STUDIES ON ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS IN LIME [*Citrus aurantifolia* (Christm) Swingle], THEIR ANTIOXIDANT AND ANTICANCER PROPERTIES

Thesis submitted to the
University of Agricultural Sciences, Dharwad
in partial fulfillment of the requirements for the
Degree of

DOCTOR OF PHILOSOPHY

IN

CROP PHYSIOLOGY

By

JAIPRAKASH R. PATIL

DEPARTMENT OF CROP PHYSIOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES
DHARWAD – 580 005

JULY, 2009

ADVISORY COMMITTEE

Dharwad
July, 2009

(M. B. CHETTI)
Major Advisor

Approved by :
Chairman :

_____
(M. B. CHETTI)

Co-Chairman:

_____
(B. S. PATIL)

Members :

1. _____
(P. M. SALIMATH)

2. _____
(B. C. PATIL)

3. _____
(C. P. MANSUR)

4. _____
(D. S. UPPAR)

CONTENTS

LIST OF TABLES

Contd……

LIST OF FIGURES

Contd……

Figure No.	Title
14.	Effect of Lime peel extracts on expression levels of apoptosis related proteins (p21, Bax, bcl_2, Caspase-3 and AKT) of Panc-28 cells
15.	Effect of Lime volatile oil on proliferation of normal (NIH 3T3) cells. Mouse embryonic fibroblast cells were treated with different concentrations of Lime volatile oil and viable cells were counted after 24 and 48 h of incubation using Z_1 beckman counter (0 indicates with out volatile oil)
16.	DNA fragmentation in lime volatile oil treated SW-480 cells, M-Marker; C- control (untreated cells); Ca-Camptothecin at (50 µg/ml), for 12 h: DNA disintegration after treatment of lime volatile oil (100 µg/ml) for 6, 12 and 24 h
17.	Effect of lime volatile oil treatment (24 and 48 h) on the Caspase-3 content of SW480 cells, Caspase-3 was measured using a specific substrate Ac-DEVD-AMC AMC [N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)] and results were expressed in terms of fluorescent units related to 7-amino-4-methylcoumarin
18.	Expression of apoptosis related proteins by treatment of lime volatile oil for 24 and 48 h
19.	Fluorescent images showing morphology of Control cells, Camptothecin and Lime volatile oil (100 µg/ml)

1. INTRODUCTION

Lime [*Citrus aurantifolia* (Christm) Swingle] is a polyembryonic species with greenish yellow, smooth surfaced, thin-skinned fruits, and solid core at maturity with highly acidic juice. Lemons and limes are cultivated in many countries all over the world. Lemons grow in regions with temperate summers and mild winters, particularly in Mediterranean countries, Southern California, and Argentina ; whereas, limes grow in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and the West Indies. According to the classification by Swingle, lemons and limes belong to two species: *Citrus limon* (L.) Burm. and *Citrus aurantifolia* (Christm.) Swingle (family: Rutaceae). Conversely, in the Tanaka system, they are divided into several species characterized by botanical variability (Anon., 1992).

Citrus production worldwide in selected major producing countries in 2006-2007 was 73.10 million metric tons. India produced about 4.6 m.t. of citrus from a total area of about 0.56 million ha. India ranks sixth in the production of citrus fruits in the world. Other major citrus producing countries are Spain, USA, Israel, Morocco, South Africa, Japan, Brazil, Turkey and Cuba. It occupies third position after mango and banana in the production of fruits in India. It is of particular interest because of its high vitamin C content and used as a refreshing juice. Citrus is grown almost all over India. However, the states of Andhra Pradesh, and Maharashtra have the largest share. Of the various types of citrus fruits grown in India, mandarin orange (*Citrus reticulata* Blanco), sweet orange (*C. sinensis* Osbeck) and lime / lemon (*C. aurantifolia* Swingle) are of commercial importance. Lime or acid lime is also commercially known as 'Pati lime' or 'Kagzi lime'.

Pancreatic cancer is one of the most devastating of all malignancies with the highest mortality compared to other cancers (Li *et al*., 2004), and is the fourth leading cause of cancer death in the USA (Jemal *et al*., 2008). Late diagnosis of cancer is the main cause for limited option for successful treatment and also development of resistance to most of chemotherapy and radiotherapy (Lowenfels and Maisonneuve, 2005). Hence, prevention seems to be the most promising strategy to reduce the mortality rates of pancreatic cancer (Bobe *et al.,* 2008).

Based on the success rate and complications from currently available synthetic drugs for pancreatic cancer, treatment using natural compounds have gained considerable attention due to their safety and efficacy in overcoming tumor cell resistance to apoptosis (Bharti and Aggarwal, 2002). Current research information available suggest that few natural compounds have demonstrated potential benefits in pancreatic cancer prevention including, curcumin (Aggarwal and Sung, 2008), flavonoids (Zhang *et al*., 2008) and isoflavones (Awale *et al.,* 2008). Hence, screening of naturally derived compounds may be one of the promising approaches in prevention of pancreatic cancer.

Colon cancer is another prevalent cancer throughout the world and especially in western countries. This is continuously increasing worldwide due to rapid changes in dietary pattern and preferences. Many epidemiological studies indicated that western-style diet, primarily, the consumption of red meat is positively associated with a high colon cancer incidence (Abeysinghe *et al.,* 2007). Continuous efforts are being made for search of novel source of bioactive compounds to prevent colon carcinoma. In this direction, bioactive compounds of natural origin, particularly from dietary source are gaining significance. In recent years, citrus has gained importance due to their ability to provide multitude health benefits not only from vitamin-C but also from other bioactive compounds. An animal study has revealed that freeze dried grapefruit juice powder (13.7 g/kg) and its bioactive compounds naringin and limonin (200 mg/kg) are capable of inhibiting aberrant crypts through the suppression of cyclo-oxygenase-2 and inducible nitric oxide synthase (iNOS) in azoxymethane treated animals (Vanamala *et al.,* 2006).

Until recently, citrus health promoting properties have always been associated with their content of vitamin C. Only in the last decade studies have focused on several bioactive compounds, specifically limonoids and flavonoids which play a major role in preventing chronic diseases (Tian *et al*., 2001). Certain citrus fruits are not bitter when freshly squeezed, but they become bitter after a few hours at room temperature or when refrigerated overnight. The bitterness comes from certain limonoids, which are characteristic of plants in Rutaceae family (Emerson, 1948). Limonoids are compounds in citrus fruits, generally found in the peel,

which produce the familiar bitter taste and zesty aroma. Limonoids are highly oxygenated modified triterpenes, derived from a precursor with 4, 4, 8-trimethyl-17-furanylsteroid skeleton. An important characteristic of this class of compounds is a substituted furan moiety. Citrus limonoids appear in large amounts in citrus juices and citrus tissues as water-soluble limonoid glucosides or in seeds as water-insoluble limonoid aglycones. In addition to vitamin-C, carotenoids, flavonoids, limonoids, phenolic acids in addition to coumarins, furanocoumarins, pyranocoumarins alkaloids and phytosterols when consumed in appropriate quantities are beneficial to human health (Gray and Waterman, 1978; Macheix *et al*., 1990; McHale and Sheridan, 1989; Tatum and Berry, 1977, Kane and Lipsky, 2000; Poulose *et al*., 2005; Tian *et al.,* 2001, and Wang *et al.,* 2007). Limes are also used for the preparation of beverages and pickles

About 56 limonoids have been identified in citrus, some of the commonly found limonoids in citrus are limonin, nomilin, LG and deacetyl nomilinic acid glucoside (Jayaprakasha *et al*., 2008). Limonin has shown to possess anti-carcinogenic properties in both cell culture and *in vivo* rodent models. Limonin reduced the incidence of 7,12-dimethylbenz[a]anthracene (DMBA)-induced buccal pouch epidermoid carcinomas in female Syrian hamsters (Miller *et al.,* 1989). Treatment with limonin effectively inhibited DMBA-induced neoplasia in experimental mice (Lam and Hasegawa, 1989). However, in a chronic study, limonin and obacunone have decrease the frequency of colonic adenocarcinoma (Tanaka *et al.,* 2001).

Growing body of evidence seems to suggest that limonoids and flavonoids have different biological functions, including antioxidative, anti-inflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticarcinogenic activities (Hasegawa *et al*., 1996; Benavente *et al.,* 1997; Poulose *et al*.,2006; Vanamala *et al*., 2006, Haaz *et al.,* 2006 and Jayaprakasha *et al*., 2008). Therefore, new Citrus cultivars have been developed for fresh consumption. Furthermore, the particular characteristics of these bioactive compounds have the potential to be used in the pharmacological and food technology area (Del *et al.,* 1997; Ortuno *et al.,* 1997).

Citrus as source of flavonoids and are a large class of low molecular weight polyphenolic compounds that occur ubiquitously in plants. Flavionoids have antioxidant properties and their involvement in antiproliferation processes, cell cycle arrest and apoptosis, antioxidation, induction of detoxification enzymes, regulation of host immune functions has been demonstrated. Consumption of food rich in flavonoids prevents several degenerative pathologies, including cardiovascular diseases, atherosclerosis, cataract and several forms of cancer (Federica and Sergio, 2005). Apart from antioxidant activity, hesperidin is known to act as anticancer agent through prostaglandin (PG) and chemical carcinogens inhibition (Kupfer and Bulger, 1987). The other major flavonoid reported in lime fruits is rutin, also known as quercetin-3-rutinoside. Rutin has shown significant scavenging properties on oxidizing species, such as hydroxyl radical, superoxide radical, and peroxyl radical. Furthermore, it has shown antiallergic, anti-inflammatory and antitumor, antibacterial, antiviral and anti-protozoal properties (Deschner *et al.,* 1991).

Citrus phytochemicals have shown to inhibit colon (Jayaprakasha *et al*., 2008), breast (Sergeev *et al*., 2006), neuroblastoma (Poulose *et al.,* 2005) and prostate cancer cells (Gao *et al*., 2006). Recently, citrus compounds are known to inhibit the colon cancer cells proliferation in both cell culture and animal studies (Poulose *et al*., 2006, Poulose *et al*., 2007; Vanamala *et al*., 2006). The antiproliferative effects of limonoids have been reported in various cancer cells such as, MCF-7 (breast cancer) (Tian *et al*., 2001), HT-29 (colon cancer) (Jayaprakasha *et al.,* 2008). Kaffir lime (*Citrus hystrix*) volatile oil has been demonstrated to reduce blood pressure and relieve depression in human studies (Hongratanaworakit and Buchbauer, 2007), which provides strong evidence on potent health benefits of citrus volatile oils.

Apart from anticancer property of citrus fruits, several antioxidant compounds have been identified in the seeds of citrus (Gorinstein *et al.,* 2004; Jayaprakasha and Patil, 2007). These compounds include flavonoids, which can scavenge free radicals and also chelate metal ions, and hence they are potential antioxidant. Hesperidin is a type of flavonoid present in several vegetables and fruits, but mainly in citrus (Cserhati, 1995). Hesperidin is known to posses certain biological activities including antioxidant property, and inhibition of

prostaglandin biosynthesis, and also known to inhibit chemical carcinogenesis (Kupfer and Bulger, 1987).

It must be emphasized here that, inspite of lime being one of the important citrus fruits and also widely available and popular among consumer worldwide, there is hardly any information available with respect to bioactive compounds available from limes and their effect on human pancreatic cancer cells, antioxidant potential of the phytochemicals in limes, the effects of citrus compounds on pancreatic carcinoma cells, proliferation inhibitory activity of lime juice extracts on human pancreatic cancer cells and mechanisms involved in cell suppression.

In view of the above, "**Studies on isolation and characterization of bioactive compounds in lime [*Citrus aurantifolia* (Christm) Swingle], their antioxidant and anticancer properties**" was formulated with the following objectives.

1. Isolation and identification of bioactive compounds from limes by using chromatographic techniques and spectroscopic studies.
2. Studies on antioxidant activities of bioactive compounds.
3. Analysis of chemical composition of lime volatile oil by GC-MS.
4. Studies on anticancer activity of purified compounds using cell lines.

2. REVIEW OF LITERATURE

Lime [*Citrus aurantifolia* (Christm.) Swingle] is a polyembryonic species with greenish yellow, smooth surfaced, thin-skinned fruits, and solid core at maturity with highly acidic juice (Morton, 1987). Lime is used for the extraction of juice, preparation of squash, concentrates, beverages, and byproducts, such as citric acid and pectin (Gorinstein *et al*., 2005). Limes are also used for the preparation of beverages and pickles. In addition to vitamin C, other major classes of phytochemicals found in lime are limonoids, flavonoids, phenolic acids, coumarins, alkaloids and phytosterols (Wang *et al*., 2007)

Citrus fruits are consumed globally in the form of fresh as well as processed juices. Recently, citrus fruits have been studied for various health benefits. This includes cardiovascular disease (Lowe *et al*., 2003), obesity (Haaz *et al*., 2006) and cancer (Jayaprakasha *et al*., 2008; Poulose *et al*., 2006; Tian *et al*., 2001). Kagzi limes (*Citrus aurantifolia* Swingle) are popular for their attractive and unique flavor, as well as vitamin C, flavonoids and other biologically active compounds.

Health benefits of phytochemicals from citrus juice are known world-wide for their tart, tangy-flavored juice and especially for their unique flowery, characteristic aroma. Limes are popular for use in juice mixtures, carbonated beverages, and as a component of alcoholic drinks and mixes. In some countries, they are used in pickling, culinary, and medical applications. The juice (100 ml) supplies 110–140 KJ (26 Kcal) of energy, 50 mg of ascorbic acid (vitamin C) and a trace of dietary fiber (Berry and Benjamin, 2003). Further, the most widely consumed product of citrus fruits is juice and it accounts for approximately more than 50 per cent of the total mass of the whole fruit (Lario *et al*., 2004).

Consumption of fruits and vegetables is associated with a lower risk of degenerative diseases including cancer, cardiovascular disease, cataracts, and brain dysfunction (Ames *et al*., 1993). There are more than 200 epidemiological studies which indicate that an association between low consumption of fruits and vegetables and the incidence of cancer (Block *et al*., 1992; Steinmetz and Potter, 1996). In addition, citrus species have been widely used in the ethnomedicine, with their broad range of bioactive ingredients, and have been found to possess anti-infection and anti-inflammatory properties (Murakami *et al*., 2000; Rodrigues *et al*., 2000).

Citrus fruits are known for their potential in prevention of cancer in an epidemiological survey (Wattenburg, 1985). Also the potential use of citrus flavonoids in cancer treatment has been suggested by some investigators (Rooprai *et al*., 2001).

2.1 Isolation of bioactive fractions from limes using chromatographic techniques

The composition of phytochemicals in citrus fruits are extensively studied, apart from ascorbic acid, some of the major class of compounds includes, flavonoids, limonoids, coumarins and phytosterols (Wang *et al*., 2007)

Until recently, citrus health promoting properties have always been associated with their content of vitamin C; only in the last decade studies have focused on several bioactive compounds, specifically limonoids and flavonoids which play a major role in preventing chronic diseases (Tian and Ding, 2000).Certain citrus fruits are not bitter when freshly squeezed, but they become bitter after a few hours at room temperature or when refrigerated overnight. The bitterness comes from certain limonoids, which are characteristic of plants in the Rutaceae family (Emerson, 1948)

Limonoids are compounds in citrus fruits, generally found in the peel, which produce the familiar bitter taste and zesty aroma. Limonoids are highly oxygenated modified triterpenes, derived from a precursor with 4, 4, 8-trimethyl-17-furanylsteroid skeleton. An important characteristic of this class of compounds is a substituted furan moiety. Citrus limonoids appear in large amounts in citrus juices and citrus tissues as water-soluble limonoid glucosides or in seeds as water-insoluble limonoid aglycones. Citrus species are known for

unique limonoids. About 37 limonoid aglycones and 19 limonoid glucosides have been isolated from citrus and their hybrids (Hasegawa *et al.*, 2000).

Growing evidence seems to suggest that limonoids and flavonoids have different biological functions, including antioxidative, anti-inflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticarcinogenic activities (Hasegawa *et al.*, 1996; Miller *et al.*, 1989; Poulose *et al.*, 2005; Vanamala *et al.*, 2006).

2.1.1 Limonoids, Aglycons and Glucosides

Limonoids are mostly present in Rutaceae and Maleaceae family in the form of aglycones and glucosides (Hasegawa *et al.*, 1989). Most of the aglycones are bitter in taste, while glucosides are tasteless, odorless and water soluble (Bennett *et al.*, 1989). About 56 limonoids have been identified in citrus, some of the commonly found limonoids in citrus are limonin, nomilin, LG and deacetyl nomilinic acid glucoside (Jayaprakasha *et al.*, 2008)

The seeds of *Citrus reticulata* afforded the new limonoid derivative, isolimonexic acid methyl ether, in addition to the previously isolated limonin, deacetylnomilin, obacunone and ichangin. The structure elucidation achieved primarily through 1D and 2-D-NMR analyses. The marginal antimalarial activity of isolimonexic acid methyl ether is reported (Ashraf *et al.*, 2002).

Limonin has shown to possess anticarcinogenic properties in both cell culture and *in vivo* rodent models. Limonin reduced the incidence of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced buccal pouch epidermoid carcinomas in female Syrian hamsters (Miller *et al.*, 1989). Treatment with limonin has effectively inhibited DMBA-induced neoplasia in experimental mice (Lam and Hasegawa, 1989).

Obacunone and limonin were tested using azoxymethane induced colon tumourigenesis in male F344 rats. Feeding of these compounds at 200 ppm, significantly inhibited the formation of aberrant crypt foci (ACF) in both the initiation (during AOM exposure) and post-initiation (after AOM treatment) (Tanaka *et al.*, 2000). However, in a chronic study, limonin and obacunone have shown decrease in the frequency of colonic adenocarcinoma (Tanaka *et al.*, 2001).

Limonin, nomilin and LG were tested for their ability to inhibit proliferation of MDA-MB-435 estrogen receptor-negative human breast cancer cells, by the incorporation of [^{3}H] thymidine. Among the compounds tested, nomilin was the most effective with IC_{50} value of 0.4 µg/ml, followed by limonin (12.5 µg/ml) and LG (75 µg/ml) (Guthrie *et al.*, 1997).

The LNA, isolated form *Raulinoa echinata* has shown antinociceptive activity in rodent model. It was speculated that the activity may involve GABAergic and nitroxidergic pathway (Biavatti *et al.*, 2007). ILNA isolated form *Citrus reticulata* has shown antifungal activity against *Candida albicans, Cryptococcus neoformans* and antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* at 500 µg/ml (Khalil *et al.*, 2003).

Citrus limonoids are capable of inducing cytotoxicity in both, cultured human cancer cells and animal models (Poulose *et al.*, 2006; Tian *et al.*, 2001; Vanamala *et al.*, 2006). The antiproliferative effects of limonoids have been reported in various cancer cells such as, MCF-7 (breast cancer) (Tian *et al.*, 2001), HT-29 (colon cancer) (Alexandra *et al.*, 1998; Jayaprakasha *et al.*, 2008), and SHSY5Y (neuroblastoma) (Poulose *et al.*, 2005). Further, antiproliferative activity of limonoids through caspase mediated apoptosis has been demonstrated (Poulose *et al.*, 2005).

2.1.2 Sitosterols

Sitosterol glucoside(SG) is chemically (3S,8S,9S,10R,13R,14S,17R)-17-[(2S,5S)-5-ethyl-6-methyl-heptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17 dodecahydro-1H-cyclopenta[a] phenanthren-3-ol. It is used to prevent obesity due to its ability of decreasing the absorption of cholesterol in the digestive system and decrease the amount of cholesterol produced by the liver (Sudhop *et al.*, 2002). It is also known for other biological activities such as, immunomodulation (Bouic *et al.*, 1996), anticarcinogenic (Awad *et al.*, 2007;

Jayaprakasha *et al*., 2007), inhibition of tumor invasion promoting markers (Kondegowda *et al*., 2006) , analgesic, anthelmenthic and antimutagenic activities (Villaseñor *et al*., 2002).

2.1.3 Furocoumarins in citrus peel

The citrus fruits also contain coumarins, furanocoumarins and pyranocoumarins (Gray and Waterman, 1978; Macheix *et al*., 1990; McHale and Sheridan, 1989; Tatum and Berry, 1977). The isolation and characterization of Bergamottin, 6′, 7′-dihydroxybergamottin and Paradisin A from grapefruit juice has been documented. The compound Paradisin A was found to be the most potent CYP3A4 inhibitor with an IC_{50} of 1.2 µM followed by DHB and bergamottin (Girennavar *et al*., 2006). The other coumarins such as, epoxybergamottin were isolated from peels of grapefruit using diethyl ether (Wangensteen *et al*., 2003) and paradisin C from grapefruit juice using hexane–ethyl acetate extract followed by column chromatography and HPLC separation (Ohta *et al*., 2002).

Further, bioactive compounds in citrus are carotenoids, limonoids, flavonoids, pectin, vitamin C, furocoumarins, and coumarins, when consumed in appropriate quantities are beneficial to human health (Kane and Lipsky, 2000; Poulose *et al*., 2005; Tian *et al*., 2001). The Coumarins are phytoalexins which are secondary metabolites found in some plants and their function in plants is not understood completely. However, they are speculated to play a role in defense mechanism of plants. As their biosynthesis in plants, is triggered when plants are subjected to various stresses and also their anti-oxidative and anti-microbial activities (Shimizu *et al*., 2005; Tietjen and Matern, 1984; Zobel and Brown, 1990).

Furthermore, plants belonging to Rutaceae family are known as one of the natural sources for structurally diverse coumarins (Gray and Waterman, 1978) . These compounds are known for several biological activity such as anti-platelet-aggregating (Chen *et al*., 1995), antimicrobial (Nakatani *et al*., 1987), antimutagenic (Edenharder *et al*., 1995) and anti-tumor-promoting activities (Mizuno *et al*., 1994; Murakami *et al*., 1997). In addition, inhibitory activities of coumarins against oxygen radical generation in leukocytes have been demonstrated (Murakami *et al*., 1997; Paya *et al*., 1993). The most known biological activity of furocoumarins is their potent inhibition of cytochrome P450 3A4 enzyme and transporter proteins (Girennavar *et al*., 2006; Guo and Yamazoe, 2004).

2.1.4 Flavonoids and other phytochemicals

Citrus as source of flavonoids are a large class of low molecular weight polyphenolic compounds that occur ubiquitously in plants. Citrus fruits contain multiple bioactive agents. Flavionoids have antioxidant properties and their involvement in antiproliferation processes, cell cycle arrest and apoptosis, antioxidation, induction of detoxification enzymes, regulation of host immune functions. Consumption of foods rich in flavonoids are known to prevent several degenerative pathologies, including cardiovascular diseases, atherosclerosis, cataract and several forms of cancer (Federica and Sergio, 2005).

Among the flavonoids, hesperidin was found to be the most abundant in *C. reticulata Blanco* (6.76–12.0 mg/g dry matter), *C. sinensis* (L.) *Osbeck* (6.98–10.8 mg/g dry matter), and *C. limon* (L.) (3.58 mg/g dry matter) (Kawaii *et al*., 1999). Apart from antioxidant activity, hesperidin is known to act as anticancer agent through prostaglandin (PG) and inhibitor of chemical carcinogens (Kupfer and Bulger, 1987).

The other major flavonoid reported in lime fruits is rutin, which is also known as quercetin-3-rutinoside. Rutin has shown significant scavenging properties on oxidizing species, such as hydroxyl radical, superoxide radical, and peroxyl radical. Furthermore, it has shown antiallergic, anti-inflammatory and antitumor, antibacterial, antiviral and anti-protozoal properties (Deschner *et al*., 1991).

Among the limonoids, limonin, nomilin, obacunone and their glucosides are found in most of the citrus species (Tanaka *et al*., 2000). Citrus phytochemicals have shown to inhibit colon (Jayaprakasha *et al*., 2008), breast (Sergeev *et al*., 2006), neuroblastoma (Poulose *et al*., 2005) and prostate cancer cells (Gao *et al*., 2006). Recent research has also indicated that, citrus compounds inhibit the proliferation of colon cancer cells in both cell culture and animal studies (Poulose *et al*., 2006; Poulose *et al*., 2007; Vanamala *et al*., 2006).

2.2 Studies on antioxidant activities of bioactive compounds

Antioxidants activity of citrus fraction was also ascribed to their hydrogen donating ability (Girennavar *et al*., 2007; Jayaprakasha and Patil, 2007), and may be due to the presence of flavonoids, carotenoids and ascorbic acid (Halliwell, 2001). The mechanisms of antioxidant action can include inhibition of reactive oxygen species formation by suppressing enzymes involved in free radical production; scavenging reactive oxygen species; and protecting antioxidant defenses (Halliwell and Gutteridge, 2000).

Flavonoids have been identified to fulfill most of the criteria described above and thus, their effects are twofold. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C (Hanasaki *et al*., 1994) . Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in the generation of reactive oxygen species.(Korkina *et al*., 1996). Besides scavenging, flavonoids may stabilize free radicals involved in oxidative processes by complexing with them. (Shahidi *et al*., 1992).

The antioxidant capacity of flavonoids is directly related to their structure (Cos *et al*., 1998), and in the case of hesperidin, the presence of a hydroxyl group at position 3 of ring B is responsible for the capacity of hesperidin to scavenge the hydroxyl radicals generated from hydrogen peroxide. It is already known that the ability to scavenge superoxide is due to a hydroxyl group at position C-4 of ring B (Acker *et al*., 1996; Cos *et al*., 1998). It has previously been shown that the C-4 methyl substitution at C-4 and hydroxyl at C-3 of hesperidin at ring B can activate, making hesperidin as more active scavenger to the superoxide radical (Van Acker *et al*., 1998).

Reports have shown positive correlations between the TEAC and PCL assays and total phenolics and also the phenolic compounds play a major role as a source of antioxidants in native Australian fruits where a variety of phenolic compounds such as flavonoids anthocyanins, flavan-3-ols, etc and phenolic acids (benzoic and cinnamic) contribute to antioxidant activity (Netzel *et al*., 2007). Similar other studies also reported a linear relationship between total phenolic content and antioxidant capacity in berry crops and herbs (Zheng and Wang, 2001; Zheng and Wang, 2003). This is also corroborated by the findings of other reports, which suggested that the *in vitro* ROS-scavenging activity of a rose hip water/acetone extract is mainly due to its phenolic constituents (Daels-Rakotoarison *et al*., 2002).

2.2.1 Antioxidant activity of lime juice extract

Recent reports have shown that there has been increasing interest in investigating the potential of natural antioxidants (mainly phenolics), particularly those of plant origin (Jayaprakasha *et al*., 2000) are of substantial interest as dietary supplements (Halliwell *et al*., 1995). The antioxidant activity by citrus extracts is primarily attributed to its proton donating capacity (Girennavar *et al*., 2007; Jayaprakasha and Patil, 2007). Evidences from research indicate that the activity may also be due to the presence of flavonoids, carotenoids and ascorbic acid in citrus fruits (Gorinstein *et al*., 2004).

Recent research suggest positive correlations between the TEAC and PCL assays and total phenolics (Netzel *et al*., 2007); while, the other studies reported a linear relationship between total phenolic content and antioxidant capacity in berry crops and herbs (Zheng and Wang, 2001; Zheng and Wang, 2003).

2.2.2 Antioxidant activity of lime seeds and peels extract

Phenolic are aromatic secondary plant metabolites, which play a significant role in color, sensory and nutritional qualities and antioxidant properties of food (Robbins, 2003). . Studies have reported that, some limonoids were found to be present in the chloroform extract of lemon peel and the most predominant being limonin (Baldi *et al*., 1995).

Citrus fruits are known for their rich sources of bioactive compounds, including vitamin C, phenolics and flavonoids, with potential health-promoting properties (Gorinstein *et*

al., 2001). These bioactive compounds are known to act as free radical-scavengers, to modulate enzymatic activities and to protect against a variety of diseases, particularly cardiovascular diseases and some types of cancer (Kurowska *et al*., 2000). Free radical scavenging is one of the known mechanisms of inhibition of lipid oxidation. In DPPH (1, 1,-diphenyl-2-picryl hydrazine) free radical scavenging assay, antiradical power of an antioxidant is measured as color changes from purple to yellow. This is used to evaluate hydrogen-donating ability of the compound. It is a rapid method and most widely employed to characterize antioxidant activity of plant material (Arnao, 2000). Furthermore, mechanisms of antioxidant action can include inhibition of reactive oxygen species formation by suppressing of enzymes involved in free radical production; scavenging reactive oxygen species; and protecting antioxidant defenses (Halliwell and Gutteridge, 2000).

Flavonoids have been identified as fulfilling most of the criteria described above. Thus, their effects are twofold. 1. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C (Hanasaki *et al*., 1994) . Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in reactive oxygen species generation.(Korkina *et al*., 1996). Besides scavenging, flavonoids may stabilize free radicals involved in oxidative processes by complexing with them (Shahidi *et al*., 1992).

The antioxidant capacity of flavonoids is directly related to their structure (Cos *et al*., 1998), and in the case of hesperidin, the presence of a hydroxyl group at position 3' of ring B may be responsible for the capacity of hesperidin to scavenge the hydroxyl radicals generated from hydrogen peroxide. It is already known that the ability to scavenge superoxide is due to a hydroxyl group at position C-4' of ring B (Van Acker *et al*., 1996; Cos *et al*., 1998). It has previously been shown that the ring B C-4' methyl substitution of hesperidin can activate the ring B C-3' hydroxyl, making hesperidin a more active scavenger to the superoxide radical (Van Acker *et al*., 1998).

2.2.3 Quantification of phenolics in citrus

Phenolic are aromatic secondary plant metabolites, which play a significant role in color, sensory and nutritional qualities and antioxidant properties of food (Robbins, 2003). The earlier report suggest that that the polyphenol content (chlorogenic acid equivalents) in peeled lemon (Citrus limon) and orange (Citrus sinensis) were 164 ± 10.3 and 154 ± 10.2 mg/100 g fresh fruit, respectively (Gorinstein *et al*., 2001).

The MeOH solvent was able to extract most of the phenolics due to its high polarity. Similar results with 0.48% w/w phenolics content have been reported from Valencia late juice (Rapisarda *et al*., 1999). The total polyphenols content in orange fruits ranged from 0.5 to 1.0 percent w/w in fresh juice (Vinson *et al*., 2001). Further, total polyphenols in three commercially available orange, Jaffa orange, and Florida orange ranged from 0.5 to 0.7 % (Gardner *et al*., 2000).

The phenolic compounds are reported to have anti-oxidant activity *in vitro* and observational studies also support their potential role in protecting cardiovascular health (Hertog *et al*., 1993). The highest yield of citrus peel extract (19.87%) was obtained with MeOH, followed by acetone (15.00%) and diethyl ether (12.75%) in study of use of citrus peel in preservation of oils (Zia ur, 2006). The MeOH extract of lime peel contains different phenolic antioxidant compounds (Alexandra *et al*., 1998). Recent study has shown that the yield of extractable compounds was highest in MeOH extract from pomegranate peel and also citrus in comparison with the solvents such as ethyl acetate and water (Jayaprakasha and Patil, 2007; Singh *et al*., 2002).

2.2.4 Quantification of limonoids in citrus

Previous studies have reported that some limonoids were found to be present in the chloroform extract of lemon peel and the most predominant being limonin (Baldi *et al*., 1995). Further, reports showed that the isolimonoic acid was present in significant amounts in Nova tangerine and Cleopatra mandarin seeds (Vikram *et al*., 2007).

2.2.5 Quantification of flavonoids in citrus

The flavonoid composition of various varieties of citrus fruit have been reported and hesperidin was found to be the most abundant flavonoid, in *C. reticulata* Blanco (6.76–12.0 mg/g dry matter), *C. sinensis* (L.) Osbeck (6.98–10.8 mg/g dry matter), and *C. limon* (L.) Bur (3.58 mg/g dry matter) (Kawaii *et al.*, 1999).

Research reports on flavonoids of citrus suggest that flavanone is the major class of flavonoid in oranges (Wang *et al.*, 2007). Similarly, the flavonoid analysis of extract of Pericarpium Citri Reticulatae (FEPCR) by HPLC revealed that the main flavonoid compound in FEPCR were hesperidin (68.45 ± 0.61%), nobiletin (12.11 ± 0.22%) and tangeretin (7.38 ± 0.10%) (Yi *et al.*, 2008).

The hesperidin and eriocitrin are found to be prominent flavonones of lemon, whereas only hesperidin is the major concentration in limes (Peterson *et al.*, 2006). In another study, flavonoid content of Mexican lime was found to be hesperidin (92.4% w/w), eriocitrin (1.8% w/w) and narirutin (2.0% w/w of total flavonoids) (Nogata *et al.*, 2006). In addition to flavanone, limes are known to contain flavonol such as rutin (Nogata *et al.*, 2006).

The rutin, didymin and hesperitin are the other flavonoids found in lime juice. The presence of rutin along with other class of flavonoids makes lime juice unique in nature compared to other citrus juices (Calabrò *et al.*, 2005).The major glucoside in citrus juice was limonin 17-O-β-D-glucopyianoside (LG), which constituted over 50 per cent of the total limonoid glucosides in the juices (Fong *et al.*, 1989) . Further, LG was reported as a major glucoside in a variety of citrus juices tested. Orange juice contains an average of 180 ppm or 56 per cent of the total limonoids. Grapefruit juice contains 120 ppm, which accounts for 63 per cent of the total limonoids, while lemon juice is known to contain about 54 ppm, which was 66 per cent of the total limoniod content (Fong *et al.*, 1989).

Citrus fruits are known for their rich sources of bioactive compounds, including vitamin C, phenolics and flavonoids, with potential health-promoting properties (Gorinstein *et al.*, 2001). These bioactive compounds are known to act as free radical-scavengers, to modulate enzymatic activities and to protect against a variety of diseases, particularly cardiovascular diseases and also some types of cancer (Kurowska *et al.*, 2000).

Free radical scavenging is one of the known mechanisms of inhibition of lipid oxidation. In DPPH (1, 1,-diphenyl-2-picryl hydrazine) free radical scavenging assay, anti radical power of an antioxidant is measured as color changes from purple to yellow. This is used to evaluate hydrogen-donating ability of the compound. It is a rapid method and most widely employed to characterize antioxidant activity of plant material (Arnao, 2000).

2.3 Analysis of chemical composition of volatile oils in citrus

Previously D-dihydrocarvone was reported in orange juice and calamondin peel by GC-MS (Selli *et al.*, 2004). Additionally, an ester (neryl acetate) and epoxide (2, 3-dehydro-1, 8-cineole) were also identified.

Recently, fresh and dehydrated lime chemical composition was analyzed by GC-MS. D-limonene was found to be 75.5% and 53.5% in fresh and dehydrated limes respectively (Yadav *et al.*, 2004).Furthermore, chemical composition of nine cultivars of mandarins was reported with D- limonene (>88%) as the major compound in all volatile oils (Merle *et al.*, 2004).

2.3.1 Health and other benefits of volatile oils

Volatile principles of plant origin are known to inhibit cancer cells growth. Volatile oil of black cumin has shown inhibition of 1, 2-dimethylhydrazine-induced aberrant crypt foci (ACF) in rats (Salim and Fukushima, 2003).

Patrinia scabra Bunge root volatile oil has shown cytotoxic activity in human ovarian carcinoma and hepatoma cells (Xiang *et al.*, 2005). Induction of cell death by D-limonene on mammary carcinoma cells has been reported (Haag *et al.*, 1992).

Recent reports indicate protein isoprenylation and apoptosis as the possible mechanisms of cytotoxicity. D-dihydrocarvone, a major constituent of oil, has also shown ability to inhibit microbes of oral cavity and the same is used as one of the ingredients in oral care formulation (Parikh *et al*., 2004).

Caspase-3, a major executor class of caspases is essential for the induction of DNA fragmentation as well as apoptosis (Porter and JaÈnicke, 1996). Its activity was found to be decreased upon acetylation, indicating basic chemical moiety which is essential for enhancing caspase-3 to induce apoptosis (Rochaa *et al*., 2007). Bax, a member of the Bcl-2 protein family, plays a vital role in the induction of apoptosis (Zhang *et al*., 2000). It is an integral membrane protein associated with organelles or bound to organelles by Bcl-2 or a soluble protein found in the cytosol and has shown mobility from cytosol to mitochondria during apoptosis (Wolter *et al*., 1997).

Expression levels of Bax (pro-apoptotic) and Bcl_2 (anti-apoptotic) gene level provide further information regarding apoptosis. Some of the natural compounds such as, flavonoids, and polyphenols have shown significant induction of Bax and depletion in Bcl_2 for induction of apoptosis mediated cell death (Mohan *et al*., 2007). Thymoquine, a volatile active principle has shown induction apoptosis in colon cancer cells (Gali-Muhtasib *et al*., 2006).

The ratio of Bax /Bcl_2 is considered as one of the major biochemical markers for evaluation of tumor/cancer inhibition, i.e. increase in the ratio is considered to be anti-carcinogenic and vice-versa (Zhang *et al*., 2003).

Only few natural compounds have shown to alter the ratio of Bcl_2 and Bax and those which have an effect are considered to be effective candidates for prevention of different types of cancer (Corsetti *et al*., 2008).

Apart from cell culture evidences, *in vivo* studies conducted using D-limonene, a major constituent of volatile oil has showed chemo preventive ability in both initiation and promotion stages of skin carcinoma in chemically induced rodents (Elegbede *et al*., 1986). D-limonene and its derived monoterpinoids have shown to affect $p21^{ras}$ expression by altering overall expression or through farnesylation of proteins (Gelba *et al*., 1995).

2.4 Studies on anticancer activity of purified compounds in citrus seeds using cell lines

Pancreatic cancer is the fourth most common cause of cancer mortality in USA (Anon, 2007). According to the National Cancer Institute, more than 37,680 American men and women will suffer from pancreatic cancer and 34,290 were estimated to die from pancreatic cancer during 2008 (Jemal *et al*., 2008).The high fatality is mainly due to lack of effective screening test for pancreatic cancer (Brand and Mahr, 2005). Late diagnosis of cancer is the main cause for limited option for successful treatment and also development of resistance to most of chemotherapy and radiotherapy (Lowenfels and Maisonneuve, 2005). Hence, prevention seems to be the most promising strategy to reduce the mortality rates of pancreatic cancer (Bobe *et al*., 2008).

Based on the success rate and complications from currently available synthetic drugs for pancreatic cancer, treatment using natural compounds has gained considerable attention due to their safety and efficacy in overcoming tumor cell resistance to apoptosis (Bharti and Aggarwal, 2002).

Current research information available suggest that few natural compounds have demonstrated potential benefits in pancreatic cancer prevention including curcumin (Aggarwal and Sung, 2008), flavonoids (Zhang *et al*., 2008) and isoflavones (Awale *et al*., 2008).

Modified natural compounds such as glycyrrhetinic and ursolic acid have also shown significant inhibition of pancreatic cells (Chadalapaka *et al*., 2008). Curcumin and proanthocyanidins have shown highly promising results in clinical trials at different stages by acting on different cell signaling pathways such as, p53, NF-κB, MAPK, Akt, AR and ER (Sarkar and Li, 2004).

Recent research indicated that certain citrus limonoids are found to inhibit proliferation of cancer cells, such as MCF-7 (Tian *et al*., 2001). Inhibition of proliferation of colon cancer cells and neuroblastoma by four glucosides of citrus has been reported. (Poulose *et al*., 2006). Studies have also shown that apoptosis is the major cause for inhibition of proliferation of colon cancer cells by citrus limonoids (Jayaprakasha *et al*., 2007; Jayaprakasha *et al*., 2008; Poulose *et al*., 2006).

2.5 Prevention of colon cancer by citrus peel compounds

In spite of understanding the exact sequence of genetic mutation of the adenoma to carcinoma in colorectal cancer, it continues to be the main cause of death. Every year about 78 lakhs of new cases are diagnosed world wide. Colorectal cancer is common in industrialized countries and is a public health priority because of the high incidence and mortality associated with it. Further, colorectal cancer is the third most commonly diagnosed cancer in the United States and the third most common cause of cancer death among men and women put together. In 2008, 1.49 lakh people were diagnosed with colorectal cancer and 49,960 deaths were expected from the disease. The colon cancer accounted for 72 per cent of total colorectal cancer and cases of rectum cancer was 28 per cent (Jemal *et al*., 2008).

A number of studies have shown that certain citrus fruits contain photochemicals which induce proliferation inhibition of some cancer cell (Arias and Ramón-Laca, 2005) and the cause of cell death may be due to apoptosis in case of human colon cancer cells (Kim *et al*., 2005). Citrus species are known to accumulate significant amounts of limonoids and flavonoids during the process of plant development and growth (Castillo *et al*., 1992). Recent studies indicate the presence of potential dietary bioactive compounds in citrus fruits which possess cancer prevention properties (Poulose *et al*., 2006; Vanamala *et al*., 2006).

