INVESTIGATION ON BIOACTIVE PHYTOCHEMICALS OF JAMUN (*Syzygium cumini*) FRUIT

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BY

BENHERLAL P S

AGROPROCESSING AND NATURAL PRODUCTS DIVISION

NATIONAL INSTITUTE FOR INTERDISCIPLINARY

SCIENCE AND TECHNOLOGY (CSIR)

THIRUVANANTHAPURAM-19, KERALA, INDIA

NOVEMBER 2010

DECLARATION

I hereby declare that the thesis entitled 'INVESTIGATION ON BIOACTIVE PHYTOCHEMICALS OF JAMUN (*Syzygium cumini*) FRUIT' embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram, as a full-time Research Scholar under the supervision of Dr. C. Arumughan and that no part of this thesis has been presented before for any other degree.

Benherlal. P. S

Thiruvananthapuram

November, 2010

CERTIFICATE

This is to certify that the thesis entitled 'INVESTIGATION ON BIOACTIVE PHYTOCHEMICALS OF JAMUN (*Syzygium cumini*) FRUIT ' is an authentic record of research work c arried out by Mr. Benherlal. P. S under my supervision in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biochemistry of Cochin University of Science and Technology, and further that no part of this thesis has been submitted elsewhere for any other degree.

Dr. C. Arumughan

Scientist 'G' and Head (Rtd.)

Agroprocessing and Natural Products Division

Thiruvananthapuram

November, 2010

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Dedicated to My Parents and Teachers

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CHAPTER 1

INTRODUCTION

1.1 Preamble

The history of plant based health care goes back to antiquity and as old as human civilization. Plants have been primary source of medicines in the traditional healthcare systems around the globe, till recently and even currently in most of the developing countries. The approach to characterization and isolation of active ingredients from plants started in the late 19th century. Consequently chemical substances isolated are currently used as important drugs as such or as their derivative(s) today. From 1983 to 1994, 39% of the New Approved Drug (NAD) was of natural origin i.e. original natural products, products derived semi synthetically from natural products and synthetic products based on natural molecules (Cragg et al., 1997). Parallel to synthetic drug demand, the global natural products market is growing exponentially. The global demand for botanical and plant derived drugs is expected to increase from 19.5 billion in 2008 to 32.9 billion USD in 2013, with a compound annual growth rate of 11.0% (Lawson, 2009)

The number of higher plant species on this planet is estimated at 250,000 (Ayensu and DeFilipps, 1978). Of these, only around 6% have been screened for biological activity, and of which only 15% are reported to be phytochemically characterized (Verpoorte, 2000). Asia, especially the southern region shares about 20% of the all known vascular plants in the Globe. This includes 7000-8000 species of medicinal plants. Out of the 17,500 flowering plant species found in India, over 1600 are used in traditional medicine (Pushpangathan, 2004). According to world health organization (WHO), 65-80 % of the global population use plants and plant products for their primary health care (Bagozzi, 2003; Farnsworth et al., 1985). The Investigations on therapeutic applications of plants have lead to the discovery of several clinically applicable drugs (e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine etc.). Elucidation of the structure of active principles paved the way for synthesis and derivatization for compounds with higher

efficacy and lower adverse effects (e.g., Metformin, nabilone, oxycodon, taxotere, teniposide, verapamil, amiodarone etc) (Daniel et al., 2001). Thus plants continue to engage the attention of scientists associated with drug discovery.

Evidences accumulated thus clearly show that plants are rich source of bioactive chemical entities. Many phytochemicals are capable of modulating biochemical pathways of higher animals. However phytochemicals can be beneficial or harmful. There is sufficient traditional knowledge to substantiate this but further studies are required to index plants with beneficial and adverse effects. Based on the traditional knowledge, some plants and plant products are documented to be non-toxic and therapeutically potent. This knowledge can be exploited to develop cheaper plant based product/formulation for preventive health and disease management. However scientific evidences need to be created for their efficacy through pharmacological and chemical studies. Great number of plants documented in the Indian traditional medicine yet to be investigated in this line. The present study proposes to evaluate the *Syzygium cumini* fruit (SCF) for its phytochemical profile and bioactive potentials. *S. cumini* is a tropical plant belongs to the family of myrtaceae. The plant is endemic to India Pakistan, Srilanka and Malaysia. In traditional medicine SCF has primarily been used in the treatment of diabetes mellitus. In many Indian traditional health care systems, the seed of the fruit is extracted in water and used to control of hyper glycemia. Apart from its use as antidiabetic agent, this fruit has also been used as astringent, carminative, stomachic, antiscorbutic and as diuretic agent.

1.2 Plant based traditional healthcare: an overview

According to WHO, Traditional Medicine (TM) refers to health practices, approaches, knowledge and belief incorporating plant, animal and mineral based medicine, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illness or well being

(Xiaorui Zhang et al., 2002). Naturopathic, Ayurveda, Reiki, Chinese, Native American medicine, Homeopathic medicine, etc. are some examples of traditional healthcare systems. Fossil records date, use of plants as medicines at least to the middle Paleolithic period, some 60,000 years ago (Solecki and Shanidar, 1975). One of the oldest records related to the plant based medicine is the Papyrus Eber written nearly 1500 BC and contains information of more than 500 natural ingredients (Ghalioungui, 1969). Similarly, Asia's one of the traditional systems of health care is Chinese medicine that primarily use plants for disease management. Shennong Bencao Jing is the first Chinese herbal book which was compiled during the Han Dynasty but dating back to a much earlier date, possibly 2700 B.C., that lists 365 medicinal plants and their uses. During the middle age, many medical texts have been written. Causes and Cures were written by a 12th century Benedictine nun. `Herbals and medical texts migrated from Asia to middle East and West. The knowledge and applications of herbals greatly increased during the period 15 to 17th century. The books published during that period include Great herbals, 1526; General history off plants, 1597 etc. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices (Narayana et al., 1998). "Indian traditional medicine "Ayurveda" is a store house of knowledge on traditional healthcare. A most authentic compilation of the teaching of Sushrutha, called "*Sushrutha Samhitha*" contains description about 1120 illness and 700 medicinal plants (Dwivedi and Dwivedi, 2007). Charak Samhita' and 'Sushruta Samhita' described not only the medicinal properties of individual plants but also the poly herbal and herbomineral preparations. These resources, that emphasize the doctrines for life with healthy mind and body, were evolved through day-to-day life experiences as a part of cultural heritage of India. Besides Ayurveda, there are several other complementary and alternative systems of medicine like Homeopathy, Siddha and Unani, which are also practiced and developed with the course of time in India, where plants and plant-based formulations are employed for health care and disease treatments. In "Ayurveda" and almost all

other traditional systems of medicine, the predominant raw materials used are plants that they play a major role and constitute the backbone. These systems are based on experience and interaction with nature and natural resources. Variations in geographical landscaping and biodiversities in the Indian subcontinent have helped to develop the use of a variety of plant species and other natural resources for health care and contributed to TM. The inherited cumulative knowledge and experience in Ayurveda as well as other TM may be of interest because of new leads to modern approach for diseases treatment and management.

TM is rapidly growing health care system with great economic importance. In underdeveloped countries majority of the population uses TM to meet their health care needs. In several regions viz. Asia and Latin America, people use TM as a result of their historical background and cultural beliefs. Meanwhile, in many developed countries, TM is becoming more and more popular. In developing countries, broad use of TM is often attributable to its accessibility and affordability and by concern about the adverse effects of chemical drugs used in allopathic. Therefore many people trust the gentleness of TM in managing diseases than allopathic medicine. Moreover together with demand for TM, concerns over the safety, efficacy and quality of TM, products and practices related to it have also been raised. Since TM has developed within different cultures in different regions, there has been no parallel development of standards and methods. Therefore scientific evidence to prove the rationale of using these formulations in health care is essential to develop and to preserve the cultural heritage. Approaches like high-throughput screening, phytochemical profiling, quality control and standardization of raw materials and formulations, pharmacokinetics, pharmacovigilance and clinical trials of herbal therapeutics will not only help to prove the rationale of using these systems but also to get maximum benefits of the natural resources (Mukherjee, 2005).

1.3 Traditional medicines: merits and demerits

In developing countries, 80% of the population still depends on the traditional medicine for their primary health care since these are presumed to be cheaper and affordable. These traditional medicines are developed by practices over centuries by trials and observations. However the practices and remedies drastically vary from one country to another. Though there are variations, there is a consensus that TM generated important knowledge about the therapeutically potent plant(s), plant part, preparation methods, dose etc. but, lack scientific evidences. While some practices seem to offer benefits, others remain questionable. The only remedies to answer the unanswered questions are to do further research based on evidences to address safety, efficacy, and quality. Some traditional practices for several decades have now been evaluated by modern science and found to be harmful to health (e.g. ephedra). Ephedra is used in Chinese traditional medicine to treat short-term respiratory congestion and its long term use is reported to lead to, heart attack and stroke including death (Martínez-Quintana et al., 2010). Safety evaluation of TM products and practices using modern scientific method is also problematic. This is especially true of herbal medicines, the effectiveness and quality of which can be influenced by numerous factors. It is well known that the amount of research work on validation of TM has been inadequate. Further systematic and scientific studies are required to generate sufficient data for judging the efficacy, and safety. Because of the lack of comprehensive methodologies for scientific validation, developments in drafting regulation and guidelines for TM have slowed. Besides, many TMs have still been practiced and people are claimed to have benefitted. However many herbs that used are untested by scientific methods and their use not monitored. As a result, knowledge of their potential side-effects is limited. This makes identification of the safest and most effective therapies and promotion of their rational use more difficult. Many traditional formulations currently in use are not standardized. Enormous

fluctuation is in the phytochemical profiles of raw materials for formulations due to climatic, environmental, soil chemical and genetic could influence their efficacy. If TM is to be promoted as a source of health care, developments of modern methodologies and scientific studies are very much essential

1.4 Scientific approach to plant based health care.

Several reviews are published regarding the methods for selecting plants as candidates for drug discovery (Verpoorte, 2000). Random selection followed by biological activity screening is one approach. In this approach, randomly selected plants are subjected to various biological activities and a comprehensive screening of major phytochemicals viz. cardenolides/bufadenolides, alkaloids, triterpenes, flavonoids, isothiocyanates, iridoids, etc. is performed using various analytical and quantitative tools. Based on the partial chemical analysis, biological activity is attributed to the major phytochemical class (Farnsworth, 1966). This approach has been used in the past and is currently pursued mainly in the developing countries. The tests are simple to perform, but false-positive and false-negative tests often render results difficult to assess (Farnsworth, 1966; Farnsworth et al., 1962; Roper et al., 1965; Segelman et al., 1968). More importantly, it is frequently impossible to relate one class of phytochemicals to a specific biologic activity; for example, the alkaloids or flavonoids produce a vast array of biologic effects that are usually not predictable in advance.

Another approach is the random selection of plant material against a targeted disease followed by one or more biologic assays. Crude extracts prepared from a spectrum of plants / plant materials are subjected to in vitro and in vivo analyses, and based on the promising result, more experiments are conducted to identify the active principle. National Cancer Institute (NCI) adopted this approach for anticancer drug screening using experimental animals (Douros and M., 1980; Douros and Suffness, 1981; Farnsworth et al., 1966). Taxol and camptothecin (Wall and WaniMC, 1996) were discovered in this program as

well they have reported several other compounds that were unsuccessful in human studies. In 1986 the NCI program abandoned this approach and continued to collect and screen plants using a battery of 60 human tumor cell lines and also initiated a screening of plants for anti-HIV activity in vitro. Calanolide A, currently in Phase I clinical trials, was developed from this program (Anonymous, 2000; Kashman et al., 1992).

Approaches based on prior arts utilize knowledge of traditionally practiced medicinal plants and plant products. Several types of ethnomedical information are available about the plants used in organized traditional medical systems viz. Ayurveda, Unani, Kampo, traditional Chinese medicine etc. against several ailments (Bannerman and W C Burton J, 1983). Based on the knowledge about the ethno medicine (plant species, anatomical part used, targeted disease etc.), taxonomic relations (similar therapeutical potency in related species) and current scientific data (chemical composition, limited in vitro and in vivo study results, etc.), new scientific investigations have to be redesigned in order to identify and characterize active principles.

If scientific information about phytochemical composition and activity of each of the chemical entities in a particular plant species are known, standardized health care product formulation based on the said plant species would be relatively easy. However complete phytochemical characterization of plant species is the practical difficulty associate with this method. Though a complete chemical characterization is done, if knowledge about the biological functions of the characterized chemical entities are not available, further attributions of biological activity require wide array of in vitro and in vivo screening. Scientific approach in plant based healthcare mainly focus on the selection of standardized methods, use of plants/plant products, evaluation of biological activity, identification of active principle, toxicological and pharmacological study, dose optimization etc. Fig. 1-1 is a conceptual scheme for modern approach in development of plant based healthcare.

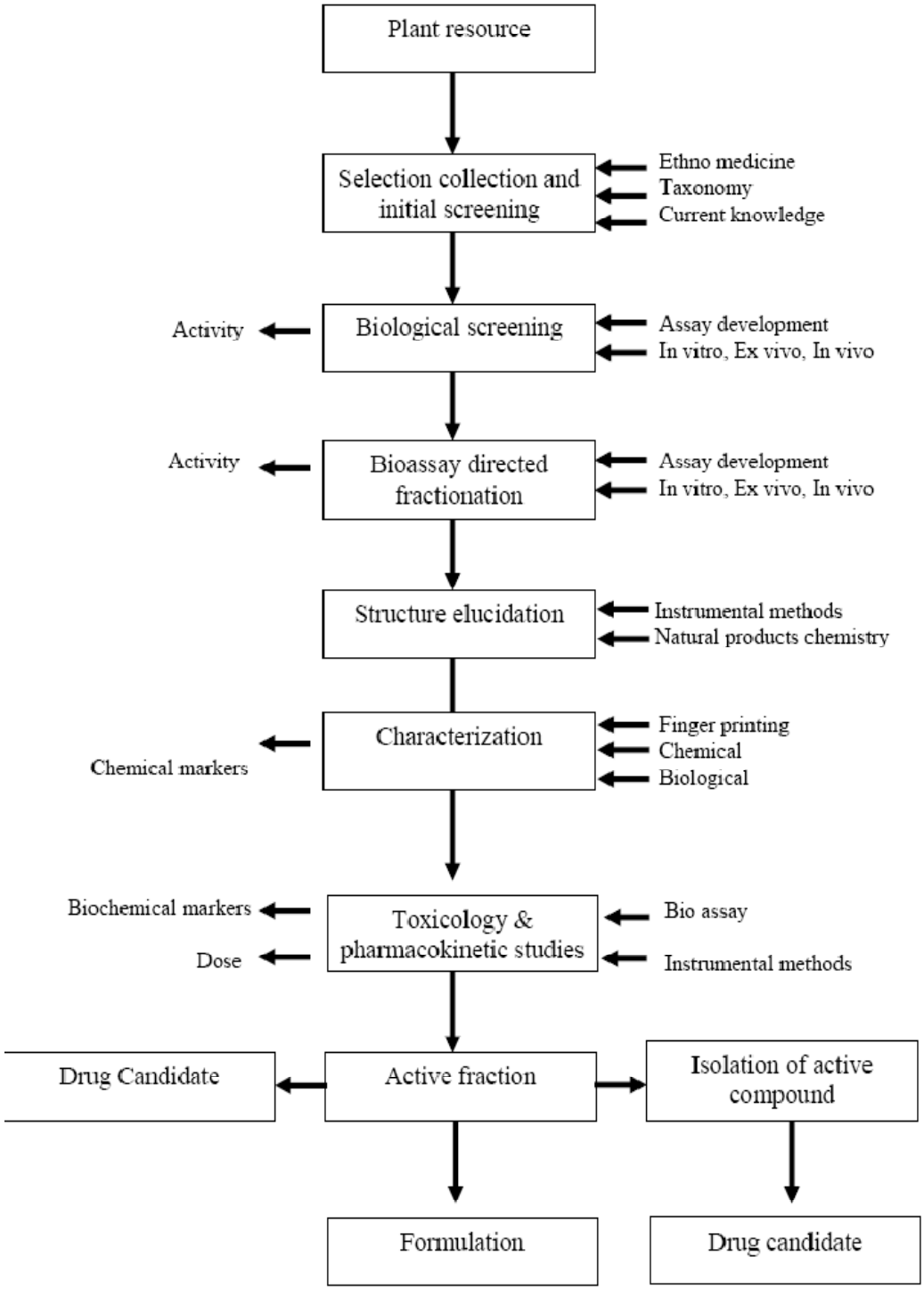

Fig. 1-1 Conceptual scheme for modern approach to plant based health care

products. According to this scheme the plant(s)/plant product is selected based on any one or all of the following criteria viz.; ethno medicinal knowledge, taxonomical relation to well-studied therapeutically important plants and the current scientific knowledge available about a plant species. After selection, a thorough bioactivity evaluation/validation has to be done by differential analysis and bioassay guided fractionation. Finally identification of active fraction/principle and chemical characterization including structure elucidation has also to be done. After establishing the chemistry of active principle, a standard drug screening protocol may be followed to validate the results that include in vivo animal studies and various clinical trials. On successful completion of these scientific methodologies, a standardized plant based healthcare product can be formulated for disease prevention, cure or management.

1.5 Plants as source of drugs

Drug is a substance that alters body function (Cohen, 2010). Drug discovery is a lengthy, expensive difficult and often an inefficient process with low success rate (Blake et al., 2009). Though there are several advancements in medical treatments, including gene therapy, immunotherapy, DNA vaccines, regenerative therapy etc., for acute diseases, effective medical treatment would be based on small organic molecules. The process of finding a new drug against a chosen target disease usually involves high throughput screening (HTS), wherein large libraries of chemicals are tested for their ability to modify the target. Despite the rise of combinatorial chemistry as an integral part of lead discovery process, natural products still play a major role as starting material for drug discovery (Feher and Schmidt, 2003). From 1981 to 2006, of the identified bioactive chemical entities, 63% were natural derived or partially synthesized based on the chemical backbone from natural products (Newman and Cragg, 2007). As it is well known, vast crude drugs used in almost all traditional healthcare systems are plant based extracts and hence plants could be an important sources of starting material for novel chemical entities for

modern drug discovery (Lewinsohn and Gijzen, 2009; Nussbaum et al., 2006). Several drugs have been isolated from plants and are practiced today as antimicrobial agent (e.g. glaucaroubin)(Efrén et al., 1956), analgesic (e.g. morphine and codeine) (Benyhe, 1994; Bosch et al., 1981), antihypertensive (e.g. romitoxin)(Liu et al., 2003), and anti-inflammatory drugs (e.g. aescin, reserpine etc.) (Bonaccorsi, 1968; Sirtori, 2001). Moreover, plant derived antitumor agents (e.g. taxol, etoposide) (Aspandiar et al., 1987; Guchelaar et al., 1994) as well as cardioprotective agents (e.g. digitalis, acetyldigox in, digoxin etc.)(Charles et al., 1953) are also being used now. Table 1-1 shows some important phytochemicals that are now used as drugs. Literature survey showed that the 122 bioactive phytochemicals identified in 1985 were all derived from only 94 plants species (Farnsworth et al., 1985) out of estimated 250000 flowering plants on the planet. Therefore there should be abundance of drugs remaining to be discovered. This is a representation of the volume of phytochemical resources available and how much has been identified. Therefore HTS of plants is essential to explore the hidden therapeutically potent phytochemicals.

Table 1-1 Few phytochemicals with therapeutic properties

Drug	Action or clinical use	Plant source
Trichosanthin	Abortifacient	*Thymus vulgaris*
Glaucaroubin	Amoebicide	*Simarouba glauca*
Emetine	Amoebicide; emetic	*Cephaelis ipecacuanha*
Picrotoxin	Analeptic	*Anamirta cocculus*
Morphine	Analgesic	*Papaver somniferum*
Codeine	Analgesic; antitussive	*Papaver somniferum*
Rotundine	Analgesic; sedative	*Stephania sinica*
Agrimophol	Anthelmintic	*Agrimonia eupatoria*
Atropine	Anticholinergic	*Atropa belladonna*
Protoveratrines	Antihypertensive	*Veratrum album*
Reserpine	Antihypertensive	*Rauvolfia serpentina*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Quinine	Antimalarial	*Cinchona ledgeriana*
Monocrotaline	Antitumor agent	*Crotolaria sessiliflora*
Colchicine	Antitumor agent; antigout	*Colchicum autumnale*

Etoposide	Antitumour agent	*Podophyllum peltatum*
Noscapine	Antitussive	*Papaver somniferum*
Kainic Acid	Ascaricide	*Digenea simplex*
Santonin	Ascaricide	*Artemisia maritima*
Andrographolide	Bacillary dysentery	*Andrographis paniculata*
Khellin	Bronchodilator	*Ammi visnaga*
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Deslanoside	Cardiotonic	*Digitalis lanata*
Digitalin	Cardiotonic	*Digitalis purpurea*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis lanata*
Gitalin	Cardiotonic	*Digitalis purpurea*
Scillarin A	Cardiotonic	*Urginea maritim*
Vincamine	Cerebral stimulant	*Vinca mino*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum*
Caffeine	CNS stimulant	*Camellia sinensis*
Podophyllotoxin	Condylomata acuminata	*Podophyllum peltatum*
Theobromine	Diuretic; bronchodilator	*Theobroma cacao*
(+)-Catechin	Haemostatic	*Potentilla fragaroides*
Danthron	Laxative	*Cassia spp*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Gossypol	Male contraceptive	*Gossypium spp.*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Valepotriates	Sedative	*Valeriana officinalis*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Ephedrine	Sympathomimetic	*Ephedra sinica*

Data adopted from (Daniel et al., 2001; Farnsworth et al., 1985).

1.6 Plant based prophylactic agents

Plant based drugs are organic compounds derived from plants that alter normal body functions. Plant drugs are distinct from plant based nutrients. Apart from nutrients and molecules that have direct effect on human body, there are several non-nutritive organic compounds, in plants, having no apparent detrimental action on human body when consumed with food in the natural concentration. Many compounds belong to this group act as prophylactic agent (Prophylaxis: treatment given or action taken to prevent disease(Simpson and Weiner, 2010)) in the system and help to protect the body from several degenerative diseases caused, especially by oxidative stress in consequent to metabolism. A cross cultural population comparison study showed that plasma levels of antioxidant molecules viz. vitamin C, β carotene, vitamin E and selenium were significantly higher in men, 40-49 aged, from Switzerland and Italy compared to their counterpart in Finland and Scotland (Gey, 1987). In that study, the author highlighted that there is an antioxidant index with respect to antioxidant molecule level in each subjects. The antioxidant molecule's levels in such subjects correlates with a positive antioxidant index and this relation was inversely related to mortality rates by ischemic heart diseases (IHD). The antioxidant hypothesis evolved from this study with the proposal that high intake of dietary antioxidants prevent oxidation of plasma and thereby oxidative stress (Gey, 1987). In the last decade, considerable worldwide attention has been given to plant phenolic antioxidants including flavonoids and various other phytochemicals found in many fruits and vegetables, red wine, etc. for their protective effect against the damage from oxidative stress. The literature on antioxidants has expanded tremendously because of accumulation of evidence that they may contribute to the recognized extra nutritional benefits of food and beverages containing phenolic compounds (Frankel and German, 2006; Kroon and Williamson, 2005). Prophylactic activities of phytochemicals are attributed to their antioxidant/radical

scavenging activities to retard oxidative stress caused by free radicals in vivo. Free radicals are highly reactive chemical entities normally produced but often overproduced in all higher organisms. When free radicals are excessively produced, it can damage biomolecules viz. fatty acid, protein and DNA and can be one among many reasons for the early incidence of degenerative disease.

1.6.1 Etiology of chronic degenerative diseases; special attention to free radicals

With increase in life expectancy, chronic degenerative diseases have become by far the principal cause of death world over. The highest mortality (26.3%) was attributed to cardiovascular diseases (CVD). The next highest mortality was due to malignant neoplasms (24.1%). Other causes were; chronic lower respiratory diseases (4.9%), Diabetes mellitus (3%), and Alzheimer's disease (1.8 %) followed by others (Heron, 2010). In most cases, the etiology is highly complicated and multi-factorial such as genetic, environmental, occupational, dietary habit, lifestyle etc. In all above said factors, free radical production is a common phenomenon that speeds up the onset and progression of degenerative diseases. Free radical theory of aging argues that oxygen free radicals produced during normal respiration would cause cumulative damage which would eventually lead to organism's loss of functionality and ultimately death (Harman, 1972; Harman, 1992). However evidences to substantiate this hypothesis lack at present. During the last 3 decades huge body of literatures have been published that correlate free radicals and onset of cancer, CVD, diabetes, cataract etc. It can be presumed that free radicals along with several other risk factors may accelerate the onset and progression of several degenerative diseases. One of the approaches thus could be to use free radical scavengers / antioxidants to prevent or retard onset and progression.

1.6.2 Chemistry and biochemistry of production of endogenous free radicals and reactive oxygen species (ROS)

Various free radicals are produced in intracellular and extracellular matrices of higher animals and their excess production accelerate the onset and progression

of degenerative diseases (Abd Ellah, 2010). Free radicals are chemical entities with one or more unpaired electrons and capable of independent existence and are more reactive to various biomolecules in the site of production. Other than radicals there are highly reactive oxygen species (ROS) which can oxidize sensitive chemical entities in the vicinity (e.g. peroxy nitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) (kappus, 1985)**)**. Free radicals, and ROS are continuously produced as intermediate and byproduct in all living beings, especially in higher organisms, by normal cellular metabolism, phagocytosis, inflammation and by other exogenous factors such as ionizing radiations and xenobiotics (Beckman and Ames, 1998). Several initiators such as transition metals, pollutants, drugs, food components, radiation etc. promote oxidation of biomolecules viz. lipids, DNA and proteins and hence ultimately cellular and tissue damage happens that leads to early onset of various diseases. The important free radicals produced in vivo are superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($HO^\bullet$), peroxyl radicals ($ROO^\bullet$) and nitric oxide ($NO^\bullet$).

Superoxide radical: The discovery of superoxide as the enzymatically produced diffusion-free dioxygen radical anion was the crucial break-through in the study of free radical processes in biology (McCord and Fridovich, 1968). In a biological system, superoxide is a by-product of normal respiration through the one-electron reduction of molecular oxygen (Chance et al., 1979; Raha and Robinson, 2000). Superoxides are also produced by NADPH oxidases (Kussmaul and Hirst, 2006), xanthine oxidase (Galbuseraa et al., 2006) etc.

$$\mathrm{Enz-Flavin-H_2 + O_2 \rightarrow Enz-Flavin-H + O_2^{\bullet-}}$$

Equation 1-1

The findings associated with the biological role of superoxide are linked with their signaling functions (Finkel, 1998). Superoxides enhances the migration of monocytes across blood-brain barrier (Goes et al., 2001). Together with hydrogen peroxides superoxide activates phosphorylation of p38 MAP kinase

during hypoxia in cardio myocytes (Kulisz et al., 2002)

Hydroxyl radical: For long time it has been thought that the only route of highly reactive hydroxyl radical is the superoxide dependent Fenton's reaction.

$$O_2^{\bullet -} + Fe^{3+} \Longrightarrow O_2 + Fe^{2+}$$

Equation 1-2

$$Fe^{2+} + H_2O_2 \Longrightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

Equation 1-3

Another possible pathway of accelerating the in vivo Fenton's reaction is by enhancing the catalytically active ferrous ion as a result of the interaction of $O_2^{\bullet -}$ with [4Fe-4S] clusters of dehydratase such as aconitase (Liochev and Fridovich, 1994). According to this mechanism, superoxide reacts with aconitase to oxidize ferrous ion which is released from the cluster and enabling it to participating the Fenton's reaction (Equation 1-4)

$$O_2^{\bullet -} + [2Fe^{2+}2Fe^{3+}-4S] + 2H^{+} \Longrightarrow H_2O_2 + [Fe^{2+}3Fe^{3+}-4S]$$

Equation 1-4

$$[Fe^{2+}3Fe^{3+}-4S] \Longrightarrow [3Fe^{3+}-4S] + Fe^{2+}$$

Equation 1-5

Hydroxyl radicals are most reactive and can cause damage to any organic compounds in the vicinity. In biological system, hydroxyl radicals damage membrane integrity, protein folding, DNA structure etc. (Halliwell and Gutteridge, 1989).

Peroxyl radical: Lipid peroxidation is one of the inevitable biological events that lead to the formation of peroxyl radical. Generally, peroxyl radicals are very low in a physiological system compared to that of superoxide. Despite the low concentration, peroxyl radicals could be even more important radical than superoxide due to its greater reactivity in hydrogen abstraction reaction.

Peroxyl radicals readily abstract hydrogen from the bis-allylic positions of unsaturated fatty acids such as linolic, linolenic and arachidonic (Bielski et al., 1983)

$$HOO^{\bullet} + NADH \Longrightarrow H_2O_2 + NAD^{\bullet}$$

Equation 1-6

$$HOO^{\bullet} + GAPDH{-}NADH \Longrightarrow H_2O_2 + GAPDH{-}NAD^{\bullet}$$

Equation 1-7

Other two major biochemical intermediates, where the participation of peroxyl radicals observed are NADH and GAPDH-NADH complex. Peroxyl radicals abstract hydrogen atom from NADH and produce hydrogen peroxide and NAD radical with a rate constant of $2E^5$ l $mol^{-1}s^{-1}$ (Equation 1-6). In the case of GAPDH-NADH complex also a hydrogen abstraction reaction could be seen with the production of hydrogen peroxide and NAD radical, but with a high rate constant; $2E^7$ l mol^{-1} s^{-1} (Equation 1-7).

Nitroxy radical: In a biological system, NO is an intra and inter cellular messenger (Snyder and Bredt, 1992). As a free radical, it can oxidize, reduce or forms complex with other biomolecules (Gaston et al., 1994). The uncharged NO composed of seven electrons from nitrogen and eight electrons from oxygen. This particular combination results in the presence of an unpaired electron and makes NO paramagnetic and radical (Kerwin and M, 1994). In human body, nitric oxide radicals are produced in three major sites; by vascular endothelial nitric oxide synthase, inducible nitric oxide synthase in macrophage and neuronal nitric oxide synthase. Biologic relevant oxides of nitrogen include elemental nitrogen in five oxidation states (NO_x: N_2O, NO., NO_2^-, $NO_2^{\bullet}$, NO_3^-) (Gaston et al., 1994). Normally NO does not remain as $NO^{\bullet-}$ moiety in biological environment and hence the most stable form is the NO. In aqueous systems and in air liquid interfaces, $NO^{\bullet-}$ generation yields nitrite (NO_2^-) and nitrate (NO_3^-) as end products (Stamler et al., 1992). Nitric oxide reacts with superoxide and forms a highly toxic peroxynitrite anion (ONOO-) (Huie and

Padmaja, 1993). Though Peroxy nitrite has important microbicidal and tumoricidal function, the excess production leads to oxidative injury (Ischiropoulos et al., 1995). Peroxynitrite specifically nitrates phenolic residues of tyrosine and forms nitrotyrosine, a marker for NO induced oxidative injury (Beckman and Koppenol, 1996). Being a free radical NO has both pro and antioxidant properties (Hallman and Bry, 1996). Depending on the specific condition and concentration either it can act as an antioxidant or promote oxidative stress. Pro-oxidant action occurs in the presence of superoxide radicals, however in consequent to the reaction with peroxyl and alkoxyl radicals intermediate during lipid oxidation, it terminates the chain reaction (Rubbo et al., 1994).

Hydrogen peroxide: Incomplete reduction of oxygen during respiration produces superoxide anion, which is spontaneously or enzymatically dismutated to hydrogen peroxide. Apart from this pathway many cell types including phagocytic cells produce hydrogen peroxide in response to a broad range of extra cellular stimuli such as cytokines (TGF-B1, TNF-α, IL), peptide growth factors (PDGE, EGF, VEGF, bFGF and Insulin), the agonists of heterotrimeric G protein-coupled receptor (GPCR, angiotensin 2, thrombin, histamine, bradykinin) via NADPH oxidase, and shear and stress (Rhee et al., 2000). Hydrogen peroxide is also produced in vivo by the enzyme glyoxylate oxidase. Hydrogen peroxide is a major reactant in Fenton's reaction that produces hydroxyl radicals and hence it is a major source for hydroxyl radical mediated biomolecule damage.

