
<td>Energy</td>
<td>20 Kcal</td>
<td>287 Kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>0.6 g</td>
<td>28.7 g</td>
</tr>
<tr>
<td>Total lipid (fat)</td>
<td>0.54 g</td>
<td>5.2 g</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>3.59 g</td>
<td>49.6 g</td>
</tr>
<tr>
<td>Dietary Fiber</td>
<td>1.6 g</td>
<td>9.6 g</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
<td>-------</td>
</tr>
</tbody>
</table>

### Minerals

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Amount 1</th>
<th>Amount 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>21 mg</td>
<td>1913 mg</td>
</tr>
<tr>
<td>Potassium</td>
<td>232 mg</td>
<td>4348 mg</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>18 mg</td>
<td>261 mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>24 mg</td>
<td>956 mg</td>
</tr>
<tr>
<td>Iron</td>
<td>0.29 mg</td>
<td>35.7 mg</td>
</tr>
<tr>
<td>Magnesium</td>
<td>9 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.30 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.70 mg</td>
<td>0 mg</td>
</tr>
</tbody>
</table>

### Vitamins

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount 1</th>
<th>Amount 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>22.8 mg</td>
<td>704 mg</td>
</tr>
<tr>
<td>B-1 (thiamin)</td>
<td>0.005 mg</td>
<td>0.7 mg</td>
</tr>
<tr>
<td>B-2 (riboflavin)</td>
<td>0.045 mg</td>
<td>2.43 mg</td>
</tr>
<tr>
<td>B-3 (niacin)</td>
<td>0.3 mg</td>
<td>34.8 mg</td>
</tr>
<tr>
<td>B-5 (pantothenic acid)</td>
<td>0.088 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>B-6 (pyridoxine)</td>
<td>0.071 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>Folate</td>
<td>27 µg</td>
<td>0 mg</td>
</tr>
<tr>
<td>B-12</td>
<td>0</td>
<td>0 mg</td>
</tr>
<tr>
<td>Vitamin A Equivalent</td>
<td>8.0 IU</td>
<td>21 mg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.001 µg</td>
<td>0 mg</td>
</tr>
</tbody>
</table>

### 2.3.5. Health benefits of *R. sativus*

According to Hakeem Hashmi, an eminent Unani physician from India, *R. sativus* has unparalleled capacity to cure any kind of ailments. All the parts of *R. sativus* such as seed, root, stem and leaves are used in food and medicine. *R. sativus*, like other members of cruciferous family contains cancer preventive properties.

**a) Liver and gall bladder disorders**

Throughout history, *R. sativus* root and seeds have been effectively used as medicines for several liver disorders. They contain sulfur-based compounds such as GLs...
and ITCs that increase bile flow and help to maintain healthy gallbladder and liver (Chevallier, 1996). *R. sativus* is also an excellent remedy for gall bladder stone.

**b) Kidney disorders**

*R. sativus* root, seeds and leaves are diuretic in nature and increase urine output. Their diuretic properties help to flush out toxins accumulated in kidneys and protect them from infections and inflammatory conditions. It is an old belief that *R. sativus* can help in treatment as well as prevention of kidney stones (Chopra et al, 1986).

**c) Respiratory disorders**

*R. sativus* is an anti-congestive agent and alleviates congestion of respiratory system. It has found to be beneficial in problems associated with bronchitis (Bown, 1995) and asthma (Duke and Ayensu, 1985).

**d) Skin disorders**

*R. sativus* helps to cure skin disorders such as leucoderma, rashes, cracks, etc and also refreshes skin by maintaining its moisture content (Duke and Ayensu, 1985).

**e) Digestive disorders**

*R. sativus* root, seeds and leaves are rich in roughages (dietary fibres) which facilitate digestion, retain water and relieve constipation (Chopra et al, 1986). They also soothe digestive system and stimulate appetite (Chevallier, 1996).

**f) Nervous and vascular disorders**

*R. sativus* decreases nervous tension and enhances blood circulation. It is a remedy for insomnia, hypochondria and irritative condition of the central nervous system (Panda, 1999).

**g) Other benefits**

*R. sativus* is germicidal and suppresses phlegm. It is a good appetizer, mouth freshener, laxative, remedy for headache, acidity, piles, nausea, obesity, sore throat, whooping cough, dyspepsia, etc (Nadkarni, 1976; Kapoor, 1990).

**2.3.6. Chemical constituents of R. sativus**

Glucosinolate (GL) is an important and unique class of secondary plant metabolite found in seeds, roots and leaves of *R. sativus* (Daxenbichler et al, 1991; Blazevic and Mastelic, 2009). GLs include several naturally occurring thioglucosides with a common structure (Figure 2.2) characterized by side chains (R) with varying
aliphatic, aromatic and heteroaromatic carbon skeletons, all presumably derived from amino acids by a chain-lengthening process and hydroxylation or oxidation (Larsen, 1981).

In an intact cell, GLs are separated from thioglucosidase (EC 3.2.3.11), an enzyme generally known as myrosinase. When plant cell structure is damaged, myrosinase catalyzes the hydrolysis of GLs to yield D-glucose, sulfate and a series of compounds such as isothiocyanates, thiocyanates and nitriles, which depends both on substrate and pH of the reaction (Figure 2.2). GLs are also hydrolyzed by thioglucosidase activity of the intestinal microflora (Jeffery and Jarrell, 2001).

4-(methylthio)-3-butenyl glucosinolate (glucoraphasatin), 4-(methylsulfinyl)butyl glucosinolate (glucoraphanin) and 4- (methylsulfinyl)-3-butenyl glucosinolate (glucoraphenin) are the most predominant GLs in root and seeds of *R. sativus* (Daxenbichler *et al*, 1991; Carlson *et al*, 1985). These GLs on hydrolysis by myrosinase yield MTBITC, sulforaphane and sulforaphene respectively. GLs are not uniformly distributed; highest in the distal end of root, decreasing in upper root section with lowest level in vegetative tops (Esaki and Onozaki, 1980).

Apart from GLs and their hydrolyzed products, *R. sativus* also contains polyphenolics such as phenolic acid, flavonoids and anthocyanins. Several polyphenolic compounds including sinapic acid esters and kaempferol are isolated from *R. sativus* sprouts (Takaya *et al*, 2003). Twelve acylated anthocyanins (pelargonidin) are isolated from *R. sativus* red variety (Otsuki *et al*, 2002). Phytochemical screening has shown the presence of other phytochemicals such as triterpenes, alkaloids, saponins and coumarins in *R. sativus* seeds (Mohamed *et al*, 2008).

Novel classes of plant defensins (small basic cysteine rich peptides) such as *Raphanus sativus* antifungal peptide 1 and 2 (RsAFP1 and RsAFP2) are isolated from seeds of *R. sativus* (Terras *et al*, 1992a). RsAFP1 and RsAFP2 are highly basic oligomeric proteins composed of small (5 KDa) polypeptides that are rich in cysteine. Both RsAFP1 and RsAFP2 show broad spectrum antifungal activity with high degree of specificity to filamentous fungi (Terras *et al*, 1992b). They are active against both phytopathogenic fungi such as *Fusarium culmorum* and *Botrytis cinerea* (Terras *et al*, 1992b), human pathogenic fungi such as *Candida albicans* (Aerts *et al*, 2007) and occasionally possess antibacterial activity. However, they are non-toxic to humans and plant cells. *R. sativus*
25 storage albumins are identified as second novel class of antifungal protein (Terras et al, 1992a). They inhibit the growth of different plant pathogenic fungi and certain bacteria (Terras et al, 1992a).