Recent research report showed that grape fruits juice are a rich source of bergamottin, paradisin A, and paradisin B (Fukuda *et al*., 2000). Further, the composition of furocoumarins in various juice fractions using LC–MS was reported.(Manthey and Buslig, 2005). A similar compound such as epoxybergamottin was isolated from grapefruit peel using diethyl ether (Wangensteen *et al*., 2003). Similarly, column chromatography along with HPLC was used to isolate paradisin C from hexane–ethyl acetate extract of grapefruit juice (Ohta *et al*., 2002). The spectroscopic behaviour of coumarin derivatives have been studied and reported. Both proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopic properties of coumarins have been reported (Cussans and Huckerby, 1975; Stect and Mazurek, 1972).

Mass spectrometry has been found to be an important tool in the characterization of natural as well as synthetic coumarins. Earlier reports of electron impact-mass spectrometry (Corda *et al*., 1998; Kutney *et al*., 1971) and recently electrospray ionisation mass spectrometry (Concannon *et al*., 2000) have reported to be helpful in structural characterisation of coumarins. Studies on the separation and identification of coumarin derivatives by capillary electrophoresis have been reported (Ochocka *et al*., 1995; Suntornsuk, 2002). Their voltammetric behaviour has also been reported(Wu and Dewald, 2001).

2.6 Prevention of pancreatic cancer by citrus juice extracts

Pancreatic cancer is one of the most devastating of all malignancies with the highest mortality compared to other cancer (Li *et al*., 2004), and is the fourth leading cause of cancer death in the USA (Jemal *et al*., 2008). Pancreatic cancer is known to exhibit resistance to the most of chemotherapeutic agents such as, 5-fluorouracil, taxol, doxorubicin, cisplatin and camptothecin, and persist to be therapeutic problem (Li *et al*., 2004). Recently, new strategies for suppressing the pancreatic tumor cells with safe and naturally occurring dietary chemopreventive biomolecules are attracting attention due to their ability in limiting the tumor cell resistance to apoptosis (Bharti and Aggarwal, 2002).

Epidemiological studies have clearly demonstrated inverse relation between consumption of fruits and vegetables with incidences of pancreatic cancer (Chung *et al*.,

2004). A large number of case-control studies and one cohort study have also reported similar findings (Baghurst *et al*., 1991; Howe *et al*., 1992; Shibata *et al*., 1994).

Recent results have demonstrated the inhibitory activity of the proliferation of number of cancer cells by citrus limonoids(Tian *et al*., 2001). Studies have also shown that the major cause for inhibition of proliferation of colon and neuroblastoma cancer cells by citrus limonoids is due to apoptosis (Jayaprakasha *et al*., 2007; Jayaprakasha *et al*., 2008; Poulose *et al*., 2006).

The induction of tumor suppressor protein (p53) in cells treated with different extracts of lime juice suggests the potential of lime juice in p53 mediated apoptosis induction. Mutations of these proteins or dysfunctions is one of the major causes for malignant transformation (Baudier *et al*., 1992; Lavin and Gueven, 2006).

Failure of the p53 tumor suppressor protein is a causal incident in the pathogenesis of a large portion of human malignancies (Fridman and Lowe, 2003). The p53 is a transcription factor co-ordinating cellular responses to stresses, such as DNA damage and oncogene activation after induction, p53 alters the expression of a huge set of target genes leading to cell-cycle arrest, apoptosis, increased DNA repair, and/or inhibition of angiogenesis (Giono and Manfredi, 2006; Vogelstein *et al*., 2000).

Apoptosis is controlled by the complex interaction between regulatory proteins from the Bcl-2 family. The death inducing intrinsic pathway of apoptosis are controlled by these pro and anti apoptotic protein (Marzo and Naval, 2008). The caspase-3, which is also known as executor caspase is believed to serve as a general mediator of apoptosis in the pathway and is activated early during apoptosis. Activated caspase-3 is often considered the key executioner of apoptosis because of its ability to cleave a vast array of proteins (Green and Reed, 1998).

Research has shown that five flavonoids, luteolin, quercetin, kaempferol, apigenin, and taxifolin, markedly inhibited cancer cell lipogenesis, in both prostate and breast cancer cells, and a remarkable dose-dependent response was observed between flavonoid-induced inhibition of cell growth, and induction of apoptosis (Brusselmans *et al*., 2005). In addition, reports also suggest treatment with kaempferol resulted in a dose- and time-dependent reduction in cell viability and DNA synthesis (Nguyen *et al*., 2003).

The caspase-3, which is also known as executor caspase is believed to serve as a general mediator of apoptosis in the pathway and is activated early during apoptosis. Activated caspase-3 is often considered the key executioner of apoptosis because of its ability to cleave a vast array of proteins (Green and Reed, 1998).Apoptosis is an active physiological process leading to cellular self-destruction that involves precise morphological and biochemical changes in the nucleus and cytoplasm (Khan and Mlungwana, 1999).

Natural compounds that inhibit the proliferation of malignant cells through induction of apoptosis may represent a helpful holistic approach to both cancer chemoprevention and chemotherapy. Though several anticancer agents have been developed but have serious shortcomings like adverse side effects and resistance (Panchal, 1998).

There is need for more attention towards research in the use of plant materials for the treatment/prevention of cancers and also development of safer and more effective therapeutic agents (Ramos, 2007). Normally, apoptosis is a result of a complex interplay between regulatory proteins from the Bcl-2 family (Williams and Smith, 1993). These pro- and anti-apoptotic proteins are key regulators of the intrinsic pathway of apoptosis, controlling the point of no return and setting the threshold for engagement of the death machinery (Marzo and Naval, 2008).

Previous reports have shown that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals (Chang *et al*., 2005). Therefore, Bcl-2 proteins have emerged as an attractive target for the development of novel anticancer drugs (Mohammad *et al*., 2008). Changes in the Bcl-2/Bax ratio have been reported to be caused by downregulation of Bcl-2 and slight downregulation of Bax (Cha *et al*., 2004) downregulation of Bcl-2 and upregulation of Bax (Han, 2008; Paris *et al*., 2007) and downregulation of Bcl-2 with no change in the level of Bax (Motomura *et al*., 2008).

2.7 Prevention of pancreatic cancer by citrus seed and peel extracts

Citrus fruits are widely used for juice purpose and the average juice yield of processed citrus fruits is about 50 per cent of total, on weight basis (Bovill, 1996). The primary waste fraction after processing the fruits for juice extraction includes seed and peel. The seeds can be utilized to recover limonoids, which are known for their anticarcinogenic/chemopreventive activities (Braddock, 1995). Further these fractions are also a good source of phenolic compounds, which are rich in flavonoids and hence make them very good markers of adulteration in commercial juices (Marini and Balestrieri, 1995; Mouly *et al*., 1994).

Apart from anticancer property of citrus fruits, several antioxidant compounds have been identified in the seeds of citrus (Gorinstein *et al*., 2004; Jayaprakasha and Patil, 2007; Mandadi *et al*., 2007). These compounds include flavonoids, which can scavenge free radicals and also chelate metal ions, and hence they are potential antioxidants. Hesperidin is a type of flavonoid present in several vegetables and fruits but mainly in citrus (Cserhati, 1995).

Hesperidin is known to possess certain biological activities including antioxidant property, and inhibition of prostaglandin biosynthesis, and also known to inhibit chemical carcinogenesis (Kupfer and Bulger, 1987). In addition, these naturally-occurring antioxidants can be formulated to give neutraceuticals that can help to prevent oxidative damage from occurring in the body (Moller *et al*., 1999).

Recent human cancer cell culture and animal studies have shown citrus flavonoids and limonoids to protect against a variety of chronic diseases such as atherosclerotic plaque formation and cancer (Poulose *et al*., 2006; Poulose *et al*., 2007).

The p53 is known as death inducing protein, which play a vital role in cancer initiation. Mutation of p53 has been reported in cancers such as, colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues, and hemopoietic tissues (Hollstein *et al*., 1991). Mutation of K-ras and p53 genes are known to play a role in pancreatic cancers (Pellegata *et al*., 1994).

In another study, p53 gene transduction has shown direct inhibition of telomerase activity, independent of its effects on cell growth arrest, cell cycle arrest, and apoptosis in human pancreatic cells (Kusumoto *et al*., 1999). Lectin from Polygonatum cyrtonema has shown induction of p53 mediated apoptosis in human melanoma A375 cell (Liu *et al*., 2009).

Other natural compounds which have shown induction of p53 mediated apoptosis are, curcumin, resveratrol, anthocyanins and capsaicin (Khan *et al*., 2008; Kuramori *et al*., 2009; Qin *et al*., 2009). Expression of p21 is regulated by p53 in both dependant and independent manner during cell differentiation and DNA damage (Macleod *et al*., 1995). The gene p21 has been known for regulation independent of p53 in situation such as, tissue development, serum stimulation and cell differentiation. Such independent expression of p21 has been reported in pancreatic cancer cells (Digiuseppe *et al*., 1995).

Members of bcl-2 family proteins are key regulators of the process of apoptosis. The bcl-2 is an upstream effectors molecule in the apoptotic pathway and is identified potentially anti-apoptotic. The bcl-2 proteins seem to form a heterodimer complex with Bax, which results in neutralizing its proapoptotic effects of inducing cell death (Khan *et al*., 2007). Hence, the ratio of Bax/bcl_2 is considered as one of the major markers of apoptosis. Very few compounds of natural origin, such as EGCG (present in pomegranate and tea) and resveratrol from grape skin are known to affect the ratio (Kalra *et al*., 2008).

Recent research evidence suggest that citrus fruits have anticancer effects, including reducing the proliferation of some cancer cells (Arias and Ramón-Laca, 2005), and the induction of apoptosis in human gastric and colon cancer cells (Kim *et al*., 2005). Further, recent research has shown that citrus contains several possible anti-cancer agents such as flavonoids and limonoids (Poulose *et al*., 2006; Vanamala *et al*., 2006).

2.8 Prevention of colon cancer by citrus volatile oils

Colon cancer is one of the most prevalent cancers throughout world and especially in western countries. This is continuously increasing worldwide due to rapid changes in dietary pattern and preferences. Many epidemiological studies indicated that western-style diet, primarily, the consumption of red meat, is positively associated with a high colon cancer incidence (Abeysinghe *et al*., 2007).

.Investigations have also provided evidences for health-promoting properties of these compounds, such as *in vitro* antioxidant activity, prevention of cancer in animal and cell culture studies (Jayaprakasha *et al*., 2007; Jayaprakasha *et al*., 2008).

Recent research has also demonstrated that citrus bioactive compounds are capable of inhibiting human neuroblastoma and colon cancer cells (Poulose *et al*., 2006). An animal study has revealed that freeze dried grapefruit juice powder (13.7 g/kg) and its bioactive compounds naringin and limonin (200 mg/kg) are capable of inhibiting aberrant crypts through the suppression of cyclo-oxygenase-2 and inducible nitric oxide synthase (iNOS) in azoxymethane treated animals (Vanamala *et al*., 2006).

Pharmaceutical industries also use lime volatile oil as flavoring agent in syrups and suspensions (Porta *et al*., 1997). In perfumery, lime volatile oils have been used as base for many compositions, which have higher market value per pound than other citrus varieties like orange, grapefruit, or tangerine volatile oils. Some of the reported chemical compositions of lime volatile oil consists of D-limonene, α-terpineol, 4-terpineol, 1,4-cineole, 1,8-cineole, p-cymene, β-pinene, β-bisabolene, citral, geranial and neral (Ranganna *et al*., 1983).

Recently some more additional flavour compounds, such as neryl acetate, α-bergamotene, valencene and germacrene-D have been reported (Veriotti and Sacks, 2001). Kaffir lime (*Citrus hystrix*) volatile oil has demonstrated to reduce blood pressure and relieve depression in human studies (Hongratanaworakit and Buchbauer, 2007), which provides strong evidence on potent health benefits of citrus volatile oils. The above studies have demonstrated that citrus fruits and their bioactive compounds have significant role in human disease prevention.

Most of the principle components present in lime volatile oil are monoterpenes. Monoterpenes have shown prevention of mammary, lung, skin, liver and forestomach cancer in rat models (Haag *et al*., 1992). D-limonene is metabolized into perillic acid, dihydroperillic acid and limonin 1,2-diol and these have higher bioavailability (Crowell *et al*., 1994).

Volatile principles of plant origin are known to inhibit cancer cells growth. Volatile oil of black cumin has shown inhibition of 1, 2-dimethylhydrazine-induced aberrant crypt foci (ACF) in rats (Salim and Fukushima, 2003).

Patrinia scabra Bunge root volatile oil has shown cytotoxic activity in human ovarian carcinoma and hepatoma cells (HongXiang *et al*., 2005). Induction of cell death by D-limonene on mammary carcinoma cells was reported (Haag *et al*., 1992). D-dihydrocarvone, a major constituent of oil, has also shown ability to inhibit microbes of oral cavity and the same is used as one of the ingredients in oral care formulation (Parikh *et al*., 2004). The next major compound identified was m-Mentha-6, 8-diene R (+) which constituted 9.31 per cent. This component has also been reported from ginger and *Piper nigrum* and demonstrated antioxidant and antiproliferation activity (Ma and Gang, 2004).

Caspase-3, a major executor class of caspases, is essential for the induction of DNA fragmentation as well as apoptosis (Porter and JaÈnicke, 1996). Few natural compounds are known to alter caspase-3 to induce apoptosis in cancer cells. Triterpenoids of natural origin are known for elevating levels of caspase-3 in leukemia cells. Activity was found to be decreased upon acetylation, indicating basic chemical moiety is essential for enhancing caspase-3 to induce apoptosis (Rochaa *et al*., 2007).

Bax, a member of the Bcl-2 protein family, plays a vital role in the induction of apoptosis (Zhang *et al*., 2000). It is an integral membrane protein associated with organelles or bound to organelles by Bcl-2 or a soluble protein found in the cytosol and has shown mobility from cytosol to mitochondria during apoptosis (Wolter *et al*., 1997).

Expression levels of Bax (pro-apoptotic) and Bcl_2 (anti-apoptotic) gene level provide further information regarding apoptosis. Some of the natural compounds such as, flavonoids, polyphenols have shown significant induction of Bax and depletion in Bcl_2 for induction of apoptosis mediated cell death (Mohan *et al.*, 2007). Thymoquine, a volatile active principle has shown induction of apoptosis in colon cancer cells (Gali-Muhtasib *et al.*, 2006).

The ratio of Bax /Bcl_2 is considered as one of the major biochemical marker for evaluation of tumor/cancer inhibition, i.e. increase in the ratio is considered to be anti-carcinogenic and vice-versa (Zhang *et al.*, 2003). Only few natural compounds have shown to alter the ratio of Bcl_2 and Bax and those which have an effect are considered to be effective candidates for prevention of different types of cancer (Corsetti *et al.*, 2008).

Morphology of cells treated with volatile oil was similar to that of camptothecin with irregular shape and most of the cellular contents was found as small fractions confirming the cells death. Apart from cell culture evidences, *in vivo* studies conducted using D-limonene, a major constituent of volatile oil has showed chemopreventive ability in both initiation and promotion stages of skin carcinoma in chemically induced rodents (Elegbede *et al.*, 1986).

D-limonene and its derived monoterpinoids have shown to affect $p21^{ras}$ expression by altering overall expression or through farnesylation of proteins (Gelba *et al.*, 1995).

3. MATERIAL AND METHODS

Citrus aurantifolia (Lime) is a major citrus fruit and widely consumed and are known world-wide for their tart, tangy-flavored juice and especially for their unique flowery, characteristic aromas. Limes are popular for use as juice and carbonated beverages. In some Asian countries, they are used in pickling, culinary, and medical applications. Citrus fruits are a rich resource of biologically active compounds that help prevent lifestyle-related diseases such as diabetes, high blood pressure and cancer. Several studies have reported that citrus fruit have anticancer effects, including the reduced proliferation of some cancer cells and the induction of apoptosis in human gastric and colon cancer cells. But there is limited evidence about heath promoting ability of lime fruits. Hence an investigation, to isolate and purify bioactive compounds in lime seed and peel and also study the chemopreventive effects of isolated compounds along with extracts of edible (juice) and un edible parts (seed and peel) of lime fruits. An attempt has been made in the present investigation to isolate the bioactive compound from lime and study their antioxidant properties and also anticancer activity of purified compound and extracts by different methods. The research was carried out at Vegetable and Fruits Improvement Centre, Department of Horticultural Sciences, Texas A&M University, College Station, Texas, USA.

It was also intended to distil the volatile oil from lime and study the chemical composition and anticancer activity from the volatile oil. The material used and methods employed are presented in this chapter.

3.1. Isolation of bioactive fractions from limes using chromatographic techniques

3.1.1. Plant material

Lime fruits were harvested during late March, 2006 from the farmer's orchard of Bijapur (Karnataka, India). The seeds were separated manually and dried under shade at > 25°C. Dried seeds containing less than 8 per cent moisture were then powdered to a mesh size of 20 using a blender.

3.1.2 Chemicals and Reagents

All the solvents/chemicals used were of analytical grade except HPLC analysis were HPLC grade solvents, were used and obtained from Fisher Scientific (Somerville, NJ, USA). Silica gel (200 - 400 mesh) and dowex-50 (50-100 mesh) were purchased from Aldrich (Saint Louis, MO, USA). Sepabeads adsorbent resin was purchased from Supelco (Bellefonte, PA, USA). TLC plate's Silica gel 60 F-254, thicknesses 0.20 mm (20 cm x 20 cm) were obtained from Alltech Associates, Inc. (Brentwood, TN, USA). *Para-N*, *N*-dimethyl amino benzaldehyde (Ehrlich reagent) was obtained from Sigma Chemical Co., (St. Louis, MO, USA). Chemicals and media for cell culture were obtained from Hyclone (Logon, UT, USA) and ATCC (Manassas, VA, USA). Anti-caspase-3 3 (34 kDa), anti-cytochrome-c, anti-bcl2, p53, p21anti-Bax and anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and HRP-conjugated goat anti-mouse secondary antisera was from Pierce Biotechnology Inc.(Rockford, IL, USA). Annexin-V FITC kit was from BioVision Research Products (Mountain View, CA , USA).

3.1.3 Extraction

Lime seed powder (2.459 kg) was extracted in a Soxhlet type apparatus with hexane for 8 h for the removal of fatty matter. The defatted powder was extracted successively for 8 h, each with ethyl acetate, acetone, MeOH, MeOH: water (80:20) at 60-70°C. The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) separately to obtain viscous concentrate. The yields of these extracts were 11.0 (ethyl acetate), 15.9 (acetone), 61.2 (MeOH), 44.4 (MeOH: water) g/kg of seed. This concentrate was subjected to freeze drying and the dried material was stored in -20°C until further use.

3.1.4 Purification of ethyl acetate extracts (Limonoids -aglycons and sitosterols)

Freeze dried extract (21.8 g) was impregnated with silica gel and loaded onto silica gel (100 cm x 35 mm) column. The column was washed thoroughly with hexane and eluted with linear gradient solvent of hexane and mixture of hexane-chloroform, acetone and MeOH with increasing polarity. Fractions (1000 ml each) were collected and analyzed by TLC and HPLC. Fractions containing same spots/peaks were pooled and concentrated under vacuum and crystallized. Compounds 1, 2, 3 and 4 were eluted with chloroform (100 per cent), acetone: chloroform (15:85), acetone: chloroform (20:80), acetone: chloroform (25:75) and yielded 4.1, 0.33, 0.51 and 0.26 g, respectively.

3.1.5 Purification of MeOH and MeOH: water extracts (Glucosides)

Freeze dried MeOH (260 g) extracts and MeOH: water (124 g) were combined and loaded onto activated dowex [H^+] resin column. The column was washed thoroughly with excess water. Elute from dowex column was passed through sepabeads resin (120 cm x 10 cm), which was then eluted with a linear gradient solvent of 1per cent acetonitrile in water to 15 per cent acetonitrile in water. Fractions (1000 ml each) were collected at a flow rate of 30 ml/min. All the fractions were analyzed by HPLC. Fractions containing similar peaks were pooled and concentrated under vacuum. The concentrated fractions were stored for crystallization at 3-4 ℃. Fractions eluted with 7.5 per cent, 10.0 per cent and 12.5 per cent acetonitrile in water gave compound 5. This compound was collected by filtration and was dried under vacuum desiccator to obtain pure compound 5 with an yield of 1.36 g.

3.1.6 Identification of bioactive compounds

3.1.6.1 TLC analysis

Purified compounds (1-5) were spotted on silica gel 60 F-254 plates. The plates were developed using hexane: chloroform (1:9), acetone: chloroform (1.5: 8.5) acetone: chloroform (2:8), acetone: chloroform (2.5:7.5) and chloroform: MeOH (1: 1). The plates were sprayed with Ehrlich's reagent (N, N, dimethyl amino benzaldehyde in ethyl alcohol) @2.0 per cent and developed in an HCl gas chamber. Typical pink / reddish coloured spots were obtained for limonoids. In addition, the plates were sprayed with 10 per cent sulfuric acid in MeOH followed by heating at 100 ℃ for 10 minutes to detect any other impurities.

3.1.6.2 HPLC analysis

All the column fractions and compounds (1-5) were subjected to HPLC analysis using Agilent Technologies 1200 series (Santa Clara, CA, USA). All the compounds were separated on C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 mm × 4.6 mm) and detected at 210 nm. All the compounds were quantified using Chem Station software. The gradient mobile phase consisted of (A) 3 mM phosphoric acid (B) acetonitrile at a flow rate of 1.0 ml/min. The elution of binary solvent was conducted in gradient fashion, starting at 85% of solvent A, reduced to 77% in 5 min, 74% after 25 min, which was further reduced to 60% at 30 min and completing the gradient at 54% at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run. The flow rate was kept at 1.0 ml/min. All the standards and samples were filtered through 0.45 µm millipore filter.

3.1.6.3 Calibration curve

The linearity of the method was evaluated by analyzing a series of limonoids such as limonin, nomilin, isolimonic acid, ichangin, isoobacunoic acid, limonin glucoside and deacetyl nomilinic acid glucoside. About 20 µL of each of the six working standard limonoids solutions containing 0.5–8.0 µg were injected on to the HPLC and elution was carried out as discussed above and peak area responses were obtained. The calibration curve for each limonoid was prepared by plotting concentration of limonoid versus peak area (average of three runs).

3.1.6.4 Mass spectral analysis

Purified compounds were identified by mass spectra using ThermoFinnigan LCQ-DECA instrument (Thermo, San Jose, CA, USA). MS conditions were 450 ºC vaporizer with 300 µl of flow of MeOH with 5 µA current, 30 PSI sheath and 15 PSI aux.

3.2 Isolation of bioactive fractions (coumarins) from lime peel using flash chromatography techniques

3.2.1 Extraction

Mature limes were collected from Texas A&M University-Kingsville, Citrus Center, Weslaco, Texas, USA in the month of December 2006. Juice, peel and seeds were separated manually and the peel was dried under shade to complete dryness. Further, the peel was powdered to 20 mesh. Lime peel powder was successively extracted using a Soxhlet type extractor with hexane for 8 h, the extracts were cooled, filtered, concentrated to remove 95 per cent of solvent and freeze-dried and stored at -20 º C for further use.

3.2.2 Purification of Coumarins

Flash chromatography

Sample preparation**:** 25 g of lime peel hexane extract (freeze dried) was dissolved in 10 ml of chloroform, and 10.0 g of silica gel was added and thoroughly mixed over shaker and kept in hood for drying.

Details of column used and solvents:

a) Column: 330 g

b) Solvents: Acetone and Hexane

The column was eluted with hexane and acetone, fractions 33,44 and 48 were collected and analyzed by TLC and HPLC for furocoumarins. Fraction number four showed furocoumarins and it was subjected to concentration under vacuum.

3.2.3 TLC analysis

Purified compounds were dissolved in methanol, spotted on TLC plates, and separated using hexane/ethyl acetate (4:1) as mobile phase. The compounds were visualized as black spots when sprayed with 10% sulfuric acid in methanol, followed by heating at 110 ºC for 10 min.

3.2.4. HPLC analysis

Quantification of bioactive compounds by HPLC analysis

The HPLC system consisted of Perkin Elmer Series pump 2000, coupled with Perkin Elmer Series 2000 autosampler and Perkin Elmer Diode Array detector 235C. Limonoids were separated on C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 mm × 4.6 mm) and detected at 210 nm. Binary solvent system used was 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B), other parameters were maintained as per published method (Vikram *et al.,* 2007). The compounds were quantified using TurboChrome Navigator Software ver. 6.1.2.0.1 (Perkin Elmer, Boston,MA, USA).

3.2.5 Identification

^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a JEOL AMX 400 FT instrument (JEOL-USA, Inc., Peabody, MA). TMS was used as internal standard. ^{13}C NMR assignments were given on the basis of 2D experiments.

3.3 Studies on antioxidant activities of bioactive compounds in lime juice, peels and seeds extracts

3.3.1 Plant material

Limes (*Citrus aurantifolia* Swingle) were collected from citrus orchard, located at the Texas A&M University-Kingsville Citrus Center, Weslaco, Texas, USA, during December 2006. Limes were hand squeezed to obtain juice and juice was freeze-dried using Labconco freeze drier (Labconco Corporation, Kansas City, MO, USA). Dried juice was stored at –20 °C until further use. Further, the seed and peels were separated manually and freeze-dried. Samples of seed and peel were then powdered using a mixer and were also stored at –20 °C until further use. These extracts were subjected for the analysis of total phenolics, limonoids and flavanoids, the details of which are indicated below. Further, the antioxidant activity of the extracts was measured.

3.3.2 Chemicals and Reagents

All the solvents used for the extraction and quantification analysis were ACS and HPLC grade, respectively. These chemicals were obtained from EDM chemicals Inc. (Gibbstown, NJ, USA). The other chemicals such as, 1,1-diphenyl-2-picryl hydrazyl (DPPH), Folin–Ciocalteu reagent, trolox, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulphate, ascorbic acid, and catechin were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.3.3 Extraction of phytochemicals

Freeze dried lime juice, seed and peel were powdered to obtain uniform mesh size powder. The powder (462.4 g) was extracted using Soxhlet type apparatus with 2000 ml of chloroform at 55-60 °C for 8 h. The extract was filtered through Whatman no. 1 filter paper in a Buchner funnel to remove juice particles. The residue from the funnel and the Soxhlet apparatus was re-extracted with other solvents such as acetone, MeOH and MeOH: water (80:20) successively 8 h each. All the extracts were pooled and evaporated under reduced pressure in a rotary evaporator (Büchi, Switzerland) at 40 °C to recover almost 95 per cent of solvent. It was further dried in a freeze drier and stored at -20 °C for further use.

3.3.4 Determination of total phenolics

The concentration of total phenolics in the lime juice peel and seed extracts were determined spectrophotometrically (Negi *et al.,* 2003) with slight modification. The freeze dried chloroform extract was dissolved in acetone and other extracts were dissolved in methanol and water (1:1 v/v). Different concentrations (10, 20, 30, 40, 50, 75 and 100 µg) of standard (+)-catechin and extracts were taken in tubes and the volume was adjusted to 0.2 ml by nanopure water. One milli liter of 10-fold diluted Folin–Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution were added to all the tubes. After 30 min.of incubation at 25 °C, the absorbance was measured at 765 nm using spectrophotometer (Beckman Coulter,DU-800, USA). The estimation of total phenolics in all the extracts was carried out in triplicates and the results were expressed as catechin equivalents.

3.3.5 Determination of Limonoids

The lime extracts of juice, seeds and peels were dissolved in MeOH and filtered with 0.45 micron filter and subjected to HPLC analysis. The HPLC system consisted of Perkin Elmer Series pump 2000 coupled with Perkin Elmer Series 2000 auto sampler and Perkin Elmer Diode Array detector 235 C. Limonoids were separated on C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 mm x 4.6 mm) and detected at 210 nm. Binary solvent system used was 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B), other parameters were maintained as per the procedures of Vikram *et al.* (2007). The compounds were quantified using TurboChrome Navigator Software ver. 6.1.2.0.1 (Perkin Elmer, Boston, MA, USA).

3.3.6 Determination of flavonoids

The HPLC system consisted of Waters 1525 binary HPLC pump coupled with Waters 717 plus autosampler and Waters 2996 photodiode array detector (Waters Corporation Milford, MA). Flavonoids were separated on C_{18} Waters Bridge Column**,** 3 µm particle size, (150 mm × 4.6 mm) and compounds were detected at 280 nm. Binary solvent system used were water: acetic acid (96:4 v/v, solvent A) acetonitrile (solvent B) and the elution of binary solvent was conducted in gradient fashion, starting at 85% of solvent A, reduced to 50% after 35 min, and completing the gradient at 85% at the end of 40 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run. The flow rate was kept at 1.0 ml min^{-1}. The calibration curve for each flavonoid was prepared by plotting concentration of flavonoid versus peak area (average of three runs).

3.3.6.1 Chromatographic conditions

Binary solvent system used was 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The elution of binary solvent was conducted in gradient fashion, starting at 85% of solvent A, reduced to 77% in 5 min, 74% after 25 min, which was further reduced to 60% at 30 min and completing the gradient at 54% at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run. The flow rate was kept at 1.0 mL min^{-1}. All standards and samples were filtered through 0.45 µm Millipore filter and 50 µL loops was used in this study. The compounds were quantified using TurboChrome Navigator Software ver. 6.1.2.0.1 (Perkin Elmer, Boston, USA).

3.3.7 Measurement of antioxidant activity of lime juice, peels and seed extract

3.3.7.1 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

Various concentrations (10, 20, 30, and 40 µl) equivalent to 208, 416, 624 and 832 µg/ml of freeze-dried extracts (juice, seed and peel), and ascorbic acid were pipetted into 96 well plates. The total volume of the samples and standards were adjusted to 40 µl by the addition of MeOH. 200 µl of solution of DPPH (100 µM) was added, shaken gently {Singh et al., 2002}. A control was prepared as described above without samples or standards. The changes in the absorbance of all the samples and standards were measured at 517 nm using microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) after 20 min. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following formula, % Radical scavenging activity = (Control absorbance – sample absorbance/control absorbance) x 100.

3.3.7.2 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay

The ABTS was prepared by reaction of 7 mmol/l aqueous ABTS solution and 2.45 mmol/l potassium persulfate solution (Jayaprakasha *et al*., 2008). The mixed solution was stored in the dark for 16 h; the radical cation solution was further diluted in MeOH until the initial absorbance value of 0.7 at 734 nm. Solutions of four extracts of lime juice and ascorbic acid were pipetted into 96 well plates as mentioned in DPPH assay. Diluted ABTS radical solution (200 µl) was added to each well and the readings were recorded after 15 min at 734 nm using micro plate reader with an interval of 3 minutes. The radical scavenging activity was calculated using the formula as mentioned in the DPPH method. All the measurements were conducted in triplicate.

3.4. Studies on chemical composition of lime volatile oil

Volatile oil of lime is widely used as flavouring agent in beverages and food products. Pharmaceutical industries also use lime volatile oil as flavouring agent to mask unpleasant tastes of drugs. Studies were conducted to find out the compounds of lime volatile oil and the details of methods followed are enumerated hereunder.

3.4.1 Isolation of volatile oil

Mature limes were collected in November 2006 from Texas A&M University-Kingsville Citrus Center, Weslaco, Texas. Limes (600 g) were blended with 300 ml of nanopure water

and subjected to hydro-distillation in a Clevenger-type apparatus for 8.0 h. The volatile oil was dried over anhydrous sodium sulfate and stored at 4 °C until further use.

3.4.1.1 Chemicals and reagents

All the chemicals used were of analytical grade and procured from Sigma (St. Louis, MO, USA). D-Limonene, β-linalool, α-terpineol, C_8-C_{20} n-alkanes and α-pinene were obtained from Sigma (St. Louis, MO, USA).

3.4.1.2 Procedure

The chemical composition of volatile oil was analyzed using a Thermofinnigan GC-MS (Thermo Fisher Scientific, Inc. San Jose, CA, USA) equipped with a DSQ (Quadrupole) mass spectrometer. Lime volatile oil samples were diluted 20 times with acetone and 1.0 µl was injected. Separation was carried out on fused silica column, DB-5MS (Restek, Bellefonte, PA, USA), (60 m × 0.25 carrier gas at a rate of 1 ml/min. The injector port temperature was 275 ℃, and column temperature was maintained at 40° C for 12 min and then increased to 330° C at the rate of 2 ℃/min and temperature of the column was maintained for 30 min. The split ratio was 1: 10 and the ionization voltage was 70 eV.

Retention indices for all the compounds were determined according to the Kovats method using n-alkanes as standards. The identification of the compounds was done by comparison of Kovats indices and by matching their fragmentation pattern in mass spectra with those of the NIST library database and published mass spectra (Yadav *et al*., 2004).

KI can play an important role in the qualitative identification of individual components. KI was calculated as follows:

$$KI = 100N + 100 \frac{\text{Log } t'R(A) - \text{log } t'R(N)}{\text{Log } t'R(N+1) - \text{log } t'R(N)}$$

where,

KI = Kovats index
CI = Chemical ionization

3.5 Studies on anticancer activity of purified compounds and extracts

Limes collected earlier and separated into juice, seed and peel for extraction of various phytochemicals present in them and used for their antioxidant properties were also subjected for the analysis of anticancer properties of various bioactive compounds present in all other parts of lime fruit. The details of the method followed are presented below

3.5.1 Culture of cells and maintenance

Pancreatic cancer (Panc-28) cells were received from Dr. Paul Chiao, Department of Molecular and Cellular Oncology, M. D. Anderson Cancer Center, (Houston, Texas, USA). The cells were cultured in DMEM contain 10 % FBS (Fetal bovine serum) and maintained in a CO_2 incubator at 37°C and 85± 5% RH. These cells were sub-cultured and used for experiment.

3.5.2 Assessment of cell viability

Cell viability was determined using [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay **(**MTT) assay according to a previously described protocol (Mossman, 1983). In order to detect the cytotoxicity of Panc-28, cells were treated with limonin, limonexic acid (LNA), isolimonexic acid (ILNA), sitosterol glucoside (SG) and limonin glucoside (LG) at different (6.25, 12.5, 25, 50,100 and 200 µM) concentrations and incubated for 24, 48 and 72 h. Gemcitabine (50 µM) a drug used in treating pancreatic cancer was used as positive control for the comparison purpose. The control group was

treated with the equivalent amount of dimethyl sulphoxide (DMSO, the maximum of 0.2% of the assay mixture was used). The intensity of formazan, a reduced product of MTT after reaction with active mitochondria of live cells, was determined by measuring the absorbance in 96-well microplate reader (Bio-Tek, Winooski, VT, USA) at a wavelength of 550 nm.

3.5.2.1 Determination of cell proliferation using cell count assay

This assay was performed in juice, seed and peel extracts using freeze dried solvent extracts (EtOAc, acetone, MeOH, and MeOH:water) and purified compounds viz., with limonin, limonexic acid (LNA), isolimonexic acid (ILNA), sitosterol glucoside (SG) and limonin glucoside (LG). Approximately 2×10^4 cells/well were cultured in 12 well sterile plates and incubated for 24 h. Media was replaced by 1.0 ml of fresh Dulbecco's Modified Eagle's Medium (DMEM) containing different concentrations of solvent extracts (25, 50 and 100 µg/ml) or purified compounds (25, 50 and 100 µM) and gemcetabine (50 µM). After 48, 96 and 144 h of treatment, the viable cells were counted using Z_1 coulter particle counter (Beckman Coulter, Miami, FL, USA). Results were expressed as per cent inhibition with respect to control (untreated). Gemcitabine, a chemotherapeutic agent was used as standard at 50 µM for comparison purpose.

3.5.2.2 Studies on the effects of lime compounds on DNA of pancreatic and colon cancer cells

Lime compounds extracted earlier were tested for their ability to inhibit the growth of pancreatic (Panc-28) and colon cancer (SW-480) cells through DNA fragmentation. Cancer cells were grown for two days in a petridish and treated with 100 µM of compounds viz., limonin, LNA, ILNA, SG and LG and incubated for 24 h. Genomic DNA was extracted using phenol : chloroform extraction method, with slight modification after 24 h of treatment. Cells were washed twice with cold PBS and incubated with lysis buffer for 30 min at 60 ºC and these lysates were incubated in ice for 60 min after addition of sodium acetate. The precipitated proteins were removed by centrifugation and clear lysate was incubated with RNAse for 30 min at room temperature. DNA was fractionated by phenol: chloroform: amyl alcohol (24:25:1,v/v/v) mixture and the phenol layer was subjected for precipitation of DNA in 100% ethanol at 4 ºC for overnight. DNA was separated by centrifugation at 5000 g and dissolved with TE buffer after removal of ethanol traces. DNA was quantified by using ND 1000 UV-visible spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) at 280 nm. Equal amount of DNA samples and marker was loaded for the separation on 1.2 per cent agarose gel electrophoresis at 55 mV current. DNA bands were identified by ethidium bromide staining. Gel image was captured using UV19 transilluminator (Fuji Life Science, Irvine, CA, USA).

3.5.2.3 Protein expression analysis

Pancreatic and colon cancer cells ($2x10^6$ cells/100 mm dish) were seeded in DME medium and incubated overnight and compounds viz., limonin, LNA , ILNA, SG and LG and extracts from lime seed, juice and peel (100 µM) were treated and incubated for 24 h. Cells were lysed in buffer (containing 130 mM NaCl, 1 mM dithiothreitol, 2 mg/ml, leupeptin, 10 mM NaF, 1 mM PMSF and 20 mM tris, pH 7.4), and lysates were centrifuged at 3000 g for 15 min (4 ºC) and the supernatant was used for the experiment. Protein content of lysates was determined by BCA (bicinchoninic acid) Protein Assays (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein equivalent to 50 mg of each sample was resolved on 12% SDS-PAGE at 110v for 75 min using Mini-PROTEAN® Tetra electrophoresis system (Bio- Rad laboratories, Hercules, CA, USA). Separated proteins were transferred into 0.45µm nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA, USA) using semi dry transfer system (TRANS-BLOT SD, Bio-Rad, Hercules, CA, USA) at 10v for 40 min. These membranes were blocked for 30 min in tris-buffered saline-tween-20 (TBST; consisting 1 of 150 mM NaCl, 10 mM Tris pH 7.4, 0.05% tween-20) containing 6% dried skimmed milk powder. Membranes were dried and probed with mouse monoclonal anti-AR antibody at 1:2500 dilutions (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for overnight at 4ºC temperature. These membranes were washed four times (10 min each) in TBST (Tris-Buffered Saline Tween-20) with gentle agitation, followed by incubation with horseradish peroxidase (HRP) -conjugated goat anti-mouse secondary antisera at 1:25000 dilutions (Pierce protein research products, Rockford, IL, USA) for 1 h at room temperature. Protein bands were visualized using SuperSignal West Femto maximum sensitivity substrate as

described by the manufacturer (Pierce Biotechnology, Inc., Rockford, IL, USA) Chemiluminescence image was captured using UV19 transilluminator (Fuji Life Science, Irvine, CA, USA). Band intensity was quantified using alpha image 5.5 software (Alpha innotech Corp., San Leandro, CA, USA).

3.6 Studies on the effect of volatile oil on prevention of colon cancer

It was intended to study the effect of volatile oil extracted from whole lime fruits for their ability to prevent the inhibition of human colon cancer cells. Although, most of the volatile oils are concentrated in the peel, the whole fruit was used for the extraction of volatile oil as mentioned in 3.4.1. After the extraction of volatile oil, they were tested for their ability to suppress the growth of colon cancer cells, the details are mentioned below.

3.6.1 Culture of cells and maintenance

Both NIH3T3 (normal cells) and SW-480 cells (colon cancer cells) were obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % Fetal bovine serum along with antibiotics. These cells were sub-cultured and stock culture obtained was used for experiment after 4-5 passage upon observing normal multiplication pattern. Cells were grown at 5% carbon dioxide at 37 ºC, RH of 85± 5 ºC and cultured in 75 cm^2 falcon cell culture flasks.

The cell viability assay was done by MTT and also the inhibition studies by cell count were done as per the method mentioned in 3.5.2 and 3.5.2.1 respectively.

3.6.2 Cytotoxicity through LDH assay

After incubation of volatile oil samples with SW480 cells for 24 and 48 h, the media (without cells) was removed without disturbing cells for LDH assay. 50 µl of the media from each well was transferred to a new plate and 50 µl of LDH reagent and catalyst (1:45) (Roche Diagnostics Corporation, Indianapolis, IN, USA) was added and incubated for 30 min in dark at room temperature. The absorbance was read at 500 nm using the Synergy HT multi detection Micro Plate Reader (BioTek Instruments, Inc., Winooski, VT, USA) . To untreated cells, 20 µl of 10% triton X-100 and the LDH reagent was added and these results were served as positive control. Media without cells served as negative control. The results were expressed as % LDH leakage, which was proportional to the number of dead cells {Schmitt, 2005 #80}.