Hypochlorous acid: Myeloperoxidase produces hypochlorous acid (HOCl) from H_2O_2 and Cl during the nutrophil's respiratory burst. Myeloperoxidase is a heme-containing enzyme present most abundantly in nutrophil granulocytes. Hypochlorous acid oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as oxidizing agent. Hypochlorous acid and tyrosine radicals are highly cytotoxic and normally used by the immune cells to kill bacteria and other pathogens Apart from these endogenous sources, some exogenous factors.

Hypochlorous acid can react with superoxide radicals and produce highly reactive hydroxyl radicals and consequently oxidative damage to biomolecules (Candeias et al., 1992).

1.6.3 Free radical mediated oxidation of biomolecules

The biomolecules are more prone to oxidative stress. These undergo structural and subsequent functional changes when exposed to oxidative stress. The changes lead to the abnormalities in the physiological homeostasis and leads to many pathological conditions.

Lipid peroxidation: Lipid is probably the most studied substrate for oxidation by free radicals in biological system. Oxidation of lipid in non enzymatic mode is mainly by free radicals and ROS. The free radicals that induce lipid oxidation are superoxide radical, peroxide radical and nitric oxide. Superoxide does not abstract hydrogen atom even from very reactive bis-allylic methylene groups (Afanas'ev, 1989; Bielski et al., 1983), although it's conjugated acid HOO• is more active in hydrogen atom abstraction and probably capable of initiating the lipid peroxidation (Bielski et al., 1983). Though the superoxide is too inert to initiate lipid peroxidation, it can initiate lipid oxidation through other ways such as by reduction of ferric to ferrous ion that catalyzes the Fenton's reaction. Subsequent studies showed that formation of hydroxyl radicals, even if it take place during lipid peroxidation, are of no real importance and that have been reported by several authors (Bast and Steeghs, 1986; Beloqui and Cederbaum, 1986; Gutteridge, 1982; Vile and Winterbourn, 1987). The possibility of hydroxyl radical dependent lipid peroxidation was studied earlier and reported that hydroxyl radicals are involved in the NADPH dependent microsomal lipid peroxidation (Lai and Piette, 1977). Peroxy radicals especially neutral, positively and negatively charged alkyl peroxyl radicals are more efficient initiators of LDL oxidation compared to that of superoxide (Bedard et al., 2001). NO is also incapable of abstracting hydrogen atom from unsaturated substrates similar to superoxide, but forms various other reactive species capable of initiating lipid oxidation. The pro-oxidant effect of

NO depends on the relative concentration of NO and oxygen, the direct interaction of NO with free radicals formed in the lipid peroxidation and conversion of NO into peroxy nitrites or other reactive NO metabolites (Bloodsworth et al., 2000; O'Donnell et al., 1997; Rubbo et al., 1994).

Amino acid oxidation: Amino acids are also more sensitive to free radicals and ROS damage. Oxidation of amino acid moieties of functional and structural protein leads to the inactivation of enzymes, receptors, hormones, loss of structural integrity etc. Free radicals cause fragmentation of protein and cross linking of amino acids by hydroxyl radicals in the absence of dioxygen.(Dean et al., 1986). Alpha-position of the simple aliphatic amino acid or amino acid residue in the polypeptide chain is more prone to the hydroxyl radical-mediated abstraction of hydrogen atom. As the number of carbon atoms in an amino acid increase, that amino acid would be more prone to free radical mediated cross link with other aliphatic amino acids. In the case of aromatic amino acids, the ring is the primary site of attack leading to ring scission, and in the case of tyrosine, to the formation of Tyr-Tyr cross-linked dimmers (Stadtman, 1993). Davies et al. reported that superoxide radical alone does not damage amino acids but, in the presence of hydroxyl radicals, it causes several fold damage to the protein compared to that of the damage caused by hydroxyl radical alone (Davies, 1987; Davies et al., 1987). ROS mediated oxidation of amino acids can also lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and to conversion of some amino acid residues to carbonyl derivatives (Stadtman and Levine, 2003)

DNA oxidation: DNA is another target for ROS, RNS, free radicals, transition metals, etc. Reactions of hydroxyl radicals with DNA have been thoroughly studied based on the effect of ionizing radiations on DNA. One important attribute of free radical mediated damage to DNA molecule is the multiple ways of free radical attack. Mostly the DNA damage happens through base

modification (Aruoma et al., 1989; Halliwell and Aruoma, 1991). Another important mode of DNA damage is the oxidative stress induced strand breaks. Hydroxyl radicals can easily abstract hydrogen atom from the ribose of sugar-phosphate backbone and causes single strand break (Breen and Murphy, 1995). Though the single strand breaks are not very harmful, double strand breaks can cause cell death. The double strand breaks are formed because of multiple hydroxyl radical attack (Ward, 1985). Mitochondrial DNA is more sensitive to ROS compared to nDNA. Both xanthine oxidase and menadione generated oxygen radicals caused severe damage to mtDNA with no significant effect on nDNA (Grishko et al., 2001). This theory is supported by the evidence that the lack of histone proteins in the mtDNA makes this more susceptible to ROS induced damage (Ballinger et al., 2000; Yakes and Houten, 1997). Reactive nitrogen species is also known to have the capacity to induce damage to DNA. Amount of nitric oxides generated by interleukin-1-B-induced nitric oxide synthase is sufficient to cause damage to DNA in many cell line in vitro (Delaney et al., 1993).

1.6.4 Consequences of free radicals and ROS mediated oxidation of biomolecules

Free radicals, ROS and RNS cause irreversible damage to biomolecules such as fatty acid, amino acid, and DNA. Oxidative modification / damage of these molecules lead to the onset of several degenerative diseases such as cancer, vascular diseases, diabetes, inflammation etc.

Cancer: The possible role of free radicals in cancer has been discussed based on the discovery of excess production of free radicals in tumor cells (Saprin et al., 1965). Further, the discovery of superoxide in biological system and superoxide dismutase attracted much attention towards the association of free radicals and carcinogenesis. Studies in expression level of SOD gene in normal and cancer cells pointed out this association because, the tumor cells express low levels of SOD (Oberley, 1982). In addition, Mn SOD is not expressed at all in cancer cells even at elevated level of superoxide (Oberley and Oberley,

1988). Consequently, elevated superoxide cause DNA damage and thus initiate carcinogenesis (Nakamura et al., 1988). During initiation process, the involvement of free radicals were emphasized based on the fact that organic peroxides promotes carcinogenesis (Floyd, 1990). Further, ROS and RNS react with guanine and forms 8-OHdG (Kim et al., 2003). The role of 8-OHdG in the process of carcinogenesis is well established (Floyd, 1990; Ogawa et al., 1995; Wei and Frenkel, 1993) by its potential to mutate a few cancer related genes and transformation of proto-oncogenes to oncogenes (Cerutti, 1994). Another study reported that enhanced production of hydroxyl radicals can initiate carcinogenesis, in particular, cologenic hydroxyl radical and other ROS generation is considered as one important factor for the cause of colorectal carcinoma (Babbs, 1990). The involvement of inflammation, especially inflammatory phagocytes, on the cancer promotion has been understood very long time back. During inflammation, stimulated macrophage induce DNA damage supposedly through the generation of free radicals (Chong et al., 1985). The ROS, RNS, and various other carcinogens together may change the normal cellular genome to neoplastic one at the onset of cancer.

Diabetes: The involvement of free radicals in the development of diabetes is a core research area in the epidemiological studies of diabetes. Much study has been done on the free radicals in biology and diabetes independently. However, the number of comprehensive studies to understand the involvement of free radicals in the etiology of diabetes is very few. Type 1 diabetes is caused by destruction of pancreatic beta cells responsible for the production of insulin. In human the diabetogenic process is caused by immune destruction of beta cells; part of this process is apparently by white cell production of ROS. Well-established evidence is the experimental diabetic inducing agents; alloxan and streptozotocin. Though the mechanism of action of these two compounds are different, both results in the production of ROS. The presence of ROS scavengers effectively inhibited the development of diabetes in these compound induced diabetic models (Oberley, 1988). Josefsen et al. demonstrated that the circulating monocytes in newly diagnosed type-1

diabetes were activated which could play a very important role in the destruction of β-cells of pancreas (Josefsen et al., 1994). These monocytes were reported to produce excess superoxide in patients with early hypertriglyceridemia and diabetes (Hiramatsu and Arimori, 1988). In type-2 DM also the plasma redox balance is disturbed and oxidative stress is observed. This is evidenced by several fold reduced plasma superoxide dismutase level and other endogenous antioxidants in type-2 DM patients compared to that of nondiabetic control (Collier et al., 1990). Thus the oxidative stress caused at the onset of type-2 DM may promote the progression of pancreatic cell damage as well as leads to higher prevalence to mortality from CVD (MacRury et al., 1993).

Vascular diseases: Vascular diseases such as atherosclerosis, peripheral artery disease, hypertension, peripheral vascular disease etc. are caused by xenobiotics, physical inactivity, unhealthy diet etc. However the free radicals and ROS in the vascular system promote the onset as well as progression. The role of free radicals in the etiology of a few vascular diseases are presented in the following sections

Ischemic reperfusion injury: Hypoxia and reoxygenation generally causes injury to cells (Li and Jackson, 2002). The major cause of circulatory shook, myocardial ischemia, and stroke are believed to be reoxygenation. During reoxygenation, large amount of ROS especially superoxide (Kim et al., 2002; Kowaltowski and Vercesi, 1999; McCord, 1985) and hydroxyl radicals (Werns et al., 1985) are formed and recognized as the cause of reoxygenation injury. Formation of ROS under this pathological conditions were established by several studies (Das et al., 1986; Hess et al., 1982; Werns et al., 1985).

Atherosclerosis: A large number of reports emphasize that excess superoxide play an important role in the onset of atherosclerosis and hence promote endothelial dysfunction (Kojda and Harrison, 1999). Moreover the oxidized proteins, lipids, LDL and nucleic acids as a result of plasma oxidative stress also promote the progression of vascular tissue damage (Beckman and

Koppenol, 1996) and atherosclerotic plaque formation (Stocker et al., 2004). Promotion of atherosclerosis as a result of reduced expression of extracellular SOD and mutation in endothelial is an important evidence for the role of ROS in vascular diseases (Faraci and Didion, 2004; Fukai et al., 2002; Landmesser et al., 2000)**.**

Hypertension: The possible role of free radicals in the pathogenesis of atherosclerosis and hypertension has been suspected for long time. This was evidenced by low serum antioxidant capacity and hypertension (Salonen et al., 1988), high serum antioxidant capacity and low level of atherogenic protein (Nyyssonen et al., 1994). correlation between antioxidant supplement and normotension (Salonen et al., 1994) etc.

Inflammation: Under chronic inflammatory condition, a large number of ROS are produced. Superoxide thus produced stimulate the release of IL-1 from blood monocytes (Kasama et al., 1989). IL-1 act as feedback booster and in turn increases the formation of excess ROS in the vicinity (Babior et al., 1973). The excess ROS thus produced oxidize lipoprotein, lipids, protein etc. and accelerate atherogenic processes in the vascular system (Zhang et al., 2002)**,** induce carcinogenesis (Coussens and Werb, 2002), neurodegeneration (Akiyama et al., 2000) etc.

Life expectancy: Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age. Among the several ageing theories proposed, the "free radical theory of aging" (Harman, 1956; Harman, 1992) has gained universal acceptance and is supported by the fact that the sum of the deleterious free radical reactions going on continuously throughout the cells and tissues constitutes the aging process (Sohal and Weindruch, 1996). The free radial theory is supported by the "rate of living" hypothesis, which links metabolic rate and subsequent free radical production with the short lifespan of organisms (Ku et al., 1993). Under vigorous metabolism, free radicals and ROS are produced and can damage proteins,

DNA and lipids and this oxidation process accelerate aging process (Barja and Herrero, 2000; Sohal et al., 1995; Sohal and Weindruch, 1996). Caloric restriction and thereby reducing free radical production and oxidative stress has been shown to increase lifespan in animal studies (Agarwal and Rao, 1998; Sohal and Weindruch, 1996). The inverse relation between free radicals and oxidative stress versus longevity is reported by several studies (Buchan and Sohal, 1981; Merry, 2004; Yan and Sohal, 2000). Other studies also supported this relation indirectly such as low cellular superoxide and hydrogen peroxide production, as a result of high antioxidant enzymes level, and maximum life span (Barja, 1998; Ku et al., 1993). Genetic studies viz. over expression of superoxide dismutase in transgenic flies, catalase enzyme in *C. elegans*, mitochondrial catalase enzyme in mice etc. support the relation between free radicals and longevity (Melov et al., 1995; Tower, 2000).

1.6.5 Scavenging of free radicals and other ROS

In a physiological system, free radicals are formed as a part of normal metabolism and by exogenous factors, and the antioxidant defense system continuously scavenges the excess oxidants, ROS and free radicals formed. The free radical production and its removal is taking place in a balanced condition. When the free radical production is more and a corresponding removal is not done, the system would undergo a state called oxidative stress. The oxidative stress can be cellular, tissue level or in organ level and can be an important cause for early onset of various degenerative diseases as discussed in the previous section. The antioxidant defense system plays a vital role in removal of excess free radicals and maintains a balanced redox state. One important line of defense is a system of enzymes, including glutathione peroxidase, superoxide dismutase and catalase, which decrease concentrations of the most harmful oxidants in the tissues. Several essential minerals including selenium, copper, manganese and zinc are necessary for the formation or activity of these enzymes. Hence, if the nutritional supply of these minerals is inadequate, enzymatic defenses against free radicals may be impaired (Bagchi and Puri,

1998). The second line of defense against free radical damage is the presence of nonenzymatic antioxidants. Antioxidants are a group of substances which when present at lower concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative process, while often being oxidized themselves (Vaya and Aviram, 2001). Antioxidants are stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Some such antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body. Other lighter antioxidants are found in the diet. Although about 4000 antioxidants have been identified, the best known are vitamin E, vitamin C and the carotenoids. Many other non-nutrient food substances, generally phenolic or polyphenolic compounds, display antioxidant properties and, thus, may be important for health (Bagchi and Puri, 1998). Both antioxidant defense systems jointly scavenge the excess free radicals produced in vivo. Therefore the antioxidant defense system helps to maintain health and protect from early onset of free radical mediated degenerative diseases.

1.6.6 Endogenous antioxidants:

ROS contribute to the formation of various pathological conditions. To counteract the effects of ROS, the body is endowed with a protective mechanism consisting of enzymatic and non enzymatic endogenous antioxidants. Up regulation of enzymatic antioxidants have been reported to minimize free radical production and oxidative stress mediated tissue damage and hence the onset and progression of degenerative disease (Li et al., 2006). Endogenous antioxidants are capable of different activities and work synergistically with exogenous antioxidants contributing the overall protective effect to the individuals by preventing or delaying the onset and progression of various free radical contributed degenerative disease (Serafini, 2006). Following section presents about various endogenous antioxidant and their importance.

Superoxide dismutase: Superoxide dismutase is the first antioxidant enzyme

involved in the antioxidant defense system found in higher organism and microbes (Fridovich, 1983; Fridovich, 1986) and the major function is the removal of superoxide radicals formed by various reasons as presented in section 0.

***Catalase**:* Catalase is a common peroxidase enzyme found virtually in all aerobic organisms that breakdown hydrogen peroxide which is produced by superoxide dismutase enzyme. Hydrogen peroxide is an important starting molecule for the production of hydroxyl radicals by Fenton's reaction. Catalase decomposes hydrogen peroxide into water and oxygen and thereby prevents the damaging effect caused by hydroxyl radicals (Deisseroth and Dounce, 1970).

***Glutathione peroxidase**:* Glutathione peroxidase (GPX) is ubiquitous selenium containing antioxidant enzyme in all higher organisms that catalyze the decomposition of hydrogen peroxide to water by utilizing reduced glutathione as hydrogen atom source. There are several isozymes differentially located in various organs. GPX-1 is present in the cytoplasm of all mammalian cells and its preferred substrate is hydrogen peroxide. GPX-2 is an intestinal and extracellular enzyme. The GPX enzyme, which is more abundant in extracellular fluid, especially plasma, is isozyme-3. isozyme-4 also expressed in all cells in less abundance whose preferred substrate is lipid hydroperoxids (Muller et al., 2007).

Glutathione reductase: Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione. Oxidation of glutathione by GPX forms two glutathione molecules, which are linked to form glutathione disulfide (GSSG), a stable molecule. The reduction of glutathione disulfide catalyzed by GR produces two molecules of GSH, which is one important substrate for GPX for decomposing hydrogen peroxide to water. The substrate, GSH, used from the pool during the detoxification of hydrogen peroxide is maintained by the glutathione reductase.

Thiols: Thiols contain highly active SH group and therefore having antioxidant property. The most studied endogenous antioxidant thiols are lipoic acid and glutathione. Lipoic acid and dihydrolipoic acids are present in most kind of

cells. Properties and therapeutic effects of LA and DHLA are well reviewed (Fuchs et al., 1997; Sies, 1997). DHLA is an efficient scavenger of all oxygen radicals; however, LA is active only in the reaction with highly reactive hydroxyl radicals.

Ubiquinones: Ubiquinones are essential electron carriers in the mitochondrial electron transport chain. They shuttle electron from NADH and succinate dehydrogenase to the cytochrome b-c1 complex. There are two types of redox interaction, in which ubiquinone can manifest their antioxidant activity: the reaction with quinone and hydroquinone formation. The antioxidant activities of ubiquinone has been demonstrated in vitro and in vivo studies (Filipe et al., 2001; Robak et al., 1986; Silva et al., 2000)

Uric acid: Uric acid is another physiologically important antioxidant. Uric acid contains two active hydroxyl groups in the purine heterocycle. The physiological level of uric acid protects erythrocyte against free radical damage (Ames et al., 1981). It is also a major antioxidant in human airway mucosal surface (Peden et al., 1990).

NADPH*:* NADPH is an indirect antioxidant due to its capacity to reduce various oxidized substrates. Recent study showed that NADPH possesses scavenging capacity against free radicals such as $CO_3^{\bullet -}$, $NO_2\bullet$, ROO• and RO• (Kirsch and Groot, 2001).

Melatonin: Melatonin is a pineal hormone, which is synthesized from tryptophan. Melatonin is an effective scavenger of hydroxyl radicals, nitric oxide and peroxy nitrite (Reiter et al., 2000). It is an effective inhibitor of iron-initiated peroxidation of brain phospholipids liposome (Marshall et al., 1996).

1.6.7 Exogenous antioxidants.

Similar to endogenous antioxidants, some exogenous dietary compounds can neutralize the free radicals as well as enhance the activities of endogenous antioxidants. When the system is under oxidative stress and the endogenous antioxidants are not sufficient enough to scavenge the free radicals and ROS,

the dietary antioxidants may be required to maintain optimal cellular functions (Rahman, 2007). Some important dietary antioxidants are presented below.

α-Tocopherol*:* α-Tocopherol is a lipid soluble phenolic antioxidant with an active hydroxyl group. Several authors reported the high antioxidant and antiradical activities of α-tocopherol (Burton et al., 1983; Doba et al., 1985; Lambelet and Loliger, 1984; Scarpa et al., 1984). However similar to many other antioxidants, α-tocopherol also shows pro-oxidant action under certain conditions (Terao and Matsushota, 1986; Upston et al., 1999; Weinberg et al., 2001).

Vitamin C*:* Ascorbic acid is a highly active free radical scavenger and strong reducing agent. Oxidation and reduction reactions of ascorbic acid with numerous oxidants and reductants are well studied (Afanas'ev, 1989). Other than its antioxidant properties pro-oxidant activities also well studied. It is known that the competition between antioxidant and pro-oxidant activities of ascorbic acid depends on the rate of reaction (Afanas'ev et al., 1987; McCay et al., 1978). Ascorbic acid at lower concentration enhanced lipid peroxidation but inhibited at higher concentration (Afanas'ev et al., 1989). Presence of other factor also promotes the pro-oxidant activity of ascorbic acid. In the presence of Fenton's reactants, ascorbic acid promotes the hydroxyl radical production by redox cycling of iron ion (Benherlal and Arumughan, 2008).

Carotenoids: Many hundreds of carotenoids are found in nature but relatively a few are found in human tissues, the five main carotenoids are; β-carotene, lutein, lycopene, β-cryptoxanthin, and α-carotene (Bendich and Olson, 1989; Rock et al., 1996; Thurnham, 1994). The antioxidant properties of the carotenoids closely relate to the extended system of conjugated double bonds, which occupies the central part of carotenoid molecules, and to the various functional groups on the terminal ring structures (Mathews-Roth, 1974; Stryker, 1988; Thurnham, 1997). The reactive oxygen species scavenged by carotenoids are singlet oxygen and peroxyl radicals (Foote and Denny, 1968; Palozza and Krinsky, 1992). In this process the carotenoid absorbs the excess

energy from singlet oxygen and then releases it as heat. Singlet oxygen is generated during photosynthesis; therefore, carotenoids are important for protecting plant tissues, but there is some evidence for an antioxidant role in humans. β-Carotene has been used in the treatment of erythropoietic Protoporphyria (Mathews-Roth, 1986). Using in vitro studies, they showed that β carotene was effective in reducing the rate of lipid peroxidation at the low oxygen concentrations found in tissues (Johnson and GR, 1993; Terao, 1989).

Flavonoids*:* Flavonoids are naturally occurring low molecular weight phenolic compounds widely distributed in plant Kingdome. Huge amount of literature is available on the antioxidant activates of flavonoids. Flavonoids are reported to have multiple biological activities such as anti-inflammatory, antidiabetic, antiallergic, antiviral, anticancer etc. (Critchfield et al., 1996; Havsteen, 1983). Since they are polyphenols, their antioxidant activities depend on the hydroxyl groups. Flavonoids are generally good scavengers of peroxyl radicals, hydroxyl radical and superoxide radicals (Denisov and Afanas′ev, 2005). Other non-flavonoid phenolic compounds also possess in vitro and in vivo antioxidant activity. One of the well studied compound is resveratrol, which has been identified as potential antioxidant , anticancer and antimutagenic agent (Jang et al., 1997).

Steroids*:* Some steroid molecules such as estrone, estradiol, and estriol has phenolic hydroxyl group and therefore are able to react with free radicals. All the above said compounds are reported to inhibited liposomal lipid peroxidation (Nakano et al., 1987; Sugioka et al., 1987). The role of phenolic hydroxyl group in the steroid molecules have been studied using various steroids. Only phenolic hydroxyl group containing steroids inhibited lipid peroxidation (Huber et al., 1990).

1.6.8 Antioxidants and disease prevention.

In vitro as well as in vivo cell culture studies showed that intra cellular and extracellular antioxidants may prolong the onset or progression of degenerative diseases such as diabetes, cancer, and cardiovascular diseases etc. the literature

evidences showing role of antioxidants in a few degenerative disease are presented below.

Cancer: In vitro and in vivo research indicated that some dietary antioxidants show anticancer activity. Strong antioxidants such as pyrrolidine dithiocarbamate (PDTC) and N-acetyl cysteine (NAC) inhibited growth of human colorectal cell in culture and when fed to mice with implanted tumors (Chinery et al., 1997). Another similar study showed that consumption of antioxidant rich tea reduced the risk of breast cancer several fold (Hirvonen et al., 2006) .Though the mechanism of action of antioxidants and low cancer risks are not clear, there are some indications about the activation of tumor suppressor genes which are inactivated in almost one half of human tumors (Chinery et al., 1997). It was shown that the possible first step in the activation of tumor suppressor genes is by antioxidant-induced activation of protein kinase A. The reduced form of protein kinase A activated by associating with plasma membrane, and triggers the downstream events. The modulation of activity of protein kinases by antioxidants is one attractive way to improve current cancer therapies and prevention strategies. Antioxidants are also reported to induce apoptosis by the activation of nuclear factor *k*B (NF*k*B). Pyrrolidinedithiocarbamate (PDTC) inhibited translocation of NF*k*B from cytoplasm to nucleus and prevented expression of several antiapoptotic proteins (Gunawardena et al., 2005; Gunawardena et al., 2002). Antioxidants also inhibit or delay the onset and progression of cancer possibly by inhibition other factors such as angiogenesis and endothelial nitric oxide synthase. (Bai et al., 1998; Hesketh, 1997; Kuzumaki et al., 1998). Moreover, when used with chemotherapy agents such as 5-fluorouracil and doxorubicin, antioxidants enhance the cytotoxicity of chemotherapy agents and cause complete remissions, where only partial remission is possible with chemotherapy agents alone (Bach et al., 2001; Chinery et al., 1997).

Diabetes: Pancreatic β-cell dysfunction together with insulin resistance is associated with the development of type 2 diabetes. Various authors have

shown the significance of hyperglycemia as a direct cause of β-cell glucose toxicity in vivo (Zangen et al., 1997) and in vitro (Olson et al., 1993; Poitout et al., 1996; Robertson et al., 1992; Sharma et al., 1995). At the onset of insulin resistance and hyperglycemia, β-cell function progressively deteriorates and subsequently glucose induced insulin secretion becomes further impaired and β-cells number decreases as a result of degranulation (DeFronzo et al., 1992; Porte, 1991; Vinik et al., 1996; Yki-Jarvinen, 1992). Impairment of β-cell function happens at the level of insulin synthesis as well as insulin secretion (Olson et al., 1993; Poitout et al., 1996; Robertson et al., 1992; Sharma et al., 1995). One important reason attributed for the hyperglycemia induced dysfunction is through hyperglycemia mediated production of free radicals and ROS by the glycation of biomolecules (Hunt et al., 1991; Sakurai and Tsuchiya, 1988). Although the induction of the glycation reaction in diabetes was originally found in neural cells and the lens crystalline, which are known targets of diabetic complications, another target that accelerate the progression of DM was shown to be the β-cell (Ihara et al., 1999; Kaneto et al., 1996). Moreover the ROS thus produced also play significant role in the development of other complications related to diabetes (Baynes, 1991). In vitro studies that simulated β-cells in hyperglycemic condition showed several glycosylation end products as well as oxidative stress markers (Ihara et al., 1999). It has also been noted that under hyperglycemic condition, expression of cellular endogenous antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are down regulated in islet cells compared with other tissues and cells (Tiedg et al., 1997). Therefore, once β-cells face oxidative stress, they may be rather sensitive to it, suggesting that glycation and subsequent oxidative stress may in part mediate the toxic effect of hyperglycemia. As direct support for this, another study showed that glycation mediated ROS production reduces insulin gene transcription (Matsuoka et al., 1997). Conclusively the antioxidant treatment is beneficial for treating diabetes and can provide protection to β-cells against glucose toxicity. This is supported by a study (Kaneto et al., 1999) in which the cells under hyperglycemic condition treated with N-acetyl-L-

cysteine (NAC), vitamins C and E showed protective activity. Though vitamin C and E did not show significant effects when used alone, they exerted some beneficial effects when used in combination with NAC. A recent study of 2285 men and 2019 women, 40-69 years aged, showed that intake of vitamin C, carotenoids and tocopherols reduced the development of type 2 diabetes (Montonen et al., 2004). Thus a sufficient supply of antioxidants may prevent or delay β-cell dysfunction in diabetes by providing protection against glucose toxicity.

Cardio vascular disease: Quite a lot of recent studies have demonstrated that altered oxygen utilization and/or increased formation of ROS contribute to atherogenesis and CVD progression. Several sources of oxygen/nitrogen species do occur in CVD. Intracellular ROS are formed during mitochondrial electron transport chain and are controlled by antioxidant defense system. However several studies suggested that oxidative stress as well as polymorphic variations in endogenous antioxidants are linked to increased risk for atherosclerosis and CVD (Hiroi et al., 1999). Immediate targets of ROS are long-chain free fatty acids in the cytosolic compartment and membrane-bound lipids however chemically vulnerable substrates to ROS are the polyunsaturated fatty acids in the lipoproteins. Free radicals attack plasma low density lipoprotein (LDL) that is oxidatively modified to oxidized low density lipoprotein (oxLDL) leading to the attraction of blood monocytes beneath the endothelium. Monocytes differentiate into macrophages that are converted to foam cells, full of cholesterol and oxidized lipids. Foam cells form the early atherosclerotic lesions, which are documented as the pathogenesis of CVD (Berliner et al., 1995). Based on the 'oxidation theory' for atherosclerosis, dietary antioxidants have attracted considerable attention as preventive and therapeutic agents. There is adequate evidence from observational, in vitro, ex vivo, controlled intervention and animal model studies that consumption of certain extracts contain vitamin C and E, β carotene, and polyphenols results to a reduction in oxidative stress and myocardial infarction biomarkers (Kalioraa et al., 2006). There is a large body of observational studies on the dietary

antioxidant intake link to prevention of CVD progression. Amongst the most established are; inverse correlation between death for myocardial infarction and Vitamin E (Stephens et al., 1996), inverse correlation between vitamin C intake and carotid wall thickening (Kritchevsky et al., 1995), vitamin C deficiency and associated increased risk of myocardial infarction (Nyyss¨onen et al., 1997), Correlation between flavonoid intake and reduction in CVD mortality (Hertog et al., 1993), inverse correlation between carotenoids level and myocardial infarction (Street et al., 1994), the relationship between adipose tissue lycopene and the risk for myocardial infarction (Kohlmeier et al., 1997) etc.

1.7 Plant-based anticancer agents

Cancer is one of the second largest health problems worldwide after CVD and the new incidence is close to 6 million per year (Heron, 2010). The development of new anticancer drugs and more effective treatment strategies are the fields of utmost importance in drug discovery and clinical therapy. Much of the research in these area is currently focused on cancer-specific mechanisms and the corresponding molecular targets (McLaughlin et al., 2003). Conventional cancer therapy includes surgery, irradiation, and chemotherapy. Chemotherapy is based on the systemic administration of anticancer drug targeting localized tumors and metastized cancer cells. Chemotherapy is a rapidly developing field of cancer treatment with new drugs continuously being tested and developed, which includes plant secondary metabolites. Some secondary metabolites are considered as metabolic waste products. However, a significant portion of the products derived from secondary pathways serve either as protective agents against various pathogens or growth regulatory molecules to the plants. The protective nature of these secondary metabolites can be attributed to their capacity to induce toxicity to invading organisms. This cytotoxicity can be exploited to treat fast proliferating cancer cells under optimized dose that are in sub-lethal levels to

normal cells. The phytochemical classes which are known to have anticancer activities are; aldehydes, alkaloids, annonaceous acetogenins, flavonoids, glycosides, lignans, lipids (unsaponified), phenols and derivatives, and terpenoids (Kintzios and Barberaki, 2004).

Several phytochemicals having anticancer property are being clinically used at present and many are under clinical evaluation. In the process of identification of novel anticancer agents from plant materials, knowledge about the possible mechanisms of action of the active principles could contribute in developing structure function relation as well as developing new drug candidates by advanced computer aided designs and chemoinformatics studies. Mechanism of action and signaling pathways of several phytochemicals are known and based on these compounds' structure, many modified compounds have been synthesized with enhanced bioavailability and efficacy. A few standard anticancer phytochemicals viz. camptothecin, Taxol, combretastatin A-4, and podophyllotoxin are reviewed below

***Camptothecin*:** Camptothecin is a naturally occurring alkaloid isolated from the wood of Chinese tree *C.acuminata* (Wall, 1998). Preliminary studies showed substantial antitumor activity in standard in vitro systems and subsequently extended the studies in animals and human volunteers. Due to the severe toxicity, this has been withdrawn from the phase III clinical trials. However, work had been continued to synthesize its analogues with low nonspecific toxicity. Presently the first generation analogues of camptothecin such as hycamtin and camptosar are marketed for the treatment of ovarian cancer and colon cancer (Gore et al., 2001; Saltz et al., 2000). Initial studies on the mechanism of antitumor activity showed that camptothecin selectively inhibited topoisomerase enzyme. Topoisomerases are enzymes involved in the uncoiling of super coiled double stranded DNA during replication (Potmesil and Kohn, 1991)

Paclitaxel: paclitaxel is a complex polyoxygenated diterpenoid isolated from the pacific yew, *Taxus brevifolia* (Wani et al., 1971). The drug Taxol was

developed from paclitaxel by the National Cancer Institute, USA and commercially produced by Bristol-Myers Squibb. Taxol exhibits a unique mode of action by stabilizing microtubulin while the other anticancer agents destabilize this process (Schiff et al., 1979). The major drawback of taxol is its poor bioavailability.