Figure 2.2
The myrosinase - catalyzed hydrolysis of glucosinolates. (Adapted from Rask et al, 2000)

R = CH$_3$-SO-(CH$_2$)$_4$  Methylsulfinylbutyl (glucoraphanin)
R = CH$_3$-SO-CH=CH-(CH$_2$)$_2$  4-Methylsulfinyl-3-butenyl (glucoraphenin)
R = CH$_3$-S-CH=CH-(CH$_2$)$_2$  4-Methylthio-3-butenyl (glucoraphasatin)
At least eight distinguishable isoperoxidases are isolated and purified to apparent homogeneity from Korean \textit{R sativus} roots. Among them are two cationic isoperoxidases such as C1 and C3 and four anionic isoperoxidases such as A1, A2, A3n and A3 (Lee and Kim, 1994). Plant peroxidases play an important role in several physiological functions such as removal of peroxides, oxidation of toxic reductants, wound healing and cell wall biosynthesis (Hammerschmidt \textit{et al}, 1982). Further, peroxidase represents an important component of an early response to pathogen attack in plants and plays a key role in the biosynthesis of lignin, which limits the extent of pathogen spread (Bruce and West, 1989). Products of this enzyme have antimicrobial and antiviral activity in the presence of a hydrogen donor and hydrogen peroxide (Van Loon and Callow, 1983). Recently, a novel heme peroxidase essentially resistant to H$_2$O$_2$ has been isolated from \textit{R. sativus}, which has shown comparatively stronger oxidative stability than that of reference horse radish peroxidase (Rodríguez \textit{et al}, 2008).

2.4. Biological activities of \textit{R. sativus}

Evidence from numerous investigations revealed that the biological and pharmacological functions of \textit{R. sativus} are mainly due to its GLs and its hydrolyzed products such as ITCs (Esaki and Onozaki, 1982; Nakamura \textit{et al} 2001; Barillari \textit{et al}, 2006; Papi \textit{et al}, 2008). These compounds provide \textit{R. sativus} its characteristic odor and flavor as well as most of its biological properties. GLs and ITCs have long been known for their fungicidal, bactericidal, nematocidal and allelopathic properties (Brown \textit{et al}, 1991) and have attracted intense research interest because of their cancer preventive attributes (Fahey \textit{et al}, 2001; Verhoeven \textit{et al}, 1997). Polyphenolics, alkaloids, saponins, isoperoxidases and antifungal peptides are also accountable for the health benefits of \textit{R. sativus}, which perhaps would work synergistically with GLs and ITCs of \textit{R. sativus}. These constituents are reported to exhibit several biological activities such as radical scavenging activity (Takaya \textit{et al}, 2003), gut stimulatory, uterotonic and spasmogenic effect (Gilani and Ghayur, 2004; Ghayur and Gilani, 2005), anti-hyperlipidemic activity (Wang \textit{et al}, 2002) and anti-atherogenic effect (Suh \textit{et al}, 2006).
2.4.1. Antioxidant activity

Damage to proteins, lipids and DNA by reactive oxygen species (ROS) and reactive nitrogen species (RNS) can lead to a variety of chronic diseases such as cancer, cardiovascular, inflammatory and age-related neurodegenerative diseases (Borek, 1997; Richardson, 1993). ROS/RNS can damage cell membranes, disrupt enzymes, reduce immunity (Ahsan et al, 2003) and induce mutation (Loft and Poulsen, 1996). ROS/RNS are by-products of normal aerobic metabolism and occur during mitochondrial/microsomal electron transport chain, phagocytic activity or generated from oxidase enzymes and transition metal ions (Nohl et al, 2003; Aruoma et al, 1989). Other sources of ROS/RNS are environmental factors such as pollution, exposure to UV rays of sun, cigarette smoke and even some kinds of food (Schroder and Krutmann, 2004). Oxidative damage induced by these reactive species is usually counteracted by antioxidant defense mechanism (Bagchi and Puri, 1998). Recent studies have shown evidence that plant-based diets, particularly those rich in fruits and vegetables provide a considerable amount of antioxidant phytochemicals which offer protection against cellular damage (Dimitrios, 2006).

2.4.1.1. Vitamins

Ascorbic acid is considered as the most efficient antioxidant in inhibiting lipid peroxidation among several types of antioxidants including α-tocopherol (Fei et al, 1989). Ascorbic acid is also capable of scavenging hydrogen peroxide, singlet oxygen, superoxide and hydroxyl radicals efficiently (Fei et al, 1989). It is also involved in regeneration and recycling of tocopherols and β-carotene (Niki et al, 1995). Numerous studies have shown that ascorbic acid is effective in lowering the risk of developing cancers (Block, 1991) and cardiovascular diseases (Trout, 1991). In spite of overwhelming evidence on its health benefits, there are reports that demonstrated pro-oxidant activity of ascorbic acid (Podmore, 1998). Tocopherols are essential vitamins with a major role as antioxidants in protecting polyunsaturated fatty acids (PUFAs) and other components of cell membranes and low-density lipoprotein (LDL) from oxidation, thereby preventing the onset of heart diseases (Rimm et al, 1993).
2.4.1.2. Polyphenolics

Polyphenolics is an extremely comprehensive word that covers different subgroups of phenols and phenolic acids. These compounds are most commonly present in fruits and vegetables. They are essential for plant physiology, being involved in diverse functions such as lignification, pigmentation, pollination, allelopathy, pathogen resistance and growth (Haslam, 1996). Polyphenolics comprise single-ring structure such as hydroxybenzoic acids and hydroxycinnamic acids and multi-ring structure such as flavonoids, which can be further classified into anthocyanins, flavan-3-ols, flavones, flavanones and flavonols. Some of the flavonoids such as flavan-3-ols can be found in their dimeric, trimeric and polymeric forms. Most of these polyphenolics are associated or conjugated with sugar moieties that further complicate the polyphenolic profile of fruits and vegetables. Polyphenolics are particularly important as antioxidants, because they have high redox potential, which permit them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelator (Kahkonen et al, 1999) and alleviate free-radical mediated cellular injury (Shahidi and Wanasundara, 1992).

The antioxidant ability of individual polyphenolics may differ, but, as a group, they are one of the strongest groups of antioxidants. The antioxidant activity of a polyphenolic compound is chiefly determined by its structure, in particular electron delocalization over an aromatic nucleus (Tsao and Akhtar, 2005). When a polyphenolic compound reacts with a free radical, delocalization of gained electron over phenolic group and stabilization of the aromatic nucleus by resonance effect take place that prevent continuation of free radical-mediated chain reaction (Tsao and Akhtar, 2005).

2.4.1.3. Sulfur-containing compounds

GLs are a group of sulfur-containing compounds found in cruciferous plants such as *R. sativus*, broccoli, cabbage, mustard, wasabi etc. GLs and their hydrolysis products such as ITCs are found to be strong antioxidants by virtue of their ability to induce detoxification enzyme system (Zhang and Talalay, 1998). This property of GLs and ITCs is considered as one of the major contributors to its anti-cancer activity (Zhang and Talalay, 1998).
2.4.1.4. Antioxidant activity of *R. sativus*

*R. sativus* is one of the major sources of dietary phenolic acids and flavonoids, which are mostly present as sugar conjugates (Takaya *et al.*, 2003). The major phenolic acids found in *R. sativus* sprout are sinapic acid and ferulic acid, which are present in conjugated form as 1-sinapoyl-1-β-D-glucopyranoside, β-D-(3-sinapoyl) fructofuranosyl -α-D-(6-sinapoyl) glucopyranoside and 1-feruloyl-β-D-glucopyranoside (Takaya *et al.*, 2003). The main flavonoid present in *R. sativus* sprouts is kaempferol that occurs in a conjugated form as kaempferol-3,7-O- α-L-dirhamnopyranoside and kaempferol-3-O- α-L-rhamnopyranosyl-(1-4)- β-D-glucopyranoside respectively (Takaya *et al.*, 2003).