3.6.3 Acridine orange staining

SW-480 cells were seeded at 10,000 - 15,000 cells/ml in 8 chambered microscopic slide (Lab-Tek, Rochester, NY, USA) and grown for 24 h. Then, media was replaced with fresh DMEM containing 100 µg/ml volatile oil and camptothecin and then incubated for 12 h at 37 ºC. Cells were treated with 0.2 µg of acridine orange, followed by incubation for 10 min in dark. Media was removed and the cells were washed with isotonic solution. These stained cells were observed under Olympus FV1000 confocal microscope with spectral imaging and photoactivation (Olympus America Inc., Center Valley, PA) with 495 nm primary and 515 nm secondary filters. A minimum of 150 cells were counted in four random fields in each slide.

3.7 Statistical analysis

The results were analysed by different statistical methods as follows.

- Results are expressed as means ± standard error mean (S.Em±). The data were analyzed by one-way ANOVA followed by Turke-Kramer multiple comparison test using GraphPad Prism software (version 5.00.288). Differences were considered significant for $P < 0.01$.
- The data obtained from the laboratory experiments in terms of values were calculated to per cent and subjected to two factor analysis of variance (ANOVA) with different checks. Further, the data was subjected to Duncan Multiple Range Test (DMRT) at $p < 0.01$ to separate the means by alphabet (Gomez and Gomez, 1984).

4. EXPERIMENTAL RESULTS

Limes are popular and grown throughout the world and are well - appreciated for their refreshing juice and health benefits. While juice is the main commercial product of limes, volatile oil is widely used as flavoring agent in beverages and food products. Pharmaceutical industries use lime volatile oils as flavoring agent to mask unpleasant tastes of drugs. In perfumery, these volatile oils are used as the base of many compositions which has higher market value per pound than orange, grapefruit, or tangerine volatile oils. Further limes are also a good source of bioactive compounds. In this study, systematic attempt has been made to isolate and study their biological significance in human health in general and pancreatic and colon cancer in particular in cell culture module. Five bioactive compounds were purified from lime seeds using chromatography techniques and identified by TLC, HPLC and MS.

Results of the investigation are categorized objective wise in different sub heads and presented in this chapter.

4.1 Isolation and identification of bioactive compounds in lime seeds

Various bioactive compounds present in lime seeds using defatted seed powder were extracted using four different polar solvents viz., ethyl acetate, acetone, MeOH and MeOH: water. The results of extraction are presented in Table 1. The data indicated there was difference in the yields of extracts among different solvents. It was observed that the yield was maximum with MeOH (6.12 per cent) and minimum with ethyl acetate (1.12 per cent). Further, these extracts were subjected for the column chromatography for isolation of different bioactive compounds. The individual extract fraction were analysed by HPLC for there composition using C18 column.

Results showed that limonoids are present in most of the solvent extracts (Table 2). Ethyl acetate extract had the highest amount of isolimonexic acid (ILNA, 1061 mg/100 g), followed by limonin (390.79 mg/100 g), and limonexic acid (LNA, 210.55 mg/100 g). It was observed that no limonin glucoside (LG) was detected in ethyl acetate extract. A similar trend was observed in acetone extract also. While, MeOH and MeOH: water extracts showed the presence of LG. With respect to the presence of aglycones viz., OBA(obacunone), limonin, LNA, and ILNA; the MeOH extract had the maximum content of OBA (285.49 mg/100 g) followed by LNA (203.85 mg/100 g). While MeOH: water had the maximum content of ILNA (463.05 mg/100 g) followed by LG (364.43 mg/100 g). The lowest limonoid content were observed with MeOH as far as ILNA and MeOH : water as far as LNA was concerned. However, the quantities estimated by HPLC could not be purified completely due to the co-elution of LNA and ILNA.

4.1.1 Separation and purification of bioactive compounds

It was intended to isolate and identify bioactive compounds in lime seeds by column chromatography technique. The mobile phase consisted of hexane, chloroform, acetone and methanol. The ethyl acetate extracts were loaded to column chromatography over silica gel and the column was eluted with hexane and mixtures of hexane, chloroform, acetone and MeOH, to separate three limonoids and a phytosterol. Compounds 1, 2, 3 and 4 were eluted with chloroform (100%), acetone and chloroform (15:85), acetone and chloroform (20:80) and acetone and chloroform (25:75) respectively (Table 3). The yields of putative compounds were 4.0476 g (limonin), 0.5072 g (ILNA), 0.3305 g (LNA) and 0.2611 g (SG).

The HPLC analysis of MeOH and MeOH: water extracts showed 4-5 peaks with different concentrations. Hence, both the extracts were loaded on to dowex-50 [H^+] resin and the column was eluted with excess water. Further, the column was eluted with water, mixtures of acetonitrile: water at different concentrations. All the fractions were analyzed by HPLC for their compositions. Fractions containing similar peaks were pooled, concentrated under vacuum and stored for crystallization at 5℃. Fractions eluted with 7.5 %, 10.0 % and 12.5 % of acetonitrile yielded LG (1.3536 g). Finally, purity of all the compounds was analyzed by TLC and HPLC. The TLC analysis of all the five compounds gave motilities, which matched to reported R_f vales of limonin, LNA, ILNA, SG and LG respectively. No additional spots were

Table 1. Per cent extractable yield and phenolic content in lime seed

Solvents	Yield (g/100g of seeds)	Phenolics (g/100 g of extract)
Ethyl acetate	1.12 ± 0.22	1.75 ± 0.02
Acetone	1.59 ± 0.91	5.52 ± 0.06
MeOH	6.12 ± 0.14	3.15 ± 0.08
MeOH :Water	4.44 ± 0.23	3.49 ± 0.05

Table 2. Limonoid content (mg/100g dry wt. of seeds) in different solvent extracts of lime seeds by HPLC analysis

Compounds	EtOAc	Acetone	MeOH	MeOH:water
OBA	194.66 ± 5.59	19.55 ± 2.68	285.49 ± 2.68	178.90 ± 6.40
LIM	390.79 ± 17.01	48.53 ± 1.98	160.49 ± 6.72	167.60 ± 2.51
LNA	210.55 ± 2.73	58.34 ± 2.63	203.85 ± 0.36	123.88 ± 2.94
ILNA	1061.39 ± 32.23	134.24 ± 53.64	32.47 ± 0.15	463.05 ± 13.98
LG	--	--	235.21 ± 0.92	364.43 ± 2.42

Note: LG: Limonin glucoside, LNA: Limonexic acid
ILNA: Isolimonexic acid, LIM,: Limonin, OBA: Obacunne.
Values are mean of three independent replicates

Table 3. Details of mobile phase, fraction number and compounds isolated from lime seed

A. Results of Ethyl acetate extracts of lime seed in silica column						
Sl. No.	Fraction No.	Mobile phase	Conc.	Compound isolated	Wt. in (g)	Per cent of lime seed
1	47-60	Chloroform	100%	Limonin	4.0476	0.162
2	88-89	Acetone : $CHCl_3$	15 : 85	ILNA	0.5072	0.020
3	91-94	Acetone : $CHCl_3$	20 : 80	LNA	0.3305	0.013
4	98-104	Acetone : $CHCl_3$	25 : 75	SG	0.2611	0.011
B. Results of Methanol extracts of lime seed dowex column						
5	45-70	Acetonitrile	7.5-15%	LG	1.3536	0.020

ILNA = Isolimonexic acid; LNA= Limonexic acid;
SG = sitosterol glucoside ; LG = Limonin glucoside

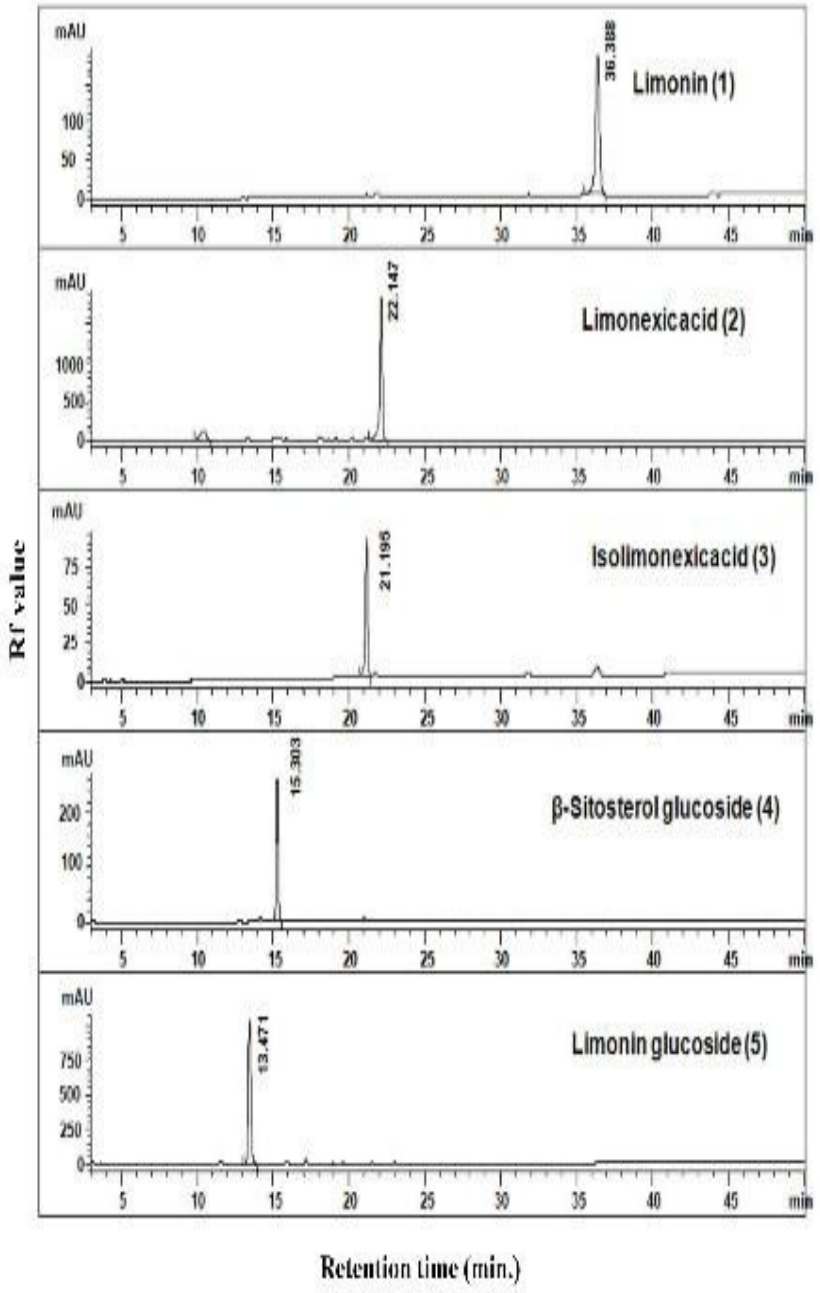


Figure 1. HPLC chromatogram of compounds (1-5) isolated from lime seeds. The reverse phase HPLC was conducted using C_{18} column (250 mm)< 4.6 mm; 5.0µM) using gradient mobile phase consisting of 3 mM phosphoric acid and acetonitrile at a flow rate of 1.0 ml/min. Detection was done at 210 nm

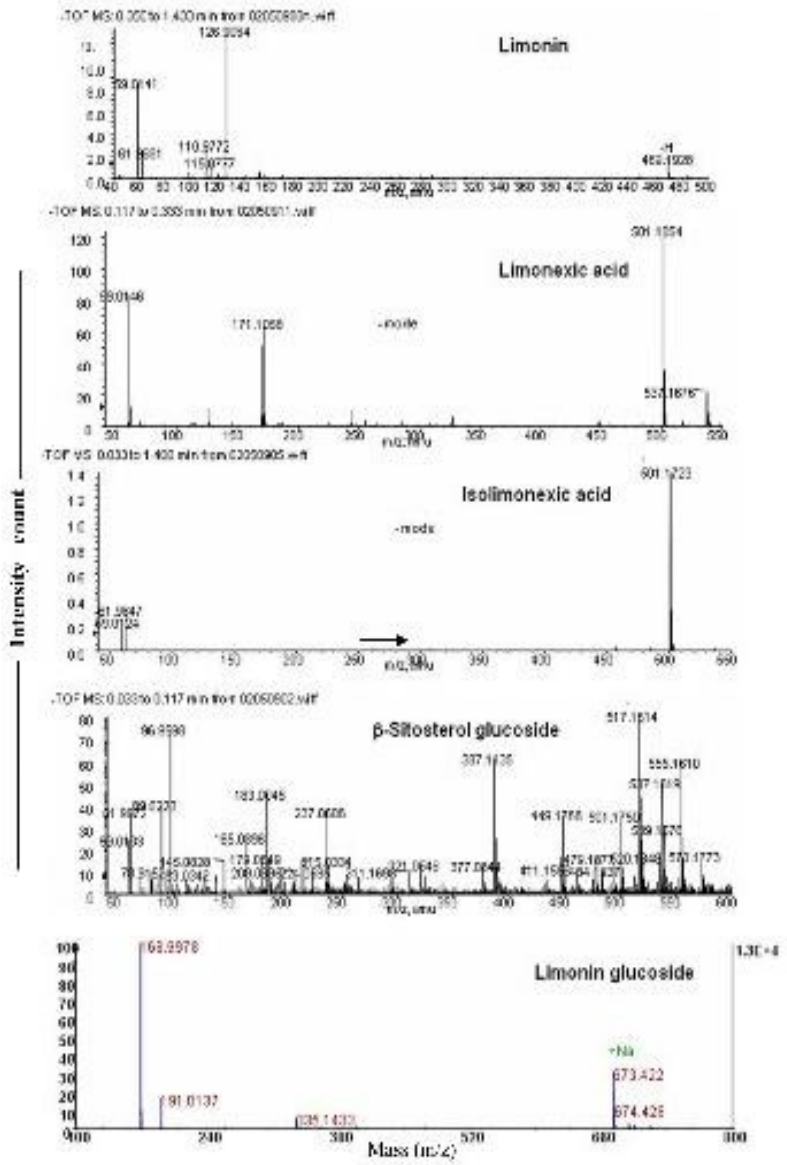


Figure 2. Mass spectra of compounds isolated from lime seeds. MS was recorded using ThermoFinnigan LCQ-DECA instrument, conditions were 450 °C vaporizer with 300 µ1 of flow of MeOH with 5 µÀ current and 30 PSI sheath and 15 PSI aux

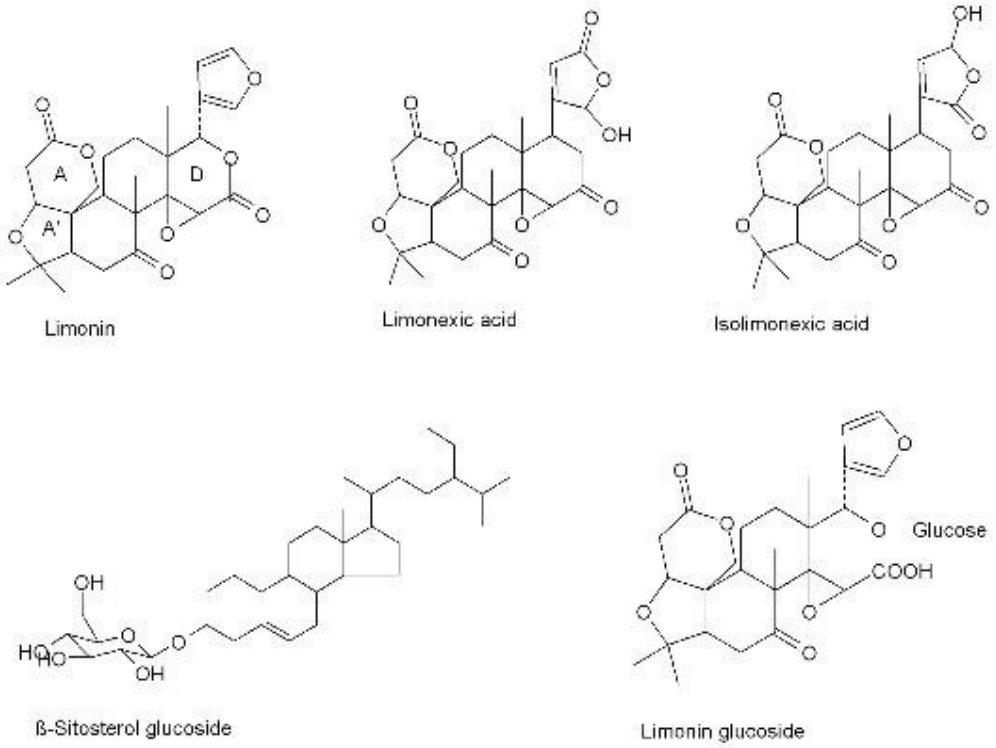


Fig.3. Structures of compounds isolated from lime seeds

visualized with either Ehrlich's reagent or methanolic sulfuric acid followed by heating at 100 ℃ for 10 min, thus confirming the purity of the isolated compounds. Furthermore, the purity of the isolated compounds was confirmed by gradient HPLC. The HPLC chromatograms of purified compounds are depicted in Figure 1. The identity of limonin, LNA, ILNA, SG and LG were confirmed by relative retention times of authentic standards and mass spectra (Figure 2). Compounds limonin, LNA, ILNA, SG showed [M-1] ion at m/z 469.17, 501.18, 501.17 and 575.17. While LG showed [M+Na] ion at 673.428. On the basis of HPLC and mass spectral analysis, all the five compounds were identified as limonin, LNA, ILNA, SG and LG, the chemical structures of which are indicated in Figure 3. Further, the percentage of different bioactive compounds were limonin (0.162), ILNA (0.020), LNA (0.013), SG (0.010) and LG (0.021).

4.1.2 Coumarins in lime peel

The dried lime peel powder (500 g) was extracted with hexane by a Soxhlet type extractor and freeze dried. The extract was loaded on C 18 column of the flash chromatography instrument, and elution was carried out using hexane and acetone to obtain purified fractions of limettin , 5,7, dimethoxy coumarin and isopimpinellin, designated as fractions 1-3 (Figure 4). The purity of compounds (1–3) was analyzed by analytical HPLC. The relative retention times of compounds 1, 2 and 3 were found to be 20.76± 0.12, 36.42 ± 0.32, and 39.54 ± 0.36 min, respectively. All the three compounds were observed as a bluish-white fluorescent spot under UV light on the TLC plates. Further, compounds 1–3 showed UV absorption maxima at 321 and 231 nm under UV light (366 nm), indicating the presence of coumarins. Compounds 1–3 were characterized and identified as Limettin , 5,7, Dimethoxy coumarin and Isopimpinellin, using ^{1}H and ^{13}C NMR spectra. Chemical shifts of the furocoumarins were found to be in accordance with the reported values. NMR spectral assignments were confirmed with the help of 2D NMR such as HMBC, DEFT, DQFCOSY, and HSQC spectra (Figure 5). Further, the percentage of different coumarins isolated from lime were limettin (0.09261), 5, 7-dimethoxy coumarin (0.10237) and isopimpinellin (0.10941) (Table 4).

4.2 Studies on antioxidant activities of bioactive compounds from lime juice, seed and peel

Oxidative stress has been linked to such degenerative diseases as atherosclerosis, and it has been suggested that increased dietary intake of antioxidants may reduce its progression. Antioxidants activity of citrus fraction is also ascribed to their hydrogen donating ability, and may be due to the presence of flavonoids, carotenoids and ascorbic acid. The mechanisms of antioxidant action can include inhibition of reactive oxygen species formation by suppressing enzymes involved in free radical production; scavenging reactive oxygen species; and protecting antioxidant defenses.

In this study, the main objectives was quantification of limonoids and flavonoids by use of high-performance liquid chromatography of lime juice, seed and peel extracts and also explore their radical scavenging potential.

4.2.1 Yield and phenolic content of lime juice

Freeze dried lime juice was extracted with four different solvents viz., chloroform, acetone, MeOH, and MeOH: water. The yield and phenolic content of various extracts presented in Table 5 indicated that among the solvents used, acetone extraction gave maximum yield (16.20 %). However, the highest phenolics were found in MeOH extract (4.20 %) and lowest by MeOH: Water extract (0.66 %). The results suggest that, extracts of lime juice differ in their phenolic content with respect to each solvent. The lowest yield of 3.33 g / 100 g was also observed with MeOH: water.

4.2.3 Quantification of limonoids in lime juice

HPLC analysis of lime juice indicted that chloroform extract contained four aglycones and one glucoside (Table 6; Figure 6). The aglycones were found to be ILNA, LNA, and limonin. LG (36.92 mg/100g) was the only glycoside found in the lime juice. However, among the solvents used, maximum LNA (67.59 mg/100 g) and LG (35.20 mg/100 g) were found in

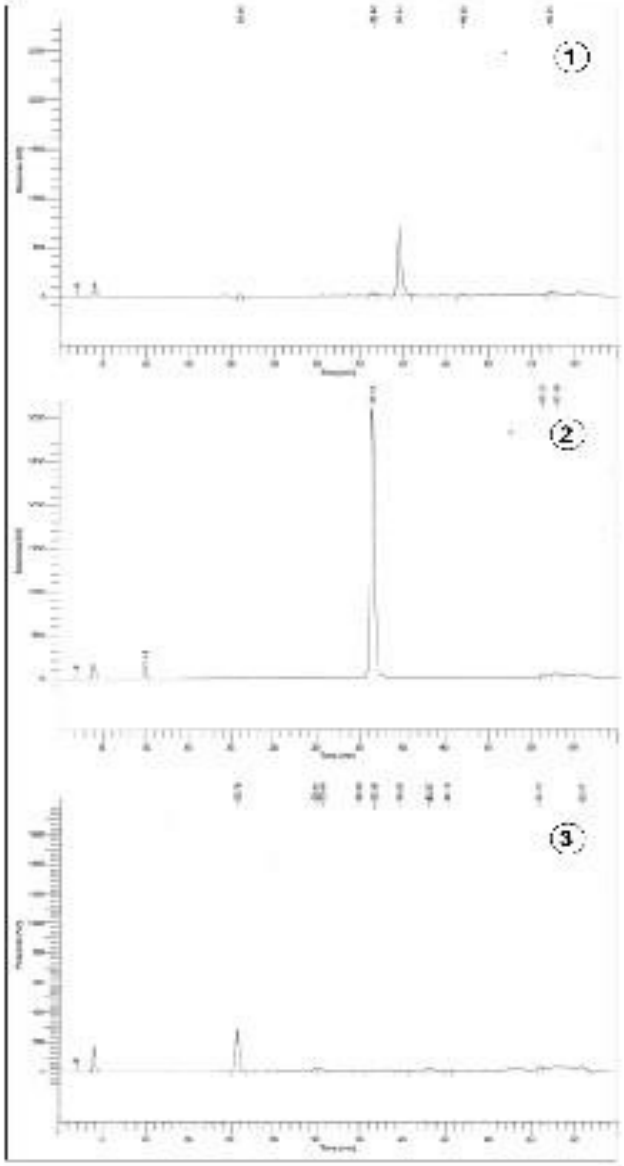


Figure 4. HPLC chromatogram of compounds (1-3) (LimeHin, 5,7 Dimethoxy coumarin and Isopimpinellin) isolated from lime peel. The reverse phase HPLC was conducted using C_{18} column (250 mm x 4.6 mm; 5.OμM) using gradient mobile phase consisting of 3 mM phosphoric acid and acetonitrile at a flow rate of 1.0 ml/min. Detection was done at 210 nm

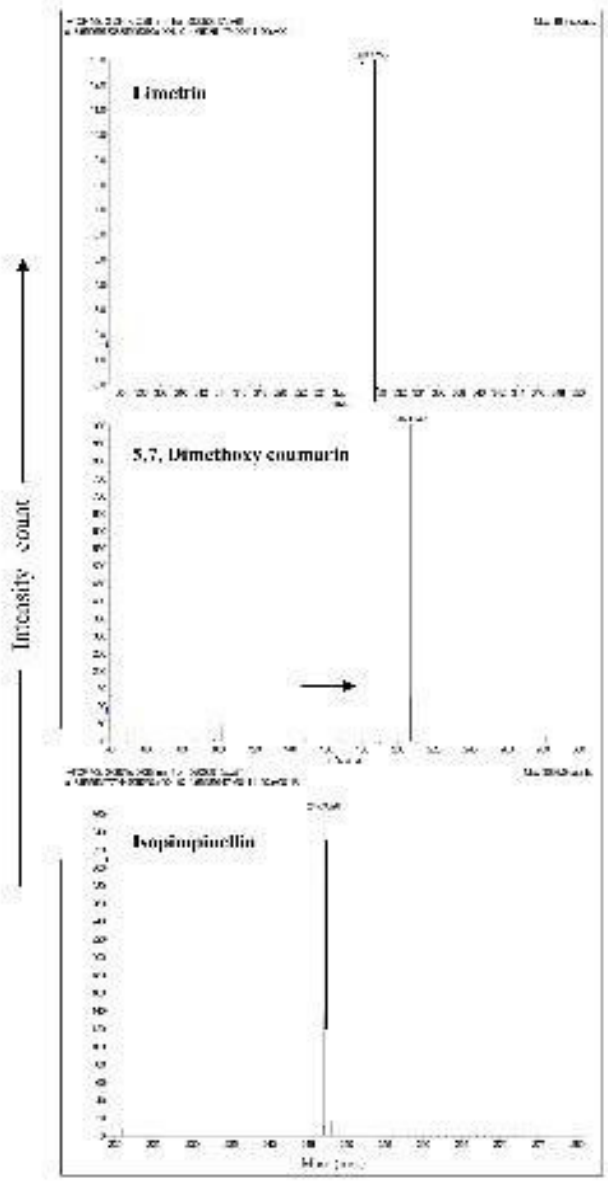


Figure 5. Mass spectra of compounds isolated from lime peel. MS was recorded using ThermoFinnigan LCQ-DECA instrument, conditions were 450 ℃ vaporizer with 300 µ1 of flow of MeOH with 5 µA current and 30 PSI sheath and 15 PSI aux

Table 4. Details of mobile phase, fraction number and coumarins isolated from lime peel

Results of hexane extracts of lime peel by C 18 column of flash chromatography						
Sl. No.	Fraction No.	Mobile phase	Conc.	Compound	wt., (g)	Per cent of lime peel
1	33	Hexane : acetone	40 : 60	Limettin	0.404	0.09261
2	34	Hexane : acetone	15 : 85	5,7, Dimethoxy coumarin	0.446	0.10237
3	42-48	Hexane : acetone	20 : 80	Isopimpinellin	0.477	0.10941

Table 5. Per cent extractable yield and phenolic content of lime juice

Solvents	Yield (g/100 g)	Phenolic in terms of catechin equivalents (g/100 g) *
Chloroform	4.39	3.65 ± 0.21
Acetone	16.20	0.69 ± 0.19
MeOH	8.58	4.20 ± 0.16
MeOH: water (80:20)	3.33	0.66 ± 0.03

* Average of three independent experiments (n =3)
MeOH = Methanol

acetone extract. In both MeOH and MeOH: water none of the limonoids viz., LG, LNA, ILNA, LIM were detected.

4.2.4 Quantification of flavonoids in lime juice

Flavonoid content present in lime juice extracted by reverse phase HPLC indicated the presence of flavanone (hesperidin, hesperitin and didymin) and only one flavonol i.e., rutin (Table 7; Figure 7). Different solvent like chloroform, acetone, MeOH and MeOH : water were used in the extraction of these bioactive compounds and it was observed that three solvents viz., acetone, MeOH and MeOH : water did not yield rutin and hesperitin and didymin. While chloroform extract yielded all the four flavonoid compounds, with a maximum content of 464.48 mg/100 g of rutin and the content of hesperitin (23.87 mg/100 g). The other solvents yielded only hesperidin to the tune of 104.29 mg/100 g with acetone, MeOH (386.24 mg/100 g), and 206.87 mg/100 g for MeOH: water.

4.2.5 Antioxidants activity of lime juice extracts by DPPH assay and ABTS assay

Measurement of antioxidant activity of the lime juice extracts by DPPH and ABTS assays indicated significant difference between the different solvent extract and the concentrations (Table 8; Figure 8). It was further observed that the antioxidant activity of the standard ascorbic acid was significantly superior over different extracts by both the methods. Among extracts, significantly higher antioxidant activity was noticed with chloroform extract by both DPPH and ABTS methods. However, ABTS method recorded higher antioxidant activity in comparison to control but was non-significant. While, no significant differences were observed between acetone extract and methanol extract with respect to antioxidant activity by DPPH method. With an increase in the concentration of the extracts, there was a significant increase in the antioxidant activity with 832 µg/ml recording, the maximum activity irrespective of DPPH or ABTS assay. Significantly lower antioxidant activity was noticed with 208 µg/ml by both the methods. The interaction of the bioactive compound and the concentration indicated that among the extracts chloroform extract at 624 µg/ml recorded significantly higher activity over rest of the treatments except chloroform extract at 832 µg/ml. Similarly, no significant differences were observed between methanol: water extract at 416 µg/ml and 624 µg/ml and acetone extract at 416 µg/ml.

With ABTS assay, it was observed that no significant differences were noticed between different concentrations of the standard and were significantly superior over other extracts except chloroform extract at 624 µg/ml and 832 µg/ml. By this assay also, chloroform extract at 832 recorded significantly higher antioxidant activity compare to all other extracts except chloroform extract at 624 µg/ml. The acetone extract at 208 µg/ml, chloroform extract at 416 µg/ml, methanol extract at 624 µg/ml and 832 µg/ml were at par with each other. Similarly, the chloroform extract at 208 µg/ml, methanol extract at 416 µg/ml and methanol:water extract at 832 µg/ml did not differ significantly among themselves.

4.2.6 Correlation between radical scavenging potential and phenolic and flavonoid contents of extracts

The contribution of total phenolics and flavonoids to the observed radical scavenging activity was investigated. The total phenolic content and flavonoids of the extracts was set in correlation with their DPPH and ABTS radical-scavenging capacity (Table 9). The total flavonoids content was positively correlated with DPPH and ABTS values (coefficients 0.900 and 0.832, respectively) indicating the activity exhibited by lime juice is mainly because of it high flavonoid content

4.2.7 Yield and Phonelic content of extracts of lime seed and peel

Powder of lime seed and peel were extracted with four different solvents. The extracts were separately concentrated under vacuum, lyophilized and stored at –20 ℃ until further use, the yield and phenolics content of various extracts from lime, seed and peel are presented in Table 10. Among the solvents used, ethyl acetate (9.2 g /100 g) extraction of lime seed and methanol extract (24.56 g /100 g) of lime peel gave the maximum yield. The highest phenolic content was recorded by ethyl acetate extract (3.9 g /100 g) in lime seed;

Table 6. Limonoid content of freeze dried lime juice extracts (mg/100 g dry weight) using in different solvents

Extract	LG	LNA	ILNA	LIM	Total
Chloroform	1.72 ± 0.06	53.56 ± 1.53	134.98 ± 4.07	4.25 ± 0.03	194.41
Acetone	35.20 ± 0.63	67.59 ± 2.17	ND	ND	102.79
MeOH	ND	ND	ND	ND	
MeOH:water	ND	ND	ND	ND	

(Quantification is based on reverse phase HPLC. Average of three independent experiments, N= 3)
ND: Not detected, LG: limonin glucoside, LNA: Limonexic acid, ILNA: Isolimonexic acid, LIM: limonin

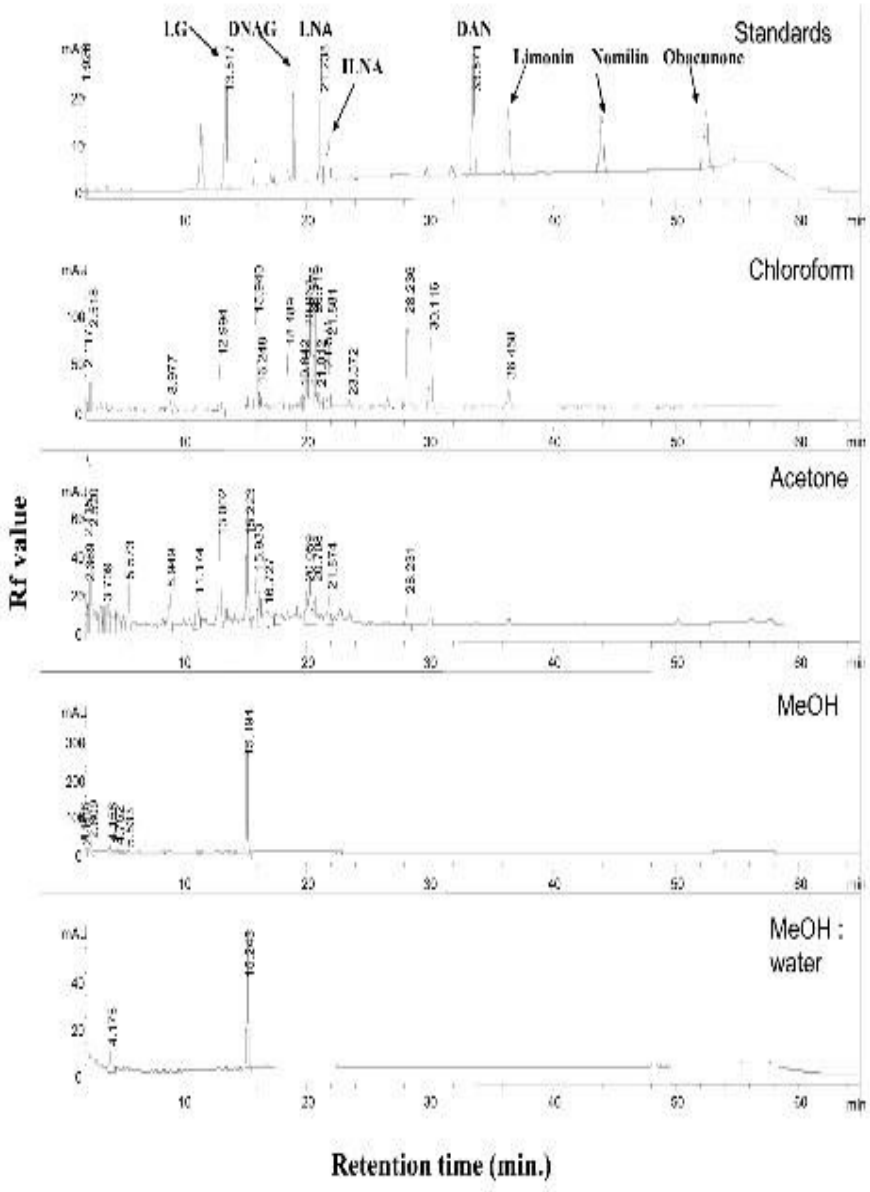


Figure 6. HPLC chromatograms of standard limonoids, different extracts from lime juice was separated using C-18 column

Table 7. Flavonoid content present of lime juice extracts (freeze dried) (mg/100 g dry weight) by reverse phase HPLC

Solvent	Rutin	Hesperidin	Didymin	Hesperitin	Total
$CHCl_3$	464.89 ± 7.20	46.18 ± 3.43	44.14 ± 1.25	23.87 ± 2.30	579.08
Acetone	ND	104.29 ± 2.42	ND	ND	104.29
MeOH	ND	386.24 ± 3.87	ND	ND	386.24
MeOH: Water	ND	206.83 ± 5.89	ND	ND	206.83

ND: Not detected

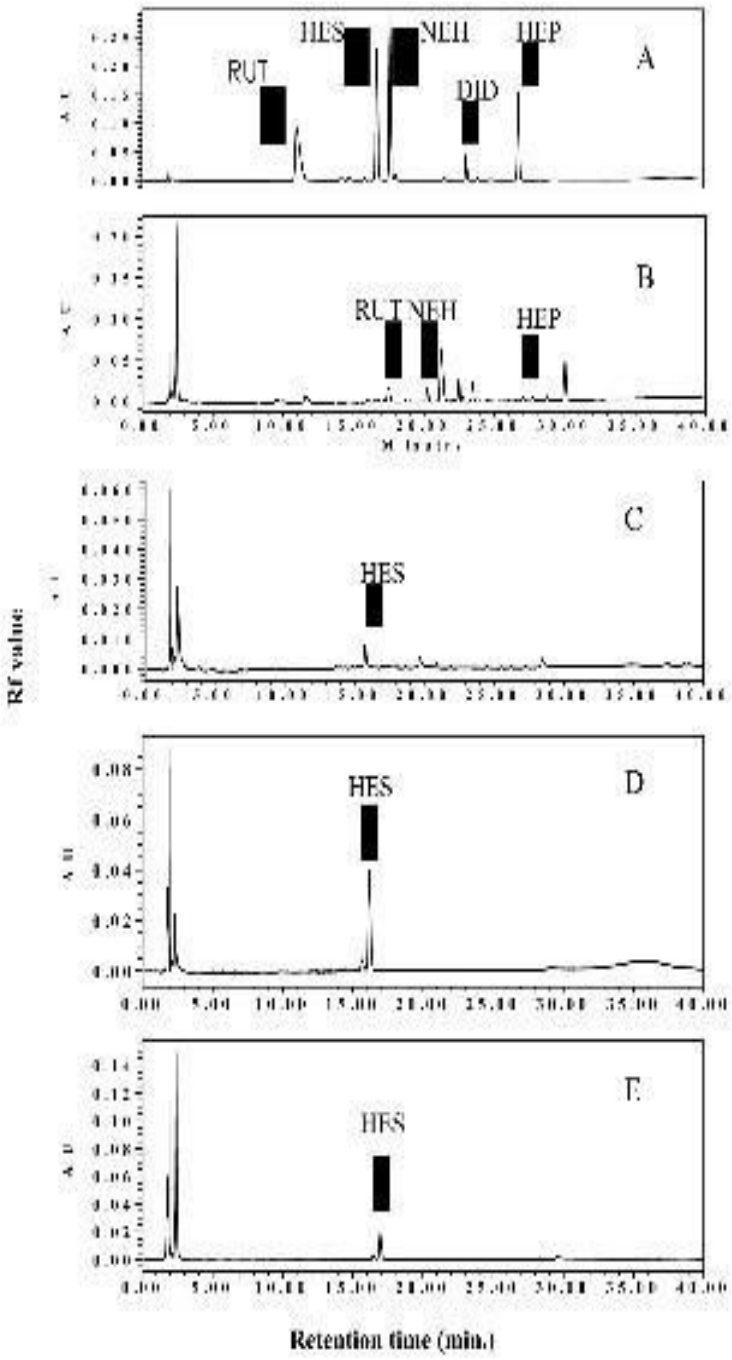
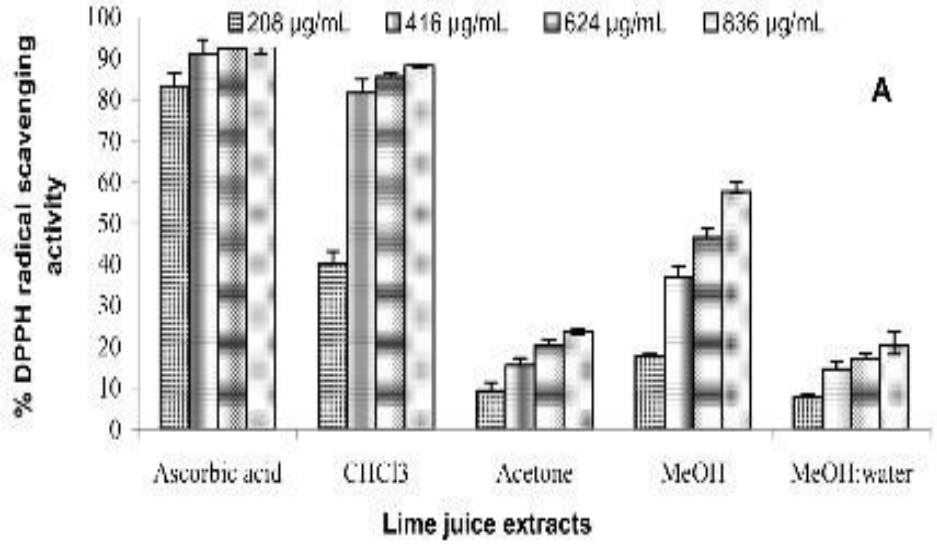


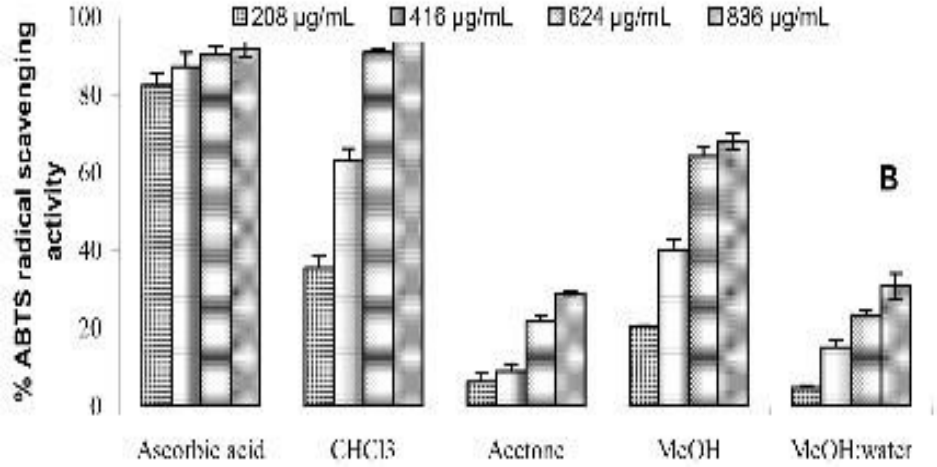
Figure 7. HPLC separation of (A) standard flavonoids, lime juice extracts of (B) chloroform, (C) acetone (B) MeON and (E) MeOH:water

Table 8. Radical scavenging potential of lime juice extracts by DPPH and ABTS assay

Sl. No.	Treatment details			Antioxidant activity (%)	
	Interaction (Main x Sub factor)				
	Bioactive compound		Concentration	DPPH	ABTS
	(Main factor)	x	(Sub factor)		
1	Chloroform extract	x	208 µg/ml	43.90 ^{f}	37.22 ^{c}
2	Chloroform extract	x	416 µg/ml	83.08 ^{d}	64.20 ^{b}
3	Chloroform extract	x	624 µg/ml	89.31 ^{bc}	87.56 ^{a}
4	Chloroform extract	x	832 µg/ml	88.56 ^{c}	93.40 ^{a}
5	Acetone extract	x	208 µg/ml	11.62 ^{k}	10.66 ^{bg}
6	Acetone extract	x	416 µg/ml	17.47 ^{j}	18.01 ^{efg}
7	Acetone extract	x	624 µg/ml	23.89 ^{hi}	30.88 ^{cd}
8	Acetone extract	x	832 µg/ml	26.13 ^{h}	35.73 ^{c}
9	Methanol extract	x	208 µg/ml	20.56 ^{ij}	22.07 ^{def}
10	Methanol extract	x	416 µg/ml	38.63 ^{gf}	36.13 ^{c}
11	Methanol extract	x	624 µg/ml	47.06 ^{f}	57.34 ^{b}
12	Methanol extract	x	832 µg/ml	55.75 ^{e}	64.57 ^{b}
13	Methanol Water extract	x	208 µg/ml	9.85 ^{k}	6.48 ^{g}
14	Methanol Water extract	x	416 µg/ml	18.18 ^{j}	8.87 ^{g}
15	Methanol Water extract	x	624 µg/ml	18.82 ^{j}	18.21 ^{efg}
16	Methanol Water extract	x	832 µg/ml	24.18 ^{h}	26.62 ^{cde}
17	Ascorbic acid	x	208 µg/ml	84.46 ^{d}	82.41 ^{a}
18	Ascorbic acid	x	416 µg/ml	92.56 ^{ab}	90.26 ^{a}
19	Ascorbic acid	x	624 µg/ml	90.63 ^{bc}	87.28 ^{a}
20	Ascorbic acid	x	832 µg/ml	94.37 ^{a}	91.72 ^{a}
			SEm±	0.887	2.817
			CD (1 %)	3.400	10.801
	Bioactive compound (Main factor)				
1	Chloroform extract			76.21 ^{b}	93.40 ^{a}
2	Acetone extract			19.77 ^{c}	35.73 ^{c}
3	Methanol extract			40.50 ^{c}	64.57 ^{b}
4	Methanol Water extract			17.75 ^{e}	26.62 ^{d}
5	Ascorbic acid (Control)			90.50 ^{a}	91.72 ^{a}
			SEm±	0.443	1.409
			CD (1 %)	1.700	5.400
	Concentration (Sub factor)				
1	208 µg/ml			34.08 ^{d}	31.77 ^{d}
2	416 µg/ml			49.98 ^{c}	43.49 ^{c}
3	624 µg/ml			53.94 ^{b}	56.25 ^{b}
4	832 µg/ml			57.79 ^{a}	62.41 ^{a}
			SEm±	0.397	1.260
			CD (1 %)	1.521	4.830

Means followed by same alphabet do not differ significantly by DMRT (P=0.01)





$CHCl_3$ Chloroform, MeOH : Methanol

Figure 8. Radical scavenging activity of lime juice (freeze dried) by (A) DPPH and (B) ABTS assay (Values are mean of three independent experiments, n=9

Table 9. Pearson correlation coefficients between the radical scavenging activity of lime juice extracts and total phenolics and flavonoids

Methods	Antioxidant activity methods		Total phenolics	Total flavonoids
	ABTS	DPPH		
ABTS	1.000	0.792	0.624	0.832
DPPH			0.539	0.900
Total Phenolics				0.477

Correlation is significant at the 0.05 level (two- tailed).