Combretastatins: combretastatins are mitotic agents isolated from the bark of the South African tree *Combretum caffrum*. It has been found to be a potent cytotoxic agent that strongly inhibits the polymerization of tubulin by binding to the colchicine site. In vitro studies have shown that combretastatins competes with colchicine for binding sites on tubulin. Hence, it is a member of the colchicine-like inhibitors of microtubulin assembly (Hamel, 1996)

Podophyllotoxin: podophyllotoxin is a well-known naturally occurring aryltetralin lignans. Podophyllotoxin was first isolated in 1880 from the North American plant *Podophyllum peltatum* (Mukherjee et al., 2006). Podophyllotoxin shows strong cytotoxic activity against various cancer cell lines. It is effective in the treatment of Wilm's tumors, various genital tumors and in non-Hodgkin's and other lymphomas and lung cancer (Subrahmanyam et al., 1998; Utsugi et al., 1996). Podophyllotoxin acts as an inhibitor of assembly of microtubules and arrests the cell cycle in metaphase (Buss and Waigh, 1995; Gordaliza et al., 2000).

Other than the above discussed phytochemicals, several cytotoxic compounds have been isolated from plant sources. Many of them are currently in clinical trials or preclinical trials or undergoing further investigation. Betulinic acid, a pentacyclic triterpene, a secondary metabolite isolated from *Betula* species (Cichewitz and Kouzi, 2004), and *Zizyphus* species (Nahar et al., 1997; Pisha et al., 1995) is a potent selective cytotoxic agent against human melanoma cell lines (Balunas and Kinghorn, 2005). The development of systemic and topical formulations of the agent for potential clinical trials by the NCI is ongoing. Pervilleine A was isolated from the roots of *Erythroxylum pervillei* Baill (Silva et al., 2001). Pervilleine A is also a selective cytotoxic against multidrug

resistant (MDR) oral cancer cell in the presence of other anticancer agents. Pervilleine A is currently in preclinical development. Silvestrol was first isolated from the fruits of *Aglaila sylvestre* (Hwang et al., 2004). Silvestrol exhibited cytotoxicity against lung and breast cancer cell lines (Cragg et al., 1997). Biological studies are ongoing to determine the mechanism(s) of action for silvestrol. Two novel alkaloids, schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea montana* (Shoeb, 2005; Shoeb et al., 2005). Both of the alkaloids exhibited significant cytotoxicity against human cancer cells. Some important phytochemicals with potent cytotoxic activity in various cancer cells are presented below (Table 1-2).

Several plant-based synthetic compounds are also known to have potent anticancer activity. Flavopiridol is a rohitukine derived synthetic flavone, which was isolated from *Dysoxylum binectariferum* (Kelland, 2000), is a potent cytotoxic agent that enhances radioresponsiveness in various tumors (Christian et al., 1997; Kathy et al., 2004). Synthetic roscovitine, a derivative of olomucine originally isolated from *Raphanus sativus*, is a strong cytotoxic agent which is now under Phase II clinical trials in Europe (Cragg and Newman, 2005; Cragg et al., 1997). Importance of phytochemicals as anticancer drug is evidenced by their huge demand. it is estimated that camptothecin, a plant derived cytotoxic agent, accounts for nearly one-third of global anticancer market (Oberlines and Kroll, 2004). Though there are more than 270000 higher plants in this planet. Only a small portion has been explored phytochemically for anticancer potential. Therefore it is assumed that plants can provide potential chemical entities for development of new anticancer drugs.

Table 1-2 Anticancer activities of a few phytochemicals in various cancer cells

Cancer type	Cell type	Compounds	References
Human oral cancer	HSC-2, HSG, SCC-25	Flavanones, isoflavans, EGC, chalcones, EGCG, curcumin, genistein, ECG, quercetin, cisplatin	(Elattar and Virji, 2000; Elattar and Virji, 2000; Fukai et al., 2000; Sakagami et al., 2000; Shi et al., 2001)
Human breast cancer	MCF-7	Flavanones, daidzein, genistein, quercetin, luteolin	(Han et al., 2001; Pouget et al., 2001)
Human thyroid cancer	ARO, NPA,WRO	Genistein, apigenin, kaempferol, chrysin, luteolin, biochanin A	(Yin et al., 1999; Yin et al., 1999)
Human lung cancer	SK-LU1, SW900, H441, H661, haGo-K-1, A549	Flavone, quercetin	(Bai et al., 1998; Caltagirone et al., 1997)
Human prostate cancer	LNCaP, PC3, DU145	Catechin, epicatechin, quercetin, kaempferol, luteolin, genistein, apigenin, myricetin, silymarin	(Agarwal, ; Bhatia and Agarwal, 2001; Kampa et al., 2000; Knowles et al., 2000)
Human colon cancer	Caco-2, HT-29, IEC-6, HCT-15	Flavone, quercetin, genistein, anthocyanin	(Kamei et al., 1998; Kuntz et al., 1999; Kuo, 1996; Kuo et al., 1997; Wenzel et al., 2000)
Human	HL-60, K562,	Apigenin, quercetin,	(Chung et al., 1999; Csokay et

leukaemia	Jurkat	myricetin, chalcones	al., 1997; Vincenzo et al., 2000; Wang et al., 1999)
B16 mouse melanoma	4A5	Chalcones	(Iwashita et al., 2000)
Colon cancer	SW620	2-hydrocycinnaldehyde	(Lee et al., 2007)
Prostate cancer	LNCaP, PC-3/AR	camptothecin	(Liu et al., 2010)
nasopharyngeal	KB-VIN	quassinoids	(Murakami et al., 2004)
Leukemia, fibrosarcoma, lung cancer, colon cancer, melanoma, breast cancer	P-388,KB, HT-1080, LU-1, COL-2, MEL-2, BC-1	22-Hydroxytingenone	(Bavovada et al., 1990)
leukemia	P388	4-Hydroxy-2-cyclopentenone	(Perry et al., 1991)
Cervix	HeLa	6,7-dehydrototarol	(Moujir et al., 2008)
leukemia	P-388	wikstromol	(Torrance et al., 1979)
Larynx and Lung cancer	HEp-2, PC-13	3-(3,3-dimethylallyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone	(Arisawa et al., 1990)

1.8 Plant-based antidiabetic agents

Diabetes mellitus (DM) is a growing public health concern worldwide. DM is a chronic metabolic disease primarily due to the disorder of carbohydrate metabolism. Cause of which is the deficiency or diminished effectiveness of insulin resulting in hyperglycemia, as a result of uncontrolled hepatic glucose output and reduced uptake of glucose by skeletal muscle (fasting plasma glucose > 7.0mM/l or post prandial plasma glucose > 11.1 mM/l), and glycosuria. Secondary abnormalities of hyperglycemia may occur in the metabolism of proteins, fats, water, and electrolytes in tissues/organs (León and Stanley, 2009), sometimes with serious consequence including ophthalmic, cardiovascular, and renal diseases (Dean et al., 2006). Several statistical reports on the risks of diabetic death warrant the need for new and alternative therapeutical/management methods to control this metabolic disorder. According to the National Vital Statistics Report (NVSR), more than 180 million people worldwide have diabetes and the number is likely to more than double by 2030. It was estimated that in 2005, 1.1 million people died from diabetes and almost 80% of diabetes occurred in low and middle income countries. NVSR projects that diabetes death will increase over 80% in upper-middle income countries by 2015 (Heron, 2010).

Several antidiabetic drugs have been isolated from plants and are currently being practiced; e.g. the α-glucosidase inhibitor acarbose and galegine that contributed to the discovery and development of the biguanides (Alan, 2010). On the other hand though we have evidences for the antidiabetic properties of traditionally used herbs, often, neither their mechanism nor their active components have been defined. Despite the lack of robust scientific data to support the efficacy of many such plants, they are still the main source of medication for patients with diabetes in many parts of the world. But currently, there is considerable interest in exploring traditionally known plant for identifying active principle. Some recent studies validated the antidiabetic

potential of plants that are used in traditional medicine viz. *Melothria maderaspatana* (Balaraman et al., 2010), *Dichrostachys glomerata* (Kuate et al., 2010), *Oreocnide integrifolia* (Ansarullah et al., 2010), *Moringa. Oleifera* (Jaiswal et al., 2009), *Ricinus communis* (Shokeen et al., 2008) etc. Some active principles viz. phenylethyl cinnamaldehyde (Phuwapraisirisan et al., 2008) and aegline-2 (Narender et al., 2007) have also been recently isolated from *Aegle marmelos,* a traditionally used plant to treat diabetes. The active principles thus isolated can improve glucose metabolism as well as the overall condition of individuals with diabetes not only by hypoglycemic effects but also by improving lipid metabolism, antioxidant status etc. (Bailey and Day, 1989). Therefore investigations on antidiabetic agents from traditionally known plant might be useful in the clinic or that might have novel effects, such as stimulation of β-cell proliferation and therefore it is possible that novel mechanisms of action and novel compounds will be discovered.

Table 1-3 Anti diabetic activity of a few plants and their parts

Plant	Parts	Reference
A. marmelos .	Seed and fruit	(Kamalakkannan and Prince, 2003; Kesari et al., 2006)
Melothria maderaspatana.	aerial parts	(Balaraman et al., 2010)
Ricinus communis	root	(Shokeen et al., 2008)
Cynodon dactylon	leaves	(Singh et al., 2007)
Coccinia indica.	aerial parts	(Balaraman et al., 2010)
Murraya koenigii.	leaves	(Kesari et al., 2005)
S. cumini	**seeds**	**(Anandharajan et al., 2006)**
Ficus benghalensis.	stem bark	(Kar et al., 2003)

Dichrostachys glomerata.	Stem bark	(Kuate et al., 2010)
G. sylvestre.	leaves	(Kar et al., 2003)
Momordica charantia.	fruits	(Reyes et al., 2006)
Pterocarpus marsupium.	bark	(Vats et al., 2002)
Swertia punicea.	whole plant	(Tian et al., 2010)
Terminalia. bellirica.	fruit pulp	(Kar et al., 2003)
Tinospora. cordifolia	root	(Stanely et al., 2000)
Trigonella. foenum.	seed	(Zia et al., 2001)
Moringa. oleifera.	stem bark	(Jaiswal et al., 2009)
Ocimum sanctum.	leaves	(Vats et al., 2004)
Carissa edulis	leaves	(El-Fiky et al., 1996)
Oreocnide integrifolia	leaves	(Ansarullah et al., 2010)
Luffa aegyptiaca	seeds	(El-Fiky et al., 1996)
Tamarindus indica	seed	(Maiti et al., 2004)
Premna integrifolia	Whole plant	(Alamgir et al., 2001)
Tragia involucrata	leaf	(Kar et al., 2003)

1.9 *Syzygium. cumini*

1.9.1 Botanical description

S. cumini is an evergreen tropical tree, which can grow above 30 m height (Fig. 1-2). The bark is generally grayish, the leaves are simple, oriented in opposite direction; the shape is elliptic to broadly oblong smooth glossy and lathery. Leaves are 5-10 cm long and pointed at tip. The leaf mid rib is slightly yellow in color. The flowers are seen in clusters at the stem tips. It is white to pinkish and the calyx is cup shaped. The flower has four petals and many stamens. The fruit is fleshy, dark purple colored and ovoid in shape with a hard single seed inside. The plant propagation is through seeds. Flowering season is January to December in South Kerala. The individual fruit weight ranged from 4.8-17.6 g, length; 2.22-4.51 cm, diameter; 1.66-3.04 cm, seed weight; 1.3-2.36 g, pulp content; 68.75-86.59% (Singh et al., 2007). This species is endemic to South-East Asia and India, but also reported to be grown in Hawaii, Australia, Philippines, Zanzibar, Pemba, Mombassa, Kenya, Florida etc.

1.9.2 Taxonomy

Common names: Java plum, Jamun, Jambolan, Duhat, Java plum, duhat, Jambolan plum, Jamelonguier, Kavika, Mesegerak. **Botanical names**: *Syzygium cumini* (L.) Skeels, *Syzygium jambolana* Lam, *Eugenia cumini* (L.) Druce, *Eugenia jambolana* Lam (NCBI, 2010).

Kingdom	: Plantae – Plants
Subkingdom	: Tracheobionta – Vascular plants
Super division	: Spermatophyta – Seed plants
Division	: Magnoliophyta – Flowering plants
Class	: Magnoliopsida – Dicotyledons
Subclass	: Rosidae
Family	: Myrtaceae – Myrtle family
Genus	: *Syzygium* P. Br. ex Gaertn – Syzygium
Species	: *Syzygium cumini* (L.) Skeels

Fig. 1-2 A, *Syzygium cumini* plant; B, flower and C, fruit

1.10 Scientific investigations on *S. cumini*

1.10.1 Antioxidant activity

In vitro and in vivo antioxidant activities of various extracts from *S. cumini* fruit, its anatomical parts viz. pulp, kernel and seed coat have been reported. Aqueous extract of *S. cumini* kernel (SCK) has been reported to inhibit plasma oxidative stress and ameliorate antioxidant status by increasing expressions of endogenous antioxidant enzymes several folds above the base line (Prince and Menon, 1998). Antioxidant activity of SCK aqueous extract to retarded oxidative events consequent to streptozotocin (STZ) treatment in experimental animals have been evidenced by decreased plasma lipid peroxides, low level of thiobarbituric acid reactive species (TBARS) and conjugated dienes (CD). This study also showed the efficacy of SCK extracts to enhance plasma antioxidant capacity by increasing levels of enzymatic antioxidants such as superoxide dismutase and catalase (D'Mello et al., 2000; Mandal et al., 2008). Antioxidant properties of SCK has been shown to protect pancreatic beta cells from alloxan induced oxidative stress in vivo and reversed hyper glycemia by regenerating beta cells and enhanced other endogenous antioxidant levels in plasma (Mallick et al., 2006; Ravi et al., 2004). Banerjee et al. evaluated the antioxidant properties of anthocyanin rich skin and reported its potency to scavenge various free radicals such as $HO^{\bullet}$, $O_2^{-\bullet}$, $DPPH^{\bullet}$ and $LOO^{\bullet}$ and the radical scavenging capacity was found to be proportional to phenolic compounds present in the fruit skin (Banerjee et al., 2005). Fruit pulp is also reported to have antioxidant activity evidenced by the results of Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP) (Luximon-Ramma et al., 2003). Antioxidant potency of *S. cumini* is more evident from its capacity to scavenge free radicals and reactive oxygen species produced in consequent to ionizing radiation exposure. Leaf extracts of *S cumini* was found to have radio-protective activity in the γ radiation induced micro nuclei formation assay in cultured human peripheral blood lymphocytes (Jagetia and Baliga, 2002). Radiation exposed experimental animals fed with *S.cumini* leaf extract also reduced the symptoms of radiation

sickness, bone marrow cell death, delayed onset of radiation induced mortality and radiation induced intestinal damage (Jagetia and Baliga, 2003; Jagetia and Baliga, 2003; Jagetia et al., 2005; Jagetia et al., 2008). However a comprehensive antioxidant activity evaluation of fruit based on anatomical parts, and fractions have not been reported

1.10.2 Anticancer activity

Detailed literature survey showed that no much works has been reported regarding anticancer potency of *S. cumini*. The only available lead is the report on cytotoxic potential of *S.cumini* pulp. The report says that pulp extract at higher concentration is capable of retarding the proliferation of several cancer cells such as MCF-7, HeLa, HEP G2, HL60 and U251in vitro (Nazif, 2007). However no attempts have been reported thereafter to understand the nature of active principle in pulp or the potency of other parts of the plant or fruit. Therefore further studies are required to evaluate the anticancer potency of *S. cumini* fruit.

1.10.3 Antidiabetic activity

Reports on anti diabetic properties of *S. cumini* fruit and its anatomical parts are summarized below. Studies conducted in type 2 diabetic human volunteers showed that administration of whole *S. cumini* fruit extract decreased serum glucose level significantly in a dose dependent manner (Safdar et al., 2006). Several studies reported the antidiabetic potential of the anatomical parts of fruit. Water and alcohol extracts of edible portion of fruit (pulp) were reported to have antihyperglycemic activity (Pepato et al., 2005; Sharma et al., 2006). There is more number of reports on the antidiabetic properties of seed than that of pulp. *S. cumini* seed aqueous extract has been studied for the capacity to ameliorate glucose metabolizing enzymes in alloxan induced diabetic rats (Prince et al., 1997). In STZ induced diabetic rats also, methanol extract of kernel ameliorated glucose metabolism evidenced by recovery in the activities of hexokinase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activity in liver besides normalizing fasting blood glucose, liver and skeletal muscle glycogen level and plasma insulin level (Mallick et al., 2006). In another advanced study, diabetic and normal rats were fed with the diet containing 15% seed powder, 15%

defatted seed powder and 6% water soluble gummy fiber isolated from seed (Pandey and Khan, 2002). The treatment with 6% water soluble gummy fiber significantly lowered blood glucose level and showed improved oral glucose tolerance test. Whereas feeding diet containing 15% powdered degummed seeds, 2.25% water insoluble neutral detergent fiber neither lowered blood glucose level nor improved oral glucose tolerance test. Based on this data, authors suggested that in vivo hypoglycemic effect might be due to the fraction containing gummy fiber. *S. cumini* bark was reported to have anti-diabetic activity substantiated with positive oral glucose tolerance test in mouse model (Villasen?or and Lamadrid, 2006) and STZ induced diabetic rat model (Saravanan and Pari, 2008). Leaves also reported to have hypoglycemic effect (Damasceno et al., 2002) but, seed coat does not have antidiabetic activity (Ravi et al., 2004). It is generally observed that antidiabetic activity of *S. cumini* is mainly found in seed alcohol extract (Prince et al., 2004; Ravi et al., 2003; Sharma et al., 2003; Singh and Gupta, 2007; Sridhar et al., 2005; Srivastava et al., 1983; Teixeira et al., 2004). Flavonoid rich extracts prepared from *S. cumini* seed alcohol extract was found to have comparatively better hypoglycemic activity than that of the kernel crude methanol extract (Sharma et al., 2008; Sharma et al., 2008). Administration of lyophilized S.cumini plant powder has been reported to have antihyperglycemic effect in STZ induced diabetic rat (Grover et al., 2000). Apart from antihyperglycemic effect, the whole plant is known to reduce renal hypertrophy and urinary albumin level in STZ induced diabetic rat models (Grover et al., 2001). It has been reported that feeding rats with 400 mg plant extract/day for 15 days prevented hyperglycemia and hyperinsulinemia induced by high fructose diet (Vikrant et al., 2001). Hypoglycemic effect of plant extract has also been shown in STZ induced diabetic rats (Grover et al., 2002; Grover et al., 2002). Advanced molecular studies showed that methanol extract of *S. cumini* plant modulate the expression of glucose transporter (Glut-4), peroxisome proliferator activator receptor gamma (PPARγ) and phosphatidylinositol-3-kinase (PI3 kinase) comparable with insulin and rosiglitazone (Anandharajan et al., 2006). Inorganic contents such as Zn, Cr, V, K and Na in *S. cumini* seed has been reported to exhibit normoglycemia and better glucose tolerance in STZ induced diabetic rats (Ravi et al., 2004).

S. cumini containing herbomineral formulations viz. "hyponidd", "Dianex", "Diakyur", have been reported to exhibit anti-hyperglycemic activity in a double blind placebo controlled studies (Babu and Prince, 2004; Joshi et al., 2007; Mutalik et al., 2005). Evaluation of *S. cumini* containing anti-diabetic poly herbal formulation in alloxan induced diabetic rats also showed significant hypoglycemic activity, positive glucose tolerance activity and reduced lipid peroxidation in various organs compared to that of the diabetic control animals (Joshi et al., 2007). Another study that evaluated four classical antidiabetic formulations, all of them contained *S. cumini* as one of many ingredients, and reported that all formulations exhibited significant hypoglycemic activity (Rafeeuddin et al., 2004).

Absence of antidiabetic activity was also reported. Oleveira et al. evaluated crude ethanol extract, water and butanol fractions of *S. cumini* leaves and reported no significant reduction in glycemia compared to that of control animals (Oliveira et al., 2005). Absence of antihyperglycemic activity of *S.cumini* leaf has also been reported by several authors (Pepato et al., 2001; Teixeira et al., 2006; Teixeira et al., 1997; Teixeira et al., 2000).

1.10.4 Other therapeutically significant properties of *S. cumini* fruit

Other therapeutically significant properties of *S. cumini* plant parts have also been reported. *S. cumini* pulp is shown to have gastro protective and anti-ulcerative activity (Chaturvedi et al., 2007; Ramirez and Roa Jr, 2003). Central nerve system stimulation activity by the alcohol extract of *S. cumini* seed were also reported (Kumar et al., 2007). Anti human immuno deficiency virus (HIV) type-1 protease inhibitor activity was observed in the *S. cumini* bark extract (Kusumoto et al., 1995). *S. cumini* plant extract also reported to have hypotensive and diuretic effect (Cirqueira and Alves, 2005). Complications associated with diabetes such as hyperlipidemia has been reversed to normal level in the STZ induced diabetic rat treated with *S. cumini* seed extract (Mallick et al., 2006; Prince et al., 2004; Prince and Menon, 1997; Ravi et al., 2005; Safdar et al., 2006; Sharma et al., 2008; Sharma et al., 2003). *S. cumini* bark extract has been known to have mutagenic effect in cultured human leukocytes in vitro by reduced mitotic index and

chromosomal aberrations (Mendioro et al., 2001)

S. cumini was found to have anti-inflammatory activity. Chloroform extract of *S. cumini* seeds was reported to inhibit carrageenan, kaolin and other mediator induced oedema in experimental animals. The extract also inhibited potency to inhibit inflammation, migration of leucocytes, granuloma formation (Chaudhuri et al., 1990). Another recent study also reported the anti-inflammatory activity of *S. cumini* seed alcohol extract on carrageenan induced rat paw oedema (Kumar et al., 2008). Ethanol extract of *S. cumini* bark exhibited significant anti-inflammatory activity in acute (carrageenan), sub acute (Kaolin-carrageenan) and chronic (cotton pellet granulation) inflammatory models in experimental animals (Muruganandan et al., 2001) (Muruganandan et al., 2002). *S. cumini* leaves also reported to have anti-inflammatory activities in animal models (Brito et al., 2007; Lima et al., 2007).

The whole plant is reported to have antileishamial and antifungal activity (Braga et al., 2007). Methanol and water extracts of *S. cumini* seed were found to inhibit growth of several species of gram positive, gram negative bacteria and fungus (Chandrasekaran and Venkatesalu, 2004; Deshpande et al., 2005). *S. cumini* leaf was reported to have better inhibitory activity against many multidrug resistant bacterial species (De Oliveira et al., 2007). Tincture prepared from the bark of *S. cumini* plant was reported to have strong fungicidal activity (Dutta et al., 2000). Shafi et al. reported the anti bacterial activity of *S. cumini* essential oil (Shafi et al., 2002). Vibriocidal activity of *S. cumini* bark and responsible active principles have also been reported recently (Sharma et al., 2008)

1.10.5 Phytochemical profiling of *S.cumini* fruit

S. cumini fruit is reported to contain a broad spectrum of phytochemicals which are belongs to the phytochemical class such as flavonoids, coumarins terpenoids etc. a comprehensive data recompiling and “napraalert” search is presented in Table 1-4

Table 1-4 Phytochemical profiling of *S. cumini* fruit

Compound isolated	Class	Source	Region	Reference
Chrysanthemin	Flavonoid	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Citric acid	Alkane	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Fructose	Carbohydrate	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Glucose	Carbohydrate	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Malic acid	Alkane to c4	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Mannose	Carbohydrate	Fruit	Brazil	(Bobbio-adilma and Scamparini, 1982)
Delphinidin-3-o-beta-d-gentiobiosid	Flavonoid	Fruit	India	(Jain and Seshadri, 1975)
Malvidin-3-o-beta-d-laminaribioside	Flavonoid	Fruit	India	(Jain and Seshadri, 1975)
Petunidin-3-o-beta-d-gentiobioside	Flavonoid	Fruit	India	(Jain and Seshadri, 1975)
Cinnamaldehyde,cis	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Cinnamaldehyde,trans	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Cinnamyl acetate,cis	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Cinnamyl acetate,trans	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)

Cinnamyl alcohol,cis	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Cinnamyl alcohol,trans	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Citronellol	Monoterpene	Fruit	France	(Vernin et al., 1991)
Geraniol	Monoterpene	Fruit	France	(Vernin et al., 1991)
Herol oxide	Monoterpene	Fruit	France	(Vernin et al., 1991)
Hotrienol	Monoterpene	Fruit	France	(Vernin et al., 1991)
Linalool	Monoterpene	Fruit	France	(Vernin et al., 1991)
Linalool oxide	Monoterpene	Fruit	France	(Vernin et al., 1991)
Nerol	Monoterpene	Fruit	France	(Vernin et al., 1991)
Phenylethanol,beta	Benzenoid	Fruit	France	(Vernin et al., 1991)
Phenylpropanal,3	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Phenylpropanol,3	Phenylpropanoid	Fruit	France	(Vernin et al., 1991)
Roes oxide	Oxygen heterocycle	Fruit	France	(Vernin et al., 1991)
Corilagin	Tannin	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Diphenic acid,hexahydroxy	Benzenoid	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Ellagic acid,3-3'-4-tri-o-methyl	Coumarin	Seed	Not stated	(BHATIA and BAJAJ, 1975)

Ellagic acid,3-3'-di-o-methyl	Coumarin	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Glucose,1-galloyl	Tannin	Seed		(BHATIA and BAJAJ, 1975)
Glucose,3-6-hexahydroxy-diphenoyl:	Benzenoid	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Glucose,3-galloyl	Benzenoid	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Glucose,4-6-hexahydroxy-diphenoyl	Tannin	Seed	Not stated	(BHATIA and BAJAJ, 1975)
Quercetin	Flavonol	Seed	Not stated	(Bhatia and Bajaj, 1975)
Taxifolin	Flavonoid	Seed	Not stated	(Bhatia and Bajaj, 1975)
Gallic acid	Benzenoid	Seed	Brazil	(De lima et al., 1998)
Coniferyl alcohol	Phenylpropanoid	Seed	Philippines	(Martin et al., 1998)
Furfural,5-(hydroxy-methyl)	Oxygen heterocycle	Seed	Philippines	(Martin et al., 1998)
Medioresinol-4''-o-beta-glucoside	Lignan	Seed	Philippines	(Martin et al., 1998)
Pinoresinol-o-beta-glucoside,(+)	Lignan	Seed	Philippines	(Martin et al., 1998)
Syringaresinol-o-beta-glucoside,(+)	Lignan	Seed	Philippines	(Martin et al., 1998)

Ellagic acid	Coumarin	Seed	India	(Steinmetz, 1961)
Jambolin structure unknown		Seed	India	(Steinmetz, 1961)
Lectin(eugenia jambos)	Proteid	Seed	Honduras	(Tabora and De Bertrand, 1980)
Elaeostearic acid	Lipid	Seed	India	(Das and Banerjee, 1995)
Lauric acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Linoleic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Malvalic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Myristic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Oleic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Palmitic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Stearic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Sterculic acid	Lipid	Seed	India	(Daulatabad et al., 1988)
Vernolic acid	Lipid	Seed	India	(Daulatabad et al., 1988)

1.11 Relevance and objectives of the present study

Epidemiological studies show a positive correlation between consumption of fruits and vegetables and delayed onset and progression of degenerative diseases. Therefore the plant-based healthcare is getting more popular. Recent evidences in terms of growing related literature volume and demand for plant based healthcare products also establish the importance. However many plants that are traditionally known to have prophylactic or therapeutic properties are not scientifically validated and hence they are not standardized and validated plant based healthcare products with chemopreventive or therapeutic properties. This demand scientific investigation on traditionally used plants to validate known therapeutic properties and identification of active principles/fractions.

The phytochemical content of active fraction are subject to large variations due to variety, age, maturity of the plants used, season, geo-agro-climatic conditions, agronomical practices, post harvest handling, storage, processing etc. The active principle thus can vary tremendously and that in turn would affect the biopotency. It is in this context that chemical profiling of the plants is important to produce products with consistent quality.

The plant selected for the present study, *S. cumini*, is a tropical plant of Myrtacea family, endemic to India, Pakistan, Sri Lanka and Malaysia. Traditionally the plant products have been used in the treatment of diabetes and various other ailments. Prophylactic properties of the fruit and fruit parts have been reported by various authors but, most of the studies limited to crude extracts. No comprehensive evaluation is reported to validate bioactivity of fruit following modern methods and modern instrumental analyses.

Though only kernel has traditionally been used in the treatment of diabetes, recent studied reported the antidiabetic activity in whole plant and other parts viz. leaf, bark, and fruit pulp. Among the several anatomical parts and whole plant studied by various researchers, seed is found to be the most important as evidenced by the volume of data generated by standardized analytical methods. However, the major

literature gap found in the literature study is the lack of knowledge about the active principle or purified active fraction associated with antidiabetic or anticancer properties.

Study of traditional as well as modern literature showed no indications about the anticancer properties of *S. cumini* fruit. However a recent study evaluated the cytotoxic potential of edible portion of SCF in a variety of cancer cell lines. But, no information is available till date about the cytotoxic potential of other anatomical parts of fruit.

Based on the literature gap as well as extending the possibility of SCF for prophylactic and therapeutic applications, the objectives of present study therefore were framed to investigate the chemical composition of SCF , detailed antioxidant activity mapping , antidiabetic and anticancer activity study and subsequent activity guided fractionation. The major objectives undertaken by this study were the followings:

1. **Proximate and detailed phenolic composition analysis of *S. cumini* fruit**
2. **Prophylactic property evaluation of fruit:**
    a. **Preliminary in vitro antioxidant activity evaluation anatomically distinct parts of fruit.**
    b. **Antioxidant activity guided fractionation of fruit.**
3. **Antidiabetic activity evaluation of SCF:**
    a. **Preliminary in vitro anti diabetic activity evaluation of anatomically distinct parts of fruit using cell culture.**
    b. **Activity guided fractionation of seed kernel based on in vitro cell culture assay for diabetes**
4. **Anticancer activity evaluation of SCF**
    a. **Preliminary in vitro cytotoxic activity evaluation of anatomically distinct parts of fruit using cancer cell lines**
    b. **Activity guided fractionation of fruit based on in vitro cell culture assay and various fractionation for cancer**
5. **Data analyses:**
    a. **Association of antioxidant activity and antidiabetic/cancer.**

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemical composition analyses of SCF.

2.1.1 Materials required

HPLC grade methanol, water, acetic acid, C18- reverse phase column etc. were obtained from Merck chemicals, Bangalore. Biochemical standards such as quinic acid (QA), gallic acid (GA), dihydroxybenzoic acid (DBA), *p*-hydroxybenzoic acid (PHBA), chlorogenic acid (ClA), caffeic acid (CA), *p*-coumaric acid (PCA), ferulic acid (FA), sinapic acid (SA), quercetin (Qtn), kaempferol (Kmp) etc. were sourced from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade chemicals viz. Dinitrophenylhydrazine, narengenin, methanol, potassium hydroxide, aluminium chloride, potassium chloride buffer (pH 1.0 and 0.4 M), sodium acetate buffer (pH 4.5), Folins-Ciocalteu reagent, $CaCl_2$, NaCl, KCl and $FeCl_3$, ammonium vanadate-molybdate, anthrone, thiourea, perchloric acid, D-glucose, ethyl alcohol, components of Kjeldahl digestion mixture, boric acid, chloroform, sodium sulphate etc. were procured from SD fine chemicals, India. Other instruments used for chemical composition analyses were; UV-Vis spectrophotometer (Shimadzu), analytical HPLC with binary gradient pump and photodiode array detector (Shimadzu), tissue homogenizer, laboratory blender, Kjeldahl apparatus, vacuum rotary evaporator etc.