Lugasi *et al.* (1998) demonstrated the strong antioxidant property of squeezed juice extracted from black *R. sativus* root, through its ability to donate electrons, chelate metal ions and scavenge free radicals in a $\text{H}_2\text{O}_2$/OH-luminol system. Since HPLC analysis revealed the presence of considerable amount of GLs degradation products and polyphenols in the squeezed juice, antioxidant activity of black *R. sativus* root could be attributed to these compounds.

Takaya *et al.* (2003) tested methanolic extracts from 11 different plants including Daikon *R. sativus* sprouts for their ability to scavenge free radicals. Daikon *R. sativus* sprouts proved to be the most potent, almost 1.8 times more effective than Vitamin C.

Souri *et al.* (2004) studied antioxidant activity of 26 commonly used vegetables of Iranian diets and found that methanolic extract of *R. sativus* leaves significantly inhibited the peroxidation of linoleic acid as compared to standard antioxidants such as α-tocopherol and quercetin.

Katsuzaki *et al.* (2004) found that hot water extract of Daikon *R. sativus* extract showed more significant antioxidant activity than the extract obtained at an ambient temperature. L-tryptophan was isolated and identified as the compound responsible for the antioxidant activity. They also found that L-tryptophan converted to 5-hydroxy tryptophan (5-HTP), a precursor to serotonin in rat liver microsome model system. A plant-based 5-HTP supplement is popular for its anti-depressant, appetite suppressant and sleep aiding properties.

Lugasi *et al.* (2005) further demonstrated that squeezed juice from black *R. sativus* significantly alleviated the free-radical mediated reaction in hyperlipidaemic rats by decreasing lipid peroxidation and improving their antioxidant status. Recent study also
showed that *R. sativus* extract reduced the extent of lipid peroxidation in a dose dependent manner in rat liver homogenate treated with cumene hydroperoxide by increasing reduced glutathione level and thereby protecting liver from the toxin-induced oxidative damage (Chaturvedi, 2008).

Salah-Abbes *et al* (2008a) showed the protective effect of Tunisian *R. sativus* root extract against toxicity induced by zearalenone in mice by virtue of its ability to alleviate oxidative stress through stimulation and improvement of their antioxidant status.

Polyphenolics in *R. sativus* may act in a synergistic or additive manner with GLs and/or ITCs and exert their antioxidant activity through inhibition of lipid peroxidation and induction of cellular antioxidant enzymes. Apart from these phytochemicals, *R. sativus* also contains several classes of peroxidases that could play a significant role in the elimination of toxic peroxides and thus reduce the impact of free-radical mediated cellular injury (Wang *et al*, 2002).

### 2.4.2. Antimicrobial activity

Infectious diseases are the world’s leading cause of untimely death, killing approximately 50,000 people every year. Bacteria have remarkable ability to develop resistance to most of the pharmaceutical antibiotics. An increase in such antibiotic-resistant bacteria are menacing human population with recurrence of infectious diseases that were once thought to be under control, at least in developed countries (Pinner *et al*, 1996). These antibiotic-resistant bacteria have also caused unique problems in treating infections in patients with cancer and AIDS (Dennesen *et al*, 1998). Since tenacious and virulent bacteria develop immunity to solitary antibiotics at an alarming speed, there is an imperative need for a holistic targeted approach to search for novel antimicrobials from natural sources, especially from plant kingdom.

Long before mankind ascertained the existence of microbes, the fact that certain plants have therapeutic potential is very well accepted. Since ancient time, man has used plants as widespread remedial tool to treat common infectious diseases. Some of these traditional medicines are still included as part of the habitual treatment of various maladies. Bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) are employed to treat urinary tract infections, while species such as lemon balm (*Melissa*
*officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described to possess broad-spectrum antimicrobial activity (Heinrich *et al*, 2004).

Plant-based antimicrobials represent a vast unexploited source for medicines, which need to be explored further. They have an immense therapeutic potential as they are effective in the treatment of infectious diseases while concomitantly alleviating side effects that are frequently associated with synthetic antimicrobials (Cowan, 1999). Generally, plant based anti-infective agents have manifold effect on the body and often act beyond symptomatic treatment of infectious diseases. Plants have virtually unlimited capacity to produce secondary metabolites, especially for their defense against predation by microorganisms, insects and herbivores. Antimicrobial phytochemicals are divided into several categories based on their structural similarity as follows:

### 2.4.2.1. Phenolic acids

Phenolic acids are the simplest bioactive phytochemicals consisting of a single substituted phenolic ring. Cinnamic acid and caffeic acids are the common representatives of this group. Phenolic acids are reported to be effective against viruses (Wild, 1994), bacteria (Brantner *et al*, 1996) and fungi (Duke, 1985). The number and site of hydroxyl group on their phenolic ring are believed to be connected to their relative toxicity to microorganisms. Phenolic acids, which are in higher oxidized state are more inhibitory to microorganisms than the one with lower oxidation state (Scalbert, 1991). Mechanisms appeared to be responsible for the antimicrobial activity of phenolic acids could include enzyme inhibition through interaction with –SH groups or non-specific interaction with microbial proteins (Mason and Wasserman, 1987).

### 2.4.2.2. Quinones

Quinones are aromatic compounds with two ketone substitutions in their phenolic ring. They are ubiquitous in nature and show broad-spectrum antimicrobial properties (Duke, 1997). They are exceptionally active, as they can switch between hydroquinone and quinone through oxidation/reduction reactions. Quinones bind with proteins irreversibly and lead to inactivation and loss of function of proteins (Stern *et al*, 1996). They may also make substrates inaccessible to microbes.
2.4.2.3. Flavonoids

Flavonoids are phenolic structures containing hydroxyl groups. They are ubiquitous and are commonly found in fruits, vegetables, nuts, tea, wine, honey, etc. They are effective antimicrobial agents against a wide variety of microorganisms (Cushnie and Lamb, 2005). Catechins are the most extensively studied flavonoids for their possible antimicrobial activity due to their occurrence in green tea (Toda et al, 1989). Flavonoids have the ability to complex with extracellular proteins as well as with bacterial cell walls, rendering them inactive (Cushnie and Lamb, 2005). More lipophilic flavonoids may also have the ability to disrupt microbial membrane (Tsuchiya et al, 1996).

2.4.2.4. Terpenoids and essential oils

Essential oils are secondary metabolites that are highly supplemented in compounds based on isoprene structures (Cowan, 1999). They are called as terpenes and usually occur as di, tri, tetra, hemi and sesquiterpenes. When such compounds contain extra elements such as oxygen, they are called as terpenoids. Camphor, farnesol, artemisinin and capsaicin are common examples of terpenoids. Terpenes and terpenoids are active against an array of bacteria (Habtemariam et al, 1993) and fungi (Rana et al, 1997). Previous research showed that terpenoids present in the essential oils of plants could be useful in the control of Listeria monocytogenes (Aureli et al, 1992). The mechanism action of terpenes is not yet established precisely, but is speculated to be due to disruption of bacterial cell membrane by lipophilic terpenoids (Mendoza et al, 1997).

2.4.2.5. Alkaloids

Alkaloids constitute a large group of compounds containing a nitrogen atom in their heterocyclic ring, with broad range of biological activities. The first medically functional alkaloid is morphine isolated from Papaver somniferum (Fessenden and Fessenden, 1982). Alkaloids are generally found to have potent antimicrobial activity (Ghoshal et al, 1996). Solamargine, a glycoalkaloid from the berries of Solanum khasianum reported to be useful against HIV infection and intestinal infections associated with AIDS (McMahon et al, 1995). Berberine is an important and frequently studied member of the alkaloid group. It is potentially efficient against trypanosomes (Freiburghaus et al,
1996) and plasmodial infections (Wright et al, 1992). The mode of action responsible for antimicrobial activity of alkaloids may be attributed to their ability to intercalate with DNA and arresting metabolic activity of bacterial cells (Phillipson and O'Neill, 1987).