Table 10. Influence of solvents on per cent yield of extractable and phenolic content of lime seed and peel extracts

Samples	Solvents	Yield (g/100 g)	Phenolics content (catechin equivalents g/100g)
Seed	EtOAc	9.2	3.90 ± 0.63
	MeOH	4.8	2.48 ± 0.12
	MeOH: water (8:2)	1.2	3.40 ± 0.45
	EtOAc	7.34	3.40 ± 0.45
	Acetone	8.02	2.59 ± 0.30
Peel	MeOH	24.56	1.06 ± 0.14
	MeOH:water (8:2)	5.34	4.20 ± 0.25

(Average extraction time 8 h)
* Average of three independent experiments (n=3)

were as, MeOH: water (8:2) extract of lime peel contained higher amount of phenolics (4.20 g /100 g). The lowest yield was observed with MeOH : water both in the seed and peel (1.2 and 5.34 g/100 g, respectively).

4.2.8 Quantification of limonoids from lime seed and peel by HPLC

The limonoid profile of seed revealed that, limonin was the major compound in lime seeds (100.32 mg/100g), followed by ILNA (isolimonexic acid) (55.29 mg/100g) and LNA (limonexic acid) (25.50 mg/100g) (Table 11). The ethyl acetate fraction accounted for maximum limonin (80.89 mg/100 g) followed by MeOH extract (19.43 mg/100 g). The other minor limonoids in seed were obacunone (11.60 mg/100 g) and LG (limonin glucoside,2.63 mg/100 g). The most abundant limonoid in peel was ILNA (111.15 mg/100g) closely followed by LNA (100.7 mg/100 g) and other limonoids were LG (55.42 mg/100 g), and limonin (6.78 mg/100g). Among the solvents used, methanol extract had the maximum LNA (39.63 mg/100 g) and the highest ILNA (40.15 mg/100 g) was present in EtOAc extract. Further, the lime peel had a total limonoid content of 274.05 mg/100g compared to 198.34 mg/100g in seed. This clearly indicates that peels are also a good source of limonoids in addition to seeds.

4.2.9 Quantification of flavonoids from lime seed and peels by HPLC

The major flavonoid identified in lime seed was hesperidin and the maximum content was in MeOH extract followed by EtOAc and MeOH: water (Table 12). However, the peel contained an additional flavonoid neohesperidin (204.34 mg/100g) along with hesperidin. Further, the total hesperdin content was 3105.95 mg/100g in peel which was almost nine times more than seeds (394.49 mg/100g), suggesting that peel contains a very high amount of flavonoids. The seed, however, did not show the presence of neohesperidin.

4.2.10 Radical scavenging potentials of various extracts from lime seeds by DPPH and ABTS assay

The Antioxidant activity of various extracts from lime seeds was estimated by using DPPH and ABTS methods and were compared with the standard ascorbic acid at different concentrations viz. 208, 416, 624 and 832 µg/ml (Table-13), the results indicated that, among all the solvent extracts tested, methanol extract recorded maximum antioxidant activities (54.71%) by DPPH method, while ethyl acetate extract had the maximum antioxidants activity with ABTS method (64.70%). The lowest antioxidant activity was however observed with MeOH: water extract both by DPPH ad ABTS methods. The antioxidant activity of the standard didn't vary by both the methods and was the same (91.60%). The extracts of bioactive compounds varying significantly among themselves and with the control.

It was further observed that with an increase in the concentration of the bioactive compounds, the antioxidants activity also increased linearly from 208 to 832 µg/ml irrespective of the methods tested. This variation in the activity was significant between the concentration with 832 µg/ml recordings significantly higher activity and 208 µg/ml recording significantly lower activity compare to other concentrations by both the methods. The interaction between the bioactive compounds and the concentration was also significant by both the methods with methanol:water extract at 832 µg/ml having the maximum antioxidant activity (72.12%) and methanol extract having the lowest activity (31.55%) by DPPH assay. While the results of ABTS assay indicated that ethyl acetate extract at 832 µg/ml recording significantly higher activity and methanol : water extract at 208 µg/ml recording significantly lower antioxidant activity compare to all other treatment combinations.

4.2.11 Radical scavenging potentials of various extracts from lime peel by DPPH and ABTS assay

The antioxidant activity of the lime peel extracts indicated significant difference between the various extracts and the concentrations and their interactions by both the methods (Table 14). There was a variation in the percent antioxidant activity of the bioactive compounds with different methods and in general the antioxidant activity was the maximum with ABTS method compare to DPPH method. Among the various extracts, the antioxidant activity was maximum in ethyl acetate extract by both DPPH and ABTS methods which differs significantly with rest of the extracts but was significantly lower compare to the standard i.e.

Table 11. Influence of solvents extraction on limonoid content of lime seed and peel (mg/100 g dry weight)

Samples	Extract	LG	LNA	ILNA	LIM	OBA
Seed	EtOAc	ND	23.51 ± 1.69	40.44 ± 2.93	80.89 ± 1.22	ND
	MeOH	2.63 ± 0.06	4.99 ± 0.23	14.47 ± 1.08	19.43 ± 0.29	11.60 ± 0.89
	MeOH: water (8:2)	ND	ND	0.38 ± 0.01	ND	ND
	Total	2.63	28.50	55.29	100.32	11.6
Peel	EtOAc	22.57 ± 0.80	37.97 ± 2.47	46.43 ± 2.60	4.80 ± 0.03	ND
	Acetone	16.72 ± 0.16	12.66 ± 0.74	15.00 ± 0.27	1.98 ± 0.11	ND
	MeOH	13.70 ± 0.87	39.63 ± 1.46	40.15 ± 1.25	ND	ND
	MeOH: water (8:2)	2.43 ± 0.26	10.44 ± 0.08	9.57 ± 1.14	ND	ND
	Total	55.42	100.7	111.15	6.78	

(Quantification is based on reverse phase HPLC and are based on average of three independent experiments=3)

ND: Not detected, LG: limonin glucoside, LNA: Limonexic acid,
ILNA: Isolimonexic acid, LIM: limonin, OBA: obacunone.

Table 12. Influence of solvent extract on flavonoid content of lime seed and peel extracts (mg/100 g dry weight)

Samples	Extract	Hesperidin	Neohesperidin
Seed	EtOAc	134.00 ± 5.48	ND
	MeOH	179.26 ± 7.05	ND
	MeOH :water(8:2)	81.24 ± 1.67	ND
	Total	394.49	—
Peel	EtOAc	613.11 ± 19.11	98.24 ± 4.77
	Acetone	610.32 ± 54.46	51.92 ± 5.34
	MeOH	1063.07 ± 16.40	54.18 ± 6.44
	MeOH : water(8:2)	819.45 ± 12.16	ND
	Total	3105.95	204.34

(Quantification is based on reverse phase HPLC, and are based on average of three independent experiments, N= 3

* ND: Not detected

Table 13. Radical scavenging potential of lime seed extracts by DPPH and ABTS assay

Sl. No.	Treatment details			Antioxidant activity (%)	
	Interaction (Main x Sub factor)				
	Bioactive compound		Concentration	DPPH	ABTS
	(Main factor)	x	(Sub factor)		
1	Ethyl acetate extract	x	208 µg/ml	32.29 ^{hi}	56.76 ^{e}
2	Ethyl acetate extract	x	416 µg/ml	40.18 ^{g}	62.01 ^{d}
3	Ethyl acetate extract	x	624 µg/ml	48.15 ^{f}	68.73 ^{c}
4	Ethyl acetate extract	x	832 µg/ml	60.08 ^{e}	71.30 ^{c}
5	Methanol extract	x	208 µg/ml	31.55 ^{hij}	26.15 ^{i}
6	Methanol extract	x	416 µg/ml	51.42 ^{f}	45.40 ^{g}
7	Methanol extract	x	624 µg/ml	63.76 ^{d}	63.15 ^{d}
8	Methanol extract	x	832 µg/ml	72.12 ^{c}	81.54 ^{b}
9	Methanol Water extract	x	208 µg/ml	22.96 ^{k}	7.95 ^{j}
10	Methanol Water extract	x	416 µg/ml	33.76 ^{h}	31.20 ^{h}
11	Methanol Water extract	x	624 µg/ml	29.11 ^{ij}	50.15 ^{f}
12	Methanol Water extract	x	832 µg/ml	28.41 ^{j}	54.38 ^{e}
13	Ascorbic acid	x	208 µg/ml	83.50 ^{b}	83.50 ^{b}
14	Ascorbic acid	x	416 µg/ml	92.93 ^{a}	92.93 ^{a}
15	Ascorbic acid	x	624 µg/ml	94.01 ^{a}	94.01 ^{a}
16	Ascorbic acid	x	832 µg/ml	95.95 ^{a}	95.95 ^{a}
			SEm±	0.882	1.014
			CD (1 %)	3.429	3.943
	Bioactive compound (Main factor)				
1	Ethyl acetate extract			45.18 ^{c}	64.70 ^{b}
2	Methanol extract			54.71 ^{b}	54.06 ^{c}
3	Methanol Water extract			28.56 ^{d}	35.92 ^{d}
4	Ascorbic acid (Control)			91.60 ^{a}	91.60 ^{a}
			SEm±	0.441	0.507
			CD (1 %)	1.714	1.971
	Concentration (Sub factor)				
1	208 µg/ml			42.58 ^{d}	43.59 ^{d}
2	416 µg/ml			54.57 ^{c}	57.88 ^{c}
3	624 µg/ml			58.75 ^{b}	69.01 ^{b}
4	832 µg/ml			64.14 ^{a}	75.79 ^{a}
			SEm±	0.441	0.507
			CD (1 %)	1.714	1.971

Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

Table 14. Radical scavenging potential of lime peel extracts by DPPH and ABTS assay of lime peel extracts

Sl No.	Treatment details			Antioxidant activity (%)	
	Interaction (Main x Sub factor)				
	Bioactive compound		Concentration	DPPH	ABTS
	(Main factor)	x	(Sub factor)		
1	Ethyl acetate extract	x	208 µg/ml	35.30 ^{f}	41.14 ^{f}
2	Ethyl acetate extract	x	416 µg/ml	50.34 ^{d}	60.59 ^{d}
3	Ethyl acetate extract	x	624 µg/ml	56.42 ^{c}	73.59 ^{c}
4	Ethyl acetate extract	x	832 µg/ml	59.18 ^{c}	81.78 ^{b}
5	Acetone extract	x	208 µg/ml	27.75 ^{hi}	25.61 ^{h}
6	Acetone extract	x	416 µg/ml	35.12 ^{f}	44.56 ^{f}
7	Acetone extract	x	624 µg/ml	48.07 ^{d}	63.94 ^{d}
8	Acetone extract	x	832 µg/ml	56.65 ^{c}	74.76 ^{c}
9	Methanol extract	x	208 µg/ml	18.31 ^{k}	10.82 ^{j}
10	Methanol extract	x	416 µg/ml	22.01 ^{j}	18.29 ^{i}
11	Methanol extract	x	624 µg/ml	25.01 ^{ij}	27.81 ^{h}
12	Methanol extract	x	832 µg/ml	30.18 ^{gh}	35.25 ^{g}
13	Methanol Water extract	x	208 µg/ml	31.11 ^{g}	41.52 ^{f}
14	Methanol Water extract	x	416 µg/ml	43.72 ^{e}	58.63 ^{e}
15	Methanol Water extract	x	624 µg/ml	50.82 ^{d}	61.64 ^{de}
16	Methanol Water extract	x	832 µg/ml	57.90 ^{c}	83.13 ^{b}
17	Ascorbic acid	x	208 µg/ml	83.50 ^{b}	92.84 ^{a}
18	Ascorbic acid	x	416 µg/ml	92.93 ^{a}	93.84 ^{a}
19	Ascorbic acid	x	624 µg/ml	94.01 ^{a}	95.91 ^{a}
20	Ascorbic acid	x	832 µg/ml	95.95 ^{a}	83.13 ^{b}
			SEm±	0.842	1.125
			CD (1 %)	3.228	4.314
	Bioactive compound (Main factor)				
1	Ethyl acetate extract			50.31 ^{b}	81.78 ^{b}
2	Acetone extract			41.90 ^{d}	74.76 ^{c}
3	Methanol extract			23.88 ^{e}	35.25 ^{e}
4	Methanol Water extract			45.88 ^{c}	69.93 ^{d}
5	Ascorbic acid (Control)			91.60 ^{a}	95.91 ^{a}
			SEm±	0.421	0.563
			CD (1 %)	1.614	2.157
	Concentration (Sub factor)				
1	208 µg/ml			39.19 ^{d}	40.44 ^{d}
2	416 µg/ml			48.82 ^{c}	54.98 ^{c}
3	624 µg/ml			54.86 ^{b}	64.17 ^{b}
4	832 µg/ml			59.97 ^{a}	71.52 ^{a}
			SEm±	0.376	0.503
			CD (1 %)	1.444	1.929

Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

ascorbic acid . However, the antioxidant with the standard was maximum by ABTS assay compare to DPPH assay. The lowest antioxidant activity was noticed with methanol extract by both the methods. It was further observed that with an increase in the concentration of the extract, there was a significant increase in the antioxidant activity by both the methods. Significantly higher antioxidant activity was noticed with 832 µg/ml in both the methods compare to all other concentrations and significantly lower activity was noticed with 208 µg/ml. The interaction effect indicated that the antioxidant activity was maximum with ethyl acetate extract at 832 µg/ml and differed significantly with rest of the treatment combinations except AEE at 624 and AE at 832 µg/ml by DPPH assay. While results of ABTS was indicated that again MeOH:water extract recorded significantly higher antioxidant activity at 832 µg/ml (83.13%) followed by ethyl acetate extract again at 832 µg/ml and were significantly superior over rest of the treatments. Significantly lower antioxidant activity was observed with methanol extract at 208 µg/ml compare to all other treatments. It was further noted that the antioxidant activity of the standard ascorbic acid was significantly superior over the antioxidant activity of the bioactive compounds by different extracts and different methods.

4.2.12 Correlation coefficients

In order to investigate the contribution of phenolic and flavonoid constituents to the observed activity, the total phenolic content of the extracts was set in correlation with their DPPH and ABTS radical-scavenging capacity (Table 15). A high positive correlation was found (coefficient of determination 0.899) between total flavonoids in seed and DPPH activity. And a similar trend was observed in ABTS assay (0.832). The correlation values for phenolics were 0.540 with DPPH and 0.624 with ABTS. The results of peel indicated both the assay had very good correlation between phenolic content and activity. The correlation co-efficient between DPPH and phenolics was 0.903 and with ABTS it was 0.910. From these results it can be concluded that the total phenolics and flavonoids present in the lime seed and peel extracts play a prominent role for the observed DPPH and ABTS radical-scavenging activity.

4.3 Analysis of chemical composition of lime volatile oil

Lime volatiles are well known for their application in pharmaceuticals and cosmetics. However, there are no reports on health benefits; this may be due to lack of scientific evidences. In the present study an attempt has been made to find out the chemical composition of lime volatile oil and the results are presented in Table 16.

Volatile oil from limes was isolated by hydro-distillation using Clevenger type apparatus. The percentage of yield (v/w) obtained was 0.46 ml on fresh weight basis. GC-MS analysis was carried out using DB-5 MS column and total ion chromatogram along with structures of major compounds has been presented in Figure 9. Retention indices for all the compounds were determined according to the Kovats method using *n*-alkanes as standards. The individual compounds were identified by their Kovats indices relative to C_8-C_{23} n-alkanes. In addition, the fragmentation pattern in mass spectra was compared and identified using Wiley library database and published mass spectra. Standard D-limonene, β-linalool, α-terpineol and α-pinene were used for authentication. The retention times and mass spectra of these standards and the corresponding peaks in samples were matched. The GC–MS analysis of lime volatile oil showed the presence of 22 compounds, constituting 90 % of the volatile principles (Table 16). Total hydrocarbons in the lime volatile oil accounted for 45.02% and the major identified compounds are D-limonene (30.13%) along with another nine hydrocarbons. In addition, lime volatile oil has 44.9% oxygenated hydrocarbons. The alcohols (11.5%) represented seven compounds such as trans-p-mentha-2, 8-dienol, linalool, fenchol, *p*-menth-8-en-1-ol, myrtenol and terpineol. Ketones (32.72%) were represented by three compounds and D-dihydrocarvone (30.47%) was found to be the one of the major compounds present in the volatile oil. In the present study, D-limonene and D-dihydrocarovone were found to be 30.13 and 30.47%, respectively.

4.4 Studies on anticancer activity of purified compounds using cell lines

Anticancer properties were studied from the purified compounds viz., limonin isolimonexic acid,limonexic acid, Sitosterol glucoside and limonin glucoside isolated from lime

Table 15. Pearson Correlation Coefficients between the radical scavenging activity (DPPH, ABTS) of extracts and total phenolic and flavonoids

Activity/ Content	Seed				Peel		
	DPPH	ABTS	Total Phenolic	Total Flavonods	ABTS	Total Phenolic	Total Flavonoids
DPPH		0.792	0.540	0.899	0.947	0.903	0.306
ABTS			0.624	0.832		0.910	0.217
Total Phenolic				0.477			0.410

a) Correlation is significant at 0.05 level (two- tailed)

Table 16. Chemical composition and retention index of *Citrus aurantifolia* volatile oil

Sl. No.	Retention time (min.)	Compound name	Kovats index (KI)	Per cent of total	Method of Identification
1	49.94	α-Pinene	811	1.07	MS, KI, CI
2	53.24	2,3-Dehydro-1,8-cineole	829	0.34	MS, KI
3	54.72	Camphene	996	0.58	MS, KI
4	57.20	p-Cymene	1012	0.44	MS, KI
5	60.2	m-cymene	1035	0.22	MS, KI
6	63.27	m-Mentha-6,8-diene R(+)	1038	9.31	MS, KI
7	64.45	D-Limonene	1041	30.13	MS, KI, CI
8	69.27	β-cis-ocimene	1061	0.20	MS, KI
9	74.20	Trans-p-mentha-2,8-dienol	1078	0.32	MS, KI
10	83.29	β-Linalool	1111	2.05	MS, KI, CI
11	85.58	Fenchol	1121	1.99	MS, KI
12	94.02	P-Menth-8-en-1-ol	1153	0.82	MS, KI
13	98.02	D-Verbenone	1168	0.18	MS, KI
14	103.15	α-Terpineol	1186	5.86	MS, KI, CI
15	108.75	D-Dihydrocarvone	1205	30.47	MS, KI
16	109.04	Myrtenol	1206	0.15	MS, KI
17	109.41	γ-Terpineol	1207	0.27	MS, KI
18	128.15	Verbenone	1279	2.07	MS, KI
19	145.01	δ-Elemene	1346	0.21	MS, KI
20	153.37	Neryl acetate	1378	0.37	MS, KI
21	164.61	Caryophyllene	1423	0.60	MS, KI
22	169.95	Trans-α-Bergamotene	1445	2.26	MS, KI

MS = Mas spectra; KI = Kovats index ; CI =Co-injection

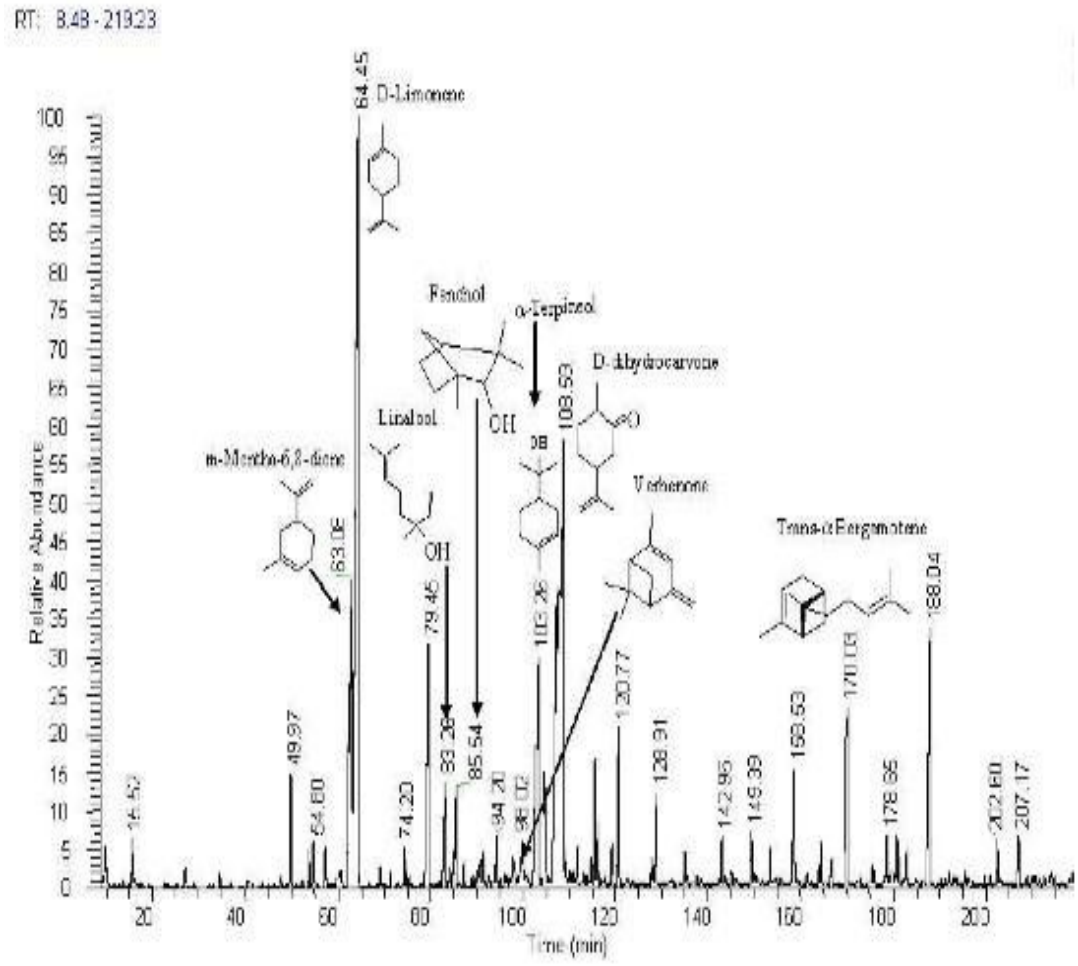


Figure 9. GC-MS total ion chromatogram of lime volatile oil showing the structure of major compounds along with their retention time

seeds using pancreatic cancer cells (Panc 28). The assays consisted of MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay, viable cell count, DNA fragmentation , and Immunobloting (protein expression). The purified compounds from of lime peel were also tested for their proliferation inhibition by use of Colon cancer cells (SW480).

Further, lime fruits were separated into edible (juice) and non edible (seed and peel) and the extracts of these parts were subjected for inhibition of proliferation of Pancreatic cancer cells. The volatile oil extracted from lime was also tested for their proliferation inhibition ability of colon cancer cells. The results obtained from these studies are discussed under different sub heads.

4.4.1 Proliferation inhibition of Panc-28 cells of purified compounds from lime seed

Five of the purified compounds from lime seeds were dissolved in DMSO and subjected to proliferation inhibition assay on Panc-28 cells (maximum amount of DMSO in media was <0.2%). The proliferation inhibition was measured using spectrophotometric MTT assay. This assay is based on the formation of insoluble formazan by the reaction of MTT dye with active mitochondrial of live cells. The intensity of formazan dye is measured at 550 nm with corrections at 515 and 650 nm; the purple intensity of formazan is directly proportional to number of live cells.

4.4.1.1 MTT assay

The IC_{50} value of compounds after incubation for 24 h indicates the potency in inducing cytotoxicity at 50 µM and higher concentrations (Table 17). The IC_{50} value of SG was minimum (68.26 µM) followed by LG (74.53 µM), ILNA (90.86 µM), where as limonin and LNA showed >100.0 µM). Further, results of 48 and 72 h of incubation have also showed similar trend to induce cytotoxicity. The IC_{50} values of the compounds after 72 h of incubation were in the range of 18.1to 42.40 µM. Differential inhibitions of compounds with incubation time could be due to their solubility, which determines their bio-accessibility.

4.4.1.2 Viable cell count assay

To confirm the proliferation inhibition activity of lime putative compounds by MTT assay, another experiment was conducted based on counting the number of viable cells after incubating with samples for specific period. Results of the studies on per cent reduction in proliferation of pancreatic cancer cells treated with isolated compounds from lime seeds indicated significant differences between the bio-active compounds and concentration at all the incubation periods (Table 18). It was further observed that the per cent reduction increased from 48 h of incubation to 144 h of incubation with respect to all the compounds and concentrations including the control. However, the per cent reduction almost doubled between 48 and 96 h of incubation while there was a marginal increase in the per cent reduction between 96 and 144 h of incubation. Among various bio-active compounds tested, the per cent inhibition of proliferation was highest with respect to LG followed by SG and LNA. However, no significant differences were observed between ILNA and SG; limonin and LNA at 48 h of incubation. Similarly, ILNA and SG; LNA and LG did not differ significantly among themselves at 96 h of incubation. While, ILNA and SG were at par with each other at 144 h of incubation.

Among the concentrations, the maximum per cent reduction was observed with 100 µM at all the periods of incubation. With an increase in the concentration from 25 µM to 100 µM, there was gradually increase in the per cent reduction in the proliferation inhibition of pancreatic cancer cells. These concentrations differed significantly at all the periods of incubation. Among the interaction, the maximum per cent reduction was observed with ILNA at 100 µM followed by SG and LG at 100 µM. The lowest per cent reduction was however observed with limonin at 25 µM which was significantly lower compared to all the bio-active compounds and the concentrations. These bio-active compounds were compared with temoxifen citrate (positive control) at 50 µM which obviously recorded the maximum per cent reduction at all the incubation periods.

Table 17. The IC_{50} values (µM) of MTT assay of purified compounds of lime seeds on Pancreatic cancer cells (Panc 28)

Compounds	24 h	48 h	72 h
Limonin	>100	49.84	42.40
LNA	>100	44.81	21.91
ILNA	90.86	41.86	18.01
SG	68.26	42.62	32.32
LG	74.53	43.40	20.49

Values are mean of three biological replicates.

ILNA = Isolimonexic acid ; LNA= Limonexic acid ;
SG = sitosterol glucoside ; LG = Limonin glucoside

Table 18. Per cent reduction in proliferation inhibition of pancreatic cancer cells (Panc-28) treated with isolated compounds from lime seeds with respect to control

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Bioactive compound		Concentration	48	96	144
	(Main factor)	x	(Sub factor)			
1	Limonin	x	25 µM	27.70 ^{j}	32.66 ^{h}	83.90 ^{fg}
2	Limonin	x	50 µM	44.90 ^{ef}	48.14 ^{f}	87.55 ^{cdef}
3	Limonin	x	100 µM	46.21 ^{de}	88.65 ^{bc}	90.19 ^{bcd}
4	Limonexic acid	x	25 µM	35.23 $^{h i}$	42.89 ^{g}	69.70 ^{h}
5	Limonexic acid	x	50 µM	39.75 ^{g}	76.94 ^{d}	80.88 ^{g}
6	Limonexic acid	x	100 µM	46.87 $^{d e}$	87.46 ^{c}	88.41 ^{cd}
7	Isolimonexic acid	x	25 µM	40.61 $^{f g}$	79.54 ^{d}	80.67 ^{g}
8	Isolimonexic acid	x	50 µM	47.57 ^{de}	87.87 ^{bc}	86.42 ^{def}
9	Isolimonexic acid	x	100 µM	50.22 ^{d}	91.85 ^{b}	93.16 ^{ab}
10	Sitosterol glucoside	x	25 µM	32.48 ^{i}	78.31 ^{d}	80.63 ^{g}
11	Sitosterol glucoside	x	50 µM	50.09 ^{d}	85.79 ^{c}	84.40 ^{efg}
12	Sitosterol glucoside	x	100 µM	59.07 ^{c}	91.89 ^{b}	92.85 ^{ab}
13	Limonin glucoside	x	25 µM	37.61 ^{gh}	61.06 ^{e}	87.93 ^{cde}
14	Limonin glucoside	x	50 µM	49.91 ^{d}	88.82 ^{bc}	91.19 ^{bc}
15	Limonin glucoside	x	100 µM	65.14 ^{b}	91.78 ^{b}	92.83 ^{ab}
16	Temoxfin citrate (control)		50 µM	84.60 ^{a}	96.12 ^{a}	96.45 ^{a}
			SEm±	1.112	0.941	0.948
			CD (1 %)	4.290	3.632	3.658
	Bioactive compound (Main factor)					
1	Limonin			39.60 ^{d}	56.49 ^{e}	87.21 ^{c}
2	Limonexic acid			40.61 ^{d}	69.09 ^{d}	79.66 ^{d}
3	Isolimonexic acid			46.13 ^{c}	86.42 ^{b}	86.75 ^{c}
4	Sitosterol glucoside			47.21 ^{c}	85.33 ^{b}	85.96 ^{c}
5	Limonin glucoside			50.89 ^{b}	80.55 ^{c}	90.65 ^{b}
6	Temoxfin citrate (control)			84.60 ^{a}	96.12 ^{a}	96.45 ^{a}
			SEm±	0.642	0.544	0.547
			CD (1 %)	2.477	2.097	2.112
	Concentration (Sub factor)					
1	25 µM			43.04 ^{c}	65.10 ^{c}	83.21 ^{c}
2	50 µM			52.80 ^{b}	80.61 ^{b}	87.82 ^{b}
3	100 µM			58.69 ^{a}	91.29 ^{a}	92.32 ^{a}
			SEm±	0.454	0.384	0.387
			CD (1 %)	1.751	1.483	1.493

Multimix =Mixture of five standards compared
Means followed by same alphabet do not differ significantly by DMRT (P=0.01)
Values are mean of three biological replicates

4.4.1.3 DNA fragmentation of Panc-28 cells

The Panc-28 cells treated with compounds were analyzed to determine possible cause of cytotoxicity. The genomic DNA was found to be intact after treatment with sample for 24 h and there were no fragmentation observed (Figure 10). The results suggest that cell death is either due to non-apoptotic pathway or apoptotic pathway which does not involve DNA fragmentation.

4.4.1.4 Apoptosis related proteins expression of Panc-28 cells

In order to confirm the involvement of apoptosis, expressions of major apoptotic proteins like, p53, Bax, bcl-2, casapse-3, cytochrome-c (both cytosolic and mitochondrial) were studied using western blot analysis. Results of the western analysis, as depicted in Figure 11 have showed clear evidence of apoptosis involvement in the induction of cytotoxicity by treated compounds. Expression of p53 (tumor suppressor) has shown higher expression of proteins in the cells treated with putative lime compounds. Expression was found to be highest in cells treated with limonin, followed by LNA and ILNA. Expression of cyclin dependant kinase inhibitor p21 was depleted in the cells treated with Limonin, however the same was higher with treatment of other compounds. The extent of enhanced expression of p21 were in the order of, SG>LG> LNA>ILNA.

The results indicate that the apoptosis induced by these compounds are both p21 and p53 dependant pathway. Expression of caspase-3 (procasaspe-34 kDa), an apoptosis executor protein has shown that treatment of compounds has decreased in expression of pro-caspase-3, This indicated that with treatment of lime seed compounds casaspe-3 was activated to induce cell death through apoptosis. Further, expression of pro-apoptotic Bax and anti-apoptotic bcl_2 has shown that compounds favor the induction of apoptosis. Expression of Bax was found to be elevated in cells treated with compounds except SG. Interestingly, expression of bcl_2 was down regulated in cells treated with lime compounds except in case of limonin. This variation in the expression may be due to dimerization and hence the ratio of Bax/bcl_2 was measured. Results of the ratio has shown that cells treated with ILNA has maximum ratio, which is 2.35 fold higher compared to control cells, followed by LNA (2.04 fold), SG (1.89 fold) and Limonin (1.51) and LG (1.34).

4.4.2 Prevention of colon cancer by lime peel compounds

Percent inhibition of colon cancer cells tested with compound extracted from lime peel indicated significant difference between them and between the concentrations and their interactions at all the incubation period (Table 19). The percent inhibition of bioactive compound was compared with the control gemcitabine, a drug used in the treatment of pancreatic cancer. It was observed that the percent inhibition of pancreatic cancer cells was maximum with the gemcitabine at all incubation period and the percent inhibition increases progressively with an increase in the incubation period. Among the bioactive compounds, the percent inhibition of pancreatic cancer cells was significantly higher with 48 h at all the incubation periods compare to other bioactive compounds. Significantly lower percent inhibition was noticed with Limettin at all the incubation period. With an increase in the concentration of bioactive compound, there was a progressive increase in percent inhibition of pancreatic cancer cells at all the incubation period with 200 µM recording significantly higher percent inhibition over other concentrations. However, significantly lower percent inhibition was noticed with 12.5 µM. The interaction between the bioactive compound and the concentration revealed with 48 µM at 200 µM had a maximum percent inhibition (68.48%) at 48 h incubation compare to all other treatments. However, no significant differences were observed between Limettin at 12.5 µM and 48 at 12.5 µM; Limettin at 50 µM and 48 at 100 µM; Limettin at 25 µM and 48 at 25 µM at 48 h of incubation. At 96 h of incubation, among the bioactive compounds, (44) at 200 µM recorded significantly higher percent inhibition over all other bioactive compounds and concentrations. However, no significant differences were observed between Limettin at 50 µM and 44 at 100 µM; Limettin at 100 µM and 48 at 100 µM and 200 µM at 96 h of incubation. While at 144 h of incubation, Limettin at 200 µM recorded significantly higher percent inhibition (88.76%) over all other treatment combinations but was significantly lower compare to the control. Similarly, no significant differences were observed between 44 at 12.5 µM and 25 µM, Limettin at 12.5 µM; 44 at 100 µM and 200 µM, Limettin at 25 µM and 48 at 25 µM.