2.1.2 Collection of *S. cumini* fruit.

Fresh and fully ripened *S. cumini* fruits were collected from Thiruvananthapuram district of Kerala, India. The fruits were graded based on maturation and wrinkled fruits were discarded. Fully matured and ripened fruits were then immediately shifted to lab. The fruits were washed and dried using an air blower. Fully dried and undamaged fruits were then sorted and packed in polypropylene bag. The sealed bags were stored at -20°C for future use.

2.1.3 Preparation of samples for composition analyses.

The sample preparation scheme for composition analysis and antioxidant studies is depicted in Fig. 2-1. Anatomically distinct part of fruit such as pulp, kernel and seed coat were separated manually and prepared for composition

analysis. Methanol extracts were prepared from the three anatomical parts for composition analysis. Methanol extraction was performed for each part with a material solvent ratio of 1:2 for four hours, five times. The extracts were pooled and suspended particles were removed by centrifugation (7500g for 10 min) followed by filtration. The pulp methanol extract (PM), kernel methanol extract (KM) and seed coat methanol extract (SCM) thus obtained were concentrated to appropriate strength using rotary evaporator at 45°C (Heidolf rotavac) under the pressure of -600 mm Hg. The residual parts namely residual pulp (RP), residual kernel (RK) and residual seed coat (RSC) obtained after extraction were dried in shade and subjected to composition analysis.

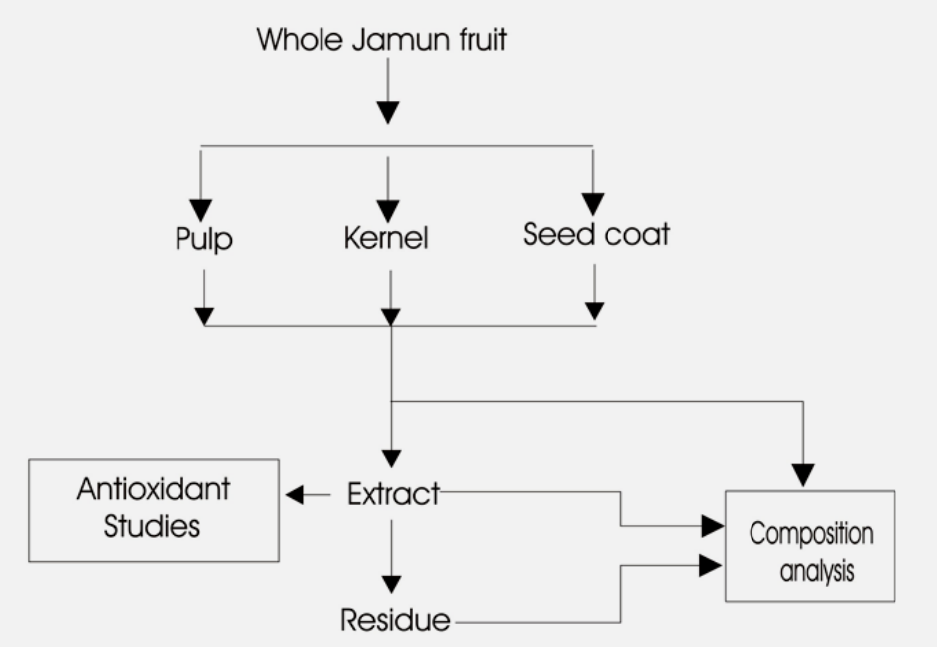

Fig. 2-1Scheme for separation and extraction of anatomical parts of SCF, for composition analysis.

2.1.4 Gravimetric estimation of anatomical components of SCF

Anatomical composition of fresh SCF was estimated gravimetrically. 50 g of fully ripened fruits with standard size were weighed and the pulp and seed was separated manually. The seed coat was then separated from seed by gently scratching on a wire mesh. The fresh pulp, seed coat and kernel thus prepared were quantified gravimetrically.

2.1.5 Estimation of moisture

Moisture content of fruit parts were estimated by AOAC method (No. 930.15). 10 g of fruit parts were weighed separately in pre weighed glass Petri dishes and dried in hot air oven at 80-95^{o}C for 3 hours. The dishes were then transferred to desiccator, cooled to room temperature and recorded the weight. The drying was continued till the final weight become stable. The weight differences were noted and the moisture contents were expressed in percentage weight of fresh fruit parts (Horowitz, 2000).

2.1.6 Estimation of total fatty matter (TFM)

Total fatty matter (TFM), including polar and nonpolar lipids, was estimated by Bligh's method (Bligh and Dyer, 1959). Fresh fruit parts (50g) were ground well in a tissue homogenizer with 100 ml methanol, 100 ml chloroform and 50 ml water. The homogenate was centrifuged (4000 x g, 25^{o}C) and supernatant was collected separately. The pooled supernatant was then transferred to a separating funnel and the chloroform layer was collected in a beaker and dehydrated with anhydrous sodium sulphate. Dehydrated chloroform fraction was then desolventized using vacuum thin film rotary evaporator and the amount of lipid was quantified gravimetrically.

2.1.7 Estimation of crude proteins

Total protein content of fruit parts were estimated indirectly based on total nitrogen content by Kjeldahl method, AOAC No.988.01 (Horowitz, 2000). Dried fruit samples (1.0g) were digested in a Kjeldahl's flask containing 0.5g digestion mixture (2.5g SeO_2, 100g K_2SO_4, 20g $CuSO_4$) and 10 ml of con H_2SO_4 under heating condition. The digested samples were made up to 100 ml is standard measuring flask. The digested sample (5 ml) with 20ml of 40% NaOH and 10 ml water was loaded into the Kjeldahl apparatus and steam was allowed to bubble through it to release ammonia. The released ammonia was trapped in a beaker containing 20 ml boric acid and 1 ml mixed indicator (bromocresol green and methyl red). Entrapment of ammonia was continued for 20 min. The blue colored ammonia trapped boric acid was titrated with 0.1N HCl till the disappearance of blue color. Crude protein content was calculated by multiplying the % nitrogen

(calculated from titer value) in the sample with the factor 6.25.

2.1.8 Estimation of total soluble carbohydrates

Total soluble carbohydrate in fruit part was estimated by anthrone method (Roe, 2000). 2.0 g of defatted and dried fruit parts were ground separately and refluxed with 20.0 ml of 80% ethyl alcohol for 6.0 h. The extracts were filtered and made up to 25 ml. The samples (0.2 and 0.4 ml) and standard glucose (0.2-1.0 ml from 0.1mg/ml D-glucose stock) were taken in a series of test tube and the volume was made up to 1.0 ml. A reagent blank was prepared by taking 1.0 ml water alone. 4.0 ml of anthrone reagent (0.5g anthrone and10g thiourea, in 1L 66% H_2SO_4) was added to all test tubes and heated in a 90^{o}C water bath for 15 min. The blue color developed was read at 620 nm in a UV-Vis spectrophotometer against the reagent blank. The amounts of soluble carbohydrates in samples were calculated from the glucose standard curve.

2.1.9 Estimation of starch

Sugar removed (soluble) fruit parts were washed several times in 80% ethanol. The residues were then treated with 10 ml water and 15 ml perchloric acid and extracted for 20 min. Extraction was repeated 3 times with fresh water and perchloric acid. The extracts were pooled and made up to 100 ml. Carbohydrate content in the extracts were quantified by anthrone method using glucose calibration curve. Starch was quantified by multiplying the result with factor 0.9 (Hodg and Hofreiter, 1962; Thayumanavan and Sadasivam, 1984).

2.1.10 Estimation of crude fiber

Crude fiber from fruit parts were estimated by consecutive acid and alkali digestion. 5.0 g of defatted and dried fruit parts were mixed with 200 ml of 1.25% sulfuric acid and boiled for 30 min. The contents were filtered and residue was washed several times in boiling distilled water to remove traces of acid. Then the residue was treated with 200 ml of 1.25% boiling NaOH for 30 min. The digest was filtered to obtain the residue. This was washed in boiling water and finally with ethanol. The washed residue was dried in an oven at 130ºC to constant weight and cooled in desiccators. The residue was scraped into a pre–weighed

porcelain crucible, weighed, ashed at 550ºC for two hours, cooled in a desiccator and reweighed. Crude fiber content was expressed as percentage loss in weight on ignition (Triebold and Aurand, 1982).

2.1.11 Estimation of ash and mineral composition (Ca, Na, K, P and Fe)

Total inorganic material in fruit parts were estimated by AOAC method (AOAC, 1990). The minerals viz. Ca, Na, K and Fe contents in fruit parts were estimated by flame photometry (AOAC, 1975) using Systronics-128 flame photometer. Standard solutions of $CaCl_2$, NaCl, KCl and $FeCl_3$ were used at 10 ppm levels. The phosphorus was analyzed photometrically by ammonium vanadate-molybdate method (AOAC, 1991).

2.1.12 Estimation of total phenolic content.

Total phenolic composition was determined using Folins-Ciocalteu reagent and expressed as gallic acid equivalent (GAE) (Jayasinghe et al., 2003). The samples and standard gallic acid were appropriately diluted in 2.0 ml distilled water and 2.0 ml of 2.0 N Folins-Ciocalteu reagent was added. The contents were mixed well and kept for 5 min at room temperature; followed by 2.0 ml of 10% aqueous sodium carbonate was added and incubated at room temperature for 1 h. Absorbance of the developed blue color was read at 760 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against a reagent blank.

2.1.13 Estimation of total anthocyanins.

Anthocyanin content of the whole fruit was extracted with acidic methanol (0.1% HCL) (Giusti et al., 1999). Total monomeric anthocyanins in the extract was estimated by the pH differential method and expressed in cyandin-3-glucoside equivalency; where MW of cyaniding-3-glucoside is 449.2 and molar extinction coefficient is 26900 (Wrolstad et al., 1970). A known volume of extracted anthocyanin was made up to 50.0 ml using 0.025 M potassium chloride buffer, pH 1.0 and 0.4 M sodium acetate buffer, pH 4.5; separately. The buffered anthocyanin extract was allowed to equilibrate for 15 min at room temperature. Absorbance of each buffered sample was measured at 520 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against a blank cell with distilled

water. The concentration of monomeric anthocyanin pigment in final solution was calculated by the formula.

$$\text{Monomeric anthocyanin pigment (mg/L)} = \frac{(\text{A x MW x DF x } 1000)}{(\varepsilon \text{ x } 1)}$$

A, absorbance; MW, molecular weight; DF, dilution factor; ε, molar extinction coefficient.

2.1.14 Estimation of total flavonoids.

Quantitative determination of flavonoids was performed by two complementary colorimetric methods viz. aluminium chloride method and 2, 4-dinitro phenyl hydrazine method (2, 4-DNPH). For the quantitative estimation of total flavonoids in the whole Jamun fruit, the extraction procedure described by Chia Chi Chang et al. was performed (Chang et al., 2002).

Aluminium chloride method: Ten to 100 µg/ml of quercetin standard and appropriately diluted samples in 80% ethanol were taken in different test tubes (0.5 ml) and made up to 2 ml with 95% ethanol followed by the addition of 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and incubated in room temperature (30-34°C) for 30 min. The intensity of color developed was read at 415 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against reagent blank.

Dinitrophenyl hydrazine method: The reference standard used in this assay was narengenin. Then 2.0 ml of 1% 2,4-Dinitrophenylhydrazine reagent and 2.0 ml of methanol were added to the reaction system and the constituents were mixed thoroughly. The tubes were stoppered and incubated at 50°C for 50 min in a constant temperature water bath. After incubation the tubes were cooled and added 5.0 ml of 1% potassium hydroxide in 70% Hundred to thousand micro grams of appropriately diluted standard and samples were taken in separate test tubes and the volume was made up to 4.0 ml with methanol and kept at room temperature (30-34°C) for 2 min. Finally 1.0 ml of the reaction mixture was taken from each tube and mixed with 5.0 ml methanol. The precipitates formed were removed by centrifugation at 7500g for 10 min. The supernatant was collected

and adjusted to 25.0 ml and the absorbance of the final solution was measured at 415 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against the blank.

Total flavonoid was expressed as the sum of % flavonoid obtained in each method.

2.1.15 Phenolic composition analyses of SCF by HPLC.

Preparation of sample: For HPLC profiling of SCF phenolic compounds, methanol extracts from three anatomically distinct parts were prepared as described in section 2.1.1 and Fig. 2-1. The methanol extracts (PM, KM and SCM) thus prepared were clarified by low speed centrifugation followed by filtration through 0.4 µ syringe filter before loading in HPLC system. The final concentrations of the samples were set at 10 µg ml on the basis of total solids.

Preparation of standards: Stock standard solution of all 12 reference standards viz. quinic acid (QA), gallic acid (GA), dihydroxybenzoic acid (DBA), *p*-hydroxybenzoic acid (PHBA), chlorogenic acid (ClA), caffeic acid (CA), *p*-coumaric acid PCA, ferulic acid (FA), sinapic acid (SA), quercetin (Qtn), and kaempferol (Kmp) were prepared in HPLC grade methanol at 1 mg/ml concentration. All stock standards were filtered through 0.4 µ PTFE membrane before injection.

HPLC conditions: HPLC analyses were carried out in a binary gradient HPLC system equipped with C18 ODS column (Phenomenex, 5 µ, 250 x 4.6 mm dia). The HPLC system details are as follows; Pump, LC-8A (high pressure binary gradient system); detector, SPD-10A photodiode array. HPLC conditions for the analyses were; Solvent A, methanol; solvent B, 2% acetic acid; total flow rate, 1 ml/min; gradient condition: 0-5 min, 100% B; 5-100 min, 100% A (linear gradient); 100-140 min 100% A; post run, 100% B (20 min); PDA wavelength range, 200-450 nm. All solvents used were HPLC grade. Samples were filtered through a 0.4 µ syringe filter and 20 µl of the sample was injected into the system. Data acquisition and analyses were done using ClassVP LC software.

Calibration and preparation of dose response curve: For calibration purpose, a standard dose response curve was developed using reference standards. Working

stock solution of each standard was prepared in 10 different concentrations (2 to 20 µg/ml, step 2) and 20 µl from each working stock was injected in HPLC system. The chromatogram retention time and dose dependent peak area of each standard was optimized individually. For each standard, corresponding λ max was used to pick retention time and area under curve.

Preparation of spectral library and spectral correlation analysis: UV-Vis. Spectra of each standard compound, under the gradient mobile phase, was recorded using photo diode array detector and created a spectral library of all standard compounds used in the study. This Spectral library was used for identification of peaks in the sample using retention time and spectral correlation analysis

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2.2 Antioxidant activity evaluation of SCF

2.2.1 Materials required

Antioxidant reference standards such as gallic acid, catechin, Trolox, vitamin C, BHT, quercetin and reagents for antioxidant assay viz. $DPPH^{\bullet}$, xanthine, xanthine oxidase, nitro-blue tetrazolium (NBT), tertiary butyl hydroperoxide, thiobarbituric acid etc. were purchased from Merck, India, Other chemicals, reagents and solvents such as potassium ferricyanide, ferric chloride, ferrous ammonium sulfate, EDTA, ethanol, phosphate buffered dimethyl sulphoxide, trichloroacetic acid, nash reagent, heparin, isotonic saline, sodium hydroxide, phosphate buffer etc.were procured from SD fine chemicals. Red blood cell suspension was prepared in lab.

2.2.2 Sample preparation

Preliminary antioxidant activity of SCF was evaluated on methanol extracts of fruit parts. The sample preparation details are presented in section 2.1.3 (Fig. 2-1).

2.2.3 DPPH radical scavenging activity.

DPPH radical scavenging activity was estimated according to the method of

Brand-William (Brand-Williams et al., 1995). 2.9 ml of 0.1 mM DPPH in ethanol and 0.1 ml of various concentrations of extracts and standards (gallic acid, catechin, Trolox etc) in the same solvent were taken in a glass cuvette. The contents were mixed well immediately and the degree of reduction of absorbance was recorded for 30 min in an UV Visible spectrophotometer at 517 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.). Optical densities at time zero (OD t_0) and at 30th min (OD t_{30}) were used for calculating percentage radical scavenging activity. Percentage radical scavenging activity was plotted against the corresponding antioxidant substance concentration to get IC50 values.

2.2.4 Superoxide radical scavenging activity.

Superoxide radical scavenging activity study was performed according to the method of Parejo et al. using Xanthine-Xanthine oxidase system (Parejo et al., 2002). 50-250 µg of appropriately diluted samples and standards viz. catechin and Trolox and were taken in a 1.0 ml cuvette separately and added xanthine at a final concentration of 0.2 mM. 63.0 µL of 1.0 mM nitro-blue tetrazolium (NBT) was added to the reaction system and the final volume was made up to 1.0 ml with phosphate buffer (50 mM, pH 7.5) excluding the volume of enzyme. 63.0 µL of xanthine oxidase enzyme (1.2 U/µL) was added to the system and mixed well to start the reaction. The blue color developed by the reduction of NBT by superoxide radicals were measured at 560 nm for 15 min in a spectrophotometer (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.). A blank was prepared without sample and standards which was considered as 100% radical production. A decrease in NBT reduction in the presence of added antioxidant extract and standard compounds were monitored and % radical scavenging activity (RSA) was calculated by the following formula.

$$\%RSA = (1 - \frac{OD\ of\ sample\ or\ standard}{blank\ OD}) \times 100$$

2.2.5 Hydroxyl radical scavenging activity.

Hydroxyl radical scavenging activity was studied according to the method of Klein et al. (Klein et al., 1991). Different concentrations of appropriately diluted extracts and standards viz. vitamin C, BHT, gallic acid, Trolox, catechin and

quercetin were taken in a series of test tubes and added the following reagents. 1.0 ml iron EDTA solution (0.13% ferrous ammonium sulfate and 0.2% EDTA), 0.5 ml EDTA (0.018%) and 1.0 ml phosphate buffered dimethyl sulphoxide (DMSO) (0.855% DMSO in 0.1mM phosphate buffer, pH 7.4. v/v). The contents were mixed well and the reaction was started by adding 0.5 ml 0.22% ascorbic acid. All tubes were closed and heated in a constant temperature water bath at 90º C for 15 min. The reaction was stopped by adding 1.0 ml 17.5% ice cold trichloroacetic acid (TCA). Finally 3.0 ml of Nash reagent (75.0 g ammonium acetate, 3.0 ml glacial acetic acid and 2.0 ml acetyl acetone were mixed and made up to 1.0 L with distilled water) was added and kept at room temperature (30-34º C) for 15 min to develop color. The yellow color developed was read at 412 nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against reagent blank. Percentage of radical scavenging activity was calculated by measuring decrease in optical density in the presence of added radical scavenger with reference to blank.

2.2.6 Inhibition of lipid peroxidation.

Inhibition of lipid peroxidation was assessed using red blood cell model system as described by Manna et al. (Manna et al., 2002). Heparinized whole blood was collected from healthy volunteers. The blood was centrifuged for 10 min at 1000g to separate plasma and red blood cells (RBCs). After removing plasma and Buffy coat, the packed RBCs were resuspended in isotonic saline and washed several times to remove plasma protein. Finally the RBCs were resuspended to a final concentration of 5% (v/v) in isotonic saline. The assay system contains a final strength of 2% RBC suspension, appropriately diluted extract and 500 µM *t*-BHP. The final volume was made up to 5.0 ml with isotonic saline and incubated at 37º C in a water bath for 2 h. After oxidative treatment, the tubes were centrifuged at 1000g for 10 min to separate RBCs. Two ml of the cell free supernatant was collected and mixed with 1.0 ml of 30 % (w/v) trichloroacetic acid. The tubes were gently mixed and further centrifuged for 15 min at 5000g. Two ml of the supernatant was collected and added 0.5 ml 1% (w/v) thiobarbituric acid (TBA) in 0.05 N NaOH. The mixture was heated in a boiling water bath for 10 min to develop color. The absorbance of pink chromogen developed was read at 532 nm

(Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against a reagent blank. Percentage reduction of pink color (inhibition of lipid peroxidation) in the presence of added standard antioxidants and samples with reference to blank was plotted against the concentration to get IC50 values

2.2.7 Total reducing power.

Total reducing power was estimated based on the method reported by Zhu et al. (Zhu et al., 2002). The samples and standards (100-500 µg dry weight) were dissolved in 1.0 ml of water; to this, 2.5 ml of phosphate buffer (0.2mM pH. 6.6) and 2.5 ml of 1% potassium ferricyanide were added. The reaction system was closed and incubated at 50^{o} C in a water bath for 30 min. After the incubation period, 2.5 ml 10% TCA was added to the assay system and the contents were mixed well. The mixture was centrifuged at 3000x g for 30 min to remove precipitate. 2.5 ml supernatant was collected and mixed with 2.5 ml of distilled water and 0.5 ml 0.1% ferric chloride. The color developed was read at 700nm (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) against a reagent blank. The color developed is directly proportional to reducing power of samples

2.3 Sequential extraction of SCF for antioxidant activity evaluation

2.3.1 Sample preparation

The fruits were separated into anatomically distinct parts viz. pulp, kernel, and seed coat. The separated fruit parts were freeze-dried and ground to an average particle size that ranged from 0.01 to 0.1 mm^3. The powdered fruit parts were extracted with different solvent of increasing polarity viz. hexane, dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH) and water sequentially in the order of increasing polarity. The extraction using each solvent was done five times at a material solvent ratio of 1:5 (w/v). After each solvent extraction, the residual solvent in the raw material was removed by evaporating at

55°C, under low pressure (-700mm Hg) in a vacuum oven. The extracts obtained from each solvent were filtered (Whatman No.1filter paper), pooled and concentrated to an appropriate strength using a rotary evaporator at 55°C, -700 mm Hg. Extraction of three anatomically distinct part of the fruit using different solvent yielded the following extracts viz. pulp hexane (PHs), pulp DCM (PDs), pulp EtOAc (PEs), pulp MeOH (PMs), pulp water (PWs), kernel hexane (KHs), kernel DCM (KDs), kernel EtOAc (KEs), kernel MeOH (KMs), kernel water (KWs), seed coat hexane (SCHs), seed coat DCM (SCDs), seed coat EtOAc (SCEs) seed coat MeOH (SCMs) and seed coat water (SCWs) (Fig. 2-2).

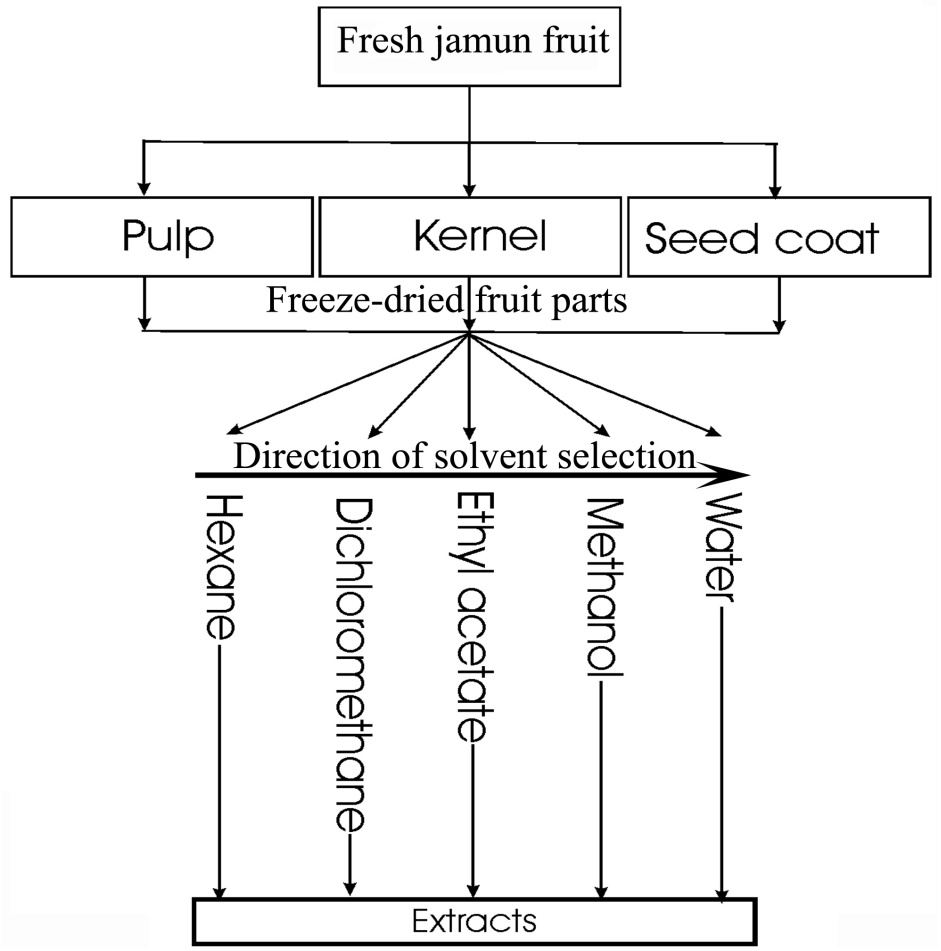

Fig. 2-2 Extraction scheme of anatomically distinct parts of Jamun fruit using different solvents of increasing polarity.

2.3.2 Quantification of yield of extracts, TPC, flavonoids and anthocyanins

Yield of all extracts were quantified by gravimetric methods (Sec. 2.1.5). Total

phenolic content, flavonoids and cyaniding-3-glucoside equivalent anthocyanin were estimated by the methods mentioned in sections 2.1.12, 2.1.14 and 2.1.13 respectively.

2.3.3 Method used for antioxidant activity studies

Antioxidant evaluation methods described in previous sections were used for estimating DPPH radical scavenging activity (2.2.3), total reducing power (2.2.7), hydroxyl radical scavenging activity (2.2.5), superoxide radical scavenging activity (2.2.4) and inhibition of lipid peroxidation (2.2.6)

2.4 Antioxidant activity guided fractionation of kernel methanol extract (KM)

2.4.1 Preparation of KM

For antioxidant activity guided fractionation, methanol extract was prepared from *S. cumini* kernel. Kernel was freeze-dried and powdered to an average particle size of 0.5mm and extracted with distilled methanol at a substance solvent ratio 1:5 (v/v) under stirring condition for 30 min. The extraction was repeated for 3 times and pooled extracts were concentrated to a final strength of 10 mg/ml using thin film vacuum rotary evaporator.

2.4.2 Liquid-liquid partition of sample

The KM was separated based on polarity of phytochemicals in it by solvent-solvent fractionation. In solvent-solvent fractionation, the aqueous parent extract was fractionated sequentially with various immiscible solvents in the order of increasing polarity. To achieve immiscibility between the parent extract and fractionating solvents, 10g of the parent methanol extract (KM) was desolventized and redissolved in 500 ml of water. Water suspension containing methanol free KM was taken in a separating funnel and partitioned with different immiscible solvents sequentially in the order of increasing polarity as follows; hexane>dichloromethane>ethyl acetate> butanol (**Error! Reference source not**

found.). For each partition, 200 ml of solvent was used and fractionated 3 times. The residue obtained after butanol fractionation was considered as water fraction. The fractions obtained in each partition were pooled and concentrated to an appropriate final strength ranging from 1-10 µg/ml and designated as follows; KH, hexane fraction of KM; KD, dichloromethane fraction of KM; KE, ethyl acetate fraction of KM; KB, butanol fraction of KM; KW, water fraction of KM.

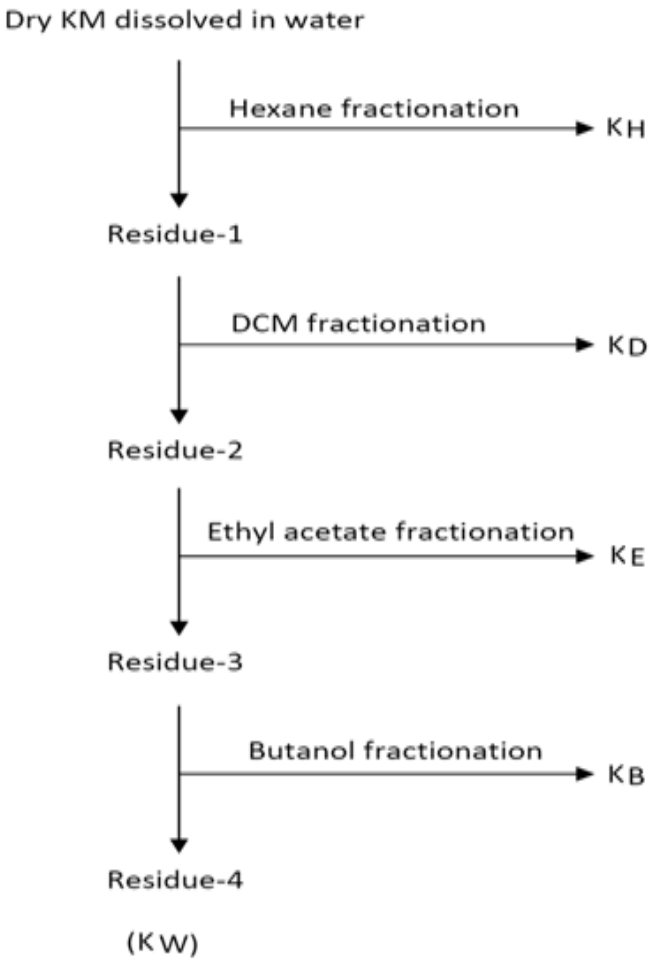

Fig. 2-3 Solvent-Solvent fractionation scheme of *S. cumini* kernel methanol extract using different solvents of increasing polarity sequentially

2.4.3 HPLC fractionation

Ethyl acetate fraction from kernel methanol extract was further fractionated using preparative HPLC equipped with high-pressure binary gradient pump and photodiode array detector. The column specifications and gradient solvent

systems are as follows; Column, Phenomenex C18 preparative, 21x250 mm size, particle size 15 µ. Mobile phase: solvent A, 2% acetic acid; solvent B, methanol. Gradient conditions: 0-5 min A=100%, 5-60 min B=100% (linear gradient), 60-80 min B=100%, 81 min A=100%, 81-100 min A=100, total flow rate: 25 mL/min. About 100 mg of KE in 5 ml was loaded into the column and the fractions were collected based on the chromatogram using the programmable fraction collector.

2.5 Modulation of DNA integrity in Fenton's system by KM

2.5.1 Materials

Phytochemical standards such as catechin, apigenin, naringin, naringenin, vitamin C, gallic acid etc. were procured from Sigma-Adrich. Lambda DNA and ethidium bromide were sourced from Bangalore Genei, Bangalore. Ferrous chloride, ferrozine, hydrogen peroxide, dimethyl formamide, tris buffer and agarose gel were purchased from SD fine chemicals, India. Samples viz. methanol extracts of *Syzygium cumini* kernel, *Hipaphea rahmnoides* kernel, *Punica granatum* pericarp were prepared in laboratory. Important instruments used were; horizontal gel electrophoresis system with switched mode power supply, gel documentation system with 254 and 360 nm illumination source and high resolution digital camera. The software used for image processing and quantification was "ImageJ", kindly provided by NIH.

2.5.2 Sample preparation

The standards (catechin, apigenin, naringin, naringenin, vitamin C, and gallic acid) were prepared in tris buffered (50 mM, pH 7.0) 0.01% dimethyl formamide (TBDMF) as stock solution of 5.0 mM concentration. The plant material used here were methanol extracts of *Syzygium cumini* kernel (SCK) *Hipaphea rahmnoides* kernel (HRK), and *Punica granatum* pericarp (PGP). The plant materials were selected based on their high antioxidant activity obtained from the

studies conducted in our laboratory. The methanol extracts were prepared from powdered raw materials. The extraction was performed with material to solvent ratio of 1:5 (w/v) for five times. The extracts thus obtained from each material were pooled and concentrated to dryness using vacuum rotary evaporator at reduced temperature and pressure (50°C, -700 mm Hg). The alcohol free extracts were then dissolved in TBDMF, filtered, and adjusted to 5.0 mM phenolic content. Phenolic content of extracts was estimated using Folin's reagent(Jayasinghe et al., 2003).