2.4.2.6. Sulfur-containing compounds

Sulfur-containing compounds encompass a wide range of compounds and usually found in plants as glucosides (glucosinolates, alliin, etc). These glucosides, during the rupturing of plant cell wall, are hydrolyzed into volatile sulfur compounds such as ITCs, allicin, allyl sulfide, diallyl disulfate, etc. Biological activity of sulfur-containing compounds is considered to be chiefly due to their degradation products, as intact glucosides usually display much less biological activities than their subsequent hydrolysis products (Donkin et al, 1995).

The mechanism of action responsible for antimicrobial activity of sulfur-containing compounds varies. Antimicrobial activity of ITC is thought to be related to its –NCS group, in which central carbon atom is highly electrophilic, which could interact irreversibly with nucleophilic targets of a microbial cell wall. Allicin does not initiate leakage of cellular content and fusion or aggregation of membrane, but can permeate into cells through phospholipid bilayer and interact with –SH groups of enzymes and proteins thereby modifying their activities (Rabinkov et al, 1998).

2.4.2.7. Antimicrobial activity of cruciferous vegetables

Antimicrobial activity of cruciferous vegetables was first reported in early 1900s. However, interest in their use as food preservatives has developed only during the past decade (Delaquis and Sholberg, 1997). Inhibition of microbial growth by cruciferous vegetables is linked to biologically-active ITCs. Particular attention has been centered on AITC, which contributes approximately to 90% of volatile essential oil isolated from Amoracia lapathifolia and constantly shown antimicrobial activity in both liquid and vapor phases (Shofran et al, 1998; Ward et al, 1998). The degree of inhibition of AITC seems to be species-specific and concentration-dependent. Interestingly, AITC significantly inhibited the growth of pathogenic bacteria without affecting the growth of lactic acid bacteria which themselves inhibit unwanted growth of bacteria, in the guts (Ward et al, 1998). When AITC has been investigated for its bactericidal activity, it is
found to be effective at all growth phases of bacteria (Lin et al., 2000). Thus, the ability of AITC to inhibit bacteria, which are in the stationary phase, is pertinent to its application in food preservation, as processing and temperature conditions normally diminish the metabolic activity of bacteria in food system.

6-methylsulfinyl hexyl ITC, a volatile compound extracted from *Wasabi japonica* is another ITC with considerable antibacterial activity, especially against *Escherichia coli* and *Staphylococcus aureus* (Ono et al., 1998). Similarly, sulphoraphane, an ITC from broccoli displayed a significant bactericidal effect against intracellular *Helicobacter pylori* in human epithelial cell line (Haristoy et al., 2005).

Recently, Shin et al. (2004) studied the bactericidal activity of Korean and Japanese wasabi roots, stem and leaves against *Helicobacter pylori* and found that leaves exhibited more potent bactericidal activity than roots, even though AITC level of the leaves was lesser than that of the roots. These results suggest that certain components other than AITC in wasabi are effective in inhibiting the growth of *Helicobacter pylori*.

### 2.4.2.8. Antimicrobial activity of *R. sativus*

There have been very few studies on the antibacterial activity of *R. sativus*. Abdou et al. (1972) described antibacterial activity of an aqueous extract of *R. sativus* root against *Escherichia coli*, *Pseudomonas pyocyaneus*, *Salmonella typhi* and *Bacillus subtilis*. However, *R. sativus*, when extracted with isopropyl alcohol and ethanol inhibited only *E. coli* and showed no inhibitory activity towards the remaining bacteria. Similarly, when extracted with ether, petroleum ether and chloroform, *R. sativus* failed to reduce the growth of any of the bacteria studied.

Esaki and Onozaki (1982) identified the pungent principle of *R. sativus* root (MTBITC) as antimicrobial to *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Aspergillus oryzae*. Additional research revealed that 2-thioxo-3-pyrrolidin-carboxaldehyde, produced by the degradation of MTBITC could be the actual component responsible for the antimicrobial activity of *R. sativus*. Khan et al. (1985) reported the antibacterial activity of roots, flowers and pods against bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*.

Recently, Rani et al. (2008) demonstrated the antimicrobial activity of crude water extract, supernatant and methanolic extract of *R. sativus* seeds against a variety of
bacteria and fungi. All extracts exhibited significant antibacterial activity against *Hafnia alvei*, *Enterobacter agglomerans*, *Lactobacillus* and *Bacillus thuringiensis*, whereas fungal species such as *Penicillium lilacinum*, *Paecilomyces variotii*, *Spadicoides stoveri*, *Penicillium funiculosum* displayed variable degree of inhibition.

### 2.4.3. Chemoprevention of cancer

Cancer, presently the second leading cause of death may outrank cardiovascular disease both in the developed and developing countries in a few decades (Oliveria *et al.*, 1997). Cancer is a dynamic process that entails many intricate factors (Nowell, 1986). Hanahan and Weinberg, (2000) implied that cancer is a sign of significant alterations in cellular physiology, which result in unrepressed malignant growth. Carcinogenesis can be regarded as a gradual accumulation of genetic and biochemical changes, which occur through three stages (initiation, promotion and progression) that represent progressive transformation of normal cells into highly aggressive malignant cells (Pitot and Dragan, 1994). Initiation is defined as a mutagenic event resulting from exposure and interaction of carcinogens with cellular constituents, especially DNA. Promotion is characterized by persistence and replication of a clone of abnormal cells that ultimately grow into a definable focus of preneoplastic cells. Progression is considered as a final stage of cancer development that alters the preneoplastic cells into an invasive and metastatic cell population.

The recent development in understanding the carcinogenic process at the cellular and molecular level has enhanced the probability that cancer prevention, either primary or secondary will rely increasingly on intervention collectively termed “chemoprevention”. Chemoprevention is described as a pharmacological approach that intends to arrest or reverse the development and progression of precancerous cells through the use of nutrients and/or pharmacological agents during the stage between tumor initiation and progression (Kelloff *et al.*, 1994). Since carcinogenesis is a multistage process often having a latency of many years or decades, there are considerable opportunities for intervention with innovative approaches and potential to interrupt this process at different steps during initiation, promotion or progression (Kelloff *et al.*, 1996; Greenwald, 2001). Molecular advances have led to the detection of genetic lesions and cellular components that may be involved in the initiation and progression of
malignancies and thus constitute probable targets for chemoprevention. The emerging field of cancer prevention by chemopreventive agents offers substantial potential for reducing the incidence and mortality of cancer. Because of its promising effects, chemoprevention has been largely identified as a powerful treatment strategy by clinicians. Chemopreventive agents may exert their effect either by blocking or metabolizing carcinogens or by inhibiting the growth of cancerous cells. The most significant beneficial effects of chemopreventive agents are their non-toxic nature and negligible chemoresistance. Further, they display selective activity by initiating apoptotic pathways to impede cancer in cancer cells and at the same time induce detoxifying enzymes, which render them non-toxic to normal cells.

2.4.3.1. Phytochemicals as chemopreventive agents

Majority of human cancers are induced by environmental factors existing in the milieu. It has been projected that more than two-third of human cancers could be averted by lifestyle modifications including dietary changes (Surh, 2003). Epidemiological studies have strongly indicated that certain daily-consumed dietary phytochemicals could have cancer preventive effects against several forms of human cancers (Block et al, 1992). Diet has been regarded as a potential source of chemopreventive agents because of its likely protection following long-term administration to humans. Indeed, a number of natural compounds with inhibitory effect on cancer formation have been identified from diet or source of diet.