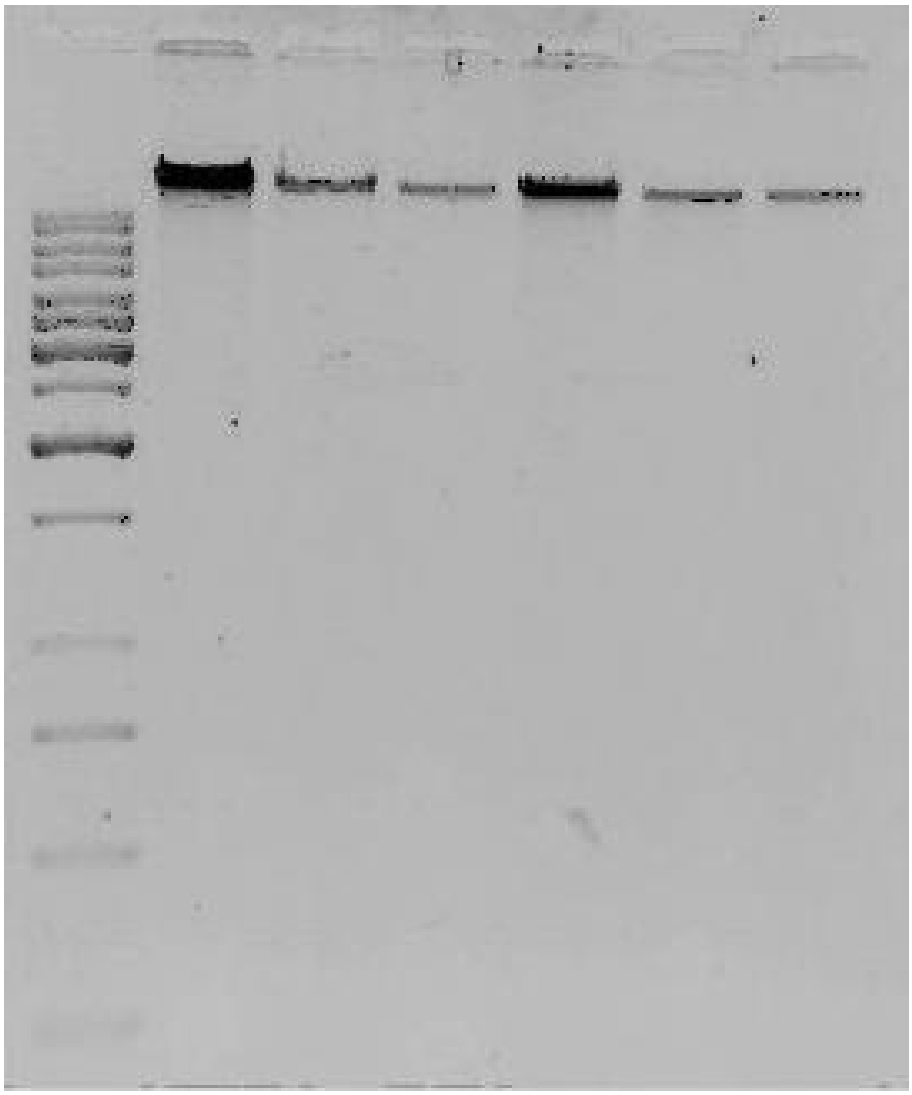


Figure 10. Image of DNA of pancreatic cells (Panc-28) treated with lime bioactive compounds. The lanes are in the order of marker, control, limonin, LG, LNA, ILNA and SG treatment (from left). DNA from Panc-28 cells treated with compounds were isolated using phenol: chloroform method. The iso lated DNA was electrophoresed on 1.2% agarose gel and Ethidium bro mide was used to visualize the bands

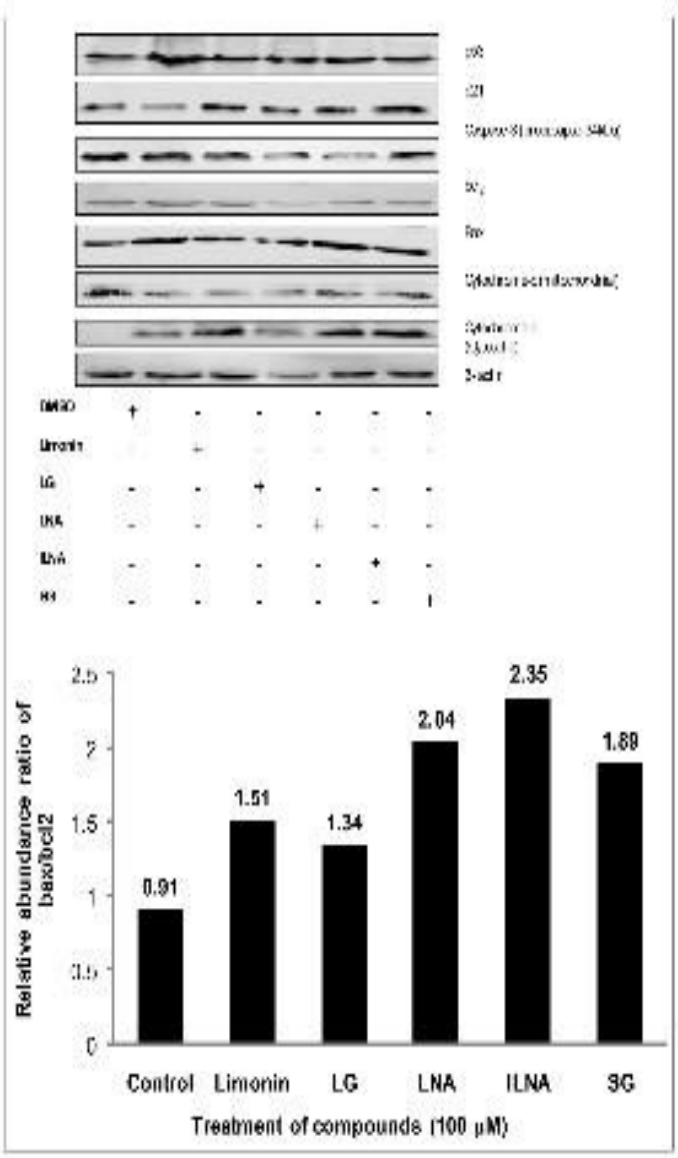


Figure 11. Effect of purified lime compounds (Limonin; LG; LNA; lLNA and SG) on expression levels of apoptosis related proteins (p53, p21, Bax, bcl_2, Caspase3) of Panc-28 cells. Cells were treated with 100 gM concentrations of compounds for 24 h and total protein was separated on 10% SDS-PAGE. The separated proteins band was incubated with specific primary antibodies overnight at 4℃ and tagged with secondary HRP-conjugated anti-mouse antibody for 2 h. The binding efficiency was detected using Super Signal west femto-maximum sensitivity substrate and the chemiluminescence image was captured using LAS4000 image analyzer at -30℃. The â-actin was used as a reference to ensure uniform protein loading (top). Results of the ratio of expression of Bax/bcl2 proteins as measured by relative abundance (bottom)

4.4.3 Prevention of pancreatic cancer by lime juice extracts

4.4.3.1 MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay

The IC50 value at 72 h of lime juice extracts seems to suggest that these treatments are capable of inducing cytotoxicity of Panc-28 cells at lower concentrations. The IC50 value of all the extracts were>200 µg/mL at 24 h. Results of 48 and 72 h have shown that IC50 value of MeOH extract was minimum followed by MeOH/water and acetone. The MeOH (109.67 µg/mL) showed a minimum value at 48 h. However, the IC50 values of the pure compounds after 72 h of incubation were in the 16.7-89.3 µg/mL. Among the pure compounds tested, the IC50 values of flavonoids, such as rutin (41.73 µg/mL) and hesperidin (16.7 µg/mL), were lower compared to those of limonoids, such as limonin (89.3 µg/mL) and LG (31.7 µg/mL), suggesting the potential of certain flavonoids from lime juice in suppressing the pancreatic cancer cells

4.4.3.2 Cell growth inhibition by viable cell count

Major Phytochemicals present in lime juice were quantified and all the solvent extracts extracted were compared with standards on the proliferation inhibition of pancreatic cancer cells and the results are presented in Table 21. Among various extracts, maximum per cent inhibition was observed with chloroform extract followed by multimix at 48 h of incubation. At this stage, no significant differences were observed between acetone extract and methanol: water extract. The least per cent inhibition was however noticed with methanol extract. The per cent inhibition increased from 48 to 96 h of incubation. However, the per cent inhibition almost doubled between 96 and 144 h of incubation in acetone extract, methanol extract and methanol: water extract. At 96 h of incubation, multimix recorded the maximum per cent inhibition followed by chloroform extract and no significant differences were observed between acetone extract, methanol extract and methanol: water extract. While, at 144 h of incubation, the maximum inhibition was observed with multimix followed by methanol: water extract and the least per cent inhibition was observed with methanol extract. In fact, no significant differences were observed between multimix and standards (rutin and temoxifen citrate) at 144 h of incubation. Similarly, no significant differences were observed between standards ILNA and hespridin at this stage.

Among the concentrations studied, there was increase in the per cent inhibition with an increase in the concentration from 25 µM to 100 µM at all the incubation periods and the per cent inhibition increased with an increase in the incubation period in all the concentrations. However, no significant differences were observed between 50 µM and 100 µM at 144 h of incubation. Among the concentrations, the lowest per cent inhibition was observed with 25 µM at 48 h and the highest with 100 µM at 144 h of incubation. Similarly, with respect to bio-active compounds, the lowest per cent inhibition was noticed with the ILNA (29.25 per cent) followed by LG (29.53 per cent) at 48 h of incubation and the maximum per cent inhibition was noticed with routine and temoxifen citrate which did not differ significantly with multimix. The interaction between the bio-active compounds and the concentrations revealed that the per cent inhibition was minimum at 25 µM at 48 h of incubation. While, at 96 h of incubation, the maximum was cutin in routine which did not differ significantly with multimix and temoxifen citrate (positive control). The lowest per cent inhibition was however noticed with acetone extract (15.4 per cent). A similar trend continued at 144 h of incubation with acetone extract recording the lowest per cent inhibition but the maximum inhibition was recorded with multimix at 100 µM followed by multimix at 50 µM.

4.4.3.3 Apoptosis related protein expression of Panc-28 cells

In order to know the possible mechanism of cytotoxicity, immunoblotting studies were conducted to know the level of major apoptotic related proteins. The protein level of p53 (tumor suppressor protein) has exhibited higher level of proteins in the cells treated with lime juice extracts (Figure 12). The level of p53 protein was found to be highest in cells treated with MeOH extract, followed by MeOH: water and acetone extract. Further, protein level of pro-apoptotic Bax and anti-apoptotic bcl_2 has shown that lime juice favour the induction of apoptosis. The protein level of Bax was found to be elevated in cells treated with all the extracts. Interestingly, protein level of bcl_2 was down regulated in cells treated with lime juice extracts. The ratio of Bax/bcl_2 was also measured, cells treated with acetone extract had

Table 19. Per cent reduction in proliferation inhibition of colon cancer cells (SW 480) treated with isolated compounds from lime peel as measured by viable cell count method

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Bioactive compound		Concentration	48	96	144
	(Main factor)	x	(Sub factor)			
1	Limettin	x	12.5 µM	39.13 ^{g}	43.38 ^{g}	51.13 ^{g}
2	Limettin	x	25 µM	47.01 ^{f}	61.03 ^{e}	70.86 ^{e}
3	Limettin	x	50 µM	57.61 ^{de}	64.95 ^{d}	78.38 ^{d}
4	Limettin	x	100 µM	58.42 ^{d}	71.07 ^{c}	85.80 ^{c}
5	Limettin	x	200 µM	63.59 ^{c}	81.37 ^{b}	88.76 ^{b}
6	5,7, Dimethoxy coumarin	x	12.5 µM	20.11 ^{i}	26.47 ^{i}	48.70 ^{gh}
7	5,7, Dimethoxy coumarin	x	25 µM	34.24 ^{h}	35.66 ^{h}	49.84 ^{g}
8	5,7, Dimethoxy coumarin	x	50 µM	38.58 ^{g}	57.84 ^{e}	63.98 ^{f}
9	5,7, Dimethoxy coumarin	x	100 µM	45.11 ^{f}	61.64 ^{de}	71.39 ^{e}
10	5,7, Dimethoxy coumarin	x	200 µM	64.31 ^{c}	78.43 ^{b}	84.73 ^{c}
11	Isopimpinellin	x	12.5 µM	35.86 ^{gh}	41.54 ^{g}	46.77 ^{h}
12	Isopimpinellin	x	25 µM	47.28 ^{f}	52.69 ^{f}	63.87 ^{f}
13	Isopimpinellin	x	50 µM	54.89 ^{e}	60.04 ^{e}	72.04 ^{e}
14	Isopimpinellin	x	100 µM	60.14 ^{d}	70.22 ^{c}	76.45 ^{d}
15	Isopimpinellin	x	200 µM	68.48 ^{b}	73.28 ^{c}	84.19 ^{c}
16	Gemcitabine	x	50 µM	71.74 ^{a}	90.69 ^{a}	97.20 ^{a}
			SEm±	0.834	0.952	0.636
			CD (1 %)	3.198	3.649	2.437
	Bioactive compound (Main factor)					
1	Limettin			53.15 ^{d}	64.36 ^{d}	74.98 ^{d}
2	5,7, Dimethoxy coumarin			40.47 ^{c}	52.00 ^{c}	63.73 ^{c}
3	Isopimpinellin			53.33 ^{b}	59.55 ^{b}	68.66 ^{b}
4	Gemcitabine			71.74 ^{a}	90.69 ^{a}	97.20 ^{a}
			SEm±	0.373	0.426	0.284
			CD (1 %)	1.430	1.632	1.090
	Concentration (Sub factor)					
1	12.5 µM			41.71 ^{e}	50.52 ^{e}	60.95 ^{e}
2	25 µM			50.06 ^{d}	60.01 ^{d}	70.44 ^{d}
3	50 µM			55.70 ^{c}	68.38 ^{c}	77.90 ^{c}
4	100 µM			58.85 ^{b}	73.40 ^{b}	82.71 ^{b}
5	200 µM			67.03 ^{a}	80.94 ^{a}	88.72 ^{a}
			SEm±	0.417	0.476	0.318
			CD (1 %)	1.599	1.825	1.219

Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

Table 20. IC_{50} values (µg/ml) of extracts from lime juice showing inhibition against Pancreatic cancer cells (Panc-28) by MTT assay

Extracts	24 h	48 h	72 h
Chloroform	>200	198.48 ± 8.45	123.31 ± 4.0
Acetone	>200	161.69 ± 4.28	129.32 ± 5.84
MeOH	>200	109.67 ± 4.15	81.20 ± 5.75
MeOH: water	>200	121.82 ± 1.02	101.6 ± 5.30
Limonin	>200	106.0 ± 1.81	89.31 ± 1.72
LG	116 ± 3.40	66.34 ± 2.46	31.69 ± 1.23
Rutin	187.20 ± 5.58	49.47 ± 6.73	41.73 ± 3.25
Hesperidin	147.28 ± 5.73	26.29 ± 0.48	16.68 ± 0.81

* Values are means of three biological replicas

Table 21. Comparison of lime juice extracts with standards on proliferation inhibition of pancreatic cancer cells (Panc-28)

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Bioactive compound		Concentration	48	96	144
	(Main factor)	x	(Sub factor)			
1	Chloroform extract	x	25 µM	40.69 ^{l}	45.83 ^{k}	72.60 ^{l}
2	Chloroform extract	x	50 µM	75.35 ^{l}	77.92 ^{d}	95.77 ^{de}
3	Chloroform extract	x	100 µM	82.44 ^{b}	88.89 ^{c}	98.32 ^{ab}
4	Acetone extract	x	25 µM	13.10 ^{k}	15.45 ^{l}	67.04 ^{m}
5	Acetone extract	x	50 µM	42.06 ^{h}i	49.57 ^{j}	83.38 ^{i}
6	Acetone extract	x	100 µM	46.45 ^{g}h	72.89 ^{efg}	90.05 ^{h}
7	Methanol extract	x	25 µM	13.56 ^{k}	16. 38 ^{l}	77.90 ^{k}
8	Methanol extract	x	50 µM	45.21 ^{h}i	52.42 ^{j}	90.32 ^{h}
9	Methanol extract	x	100 µM	55.49 ^{e}	73.93 ^{def}	94.16 ^{ef}
10	Methanol Water extract	x	25 µM	14.89 ^{k}	16.95 ^{l}	81.14 ^{j}
11	Methanol Water extract	x	50 µM	41.75 ^{hi}	52.13 ^{j}	96.03 ^{d}
12	Methanol Water extract	x	100 µM	46.27 ^{gh}	75.71 ^{de}	97.76 ^{b}c
13	Multimix	x	25 µM	45.76 ^{h}	71.51 ^{fg}	96.43 ^{cd}
14	Multimix	x	50 µM	50.71^{fg}	89.88 ^{bc}	99.42 ^{ab}
15	Multimix	x	100 µM	71.45 ^{c}	94.44 ^{a}	99.76 ^{a}
16	Limonin glucoside		(Standard 1)	29.53 ^{j}	57.72 ^{l}	93.97 ^{fg}
17	Limonexic acid		(Standard 2)	53.87 ^{ed}	62.14 ^{h}	93.97 ^{fg}
18	Isolimonexic acid		(Standard 3)	29.25 ^{j}	69.72 ^{g}	93.97 ^{fg}
19	Hespridin		(Standard 4)	66.66 ^{d}	77.49 ^{d}	92.24 ^{g}
20	Rutin		(Standard 5)	66.66 ^{d}	95.72 ^{a}	92.24 ^{g}
21	Temoxifen citrate		(Control)	91.13 ^{a}	93.30 ^{ab}	92.24 ^{g}
			SEm±	1.194	0.975	0.427
			CD (1 %)	4.480	3.659	1.602
	Bioactive compound (Main factor)					
1	Chloroform extract			66.16 ^{c}	70.88 ^{d}	88.89 ^{f}
2	Acetone extract			33.87 ^{f}	45.97 ^{g}	80.16 ^{g}
3	Methanol extract			38.09 ^{e}	47.58 ^{g}	87.46 ^{h}
4	Methanol Water extract			34.30 ^{f}	48.26 ^{g}	91.64 ^{e}
5	Multi-mix			55.97 ^{d}	85.28 ^{b}	98.54 ^{a}
6	Limonin glucoside		(Standard 1)	29.53 ^{g}	57.72 ^{f}	93.97 ^{d}
7	Limonexic acid		(Standard 2)	53.87 ^{d}	62.14 ^{e}	92.24 ^{c}
8	Isolimonexic acid		(Standard 3)	29.25 ^{g}	69.72 ^{d}	93.36 ^{bc}
9	Hespridin		(Standard 4)	66.66 ^{c}	77.49 ^{c}	94.26 ^{b}
10	Rutin		(Standard 5)	85.81 ^{b}	95.72 ^{a}	98.81 ^{a}
11	Temoxifen citrate		(Control)	91.13 ^{a}	93.30 ^{a}	98.61 ^{a}
			SEm±	0.689	0.563	0.247
			CD (1 %)	2.586	2.112	0.925
	Concentration (Sub factor)					
1	25 µM			44.02 ^{c}	56.56 ^{c}	87.85 ^{c}
2	50 µM			55.57 ^{b}	70.73 ^{b}	94.20 ^{b}
3	100 µM			59.85 ^{a}	78.36 ^{a}	92.54 ^{a}
			SEm±	0.360	0.294	0.129
			CD (1 %)	1.351	1.103	0.483

Multimix =Mixture of five standards compared
Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

highest ratio of 2.1 folds, followed by cell treated with MeOH (2.13), MeOH: water (2.02) and chloroform extract (1.35) compared to control. The expression of procasaspe-3 (34 kDa) has shown decrease in the content, indicating activation of casaspe-3 the decrease in the protein expression were in the order of acetone> MeOH > MeOH:water > chloroform. Results of the immunoblotting have shown clear evidence of apoptosis involvement in the induction of cytotoxicity by lime juice extracts.

4.4.4 Prevention of pancreatic cancer by lime seed and peel extracts

4.4.4.1 MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay

MTT analysis was performed on all the extracts of seed and peel using the Pac 28 cell-line. The results represent the means of three independent runs with error bars denoting SEM and presented in Table 22. Among the four extracts of lime seed tested, MeOH: water showed least IC_{50} values of 4.11 µg/ml at 72 h. However, at 24 h the MTT values ranged from 11.34 to 13.84 µg/ml and 4.3 to 7.48 µg/ml at 48 h. The results indicate that all the extracts of lime seed showed potential in suppressing the pancreatic cancer cells and this was dose and time dependent. Further in case of lime peel, the least IC_{50} value was once again by MeOH: water extract (1.04 µg/ml) at 72 h. Further, four pure compounds such as, limonin, LG, and hesperidins were also tested to compare the results with lime extracts. Among these standards hesperidin with 0.33 µg/ml IC_{50} value seems to be most active in suppressing the pancreatic cancer cells.

4.4.4.2 Cell growth inhibition by viable cell count (seed extracts)

Based on the results of MMT assay which, indicating the potential of lime extracts in inhibiting the cells of pancreatic cancer cells another assay which is based on the viable cell count after treating the cells with the test compound and incubation for 2-6 days was attempted to further confirm the results of MMT assay. The results indicated there was a time and dose dependent inhibition of pancreatic cancer cells by all the extracts of seed (Table 23). Further, the results of the influence of various solvent extracts of lime seed on proliferation inhibition of pancreatic cancer cells indicated significant differences between the extracts and other bio-active compounds, concentrations and their interaction. The proliferation inhibition of the lime seed extracts were compared with rutin and temoxifen citrate (control) were compared. Among the extracts, maximum inhibition was observed with methanol: water at all the incubation periods. The per cent inhibition increased with an increase in the incubation period from 48 h of incubation with a marginal increase thereafter up to 144 h. While with respect to other bio-active compounds (standards), the per cent inhibition was less initially at 48 h and increased dramatically at later h of incubation. However, the lowest inhibition was observed with acetone extract at 48 h, methanol extract at 96 h and 144 h of incubation. Among the standards, the maximum per cent inhibition was noticed with rutin at 48 h, LG at 96 hous and 144 h. However, no significant differences were observed between LG and ILNA and LNA and hespridin at 48 h and between LG, LNA, ILNA, hespridin at 144 h. It was further observed that the per cent inhibition was maximum with the temoxifen citrate (control) at all the periods of incubation. Except, multimix, the per cent inhibition was less with respect to other extracts in comparison with the standards at 144 h.

With an increase in the concentration from 25 µM to 100 µM, there was a progressive increase in the per cent inhibition of proliferation of pancreatic cancer cells at all the incubation periods. The per cent inhibition was less at 48 h and almost increased to two-folds at 144 h in all the concentrations studied. The maximum per cent inhibition was however noticed with 100 µM multimix at 144 h and the lowest with 25 µM at 48 h. The interaction between the bio-active compounds and the concentration was significant at all the period of incubation with acetone extract recording the lower per cent inhibition at 48 h and multimix recording the maximum per cent inhibition at 144 h.

4.4.4.3 Cell growth inhibition by lime peel extracts by viable cell count

The results of the influence of various solvent extracts of lime peel on proliferation inhibition of pancreatic cancer cells presented in Table 24 indicated significant differences between the extracts and other bio-active compounds, concentrations and their interaction. The proliferation inhibition of the lime peel extracts were compared with multimix and

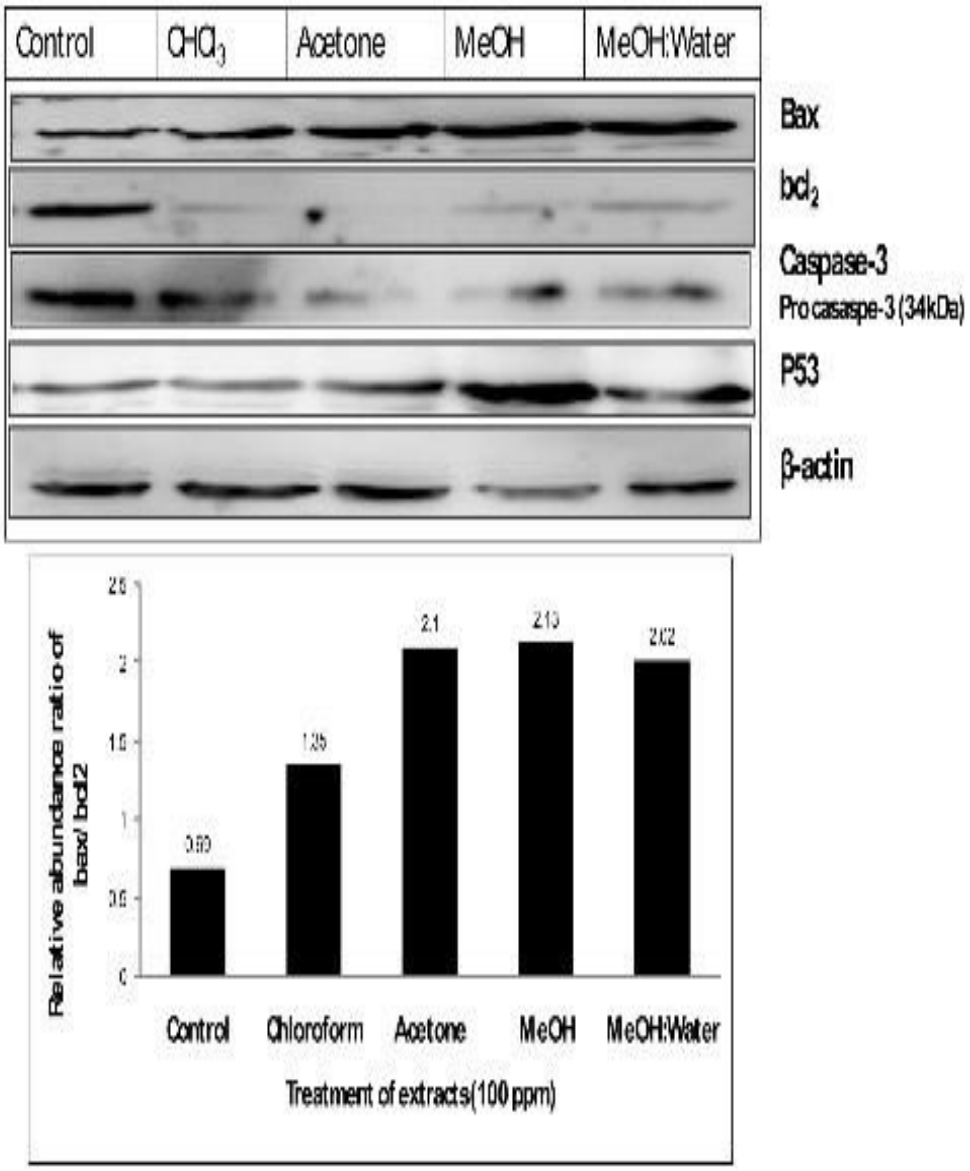


Figure 12.Effect of lime juice extracts on expression levels of apoptosis related proteins (p53, Bax, bcl_2, Caspase-3, p53, b-actin) of Panc-28 cells. Cells were treated with 100 µg/mL concentration of extracts and incubated for 24 h. Total protein was separated on 10% SDS-PAGE . The separated protein band was incubated with specific primary antibodies for overnight at 4℃ and tagged with secondary HRP-conjugated anti-mouse antibody for 2 h. The binding efficiency was detected using Super Signal west femto-maximum sensitivity substrate and the chemiluminescence image was captured using LAS4000 image analyzer at -30℃. The aactin was used as a reference to ensure uniform protein. Bar graphs represents the relative abundance ratio of bax/bcl2

Table 22. IC_{50} values (µg/mL) of MTT assay of Lime Seed and Peel extracts effect on pancreatic cancer cells (Panc-28) along pure compounds

Sample	Extract/Standards	24 h	48 h	72 h
Seed	EtOAc	12.00 ± 0.70	7.48 ± 0.52	6.19 ± 0.25
	Acetone	12.97 ± 0.50	5.05 ± 0.33	4.57 ± 0.12
	MeOH	13.84 ±1.07	6.27 ± 0.72	4.19 ± 0.16
	MeOH: water (8:2)	11.34 ±1.16	4.30 ± 0.27	4.11 ± 0.20
Peel	EtOAc	9.77 ± 0.18	5.97 ± 0.10	3.43 ± 0.19
	Acetone	9.33 ± 0.17	4.78 ± 0.12	3.69 ± 0.03
	MeOH	10.19 ± 0.52	3.91 ± 0.15	2.33 ± 0.14
	MeOH:water (8:2)	8.62 ± 0.19	1.94 ± 0.21	1.04 ± 0.10
Limonoids	Limonin	4.78 ± 0.07	2.86 ± 0.12	2.59 ± 0.96
	Limonin glucoside	4.58 ± 0.18	2.75 ± 0.10	2.30 ± 0.10
Flavonoids	Rutin	3.64 ± 0.06	2.39 ± 0.16	0.21 ± 0.12
	Hesperidin	9.91 ± 0.81	0.38 ± 0.10	0.33 ± 0.12

Table 23. Comparison of lime seed extracts, purified compounds on proliferation inhibition of pancreatic cancer cells (Panc-28)

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Bioactive compound		Concentration	48	96	144
	(Main factor)	x	(Sub factor)			
1	Ethyl acetate extract	x	25 µg/ml	23.96 ^{l}	28.32 ^{k}	49.33 ^{j}
2	Ethyl acetate extract	x	50 µg/ml	34.78 ^{i}	47.24 ^{i}	68.46 ^{g}
3	Ethyl acetate extract	x	100 µg/ml	47.25 ^{gh}	58.92 ^{j}	74.14 ^{f}
4	Acetone extract	x	25 µg/ml	9.830 ^{h}	12.28 ^{n}	53.78 ^{i}
5	Acetone extract	x	50 µg/ml	15.43 ^{m}	18.06 ^{m}	75.05 ^{ef}
6	Acetone extract	x	100 µg/ml	25.48 ^{kl}	58.07 ^{g}	87.15 ^{d}
7	Methanol extract	x	25 µg/ml	11.77 ^{mn}	24.51 ^{l}	35.78 ^{k}
8	Methanol extract	x	50 µg/ml	11.88 ^{mn}	26.93 ^{kl}	48.63 ^{j}
9	Methanol extract	x	100 µg/ml	31.65 ^{ij}	33.66 ^{j}	54.75 ^{i}
10	Methanol Water extract	x	25 µg/ml	33.25 ^{ij}	50.40 ^{h}	59.26 ^{h}
11	Methanol Water extract	x	50 µg/ml	45.44 ^{h}	59.26 ^{fg}	76.67 ^{e}
12	Methanol Water extract	x	100 µg/ml	60.46 ^{e}	78.48 ^{d}	86.34 ^{d}
13	Multimix	x	25 µg/ml	45.76 ^{h}	71.51 ^{e}	96.43 ^{b}
14	Multimix	x	50 µg/ml	50.71 ^{fg}	89.88 ^{c}	99.42 ^{a}
15	Multimix	x	100 µg/ml	71.45 ^{c}	94.44 ^{b}	99.76 ^{a}
16	Limonin glucoside		(Standard 1)	85.81 ^{b}	95.72 ^{ab}	98.81 ^{a}
17	Limonexic acid		(Standard 2)	29.53 ^{jk}	57.72 ^{g}	93.97 ^{c}
18	Isolimonexic acid		(Standard 3)	53.87 ^{f}	62.14 ^{f}	92.24 ^{c}
19	Hespridin		(Standard 4)	29.25 ^{jk}	69.72 ^{e}	93.36 ^{c}
20	Rutin		(Standard 5)	66.66 ^{d}	77.49 ^{d}	94.26 ^{bc}
21	Temoxifen Citrate		(Control)	95.58 ^{a}	98.35 ^{a}	99.34 ^{a}
			SEm±	1.121	0.771	0.571
			CD (1 %)	4.206	2.893	2.144
	Bioactive compound (Main factor)					
1	Ethyl acetate extract			35.33 ^{f}	44.82 ^{h}	63.97 ^{e}
2	Acetone extract			16.91 ^{h}	29.47 ^{i}	71.99 ^{d}
3	Methanol extract			18.43 ^{h}	28.36 ^{i}	46.38 ^{f}
4	Methanol Water extract			46.38 ^{e}	62.71 ^{f}	74.09 ^{c}
5	Multi-mix			85.81 ^{b}	95.72 ^{b}	98.81 ^{a}
6	Limonin glucoside		(Standard 1)	55.97 ^{d}	85.28 ^{c}	98.54 ^{a}
7	Limonexic acid		(Standard 2)	29.53 ^{g}	57.72 ^{g}	93.97 ^{b}
8	Isolimonexic acid		(Standard 3)	53.87 ^{d}	62.14 ^{f}	92.24 ^{b}
9	Hespridin		(Standard 4)	29.25 ^{g}	69.72 ^{e}	93.36 ^{b}
10	Rutin		(Standard 5)	66.66 ^{c}	77.49 ^{d}	94.26 ^{b}
11	Temoxifen Citrate		(Control)	95.58 ^{a}	98.35 ^{a}	99.34 ^{a}
			SEm±	0.647	0.445	0.330
			CD (1 %)	2.428	1.670	1.238
	Concentration (Sub factor)					
1	25 µg/ml			44.11 ^{c}	58.92 ^{c}	78.78 ^{c}
2	50 µg/ml			47.18 ^{b}	63.86 ^{b}	85.47 ^{b}
3	100 µg/ml			54.27 ^{a}	71.34 ^{a}	88.56 ^{a}
			SEm±	0.338	0.232	0.172
			CD (1 %)	1.268	0.872	0.646

Multimix =Mixture of five standards compared
Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

temoxifen citrate (control). Among the extracts, maximum inhibition was observed with methanol at all the incubation periods. The per cent inhibition increased with an increase in the incubation period from 48 h to 96 h of incubation with a significant increase there after up to 144 h. While with respect to other bio-active compounds (standards), the per cent inhibition was significantly higher at 48 h and increased dramatically at later h of incubation. However, the lowest inhibition was observed with acetone extract at 48 h, at 96 h and 144 h of incubation. Among the standards, the maximum per cent inhibition was noticed with rutin at 48 h, 96 h and 144 h. Further, there was significant differences observed between LG, ILNA , LNA and hespridin at 48 h. However, there was no significant difference and between LG, ILNA, hespridin at 144 h. It was further observed that the per cent inhibition was maximum with the temoxifen citrate (control) at all the periods of incubation. Except, multimix, the per cent inhibition was less with respect to other extracts in comparison with the standards at 144 h.

With an increase in the concentration from 25 µM to 100 µM, there was a progressive increase in the per cent inhibition of proliferation of pancreatic cancer cells at all the incubation periods. The per cent inhibition was less at 48 h and almost increased to two-folds at 144 h in all the concentrations studied. The maximum per cent inhibition was however noticed with 100 µM multimix at 144 h. The interaction between the bio-active compounds and the concentration was significant at all the period of incubation with ethyl acetate extract recording the lower per cent inhibition at 48 h and multimix recording the maximum per cent inhibition at 144 h.

4.4.4.4 Apoptosis related protein expression of Panc-28 cells

In order to know the possible mechanism of cytotoxicity, immunoblotting studies were attempted to know the level of major apoptotic related proteins. The protein level of Bax was found to be elevated in cells treated with all the extracts except MeOH: water in lime seed extracts (Figure 13). Interestingly, protein level of bcl_2 was down regulated in cells treated with both lime seed and peel extracts. This variation in the level of protein may be due to dimerization and hence the ratio of Bax/bcl_2 was measured. Cells treated with all the extracts of seed showed two fold increase in ratio and the highest bax/bcl2 ratio of 1.5 fold was observed in cells treated with MeOH extract of lime peel compared to control. The protein level of caspase-3 in cells treated with seed extract was in the order of EtOAc > MeOH > acetone > MeOH: water. However, in case of lime peel, it was in the order of MeOH: water> EtOAc > acetone > MeOH. Further, there was decrease in the level of AKT by acetone and MeOH: water and no change in EtOAc and MeOH extracts of lime. However, all the extracts of lime peel showed decrease in level of AKT(Figure 14). In addition, the level of p21 protein was decreased in all the lime peel extracts, in contrast, the seed extracts of EtOAc and acetone showed slight increase. In conclusion, the results of the immunoblotting have shown clear evidence of apoptosis involvement in the induction of cytotoxicity by lime seed and juice extracts.

4.4.5 Prevention of colon cancer by lime volatile oil

In order to know the effect of volatile oil on normal cells (NIH 3T3), a study was made by treating different concentrations of volatile oil and incubated for 24 h and 48 h and viable cells were counted. Results revealed that there was no appreciable difference in cell count between the control and treated cells (Figure 15).

4.4.5.1 MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay

In order to understand the effect of lime volatile oil on colon cancer cells, experiments were conducted using cultured human colon carcinoma cells. Results of the viability were measured using MTT spectrophotometric assay (Table 25). Camptothecin was used as positive control for the comparison of inhibitory potentials. Dose dependent inhibition of SW480 cells was observed at different concentrations (6.25-200 µg/ml) of lime volatile oil treatment. At 100 and 200 µg/ml, inhibition of SW480 cells was found to be 38.34 and 44%, respectively at 24 h. Further, 75% inhibition was observed after 48 h of treatment and this was found to be significant in comparison with camptothecin. Furthermore, 86.5% inhibition was observed after 72 h of incubation of cells with 200 µg/ml of volatile oil. Cell proliferation was highly significant at concentrations above 50 µg/ml ($P<0.001$). Most of the principle

Table 24. Comparison of lime peel extracts. Purified compounds on proliferation inhibition of Pancreatic cancer cells (Panc-28)

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Bioactive compound		Concentration	48	96	144
	(Main factor)	x	(Sub factor)			
1	Ethyl acetate extract	x	25 µg/ml	13.91 ^{h}	16.20 ^{J}	79.87 ^{b}
2	Ethyl acetate extract	x	50 µg/ml	25.30 ^{g}	26.75 ^{hi}	91.18 ^{a}
3	Ethyl acetate extract	x	100 µg/ml	54.19 ^{c}	73.32 ^{b}	96.94 ^{a}
4	Acetone extract	x	25 µg/ml	15.86 ^{h}	16.72 ^{J}	54.19 ^{e}
5	Acetone extract	x	50 µg/ml	30.85 ^{g}	32.06 ^{gh}	73.56 ^{bc}
6	Acetone extract	x	100 µg/ml	37.01 ^{f}	60.26 ^{d}	92.57 ^{a}
7	Methanol extract	x	25 µg/ml	37.95 ^{f}	32.29 ^{gh}	62.77 ^{d}
8	Methanol extract	x	50 µg/ml	37.22 ^{f}	37.21 ^{fg}	80.67 ^{b}
9	Methanol extract	x	100 µg/ml	49.01 ^{cd}	53.01 ^{e}	93.77 ^{a}
10	Methanol Water extract	x	25 µg/ml	18.14 ^{h}	21.13 ^{IJ}	70.44 ^{cd}
11	Methanol Water extract	x	50 µg/ml	30.43 ^{g}	41.40 ^{f}	73.39 ^{bc}
12	Methanol Water extract	x	100 µg/ml	41.87 ^{ef}	64.29 ^{cd}	77.75 ^{bc}
13	Multimix	x	25 µg/ml	44.14 ^{de}	71.51 ^{b}	96.43 ^{a}
14	Multimix	x	50 µg/ml	54.07 ^{c}	89.88 ^{a}	99.42 ^{a}
15	Multimix	x	100 µg/ml	71.45 ^{b}	94.44 ^{a}	99.76 ^{a}
16	Limonin glucoside	(Standard 1)		45.82 ^{de}	57.72 ^{de}	93.97 ^{a}
17	Limonexic acid	(Standard 2)		47.52 ^{de}	60.00 ^{d}	92.24 ^{a}
18	Isolimonexic acid	(Standard 3)		29.25 ^{g}	69.72 ^{bc}	93.36 ^{a}
19	Hespridin	(Standard 4)		66.66 ^{b}	75.78 ^{b}	94.26 ^{a}
20	Rutin	(Standard 5)		85.81 ^{a}	95.44 ^{a}	98.81 ^{a}
21	Temoxifen Citrate	(Control)		91.13 ^{a}	93.30 ^{a}	98.29 ^{a}
			SEm±	1.890	2.29	2.69
			CD (1 %)	5.410	6.54	7.69
	Bioactive compound (Main factor)					
1	Chloroform extract			31.13 ^{g}	38.76 ^{fg}	89.33 ^{b}
2	Acetone extract			27.91 ^{g}	36.35 ^{g}	73.44 ^{d}
3	Methanol extract			41.39 ^{f}	40.84 ^{fg}	79.44 ^{c}
4	Methanol Water extract			30.15 ^{g}	42.27 ^{f}	73.86 ^{d}
5	Multi-mix			56.55 ^{d}	85.28 ^{b}	98.54 ^{a}
6	Limonin glucoside	(Standard 1)		39.36 ^{f}	57.72 ^{e}	93.97 ^{ab}
7	Limonexic acid	(Standard 2)		47.52 ^{e}	60.00 ^{e}	92.24 ^{b}
8	Isolimonexic acid	(Standard 3)		29.25 ^{g}	69.72 ^{d}	93.36 ^{ab}
9	Hespridin	(Standard 4)		66.66 ^{c}	75.78 ^{c}	94.26 ^{ab}
10	Rutin	(Standard 5)		85.81 ^{b}	95.44 ^{a}	98.81 ^{a}
11	Temoxifen Citrate	(Control)		91.45 ^{a}	93.70 ^{a}	98.29 ^{a}
			SEm±	1.62	1.81	1.80
			CD (1 %)	4.60	5.15	5.40
	Concentration (Sub factor)					
1	25 µg/ml			21.47 ^{c}	21.52 ^{c}	66.82 ^{c}
2	50 µg/ml			30.95 ^{b}	34.36 ^{b}	79.70 ^{b}
3	100 µg/ml			45.52 ^{a}	62.72 ^{a}	90.26 ^{a}
			SEm±	1.97	2.29	2.56
			CD (1 %)	7.77	8.99	10.07

Multimix =Mixture of five standards compared
Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

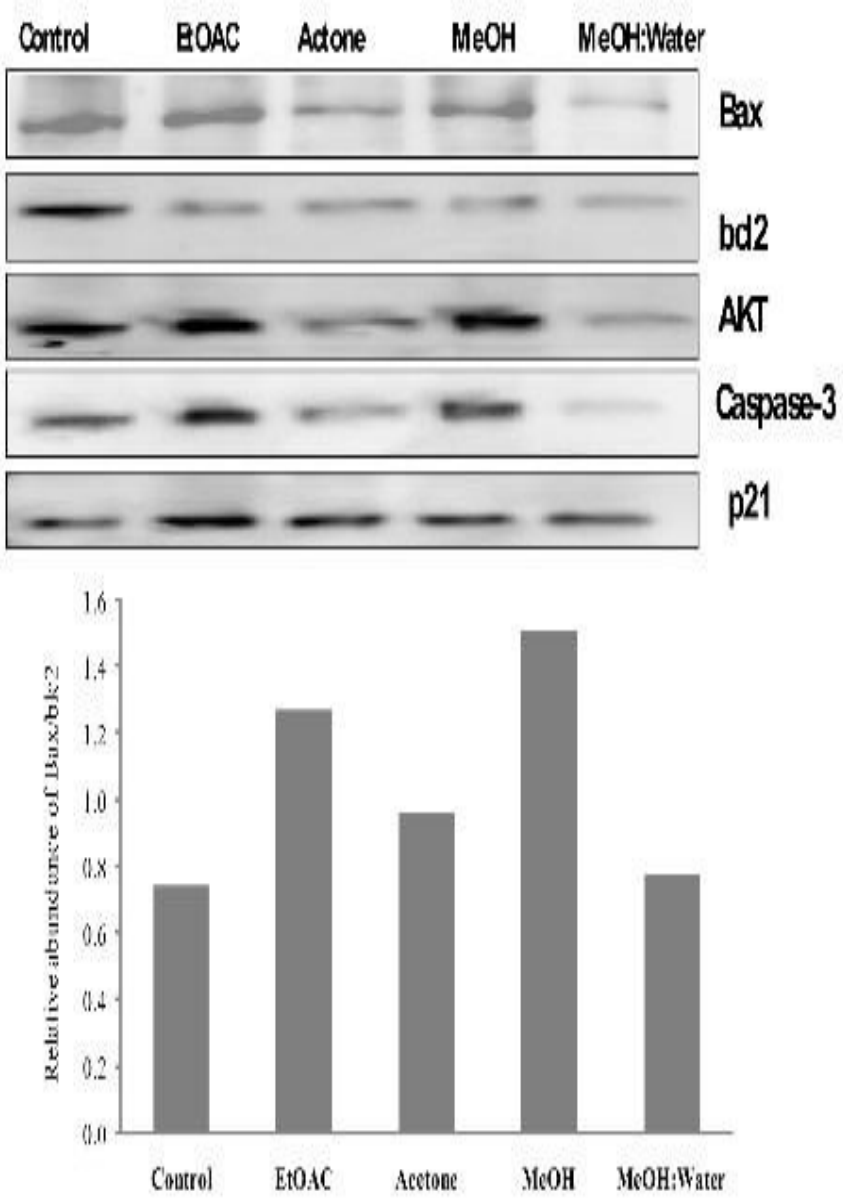


Figure 13.