2.5.3 Induction of Oxidative DNA damage and evaluation of samples

Oxidative DNA damage was induced by adopting two methods reported previously with slight modification (Musonda and Chipman, 1998; Yu and Anderson, 1997). A dose response pattern of oxidative DNA damage was standardized by treating lambda DNA with Fenton's reactants to establish the reproducibility of oxidation and quantification of DNA damage. The method in brief was as follows: the reaction system contained lambda DNA (2.5 µg), ferrous chloride (0.25mM) and various concentrations of hydrogen peroxide (0.05 to 0.35 mM; step value; 0.05). Fenton's reaction was initiated by addition of hydrogen peroxide. Final reaction volume of the assay system was maintained at 20µL. The tubes were sealed and incubated at 37^{o}C for 2.0 h in a constant temperature water bath. After incubation, the reaction was stopped by addition of 10 µL of 4 X gel loading buffer.

For evaluation, concentrations of oxidants and standard compounds/extracts were set at equimolar level. The standard antioxidant compounds and the samples were added to the Fenton's system so as to maintain a final concentration of 0.25 mM. Molarities of the extracts were calculated based on the assumption of average molecular weight of plant phenolic compounds as 350. The concentrations of ferrous chloride and hydrogen peroxide were kept at 0.25 mM in total volume of 20 µL. Working solutions of hydrogen peroxide and ferrous chloride were prepared freshly before each batch of experiments.

2.5.4 Electrophoresis

The contents of the reaction tubes were mixed well with gel loading buffer and loaded on to 0.7% agarose gel bed equilibrated with TBE buffer in a horizontal electrophoresis system (Genei, Bangalore, India). Immediately after sample loading, electrophoresis was carried out for the first 15 min. at 20 mA and continued for the next 30 min. at 40 mA. The gel was then removed and stained in 0.2 µg/mL ethidium bromide solution for 30 min. The staining conditions were kept identical for all subsequent experiments.

2.5.5 Imaging and data analysis

Uniformly stained gels were placed on a UV trans-illuminator at 254nm and photographed to tagged image file format (TIFF) with a 4 mega pixel digital camera (Canon power shot, Japan). The image captured in RGB format was imported into image processing software. The image was then transformed to 8-bit grayscale format. The gray scale image was then normalized based on the blank lane by adjusting the brightness and contrast tool. The fluorescent region of each lane was then selected using free-hand selection tool and measured the average intensity of all pixels in the selected region. The numeric values of white and black pixels are 255 and 0 respectively. The value of a pixel lies between 0 and 255 based on their signal strength. The values of all pixels in the selected area were computed and the average value per pixel was arrived at for each lane. Average fluorescence in the blank lane was considered 100 % (0 % DNA damage) and the difference between the blank and treated DNA was taken as the extent of damage in percentage

2.5.6 Iron chelating effect

Iron chelating properties of standard compounds and plant extracts were evaluated using the method reported by Pascual *et al.* (Pascual-Reguera et al., 1997) with slight modifications as follows: Standard compounds and plant extracts (2.0 mg) dissolved in 0.5 mL methanol were treated with 0.25 ml ferrous chloride (1.0 mM) and 1ml ferrozine (5mM). Volume of the assay was then made up to 2.0 ml with methanol. Final concentration of the standards and extracts in the assay thus was 1.0 mg/mL. The reaction mixture was shaken well and incubated for 10 min.

at room temperature (25-26^{o}C) for ferrous-ferrozine complex formation. Absorbance of the color developed due to the ferrous ferrozine complex was measured using spectrophotometer (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan.) at 560 nm against reagent blank. Percentage iron chelation was calculated with respect to blank, where ferrous ferrozine complex formed was taken as 100%. The extend of reduction in color was taken as iron chelating activity of the standard compounds/extracts used here under the assay conditions.

2.6 Anticancer activity studies of SCF

For conducting anticancer activity studies high quality standards, chemicals, reagents and disposable plastic wares were sourced from Sigma-Aldrich, Merck, BD Biosciences, and Cayman Chem etc. Samples were prepared from one selected lot and used for all extraction and fractionation.

2.6.1 Chemicals and reagents

Cell culture media and supplements viz. RPMI 1640 culture media, foetal bovine serum, ampiciline, streptomycine, cell culture grade glucose, pyruvate, sodium chloride, cell culture grade sodium carbonate, L-glutamine etc. were purchased from Sigma-Aldrich. Standard cytotoxic agents such as camptothecin, curcumin, and methotrexate were procured from Merck, India. Immunochemicals such as anti-Bax antibody (mouse), anti-BdXL antibody (mouse), anti-caspase 3antibody (mouse), anti-actin antibody, horseradish peroxidase conjugated goat anti-mouse antibody, LDH assay kit, MTT assay kit etc were procured from Cayman chemicals, India. Cancer cell lines viz. HeLa-S3, COLO-205, MCF-7, HL-60 were sourced from CCMB, Hyderabad, India.

2.6.2 Preparation of methanol extracts from *S. cumini* fruit parts for preliminary anticancer activity evaluation

The freeze-dried anatomical parts of fruit viz. pulp; kernel and seed coat were coarse powdered to an average particle size of 0.5mm. 10 g of the powdered fruit parts were extracted with distilled methanol at a substance solvent ratio 1:5 (v/v)

under stirring condition for 30 min. The extractions were repeated for 3 times and the extracts viz. methanol extract of pulp (PM), kernel (KM) and seed coat (SCM) were pooled separately and concentrated to a final strength of 10mg/ml using thin film vacuum rotary evaporator. For cell culture studies, samples were dried and dissolved first with minimum amount of methanol and then made up to a minimum standard volume with sterile culture media. Care has been taken to ensure that the substances are dissolved in assay system completely. The amount of methanol used to ensure solubility of substances in assay system was recorded and appropriate solvent blank assay were also conduct to address the solvent effect.

2.6.3 Fractionation of *S. cumini* kernel methanol by liquid-liquid partition and HPLC fractionation

Liquid-liquid fractionation of KM was performed as described in section 2.4.2. Ethyl acetate fraction thus obtained was further fractionated by preparative HPLC as mentioned in section 2.4.3.

2.6.4 .Cell lines and culture conditions

HeLa-S3, COLO-205, MCF-7, HL-60 etc. were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 0.25% glucose, 0.25% sodium bicarbonate, 0.03% L-glutamine and 10% fetal bovine serum (FBS). All cell lines were kept in a humidified incubator at 37^{o}C with 5% CO2 and were sub-cultured on reaching 90% confluency. A master cell bank was also maintained separately in liquid nitrogen cryo storage system.

2.6.5 MTT based cytotoxicity assay

Cytotoxicity was assessed by MTT (3,4,5-dimethylthiazol-2-yl)-5-5diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of metabolically active cells into a purple formazan product (Shi et al., 2006). Cells were seeded in 24-well plates and treated with different concentrations of sample in minimum volume of suitable solvent. After the treatment, media containing samples were carefully aspirated. Cells were washed twice with phosphate buffered saline. Subsequently 200 µl of

media containing MTT (0.5 mg/ml) was added to each well. The plate was incubated at 37^{o}C for 1 h. Then the medium was totally removed and 1.0 ml Tris-DMSO solution was added to each well. The plate was vibrated for 30 min. The absorbance, which was proportional to cell viability, was subsequently measured at 570 nm in each well using an microwell plate reader. All experiments were repeated at least three times.

2.6.6 LDH based cytotoxicity assay

Cell death was assessed by measuring the lactate dehydrogenase (LDH) activity, as described previously (Maria et al., 2006). The release of the intracellular LDH into the media was used to calculate cell viability, since released LDH is a stable enzymatic marker correlating linearly with cell death. The LDH activity was derived by measuring NADH oxidation at 340 nm during the reduction of pyruvate to lactate. Optical density values were recorded for 2 min, and the rate of reduction was calculated. Cells from control cultures were solubilized with 0.3 mL of 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed for 30 min at 37 °C. This cell-associated LDH activity was added to the LDH activity released from control cultures, and the total activity was considered to represent 100% cell death. Then, for each treatment (extracts), the amount of LDH present in the medium was calculated as a percentage of total LDH, which reflects the percent cell death in the sample.

2.6.7 Morphological analysis by bright field microscopy

Morphology was evaluated microscopically using simple bright field microscope by slightly modifying the method reported previously (Hen et al., 2005). HeLa-S3 cells were cultured on 24 well plates and then treated with different concentrations (0, 2 and 8 µg/ml) of F2 and incubated for 24 hrs. After incubation, the cells were washed with PBS twice and fixed with ethanol/acetic acid. Then, the plates were observed through an inverted bright field microscope at 40x objective. For 100x magnification, the cells after sample treatment were detached by trypsinization and then fixed using the fixative. The fixed cells were placed on a microscope slide and observed through 100X oil immersion objective. The morphology of treated cells was examined and photographed using a digital camera..

2.6.8 Dot immune blot analysis for apoptotic marker proteins

Dot blot analyses were done by modifying related methods reported previously (Delphine et al., 2004) and by following dot blot lab malual of "abcam". Briefly, the cells were harvested from the trypsinized culture plates and washed several times in 5 ml of tris buffered saline (TBS). After repeated washing the cell density from each treatment were measured micrometrically. Any differences in cell number from different well were normalized to a final cell number $10x10^6$.The cells were finally sedimented at low speed centrifugation in a 2 ml micro centrifuge tube and suspended in 1 ml of lysis buffer (0.0625 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 5% ß mercaptoethanol). Cells were boiled for 5 min and homogenized by a micro sized pestle. The cell lysate were then diluted to a standard volume and debris was removed by centrifugation. 10 µl of the diluted and cell lysate were then applied on a nitrocellulose acetate membrane using Linaomat-5 (Camag planar chromatography systems) TLC sample applicator. The cell lysate were applied at the rate of 1 µl/min on the membrane as band at a width of 10mm using nitrogen as drying gas. The membranes spotted with cell lysate containing 10 µg of protein were dried and the non-specific sites were blocked (1 h at room temperature with 5% non-fat milk in TBS). Then, the membranes were incubated overnight at 4°C with the primary antibodies separately (anti-Bax mAb, anti-BdXL- mAb, anti caspase-3 mAb and anti-actin. mAb: 10µg/ml concentration). After incubation the membranes were washed and treated with secondary antibody conjugated with horseradish peroxidase (pAb 1:200 dilution). After 2 hours of incubation, the membranes were further washed in wash buffer for 3 times and dipped in the glass tray containing 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The membranes were further incubated at room temperature for 30 min for the bands to appear. The membranes were removed from the substrate solution, air dried and scanned at 600 dpi resolution using HP scanner in TIFF format for further qualitative comparison analysis.

2.7 Antidiabetic studies on SCF

2.7.1 Chemicals and reagents

S. cumini fruit, 3T3-L1 pre-adipocytes, Isobutylmethylxanthine (IBMX), Dexamethasone (DXM), Insulin, rabbit Anti-GLUT-4 polyclonal antibody, rabbit Anti-actin polyclonal antibody, donkey anti-rabbit secondary antibody-HRP-conjugate, nitro cellulose membrane, DMSO, Oil red-O, pioglitazone, RPMI 1640 culture media, foetal bovine serum, ampiciline, streptomycine, cell culture grade glucose, pyruvate, sodium chloride, cell culture grade sodium carbonate and other common laboratory chemicals.

2.7.2 Sample preparation and fractionation

Fully mature and ripened *S. cumini* fruits were manually separated into their anatomical parts viz. pulp, kernel and seed coat. Each fruit part was freeze-dried and crude methanol extract was prepared first. The methanol extract of kernel was then separated into two fractions by liquid-liquid partition technique. The ethyl acetate fraction thus obtained was further fractionated by preparative HPLC. The detailed method for extraction, liquid-liquid partition and HPLC fractionation are presented below

2.7.2.1 Preparation of crude methanol extracts from fruit's anatomical parts for preliminary antidiabetic activity screening

For the preliminary diabetic activity screening, crude methanol extracts from anatomically distinct parts of *S. cumini* fruits were prepared and the details are described in section 2.6.2.

2.7.2.2 Fractionation of KM based on polarity of its content by liquid-liquid partition.

The KM was separated into two fractions based on polarity of phytochemicals in it by liquid-liquid partition technique. For liquid-liquid partition, 10 g (dry basis) of the methanol extract (KM) was desolventized and redissolved in 250 ml water. The water suspension containing 10g of methanol free KM was taken in a

separating funnel and partitioned with equal amount of ethyl acetate. This fractionation was repeated 5 times and the ethyl acetate fractions (KMEd) were pooled and concentrated to an appropriate strength. The residual water fractionation (KMWd) was also concentrated to an appropriate final strength. The fractions were latter diluted suitable required concentration, as mentioned in previous section.

2.7.2.3 Preparation of HPLC fractions

Ethyl acetate fraction obtained was further fractionated by preparative HPLC as mentioned in section 2.4.3.

2.7.3 Cell lines and culture conditions for in vitro antidiabetic activity evaluation

In vitro antidiabetic activity of KM fractions were studied by in vitro cell culture method (Green and Kehinde, 1974). The activity of fractions to modulate the expression of GLUT-4 protein and activation of PPARγ were analyzed to evaluate the antidiabetic potential. 3T3-L1 murine pre-adipocytes were used for GLUT-4 receptor expression and nuclear factor activation studies. 3T3-L1 pre-adipocytes were obtained from Centre for cellular and molecular biology (CCMB) and maintained in master cell banking system in several lots. The cells were revived and cultured in 24 well plates containing RPMI 1614 mammalian cell culture media supplemented with 10% foetal bovine serum (FBS), penicillin, streptomycin and high level glucose (2.5 g/l). The plates were incubated at 37^0C in a humidified, 5% CO_2, 95% air atmosphere chamber.

2.7.4 GLUT-4 receptor expression assay in differentiated 3T3 cells.

3T3-L1 pre-adipocytes were differentiated into adipocytes for GLUT-4 expression studies. The cells were proliferated in high glucose RPMI1614 containing 10% FBS and antibiotics. Upon reaching 80% confluence, differentiation was initiated by adding 250 mM 3-isobutylmethylxanthine (IBMX), 1 mM dexamethasone (DMX), and 670 nM insulin for 2 days. Differentiation was then continued in medium containing 10% FBS and 670nM insulin for approximately 6-8 days. At this point, confluence was attained and over 90% of cells contained lipid droplets

visible under phase-contrast microscopy. Differentiated adipocytes were incubated with samples at sub lethal level (KM, PM and SCM <10 µg/ml; KMEd, KMWD <7.5 µg/ml; HPLC fractions< 5µg/ml;) and pioglitazone standard (<100 µM) for 24 hrs, wherever indicated for GLUT-4 receptor expression induction (Anandharajan et al., 2006; Martineaua et al., 2006).

2.7.5 Dot Immunoblot assay for GLUT-4 receptor expression abundance

Dot blot analyses were done by slightly modifying a methods reported previously (Delphine et al., 2004) and by following dot blot lab malual of "abcam". Briefly, the sample-treated cells were harvested from the trypsinized culture plates and washed several times in tris buffered saline (TBS). After repeated washing the cell from each treatment wells were suspended in 1 ml of TBS and the density was measured using neubauer chamber. Any differences in cell number from different well were normalized to a final cell number $10x10^6$ by adjusting suspension media volume. The cells were finally sedimented at low speed centrifugation in a 2 ml micro centrifuge tube and suspended in 1 ml of lysis buffer (0.0625 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 5% ß mercaptoethanol). Cells were boiled for 5 min and homogenized by a micro pestle. The cell lysate were then diluted to a standard volume and debris was removed by centrifugation. 10 µl of the each diluted cell lysate was then applied on a nitrocellulose acetate membrane using Linaomat-5 (Camag planar chromatography systems) sample applicator. The cell lysate were applied at the rate of 1 µl/min on the membrane as band at a width of 10mm using nitrogen as drying gas. The membranes spotted with cell lysate were dried and the non-specific sites were blocked (incubation of membrane with 5% non-fat milk in TBS for 1 hr at room temperature). The membranes were then incubated overnight at 4°C with anti-GLUT-4 antibody (primary antibody) separately. After incubation, the membranes were washed and treated with secondary antibody conjugated with horseradish peroxidase (pAb 1:200 dilution). After 2 hours of incubation, the membranes were further washed in wash buffer for 3 times and dipped in the glass tray containing 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The membranes were further incubated at room temperature for

30 min for the bands to appear. The membranes were removed from the substrate solution, air dried and scanned at 600 dpi resolution using HP scanner in TIFF format for further image processing and qualitative comparison.

2.7.6 PPARγ activation assay

As an indirect method for screening thiazolidinedione-like activity (induction of differentiation to adipocytes) in SCK, fractions were evaluated in 3T3-L1 pre-adipocytes in vitro model system. PPARγ activation in 3T3-L1in the presence of extracts was assessed by its accelerated differentiation over nontreated cells by measuring the accumulation of triglycerides. (Harmon and Harp, 2001; Ljung et al., 2002; Norisada et al., 2004; Tontonoz et al.). Pre-adipocytes were grown in 24-well plates to 90% confluence. One day after attaining confluence, proliferation medium was replaced with differentiation medium containing IBMX, DXM, and insulin, with either vehicle alone, extract in vehicle, or positive control in vehicle. This medium was changed after every 24 h till the addition of next media. After 48 h, medium was replaced with differentiation medium containing only insulin, as above, with or without plant extracts or controls. This medium was changed every 24 h. Rosiglitazone, a PPARγ agonist of the thiazolidinedione family, was used as a positive control, while vehicle in proliferation medium was used as a negative control. Incubation was terminated after the first visual detection of intracellular lipid droplets by microscopy in vehicle-treated cells. The cells were then subjected to oil red assay to measure the degree of differentiation.

2.7.7 Oil red assay to measure the degree of differentiation

Oil red assay was performed by following a standard method reported previously(Kima et al., 2007) . After observing initial fat globules in blank well, the incubation was stopped and the cells were washed twice with PBS and fixed with 3.7% formaldehyde for 10 min. Formaldehyde fixed cells were stained with 0.2% Oil Red O-isopropanol for 1 h and the excess of stain was washed by 70% ethanol and water. Stained oil droplets were dissolved with isopropanol and quantified spectrophotometricaly at 510 nm. Results were represented in optical density scale

2.7.8 Ion Trap LCMS studies on active fractions

The molecular masses of chemical entities in selected fractions of SCF were studied using electrospray ionization-ion trap-tandem mass spectrometer (ESI-IT-MS-MS). The samples/fractions were filtered through a 4µ syringe filter and injected directly to the ESI-IT-MS-MS (Agilent ion trap 6300) using a programmable direct sample injection module. The direct injection flow rate was 100µl/min and other instrument parameters were as follows; nebulizer pressure, 25 PSI; dry gas flow 10 l/min; dry gas temperature, 350^{o}C; ionization mode, negative and electrospray ionization; capillary voltage, 3500. Ion trap was programmed to scan molecular ions the range of 100-2000 m/z. MS data were acquired with the Agilent 6300 series Ion Trap LC/MS Software 6.1 and data analyses were performed with post run analysis software version3.4.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Chemical Profiling of *S. cumini* fruit

Plants contain a broad spectrum of organic compounds that include common phytochemicals and species-specific secondary metabolites. Since many secondary metabolites are species specific, profiling of entire phytochemical entities in a plant species would yield chemical fingerprint unique to that species. Species specific fingerprints are useful for establishing active principles plant-based healthcare products, formulations and quality assurance. Several plant specific secondary metabolites are capable of interacting and making conformational changes to vital proteins (viz. enzymes, transport proteins, membrane receptors etc.) in living beings that can modulate several biochemical pathways. Many studies are being conducted to identify and characterize such bioactive secondary metabolites from variety of sources against targeted biochemical events. Studies on biological activities of phytochemicals have lead to the discovery of therapeutically important chemical entities. It is in this context chemical profiling of plants is significant. In the present study *S. cumini* fruit and its anatomical parts were subjected to detailed chemical analysis.

3.1.1 Anatomical composition of *S. cumini* fruit

Anatomical composition of fruit was studied gravimetrically. The mean yield of pulp, seed coat and kernel of fully ripened fruits with an average weight of 6.0 ± 3 g is shown in Table 3-1.

Table 3-1 Anatomical composition of fresh *S. cumini* fruit

Anatomical Parts	Yield (%)
Pulp	66.6 ± 11.1
Kernel	29.0 ± 4.0
Seed Coat	5.0 ± 1.5

The samples were collected randomly in batch wise from a single population based on the maturity and ripeness.. N = 20, ± SD

The fully ripened Jamun fruits studied here had; 66.0±11.1 % pulp; 29.0±4 % kernel and 5.0±1.5% seed coat. The pulp, kernel and seed coat were subjected to solvent extraction and further analysis was performed in whole material, extract and residue on dry weight basis.

3.1.2 Chemical composition anatomical parts of fruits

For composition analysis, the fruits were separated into anatomical parts as shown in the Fig. 2-1. Fractionation of *S. cumini* fruit (SCF) yielded its anatomical parts such as pulp (SCP), kernel (SCK) and seed coat (SCSC). Each part was extracted with methanol and named as extracts of pulp (PM), kernel (KM), seed coat (SCM); and residue of pulp (RP), kernel (RK) and seed coat (RSC). The yield of PM, KM and SCM were; 57±3, 15±1.5 and 21±1.5% respectively (Table 3-2).

Table 3-2 Yield of methanol extracts of *S cumini* fruit parts

Extracts	Yield (%)
PM	**57 ± 3**
KM	**15 ± 1.5**
SCM	**21 ± 1.5**

Dry fruit parts extracted with methanol yielding pulp methanol extract (PM), kernel methanol extract (KM) and seed coat methanol extract (SCM). All extracts are expressed in % dry weight of the corresponding dry fruit parts.

Chemical composition of the anatomical parts of fresh SCF is shown in Table 3-3. SCP, SCK and SCSC had moisture content of 850.0 g kg-1, 470.0 g kg-1 and 100.0 g kg-1 respectively. 80.0% methanol soluble carbohydrate content of the dry matter was; SCP, 400.0 g kg-1; SCK, 120.0 g kg-1 and SCSC, 210.0 g kg-1. Crude fiber content was 120.0 g kg-1 in SCSC; 29.0 g kg-1 in SCK and 7.0 g kg-1 in SCP. Methanol insoluble carbohydrate content was significantly more in SCK (600.0 g kg-1) and SCSC (430.0 g kg-1) than that in SCP (350.0 g kg-1). Crude protein content in SCSC was 160.0 g kg-1 whereas it was 68.0 g kg-1 in SCK and 66 g kg-1 in SCP. Total mineral content was 45.0 g kg-1 in SCP and SCSC and

SCK had lower content (25.0 g kg-1and 20.0 g kg-1). Total fatty matter (TFM) content in all samples was not very significant.

3.1.3 Mineral composition of fruit parts, their ethanol extracts and residue

Mineral analysis of SCF (Fig. 3-1) showed that calcium was the predominant mineral in all three anatomical parts. Highest calcium content (dry matter) was observed in SCSC (4.15 g kg^{-1}) followed by SCP (3.51 g kg^{-1}) and SCK (2.99 g kg^{-1}). Potassium was the next highest mineral constituent with 2.15 g kg^{-1}, 2.86 g kg^{-1} and 3.08 g kg^{-1} for SCP, SCK and SCSC respectively. A highest phosphorous content was observed in SCP (1.67 g kg^{-1}) followed by SCSC (1.26 g kg^{-1}) and SCK (1.16 g kg^{-1}). Values for Na and Fe were low in the fruit parts studied here. The results of mineral analysis indicated that SCP is rich in calcium, potassium and phosphorous. Edible part of the Jamun fruit thus showed calcium 3 fold more than that in black berries (USDA, 2004). The results of present study was compared with previous report on edible part of SCF and identified a high degree of variance that may be due to climatic and geographical conditions (Chadha, 2003).

3.1.4 Free polyphenols, anthocyanins and flavonoids content of fruit parts and, their methanol extracts

Composition of the bioactive compounds in SCF parts is shown in Table 3-4. Cyanidine-3-glucoside equivalent anthocyanin content in SCP was 1.34 g kg-1. Total flavonoids (excluding anthocyanin) estimated by the two complementary methods viz. aluminium chloride method (more specific for flavones, flavonols and flavonones) and using 2,4-dinitrophenylhydrazine reagent (more specific for flavonones) showed that SCP contained 0.07 g kg-1 of flavonoids with a corresponding phenolic content of 3.9 g kg-1. Total phenolic content in SCK and SCSC were 9 g kg-1 and 8.1 g kg-1 respectively which is almost twofold higher than that of SCP. Total flavonoid content of SCK (0.52 g kg-1) and SCSC (0.41 g kg-1) were also found to be substantially high compared to edible portion.

Table 3-3 Chemical composition of fruit parts, their methanol extract and residue.

Sample	Moisture	TFM	Protein	TESC	Crude Fiber	Starch	Ash
	($g\ kg^{-1}$)[a]	($g\ kg^{-1}$)[b]					
SCP	850.0 ±40	16.0 ±2.0	66.0 ±6.0	400.0±33.0	7.0±13.0	350.0±20.0	45.0 ±0.6
PM	-	Tr	13.0 ±0.25	520.0±9.0	ND	-	40.0 ±3.5
RP	-	36.0 ±4.0	100.0 ±7.5	139.0±7.0	20.0±2.0	672.0±23.0	26.0 ±1.8
SCK	470.0 ±30	3.5 ±0.1	68.0 ±0.4	120.0±8.0	29.0±2.0	600.0±33.0	20.0 ±1.0
KM	-	Tr	8.0 ±0.5	600.0±10.0	ND	-	17.0 ±0.3
RK	-	4.5 ±0.4	78.0 ±2.2	201.0±6.0	27.1±1.1	616.0±15.5	18.0 ±1.0
SCSC	100.0±20	5.0 ±0.7	160.0 ±5.0	210.0±7.0	120.0±6.0	430.0±15.0	25.0±0.7
SCM	-	tr	9.0 ±0.3	710.0±13.0	ND	-	16.0±0.8
RSC	-	4.7 ±0.6	138.0 ±0.4	69.0±1.3	146.0±5.0	603.0±65.0	24.0±0.4

a: fresh basis, b: dry basis. SCP: S. cumini pulp, PM: pulp methanol extract, RP: residual pulp, SCK: S. cumini kernel, KM: kernel methanol extract, RK: residual kernel, SCSC: S. cumini seed coat, SCM: seed coat methanol extract, RSC: residual seed coat, TFM: total fatty matter, TESC: total 80% ethanol soluble carbohydrate. (n=3 ± SD).

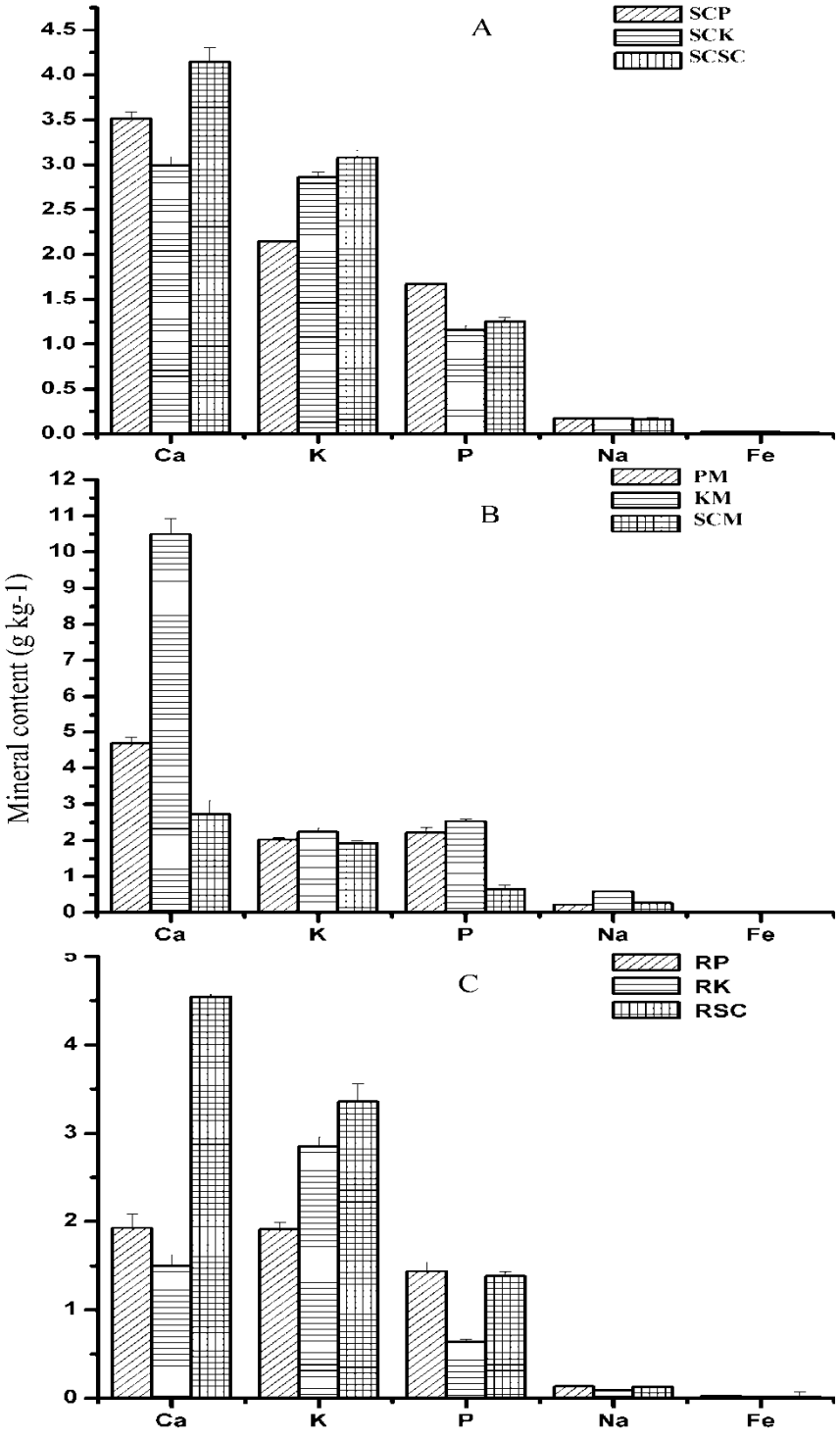

Fig. 3-1 Mineral profile of (A) *S. cumini* pulp (SCP), kernel (SCK), seed coat (SCSC); (B) pulp Methanol extract (PM), kernel methanol extract (KM), seed coat methanol extract (SCM); (C) residual pulp (RP), residual kernel (RK) and residual seed coat (RSC). All values are expressed in dry weight basis. (n=3 ± SEE)

Table 3-4 Free polyphenols, anthocyanin and flavonoid content of fresh Jamun fruit parts and their corresponding ethanol extract.

Sample Sample	Total free phenols Total free phenol	Anthocyanins Anthocyanins	Flavonoid		
			($g\ kg^{-1}$)		
	($g\ kg^{-1}$)	($g\ kg^{-1}$)	$AlCl_3$ Method	2,4-DNPH Method	Total
SCP[a]	3.9±0.5	1.34±0. 20	0.05 ± 0.03	0.02 ±0.01	0.07 ±0.04
PM[b]	340.0±1.7	3.20±0. 27	8.60 ± 0.50	1.40±0.32	10.000±0.82
SCK[a]	9.0±0.7.0	-	0.44 ± 0.17	0.08 ±0.03	0.52 ±0.20
KM[b]	370.0±7.8	-	30.00± 0.43	2.00±0.09	32.00±0.52
SCSC[a]	8.1±0.8	-	0.41 ± 0.08	ND	0.41 ± 0.08
SCM[b]	270.0±3.4	-	25.30± 0.37	ND	25.30±0.37

SCP: S. cumini pulp, PM: pulp methanol extract, SCK: S. cumini kernel, KM: kernel methanol extract, SCSC: S. cumini seed coat, SCM: seed coat methanol extract. (n=3 ± SEE). a: fresh weight basis b: dry weight basis

3.1.5 Composition of alcohol extracts.

Bulk of the dry matter of alcohol extract was soluble sugars comprising 710.0 g kg-1 in SCM, 600.0 g kg-1 in KM and 520.0 g kg-1 in PM. Among minerals, calcium was the major one with 10.49 g kg-1 in KM, 4.67 g kg-1 and 2.8 g kg-1 in PM and SCM respectively. Potassium was the next highest mineral constituent with 1.92 to 2.23 g kg-1. A significant amount of phosphorous was also observed in all three extracts.