Numerous in vitro and in vivo studies evaluated the chemopreventive effect of phytochemicals and elucidated their mode of cancer prevention. These studies have eventually resulted in the discovery of several classes of phytochemicals with cancer preventive property such as ITCs from cruciferous vegetables, catechins from green tea, resveratrol from grape seeds and red wine, curcuminoids from turmeric, procyanidins from fruits and nuts, isoflavones from soyabean and antioxidant vitamins in various foods (Surh, 2003; Greenwald, 1996; Block, 1991). Multiple cellular and molecular mechanisms appear to be accountable for the overall chemopreventive effects of these dietary phytochemicals. These include induction of cellular defense system; inhibition of cell proliferation and cell cycle progression; induction of differentiation and apoptosis; modulation of expression of genes related to apoptotic pathways and inhibition of
angiogenesis and metastasis by modulating cellular signaling pathways (Surh, 1999; Chen and Kong, 2005). These signal transduction pathways are now recognized as the potential molecular targets for chemoprevention by dietary phytochemicals (Hu and Kong, 2004).

2.4.3.2. Chemopreventive effect of cruciferous vegetables

Vegetables of Brassicaceae family, particularly those belonging to Brassica genus (broccoli, cabbage, cauliflower, radish, mustard, etc) have received much attention because they are reported to possess significant anticancer activity (Zhang and Talalay, 1994; Verhoeven et al, 1997). GLs and their hydrolysis products (ITCs) are reported to be responsible for the chemopreventive activity of cruciferous vegetables. A modulation of detoxification enzymes (inhibition of phase I enzymes and induction of phase II enzymes) has been proposed as the probable mechanism pertinent to the chemopreventive properties of ITCs (Thornalley, 2002; Zhang, 2004). These compounds have also been shown to possess anti-proliferative and apoptosis inducing activities in several cancer cell lines (Wu et al, 2005). Apart from GLs and ITCs, cruciferous vegetables contain other potentially protective constituents such as phenolic acids, flavonoids, alkaloids, vitamins, fibers and pigments whose chemopreventive effects have been reported by numerous investigations (Yang et al, 1997; Bhatt and Pezzuto, 2002; Ferguson and Harris, 1996; Albanes et al, 1995).

Earlier studies demonstrated the inhibitory effects of naturally occurring ITCs on chemically induced carcinogenesis in a variety of experimental animal models (Verhoeven et al, 1997; Hecht, 1999; Conaway et al, 2002). In line with these findings, several case-controlled epidemiological studies observed an inverse relationship between high intake of cruciferous vegetables and risk of many types of cancer (Lin et al, 1998; London et al, 2000; Spitz et al, 2000; Zhao et al, 2001; Kristal and Lampe, 2002).

Sulforaphane (SUL) is one of the most studied ITCs of cruciferous vegetables. Numerous investigations demonstrated the chemopreventive potential of sulforaphane (Zhang et al, 1992b; Zhang et al, 1994; Chung et al, 2000; Wu et al, 2005; Agudo et al, 2004; Singh AV et al, 2004; Singh SV et al, 2004; Choi and Singh, 2005). Besides sulforaphane, phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), 4-(methylthio) butyl
isothiocyanate (MTBuITC), 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) and indole-3-carbinol are other classes of ITCs with promising chemopreventive activity.

2.4.3.3. Molecular and cellular targets of ITCs

It has been ascertained that ITCs can inhibit tumor formation through multiple mechanisms such as, (i) shielding DNA from oxidative damage by modulating detoxifying enzymes; (ii) alleviating oxidative stress by inducing and maintaining cellular antioxidants; (iii) suppressing cell proliferation by blocking cell cycle progression and/or initiating apoptotic pathway, thus impeding or eliminating clonal expression of initiated, transformed and/or neoplastic cells.

2.4.3.3.1. Modulation of phase I and phase II enzymes

Most of the dietary and environmental carcinogens metabolize through an oxidative pathway called Phase I pathway, which converts procarcinogens into highly reactive intermediates. This physiological event is catalyzed by an enzyme called cytochrome P450 (CYP). CYPs occur in many isoforms and play significant role in the activation of carcinogens to electrophiles, which bind to DNA and generate DNA adduct. The DNA adduct formation is considered to be a crucial step for cancer initiation by carcinogens. Inhibition of CYPs involved in the generation of DNA adducts often results in the suppression of cancer formation.

Several lines of evidence show that ITCs may inhibit DNA adduct formation and chemical tumorigenesis through modification of the level of certain CYP isoforms in rodent models by way of competitive or mechanism-based irreversible inhibition (Yang et al, 1994; Zhang et al, 1994; Nakajima et al, 2001) or through downregulation of CYPs in target tissues (Hecht, 2000; Conaway et al, 2002). However, substantial variations occur in the inhibition of different CYPs by ITCs.

Nakajima et al (2001) showed that PEITC selectively inhibited and inactivated human CYPs expressed from baculovirus-infected insect cells. PEITC strongly inhibited CYP 1A2 and to a lesser extent, CYP 2A6 by way of competitive inhibition. CYP 2B6 and CYP 2C9 were inhibited through non-competitive inhibition and CYP 2E1 via mechanism based irreversible inhibition by PEITC (Nakajima et al, 2001). Unlike PEITC, BITC has inhibited and inactivated CYPs both from rat and human mostly by a
mechanism based irreversible inhibition, which occurred chiefly through protein modification (Goosen et al, 2000). However, efficiency of inactivating CYPs by SUL and AITC appears to be less prominent when compared with the ability of PEITC and BITC in inhibiting specific CYPs (Conaway et al, 1996).

On the other hand, inhibitory effect of ITCs on the activation of carcinogen may be carcinogen-specific. BITC has been shown to inhibit 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rat (Wattenberg, 1977) and benzo[a]pyrene (BaP)-induced pulmonary neoplasia in A/J mice (Wattenberg, 1987). However, it has showed no effect on mouse lung carcinoma initiated by tobacco-specific carcinogen, 4-(methylnitrosamo)ino-1-(3-pyridyl)-1-butanone (NNK) or by N-nitrosodiethylamine (DEN) (Morse et al, 1990), as well as on esophageal tumor induced by N-nitrosomethylbenzylamine (NMBA) in rats (Wilkinson et al, 1995).

On the contrary, PEITC has a broad inhibitory activity against neoplasia induced by N-nitrosamines that has been shown by multiple studies both in mice and rats (Morse et al, 1989; Hecht et al, 1996; Hecht et al, 1999). However, PEITC has showed an insignificant inhibitory effect on BaP-induced lung DNA adducts formation and lung carcinogenesis in A/J mice (Adam-Rodwell et al, 1993).

SUL significantly inhibited mammary tumor induced by DMBA in rats, but was ineffective as an inhibitor of BaP-induced lung tumor in mice (Zhang et al, 1994). Similarly, SUL had no effect on NNK-induced lung neoplasia in mice (Chung et al, 1997).

In contrast to diverse results on phase I enzymes; many ITCs are strong inducers of phase II enzymes. These enzymes represent an essential part of cellular defense against carcinogens and reactive oxidants through elimination of highly reactive intermediate as water soluble products in bile or urine. Several studies identified ITCs as major inducers of phase II carcinogen-detoxifying enzymes such as quinone reductase-1 (QR-1) (Brooks et al, 2001; Kirlin et al, 1999), glutathione-s-transferase (GST) (Brooks et al, 2001), UDP-glucuronosyl transferase (UGT) (Petri et al, 2003) and γ-glutamylcysteine synthetase (GCS) (Bonnesen et al, 2001; Sharf et al, 2003).

Induction of carcinogen-detoxifying enzymes by ITCs appears to occur at the transcriptional level and may be mediated through antioxidant-responsive element (ARE). Nuclear factor E2-related factor 2 (Nrf2), a member of Cap ‘n’ Collar (CNC) family of leucine zipper (bZIP) transcriptional factors binds to ARE and mediates the
transcriptional activation of an array of detoxification and antioxidant enzymes (Itoh et al., 1997).