Effect of Lime seed extracts on expression levels of apoptosis related proteins (p21, Bax, bcl_2, Caspase-3 and AKT) of Panc-28 cells. Cells were treated with the specified concentrations of extracts for 24 h and total protein was separated on 10% SDS-PAGE. The separated proteins band was incubated with specific primary antibodies overnight at 4℃ and tagged with secondary HRP-conjugated anti-mouse antibody for 2h. The binding efficiency was detected using Super Signal west femtomaximum sensitivity substrate and the chemiluminescence image was captured using LAS4000 image analyzer at -30℃.

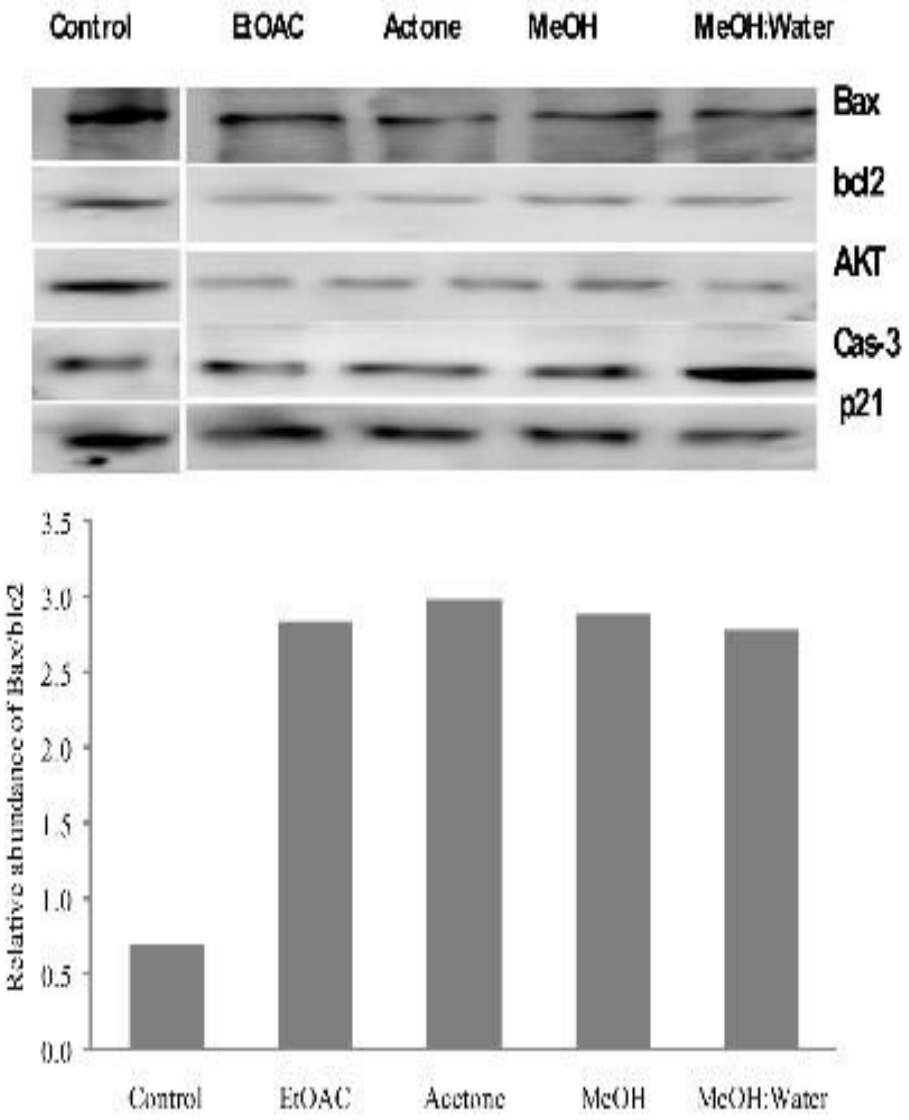


Figure14.Effect of Lime peel extracts on expression levels of apoptosis related proteins (p21, Bax, bcl_2, Caspase-3 andAKT) of Panc-28 cells. Cells were treated with the specified concentrations of extracts for 24 h and total protein was separated on 10% SDS-PAGE. The separated proteins band was incubated with specific primary antibodies overnight at 4℃ and tagged with secondary HRP-conjugated anti-mouse antibody for 2 h. The binding efficiency was detected using Super Signal west femtomaximum sensitivity substrate and the chemiluminescence image was captured using LAS4000 image analyzer at -30℃.

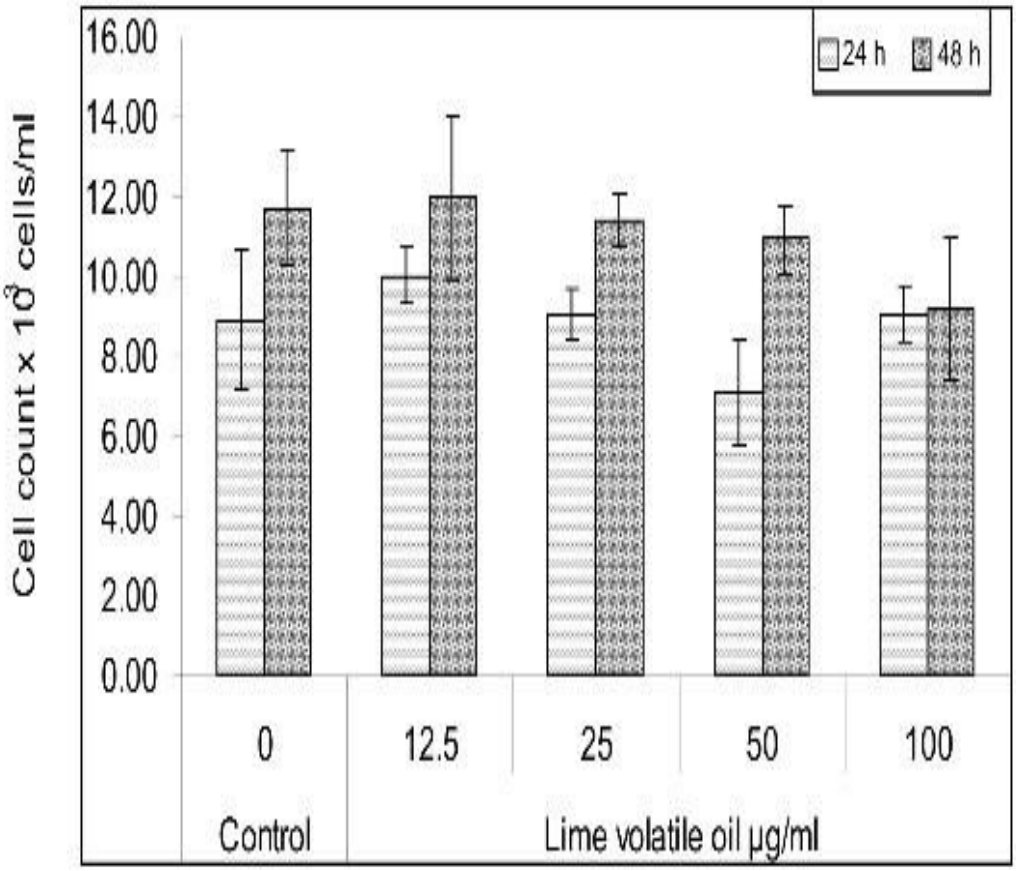


Figure 15.

Effect of Lime volatile oil on proliferation of normal (NIH 3T3) cells. Mouse embryonic fibroblast cells were treated with different concentrations of Lime volatile oil and viable cells were counted after 24 and 48 h of incubation using Z1beckman counter (0 indicates with out volatile oil)

Table 25. Per cent inhibition of colon cancer cells (SW 480) by lime volatile oil measured by MTT assay

Sl. No	Concentration of lime volatile oil (µg/ml)	Incubation period (h)		
		24	48	72
1	O1-6.25	15.21 [f]	25.85 [e]	27.63 [f]
2	O1-12.5	16.53 [f]	35.76 [d]	37.56 [e]
3	O1-25	22.62 [e]	36.12 [d]	40.92 [d]
4	O1-50	27.39 [d]	70.56 [b]	72.86 [b]
5	O1-100	35.34 [c]	76.53 [a]	86.08 [a]
6	O1-200	43.99 [b]	78.15 [a]	87.64 [a]
7	Camp-25	49.36 [a]	63.87 [c]	67.54 [c]
SEm±		0.975	1.041	0.645
CD (1%)		4.212	4.496	2.787

Means followed by same alphabet do not differ significantly by DMRT (P=0.1)

components present in lime volatile oil are monoterpenes. In the present study, the inhibition of colon cancer cells may be due to the presence of monoterpenes.

4.4.5.2 Cell proliferation using cell count assay

Results of the studies on percent inhibition of colon cancer cells (SW 480) by volatile oil extracted from lime fruits harvested from two types of lime plants (Thorny and Non thorny), there was significant differences between the plant types and the concentrations studied (Table 26). The volatile oil extracted from the lime fruits of thorny plant indicated significantly higher percent inhibition at all the incubation periods compare to non-thorny plants. With an increase in the concentration of the volatile oil from 12.5 to 100 µg/ml , there was an increase in the percent inhibition at all the incubation periods. The percent inhibition of colon cancer cells was significantly higher with the control of comptothecin at 24, 48 and 96 h of incubation. The percent inhibition also increased from 24 to 96 h of incubation not only with the control but also with other concentrations and plant types. The interaction of plant type and the concentration indicated with thorny at 100 µg/ml recorded significantly higher percent inhibition over non-thorny at the same concentration indicating its supremacy in the suppression of colon cancer cells. However, it was significantly lower compare to the control at 25 µg/ml. Significantly lower percent inhibition was noticed with non-thorny at 12.5 µg/ml over all other treatment combination. While thorny at 25 µg/ml recorded significantly lower percent inhibition of colon cancer cells among thorny types at 24 h of incubation. A similar trend continued at 48 and 96 h of incubation also with non-thorny recording significantly lower percent inhibition of colon cancer cells compare to all other treatment combinations.

4.4.5.3 DNA of SW-480 cells

Fragmentation of DNA as ~200 bp fractions is considered as one of the hallmarks in the process of apoptosis. Results of the study have shown fragmentation of DNA upon treatment of cells with volatile oil indicating involvement of apoptosis (Figure 16). Analysis of DNA from the cells treated with volatile oil has shown disintegration of DNA, which partially resembles apoptotic pattern (Figure 16). Disintegration of DNA was observed prominently after 12 and 24 h, which was not found in untreated cells (control).

4.4.5.4 Measurement of Caspase-3 content

Analysis of SW480 cells treated with lime volatile oil for 24 and 48 h for the total caspase-3 content by spectofluorimetric assay has shown elevated content of caspase-3 in cells pretreated with volatile oil. Caspase-3 was elevated up to 1.8 fold and 2 folds respectively after 24 and 48 h in comparison with untreated control cells (Figure 17). Since caspase-3 is known as an executor caspase, the result of current study serves as major support for induction of apoptosis by lime volatiles. . Induction of caspase-3 up to 2 folds by lime volatile oil indicates that casapse-3 is actively involved in apoptosis induction.

4.4.5.4 Protein expression analysis

Expression of proteins is considered as confirmation regarding execution of molecular actions and it is measured through western blotting. Results of current study have revealed elevated expression of both Bax and caspase-3 in cells pretreated with lime volatile oil for 24 and 48h Figure 18). Further, decrease in the expression levels of antiapoptotic-Bcl_2 was observed upon pre-treatment of volatile oil in both 24 and 48h. The expression ratio of Bax/Bcl_2 is considered as one of the major indicator of apoptosis, results of our study have shown that pre treatment of SW480 cells with volatile oil elevated ratio by 2 and 4.3 folds in comparison to untreated control cells after 24 and 48 h, respectively. Lime volatile oil has shown 4.3 fold enhancement in the ratio of Bax/Bcl_2, after 48 h of treatment.

4.4.5.6 Acridine orange staining

Staining of the cells pre-treated with volatile oil for 24 h showed rupture and bleeding of membrane, which is a characteristic feature of cells undergoing apoptosis (Figure 19). Morphology of cells treated with volatile oil was similar to that of camptothecin with irregular shape and most of the cellular contents was found as small fractions confirming the cell death.

Table 26. Per cent reduction in proliferation inhibition of colon cancer cells (SW 480) treated with lime volatile oil extracted from thorny and non thorny plants as and measured by viable cell count

Sl No.	Treatment details			Incubation period (h)		
	Interaction (Main x Sub factor)					
	Plant type (Main factor)	x	Concentration (Sub factor)	24	48	96
1	Thorny	x	12.5 µg/ml	23.78 ^{e}	32.51 ^{g}	39.02 ^{g}
2	Thorny	x	25 µg/ml	34.65 ^{d}	47.73 ^{e}	57.28 ^{e}
3	Thorny	x	50 µg/ml	42.50 ^{c}	57.11 ^{d}	68.53 ^{d}
4	Thorny	x	100 µg/ml	51.01 ^{b}	78.67 ^{b}	92.25 ^{b}
5	Camptothecin (control)	x	25 µg/ml	62.42 ^{a}	89.74 ^{a}	98.71 ^{a}
6	Non-thorny	x	12.5 µg/ml	16.17 ^{f}	24.65 ^{h}	29.58 ^{h}
7	Non-thorny	x	25 µg/ml	25.02 ^{e}	37.66 ^{fg}	45.19 ^{f}
8	Non-thorny	x	50 µg/ml	32.25 ^{d}	41.76 ^{ef}	50.11 ^{f}
9	Non-thorny	x	100 µg/ml	42.88 ^{c}	66.78 ^{c}	80.14 ^{c}
10	Camptothecin (control)	x	Control at 25	62.42 ^{a}	89.74 ^{a}	98.71 ^{a}
			SEm±	1.235	1.165	1.295
			CD (1 %)	4.690	4.423	4.917
	Plant type (Main factor)					
1	Thorny			42.87 ^{a}	61.15 ^{a}	71.16 ^{a}
2	Non-thorny			35.75 ^{b}	52.12 ^{b}	60.75 ^{b}
			SEm±	0.552	0.521	0.579
			CD (1 %)	2.098	1.978	2.199
	Concentration (Sub factor)					
1	12.5 µg/ml			19.98 ^{e}	28.58 ^{e}	34.30 ^{e}
2	25 µg/ml			29.83 ^{d}	42.70 ^{d}	51.24 ^{d}
3	50 µg/ml			37.38 ^{c}	49.43 ^{c}	59.32 ^{c}
4	100 µg/ml			46.94 ^{b}	72.70 ^{b}	86.19 ^{b}
5	Camptothecin (control at 25 µg/ml)			62.42 ^{a}	89.74 ^{a}	98.71 ^{a}
			SEm±	0.873	0.824	0.916
			CD (1 %)	3.317	2.34	3.477

Means followed by same alphabet do not differ significantly by DMRT (P=0.01)

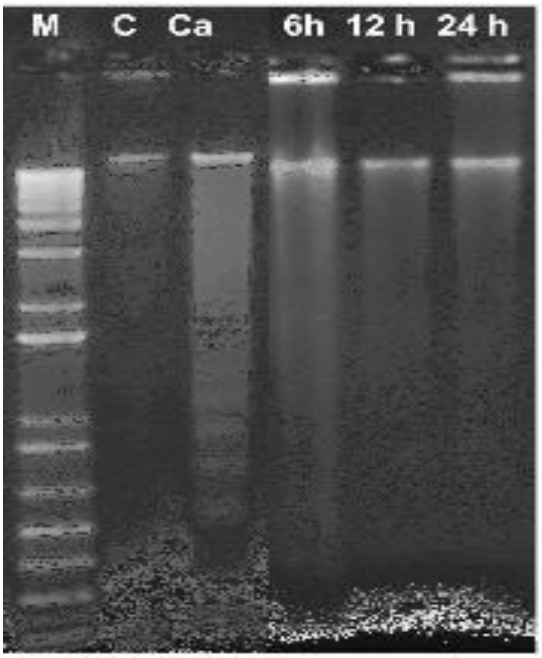


Figure 16. DNA fragmentation in lime volatile oil treated SW-480 cells, M-Marker; C-control (untreated cells); Ca-Camptothecin at (50 µg/ ml), for 12 h: DNA disintegration after treatment of lime volatile oil (100 µg/ml) for 6, 12 and 24 h

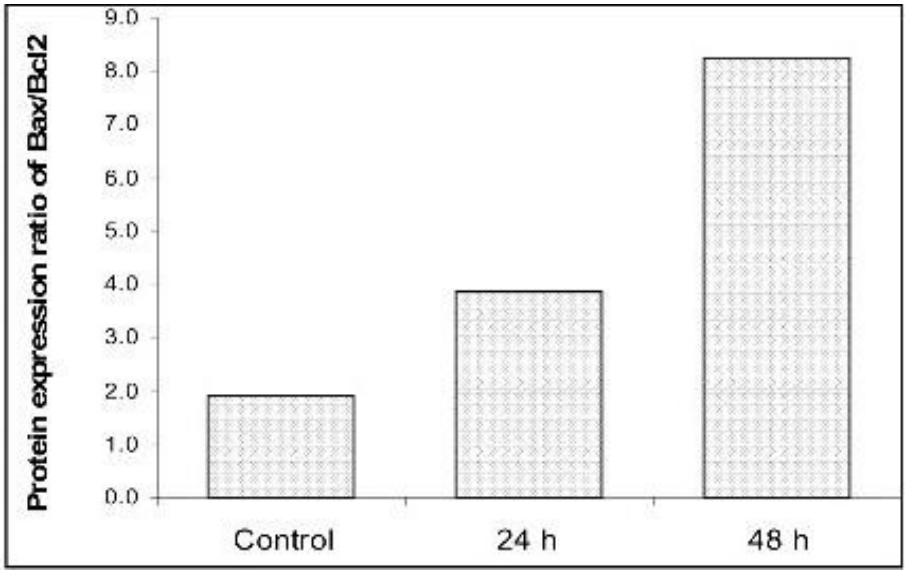


Figure 17. Effect of lime volatile oil treatment (24 and 48 h) on the Caspase-3 content of SW480 cells, Caspase-3 was measured using a specific substrate Ac-DEVD-AMC AMC IN-acetyl-Asp-Glu-Val-Asp-AMC (7-amino- 4-methylcoumarin)j and results were expressed in terms of fluorescent units related to 7-amino-4-methylcoumarin

Apart from cell culture evidences, *in vivo* studies conducted using D-limonene a major constituent of volatile oil has showed chemopreventive ability in both initiation and promotion stages of skin carcinoma in chemically induced rodents. D-limonene and its derived monoterpinoids have shown to affect $p21^{ras}$ expression by altering overall expression or through farnesylation of proteins.

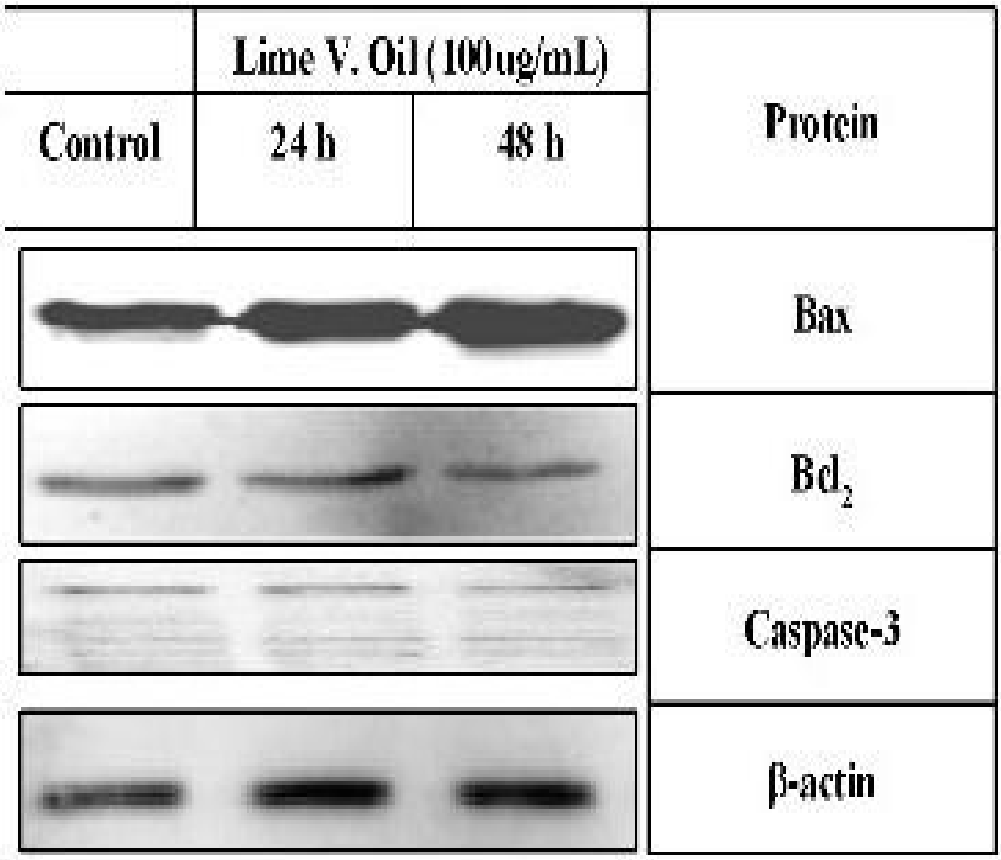


Figure 18. Expression of apoptosis related proteins by treatment of lime volatile oil for 24 and 48 h. Proteins from treated cells were separated on 12% SDS-PAGE and transferred into nitrocellulose membrane. The membrane was probed by antibodies for b- actin, Bax, Bc12 and caspase-3(Santa Cruz) and antigen-antibody complex was visualized by enhanced chemiluminescence method. Samples have shown increased expression of Bax protein, highest intensity of Bax observed in samples treated with lime oil for 48 h. Expression of $Bc1_2$ was found to decrease in both 24 and 48 h treatment, b-actin was used as loading control

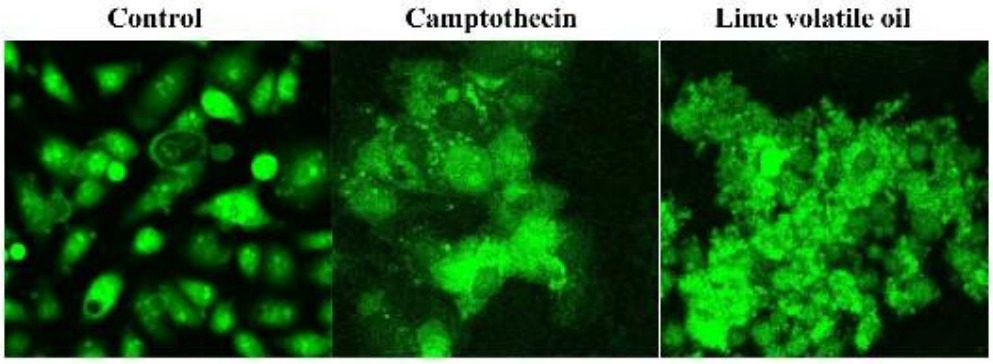


Fig.19: Fluorescent images showing morphology of control cells, Camptothecin and Lime Volatile oil (100µg/ml)

5. DISCUSSION

Citrus fruits are recognized as an important component of the human diet, providing a variety of constituents important to human nutrition, including vitamin C, folic acid, potassium, flavonoids, pectin, and dietary fiber. Citrus also contains significant amounts of highly oxygenated triterpenoid compounds (limonoids), particularly in underutilized byproducts of citrus juice production. Limonoids occur naturally only in plant species of the Rutaceae and Meliaceae plant families.

Lime (*Citrus aurantifolia* Christm), which belongs to the family Rutaceae, is widely found in India and in many other tropical and subtropical regions, and it is one of the most popular edible fruits in the world. Lime is used for the extraction of juice, preparation of squash, concentrates, beverages and byproducts, such as citric acid and pectin. Lime leaves have been traditionally used for the treatment of skin diseases and as anti-inflammatory agent. The leaf decoction is used as eye drops and to bathe a feverish patient and also as a mouthwash and gargle incases of sore throat and thrush.

In the past few years there has been an increased interest in the study of *Citrus* plants because their fruits and leaves accumulate large amounts of flavonoids that occur as either O-or C-glycosides. Flavonoids constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. However, recent interest in food phenolics has increased greatly, owing to their antioxidant capacity and their possible beneficial implications in human and mammalian health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies. Keeping this in view, the present investigations were carried out with an aim to isolate and identify the bioactive compounds present in both edible and non-edible portions of lime fruits, to study the antioxidant and anti-cancer properties of the bioactive compounds and their extracts isolated from the these portions. Results obtained from the investigation are discussed in this chapter.

5.1 Isolation of bioactive fractions from lime

Natural products have historically been a rich source of lead molecules in drug discovery. However, natural products have been de-emphasized as high throughput screening resources in the recent past, in part, because of difficulties in obtaining high quality natural products. In addition, natural products research IS based on screening of extracts, bioassayguided isolation, structure elucidation and subsequent scale up production. Fortunately, new technologies in mass spectrometry, NMR and other spectroscopic techniques can greatly facilitate structure elucidation process. Citrus species are known for unique limonoids. About 37 limonoid aglycones and 19 limonoid glucosides have been isolated from citrus and their hybrids (Hasegawa *et al*., 2000). The extensive review of information on isolation of limonoids from citrus revealed that, there are no systematic studies on isolation of limonoids from *Citrus aurantifolis* Swingle, commonly known as lime.

In the current study, three limonoid aglycones *viz.,* limomin, LNA and ILNA and one glucoside viz., LG along with a phytosterol (SG) were successfully isolated. The purity was confirmed by HPLC and MS.

Various solvents have been used for the extraction of bioactive compounds mainly because of the differential collartive of these compounds which get eluted differentially. The variation in the yield and the limonoid content also varied by different solvent extracts mainly because of this property. It was observed that the yield was maximum with methanol mainly because of the high collaret of the solvent. The per cent of different compounds isolates from the lime peel as well as the seed also varied with the mobile phases and the most effective was chloroform. This indicates that the chloroform is an effective solvent mobile phase for the extraction and separation of limonoids. Similarly (Hasegawa *et al.,* 1991; Manners, 2004; Herman *et al*., 1992) have obtained limonin glucocide, nomilin glucosy and other limonoid glucocydes chromatographically from polar extracts of dipartite citrus seeds. Further they have been kept thrice (Hasegawa *et al*., 1989; Sawabe *et al.,* 1999).

While, limonoids in citrus tissues and juice have been customarily detected by TLC (Maier and Grant, 1970). For identification purposes, we first examined R_F values of the

major standard limonoids by TLC using three solvents system. These results suggest that these HPLC conditions are sufficiently sensitive to detect the levels of the major limonoids.

The composition and relative concentrations of limonoid aglycones and their glucosides are very similar to those of the common Citrus species (Ozaki *et al*., 1991). Like the others, the ratio of aglycones to glucosides was about 2. The 17-P-Dglucopyranoside of nomilin is the predominant limonoid glucoside. This supports the previous suggestion (Fong *et al*., 1991) that limonoids and limonoid glucosides in seeds are biosynthesized there, independent from the biosynthesis occurring the fruit tissue. From these results, we recommend that HPLC procedure as a convenient method.

Limes contain coumarins, furanocoumarins (psoralens) and pyranocoumarins (Gray and Waterman, 1978; McHale and Sheridan, 1989; Macheix *et al*., 1990; Tatum and Berry, 1977). A variety of coumarins have also been isolated from lime oil (McHale and Sheridan, 1989). In the present study, three different coumarins viz., limettin, 5,7, dimethoxy coumarin and isopimpinellin have been isolated from the lime peel which varied in their concentration. Of the three, the maximum concentration was of isopimpinellin. Gray and Waterman (1978) indicated that limes also contain isopimpinellin and limettin. It is also been shown that isopimpinellin is not a photosensitizer (Ashwood-Smith *et al*., 1983; Hudson *et al*., 1987). Limettin is 200 times less photoactive than bergapten (Naganuma *et al*., 1985) although it was not photoxic at 1% on stripped human skin (Marzulli and Maibach, 1970).

Nigg *et al*. (1993) reported that in the rind of Persian limes, coumarin concentrations were in the order of limettin > bergapten > isopimpinellin > xanthotoxin > psoralen. Further, they indicated that in the rind of Key limes, psoralen and xanthotoxin were analytically absent; limettin was 10 times more concentrated than either bergapten or isopimpinellin, which were equal in concentration. While, in the Persian lime pulp it was in the order; isopimpinellin > limettin > bergapten > xanthotoxin > psoralen.

5.2 Studies on antioxidant activities of bioactive compounds

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells.

Normally, synthetic antioxidants (BHT and BHA) are used to suppress the development of rancidity in fats and oils. These synthetic antioxidants are known to have toxic and carcinogenic effects on human health (Ito *et al*., 1986; Martin & Gilbert, 1968). Therefore, there is a strong need for effective antioxidants from natural sources as alternatives to prevent several diseases.

Citrus fruits are rich sources of vitamin C (ascorbic acid), an essential nutrient with well-described antioxidant properties. However, recent studies have demonstrated that citrus also contain other bioactive compounds including flavonoids, coumarins, cartoenoids and limonoids with potential health promoting properties (Lam and Hasegawa, 1989; Miller *et al*., 2000; Tian *et al*., 2001). Accumulative evidence suggests antioxidant activities of flavonoids from a variety of plant sources (Sanz *et al*., 1994; Mira *et al*., 2002; Ng *et al*., 2000). Indeed, flavonoids possess a wide range of activities *in vitro* (Bravo, 1998; German and Walzem, 2000). For example, this class of bioactive compounds is known to act as free radical scavengers, to modulate enzymatic activities, and to inhibit cellular proliferation as well as possessing antibiotic, anti-alllergenic, anti-diarrhea, anti-ulcer, and anti-inflammatory activities (Duthier and Crozier, 2000)).

Several studies have already been realized on the antioxidant activity in food systems of several citrus fruits (sweet orange, lemon, grapefruit), used both directly (Piskur and Higgins, 1949; Williams and Harris, 1983) and as extracts (Kroyer, 1986; Pereira and Mancini-Filho, 1994; Sawamura *et al*., 1988; Ting and Newhall, 1965).

Generation of the ABTS [2, 2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid] radical cation (Wolfenden and Wilson, 1982) forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances (Rice-Evans and Miller, 1995; Rice-Evans *et al*., 1996; Miller *et al*., 1996), aqueous mixtures and beverages (Salah *et al.,* 1995; Rice-Evans, 1995). The original $ABTS^{+}$ assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants.

Free radical scavenging is one of the known mechanisms of inhibition of lipid oxidation. In DPPH (1, 1,-diphenyl-2-picryl hydrazine) free radical scavenging assay, antiradical power of an antioxidant is measured as color changes from purple to yellow. This is used to evaluate hydrogen-donating ability of the compound. It is a rapid method and most widely employed to characterize antioxidant activity of plant material (Arnao, 2000).

The current study was focused on understanding the possible role of phytochemicals present in both edible and non-edible parts of limes in antioxidant properties. The content of phytochemicals responsible for the radical scavenging activity were quantified.

5.2.1 Quantification of phenolics in lime juice

The phenolic content of lime juice extracts ranged from 0.66 to 4.20 % w/w, based on dry weight basis. The MeOH solvent was able to extract most of the phenolics due to its high polarity. Similar results, with 0.48% w/w phenolics content have been reported from Valencia late juice (Rapisarda *et al*., 1999). The total polyphenols content in orange fruits ranged from 0.5 to 1.0% w/w in fresh juice (Vinson *et al*., 2001). Further, total polyphenols in three commercially available orange, Jaffa orange, and Florida orange ranged from 0.5 to 0.75% w/w (Gardner *et al*., 2000). The phenolic compounds are reported to have anti-oxidant activity *in vitro* and observational studies also support their potential role in protecting cardiovascular health (Hertog *et al*., 1993).

5.2.2 Quantification of limonoids in lime juice

The other important groups of bioactive compounds in citrus are limonoids. Earlier studies indicate that the commercial citrus juice contained high concentration of limonoid glucosides (LG) and the contents were 320, 190 and 82 ppm in orange, grapefruit and lemon juices, respectively. The major glucoside in citrus juice was limonin 17-O-β-D-glucopyianoside (LG), which constituted over 50% of the total limonoid glucosides in the juices (Fong, 1989). Further, LG was reported as a major glucoside in a variety of citrus juices tested. Orange juice contains an average of 180 ppm or 56% of the total limonoids. Grapefruit juice contain 120 ppm, which accounts for 63% of the total limonoids, while, lemon juice is known to contain about 54 ppm, which was 66% of the total limoniod content (Fong, 1989). However, in the current study, the major limoniods such as LNA and ILNA accounted for 40 and 45 per cent, respectively. Hence, lime juice is one of the good sources for limonoids and flavonoids.

5.2.3 Quantification of flavonoids in lime juice

Citrus plants accumulate large amounts of flavonoids like other plants; however, limonoids are more specific to citrus. In the current study, hesperidin was the most abundant flavonone quantified (58.66% w/w of total flavonoids) and followed by rutin (36.67%w/w of total flavonoids). The other minor flavonoids were didymin (3.46% w/w of total flavonoids) and hespertin (1.87% w/w of total flavonoids), on dry weight basis. It has been reported that the most prominent flavanones from limes was hesperidin. The hesperidin and eriocitrin are found to be prominent flavonones of lemon, where as only hesperidin is the major concentration in limes (Peterson et al., 2006). In another study, flavonoid content of Mexican lime was found to be hesperidin (92.4% w/w), eriocitrin (1.8% w/w) and narirutin (2.0% w/w of total flavonoids) (Nogata *et al*., 2006). In addition to flavanone, limes are known to contain flavonol

such as rutin (Nogata *et al*., 2006). The rutin, didymin and hesperitin are the other flavonoids found in lime juice. The presence of rutin along with other class of flavonoids makes lime juice unique in nature compared to others citrus juices (Calabrò *et al*., 2005) .

5.2.4 Antioxidants activity of lime juice extracts by DPPH assay and ABTS assay

Recent reports have shown that there has been increasing interest in investigating the potential of natural antioxidants, particularly those of plant origin (Jayaprakasha et al., 2000). Natural antioxidants of plant origin mainly phenolics, are of substantial interest as dietary supplements (Halliwell et al., 1995). Hence, efforts were made to determine the possible antioxidant components of the extract of lime juice. Further, the antioxidant activity by citrus extracts is primarily attributed to its proton donating capacity (Girennavar *et al*., 2007; Jayaprakasha *et al*., 2007). Evidences from research indicate that, the activity may also be due to the presence of flavonoids, carotenoids and ascorbic acid in citrus fruits (Gorinstein *et al*., 2004).

The results of correlations studies in the current study are in agreement with the previous reports, which explained positive correlations between the TEAC and PCL assays and total phenolics (Netzel *et al*., 2007). Other studies reported a linear relationship between total phenolic content and antioxidant capacity in berry crops and herbs (Zheng and Wang, 2001; Zheng *et al*., 2003). The antioxidant activity of extracts demonstrated in this study clearly indicate the potential application value of the lime fruits.

5.2.5 Quantification of phenolics in lime peel and seed extracts

The phenolic content of the aqueous solutions of lime seed and peel extracts were in the range from 1.06 to 3.9 g/100 g (dry weight basis) in all the extracts. Phenolics are aromatic secondary plant metabolites, which play a significant role in colour, sensory and nutritional qualities and antioxidant properties of food (Robbins, 2003). The earlier report suggest that the polyphenol content (chlorogenic acid equivalents) in peeled lemon (*Citrus limon*) and orange (*Citrus sinensis*) were 164 ± 10.3 and 154 ± 10.2 mg/100 g fresh fruit, respectively (Gorinstein *et al*., 2001).The highest yield of citrus peel extract (19.87%) was obtained with MeOH, followed by acetone (15.00%) and diethyl ether (12.75%) was reported in study of use of citrus peel in preservation of oils (Zia ur, 2006). The MeOH extract of lime peel contains different phenolic antioxidant compounds (Alexandra *et al*., 1998). Recent study has shown that the yield of extractable compounds was highest in MeOH extract from pomegranate peel and also citrus in comparison with the solvents such as ethyl acetate and water (Jayaprakasha *et al*., 2007; Singh *et al*., 2002).

5.2.6 Quantification of limonoids in lime peel and seed extracts

Limonin was the most prominent limonoid and the other prominent limonoids were ILNA and LNA in the present investigation. Research results have also indicated that limonin was found in significant amount in all the four varieties of citrus tried. Further other reports showed the isolemonic acid was present in significant amount in Nova tangerine and Cleopatra mandarin seeds (Vikram *et al*., 2007) . In contract, the most prominent limonoids in peel was ILNA followed by LNA. Previous studies have reported that some limonoids were found to be present in the chloroform extract of lemon peel and the most predominant being limonin (Baldi *et al*., 1995).

5.2.7 Quantification of flavonoids in lime peel and seed extracts

The flavonoid composition of various varieties of citrus fruit have been reported and hesperidin was the most abundant flavonoid content in *C. reticulata* Blanco (6.76–12.0 mg/g dried matter), *C. sinensis* (L.) Osbeck (6.98–10.8 mg/g dried matter), and *C. limon* (L.) Bur (3.58 mg/g dried matter) (Kawaii *et al*., 1999). The present study indicated that hesperidin is the most prominent flavonoid in lime seed, and MeOH extract contained the maximum (45%). However, the flavonoid profile of peel showed that it contained neohesperidin in addition to hesperidin. The maximum quantity of hesperidin was found in methanol extract (34%). The earlier reports on flavonoids content of citrus suggest that flavanone is the major class of flavonoid in oranges (Wang *et al*., 2007). Similarly, the flavonoid analysis of extract of

Pericarpium Citri Reticulatae (FEPCR) by HPLC revealed that the main flavonoid compounds in FEPCR were hesperidin, nobiletin and tangeretin. The proportions of major flavonoids were hesperidin (68.45±0.61%), nobiletin (12.11±0.22%) and tangeretin (7.38±0.10%) (Yi *et al*., 2008)

5.2.8 Antioxidants activity of lime seed and peel extracts by DPPH assay and ABTS assay

Citrus fruits are known for their rich sources of bioactive compounds, including vitamin C, phenolics and flavonoids, with potential health-promoting properties (Gorinstein *et al*., 2001). These bioactive compounds are known to act as free radical-scavengers, to modulate enzymatic activities and to protect against a variety of diseases, particularly cardiovascular diseases and some types of cancer (Kurowska *et al*., 2000).

Results from recent research have suggested that certain phytochemicals from citrus are known to have antioxidant activity. Furthermore, mechanisms of antioxidant action can include inhibition of reactive oxygen species formation by suppressing of enzymes involved in free radical production; scavenging reactive oxygen species; and protecting antioxidant defenses (Halliwell *et al*., 2000). Flavonoids have been identified as fulfilling most of the criteria described above. Thus, their effects are twofold. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C (Hanasaki *et al*., 1994) . Flavonoids have also been shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in reactive oxygen species generation. (Korkina *et al*., 1996). Besides scavenging, flavonoids may stabilize free radicals involved in oxidative processes by complexing with them (Shahidi *et al*., 1992).

The antioxidant capacity of flavonoids is directly related to their structure (Cos et al., 1998), and in the case of hesperidin, the presence of a hydroxyl group at position 3' of ring B may be responsible for the capacity of hesperidin to scavenge the hydroxyl radicals generated from hydrogen peroxide. It is already known that the ability to scavenge superoxide is due to a hydroxyl group at position C-4' of ring B (Cos et al., 1998; Van Acker et al., 1996). It has previously been shown that the ring B C-4' methyl substitution of hesperidin can activate the ring B C-3' hydroxyl, making hesperidin a more active scavenger to the superoxide radical (Van Acker *et al*., 1998).