The gallic acid equivalent phenolic content on dry matter in KM was 370 g kg-1 followed by PM (340 g kg-1) and SCM (270 g kg-1). Total anthocyanin assay showed that PM contained 3.20 g kg-1 cyaniding-3-glucoside equivalent anthocyanins and it was not significant in KM and SCM. KM and SCM were rich in flavonoids. By aluminium chloride method, PM, KM and SCM showed 8.6 g kg-1, 30.0 g kg-1and 25.30 g kg-1 of quercetin equivalent flavonoids respectively. Since aluminium chloride method is more flavones, flavonols and flavonones, the samples may be rich in these flavonoids. The other method using 2,4-Dinitrophenylhydrazine reagent indicated that PM and KM had very little of these flavonoids and were not detected in SCM. Total flavonoid was exceptionally high in KM (32.00 g kg-1) and in SCM (25.30 g kg-1).

Fully ripened SCF has previously been reported to contain different anthocyanins namely cyanidin, petunidin and malvidin (Chadha, 2003). The results of our investigation on fruit anthocyanin content are comparable with the findings of Archana Banerjee et al. (Banerjee et al., 2005). Total flavonoid content of edible part is comparable with the previous findings of flavonoids in fruits (Franke et al., 2004). The results of the present study indicate that Jamun fruit contain one to three times more flavonoids than that in blue berry, straw berry, apple, grape etc. Flavonoid content of crane berry is almost similar to that of Jamun (Hakkinen et al., 1999; Marinova et al., 2005; Wang and Lewers, 2007). The residue obtained after alcohol extraction was left with most of the fat and fiber and least of soluble sugars (Table 3-3)

3.1.6 Phenolic constituents of SCF by HPLC.

Methanol extracts of three anatomically distinct parts of SCF were

evaluated for major phenolic compounds. HPLC method with reference standards were used for identification and quantification. The method used here is described in section 2.1.15. The chromatographic conditions were optimized to resolve all 12 reference standards. Fig. 3-2 shows the chromatogram of reference standards (A), pulp methanol extract (PM) (section B), seed coat methanol extract (SCM) (section C) and kernel methanol extract (KM) (section D). Good resolution was obtained for all 14 reference standards used that enabled to prepare standard chromatogram. Except quinic acid, an optimum ***k*** factor was obtained for all other standard compounds as shown in the chromatogram. The retention time of standard compounds were ranged from 2 min (quinic acid) to 52 min (kaempferol).

The standard chromatogram was compared with that of PM, SCM, and KM based on major parameters such as retention time, spectral correlation, and peak segment spectral correlation using 'CLASS-VP' chromatography data processing software bundled with Shimadzu HPLC system. Using these parameters, compounds in samples (PM, SCM and KM) were identified. The identified compounds were further validated using spectral correlation and peak purity analysis and quantified using the standard calibration curve.

Detailed chromatographic analyses of PM and SCM at 280 nm yielded more than 50 minor and major peaks. However, the number of peaks in KM was more than 150. This indicates that KM contains more UV sensitive compounds than that in PM and SCM. Peak purity analyses were conducted for all prominent peaks without shoulders using peak segment spectral correlation evaluation. This purity analyses showed that most of the compounds were resolved well. However, 20-25% of the peaks observed in the chromatogram of all samples were not pure.

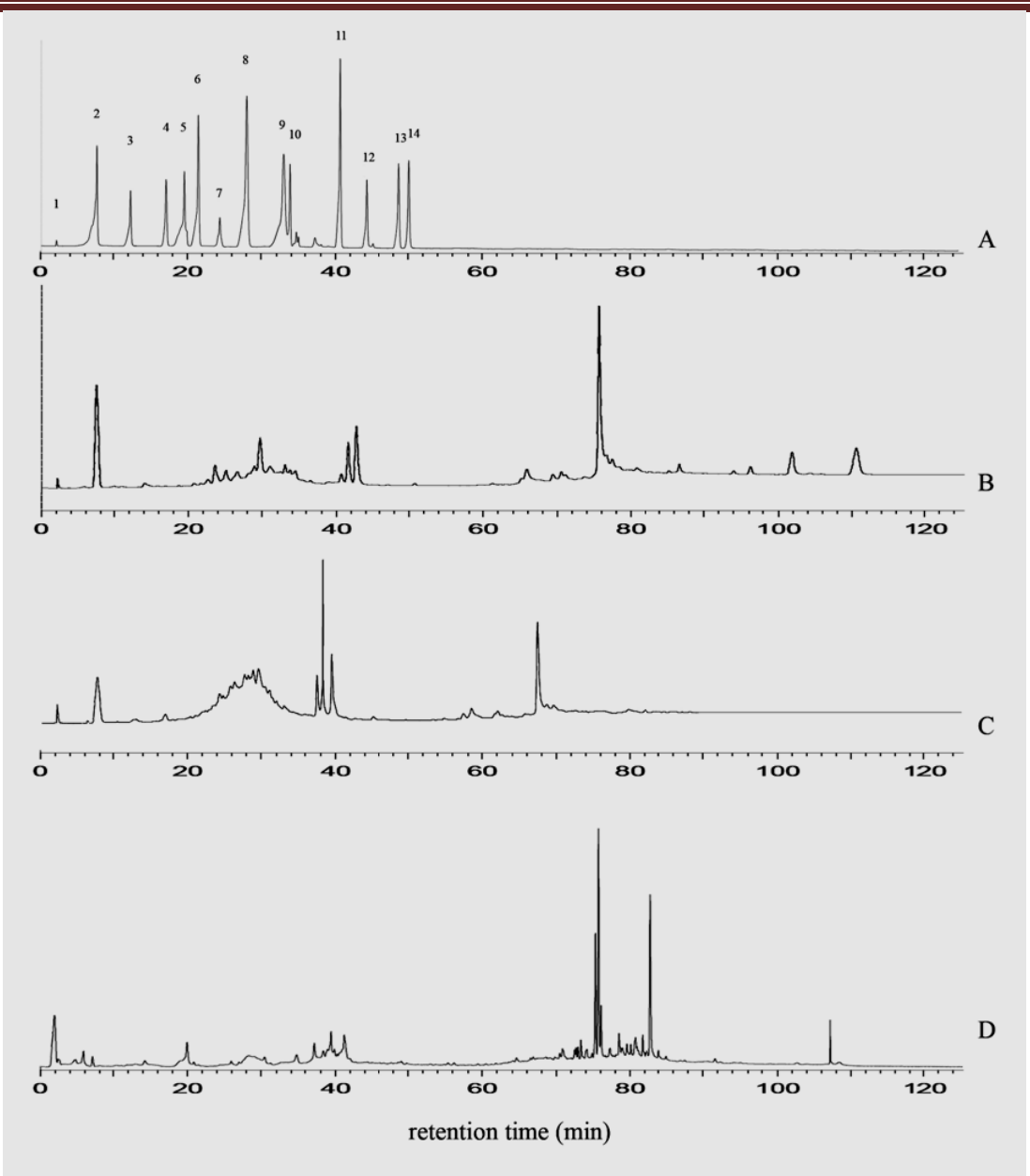

Fig. 3-2 Phenolic constituents analyses of *S. cumini* fruit parts by HPLC. A, standard chromatogram (1: QA, 2: GA, 3: DBA, 4: PHBA, 5: ClA, 6:CA, 7: Cat, 8: PCA, 9: FA, 10: SA, 11:Res, 12:Qtn, 13:Ngnn, 14: Kmp); B, pulp methanol extract (PM); C, seed coat methanol extracts (SCM), D; kernel methanol extracts (KM). HPLC conditions: Solvent A, methanol; solvent B, 2% acetic acid; flow rate, 1 ml/min; gradient condition: 0-5 min, 100% B; 5-100 min, 100% A (linear); 100-140 min 100% A; post run, 100% B (20 min); PDA wavelength range, 200-450 nm.

Table 3-5 Phenolic composition of *S. cumini* fruit parts' methanol extracts

Sl. No.	Compound	λ max (nm)	Spectral correlation (r^2)	Quantity (mg/100g of dry weight)		
				PM	KM	SCM
1	quinic acid	320	0.9993	1.2±0.1	2.1±0.17	1.6±0.21
2	**gallic acid**	270	0.9991	**4.2±0.7**	**7.4±0.89**	1.1±0.12
3	dihydroxybenzoic acid	255	0.9981	2.1±0.8	1.9±0.5	1.5±0.26
4	*p*-hydroxybenzoic acid	250	0.9392	tr.	tr	1.3±0.22
5	chlorogenic acid	325	0.9826	tr	3.7±1	tr
6	caffeic acid	325	0.9999	2.2±0.5	2.7±0.43	0.9±0.17
7	*p*-coumaric acid	310	0.9934	**4.1±1.1**	2.3±0.8	1.3±0.45
8	ferulic acid	320	0.9987	tr	2.9±0.2	ND
9	**sinapic acid**	320	0.9876	**4.3±1.1**	**3.1±0.8**	**2.3±0.5**
10	**quercetin**	260	0.9987	**4.5±1.2**	**8.5±1.5**	**2.2±0.5**
12	kaempferol	260	0.9956	1.9±0.2	3.1±0.55	1.2±0.33

tr: trace

Table 3-5 presents the content of a few phenolic compounds in PM, SCM and KM. Among the nine standard organic acids and two flavonoids studied here in different anatomical parts of SCF, PM contained GA (4.2 ±0.7 mg/100g), PCA (4.1±1.1mg/100g), SA (4.3±1.1 mg/100g), and Qtn (4.5±1.2 mg/100g). largest amount of phenolic compounds in kernel were found to be Qtn (8.5±1.5 mg/100g), GA (7.4±0.8 mg/100g), and SA (3.1±0.8 mg/100g). In SCM, the phenolic compounds found in highest concentration were SA (2.3±0.5 mg/100g) and Qtn (2.2±0.5 mg/100g). Traces of PHB was found in PM and KM but, in SCM its concentration was 1.3±0.22 mg/100g. Though ClA content in KM was 3.7±1 mg/100g, only traces could be seen in PM and SCM. FA was found to be below the detection limit in SCM, however traces could be seen in PM and in KM its amount was relatively more (2.9±0.2 mg/100g) All other phenolic compounds were in the range of 0.9 to 2.2 mg/100g in all three extracts. HPLC analyses of methanol extract of anatomically distinct parts of SCF thus showed that the amount of all phenolic compounds studied here were relatively less in SCM. However, PM and KM contained comparatively large amount of GA, SA and Qtn. Further, the unidentified compounds in the extract as observed by the number of peaks in sample chromatogram shows the complexity of its organic contents.

Composition analyses of SCF and perusal of important results indicate that it is nutritionally, prophylactically and therapeutically important one. The fatty matter content of edible and non-edible portion of fruit was less. However the edible portion (SCP) was rich in free sugars and starch (Table 3-3). Carbohydrate rich foods are primary source of energy for all body functions. Free sugars are simple carbohydrates that can be easily absorbed in intestine. Starch is complex carbohydrate with low glycemic index. Consumption of 10-15 fruit's pulp can meet $1/10^{th}$ of the daily requirement of carbohydrates. Protein content of SCP was 66 g kg^{-1}on fresh weight basis hence; 15-20 fruits can serve $1/10^{th}$ of daily requirement of proteins for adults. In the case of minerals, SCP contained a mean value of 3.5 g kg^{-1} Ca, 2 g kg^{-1} K and 1.5 g kg^{-1} of P and the corresponding recommended dietary intake are; 1200, 2000 and 700 mg (Susan et al., 2002). Consumption of 15-20 fruits can meet $1/4^{th}$, $1/10^{th}$, and $1/5^{th}$ of dietary

requirement of Ca, K and P respectively. Other than the discussed nutritional value, apparently SCF has prophylactic importance also based on its polyphenol (anthocyanins and flavonoids) content. Total anthocyanins and flavonoids are presented in Table 3-4. Anthocyanins are members of flavonoids group of plant secondary metabolites. The radical scavenging activities and other associated health benefits of anthocyanins have been reported by many authors ((Tsuda et al., 2002; Tsuda et al., 2003; Tsuda et al., 1996; Wang and Jiao, 2000). The importance of anthocyanins pigments as medicines have been well accepted in folk medicine. Anthocyanins rich extracts have been used in remedies for liver dysfunction, hypertension, vision disorder, microbial infection etc. (Rice-Evans, 1998; Smith et al., 2000; Wang et al., 2000).

The non-edible portion of SCF also contained free sugars, starch and micro mineral as seen in the edible portion (Table 3-3). However the non edible portion was superior to edible portion on the basis of total flavonoids. Total flavonoids in SCK and SCSC were 0.52±0.2 and 0.4±0.08 mg Kg^{-1}respectively. Compared to total flavonoids in SCP, SCK and SCSC contained several fold more. Consumption of flavonoids are reported to have several health benefits especially associated with decreased risk of age related diseases in several epidemiological studies (Hertog and Hollman, 1996; Hirvonen et al., 2001; Huxley and HAW, 2003). Flavonoids have powerful antioxidant activities and scavenge wide range of reactive oxygen species such as superoxide radicals, hydroxyl radical etc. (Halliwell, 2000; Mira et al., 2002; Silva et al., 2002). HPLC analyses of phenolic content of SCF showed that gallic acid, sinapic acid and quercetin were more abundant in PM and KM. Gallic acid is known to have strong antioxidant, neuroprotective (J Y Ban, 2008), anticancer (K Raina, 2008), and anti-inflammatory (B H Kroes, 1992) activities. Sinapic acid is known for anxiolytic-like (Yoon et al., 2007) and anti-inflammatory (Yun et al., 2008) activities. The major flavonoids found in SCK and SCP (quercetin) was reported to have ameliorative effect on memory dysfunction (Bhutada et al., 2010), enhancement of adiponectin secretion (Wein et al., 2010,), antioxidant and antihistaminic effect (Kahraman et al., 2003). Therefore based on the chemical analysis, SCF is a

source for nutritional, prophylactic and therapeutic important phytochemicals. Therefore further studies are required to validate prophylactic as well as therapeutic potentials

3.2 Evaluation of antioxidant properties of *S. cumini* Fruit

Chemical profiling of SCF showed that it is rich in phenolic compounds. Total phenolic content was found to be more in seed compared to that of pulp and seed coat. More than 200 prominent peaks, related to phenolic compounds, have been observed in the HPLC chromatogram developed at 280 nm. Poly phenols are structurally diverse chemical entities having the property of preventing or retarding the biological oxidation in their vicinity and many of them are also reported to have antiradical activity also. In biological context, antioxidant/antiradical activities of chemical entities/food materials refer to their ability to prevent or retard the excessive oxidation. Antioxidants/antiradicals therefore delay the onset of oxidative stress induced degenerative diseases in human. Plants and plant products that prevent or retard oxidative processes or tissue damage are said to be prophylactic agents and the potency can be assessed by evaluating their antioxidant/antiradical activity. Antioxidant activities are generally analyzed by various standard in vitro and in vivo methods. For the present study a few sensitive, reproducible and low interference standard in vitro methods were selected for analysis. The assays are;

- DPPH radical scavenging activity.
- Superoxide radical scavenging activity.
- Hydroxyl radical scavenging activity.
- Lipid peroxidation inhibition activity.
- Total reducing power.

3.2.1 Preliminary evaluation

Antioxidant activity analyses were conducted in methanol extracts of all three fruit parts viz. pulp (PM), kernel (KM) and seed coat (SCM). Direct methyl alcohol extracts from the dried fruit parts were used for the purpose. The antioxidant/antiradical activity of all three samples and standard antioxidants were

expressed graphically in the form of dose response curve and IC50 values (amount of antioxidants required to scavenge 50% of the initial radicals).

3.2.1.1 DPPH radical scavenging activity of SCF parts.

DPPH is a stable free radical and it transforms to non radical form (yellow color) by abstracting one electron from the analyte. Therefore it is widely used as measure for the electron donation capacity of the antioxidant under the assay conditions (Molyneux, 2004). DPPH radical scavenging activities of extracts and standard compounds were evaluated and the results are shown in **Fig. 3-3**. A linear relation was observed up to a certain level between percentage radical scavenging activity and sample concentrations; but in different rate with respect to the chemical composition of samples and nature of standard compounds tested. Antioxidant capacity for crude methanol extract of kernel high with an IC50 of 8.6 µg/ml. However SCM (IC50: 48 µg/ml) and PM (IC50: 158 µg/ml) also showed good antioxidant activity as compared to those of standard compounds tested here (vitamin C, 7.0 µg/ml; Trolox, 4.3 µg/ml and catechine 6.0 µg/ml) (**Table 3-6).** Though the TPC contents between the samples showed only small variation (**Table 3-4**), the antioxidant capacity of KM was 17 times more than that of PM in terms of DPPH radical scavenging activity.

3.2.1.2 Superoxide radical scavenging activity.

The major source of free radical production in-vivo is through superoxide. Superoxide are produced by the leakage of free electron during its transport in mitochondria (Vaya and Aviram, 2001). Superoxides are scavenged by the enzyme superoxide dismutase into hydrogen peroxide and subsequently inactivated by the enzyme catalase. If the electron leakage is more, more superoxide will be produced as a result of cellular respiration. If the enzymatic antioxidant defense system is not sufficient to scavenge the free radicals produced, it will leads to a condition called oxidative stress and stress-induced degenerative diseases.

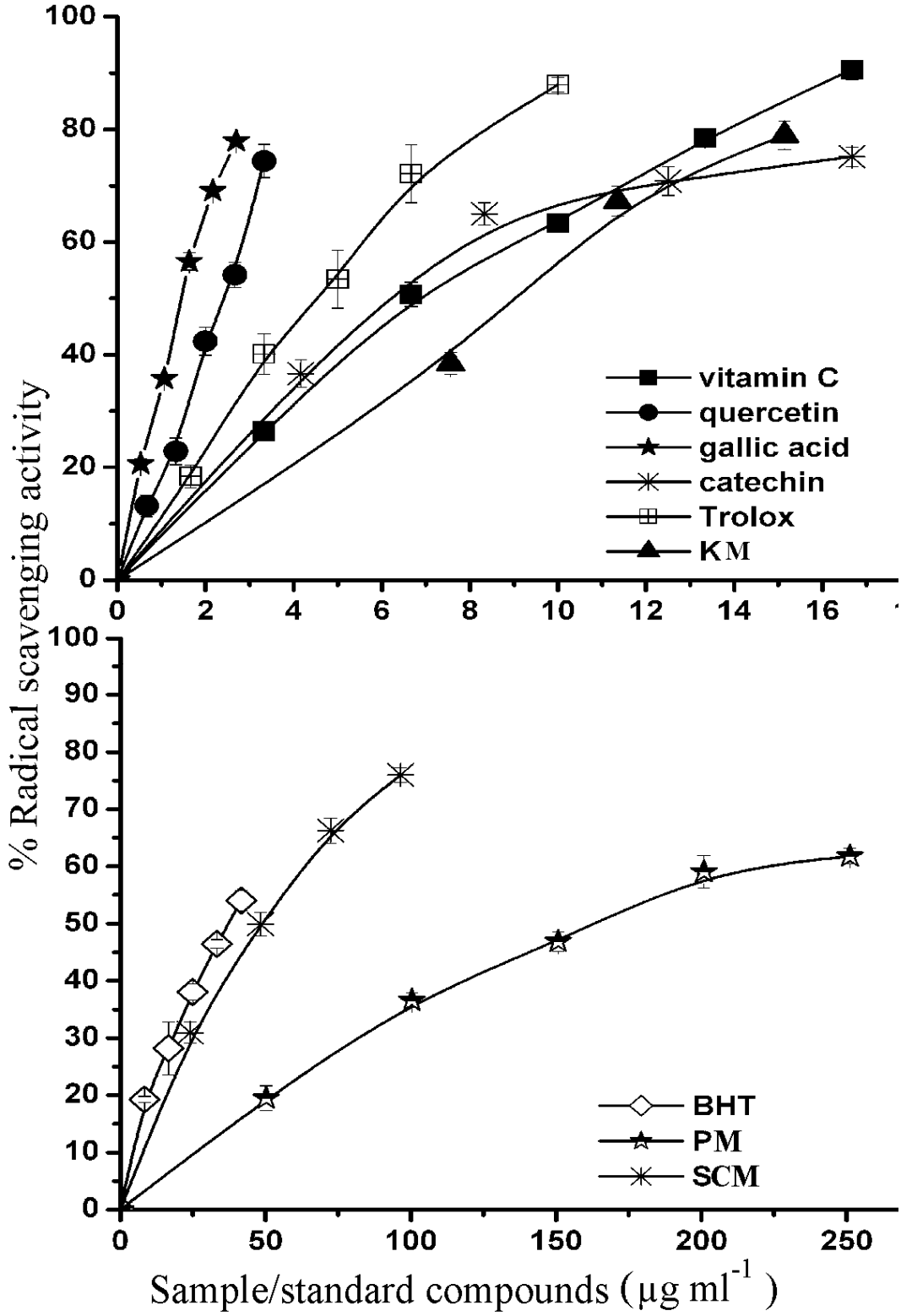

Fig. 3-3 Dose dependent DPPH radical scavenging activity of *S. cumini* fruit pulp methanol extract (PM), kernel methanol extract (KM), seed coat methanol extract (SCM) and standard BHT, vitamin C, quercetin, gallic acid, catechin and Trolox. (n=3 ± SD)

For the present study, superoxide radicals were produced by Xanthine-xanthine oxidase system in vitro. The magnitude of produced superoxide was quantified by the reduction of NBT, a tetrazolium salt. Reduction of NBT is characterized by a change in color to blue. In the presence of superoxide radical scavenging agent, the produced superoxide would be scavenged and is characterized by a reduction in blue color. A dose dependent superoxide radical scavenging activity was observed in all samples and standard molecules (**Fig. 3-4**). KM with IC50 value of 85 µg/ml was found to be very strong superoxide radical scavenger and the activity was significantly higher than those of standard compounds such as gallic acid (225 µg/ml) and catechin (296 µg/ml). The IC50 values for PM and SCM were 18 and 8 times less than that of KM. The poly phenol content of the samples did not correlate with their superoxide radical scavenging activity suggesting that the chemical structure of polyphenols may have bearing on their radical scavenging activity.

3.2.1.3 Hydroxyl radical scavenging activity.

In the present investigation hydroxyl radical scavenging activity of different samples and standard compounds were evaluated using ascorbic acid-iron-EDTA system. Hydroxyl radicals generated in the system react with DMSO to form formaldehyde. The hydroxyl radical scavenging activity of samples is related to the reduction in formaldehyde production and it is quantified using Nash reagent. A dose dependent hydroxyl radical scavenging activity was observed in KM, PM and SCM (**Fig. 3-5**).

The hydroxyl radical scavenging activity (IC50) of KM, SCM and PM were 151 µg/ml, 261 µg/ml and 310 µg/ml respectively suggesting that KM was more active than SCM and PM. Further activity of KM was comparable with those of standard quercetin (102 µg/ml), Trolox (190 µg/ml) and catechin (188 µg/ml). Activity of BHT and vitamin C was found to be substantially lower than that of the samples tested here (**Table 3-6**).

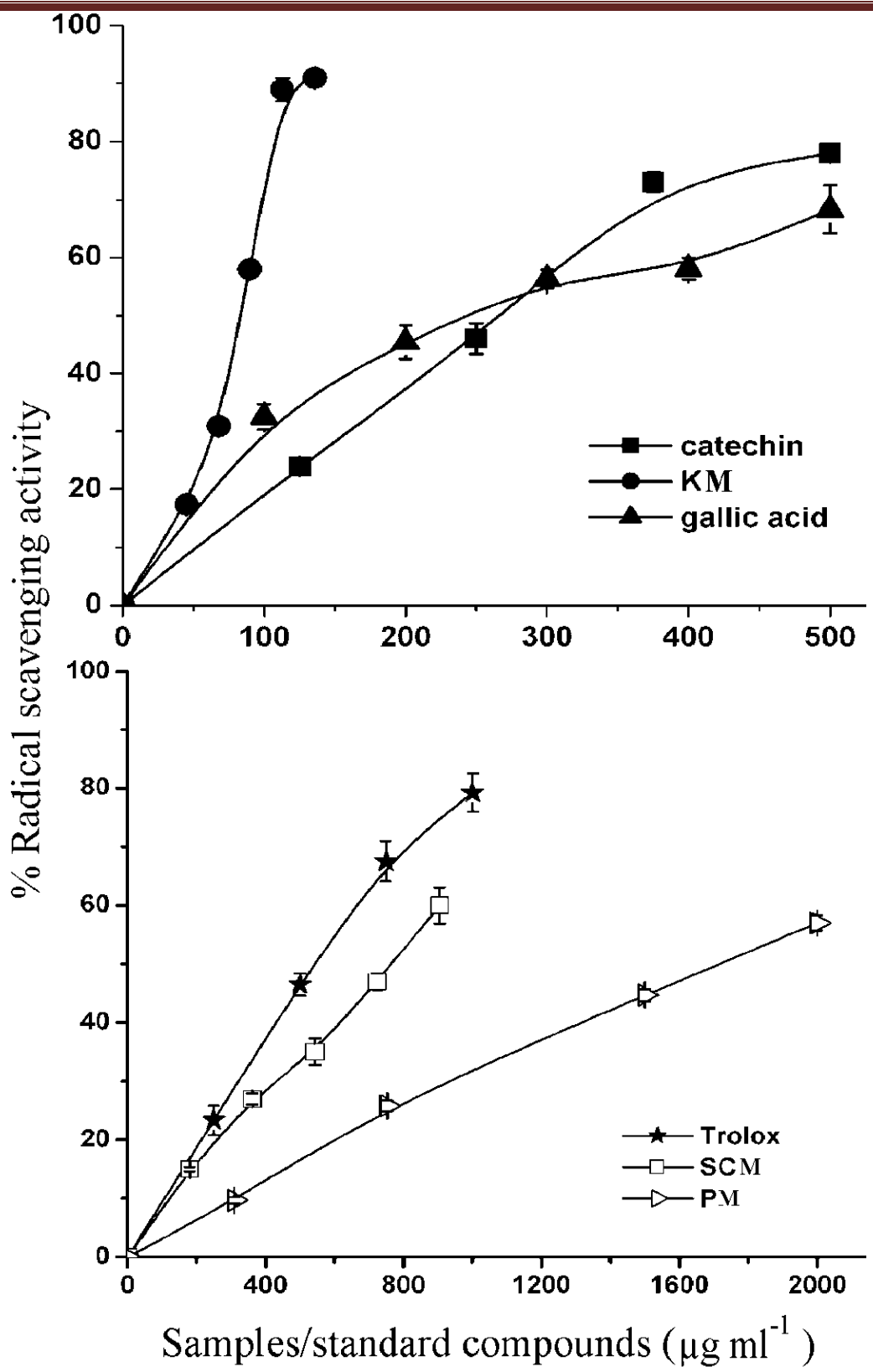

Fig. 3-4 Superoxide radical scavenging activity of *S. cumini* kernel methanol extract (KM), seed coat methanol extract (SCM), pulp methanol extract (PM), catechin, Trolox and gallic acid. (n=3 ± SD)

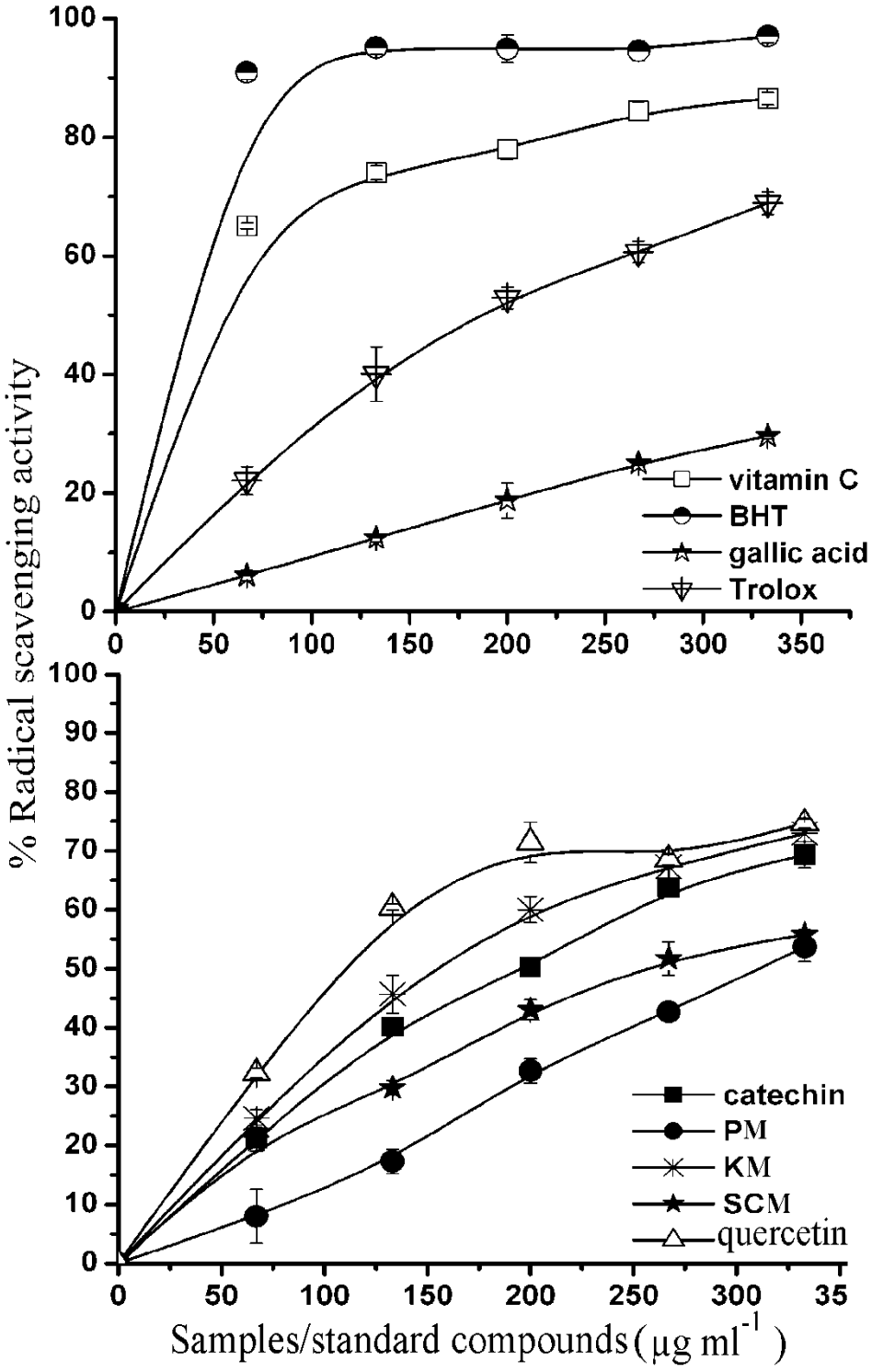

Fig. 3-5 Hydroxyl radical scavenging activity of *S. cumini* pulp methanol extract (PM), kernel methanol extract (KM), seed coat methanol extract (SCM), vitamin C, BHT, gallic acid, Trolox, catechin and quercetin. (n=3 ± SD)

3.2.1.4 Lipid peroxidation inhibition activity of SCF parts.

Oxidation of membrane phospholipids leads to loss of membrane integrity and hence that could affect normal cellular function such as membrane transport, signaling etc. Oxidized LDL is reported to initiate plaque formation in the inner lining of the vascular system and leading to atherosclerosis. Polyunsaturated fatty acid containing bis-allylic positions are more vulnerable to free radical attack by hydrogen abstraction. There are two principle mechanism involved in the inhibition or prevention of lipid peroxidation. One is the chain breaking action of antioxidants which donate one electron to the free radical formed and further progression is terminated. Second is the inhibition of chain initiation by scavenging reactive oxygen and nitrogen species (Ingold, 1961). In the present investigation, lipid peroxidation was induced in blood RBCs by *t*-BHP. The inhibition of lipid peroxidation was found to be dose dependent. Percentage antiperoxidative activity of different samples (KM, PM & SCM) and standard compounds are shown in Fig. 3-6. Among different samples, KM (IC50: 202 µg/ml) was more effective than SCM (268 µg/ml) and PM (342 µg/ml). BHT showed a high degree of antiperoxidative activity (IC50:79 µg/ml) than other standard compounds. Activity IC50 values for Trolox and quercetin was 175 µg/ml and 166 µg/ml respectively (Table 3-6). The extracts evaluated here thus showed lower activity than that of standards in the case of their ability to inhibit peroxidation of membrane lipids.