A number of upstream signaling pathways leading to the transcriptional activation of Nrf2 and ARE are illustrated in Figure 2.3. The precise mechanisms through which ITCs elicit Nrf2 transactivation are rather unclear. Nrf2 is normally sequestered in the cytoplasm as an inactive complex by Keap1 protein, which is anchored to cytoskeleton proteins (Itoh et al., 1999). Upon stimulation by ARE-mediated inducers including ITCs, Nrf2 is dissociated from Keap1 protein, apparently through cleavage of disulfide bond between Nrf2 and Keap1 (Dinkova-Kostova et al., 2002). Nrf2 then translocates to the nucleus and binds to ARE through a heterodimeric combination with other factors such as Maf proteins and activates expression of detoxifying enzymes. ITCs may also regulate Nrf2-mediated gene transcription by different mechanisms. There is also a report indicating that ITCs possess pro-oxidant properties and could generate oxidative stress by themselves in cells (Zhang, 2004). However, the amount of oxidative stress produced by ITCs seems to be dose-dependent, mild at low concentrations with signal strength ample enough to trigger the cellular defense system leading to synchronized activation of Nrf2 signaling pathway (Nakamura et al., 2000). This oxidative stress generated by low dose of ITCs is supposed to be at a sub-toxic level that would not cause any adverse effects such as DNA damage, mutation or tissue degeneration as usually caused by carcinogens.

2.4.3.3.2. Inhibition of cell proliferation

Until recently, most of the studies have been focused on the ability of ITCs to block chemical carcinogenesis through inhibition of carcinogen-activating enzymes and induction of carcinogen-detoxifying enzymes. ITCs may also be important as cancer therapeutic agents. Recent studies have shown that ITCs have the ability to induce growth inhibition rapidly and are also known to induce both cell cycle arrest and apoptosis in a number of human cancer cell lines (Gamet-Payrastre et al., 2000; Xu and Thornalley, 2000, Chiao et al., 2002; Fimognari et al., 2002). Studies have also demonstrated that ITCs appear to be selective in inhibiting the cell growth and found to be more toxic to malignant cells as compared to normal cells (Gamet-Payrastre et al., 2000; Xiao et al., 2003).
Figure 2.3
Model of the signaling pathways involved in activation of Nrf2 and ARE. Nrf2 is anchored to Keap1 in the cytosol. Upon activation of upstream proteins kinases (MAPKs, PI3K, PKC and PERK), and/or direct effect on Keap1, Nrf2 is released from Keap1 and translocates into the nucleus, where Nrf2 binds to ARE with association of small Maf inside the nucleus. Isothiocyanates may also directly cause the cleavage of disulfide bond between Nrf2 and Keap1 (Keum et al, 2004).
2.4.3.3. Induction of cell cycle arrest

Cell cycle depends on DNA replication (S phase) and segregation of chromosomes to the daughter cells (M phase), which are spaced by intervals of growth and reorganization (G1 and G2 phases). The systematic progression of the eukaryotic cell cycle is controlled by a series of proteins called cyclins, which exert their effects by binding to and activating a series of specific cyclin-dependent kinases (CDKs). This process is further modulated by inhibitory proteins such as p21/WAF-1, p16/INK4 and p27/Kip-1, collectively termed as CDK inhibitors (CDIs) (Weinstein, 2000).

Cell cycle arrest occurs in response to cellular stress such as DNA damage through activation of cellular checkpoints such as G1/S or G2/M phase until errors in DNA are rectified. However, apoptosis (programmed cell death) is initiated, if the DNA damage is extensive and beyond any repair (Figure 2.4). Thus, cell cycle arrest and apoptosis are considered as closely coupled protective cellular mechanisms. While tumor cells are regarded as a clone of transformed cells, induction of cell cycle arrest or apoptosis is considered as a potential chemopreventive approach (Keum et al., 2004).

In addition to its modulatory effect on carcinogen metabolism, induction of cell cycle arrest in several cancer cell lines has also been identified as a potential mechanism underlying the chemopreventive activities of ITCs. Hasegawa et al (1993) first observed significant accumulation of cells at G2/M phase after the treatment of HeLa cells with ITCs. Similar effects are consequently, seen in a wide variety of cells. However, molecular targets of ITCs may vary and indeed molecular mechanisms precisely responsible for the cell cycle arrest by ITCs are still far from clear. SUL causes significant accumulation of Jurkat cells (Fimognari et al., 2002) and HT29 cells (Gamet-Payrastre et al., 2000) at G2/M phase, which is associated with an increase in cyclin B1 level. However, LNCaP cells treated with SUL have showed cell cycle arrest at G1 phase with a simultaneous decrease in cyclin D1 expression (Chiao et al., 2002). On the contrary, AITC-induced G2/M phase arrest in LNCaP cells is accompanied by a prominent decrease in cyclin B1 level, but comparable effect is not noticed in PC-3 cells (Xiao et al., 2003). Similarly, AITC has arrested HL60 cells in G1 phase, in contrast to G2/M phase in other cells, whereas BITC has arrested cells in both G1 and G2/M phases (Zhang et al., 2003). From these results, it can be implied that ITCs certainly have cell cycle-arresting activity, but ITCs may arrest different cells at different phases of the cell cycle, which
primarily depend on the type of ITCs and cell lines. Furthermore, ITCs have the ability to induce cell cycle arrest as early as 3 h after the cell treatment (Zhang et al, 2003).

**Figure 2.4**
Cell cycle checkpoint and cell cycle arrest. In response to DNA damage, p53 triggers activation of CDK inhibitors that leads to cell cycle arrest. If DNA damage is extensive, apoptosis is initiated via activation of pro-apoptotic genes.
2.4.3.3.4. Induction of apoptosis

Apoptosis (programmed cell death) is a highly regulated protective mechanism, through which damaged or superfluous cells are eliminated from the system. Apoptosis is recognized to be vital for normal development, turnover and replacement of cells such as skin cells. Apoptosis can be initiated either at the cell surface (death receptor or extrinsic pathway) or from internal events within the cell (mitochondrial or intrinsic pathway). Both pathways lead to activation of caspases, which are responsible for execution of cell death by cleaving cellular substrates. Extrinsic pathway depends on ligand-activated recruitment of adaptor proteins by death receptors and consequent activation of caspase-8. The intrinsic pathway entails the release of proapoptotic molecules from mitochondria to the cytosol such as cytochrome c, which then activate caspase cascade. The chief regulators of this pathway are members of the Bcl-2 family proteins (Figure 2.5).

Additionally, apoptosis of individual cells serves as a defense mechanism against cancer development in an organism by removing genetically damaged or redundant cells that have been inappropriately stimulated to divide by a mitotic stimulus. In fact, apoptosis is deranged in cancer cells, which display reduced propensity towards apoptotic stimuli. Most of the chemopreventive agents have been shown to demonstrate their inhibitory effect through induction of apoptosis.

2.4.3.3.4.1. Role of caspases in apoptosis

Caspases (cysteine-aspartic acid proteases) belong to cysteine protease family and serve as the major effectors of apoptosis. The activation of caspases leads to distinctive morphological changes of cells such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Degterev et al, 2003). Induction to commit suicide is needed for proper development of organism, to get rid of cells that pose a threat to the organism (e.g. cell infected with virus or cancer or cancer cells), and to remove cells with damaged DNA. Cells undergoing apoptosis are eventually removed by phagocytosis. There are two types of caspases: initiator (apical) caspases and effector (executioner) caspases, both of which are synthesized as inactive proenzymes. Initiator caspases are the first to be activated and include caspase – 2, 8, 9 and 10. These in turn cleave and activate effector caspases such as 3, 6 and 7. Effector
caspases consecutively cleave, degrade or activate other cellular proteins within the cell triggering the apoptotic process (Boatright and Salvesen, 2003). The initiation of this cascade reaction is regulated by caspase inhibitors (Concha and Abdel-Meguid, 2002).