In the present study, ABTS and DPPH methods are correlated strongly with each other hence, both methods could be equally useful for assessing antioxidant activities of natural extracts at physiological pH and where colour interference is not significant. The earlier reports have shown positive correlations between the TEAC and PCL assays and total phenolics (Netzel *et al*., 2007) and also the phenolic compounds play a major role as a source of antioxidants in native Australian fruits, where a variety of phenolic compounds such as flavonoids (anthocyanins, flavan-3-ols, etc. and phenolic acids (benzoic and cinnamic) contribute to antioxidant activity activity. Other studies have also reported a linear relationship between total phenolic content and antioxidant capacity in berry crops and herbs (Zheng *et al*., 2001; Zheng *et al*., 2003). This is also corroborated by the findings of other reports which suggested that the *in vitro* ROS-scavenging activity of a rose hip water/acetone extract is mainly due to its phenolic constituents (Daels-Rakotoarison *et al*., 2002).

5.3 Analysis of chemical composition of lime volatile oil

The per cent of yield volatile oil (v/w) obtained was 0.46 ml on fresh weight basis, which was found to be more than double as compared to reported data.

The GC–MS analysis of lime volatile oil showed the presence of 22 compounds, constituting 90 per cent of the volatile principles. Total hydrocarbons in the lime volatile oil accounted for 45.02 per cent and the major identified compounds were D-limonene (30.13%) along with another nine hydrocarbons. In addition, lime volatile oil has 44.9 per cent oxygenated hydrocarbons. The alcohols (11.5%) represented seven compounds viz., trans-p-mentha-2, 8-dienol, □ □-linalool, fenchol, *p*-menth-8-en-1-ol, terpineol, myrtenol□ and terpineol. Ketones (32.72%) were represented by three compounds and D-dihydrocarvone (30.47%) was found to be the one of the major compound present in the volatile oil.

Previously D-dihydrocarvone was reported in orange juice and calamondin peel by GC-MS (Selli *et al*., 2004). Additionally, an ester (neryl acetate) and epoxide (2, 3-dehydro-1, 8-cineole) were also identified.

Recently, fresh and dehydrated lime chemical composition was analyzed by GC-MS. D-limonene was found to be 75.5 and 53.5 per cent in fresh and dehydrated limes, respectively (Yadav *et al*., 2004). However, in the present study, D-limonene and D-dihydrocarovone were found to be 30.13 and 30.47 per cent, respectively. Among the identified compounds, 14 volatile components were found to be different from the previous report. This may be due to differences in their growing location, environmental factors and harvesting time. Furthermore, chemical composition of nine cultivars of mandarins was reported with D- limonene (>88%) as the major compound in all volatile oils (Merle *et al*., 2004). Interestingly, cold pressed volatile oil has shown different chemical composition than hydro-distilled volatile oil and this may be due to heat treatment.

5.4 Studies on anticancer activity of purified compounds using cell lines

5.4.1 Prevention of pancreatic cancer by lime seed compounds

In the last few years, many studies from a number of laboratories have concentrated on the anticancer activities of citrus limonoids (Ejaz *et al*., 2006). Studies indicated that certain citrus limonoids are found to inhibit proliferation of cancer cells, such as MCF-7 (Tian *et al*., 2001). Inhibition of proliferation of colon cancer cells and neuroblastoma by four glucosides of citrus has been reported by Poulose *et al*. (2006). Studies have also shown that apoptosis is the major cause for inhibition of proliferation of colon cancer cells by citrus limonoids (Jayaprakasha *et al*., 2007; Jayaprakasha *et al*., 2008; Poulose *et al*., 2006). However, until now, very little information is available about the inhibition of pancreatic cells by lime compounds.

The purified compounds along with gemcitabine were subjected to proliferation inhibition by MTT assay on Panc-28 cells. The IC_{50} values were less than 20 and 9 µM at 24 and 72 h, respectively. Further, these results were confirmed by viable cell count assay. Thus there is a clear indication of cytotoxicity by lime compounds on pancreatic cancer cells.

In order to know the mechanism of cytotoxicity, cells treated with compounds were analyzed for DNA fragmentation. However, genomic DNA was found to be intact after treatment with compounds. A study on treatment of transformed Jurkat cell (JILdm) with staurosporine has shown nuclear morphological change and induction of apoptosis without fragmentation of DNA (Sakahira *et al*., 1999). Similar results were also reported with treatment of extracts of Brazilian red propolis to Panc-1 cells (Awale *et al*., 2008). Based on theses evidences, we further explored possible involvement of apoptosis through protein expression.

Measurement of protein expression levels in tissues or cells is one of the common tools to understand the molecular mechanisms. In the current study, cell death related proteins from Panc-28 cells treated with five of the isolated compounds were analyzed to understand the possible mode of cytotoxicity. The p53 is known as death inducing protein, which play a vital role in cancer initiation. Mutation of p53 has been reported in cancers such as, colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues, and hemopoietic tissues (Hollstein *et al*., 1991). Mutation of K-ras and p53 genes are known to play a role in pancreatic cancers (Pellegata *et al*., 1994). In another study, p53 gene transduction has shown direct inhibition of telomerase activity, independent of its effects on cell growth arrest, cell cycle arrest, and apoptosis in human pancreatic cells (Kusumoto *et al*., 1999). Lectin from *Polygonatum cyrtonema* has shown induction of p53 mediated apoptosis in human melanoma A375 cell (Liu *et al*., 2009).

Other natural compounds which have shown induction of p53 mediated apoptosis are, curcumin, resveratrol, anthocyanins and capsaicin (Athar *et al*., 2009; Khan *et al*., 2008; Kuramori *et al*., 2009; Qin *et al*., 2009). In the current study, induction of p53 has shown by all the lime compounds indicating the potential of these compounds in p53 mediated apoptosis induction. Another gene cyclin-dependent kinase inhibitor is also known as p21, which mainly

binds and inhibit the activity of cyclin-CDK2 or -CDK4 complexes and functions as a regulator of cell cycle progression at G_1. Expression of p21 is regulated by p53 in both dependant and independent manner during cell differentiation and DNA damage (Macleod *et al*., 1995). The gene p21 has been known for regulation independent of p53 in situation such as, tissue development, serum stimulation and cell differentiation. Such independent expression of p21 has been reported in pancreatic cancer cells (DiGiuseppe *et al*., 1995). Results of the current study indicate that expression of p53 is upregulated and that of p21 is down regulated, which may be due to independent expression of p21.

Members of bcl-2 family proteins are key regulators of the process of apoptosis. The bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified potentially anti-apoptotic. The bcl-2 proteins seems to form a heterodimer complex with Bax, which results in neutralizing its proapoptotic effects of inducing cell death (Khan *et al*., 2007). Hence, the ratio of Bax/bcl_2 is considered as one of the major markers of apoptosis. Very few compounds of natural origin, such as EGCG (present in pomegranate and tea) and resveratrol from grape skin are known to affect the ratio (Kalra *et al*., 2008). Results of our study suggest that lime bioactive compounds elevate the expression ratio, in the following order LNA > LG > ILNA > limonin. The results of the study clearly demonstrate that lime bioactive compounds may effectively inhibit cell proliferation of human pancreatic carcinoma cells through programmed cell death. However, further *in vivo* studies may be useful for exploitation of lime in prevention of human pancreatic cancer.

5.4.2 Prevention of colon cancer by lime peel compounds

Percent inhibition of colon cancer cells tested with compound extracted from lime peel indicated significant difference between them and between the concentrations and their interactions at all the incubation period (Table 19). The percent inhibition of bioactive compound was compared with the control gemcitabine, a drug used in the treatment of colon cancer. It was observed that the percent inhibition of colon cancer cells was highest with the gemcitabine at all incubation period and the percent inhibition increases progressively with an increase in the incubation period. Among the bioactive compounds, the percent inhibition of colon cancer cells was significantly higher with 48 at all the incubation periods compare to other bioactive compounds. Significantly lower percent inhibition was noticed with limettin at all the incubation period. With an increase in the concentration of bioactive compound, there was a progressive increase in percent inhibition of colon cancer cells at all the incubation period with 200 µM recording significantly higher percent inhibition over other concentrations. However, significantly lower percent inhibition was noticed with 12.5 µM . The interaction between the bioactive compound and the concentration revealed with Isopimpinellin µM at 200 µM had a maximum percent inhibition (68.48%) at Isopimpinellin hours incubation compare to all other treatments. However, no significant differences were observed between Limettin at 12.5 µM and Isopimpinellin at 12.5 µM; Limettin at 50 µM and Isopimpinellin at 100 µM; Limettin at 25 µM and 48 h at 25 µM at Isopimpinellin hours of incubation. At 96 h of incubation, among the bioactive compounds, 5,7, Dimethoxy coumarin at 200 µM recorded significantly higher percent inhibition over all other bioactive compounds and concentrations. However, no significant differences were observed between Limettin at 50 µM and 5,7, Dimethoxy coumarin at 100 µM; Limettin at 100 µM and Isopimpinellin at 100 µM and 200 µM at 96 h of incubation. While at 144 h of incubation, Limettin at 200 µM recorded significantly higher percent inhibition (88.76%) over all other treatment combinations but was significantly lower compare to the control. Similarly, no significant differences were observed between 5,7, Dimethoxy coumarin at 12.5 µM and 25 µM, Limettin at 12.5 µM; 5,7, Dimethoxy coumarin at 100 µM and 200 µM, Limettin at 25 µM and Isopimpinellin at 25 µM.

5.4.3 Prevention of pancreatic cancer by lime juice extracts

The results of MMT assay indicate pretreatment of Panc-28 cells with lime juice extracts, exhibited significant inhibition of proliferation as measured by both MTT assay and viable cell count. Recent results have demonstrated the inhibition of proliferation of number of cancer cells by citrus limonoids (Tian *et al*., 2001). Studies have also shown that the major cause for inhibition of proliferation of colon and neuroblastoma cancer cells by citrus limonoids is due to apoptosis (Jayaprakasha *et al*., 2007; Jayaprakasha *et al*., 2008; Poulose

et al., 2006). However, there is no information about the inhibition of pancreatic cells by this class of compound.

The induction of tumor suppressor protein (p53) in cells treated with different extracts of lime juice suggests the potential of lime juice in p53 mediated apoptosis induction. Mutations of these protein or dysfunctions is one of the major causes for malignant transformation (Baudier *et al*., 1992; Lavin *et al*., 2006). Further, failure of the p53 tumor suppressor protein is a causal incident in the pathogenesis of a large portion of human malignancies (Fridman and Lowe, 2003). The p53 is a transcription factor co-ordinates cellular responses to stresses, such as DNA damage and oncogene activation after induction, p53 alters the expression of a huge set of target genes leading to cell-cycle arrest, apoptosis, increased DNA repair, and/or inhibition of angiogenesis (Giono and Manfredi, 2006; Vogelstein *et al*., 2000). Thus, expression of p53 is one of the major proliferation activities.

Apoptosis is an evolutionarily conserved process which is also known as 'programmed cell death' that plays an essential role in organism development and tissue homeostasis. Recent research utilizes this process of programmed cell death as an ultimate weapon to treat cancer. Apoptosis is controlled by the complex interaction between regulatory proteins from the Bcl-2 family. The death inducing intrinsic pathway of apoptosis are controlled by these pro and anti apoptotic protein (Marzo and Naval, 2008). The lime juice extracts, have significantly inhibited bcl-2 protein level; while, Bax protein level was increased. These results suggest the lime juice induced apoptosis by affecting Bax/Bcl-2

To further understand the mechanism(s), involvement of caspases in the cell death was examined. Results suggested that there is an elevation of caspase-3 protein level in cells treated with extracts compared to control. The caspase-3, which is also known as executor caspase is believed to serve as a general mediator of apoptosis in the pathway and is activated early during apoptosis. Activated caspase-3 is often considered the key executioner of apoptosis because of its ability to cleave a vast array of proteins (Green and Reed, 1998). The above results of the immunobloting showed clear evidence of apoptosis involvement in the induction of cytotoxicity by treated lime juice.

5.4.4 Prevention of pancreatic cancer by lime seed and peel extracts

Recent research evidence suggest that citrus fruits have anticancer effects, including the reducing the proliferation of some cancer cells (Arias and Ramon-Laca, 2005), and the induction of apoptosis in human gastric and colon cancer cells (Kim *et al*., 2005). Further, recent research has shown that citrus contains several possible anti-cancer agents such as flavonoids and limonoids (Poulose *et al*., 2006; Vanamala *et al*., 2006). Since limes are one of important citrus fruits and widely consumed, an attempt was made to study their effect on pancreatic cancer cells. The highest proliferation inhibition activity of lime seed EtOAc extracts may be due to the fact that it contained significant amount of limonoids such as , LG (22.57 mg/100 g), LNA (37.97 mg/100 g), ILNA (46.43 mg/100 g) and Limonin (4.8 mg/100 g) along with flavonoids viz., hesperdin (613.11 mg/100 g) and neohesperidin (98.24 mg/100 g). The previous research has shown that five flavonoids, viz., luteolin, quercetin, kaempferol, apigenin, and taxifolin, markedly inhibited cancer cell lipogenesis, in both prostate and breast cancer cells, and a remarkable dose-dependent response was observed between flavonoid-induced inhibition of cell growth, and induction of apoptosis (Brusselmans *et al*., 2005). In addition, reports also suggest treatment with kaempferol resulted in a dose- and time-dependent reduction in cell viability and DNA synthesis (Nguyen *et al*., 2003). Similarly, all the extracts of peel have shown to inhibit the proliferation of pancreatic cells.

The lime seed and peel extracts have shown that bcl-2 protein level was significantly inhibited; while, Bax protein level was increased. These results suggest the lime juice induced apoptosis by affecting Bax/Bcl-2. To further understand the mechanism(s), the involvement of caspases in the cell death was examined. Results suggested that there is an elevation of caspase-3 protein level in cells treated with seed extracts of EtOAC and MeOH compared to control and in case of peel all the extract had elevated. The caspase-3, which is also known as executor caspase is believed to serve as a general mediator of apoptosis in the pathway and is activated early during apoptosis. Activated caspase-3 is often considered the key

executioner of apoptosis because of its ability to cleave a vast array of proteins (Green and Reed, 1998).

Apoptosis is an active physiological process leading to cellular self-destruction that involves precise morphological and biochemical changes in the nucleus and cytoplasm (Khan and Mlunawana, 1999). Natural compounds that inhibit the proliferation of malignant cells through induction of apoptosis may represent a helpful holistic approach to both cancer chemoprevention and chemotherapy. Though several anticancer agents have been developed, but have serious shortcomings like adverse side effects and resistance (Panchal, 1998). Hence , there is need for more attention towards research in the use of plant materials for the treatment/prevention of cancers and also development of safer and more effective therapeutic agents (Ramos, 2007).

Normally, apoptosis is a result of a complex interplay between regulatory proteins from the Bcl-2 family (Williams and Smith, 1993). These pro- and anti-apoptotic proteins are key regulators of the intrinsic pathway of apoptosis, controlling the point of no return and setting the threshold for engagement of the death machinery (Marzo and Naval, 2008). Previous reports have shown that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals(Chang *et al*., 2005). Therefore, Bcl-2 proteins have emerged as an attractive target for the development of novel anticancer drugs (Mohammad *et al*., 2008). Changes in the Bcl-2/Bax ratio have been reported to be caused by downregulation of Bcl-2 and slight downregulation of Bax (Cha *et al*., 2004) downregulation of Bcl-2 and upregulation of Bax (Paris *et al*., 2007 and Han, 2008) and downregulation of Bcl-2 with no change in the level of Bax (Motomura *et al*., 2008).

In this study, it was demonstrated that Bcl-2 expression was significantly inhibited, while Bax expression was markedly increased in a concentration-dependent manner. In contrast, time-course analysis showed that Bcl-2 expression gradually decreased in a time-dependent manner; whereas Bax expression was relatively constant in lime extract treated cells. In both cases, our results suggest that the lime extract induced apoptosis by shifting the Bax/Bcl-2 ratio in favour of apoptosis. The above results of the immunobloting showed clear evidence of apoptosis involvement in the induction of cytotoxicity by treated lime extracts.

5.5. Prevention of colon cancer by lime volatile oil

5.5.1 MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)] assay

In order to know the effect of lime volatile oil on colon cancer cells, experiments were conducted using cultured human colon carcinoma cells. Camptothecin was used as positive control for the comparison of inhibitory potentials. Dose dependent inhibition of SW480 cells was observed at different concentrations (6.25-200 µg/ml) of lime volatile oil treatment. Cell proliferation was highly significant at concentrations above 50 µg/ml ($P<0.001$). Most of the principle components present in lime volatile oil are monoterpenes. Monoterpenes have shown prevention of mammary, lung, skin, liver and forestomach cancer in rat models (Haag *et al*., 1992). D-limonene is metabolized into perillic acid, dihydroperillic acid and limonin 1,2-diol and these have higher bioavailability (Crowell *et al*., 1994). In the present study, the inhibition of colon cancer cells may be due to the presence of monoterpenes.

5.5.2 Cell proliferation using cell count assay

Results of the proliferation assay revealed similar trend of inhibition as observed by MTT assay. At 100 µg/ml of volatile oil treatment, 57.6 and 67% of cells were inhibited at 24 h and 48 h, respectively. Lime volatile oil also was tested for the toxicity using non-cancerous NIH 3T3 cells (Swiss mouse embryo fibroblast). No significant inhibition of non-cancerous cells by lime volatile oil was observed.

5.5.3 Cytotoxicity through LDH assay

Measurement of LDH content in culture medium is used as an indicator for cytotoxicity in apoptosis research (Johnson and Mukhtar, 2007). Results of the current study indicated that cytotoxicity was also induced due to loss of membrane integrity by volatile oils,

which may be due to high lipophilicity of compounds. Lactate dehydrogenase (LDH) is known to be released from cells due to damage in cell membrane, which indicated cell death either due to necrosis or apoptosis. Volatile principles of plant origin are known to inhibit cancer cells growth. Volatile oil of black cumin has shown the inhibition of 1, 2-dimethylhydrazine-induced aberrant crypt foci (ACF) in rats (Salim and Fukushima, 2003). *Patrinia scabra* Bunge root volatile oil has shown cytotoxic activity in human ovarian carcinoma and hepatoma cells (HongXiang *et al*., 2005).

Induction of cell death by D-limonene on mammary carcinoma cells was reported (Haag *et al*., 1992). This study reported protein isoprenylation and apoptosis as the possible mechanisms of cytotoxicity. D-dihydrocarvone, a major constituent of oil, has also shown ability to inhibit microbes of oral cavity and the same is used as one of the ingredients in oral care formulation (Parikh *et al*., 2004). The other major compound identified was m-Mentha-6, 8-diene R (+) which constituted 9.31% of total volatile oil, This component has also been reported from ginger and *Piper nigrum* and demonstrated antioxidant and antiproliferation activity (Maa and Gang, 2004). It is possible that the presence of these major constituents is responsible for inhibition of colon cancer cell proliferation.

5.5.4 DNA of SW-480 cells

Fragmentation of DNA as ~200 bp fractions is considered as one of the hallmark in the process of apoptosis. Results of the study have shown fragmentation of DNA upon treatment of cells with volatile oil indicating involvement of apoptosis. Analysis of DNA from the cells treated with volatile oil has shown disintegration of DNA, which partially resembles apoptotic pattern. Disintegration of DNA was observed prominently after 12 and 24 h, which was not found in untreated cells (control).

5.5.6 Measurement of Caspase-3 content

Caspase-3, a major executor class of caspases, is essential for the induction of DNA fragmentation as well as apoptosis (Porter and JaEnicke, 1996). Since caspase-3 is known as an executor caspase, the result of current study serves as major support for induction of apoptosis by lime volatiles. To the best of our knowledge, there are no reports on effect of volatile components on caspase-3 of cells. However, very few natural compounds are known to alter caspase-3 to induce apoptosis in cancer cells. Triterpenoids of natural origin are known for elevating levels of caspase-3 in leukemia cells. Activity was found to be decreased upon acetylation, indicating basic chemical moiety is essential for enhancing caspase-3 to induce apoptosis (Rochaa *et al*., 2007). Induction of caspase-3 up to 2 folds by lime volatile oil indicates that casapse-3 is actively involved in apoptosis induction.

5.5.7 Protein expression analysis

Expression of proteins is considered as confirmation regarding execution of molecular actions and it is measured through western blotting. The expression ratio of Bax/Bcl_2 is considered as one of the major indicator of apoptosis. Results of our study have shown that pre treatment of SW480 cells with volatile oil elevated ration by 2 and 4.3 folds in comparison to untreated control cells after 24 and 48 h, respectively.

Bax, a member of the Bcl-2 protein family, plays a vital role in the induction of apoptosis (Zhang *et al*., 2000). It is an integral membrane protein associated with organelles or bound to organelles by Bcl-2 or a soluble protein found in the cytosol and has shown mobility from cytosol to mitochondria during apoptosis (Wolter *et al*., 1997). Expression levels of Bax (pro-apoptotic) and Bcl_2 (anti-apoptotic) gene level provide further information regarding apoptosis. Some of the natural compounds such as, flavonoids, polyphenols have shown significant induction of Bax and depletion in Bcl_2 for induction of apoptosis mediated cell death (Mohan *et al*., 2007). Thymoquine a volatile active principle has shown induction apoptosis in colon cancer cells (Gali-Muhtasib *et al*., 2006).

The ratio of Bax /Bcl_2 is considered as one of the major biochemical markers for evaluation of tumor/cancer inhibition, i.e. increase in the ratio is considered to be anti-carcinogenic and vice versa (Zhang *et al*., 2003). Only few natural compounds have shown to alter the ratio of Bcl_2 and Bax and those which have an effect are considered to be effective

candidates for prevention of different types of cancer (Corsetti *et al*., 2008). Lime volatile oil has shown 4.3 fold enhancements in the ratio of Bax/Bcl_2, after 48 h of treatment.

5.5.8 Acridine orange staining

Morphology of cells treated with volatile oil was similar to that of camptothecin with irregular shape and most of the cellular contents were found as small fractions confirming the cells death. Apart from cell culture evidences, *in vivo* studies conducted using D-limonene a major constituent of volatile oil has showed chemopreventive ability in both initiation and promotion stages of skin carcinoma in chemically induced rodents (Elegbede *et al*., 1986). D-limonene and its derived monoterpinoids have shown to affect $p21^{ras}$ expression by altering overall expression or through farnesylation of proteins (Gelba *et al*., 1995). These evidences are further supported by the results of the current study.

6. SUMMARY AND CONCLUSIONS

The genus *Citrus* includes several important fruits such as organs, mandarins, limes, lemons and grape fruits. The rapid growth of the citrus fruit industry in the past 25 years is due to improved economic conditions in consuming nations of the world and also due to the natural distinctive flavour of citrus. The large consumption of citrus fruit is also attributed to other types of food and beverage industry.

Lime [*Citrus aurantifolia* (Christm.) Swingel] is a highly polyembryonic species with the fruit surface smooth, greenish-yellow in colour and thin-skinned, core solid at maturity, and juice highly acidic. Lime is used for the extraction of juice, preparation of squash, concentrates, beverages and byproducts, such as citric acid and pectin etc. Lime is the second most important citrus fruit, in both fresh consumption and industrial uses. It is one of the main crops in India and it is the fifth largest in harvested area worldwide.

The fruit is mainly relished for its flavour. The major flavour components of the fresh lime fruit have been reported as limonene, α-terpineol, 4-terpineol, 1,4-cineole, ρ-cymene, β-pinene, β-bisabolene, citral, geranial and neral. Recent works on lime showed the presence of some more compounds, such as neryl acetate, α-bergamotene, valencene and germacrene-d.

With this background, the present investigation was aimed to isolate and purify bioactive compounds in both edible (juice) and non-edible (seed and peel) portion of the lime fruit. It was also intended to study the antioxidant properties and chemo-preventive effects of isolated compounds along with their extracts. Lime volatile oil was also extracted from fruits and subjected for characterization of compounds and their anticancer properties on colon cancer.

For this purpose, physiology mature lime fruits were harvested from the farmer field in Bijapur district, Karnataka State, India and were separated into juice seed and peel. Seeds and peel were dried in the shade and than all the edible and non-edible portion were freeze dried and stored for -20°C for further use.

In addition lime fruits were also collected from Citrus Centre, Texas A&M University, Kingsville, Weslco were also used for studies concerning lime juice and peel studies. The research was carried out at Vegetable and Fruits Improvement Centre, Department of Horticultural Sciences, Texas A&M University, College Station, Texas, USA.

Results obtained from the investigation are summarized in this chapter.

6.1 Isolation and identification of bioactive compounds

- Five putative compounds were isolated and purified viz., limonin, LNA (limonexic acid), ILNA (isolimonexic acid), SG (sitosterol glucoside) and LG (limonin glucoside) from limes seeds.
- The purity and structures of the isolated compounds were confirmed by TLC, HPLC and mass spectra.
- Further, three Coumarins were isolated from lime peel hexane fraction by flash chromatography; the purity of these compounds was confirmed by HPLC and MS, the structures were determined by NMR studies. The three identified compounds were limettin, 5, 7 Dimethoxy coumrin and isopimperillin.

6.2 Studies on antioxidant activities of bioactive compounds

- In order to study the health benefits of lime fruits, freeze dried lime juice, seed and peel were extracted by solvents of variable polarity using ethyl acetate, chloroform, acetone, MeOH and MeOH: water (8:2). These extracts were subjected to HPLC analysis to quantify the limonoids and flavonoids present in these extracts and the phenolic content were also analyzed. HPLC analysis of lime juice indicted that chloroform extract contained three aglycones ILNA, LNA and limonine and one glucoside. The flavonoid profile showed the presence of flavanone (hesperidin, hesperitin and didymin) and only one flavonol (rutin).

- The limonoid profile of seed revealed that limonin was the major compound in lime seeds followed by ILNA (isolimonexic acid) and LNA (limonexic acid). The other minor limonoids in seed were obacunone and LG (limonin glucoside).
- The most abundant limonoid in peel was ILNA closely followed by LNA and other limonoids were LG and limonin.
- The major flavonoid identified in lime seed was hesperidin and the maximum content was in MeOH extract followed by EtOAc and MeOH: water.
- The peel contained an additional flavonoid, neohesperidin along with hesperidin.
- The radical scavenging capacity was investigated by DPPH and ABTS methods and the chloroform extract of lime juice exhibited 80% radical scavenging activity in both DPPH and ABTS assay.
- The MeOH extracts of seed and EtOAc extracts of peel showed maximum antioxidant activity.
- Fruits of *Citrus aurantifolia* were subjected to hydro-distillation using Clevenger type apparatus to obtain volatile oil. Chemical composition of volatile oil was analyzed by GC-MS. Twenty-two compounds representing more than 89.5% of the volatile oil were identified. D-limonene (30.13%) and D-dihydrocarvone (30.47%) were found to be major compounds in the lime volatile oil

6.3 Studies on anticancer activity of bioactive compounds and lime extracts from seed, peel and juice

- The purified compounds from seeds exhibited significant inhibition of Panc-28 cells with IC_{50} values in the range of 5.5-20 µM in MTT assay, which was confirmed by viable cell count.
- DNA fragmentation and expression of proteins were studied in cells treated with compounds in order to understand the mechanism. Expression of apoptosis related protein showed the induction of apoptosis through p53 and caspase-3 mediated, but p21 independent pathway.
- Expression of apoptosis favoring proteins in cells treated with isolated compounds were in the order of limonin > ILNA > LNA > SG > LG. LNA elevated ratio of *bax/bcl2* expression by 1.8 folds followed by LG (1.22 folds) and ILNA (1.2 folds).
- Gemcitabine exhibited an inhibition of colon cancer by 71.74 per cent at 48 h, compared to 53.35 per cent, 40.47 per cent and 53.33 per cent by Limettin , 5, 7 Dimethoxy coumrin and Isopimperillin, respectively.
- All the extracts of lime juice inhibited cell growth and arrested Panc-28 cells. The MeOH: water extract exhibited maximum activity with IC_{50} value of 3.4 µg mL^{-1} as measured by MTT assay.
- The inhibition of Panc-28 cells was in the range of 73- 89%, when 100 µg ml^{-1} for all the treated lime juice extracts for 96 h.
- The involvement of apoptosis in induction of cytotoxicity was confirmed by expression of proteins, such as Bax, Bcl_2, casapse-3 and p53.
- The lime seed MeOH:water and EtOAc extracts showed 86 and 74 % inhibition of cells at 100 µg/ml; whereas, lime peel extracts of EtOAc and acetone showed 97 and 92 % inhibition respectively. Further the protein levels in cell treated with extracts were assessed by immunoblotting assay to know the possible mechanism of apoptosis.
- The volatile oil showed 78% inhibition of human colon cancer cells (SW- 480) with 100 µg/ml concentration at 48 h. Lime volatile oil showed DNA fragmentation and induction of caspase-3 up to 1.8 and two folds after 24 h and 48 h, respectively.

REFERENCES

Abeysinghe, D. C., Li, X., Sun, C., Zhang, W., Zhou, C. and Chen, K., 2007, Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. Food Chem., 104 (4): 1338-1344.

Acker, V., A, S. and E, B., 1996, A quantum chemical explanation of the antioxidant activity of flavonoid. Chem. Res. Toxicol., 9: 1305-1312

Acker, V., Saskia, A. B. E., Balen, V., Plemper, G., Van Den Berg, Dirk-Jan, Bast, A., Van Der Vijgh and F., W. J., 1998, Influence of iron chelation on the antioxidant activity of flavonoids. Biochem. Pharmacol., 56 (8): 935-943.

Adams, R. P., 1989, Identification of essential oils by ion trap mass spectrometry. Academic Press, New York

Aggarwal, B. B. and Sung, B., 2008, Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends in Pharmacol. Sci., 30 (2): 85-94.

Alexandra, B., Elisabeth, C. M., Hubert, R.and Clendette, B., 1998, Antioxidant activity and phenolic composition of citrus peel and seed extract. J. Agric. Food Chem., 46 (1): 2123-2129.

Ames, B. N., Shigenaga, M. K. and Hagen, T. M., 1993, Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America*, 90 (17): 7915-7922.

Anonymous, 1992, The wealth of India, A dictionary of Indian raw materials and industrial products, information and Publications Directorate, CSIR, New Delhi, 1992, 3, p.623.

Anonymous, 2007, Cancer facts and figures, American Cancer Society, Atlanta.

Arias, B. Á. and Ramón-Laca, L., 2005, Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. J. Ethnopharmacol., 97 (1): 89-95.

Arnao, M. B., 2000, Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. Trends in Food Sci. Technol., 11 (11): 419-421.

Ashraf, T. K., Galal, T. M. and Khalid, A. E., 2002, Limonoids from *Citrus reticulata*. Z. Naturforsch, 58: 165 -170.

Ashwood-Smith, M. J., Poulton, G. A., Ceska, O., Liu M. and Furniss, E., 1983, An ultrasensitive bioassay for the detection of furocoumarins and other photosensitizing molecules. Photochem. Photobiol., 38: 113-118.

Athar, M., Back, J. H., Kopelovich, L., Bickers, D. R.and Kim, A. L., 2009, Multiple molecular Targets of Resveratrol: Anti-carcinogenic Mechanisms. Archives of Biochem. Bioph. in Press, Accepted Manuscript.

Awale, S., Li, F., Onozuka, H., Esumi, H., Tezuka, Y. and Kadota, S., 2008, Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. Bioorganic Med. Chem., 16 (1): 181-189.

Baghurst, P. A., Mcmichael, A. J. and Slavotinek, A. H., 1991, A case-control study of diet and cancer of the pancreas. American J. Epidemiol, 134: 167-79.

Baldi, A., Rosen, R. T., Fukuda, E. K. and Ho, C. T., 1995, Identification of nonvolatile components in lemon peel by high-performance liquid chromatography with confirmation by mass spectrometry and diode-array detection. J. Chromatography, 718 (1): 89-97.

Baudier, J., Delphin, C., Grunwald, D., Khochbin, S. and Lawrence, J. J., 1992, Characterization of the tumor suppressor protein p53 as a protein kinase C substrate

and a S100b-binding protein. *Proceedings of the National Academy of Sciences, United States of America*, 89 (23): 11627-11631.

Benavente-Garcia, O., Castillo, J., Marin, F. R.,Ortuno, A., Del Rio, J. A., 1997, Uses and properties of *Citrus* flavonoids. J. Agric. Food Chem., 45: 4505-4515.

Bennett, R. D., Hasegawa, S. and Herman, Z., 1989, Glucosides of acidic limonoids in citrus. Phytochemistry, 28 (10): 2777-2781.

Berry, R. E. and Benjamin, C. 2003. Citrus Fruits | Limes. Encyclopedia of Food Sciences and Nutrition, Oxford, Academic Press: 1368-1371.

Bharti, A. C. and Aggarwal, B. B., 2002, Chemopreventive agents induce suppression of nuclear factor-kappaB leading to chemosensitization. Ann. N. Y. Acad. Sci, 973: 392–395.

Biavatti, M. W., Westerlon, R., Burger, C., Mora, T. C. and De Souza, M. M., 2007, Antinociceptive action of limonexic acid obtained from *Raulinoa echinata*. J. Pharm Pharmacol., 59 (11): 1573-1581.

Block, G., Patterson, B. and Subar, A., 1992, Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr. Cancer, 18 (1): 1-29.

Bobe, G., Weinstein, S. J., Albanes, D., Hirvonen, T., Ashby, J., Taylor, P. R., Virtamo, J. and Stolzenberg-Solomon, R. Z., 2008, Flavonoid Intake and Risk of Pancreatic Cancer in Male Smokers (Finland). Cancer Epidemiol Biomarkers Prev., 17(3): 553-562.

Bouic, P. J. D., Etsebeth, S., Liebenberg, R. W., Albrecht, C. F., Pegel, K. and Van Jaarsveld, P. P., 1996, Beta-sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: Implications for their use as an immunomodulatory vitamin combination. Intl. J. Immunopharmacol., 18 (12): 693-700.

Bovill, H., 1996, Orange: source of natural compounds. Aromes Ingred. Addit, 7: 41–42.

Braddock, R. J., 1995, Byproducts of citrus fruits. Food Technol., 49: 74–77.

Brand, R. and Mahr, C. O., 2005, Risk factors for pancreatic adenocarcinoma: are we ready for screening and surveillance? Curr Gastroenterol Rep., 7: 122-7.

Bravo, L. B., 1998, Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutr. Rev., 56: 317-333.

Brusselmans, K., Vrolix, R. and Verhoeven, G., 2005, Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J. Biol. Chem., 280: 5636.

Calabrò, M. L., Tommasini, S., Donato, P., Stancanelli, R., Raneri, D., Catania, S., Costa, C., Villari, V., Ficarra, P. and Ficarra, R., 2005, The rutin-cyclodextrin interactions in fully aqueous solution: Spectroscopic studies and biological assays. J. Pharmaceutical Biomedical Anal., 36 (5): 1019-1027.

Castillo, J., and, Benavente, O. and Rio, J. A. D., 1992, Naringin and neohesperidin levels during development of leaves, flower buds, and fruits of previous term Citrus next term aurantium. Pl. Physiol., pp. 66-73.

Cha, Y.-Y., Lee, E.-O., Lee, H.-J., Park, Y.-D., Ko, S.-G., Kim, D.-H., Kim, H.-M., Kang, I.. C. and Kim, S.-H., 2004, Methylene chloride fraction of Scutellaria barbata induces apoptosis in human U937 leukemia cells via the mitochondrial signaling pathway. Clinica Chimica Acta, 348 (1-2): 41-48.

Chadalapaka, G., Jutooru, I., Mcalees, A., Stefanac, T. and Safe, S., 2008, Structure-dependent inhibition of bladder and pancreatic cancer cell growth by 2-substituted glycyrrhetinic and ursolic acid derivatives. Bioorganic Med. Chem. Lett., 18 (8): 2633-2639.

Chang, J., Hsu, Y., Kuo, P., Kuo, Y., Chiang, L. and Lin, C., 2005, Increase of Bax/ Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. Life Sci., 76 (16): 1883-1893.

Chen, I.-S., Lin, Y.-C., Tsai, I.-L., Teng, C.-M., Ko, F.-N., Ishikawa, T. and Ishii, H., 1995, Coumarins and anti-platelet aggregation constituents from Zanthoxylum schinifolium. Phytochem., 39 (5): 1091-1097.

Chung, H. W., Bang, S. M., Park, S. W., Chung, J. B., Kang, J. K., Kim, J. W., Seong, J. S., Lee, W. J. and Song, S. Y., 2004, A prospective randomized study of gemcitabine with doxifluridine versus paclitaxel with doxifluridine in concurrent chemoradiotherapy for locally advanced pancreatic cancer. Intl. J. Rad. Onco. Biol. Physic, 60 (5): 1494-1501.

Concannon, S., Venkataraman, N., Ramachandran, W. and Franklin, S., 2000, A study of the electrospray ionisation of selected coumarin derivatives and their subsequent fragmentation using an ion trap mass spectrometer. Rapid Commun. Mass Spectrometry, 14 (14): 1157-1166.

Corda, L., Delogu, G., Favretto, D., Maccioni, E., Podda, G., Santana, L., Tomaselli, C., Traldi, P.and Uriarte, E., 1998, Synthesis and mass spectrometric investigations of some new coumarin derivatives. Rapid Commun. Mass Spectrometry, 12 (24): 2041-2046.

Corsetti, G., Pasini, E., Assanelli, D. and Bianchi, R., 2008, Effects of acute caffeine administration on NOS and Bax/Bcl2 expression in the myocardium of rat. Pharmacol. Res., 57 (1): 19-25.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Van Poel, B., Pieters, L., Vlietinck, A. J. and Berghe, D. V., 1998, Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. J. Nat. Prod., 61 (1): 71-76.

Crowell, P. L., Elson, C. E., Bailey, H. H., Elegbede, A., Haag, J. D. and Gould, M. N., 1994, Human metabolism of the experimental cancer therapeutic agentd-limonene. Cancer Chemotherapy Pharmacol., 35 (1): 31-37.

Cserhati, T., 1995, Alkyl ethoxylated and alkylphenol ethoxylated nonionic surfactants: Interaction with bioactive compounds and biological effects. Environ. Health Perspect, 103: 358-364.

Cussans, N. J. and Huckerby, T. N., 1975, Carbon-13 NMR spectroscopy of heterocyclic compounds-IV : A 20 MHz study of chemical shifts and carbon-proton coupling constants in a series of hydroxy, methoxy and glucosyl coumarins. Tetrahedron, 31 (21): 2719-2726.

Daels-Rakotoarison, D. A., Gressier, B., Trotin, F., Brunet, C., Luyckx, M., Dine, T., Bailleul, F., Cazin, M. and Cazin, J. C., 2002, Effects of *Rosa canina* fruit extract on neutrophil respiratory burst. Phytotherapy Res., 16 (2): 157-161.

Dallos, A., Ngo, H. S., Kresz, R. and Héberger, K., 2008, Cluster and principal component analysis for Kovats' retention indices on apolar and polar stationary phases in gas chromatography. J. Chromatography, 1177 (1): 175-182.

Davies, W., 1990, Gas chromatographic retention indices of Monoterpens and Sesquiterpenes on methyl Silicone and Carbowax 20M phases. J. Chromatography, 50: 1-24.

Del RÝ, J. A, Fuster, M. D, Sabater, F, Porras, I, Garcý, Lido, A, Ortun, A., 1997, Food Chemistry, 59: 433–437.

Deschner, E. E., Ruperto, J., Wong, G. and Newmark, H. L., 1991, Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis, 12(7): 1193-1196.

Digiuseppe, J. A., Redston, M. S., Yeo, C. J., Kern, S. E. and Hruban, R. H., 1995, p53-independent expression of the cyclin-dependent kinase inhibitor p21 in pancreatic carcinoma. Am J. Pathol, 147 (4): 884-888.

Duthier, G. and Crozier, A., 2000, Plant-derived phenolic antioxidants. Curr. Opin. Lipidol., 11: 43-47.

Edenharder, R., Speth, C., Decker, M., Kolodziej, H., Kayser, O. and Platt, K. L., 1995, Inhibition of mutagenesis of 2-amino-3-methylimidazo -quinoline (IQ) by coumarins and furanocoumarins, chromanones and furanochromanones. Mutation Res.Genetic Toxicol., 345 (1-2): 57-71.

Ejaz, S., Ejaz, A., Matsuda, K.and Lim, C. W., 2006, Limonoids as cancer chemopreventive agents. J. Sci. Food Agricu., 86 (3).

Elegbede, J. A., Maltzman, T. H., Verma, A. K., Tanner, M. A., Elson, C. E.and Gould, M. N., 1986, Mouse skin tumor promoting activity of orange peel oil and d-limonene: a re-evaluation. Carcinogenesis, 7(12): 2047-2049.

Emerson, O. H., 1948, The bitter principles of citrus fruit. I. Isolation of nomilin, a new bitter principle from the seeds of oranges and lemons. J. Am. Chem. Soc., 70: 545.