3.2.1.5 Total reducing power of SCF parts.

Several methods are available to measure the efficiency of dietary antioxidants. These methods focus on different mechanisms of the antioxidant activity, which is, scavenging active oxygen species and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions. By measuring the total reducing power, irrespective of the stage in the oxidative chain, the antioxidant action can be assessed. Most nonenzymatic antioxidative activity (scavenging of free radicals, inhibition of lipid peroxidation, etc.) is mediated by redox reactions. The reducing power of different extracts to reduce ferric ions was determined by this method (Zhu et al., 2002).

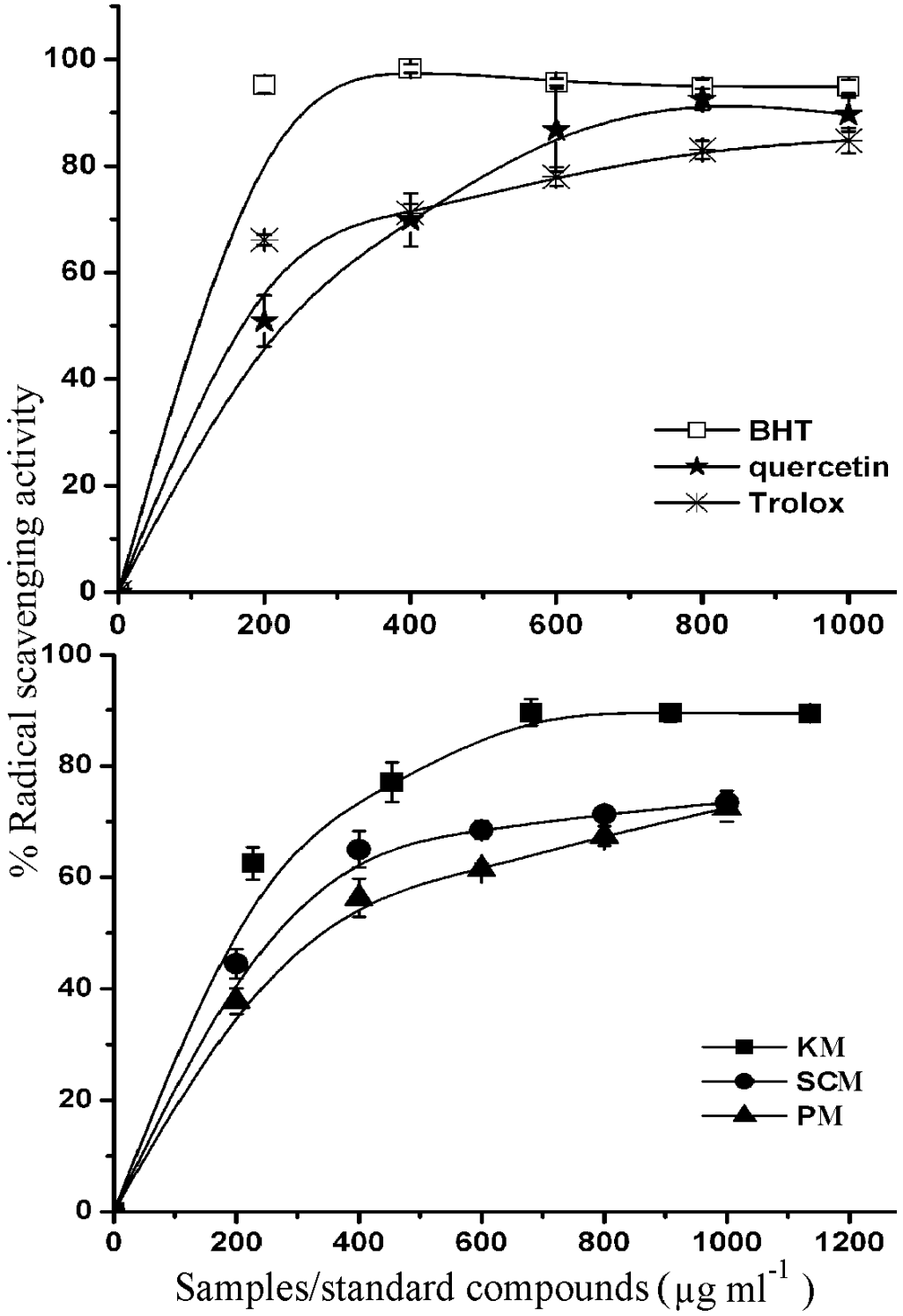

Fig. 3-6 Inhibition of lipid peroxidation in RBC membrane by *S. cumini* kernel methanol extract (KM), seed coat methanol extract (SCM), pulp methanol extract (PM), BHT, quercetin and Trolox. (n=3 ± SD)

Evaluation of total reducing power showed that KM had reducing activity greater

than SCM and PM. However vitamin C was found to be more active than the test samples. A linear relation was observed between the phenolic content and reducing activity within each samples (Fig. 3-7)

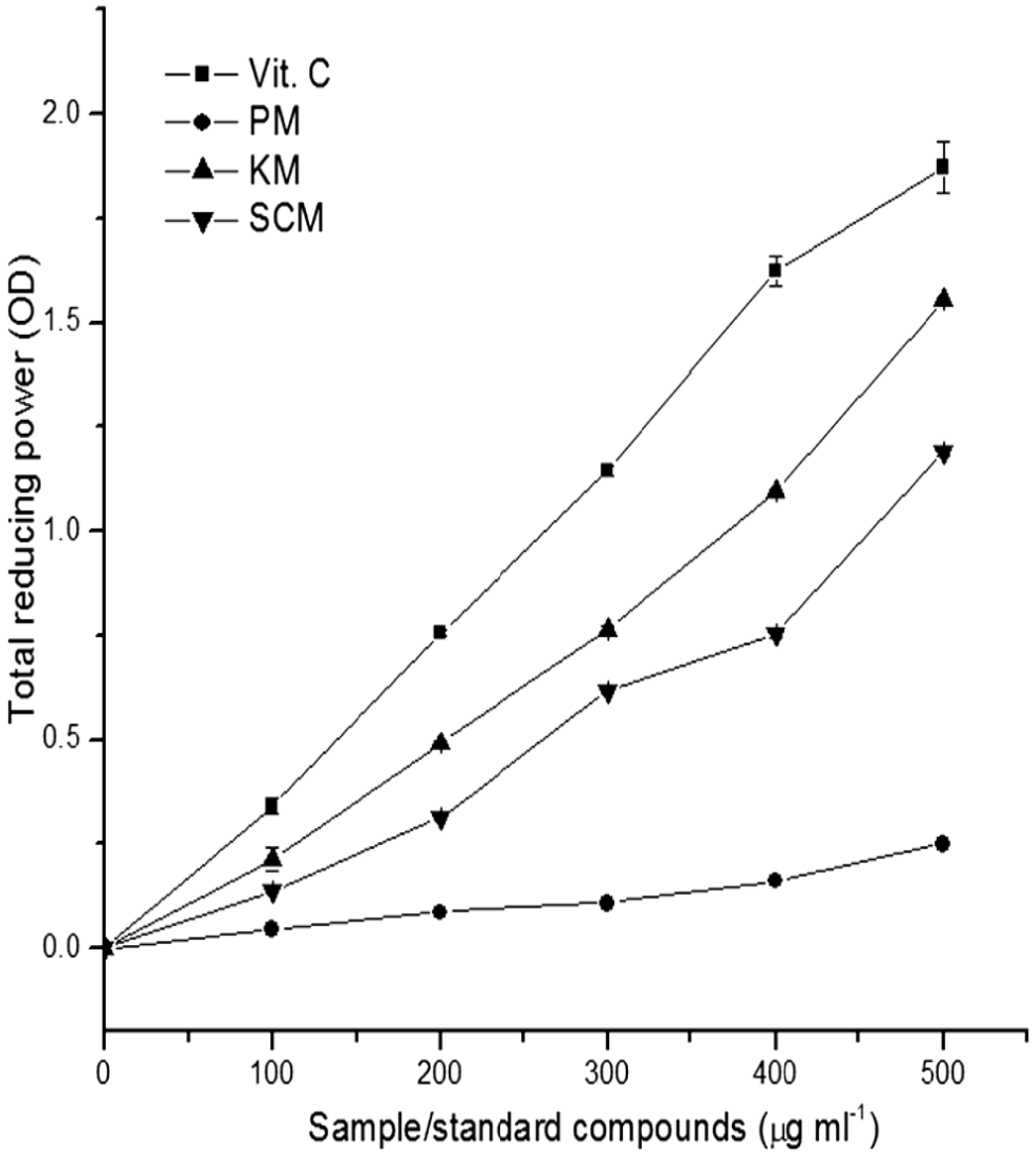

Fig. 3-7 Total reducing power of *S. cumini* kernel methanol extract (KM), seed coat methanol extract (SCM), pulp methanol extract (PM) and vitamin C. (n=3 ± SD)

Table 3-6 Antioxidant activities of PM, KM and SCM.

Sample	Radical Scavenging Assay Methods/Activity [IC50 (µg/ml)]			
	DPPH radical scavenging activity	Superoxide radical scavenging activity	Hydroxyl radical scavenging activity	Inhibition of lipid peroxidation
PM	158 ± 5	1703 ± 9	310 ± 10	342 ± 17
KM	8.6 ± 3	85 ± 5	151 ± 5	202 ± 13
SCM	48 ± 9	759 ± 14	261 ± 4	268 ± 13
gallic acid	1.3 ± 0.05	225 ± 6	--	--
vitamin C	7 ± 0.76	--	44 ± 7.2	--
BHT	40.6 ±3	--	21 ± 8	79 ± 4
quercetin	2.4 ± 2.3	--	102 ± 8	166 ± 13
Trolox	4.3 ± 1	540 ± 5	190 ± 38	175 ± 4
catechin	6 ± 0.2	296 ± 11	188 ± 6	--

PM: pulp methanol extract, KM: kernel methanol extract, SCM: seed coat methanol extract , (dry weight basis). (n=3 ± SD).

3.2.1.6 Significance of antioxidant capacity of SCF.

The chemical profiling and antioxidant evaluation of SCF parts brought out its nutritional and nutraceutical importance. The fresh pulp of SCF has slight astringency with acceptable taste and flavor. The anthocyanin in the edible part (SCP) of fruit was comparable with these of blueberry, blackberry and blackcurrant, whose nutraceutical properties are well documented, suggesting the potential nutraceutical value of SCF. Anthocyanins in these fruits are reported to be powerful antioxidant and stability studies showed that they are stable up to 6 months in dry pulps (Banerjee et al., 2005). Anthocyanins (cyanidin glucosides) have been shown to protect cell membrane lipids from oxidation (Tsuda, 1998). According to Rice-Evans, some cyanidins are many times more powerful antioxidants than tocopherols (Rice-Evans, 1995). Bertuglia et al. showed that anthocyanin supplements effectively inhibited inflammation and subsequent blood vessel damage and maintained the integrity of vascular micro capillaries in animal model (Bertuglia et al., 1995). Chemo preventive action and the molecular mechanism of anthocyanidins have been recently reviewed by Hou et al (Hou et al., 2004). Recent reports on the ability of anthocyanins to modulate insulin secretion have generated interest in fruits with deep colors such as blueberry, blackberry and raspberry (Jayaprakasam et al., 2005). However, varieties of richly colored tropical fruits is available but have not been investigated for their therapeutic properties though these fruits have been consumed for centuries. SCF fruit is one of such fruits with a deep purple color and is rich in anthocyanins, as shown in the present studies and it is grown widely in the Indian sub-continent. Very limited studies are conducted on SCF correlating its chemical composition and biological activities, though the fruit parts are used in Indian traditional medicine for management of hyperglycemia. In a recent study (Anandharajan et al., 2006) reported the ability of Jamun seed extract to modulate glucose transport through expression of specific receptors using myocytes. This finding supports the health claim of Jamun seed as antidiabetic agent by practitioner of Indian traditional medicine. However, numerous studies have been reported concerning the health benefits of anthocyanin-bearing fruits such as cranberry and raspberry

(Dvaranauskaite et al., 2006; He and Rui, 2006). The potential of black raspberry methanol extract to inhibit tumor development in mouse epithelial cells mediated by impairing signal transduction pathways leading to activation of transcription factors has been demonstrated. The factors include activator protein 1 (AP1) and nuclear factor kappa B (NF-κB) that are responsible for down regulation of vascular endothelial growth factors (VEGF) and COX-2 expressions (Lu et al., 2006). In another study a specific anthocyanin (cyanidin-3-glucoside) isolated from blueberry has been shown to inhibit UVB radiation and 12-O-tetradecanolyphorbol-13-acetate (TPA) induced transactivation of NF-kB, AP1 and expression COX-2 and tumor necrosis factor-alpha (TNF-α) and attributed these effects to the inhibition of mitogen-activated protein kinase (MAPK) activity in the cultured JB6 cell line (Ding et al., 2006). Cyanidin-3-glucoside from blackberry is further reported to suppress nitric oxide production, indicating anti-inflammatory properties of this anthocyanin (Pergola et al., 2006). The results of the in vitro models do not necessarily mean that the anthocyanins are biologically active under in vivo conditions because of the biotransformation of these molecules (Duthie et al., 2006). Studies using extracts from blackberry, blueberry and other anthocyanins containing fruits have demonstrated their effects on inflammation, neuroprotection, oxidative stress in vitro as well as in vivo models (Galli et al., 2006; Rossi et al., 2003; Wang and Lin, 2000). However, in another study the consumption of cranberry juice was not found to be effective against heart disease and cancer in healthy human volunteers (Duthie et al., 2006). Epidemiological data also suggest a strong association between consumption of fruits and vegetables and lower incidence of cancer, CVD and inflammation that could be due to chemo preventive and antioxidant properties of the phytochemicals present. The non-edible part of many fruits, particularly the kernels, is rich in polyphenols and flavonoids with high antioxidant activity. The biological properties of some of them have been validated scientifically, while many of them are yet to be studied. Most of these seeds are not palatable and therefore not consumed as food. Plant polyphenols comprise different classes of compounds, such as phenolic acids, flavonoids, anthocyanins and stilbene. Many plant derived traditional medicines are reported to contain substantial amounts of

flavonoids and are proven to have antibacterial, anti-inflammatory, anti-allergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory activities (Alan and Miller, 1996). Extracts of SCF parts evaluated in this study using four assay methods had strong antioxidant activity in the order KM > SCM > PM. Among these extracts, KM was found possesses antioxidant activity comparable or better than that of standard antioxidants in terms of DPPH, superoxide and hydroxyl radical scavenging properties. Comparison between the activities obtained in six different methods is not relevant because of the complex and diverse constituents of phytochemicals and their different mechanisms in different assay systems. The same level of phenolic content in different anatomical parts of the fruit, viz. PM and KM, thus did not show a similar antioxidant response perhaps due to the chemical structure of their constituent phytochemicals and this is supported by previous authors (Parejo et al., 2002). The present study is the first in the series to establish the possible therapeutic and chemo preventive properties of SCF which is very rich in anthocyanins and antioxidant phytochemicals that may have similar biological effects as those demonstrated in the case of blueberry and blackberry fruits. Detailed characterization of the phytochemicals based on activity-guided fractionation of SCP and SCK could lead to isolation of the active principles. SCF thus has high potential to yield products of nutraceutical value. The Jamun seed used in traditional medicine as a hypoglycemic agent requires further investigation to establish the relation, if any, between its antioxidant property and reported hypoglycemic effect.

3.2.2 Sequential extraction and antioxidant activity evaluation of SCF

Plants contain diverse phytochemicals of varying chemical structures and therefore require separation into groups employing different techniques. One simple method of grouping all phytochemicals is based on their polarity using solvents of varying polarity. The method used here in brief; freeze-dried fruit parts were sequentially extracted with different solvents in the order of increasing polarity as follows; hexane < dichloromethane < ethyl acetate < methanol < water. Extraction using each solvent was optimized The extracts thus obtained (PHs, pulp hexane extract; PDs, pulp DCM extract; PEs, pulp EtOAc extract; PMs, pulp

MeOH extract, PWs, pulp water extract; KHs, kernel hexane extract; KDs, kernel DCM extract; KEs, kernel EtOAc extract; KMs, kernel MeOH extract; KWs, kernel water extract; SCHs, seed coat hexane extract; SCDs, seed coat DCM extract; SCEs, seed coat EtOAc extract; SCMs, seed coat MeOH extract; SCW, seed coat water extract) were used to study the distribution of antioxidant activity between compounds belonging to various groups This approach is useful to understand the nature of active compounds and facilitate bioassay guided fractionation.

3.2.2.1 Yield and compositional profile of extract

Fully ripened SCFs were separated into anatomically distinct parts viz. pulp, kernel and seed coat (Table 3-1) and freeze-dried for sequential extraction. The yield of freeze-dried parts were in the following order; kernel (52± 2%) >pulp (30± 3%) >seed coat (17±3%).

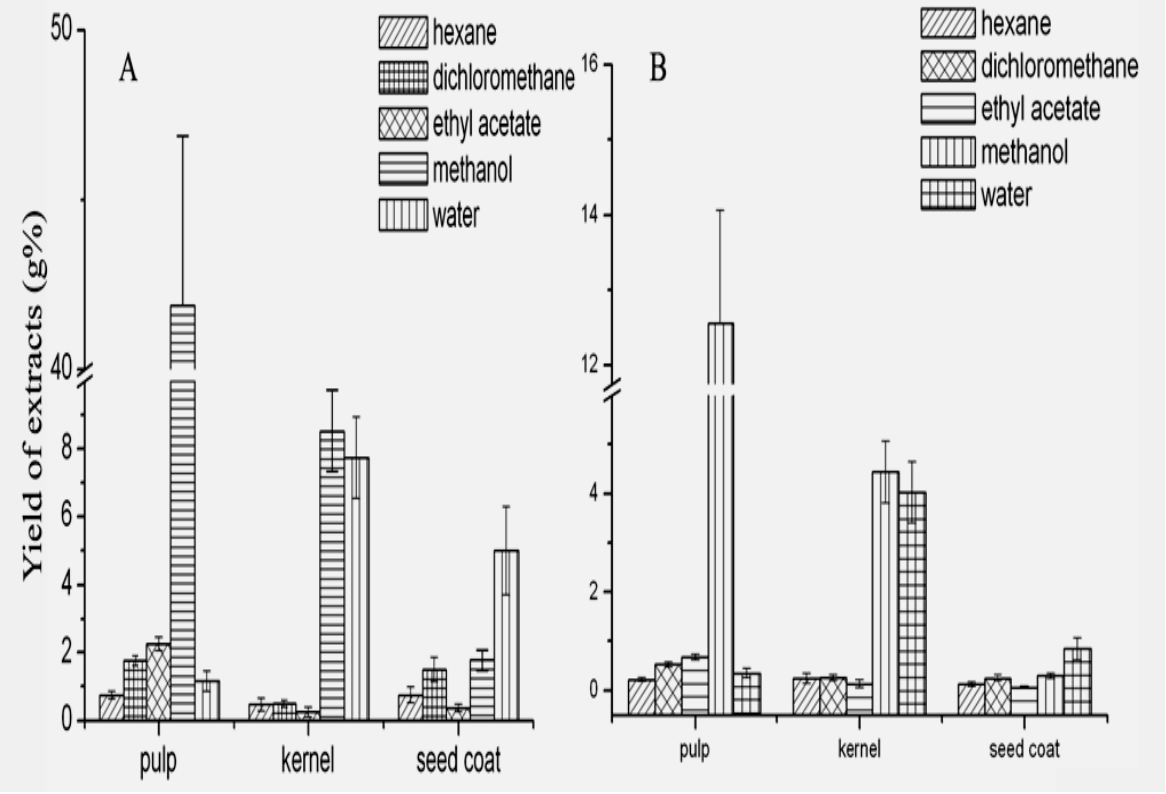

Fig. 3-8 Yield of different solvent extracts (g % on dry weight basis). A, based on freeze-dried fruit parts; B, based on whole freeze-dried fruit.

The freeze-dried fruit parts were subjected to successive extraction using five solvents in the order of increasing polarity viz. hexane, DCM, EtOAc, MeOH and water as depicted in Fig. 2-2. Fig. 3-8 (A) shows the percentage extractable substances from the three freeze dried fruit parts. Hexane, DCM and EtOAc extracted less than 2% by weight of the dry pulp. MeOH extract contained 42.0 % soluble substance and in water extract it was 1.0 %. Kernel yielded less than 1.0% solids with hexane DCM and EtOAc while MeOH and water extracts were found to be 8.0 and 7.0% respectively. Seed coat yielded 0.7%, 1.5%, 0.37% solids respectively by hexane, DCM and EtOAc. MeOH and water extracts of seed coat were 1.7%, 5.0% respectively. Fig. 3-8 (B) presents the yield of different solvent extracts based on whole freeze-dried fruit and the yield of PM, KM and KW were found to be far greater than those of less polar and non-polar extracts.

Total phenolic content of all extracts were estimated and expressed in percentage gallic acid equivalent based on total solids (Table 3-7). Relatively a high phenolic enrichment was observed in all polar extracts compared to those in the intermediate polar and non polar solvent viz. EtOAc, DCM, and hexane. Total phenolic contents in intermediately polar and less polar extracts of all three fruit parts were less than 10%. In the case of polar extracts, most of the phenolic compounds were extracted in methanol and hence in water extracts, the TPC were found to be very less. In Jamun fruit pulp, total phenolic content in PMs was 32.7% but in PWs the TPC was 4.3%. Same pattern of phenolic enrichments in methanol extracts were also seen in Jamun kernel and seed coat.

Distribution of flavonoids in the extracts was more or less similar to that of TPC. The deep purple color of the pulp is due to anthocyanins and their content was estimated in pulp alone and expressed in cyaniding-3-glucoside equivalent using the pH differential colorimetric method. Anthocyanins were not detected in PHs and PDs, but PEs, PMs and PWs contained 0.02, 0.37 and 0.64% respectively.

Table 3-7 Total phenolic, flavonoid and anthocyanin content of different extracts from anatomically distinct parts (pulp, kernel and seed coat) of freeze-dried *Syzygium cumini* fruit.

Sample		Total phenolic content	Total flavonoids	Anthocyanins
		(% dry weight of extracts)		
Pulp	PHs	0.01 ±0.00	nd --	nd --
	PDs	6.35 ±0.13	0.09 ±0.06	nd --
	PEs	2.59 ±0.12	0.25 ±0.02	0.02 ±0.006
	PMs	32.72 ±0.09	0.9 ±0.02	0.37 ±0.050
	PWs	4.43 ±0.23	0.09 ±0.04	0.064 ±0.002
Kernel	KHs	3.74 ±0.43	0 ±0.00	-- --
	KDs	7.34 ±0.57	0.02 ±0.03	-- --
	KEs	20.71 ±1.28	0.58 ±0.03	-- --
	KMs	35.54 ±1.04	3.62 ±0.05	-- --
	KWs	8.56 ±0.40	0.44 ±0.02	-- --
Seed coat	SCHs	0.63 ±0.03	0 ±0.00	-- --
	SCDs	3.27 ±0.04	0.13 ±0.19	-- --
	SCEs	2.27 ±0.00	0.65 ±0.04	-- --
	SCMs	25.56 ±0.25	2.05 ±0.15	-- --
	SCWs	11.43 ±0.21	0.75 ±0.02	-- --

PHs, pulp hexane extract; PDs, pulp dichloromethane extract; PEs, pulp ethyl acetate extract; PMs, pulp methanol extract, PWs, pulp water extract; KHs, kernel hexane extract; KDs, kernel dichloromethane extract; KEs, kernel ethyl acetate extract; KMs, kernel methanol extract; KWs, kernel water extract; SCHs, seed coat hexane extract; SCDs, seed coat dichloromethane extract; SCEs, seed coat ethyl acetate extract; SCMs, seed coat methanol extract; SCWs, seed coat water extract. nd, not detected. Extraction was performed sequentially with different solvents in the order of increasing polarity. All values are the mean of at least three experiments (n=3±SD) and expressed in % dry weight of extracts.

3.2.2.2 Antioxidant activity evaluation of the extracts

Total reducing power: A high reducing potential of the antioxidant molecule at very low concentration with respect to the oxidizable substrate is very essential to be considered as an effective antioxidant. The method adopted in the present study evaluated the efficacy of extracts at 500µg/ml concentration to reduce ferric ion by electron transfer reaction (Meir et al., 1995). The reducing potential of the extract is proportional to the color developed and expressed in absorbance unit (Table 3-8). Highest reducing potential was observed in KMs (1.34 OD) and SCMs (1.1 OD) compared to all other extracts with less polar solvents. Water extract of both kernel and seed coat showed very less reducing activity. All non-polar and polar extracts of pulp had TRP less than 0.20 OD. Among the standard antioxidant studied here, gallic acid was found to be the most active (>2.0) than vitamin C (1.82±0.01) and Trolox (1.16±0.03).

DPPH radical scavenging activity: The dose dependent DPPH radical scavenging activity of different extracts of pulp, kernel, seed coat and standard antioxidants are presented in Fig. 3-9. In the present study, up to 1000 µg (dry weight) of samples in an appropriate minimum volume was used for evaluating the activity. No significant RSA was observed for PHs and PWs up to 1000 µg/ ml. However, PDs, PEs and PMs showed a dose dependent RSA. In the case of kernel, all extracts showed a concentration dependent radical scavenging activity below 1000 µg/assay. Among the kernel extracts, KMs and KWs were the most active. In the case of extracts from seed coat, SCMs and SCWs exhibited significant RSA similar to that of kernel extract. IC50 values for the active samples viz. KEs, KMs, KWs, and SCMs were 87±1.6, 34.2±0.46, 31±2.0 and 38.3±152 µg/ml respectively (Table 3-8).

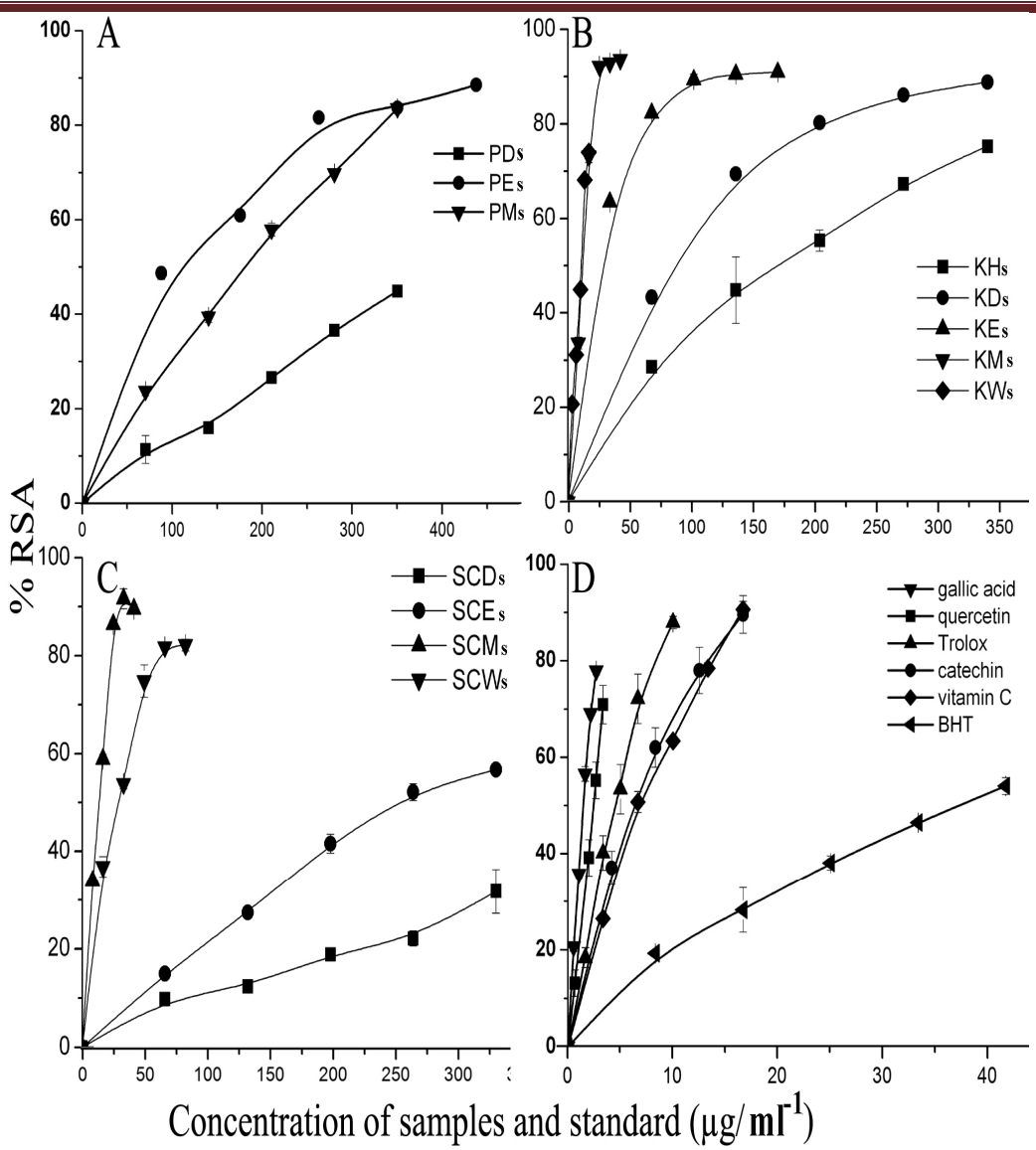

Fig. 3-9 DPPH radical scavenging activity of different solvent extracts (sequential) from anatomically distinct parts of freeze-dried SCF and standard antioxidant molecules. A, pulp extracts(PHs, pulp hexane; PDs, pulp dichloromethane; PEs, pulp ethyl acetate; PMs, pulp methanol, PWs, pulp water extract); B, kernel extracts; (KHs, kernel hexane; KDs, kernel dichloromethane; KEs, kernel ethyl acetate; KMs, kernel methanol; KWs, kernel water); C, seed coat extracts (SCHs, seed coat hexane; SCDs, seed coat dichloromethane; SCEs, seed coat ethyl acetate; SCMs, seed coat methanol; SCWs, seed coat water); D, standards,

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity of extracts and standards are presented in Fig. 3-10. Only methanol extracts of fruit parts (PMs, KMs and SCMs) showed more than 50% RSA below 1000 µg/assay system which is comparable with standard gallic acid and Trolox. Hexane, DCM and ethyl acetate extracts of all three fruit parts showed less than 30% RSA below 1000 µg/assay. But, water extract did not show any measurable response up to the highest concentration used in the study. The IC50 values were derived from dose response curve and presented in Table 3-8. Hydroxyl radical scavenging activity (IC50) of KMs, SCMs and PMs were; 228±9.86, 246 ±30 and 308±57 µg /ml respectively. Activities of standards were; BHT, 24±3; vitamin C, 46±7; quercetin, 110±9; Trolox, 188±38 and catechin, 305±21 µg/ml.

Superoxide radical scavenging activity: The superoxide radical scavenging activity of extracts from fruit parts were evaluated and presented in Fig. 3-11. Among the pulp extracts, PEs exhibited a dose dependent RSA but, PMs and PWs was not potent enough to scavenge more than 50% radicals up to 1000 µg/ml. In the case of kernel, only methanol extract showed a significant RSA. The activity of KHs, KMs and KWs were very low. KDs did not show any measurable RSA. Within the seed coat extracts, SCMs and SCEs scavenged superoxide radicals in a dose dependent manner but the activity of SCWs was very less. SCHs and SCDs did not show any measurable RSA up to 1000µg/ml concentration in the assay system. Among the various reference standards studied here, Trolox and catechin scavenged superoxide radicals in a dose dependent fashion but at higher concentration range compared to samples. The IC50 values of PEs, KMs, and SCEs were; 28.8±0.72, 58.±0.95, and 47.3±2 µg/ml respectively (Table 3-8). Among the reference standards, catechin was found to be most active (268 ± 9 µg/ml) and the above said samples were more than four times active than catechin.