Figure 2.5
Schematic representation of apoptotic pathways involving p53, Bcl-2 family and caspases.
Source: www.weizmann.ac.il/apoptoticpathways
Caspase activation can be mediated by intrinsic factors such as Bcl-2 on the mitochondrial membrane. Bcl-2 is normally found associated with Apaf-1. Damage to cells causes dissociation of Bcl-2 from Apaf-1 leading to release of cytochrome-c into the cytosol. New complex called apoptosome is formed comprising of cytochrome-c, Apaf-1, and caspase-9. Caspase-9 is cleaved and activates other caspases leading to an expanding cascade of proteolytic activity within the cell.

This eventually results in the digestion of structural proteins in the cytoplasm, chromosomal DNA degradation and phagocytosis of the cell. External signals can also affect caspase activation cascade. TNF and Fas receptors on the cell surface can be triggered upon ligand binding (TNF, Fas, certain toxins and chemicals) to cleave caspase-8 which then initiates increased proteolysis within the cell and its ultimate removal by phagocytosis (Denault and Salvesen, 2002).

2.4.3.3.4.2. Role of Bcl-2 family in apoptosis

Of late, Bcl-2 gene family has been recognized to play critical roles in the regulation of apoptosis (Cory and Adams, 2002). Study of the mechanism of apoptosis by Bcl-2-related genes presents new possibilities for prevention and treatment of several human diseases including cancer (Green, 1998; Reed, 1995). Some of the proteins within this family including Bcl-2 and Bcl-X\textsubscript{L} inhibit apoptosis. Others such as Bad, Bax, Bik, Bid and Bak promote apoptosis (Cory and Adams, 2002). They form homo-oligomers and hetero-oligomers, which act directly at the outer mitochondrial membrane. Indeed, the ratio between these two subsets determines, in part, susceptibility of cells to apoptotic stimuli (Oltvai \textit{et al}, 1993).

2.4.3.3.4.3. Role of p53 in apoptosis

p53 acts as a transcriptional activator by triggering transcription of proteins involved in DNA repair (Lohrum and Vousden, 1999). If the DNA damage is beyond repair, p53 activates apoptotic pathway, which is considered as a last resort to avoid proliferation of cells with mutated DNA (Jin and Levine, 2001). Thus, normal p53 function has been demonstrated to be crucial in the induction of apoptosis in humans following DNA damage. This result is further supported by the findings that p53 is the most commonly mutated tumor suppressor gene and found in about 50 – 55% of all
human cancers (Malkin, 2001). Lack of p53 function may contribute to the complex network of molecular events leading to tumor formation, as this may allow mutated cells to escape apoptosis. Further, loss of p53 may promptpreneoplastic cells to accumulate additional mutation by obstructing the normal apoptotic response to genotoxic damage (Bode and Dong, 2004).

In normal cells, p53 protein is latent and highly unstable with a half-life measured in minutes. It is maintained at low levels by targeted degradation mediated by its negative regulator, Mdm2 (Alarcon-Vargas and Ronai, 2002). During DNA damage and/or other stress signals, half-life increases significantly leading to the accumulation of p53 and transcription of target genes such as \( p21^{WAF1/CIP1} \) and Bax (El-Diery, 1998). The outcome of this increased transcription depends on cell type, but usually manifested as a very prolonged G1 arrest or apoptosis (El-Diery, 1998). There are several potential mediators of p53-induced apoptosis. The Bax is an apoptosis-inducing member of the Bcl-2 protein family, whose transcription is directly activated by p53-binding sites in the regulatory region of the gene (Thornborrow et al, 2002). Furthermore, p53 also participates in the initiation of apoptosis by acting directly at mitochondria. Localization of p53 to the mitochondria occurs in response to apoptotic signals and precedes cytochrome-c release and procaspase-3 activation (Haupt et al, 2003).

### 2.4.3.3.5. Mechanism of apoptosis induction

A large number of ITCs have been reported to induce apoptosis in a variety of cultured cancer cell lines as well as in animal tissues and cancer cell xenografts (Singh AV et al, 2004; Srivastava et al, 2003; Bonnesen et al, 2001; Zhang et al, 2003; Chiao et al, 2002; Gamet-Payrastre et al, 2000; Xiao et al, 2003; Xu et al, 2000; Huang et al, 1998; Fimognari et al, 2002; D’Agostini et al, 2001). Moreover, treatment of cells with ITCs leads to the activation of caspases involved in multiple apoptotic pathways. However, precise intracellular signaling pathways initiated by ITCs leading to apoptosis are likely to be complex and are only partly understood. There are substantial differences among cell lines with respect to the potential targets for apoptosis that are modulated by various ITCs. Furthermore, most of the previous studies have been focused on the mitochondrial pathway.
Chen et al (1998) have reported that ITC-induced apoptosis in HeLa and HL60 cells, is mediated by c-Jun NH$_2$-terminal kinases (JNK), whose activation may result from oxidative stress induced by ITCs. Nevertheless, Rose et al (2003) showed that treatment of HepG2 cells with ROS scavengers did not impede PEITC-induced apoptosis. In fact, another study has indicated that PEITC inhibits a phosphatase that inactivates JNK (Chen et al, 2002). Besides, JNK activation could also occur from inhibition of Bcl-2 as over-expression of Bcl-2 is found to suppress PEITC induced JNK activation in HeLa cells (Chen et al, 1998). Down-regulation of Bcl-2 is reported in various cancer cell lines treated with ITCs (Xiao et al, 2003; Fimognari et al, 2002; Singh AV et al, 2004; Srivastava et al, 2003).

ITCs may also induce apoptosis through modulation of other cellular targets. Increased expression of pro-apoptotic Bax is detected in prostate, colon and leukemia cells treated with SUL (Singh AV et al, 2004; Gamet-Payrastre et al, 2000; Fimognari et al, 2002), but not in AITC-treated prostate cancer cell lines (Srivastava et al, 2003; Xiao et al, 2003). Interestingly, increased expression of Bax, which is downstream of p53, is accompanied by an increase in p53 expression in Jurkat cells treated with SUL (Fimognari et al, 2002), but not in SUL-treated HT29 cells (Gamet-Payrastre et al, 2000). Likewise, anti-apoptotic Bcl-X$_L$ is significantly downregulated by AITC in LNCaP cells but not by AITC or SUL in PC-3 cells (Xiao et al, 2003; Singh AV et al, 2004). Even though, Huang et al (1998) have reported that upregulation of p53 is needed for PEITC-induced apoptosis, AITC, PEITC and SUL are able to induce apoptosis in p53-deficient PC-3 cell lines (Singh AV et al, 2004; Xiao et al, 2003; Xiao et al, 2002), suggesting that ITCs could initiate apoptosis through p53 independent pathway. These results are confirmed by a recent study done by Pappa et al (2006) that different ITCs have a distinct profile of cell growth inhibition, potential to induce p53 independent apoptosis and have the ability to modulate Bcl-2 family protein expression in human colon cancer cell lines.

### 2.4.3.4. Chemopreventive efficacy of *R. sativus*

Recent studies substantiate the beneficial role of *R. sativus* in the prevention of human cancers. Papi et al (2008) have reported that MTBITC extracted from Japanese *R. sativus* sprout have shown promising anti-proliferative activity in a dose-dependent manner and induced apoptosis in colon cancer cell lines (LoVo, HCT116 and HT29).
through increased expression of Bax and caspase-9 and decreased expression of Bcl-2 protein along with cleavage of PARP-1. Further, it is demonstrated that MTBITC has shown negligible toxicity to normal human lymphocytes and displayed significant antioxidant and radical scavenging activity.

Barillari et al (2008) employed standardized Kaiware Daikon extract (KDE) containing 10.5% glucoraphasatin and 3.8% glucoraphenin in combination with myrosinase, as a natural chemopreventive agent against colon cancer cell lines (LoVo, HCT116 and HT29) in comparison with pure MTBITC and sulforaphene. They found out that KDE significantly reduced cancer cell growth in a dose-dependent manner, surpassing pure MTBITC and sulforaphane at the same dose, with no significant toxicity to human lymphocytes along with induction of Bax, caspase-1 and PARP-1 protein expression and down-regulation of Bcl-2 expression.