Federica, L.and Sergio, F. D. B., 2005, Citrus flavonoids as bioactive compounds: role, bioavailability socio-economic impact and biotechnological approach for their modification" 9th International conference on agricultural biotechnology. Intl. Consortium on Agril. Biotech. Research, Yale University,Ravealloi,Italy

Fong, C. H., Hasegawa, S., Herman, Z.and Ou, P., 1989, Limonoid glucosides in commercial citrus juices. J. Food Sci., 54 (6): 1505-1506.

Fong, C. H., Hasegawa, S., Herman, Z.and Ou, P., 1991, Biosynthesis of Limonoif glucoside in lemon (*Citrus limon*). J. Sci.Food Agric, 54: 393-398.

Fridman, S. and Lowe, S. W., 2003, Control of apoptosis by p53. Oncogene, 22: 9030-9040.

Fukuda, K., Guo, L., Ohashi, N., Yoshikawa, M.and Yamazoe, Y., 2000, Amounts and variation in grapefruit juice of the main components causing grapefruit-drug interaction. J. Chromat. Biomedic. Sci., 741 (2): 195-203.

Gali-Muhtasib, H., Roessner, A.and Schneider-Stock, R., 2006, Thymoquinone: A promising anti-cancer drug from natural sources. Intl. J. Biochem. Cell Bio., 38 (8): 1249-1253.

Gao, K., Henning, S. M., Niu, Y., Youssefian, A. A., Seeram, N. P., Xu, A. and Heber, D., 2006, The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells. J. Nutri. Biochem., 17 (2): 89-95.

Gardner, P. T., White, T. A. C., Mcphail, D. B. and Duthie, G. G., 2000, The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. Food Chem., 68 (4): 471-474.

Gelba, M. H., Tamanoib, F., Yokoyamaa, K., Ghomashchia, F., Essonb, K.and Gould, M. N., 1995, The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. Cancer Lett., 91 (2): 169-175.

German, J. B. and Walzem, R. L., 2000, The health benefits of wine. Annu. Rev. Nutr., 20: 561-593.

Giono, L. E.and Manfredi, J. J.,2006, The p53 tumor suppressor participates in multiple cell cycle checkpoints. J. Cellular Physiol., 209 (1): 13-20.

Girennavar, B., Jayaprakasha, G. K., Jadegoud, Y., Nagana Gowda, G. A.and Patil, B. S., 2007, Radical scavenging and cytochrome P450 3A4 inhibitory activity of bergaptol and geranylcoumarin from grapefruit. Bioorganic Medi. Chem., 15 (11): 3684-3691.

Girennavar, B., Poulose, S. M., Jayaprakasha, G. K., Bhat, N. G.and Patil, B. S., 2006, Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes. Bioorganic Medicinal Chem., 14 (8): 2606-2612.

Gorinstein, S., Cvikrova, M., Machackova, I., Haruenkit, R., Park, Y.-S., Jung, S.T., Yamamoto, K., Leticia Martinez Ayala, A., Katrich, E.and Trakhtenberg, S., 2004, Characterization of antioxidant compounds in Jaffa sweeties and white grapefruits. Food Chem., 84 (4): 503-510.

Gorinstein, S., Leontowicz, H., Leontowicz, M., Krzeminski, R., Gralak, M., Delgado-Licon, E., Martinezayala, A. L., Katrich, E.and Trakhtenberg, S., 2005, Changes in Plasma Lipid

and Antioxidant Activity in Rats as a Result of Naringin and Red Grapefruit Supplementation. J. Agric. Food Chem., 53 (8): 3223-3228.

Gorinstein, S., Martín-Belloso, O., Park, Y.-S., Haruenkit, R., Lojek, A., Cíz, M., Caspi, A., Libman, I.and Trakhtenberg, S., 2001, Comparison of some biochemical characteristics of different citrus fruits. Food Chem., 74 (3): 309-315.

Gray, A. I.and Waterman, P. G., 1978, Coumarins in the Rutaceae. Phytochem., 17 (5): 845-864.

Green, D. R.and Reed, J. C., 1998, Mitochondria and apoptosis. Sci., 281 (5381): 1309-1312.

Guo, L. Q.and Yamazoe, Y., 2004, Inhibition of cytochrome P450 by furanocoumarins in grapefruit juice and herbal medicines. Acta Pharmacologica Sinica, 25 (2): 129-136.

Guthrie, N., Chambers, A. F. and Carroll, K. K., 1997, Inhibition of MDA-MB-435 estrogen receptor-negative human breast cancer cells by citrus limonoids. Proc.American Assoc Cancer Res., 38 : 113.

Haag, J. D., Lindstrom, M. J.and Gould, M. N., 1992, Limonene-induced Regression of Mammary Carcinomas. Cancer Res., 52 (14): 4021-4026.

Haaz, S., Fontaine, K. R., Cutter, G., Limdi, N., Perumean-Chaney, S. and Allison, D. B., 2006, *Citrus aurantium* and synephrine alkaloids in the treatment of overweight and obesity: an update. Obesity Rev., 7 (1): 79-88.

Halliwell, B. and Gutteridge, J. M. C., 2000, Free Radicals in Biology and Medicine. Claredon. Oxford, U.K Country

Halliwell, B., 2001, Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. Drugs Aging, 18 (9): 685-716.

Halliwell, B., Aeschbach, R., Löliger, J.and Aruoma, O. I., 1995, The characterization of antioxidants. Food Chem. Toxicol., 33 (7): 601-617.

Han, M., 2008, Thermally-driven nanoscale pump by molecular dynamics simulation. J. Mecha. Sci. Technol., 22 (1): 157-165.

Hanasaki, Y., Ogawa, S. and Fukui, S., 1994, The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radical Biol. Medicine, 16 (6): 845-850.

Hasegawa, S., Bennett, R. D., Herman, Z., Fong, C. H. and Ou, P., 1989, Limonoid glucosides in citrus. Phytochemistry, 28 (6): 1717-1720.

Hasegawa, S., Berhow, M. A. and Manners, G. D., 2000, Citrus Limonoid Research: An Overview, Washington DC, American Chemical Society; 1-8.

Hasegawa, S., Miyake, A. B. and Fong, C. H., Eds. 1996. Analysis of bitter principles in Citrus, Modern Methods of Plant Analysis, Fruit Analysis. Berlin, Springer-Verlag.

Hasegawa, S., Ou, P., Fong, C. H., Herman, C. W., Jr, C.and Atkin, D. R., 1991, Changes in the limonoate A-Ring lactone and Limonin 17 □-D-Dlucopyranoside content of Navel Orange During Fruit Growth and Maturation. J. Agric. Food Chem, 39 (262-265).

Herman, Z., Fong, C. H. and Hasegawa, S., 1992, Analysis of limonoids in citrus seeds. In Modern Methods of Plant Analysis: Seed Analysis. Springer, Berlin Country.

Hertog, M. G., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B.and Kromhout, D., 1993, Dietary antioxidant flavonoids and risk of coronary heart disease. The Zutphen Elderly Study Lancet, 342: 1007-1011.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C., 1991, p53 mutations in human cancers. Science, 253 (5015): 49-53.

Hongratanaworakit, T.and Buchbauer, G., 2007, Chemical composition and stimulating effect of Citrus hystrix oil on humans. Flavour Fragrance J., 22 (5): 443-449.

HongXiang, S., Cuirong, S.and Yuanjian, P., 2005, Cytotoxic activity and constituents of the volatile oil from the roots of *Patrinia scabra* Bunge. Chem. Amp. Biodiversity, 2: 1351-1357.

Howe, G. R., Ghadirian, P. and Mesquita, H. B., 1992, A collaborative casecontrol study of nutrient intake and pancreatic cancer within the search programme. Int. J. Cancer, 51: 365 - 72.

Hudson, J. B., Miki, N. and Towers, G. H. N., 1987, Isopimpinellin is not phototoxic to viruses and cells. Planta Medica, 53: 306-307.

Ito, N., Hireoze, M., Fukushima, G., Tauda, H., Shira, T. and Tatematsu, M., 1986, Studies on antioxidant : their carcinogenic and modifying effects on chemical carcinogensis. Food Chem. Toxicol., 24: 1071-1081.

Jayaprakasha, G. K., Mandadi, K. K., Poulose, S. M., Jadegoud, Y., Nagana Gowda, G. A. and Patil, B. S., 2007, Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliata* (L.) Raf. Bioorganic Med. Chem., 15 (14): 4923-4932.

Jayaprakasha, G. K., Mandadi, K. K., Poulose, S. M., Jadegoud, Y., Nagana Gowda, G. A.and Patil, B. S., 2008, Novel triterpenoid from *Citrus aurantium* L. possesses chemopreventive properties against human colon cancer cells. Bioorganic Med. Chem., 16 (11): 5939-5951.

Jayaprakasha, G. K., Negi, P. S., Sikder, S., Mohanrao, L. J.and Sakariah, K. K., 2000, Antibacterial activity of *Citrus reticulata* peel extracts. Zeitschrift fur Naturforschung - Section C. J. Biosci., 55 (11-12): 1030-1034.

Jayaprakasha, G. K.and Patil, B. S., 2007, *In vitro* evaluation of the antioxidant activities in fruit extracts from citron and blood orange. Food Chem., 101 (1): 410-418.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T.and Thun, M. J., 2008, Cancer Statistics. A Cancer J. Clinicians, 58 (2): 71-96.

Jennings, W.and Shibamoto, T., Eds. 1980. Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography. New York, Academic Press**.**

Johnson, J. J. and Mukhtar, H., 2007, Curcumin for chemoprevention of colon cancer. Cancer Lett., 255 (2): 170-181.

Kalra, N., Roy, P., Prasada, S. and Shukla, Y., 2008, Resveratrol induces apoptosis involving mitochondrial pathways in mouse skin tumorigenesis. Life Sci., 82 (7-8): 348-358.

Kane, G. C. and Lipsky, J. J., 2000, Drug-grapefruit juice interactions. *Mayo Clinic Proceedings*, 75 (9): 933-942.

Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. and Yano, M. 1999, Quantitation of Flavonoid Constituents in Citrus Fruits, 47: 3565-3571.

Khalil, A. T., Maatooq, G. T. and El Sayed, K. A., 2003, Limonoids from *Citrus reticulata*. Zeitschrift Fur Naturforschung C, 58 (3/4): 165-170.

Khan, M. R.and Mlungwana, S. M., 1999, [gamma]-Sitosterol, a cytotoxic sterol from Markhamia zanzibarica and *Kigelia africana*. Fitoterapia, 70 (1): 96-97.

Khan, N., Adhami, V. M.and Mukhtar, H., 2008, Apoptosis by dietary agents for prevention and treatment of cancer. Biochem. Pharmacol., 76 (11): 1333-1339.

Khan, N., Afaq, F.and Mukhtar, H., 2007, Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis, 28 (2): 233-239.

Kim, M. J., Hae, J. P., Mee, S. H., Park, H. J., Kim, M. S., Leem, K. H., Kim, J. B., Youn, J. K.and Hye, K. K., 2005, *Citrus reticulata* Blanco induces apoptosis in human gastric cancer cells SNU-668. Nutr. Cancer, 51 (1): 78-82.

Kondegowda, G.,Krishnan, N. N., Sridevi, N., Juand Young, H., 2006, Effect of dietary {beta}-sitosterol glucoside on the growth of estrogen-dependent human breast cancer

(MCF-7) tumors implanted in ovariectomized athymic mice. AACR Meeting Abstracts, 3: 168.

Korkina, L. G., Afanas'ev, I. B. and Helmut, S., 1996, Antioxidant and Chelating Properties of Flavonoids. Advances in Pharmacology, Academic Press, 38: 151-163.

Kroyer, G., 1986, Uber die antoixidative Aktivitat von Zitrus-fruchtschalen (Antioxidant activity of citrus peels). Z. Ernaehrungswiss, 25: 63-69.

Kupfer, D.and Bulger, W. H., 1987, Metabolic activation of pesticides with proestrogenic activity. Fed. Proc, 46: 1864-1869.

Kuramori, C., Azuma, M., Kume, K., Kaneko, Y., Inoue, A., Yamaguchi, Y., Kabe, Y., Hosoya, T., Kizaki, M., Suematsu, M. and Handa, H., 2009, Capsaicin binds to prohibitin 2 and displaces it from the mitochondria to the nucleus. Biochem. Biophysical Res. Commun., 379 (2): 519-525.

Kurowska, E. M., Borradaile, N. M., Spence, J. D. and Carroll, K. K., 2000, Hypocholesterolemic effects of dietary citrus juices in rabbits. Nutr. Res., 20 (1): 121-129.

Kusumoto, M., Ogawa, T., Mizumoto, K., Ueno, H., Niiyama, H., Sato, N., Nakamura, M.and Tanaka, M., 1999, Adenovirus-mediated p53 gene transduction inhibits telomerase activity independent of its effects on cell cycle arrest and apoptosis in human pancreatic cancer cells. Clin. Cancer Res., 5 (8): 2140-2147.

Kutney, J. P., Engendorf, G., Inaba, T.and Dreyer, D. L., 1971, Mass spectral fragmentation studies in monomeric and dimeric coumarins. Organic Mass Spectrometry, 5(3): 249-263.

Lam, L. K. T.and Hasegawa, S., 1989, Inhibition of benzo[a]pyrene-induced forestomach neoplasia in mice by citrus limonoids. Nutr. Cancer, 12 (1): 43-47.

Lario, Y., Sendra, E., García-Pérez, J., Fuentes, C., Sayas-Barberá, E., Fernández-López, J.and Pérez-Alvarez, J. A., 2004, Preparation of high dietary fiber powder from lemon juice by-products. Innovative Food Sci. Emerging Technol., 5(1): 113-117.

Lavin, M. F.and Gueven, N., 2006, The complexity of p53 stabilization and activation. Cell Death Differeniaion , 13 (6): 941-950.

Li, D., Xie, K., Wolff, R.and Abbruzzese, J. L., 2004, Pancreatic cancer. The Lancet, 363 (9414): 1049-1057.

Liu, B., Cheng, Y., Zhang, B., Bian, H.-J.and Bao, J.-K., 2009, Polygonatum cyrtonema lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS-p38-p53 pathway. Cancer Lett., 275 (1): 54-60.

Lowe, G., Woodward, M., Rumley, A., Morrison, C., Tunstall-Pedoe, H.and Stephen, K., 2003, Total tooth loss and prevalent cardiovascular disease in men and women Possible roles of citrus fruit consumption, vitamin C, and inflammatory and thrombotic variables. J. Clinical Epidemiol., 56 (7): 694-700.

Lowenfels, A. B.and Maisonneuve, P., 2005, Risk factors for pancreatic cancer. J. Cellular Biochem., 95 (4): 649-656.

Ma, X.and Gang, D. R., 2004, Metabolic profiling of in vitro micropropagated and conventionally greenhouse grown ginger (*Zingiber officinale*). Phytochem., 67 (20): 2239-2255.

Macheix, J. J., Fleuriet, A.and Biuot, J., 1990. Fruit phenolics. CRC Press Country.

Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B.and Jacks, T., 1995, p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Devp.,, 9 (8): 935-944.

Maier, V.P. and Grant, E. R., 1970, Specific thin-layer chromatography assay of limonin, a citrus bitter principle. J. Agric. Food Chem., 18: 250-252.

Mandadi, K. K., Jayaprakasha, G. K., Bhat, N. G.and Patil, B. S., 2007, Red Mexican Grapefruit: A Novel Source for Bioactive Limonoids and their Antioxidant Activity. Z Naturforsch, 62: 179-188.

Manners, G. D., 2004, Identifying citrus limonoid aglycones by HPLC-EI/MS and HPLC-APCI/MS techniques. Phytochem Anal.phytochem, 6: 372-81.

Manthey, J. A.and Buslig, B. S., 2005, Distribution of Furanocoumarins in Grapefruit Juice Fractions. J.Agril. Food Chem., 53 (13): 5158-5163.

Marini, D.and Balestrieri, F., 1995, Multivariate analysis of flavanone glycosides in citrus juices. Ital. J. Food Sci., 3: 255–264.

Martin, A. D. and Gilbert, D., 1968, Enzyme changes accompanying liver enlargement in rats treated with 3-*ter* butyl-4-hydroxyanisole. Biochem. J., 106: 22-27.

Marzo, I.and Naval, J., 2008, Bcl-2 family members as molecular targets in cancer therapy. Biochem. Pharmacol., 76 (8):939-946.

Marzulli, F.and Maibach, H., 1970, Perfume Phytotoxicity J.soc. Cosmet. Chem, 21: 686-715.

McHale, D.and Sheridan, J. B., 1989, The oxygen heterocyclic compounds of citrus peel oils. J. Essential Oil Res., 1:139-149.

Merle, H., Moron, M., Blazquez, M. A.and Boira, H., 2004, Taxonomical contribution of essential oils in mandarins cultivars. Biochem. Systemat. Ecol., 32 (5): 491-497.

Miller, E. G., Fanous, R., Rivera-Hidalgo, F., Binnie, W. H., Hasegawa, S.and Lam, L. K. T., 1989, The effect of citrus limonoids on hamster buccal pouch carcinogenesis. Carcinogenesis, 10 (8): 1535-1537.

Miller, E. G., Record, M.T., Binnie, W. H. and Hasegawa, S., 2000, Limonoid glucosides : systematic effects on oral carcinogenesis. In: *Phytochemicals and Phytopharamaceuticals*, AOAC, Press, Champaign, I..L., 95-105.

Millers,N.J.,Sampson,j.,Candeias,L.P.,Bramley,P.M.and Rice-Evans,C.A.,1996,Antioxidant activities of carotenes and Xanthophylls ,FE B S Lett,384:240-242

Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florencio, M. H. and Jennnings, K. R., 2002, Interactions of flavonoids with iron and copper ions : mechanism for their antioxidant activity. Free Radical Res., 36: 1990-2008.

Mizuno, A., Takata, M., Okada, Y., Okuyama, T., Nishino, H., Nishino, A., Takayasu, J. and Iwashima, A., 1994, Structures of new coumarins and antitumor-promoting activity of coumarins from *Angelica edulis* L. Planta Med., 60 (04): 333-336.

Mohammad, R., Giri, A.and Goustin, A. S., 2008, Small-molecule inhibitors of Bcl-2 family proteins as therapeutic agents in cancer. Recent Patents on Anti-Cancer Drug Discovery, 3 (1): 20-30.

Mohan, K. V. P. C., Gunasekaran, P., Varalakshmi, E., Hara, Y.and Nagini, S., 2007, In vitro evaluation of the anticancer effect of lactoferrin and tea polyphenol combination on oral carcinoma cells. Cell Biol. Intl., 31 (6): 599-608.

Moller, J. K. S., Lindberg Madsen, H., Aaltonen, T.and Skibsted, L. H., 1999, Dittany (*Origanum dictamnus*) as a source of water-extractable antioxidants. Food Chem., 64 (2): 215-219.

Morton, J., 1987. Mexican Lime, Fruits of Warm Climates, Miami FL, Morton JF.

Mosman T., 1983. Rapid colory metric assay for cellular growth and survival : an application to proliferation and cytotoxicity assayes. *J. immunol. Methods*, 65 : 55-63.

Motomura, M., Kwon, K. M., Suh, S.-J., Lee, Y.-C., Kim, Y.-K., Lee, I.-S., Kim, M.-S., Kwon, D. Y., Suzuki, I.and Kim, C.-H., 2008, Propolis induces cell cycle arrest and apoptosis in human leukemic U937 cells through Bcl-2/Bax regulation. Environ. Toxicol. Pharmacol., 26 (1): 61-67.

Mouly, P. P., Arzouyan, C. R., Gaydou, E. M.and Estienne, J. M., 1994, Differentiation of citrus juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides. J. Agric. Food Chem., 42 (1): 70-79.

Murakami, A., Kuki, W., Takahashi, Y., Yonei, H., Nakamura, Y., Ohto, Y., Ohigashi, H. and Koshimizu, K., 1997, Auraptene, a citrus coumarin, inhibits 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in ICR mouse skin possibly through suppression of superoxide generation in leukocytes. Japan J. Cancer Res., 88: 443–452.

Murakami, A., Nakamura, Y., Ohto, Y., Yano, M., Koshiba, T., Koshimizu, K., Tokuda, H., Nishino, H.and Ohigashi, H., 2000, Suppressive effects of Citrus fruits on free radical generation and nobiletin, an anti-inflammatory polymethoxyflavonoid. Biofactors, 12: 187-192.

Naganuma, M., Hirose, S., Nakayama, Y., Nakajima, K.and Someya, T., 1985, A study of the phototoxicity of lemon oil, Archives of Dermatological Research, 278: 31-36.

Nakatani, N., Tamada, Y.and Fuwa, H., 1987, 7-Geranyloxycoumarin from juice oil of hassaku (*Citrus hassaku*) and antimicrobial effects of related coumarins. Agric. Biol. Chem, 51: 419–423.

Netzel, M., Netzel, G., Tian, Q., Schwartz, S.and Konczak, I., 2007, Native Australian fruits -- a novel source of antioxidants for food. Innovative Food Sci. Emerging Technol., 8 (3): 339-346.

Negi, P. S., Jayaprakasha, G. K. and Jena, B.S., 2003, Antioxidant and antimutagenic activities of pomegranate peel extract. *Food Chemistry*, 80 : 393-397.

Ng, T.B., Liu, F. and Wang, Z. T., 2000, Anti-oxidative activity of natural products from plants. Life Sci., 2000, 66: 709-723.

Nguyen, T. T., Tran, E., Ong, C. K., Lee, S. K., Do, P. T., Huynh, T. T., Nguyen, T. H., Lee, J. J., Tan, Y., Ong, C. S.and Huynh, H., 2003, Kaempferol-induced growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK. J. Cellular Physiol., 197 (1): 110-121.

Nigg, H. N., Nordby, H. E., Beier, R. C., Dillman, A., Macias, C.and Hansen, R. C., 1993, Phototoxic coumarins in limes. Food Chem. Toxicol., 31 (5): 331-335.

Nogata, Y., Sakamoto, K., Shiratsuchi, H., Ishii, T., Yano, M.and Ohta, H., 2006, Flavonoid composition of fruit tissues of citrus species. Biosci. Biotech. Biochem., 70 (1): 178-192.

Ochocka, R. J., Rajzer, D., Kowalski, P.and Lamparczyk, H., 1995, Determination of coumarins from *Chrysanthemum segetum* L. by capillary electrophoresis. J. Chromat., 709 (1): 197-202.

Ohta, T., Maruyama, T., Nagahashi, M., Miyamoto, Y., Hosoi, S., Kiuchi, F., Yamazoe, Y. and Tsukamoto, S., 2002, Paradisin C: a new CYP3A4 inhibitor from grapefruit juice. Tetrahedron, 58 (33): 6631-6635.

Ortuno, A., Reynaldo, I., Fuster, M. D., Botý, J. M., Garcý, Puig, D., Sabater, F., Garcý´ A-Lido, A., Porras, I.. and Del rý´ J. A., 1997**,** Citrus cultivars with high flavonoid contents in the fruits, Scientia Horticulturae, 68: 231–236

Ozaki, Y., Miyake, M., Maeda, H., Ifuku, Y., Bennett, R. D., Herman, Z., Fong, C. H.and Hasegawa, S., 1991, Ichangensin glucoside in *Citrus junos*, *Citrus sudachi* and *Citrus sphaerocarpa*. Phytochemistry, 30 (8): 2659-2661.

Panchal, R. G., 1998, Novel Therapeutic Strategies to Selectively Kill Cancer Cells. Biochem. Pharmacol., 55 (3): 247-252.

Parikh, Rita, Mayur, Kumar and Loriand Dee, 2004. Oral care capsules. USA, Pfizer Inc. 201 Tabor Road, Morris Plains, NJ 07950.

Paris, C., Bertoglio, J. and Bréard, J., 2007, Lysosomal and mitochondrial pathways in miltefosine-induced apoptosis in U937 cells. Apoptosis, 12 (7): 1257-1267.

Paya, M., Ferrandiz, M. L., Miralles, F., Montesinos, C., Ubeda, A. and Alcaraz, M. J., 1993, Effects of coumarin derivatives on superoxide anion generation. Arzneimittelforschung, 43: 655–658.

Pellegata, N. S., Sessa, F., Renault, B., Bonato, M., Leone, B. E., Solcia, E. and Ranzani, G. N., 1994, K-ras and p53 Gene Mutations in Pancreatic Cancer: Ductal and Nonductal Tumors Progress through Different Genetic Lesions. Cancer Res., 54 (6): 1556-1560.

Pereira, R. B. and Mancini-Filho, J., 1994, Avaliacao da activade antioxidante em sementes de frutas citricas (Antioxidant activity of citrus seeds). Cien. Tecnol. Aliment., 14: 160-167.

Peterson, J. J., Beecher, G. R., Bhagwat, S. A., Dwyer, J. T., Gebhardt, S. E., Haytowitz, D. B. and Holden, J. M., 2006, Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. J. Food Composition Anal., 19 (Supplement 1): S74-S80.

Piskur, M. M. and Higgins, J. W., 1949, Antioxidants for fats. U.S. Pat. 2461080.

Porta, E. D., Reverehon, D.and Barth, D. C., 1997, Mandarin and lime peel oil processing by supercritical CO2 desorption; deterpenation and high molecular weight compound elimination. J. Essential Oil Res., 9: 515-522.

Porter, A. G.and JaEnicke, R. U., 1996, Emerging roles of caspase-3 in apoptosis. Cell Death Differentiation, 6 (2): 99 - 104.

Poulose, S. M., Harris, E. D. and Patil, B. S., 2006, Antiproliferative Effects of citrus limonoids against auman neuroblastoma and colonic adenocarcinoma cells. Nutri. Cancer, 56 (1): 103-112.

Poulose, S. M., Harris, E. D.and Patil, B. S., 2005, Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. J. Nutr., 135 (4): 870-877.

Poulose, S. M., Jayaprakasha, G. K., Mayer, R. T., Girennavar, B. and Patil, B. S., 2007, Purification of citrus limonoids and their differential inhibitory effects on human cytochrome P450 enzymes. J. Sci. Food Agric., 87 (9): 1699-1709.

Qin, L., Yang, Y.-B., Tuo, Q.-H., Zhu, B.-Y., Chen, L.-X., Zhang, L.and Liao, D.-F., 2009, Effects and underlying mechanisms of curcumin on the proliferation of vascular smooth muscle cells induced by Chol:M[beta]CD. Biochemical Biophysical Res. Commn., 379 (2): 277-282.

Ramos, S., 2007, Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. J. Nutr. Biochem., 18 (7): 427-442.

Ranganna, S., Govindrajan, V. S.and Ramana, K. V. R., 1983, Citrus fruits–varieties, chemistry, technology and quality evaluation. Part II. Chemistry, technology and evaluation. CRC Critical Rev. Food Sci. Nutr., 18: 313-316.

Rapisarda, P., Tomaino, A., Lo Cascio, R., Bonina, F., De Pasquale, A.and Saija, A., 1999, Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. J. Agric. Food Chem., 47 (11): 4718-4723.

Rice-Evans, C. A. and Miller, N. J., 1995, Antioxidants-the case for fruit and vegetables in the diet. Brit. Food J., 97: 35-40.

Rice-Evans, C. A., Miller, N. J. and Paganga, G., 1996, Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med., 20: 933-956.

Robbins, R. J., 2003, Phenolic Acids in Foods: An Overview of Analytical Methodology. J. Agric. Food Chem., 51 (10): 2866-2887.

Rochaa, G. D. G., Simõesa, M., Lúcioa, K. A., Oliveirab, R. R., Kaplanb, M. A. C.and Gattass, C. R., 2007, Natural triterpenoids from *Cecropia lyratiloba* are cytotoxic to both sensitive and multidrug resistant leukemia cell lines. Bioorganic Medi. Chem., 15 (23): 7355-7360.

Rodrigues, A., Sandström, T., Cá, H., Steinsl, H.and Aaby, J. P.,2000, Protection from cholera by adding lime juice to food. Results from community and laboratory studies in Guinea-Bissau, West Africa. Tropical Med. Intl. Health, 5: 418-422.

Rooprai, H. K., Kandanearatchi, A., Maidment, S. L., Christidou, M., Trillo-Pazos, G., Dexter, D. T., Rucklidge, G. J., Widmer, W. and Pilkington, G. J., 2001, Evaluation of the effects of swainsonine, captopril, tangeretin and nobiletin on the biological behavior of brain tumor cells in vitro. Neuropathol Appl. Neurobiol., 27: 29-39.

Sakahira, H., Enari, M., Ohsawa, Y., Uchiyama, Y.and Nagata, S., 1999, Apoptotic nuclear morphological change without DNA fragmentation. Curr. Biol., 9 (10): 543-546.

Salah, H. A., Masood, S.and Khatib, G., 1995, Percuteneous and intraoperative aspiration biopsy cytology of pancreatic neuroendocrine tumors. Acta Cytol, 40 (182-90).

Salim, E. I.and Fukushima, S., 2003, Chemopreventive potential of volatile oil from black cumin (*Nigella sativa* L.) seeds against rat colon carcinogenesis. Nutr. Cancer, 45 (2): 195 - 202.

Sanz, M. M., Ferrandiz, M. L., Cejudo, M., Terencio, M. C., Gill, B., Bustos, G., Ubeda, A., Gunasegaran, R. and Alcaraz, M. J., 1994, Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. Xenobiotica, 24: 689-699.

Sarkar, F. H.and Li, Y., 2004, Cell signaling pathways altered by natural chemopreventive agents. Mutation Res. Fundamental Molecular Mech. Mutagenesis, 555 (1-2): 53-64.

Sawabe, A., Matsubara, Y., Chong-Ren, Y.and Osamu, T. 1999. Bioactive glycosides in citrus fruit peels. Studies in Plant Science, Elsevier, 6**:** 261-274.

Sawamura, M., Kuriyama, T. and Li, Z., 1988, Rind spot, antioxidative activity and tocopherols in the flavedo of citrus fruits. J. Hortic. Sci., 63: 717-721.

Selli, S., Cabaroglu, T.and Canbas, A., 2004, Volatile flavour components of orange juice obtained from the cv. Kozan of Turkey. J. Food Composition Anal., 17: 789-796.

Sergeev, I. N., Li, S., Colby, J., Ho, C.T. and Dushenkov, S., 2006, Polymethoxylated flavones induce Ca2+-mediated apoptosis in breast cancer cells. Life Sci., 80 (3): 245-253.

Shahidi, F., Wanasundara, P. K.and Hong, C., 1992. Antioxidant activity of phenolic compounds in meat model systems. In: Phenolic compounds in foods and their effect on healthC. ACS Symp., Washington D.C. American Chemical Society, . 214-222.

Shibata, A., Mack, T. M., Paganini-Hill, A., Ross, R. K.and Henderson, B. E., 1994, A prospective study of pancreatic cancer in the elderly. Int. J. Cancer, 58: 46–9.

Shimizu, B. I., Miyagawa, H., Ueno, T., Sakata, K., Watanabe, K.and Ogawa, K., 2005, Morning glory systemically accumulates scopoletin and scopolin after interaction with *Fusarium oxysporum*. Zeitschrift fur Naturforschung - Section C. J. Biosci., 60 (1-2): 83-90.

Singh, R. P., Chidambara Murthy, K. N.and Jayaprakasha, G. K., 2002, Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J. Agric. Food Chem., 50 (1): 81-86.

Stect, W. and Mazurek, M., 1972, Identification of natural coumarins by NMR spectroscopy. Lloydia, 35 (4): 418-439.

Steinmetz, K. A. and Potter, J. D., 1996, Vegetables, fruit and cancer prevention: A review J. American Dietetic Assn., 96 (10): 1027-1039.

Sudhop, T., Lutjohann, D., Kodal, A., Igel, M., Tribble, D. L., Shah, S., Perevozskaya, I. and Von Bergmann, K., 2002. Inhibition of intestinal cholesterol absorption by ezetimibe in humans, American Heart Assn., 106: 1943-1948.

Suntornsuk, L., 2002, Capillary electrophoresis of phytochemical substances. J. Pharmaceutical Biomedical Anal., 27 (5): 679-698.

Tanaka, T., Katada, Y., Higa, S., Fujiwara, H., Wang, W., Saeki, Y., Ohshima, S., Okuda, Y., Suemura, M.and Kishimoto, T., 2001, Enhancement of T Helper2Response in the Absence of Interleukin (IL-)6; An Inhibition of IL-4-Mediated T Helper2Cell Differentiation by IL-6. Cytokine, 13 (4): 193-201.

Tanaka, T., Kohno, H., Kawabata, K., Honjo, S., Miyake, M.and Wada, K., 2000, Citrus limonoids obacunone and limonin inhibit the development of a precursor lesion, aberrant crypt foci, for colon cancer in rats. ACS Symposium Series, 758: 145-163.

Tatum, J. H.and Berry, R. E., 1977, 6,7-Dimethoxycoumatin in the peels of Citrus. Phytochem., 16: 1091-1092.

Tian, Q., Miller, E. G., Ahmad, H., Tang, L.and Patil, B. S., 2001, Differential inhibition of human cancer cell proliferation by citrus limonoids. Nutr. Cancer, 40 (2): 180-184.

Tian, Q.and Ding, X., 2000, Screening for limonoid glucosides in *Citrus tangerina* (Tanaka) Tseng by high-performance liquid chromatography-electrospray ionization mass spectrometry. J. Chromatography, 874 (1): 13-19.

Tietjen, K. G.and Matern, U., 1984, Induction and suppression of phytoalexin biosynthesis in cultured cells of safflower, *Carthamus tinctorius* L., by metabolites of *Alternaria carthami* Chowdary. Archives Biochem. Biophysics, 229 (1): 136-144.

Ting, S. V. and Newhall, W. F., 1965, Occurrence of a natural antioxidant in citrus fruit. J. Food Sci., 30: 57-63.

Van Acker, Saskia, A. B., Berg, V. D., Tromp, D.-J., Michèl, N. J. L., H Griffioen, D., P Van Bennekom, W., Van Der Vijgh, W. J. F.and Bast, A., 1996, Structural aspects of antioxidant activity of flavonoids. Free Radical Biology and Medicine, 20 (3): 331-342

Van, Acker, Saskia, A. B. E., Balen, V., Plemper, G., Van Den Berg, Dirk-Jan, Aalt, B., Vijgh, V. D.and Wim, J. F., 1998, Influence of iron chelation on the antioxidant activity of flavonoids. Biochemical Pharmacology, 56 (8): 935-943.

Vanamala, J., Leonardi, T., Patil, B. S., Taddeo, S. S., Murphy, M. E., Pike, L. M., Chapkin, R. S., Lupton, J. R.and Turner, N. D., 2006, Suppression of colon carcinogenesis by bioactive compounds in grapefruit. Carcinogenesis, 27 (6): 1257-1265.

Veriotti, T.and Sacks, R., 2001, High-speed GC and GC time-of-flight MS of lemon and lime oil samples. Anal. Chem., 73 (18): 4395-4402.

Vikram, A., Jayaprakasha, G. K.and Patil, B. S., 2007, Simultaneous determination of citrus limonoid aglycones and glucosides by high performance liquid chromatography. Analytica Chimica Acta, 590 (2): 180-186.

Villaseñor, I. M., Angelada, J., Canlas, A. P.and Echegoyen, D., 2002, Bioactivity studies on beta-sitosterol and its glucoside. Phytotherapy Res., 16 (5): 417-421.

Vinson, J. A., Su, X., Zubik, L.and Bose, P., 2001, Phenol antioxidant quantity and quality in foods: fruits. J. Agric. Food Chem., 49 (11): 5315-5321.

Vogelstein, B., Lane, D.and Levine, A. J., 2000, Surfing the p53 network. Nature, 408 (6810): 307-310.

Wang, Y.-C., Chuang, Y.-C.and Ku, Y.-H., 2007, Quantitation of bioactive compounds in citrus fruits cultivated in Taiwan, Food Chem., 102 (4): 1163-1171.

Wangensteen, H., Molden, E., Christensen, H.and Malterud, K. E., 2003, Identification of epoxybergamottin as a CYP3A4 inhibitor in grapefruit peel. European J. Clinical Pharmacol., 58 (10): 663-668.

Wattenburg, L. W., 1985, Chemoprevention of cancer. Cancer Res, 45: 1-8.

Williams, G. T.and Smith, C. A., 1993, Molecular regulation of apoptosis: Genetic controls on cell death. Cell, 74 (5): 777-779.

Williams, N. S. and Harris, N. D., 1983, Antioxidant activity in dried orange. J. Food Sci., 48: 644-645.

Wolfenden, B. S. and Willson, R.L., 1982, Radical-cation as reference chromogens in kinetin studies of one-electron ransfer reactions : pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). J. Chem. Soc. Perkin Trans., 2: 805-812.

Wolter, K. G., Hsu, Y.-T., Smith, C. L., Nechushtan, A., Xi, X.-G.and Youle, R. J., 1997, Movement of bax from the cytosol to mitochondria during apoptosis. J. Cell Biol., 139 (5): 1281-1292.

Wu, Q.and Dewald, H. D., 2001, Voltammetry of coumarins. *Electroanalysis*, 13(1): 45-48

Xiang, S. H., Rong, S. C.and Yuanjian, P., 2005, Cytotoxic activity and constituents of the volatile oil from the roots of *Patrinia scabra* Bunge. Chemistry Biodiversity, 2: 1351-1357.

Yadav, A. R., Chauhan, A. S., Rekha, M. N., Rao, L. J. M.and Ramteke, R. S., 2004, Flavour quality of dehydrated lime [*Citrus aurantifolia* (Christm.) Swingle]. Food Chem., 85 (1): 59-62.

Yi, Z., Yu, Y., Liang, Y.and Zeng, B., 2008, In vitro antioxidant and antimicrobial activities of the extract of *Pericarpium Citri Reticulatae* of a new Citrus cultivar and its main flavonoids. LWT - Food Sci. Technol., 41 (4): 597-603.

Zhang, L., Yu, J., Park, B. H., Kinzler, K. W.and Vogelstein, B., 2000, Role of bax in the Apoptotic response to anticancer agents. Sci., 290 (5493): 989-992.

Zhang, Y., Chen, A. Y., Li, M., Yao, Q.and Chen, C. J., 2008, QS302. Ginkgo Biloba extract kaempferol inhibits cell proliferation and induces apoptosis in panceratic cancer cells. J. Surgical Res., 144 (2): 387-387.

Zhang, Z., Li, M., Wang, H., Agrawal, S.and Zhang, R., 2003, Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proceedings of the National Academy of Sciences*, 100 (20): 11636.

Zheng, W.and Wang, S. Y., 2001, Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem., 49 (11): 5165-5170.

Zheng, W.and Wang, S. Y., 2003, Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem., 51 (2): 502-509.

Zia Ur, R., 2006, Citrus peel extract - A natural source of antioxidant. Food Chem., 99 (3): 450-454.

Zobel, A. M.and Brown, S. A., 1990, Seasonal changes of furanocoumarin concentrations in leaves of *Heracleum lanatum*. J. Chem. Ecol., 16 (5): 1623-1634.

STUDIES ON ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS IN LIME [*Citrus aurantifolia* (Christm) Swingle], THEIR ANTIOXIDANT AND ANTICANCER PROPERTIES

JAIPRAKASH R. PATIL 2009 Dr. M. B. CHETTI
Major Advisor

ABSTRACT

Investigations were conducted to isolate, purify and study the antioxidant and chemo-preventive effects of bioactive compounds in both juice, seed and peel of lime fruit. Lime volatile oil was also extracted from fruits and subjected for characterization of compounds and their anticancer properties. Physiologically mature fruits from India and USA were used for the investigation. The research was carried out at Vegetable and Fruits Improvement Centre, Texas A&M University, College Station, Texas, USA.

Results of HPLC analysis revealed that hesperidin and rutin were the major flavonoids and Limonexic acid and Isolimonexic acid were the prominent limonoids in lime juice. While, limonin was the major compound in lime seeds, followed by Isolimonexic acid and Llimonexic acid. Further, the lime juice, seed and peel extracted by various solvents indicated radical scavenging activity comparable to ascorbic acid.

Twenty-two volatile components were identified from *Citrus aurantifolia* using GC-MS, of which the major compounds were D-limonene, D-dihydrocarvone, verbena, β-linalool, α-terpinol, trans-α-bergamotene. Further, the bioactive compounds isolated from seeds were found to posses the potential of inhibiting human pancreatic cancer cells. While, the compounds purified from peel had the potential of suppressing the colon cancer cells.

The purified compounds from seeds exhibited significant inhibition of Panc-28 cells with IC_{50} values in the range of 18.1 - 100 µM, which was confirmed by viable cell count. DNA fragmentation and expression of proteins in cells treated with compounds showed the induction of apoptosis through p53 and caspase-3 mediated, but p21 independent pathway. The volatile oil showed 78 per cent inhibition of human colon cancer cells (SW-480) with 100 µg/ml concentration at 48 h. Lime volatile oil showed DNA fragmentation and induction of caspase-3 up to 1.8 and two folds after 24 and 48 h, respectively.