Hanlon et al (2007) shown that crude aqueous extract of Spanish black radish increased the activity of both phase I and II detoxification enzymes in HepG2 cells with maximal effect at a concentration of 1mg/ml. However, addition of glucoraphasatin showed no significant effect on the induction of detoxification akin to MTBITC, which significantly induced the phase II enzymes at a concentration of 10mM.

Kim et al (2006) reported that young R. sativus cultivated with sulfur inhibited the growth of B16-F10 melanoma cells and suppressed pulmonary tumorigenesis in mice, possibly, due to the induction of detoxification enzymes. They further proposed sulforaphane as a possible active compound responsible for the biological activity of young R. sativus.

However, most of these studies highlight the significance of ITCs as the prospective phytochemicals with biological activity. ITCs are, nevertheless, one class of compounds among large number of other phytochemicals occurring in the vegetal matrix of R. sativus. Herbal drugs derived from medicinal plants usually contain several classes of compounds endowed with a polyhedric action, which frequently act on similar target with synergistic and/or additive mode of action. Recent investigations have shown that advantage of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in it, rather than to a single phytochemical, because no confirmed health benefit of any single phytochemical has been detected in large-scale intervention studies (Goodman et al, 2003). Such evidence hints that whole
food, not a single compound, should be characterized for its effect on reduced cancer risk.

2.4.4. Other beneficial effects of *R. sativus*

Japanese *R. sativus* sprout have been shown to influence carbohydrate and lipid metabolism in normal and streptozotocin-induced diabetic rats. *R. sativus* sprout had a hypoglycemic activity in both normal and diabetic rats and also improved lipid metabolism in the normal rats. These results suggest that *R. sativus* sprout has significant anti-diabetic effect in experimental rats and thus could be viewed as a prospective agent in the primary prevention of diabetes mellitus (Taniguchi *et al*, 2006). Further study on the anti-diabetic effect of *R. sativus* revealed the difference in the ability of water-soluble and fat-soluble extract to produce hypoglycemic effect in the diabetic rats. Fat-soluble extract suppressed insulin secretion and improved the lipid metabolism, whereas water-soluble extract decreased blood glucose level without increasing insulin secretion in the diabetic rats, which suggested the potential of water-extract of *R. sativus* as the functional food component with hypoglycemic effect (Taniguchi *et al*, 2007).

*R. sativus* root extracts showed hepatoprotective effect on paracetamol-induced heptotoxicity in experimental animals. *R. sativus* extract significantly alleviated oxidative stress generated by paracetamol through induction of antioxidants such as catalase and glutathione (Chaturvedi *et al*, 2007). However, *R. sativus* extract was able to reverse paracetamol-induced lipid peroxidation and hepatotoxicity, when administered along with paracetamol, however failed to mitigate the oxidative stress if paracetamol administration continued for an extended period (Chaturvedi and Machacha, 2007). Baek *et al* (2008) demonstrated that sulfur-rich *R. sativus* extract and sulforaphane could partly diminish CCl₄-induced hepatotoxicity in mice, perhaps, by acting indirectly as antioxidants and improving the detoxification system.

Ghayur and Gilani (2005) demonstrated gastrointestinal and uterine tone modulatory activities of *R. sativus* leaves in isolated guinea-pig ileum, rabbit jejunum and rat stomach fundus and uterus. This study showed species-specific gastrointestinal effect of *R. sativus* leaves, where it is mediated partly through cholinergic receptors in rabbit and rat tissues, but through histaminergic receptors in guinea-pigs.
Ghayur and Gilani (2006) studied hypotensive, cardio-modulatory and endothelium-dependent vasodilator effect of *R. sativus* seed extract in normotensive rats and isolated guinea pig atria. Results from this study justified the traditional usage of *R. sativus* in hypertension as it has mediated cardiovascular inhibitory effect through activation of muscarinic receptors. Phytochemical analysis of *R. sativus* seed extracts showed the presence of saponins, flavonoids, tannins, phenols and alkaloids, which components could thus be responsible for the observed cardiomodulatory activity.

Previous studies also demonstrated anti-mutagenic and anti-genotoxic activity of *R. sativus* extracts. Nakamura *et al* (2001) reported that MTBITC extracted from Japanese *R. sativus* root showed significant anti-mutagenic effect in UV-induced mutation assay of *Escherichia coli* B/r WP2. However, authors ascertained that crude n-hexane extract of *R. sativus* exhibited more potent anti-mutagenic effect than isolated MTBITC, suggesting the presence of synergistic components in *R. sativus*. Similarly, Shishu *et al* (2003) isolated sulforaphene from *R. sativus* seed for its prospective anti-mutagenic effect and suggested that sulforaphene was a potent inhibitor of S9-mediated mutagenicity of all the food-derived heterocyclic amines.

Salas – Abbes *et al* (2009a) reported that MTBITC extracted from Tunisian *R. sativus* alleviated genotoxicity and DNA damage induced by Zearalenone (ZEN) in Balb/c mice through prevention of DNA fragmentation and attenuation of structural chromosome aberrations and micronuclei associated with augmentation of mitotic index. Similarly, Tunisian *R. sativus* extract showed protective effect against ZEN-induced reproductive toxicity, oxidative stress and mutagenic modifications (Salas – Abbes *et al*, 2009b) as well as the ZEN-induced immunotoxicity in male Balb/c mice (Salas – Abbes *et al*, 2008b).

Korean *R. sativus* root extract and its ITCs showed an inhibitory effect on growth of vascular smooth muscle cells, which is considered to be a prominent feature of vascular diseases including atherosclerosis. Elucidation of mechanism revealed the role of cell cycle check points, whereby *R. sativus* and its ITCs induced apoptotic cell death through cell cycle arrest in G1 phase, down-regulation of cyclins and CDKs and upregulation of CDK inhibitor p21 expression (Suh *et al*, 2006).
2.5. Problem Statement

The health benefits of *R. sativus* have been promoted for centuries, but very few studies have been conducted to prove its medicinal and pharmaceutical value. As of now, there has been very limited information on the phytochemical profile and biological activity of *R. sativus* of Indian origin. Abdou *et al* (1972), Esaki and Onozaki (1982) and Khan *et al* (1985) described the antibacterial activity of *R. sativus*. However, the number of bacterial species tested in these studies is limited. Furthermore, there has been hardly any research on antibacterial activity of stem and leaves of *R. sativus*.

Takaya *et al* (2003) characterized the radical scavenging activity of Japanese *R. sativus* sprouts and detected the presence of polyphenolics such as sinapic acid and kaempferol. Until now, information regarding polyphenolics profile and antioxidant property of *R. sativus* root, stem and leaves is almost lacking.

Papi *et al* (2008) and Barillari *et al* (2008) reported a dose-dependent chemopreventive effect of Japanese *R. sativus* sprouts against human colon cancer cells. The ability of *R. sativus* sprouts to protect against neoplastic disease has been credited especially to MTBITC. Despite these reports, the exact mechanism by which *R. sativus* acts as a chemopreventive agent in humans is not yet fully defined. These studies are limited to *R. sativus* sprouts and scarcely any research has been done on chemopreventive effect of *R. sativus* root, stem and leaves. Besides, evidence for protective effect of *R. sativus* against oxidative damage in normal human cells is almost absent.

Hence, a well documented and comprehensive study on biological activity of *R. sativus* L., root and aerial part (often under-utilized parts of this vegetable) and their constituents would substantiate their value in human nutrition as well as food and pharmaceutical supplements. In this study, phytochemicals from root and aerial part (stem and leaves) of *R. sativus* were extracted with solvents of varying polarity, analyzed for the presence of phytochemicals (using HPLC-DAD and GC-MS analysis) and evaluated for biological activities such as antioxidant, antimicrobial, chemoprotective and chemopreventive activity.