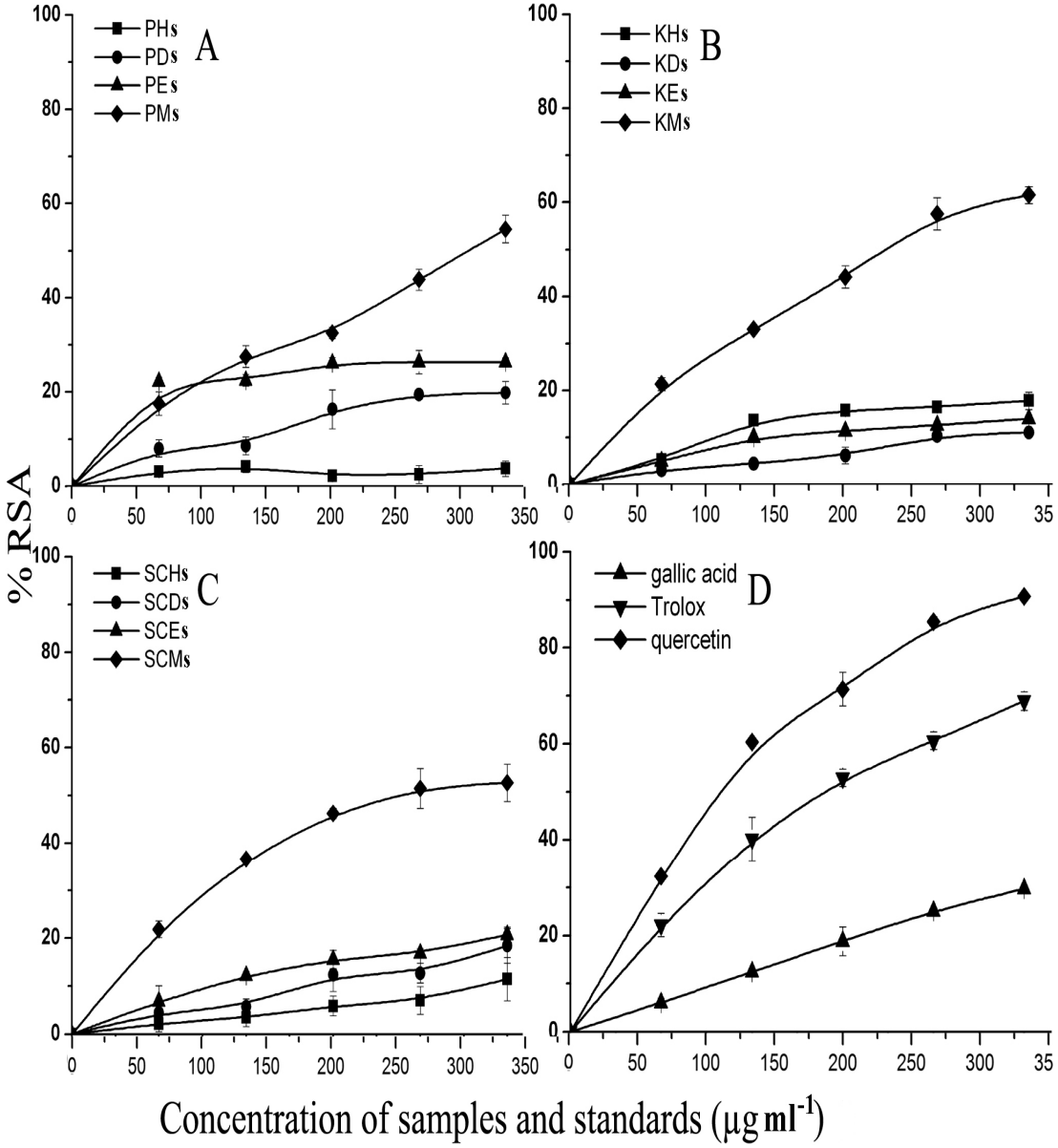

Fig. 3-10 Hydroxyl radical scavenging activity of different solvent extracts (sequential) from anatomically distinct parts of freeze-dried SCF and standard antioxidant molecules. A, pulp extracts(PHs, pulp hexane; PDs, pulp dichloromethane; PEs, pulp ethyl acetate; PMs, pulp methanol, PWs, pulp water extract); B, kernel extracts; (KHs, kernel hexane; KDs, kernel dichloromethane; KEs, kernel ethyl acetate; KMs, kernel methanol; KWs, kernel water); C, seed coat extracts (SCHs, seed coat hexane; SCDs, seed coat dichloromethane; SCEs, seed coat ethyl acetate; SCMs, seed coat methanol; SCWs, seed coat water); D, standards,

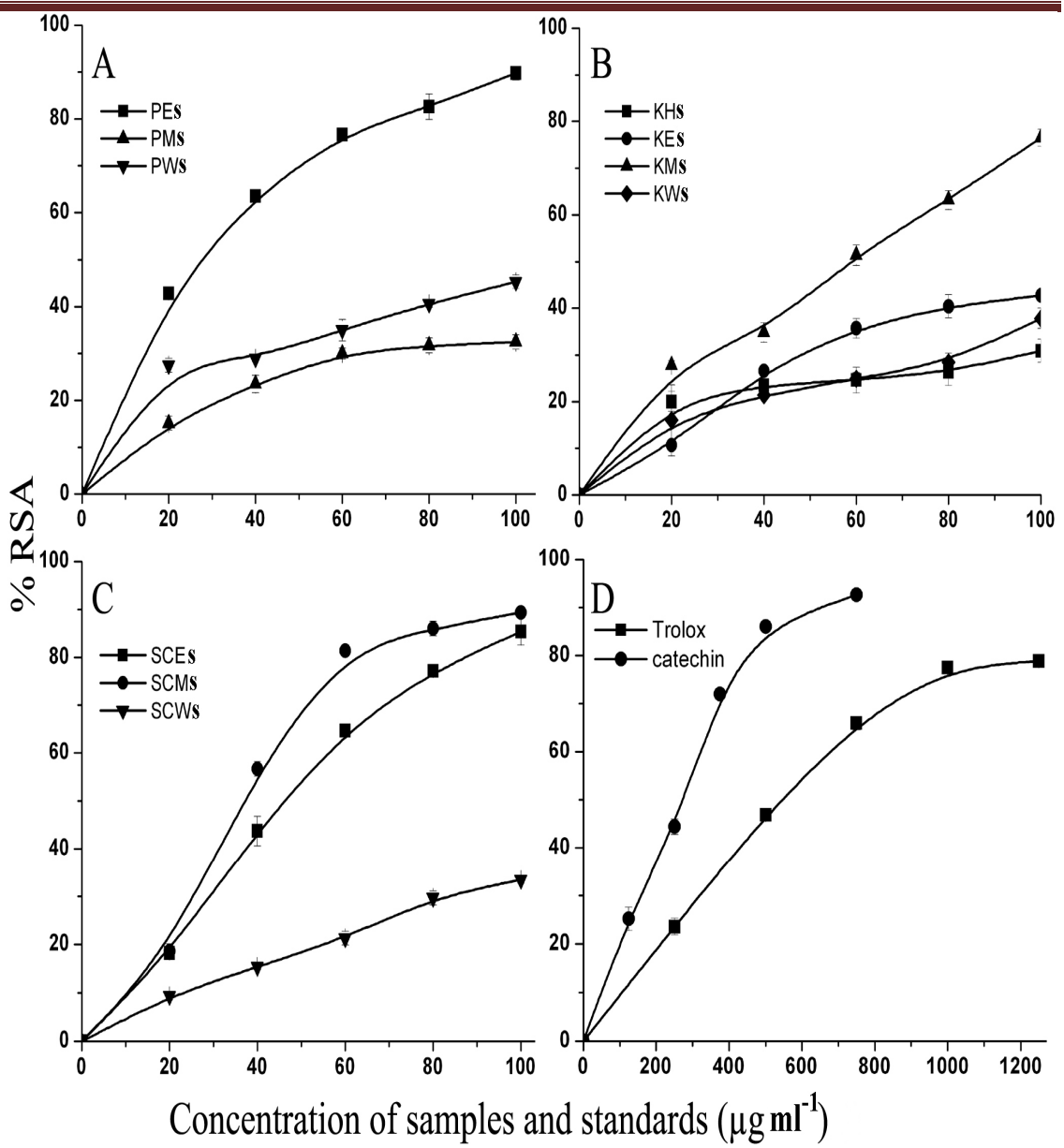

Fig. 3-11 Superoxide radical scavenging activity of different solvent extracts (sequential) from anatomically distinct parts of freeze-dried SCF and standard antioxidant molecules. A, pulp extracts(PHs, pulp hexane; PDs, pulp dichloromethane; PEs, pulp ethyl acetate; PMs, pulp methanol, PWs, pulp water extract); B, kernel extracts; (KHs, kernel hexane; KDs, kernel dichloromethane; KEs, kernel ethyl acetate; KMs, kernel methanol; KWs, kernel water); C, seed coat extracts (SCHs, seed coat hexane; SCDs, seed coat dichloromethane; SCEs, seed coat ethyl acetate; SCMs, seed coat methanol; SCWs, seed coat water); D, standards,.

Inhibition of lipid peroxidation: The ability of extracts from Jamun fruit parts and standard compounds to inhibit the lipid oxidation under the controlled peroxidation condition is presented in Fig. 3-12. Among the five extracts of pulp, ethyl acetate and methanol extracts showed more than 50% RSA below 1000 µg/assay system. Other non polar extracts of pulp and PWs did not show a measurable radical scavenging activity at this concentration range. In the case of extracts from kernel; KDs, KEs and KMs showed a dose dependent RSA below 1000 µg/ml. Most of the extracts from seed coat (SCHs, SCDs, SCEs and SCMs) inhibited lipid oxidation below1000 µg/assay. SCM was found to be more potent among all samples. The IC50 values obtained from the dose response curve of different samples and standards are presented in Table 3-8. Evaluation of IC50 values obtained from the various extracts showed that, methanol extracts of pulp, kernel, and seed coat were relatively more active in inhibiting lipid oxidation with IC50 value less than 200µg/ml. For all other extracts, the IC50 values were above 500 µg/ml. All other extracts with less activity was observed as non polar extracts of fruit parts. Methanol extracts of all three fruit parts were comparable with that of the reference standards Trolox (130±4 µg/ml) and quercetin (180±24 µg/ml). BHT (70±4 µg/ml) was found to be higher than that of all standards and extracts.

The results of all antioxidant assays on all extracts are summarized in Fig. 3-13 and Table 3-8. Fig. 3-13 is a graphical representation of relative antioxidant activity of all extracts in different assay systems with respect to the reference standard Trolox. Though different standards were used in the study, Trolox was selected for calculating relative antioxidant activity because of reproducibility and low interference in all assay system. For simplicity, the ratios of IC50 values of samples and Trolox $\left(\frac{IC50Trolox}{IC50Sample}\right)$ were computed and presented as relative antioxidant activity. Assuming 1.0 as middle value for the reference standard, Trolox, the computed ratios of all extracts were graphically transformed that ranged from 0.0 (green) to 19.1 (red) with the middle value 1.0 (black). From the color graphics, SCM and KM were found to be active in all radical scavenging assay systems (the row is shaded). The activities of PM was prominent against $OH^{\bullet}$ and $ROOH^{\bullet}$. However, the activity of PE against $O2^{\bullet-}$ was high.

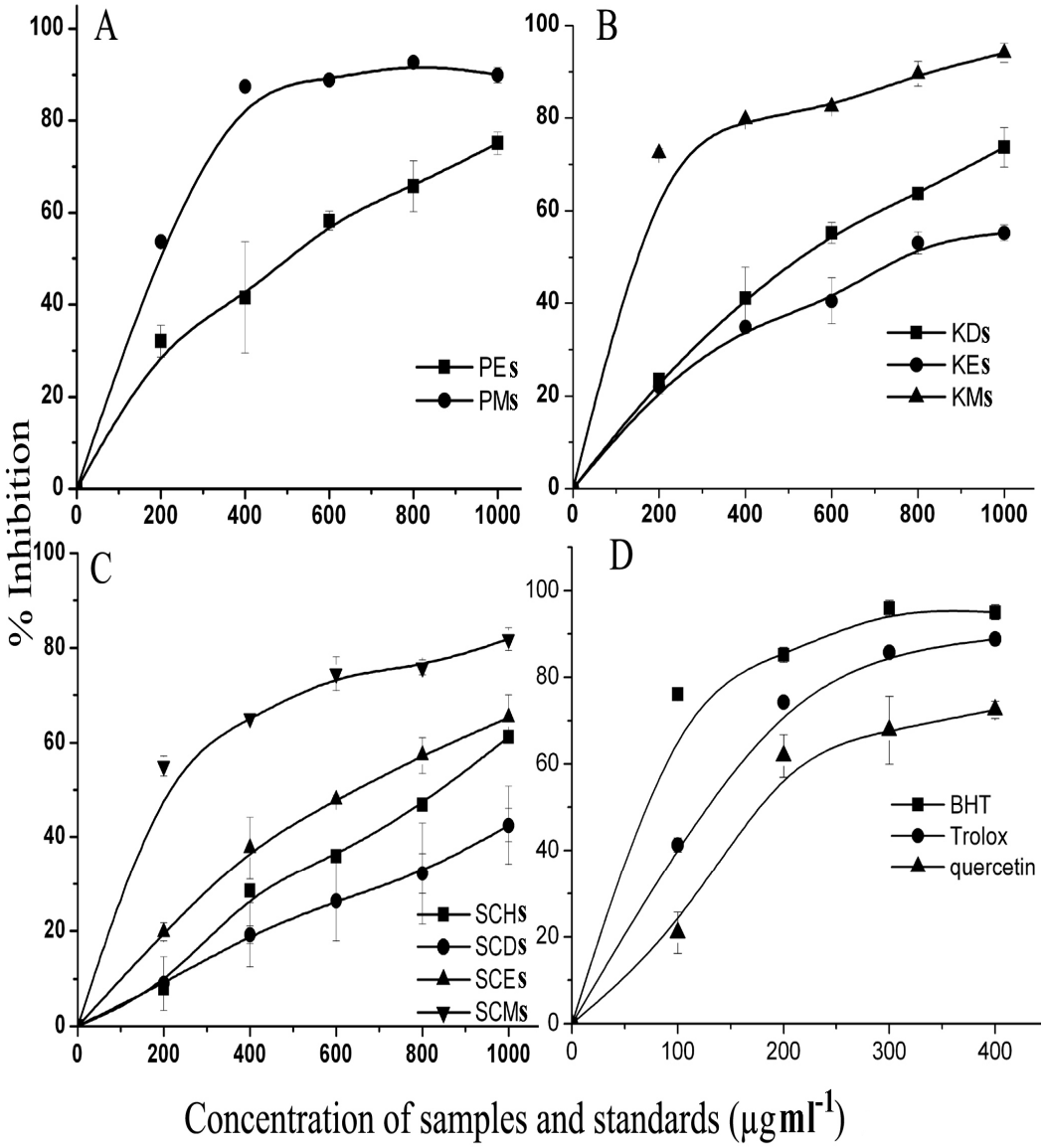

Fig. 3-12 Inhibition of lipid peroxidation by different solvent extracts (sequential) from anatomically distinct parts of freeze-dried SCF and standard antioxidant molecules. A, pulp extracts(PHs, pulp hexane; PDs, pulp dichloromethane; PEs, pulp ethyl acetate; PMs, pulp methanol, PWs, pulp water extract); B, kernel extracts; (KHs, kernel hexane; KDs, kernel dichloromethane; KEs, kernel ethyl acetate; KMs, kernel methanol; KWs, kernel water); C, seed coat extracts (SCHs, seed coat hexane; SCDs, seed coat dichloromethane; SCEs, seed coat ethyl acetate; SCMs, seed coat methanol; SCWs, seed coat water); D, standards,.

Sample	Total reducing power	DPPH radical scavenging acyivity	Hydroxyl radical scavenging activity	Superoxide radical scaveging activity	Inhibition of lipid peroxidation
PHs	0.009	0.000	0.000	0.000	0.000
PDs	0.043	0.000	0.000	0.000	0.000
PEs	0.103	0.040	0.000	19.028	0.257
PMs	0.129	0.025	0.606	0.000	0.658
PWs	0.155	0.000	0.000	0.000	0.000
KHs	0.086	0.026	0.000	0.000	0.000
KDs	0.026	0.050	0.000	0.000	0.245
KEs	0.681	0.148	0.000	0.000	0.169
KMs	1.155	0.391	0.820	9.448	0.861
KWs	0.138	0.430	0.000	0.000	0.000
SCHs	0.034	0.000	0.000	0.000	0.154
SCDs	0.052	0.000	0.000	0.000	0.000
SCEs	0.112	0.017	0.000	11.586	0.199
SCMs	0.948	0.235	0.758	0.000	0.602
SCWs	0.198	0.159	0.000	0.000	0.000
Trolox	1.000	1.000	1.000	1.000	1.000

Fig. 3-13 Antioxidant activity ratio table of different solvent extracts of anatomically distinct parts of freeze-dried SCF under various assay systems. The color scale ranges from zero (green) to 19.1 (red). The middle value is 1.0 (black) that represents the activity or activity equivalent to Trolox standard. The values below 1.0 (color toward green from black) shows the low activity and values above 1.0 (color towards red from black) shows a higher activity with respect to Trolox. Different antioxidant activity studies are as follows, A; Total reducing power, B; DPPH•, C; OH•, D; O2•- and E; ROO• scavenging assay. PHs, pulp hexane extract; PDs, pulp DCM extract; PEs, pulp EtOAc extract; PMs, pulp MeOH extract, PWs, pulp water extract; KHs, kernel hexane extract; KDs, kernel DCM extract; KEs, kernel EtOAc extract; KMs, kernel MeOH extract; KWs, kernel water extract; SCHs, seed coat hexane extract; SCDs, seed coat DCM extract; SCEs, seed coat EtOAc extract; SCMs, seed coat MeOH extract; SCW, seed coat water extract

Table 3-8. Antioxidant activities of different extract of *Syzygium cumini* (Jamun) fruit parts (pulp, kernel and seed coat) and standard antioxidants in different radical scavenging assay systems

Sample/Standard		Radical scavenging assay methods				
		Total reducing power	DPPH radical scavenging activity	Hydroxyl radical scavenging activity	Superoxide radical scavenging activity	Inhibition of lipid peroxidation
Sample/ standards		Absorbance unit	IC50 (µg/ corresponding assay system)			
Pulp	PHs	0.01±0.00	-	-	-	-
	PDs	0.05±0.01	-	-	-	-
Pulp	PEs	0.12±0.00	108±10.21		28.8±0.72	505.3±85.5
	PMs	0.15±0.01	172±3.7	308.6±57	-	197.6±36.1
	PWs	0.18±0.01	-	-	-	-
Kernel	KHs	0.10±0.01	168±54.1	-	-	-
	KDs	0.03±0.00	86±9.5	-	-	530±22
Kernel	KEs	0.79±0.01	29±1.6	-	-	771±16
	KMs	1.34±0.04	11±0.46	228±9.86	58.±0.95	151±11.5
	KWs	0.16±0.02	10±2.0	-	-	-
Seed coat	SCHs	0.04±0.00	-	-	-	845.3±30.2
	SCDs	0.06±0.01	-	-	-	-
Seed coat	SCEs	0.13±0.00	256±40.2	-	47.3±2	652.3±33.5
	SCMs	1.10±0.02	18.3±152	224 ±30	-	216±11.5
	SCWs	0.23±0.01	27±2.6	-	-	-
	Trolox	1.16±0.03	4.3±1.15	188±38	548 ± 2.5	130±4

PHs, pulp hexane extract; PDs, pulp DCM extract; PEs, pulp EtOAc extract; PMs, pulp MeOH extract, PWs, pulp water extract; KHs, kernel hexane extract; KDs, kernel DCM extract; KEs, kernel EtOAc extract; KMs, kernel MeOH extract; KWs, kernel water extract; SCHs, seed coat hexane extract; SCDs, seed coat DCM extract; SCEs, seed coat EtOAc extract; SCMs, seed coat MeOH extract; SCW, seed coat water extract. All fields marked with hyphen is either not active in the corresponding antioxidant activity assay or IC50>1000 µg/assay system. Extraction was performed sequentially with different solvents in the order of increasing polarity. All values are the mean of at least three experiments (n=3±SD).

Evaluation of the results of all assay systems used here, kernel was most active compared to pulp and seed coat. Among the different extracts of the fruit parts evaluated here, KM was found to be a rich source of phenolic compounds and showed relatively a high antioxidant activity against different free radicals. Though PM was not active against all radicals, it was rich in anthocyanins and showed relatively high hydroxyl and peroxyl radical scavenging activity. Seed coat also showed relatively a high antioxidant activity comparable with that of kernel, but its total yield limits as a major source for phenolic compounds. Extraction of fruit parts based on polarity showed that the maximum yield and activity was with polar solvents. Further work therefore should be based on KM considering the overall antioxidant activity and yield of polyphenols.

3.2.3 Activity guided fractionation.

Among the five extracts prepared following sequential extraction method as discussed before, methanol extracts of all fruit parts showed highest activity. Based on these studies two major conclusions could be drawn; 1, kernel is rich in antioxidants. 2, most antioxidant phytochemicals in SCF are polar in nature. Based on these findings, further studies were conducted on SCF focusing on methanol extract. Antioxidant activity guided fractionation thus on this fraction was conducted at two stages; first; solvent-solvent fractionation of methanol extracts to obtain a phenolics enriched fraction and second; fractionation of this fraction by using preparative HPLC to obtain semi pure antioxidant rich fraction.

3.2.3.1 Solvent- solvent fractionation

To prepare antioxidant enriched portion from SCF, Methanol extracts of freeze-dried fruit parts were fractionated based on solvent-solvent partitioning technique. The method briefly, methanol extract of SCF parts were desolventized and resuspended in water. The water suspension was then fractionated with other immiscible solvents viz. hexane dichloromethane, ethyl acetate, and butanol in the order of increasing polarity sequentially (Section 2.4.2). The fractions thus obtained were desolventized, redissolved in methanol and evaluated for antioxidant activity.

Yield of fractions of PM, KM and SCM: Yield of each fraction was quantified as dry matter gravimetrically and presented as percentage of parent extract (methanol extract of freeze-dried fruit parts) in Fig. 3-14. Hexane and dichloromethane fractions of KM, PM and SCM were found to be less than 5% of the total solids of corresponding methanol extracts. However, the intermediate polar ethyl acetate fractions from KM and SCM contained more dissolved matter (>15%). The butanol fraction from all three fruit parts were found to be more than 30% of total solids present in corresponding methanol extracts. Yield of residual water fraction was the highest (50-60%)

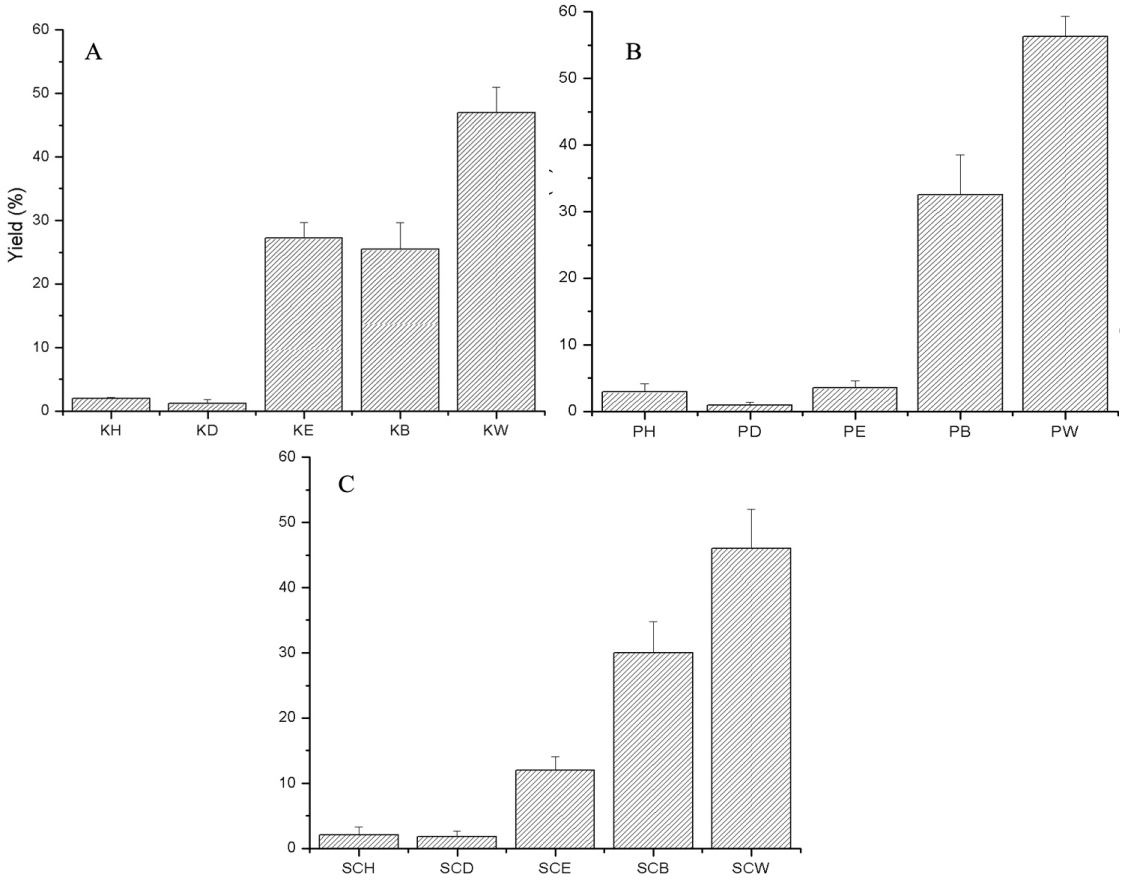

Fig. 3-14 Yield of various fractions (liquid-liquid) from *S. cumini* fruit part methanol extracts. A: hexane (KH), dichloromethane (KD), ethyl acetate (KE), butanol (KB) and water (KW)) fractions of kernel methanol extract (KM); B: fractions (PH, PD, PE, PB AND PW) of pulp methanol extract (PM); C: fractions (SCH, SCD, SCE, SCB and SCW) of seed coat methanol extract (PM)

Distribution of total phenolic contents (TPC) of KM in its various fractions: Total phenolic content in crude methanol extracts (CME) of fruit parts were evaluated and discussed in section **Error! Reference source not found.** (Table 3-4). On solvent-solvent fractionation, based on polarity, the phytochemicals in CME were fractionated and the yields of fractions were depicted in Fig. 3-14. Total phenolic contents of fractions were evaluated and the distributions of TPC in them were calculated based on the corresponding yield and the results are presented in Table 3-9. Upon fractionation of KM using different solvents, based on polarity, the phenolic compounds were partitioned in different solvents. Out of the 38% phenolic content in KM (Table 3-4), 57.8% was partitioned in ethyl acetate fraction (KE).

Table 3-9 Distribution of total phenolics of KM in its various solvent fractions.

Solvent fractions	Yield [a] (% of KM)	%.phenolic content [b]	% distribution of TPC [c]
KH	2.0	5.0	00.26
KD	1.2	16.0	00.50
KE	28.0	80.0	57.80
KB	26.0	24.0	16.4
KW	47.0	29.0	35.86

Total phenolic content in the kernel crude methanol extract: 38%; a, % yield of fractions with respect to kernel methanol extract; b, phenolic content in each fractions (% dry weight basis); c, distribution of total phenolic content in fractions with respect to the phenolic content in crude methanol extract. KH, kernel hexane fraction; KD, kernel dichloromethane fraction; KE, kernel ethyl acetate fraction; KB, kernel butanol fraction and KW, kernel water fraction from methanol extract of kernel.

Percentage phenolics partitioned in KW and KB were; 35.9 and 16.4 respectively. Negligibly small portion of TPC was partitioned with hexane (0.26%) and dichloromethane (0.5%) solvents. Perusal of Table 3-9 shows that 80% of the total solids in KE were phenolic compounds and this figure was 57% of the total

phenols found in KM. Based on this high phenolic enrichment and partitioning of major portion of TPC of KM, KE is recommended for further fractionation after confirming the antioxidant efficacy. However, antioxidant evaluations of all fractions are envisaged to find the efficacy of other fractions viz. KB and KW.

Distribution of total phenolic contents (TPC) of PM in its various fractions: Total phenolic content of each fractions from PM were estimated. Table 3-10 presents the yield of fractions and corresponding distribution of phenolics found in PM. Out of 39% total phenolic content in PM, 47.5% was partitioned in water fraction. Next highest portion of pulp phenolics were partitioned with butanol fraction (37.5%). Though the portion of total phenolics in PM partitioned PW and PB seems to be high, percentage phenolics based on total solids in these fractions were 27 and 37 respectively.

Table 3-10 Distribution of phenolic compounds in the various solvent fractions of *S. cumini* pulp crude methanol extract

Solvent fractions	Yield [a] (% of PM)	%.phenolic content [b]	% distribution of TPC [c]
PH	2.94	4.1	0.38
PD	0.94	5.3	0.16
PE	3.53	62	6.83
PB	32.5	37	37.63
PW	56.3	27	47.50

Total phenolic content in the pulp crude methanol extract: 39%; a, % yield of fractions with respect to kernel methanol extract; b, phenolic content in each fractions (% dry weight basis); c, distribution of total phenolic content in fractions with respect to the phenolic content in crude methanol extract. PH, pulp hexane fraction; PD, pulp dichloromethane fraction; PE, pulp ethyl acetate fraction; PB, pulp butanol fraction; PW, pulp water fraction from methanol extract of pulp.

Therefore rest of the solids in these fractions could be nonphenolics with less

antioxidant activity. In contrast to PW and PB, PE's 63% of total solids was found to be phenolics but, this figure is less significant because it is only 6.8% of the TPC in PM. Percentage of TPC distributed in PH (0.38%) and PD (0.16%) was negligibly less. Therefore antioxidant activity evaluation is envisaged as a criterion for further fractionation.

Distribution of total phenolic contents (TPC) of SCM in its various fractions: Total phenolic content of various fractions from SCM were estimated and calculated the distribution of TPC with reference to yield of fractions and TPC in SCM (Table 3-11).

Table 3-11 Distribution of phenolic compounds in the various solvent fractions of *S. cumini* seed coat crude methanol extract

Solvent fractions	Yield [a] (% of SCM)	%.phenolic content [b]	% distribution of TPC [c]
SCH	2.1	0.0924	0.34
SCD	1.8	0.108	0.4
SCE	12	6.6	24.4
SCB	30	7.8	28.8
SCW	46	10.58	39.1

Total phenolic content in the seed coat crude methanol extract: 27%; a, % yield of fractions with respect to crude methanol extract; b, phenolic content in fractions (dry weight basis); c,Calculated from the %content of TPC in fractions and their yield; d, phenolic distribution in fractions with respect to the phenolic content in crude methanol extract. SCH, seed coat hexane fraction; SCD, seed coat dichloromethane fraction; SCE, seed coat ethyl acetate fraction; SCB, seed coat butanol fraction; SCW, seed coat water fraction.

Percentage phenolic content in SCH and SCD were negligibly less and hence the TPC of SCM (27%) distributed in these fraction were 0.34 and 0.4 respectively. In the case of SCE, SCB and SCW, %phenolic distribution was proportional to their yields. However most of the phenolics (10.58%) of SCM were partitioned in SCW followed by SCB (7.8) and SCE (6.6). However no significant enrichment

proportional to the yield of fraction was observed in any of the fractions.

3.2.3.2 Antioxidant activity evaluation of solvent fractions of crude methanol extracts (CME) from kernel, pulp and seed coat

Apart from yield and TPC analyses, fractions were also evaluated for antioxidant activity. Dose dependent DPPH radical scavenging activities of fractions were observed (Fig. 3-15). Among the fractions studied here, highest activity was observed for ethyl acetate fractions followed by butanol fractions from fruit parts. However, ethyl acetate fraction from kernel methanol extract showed highest activity

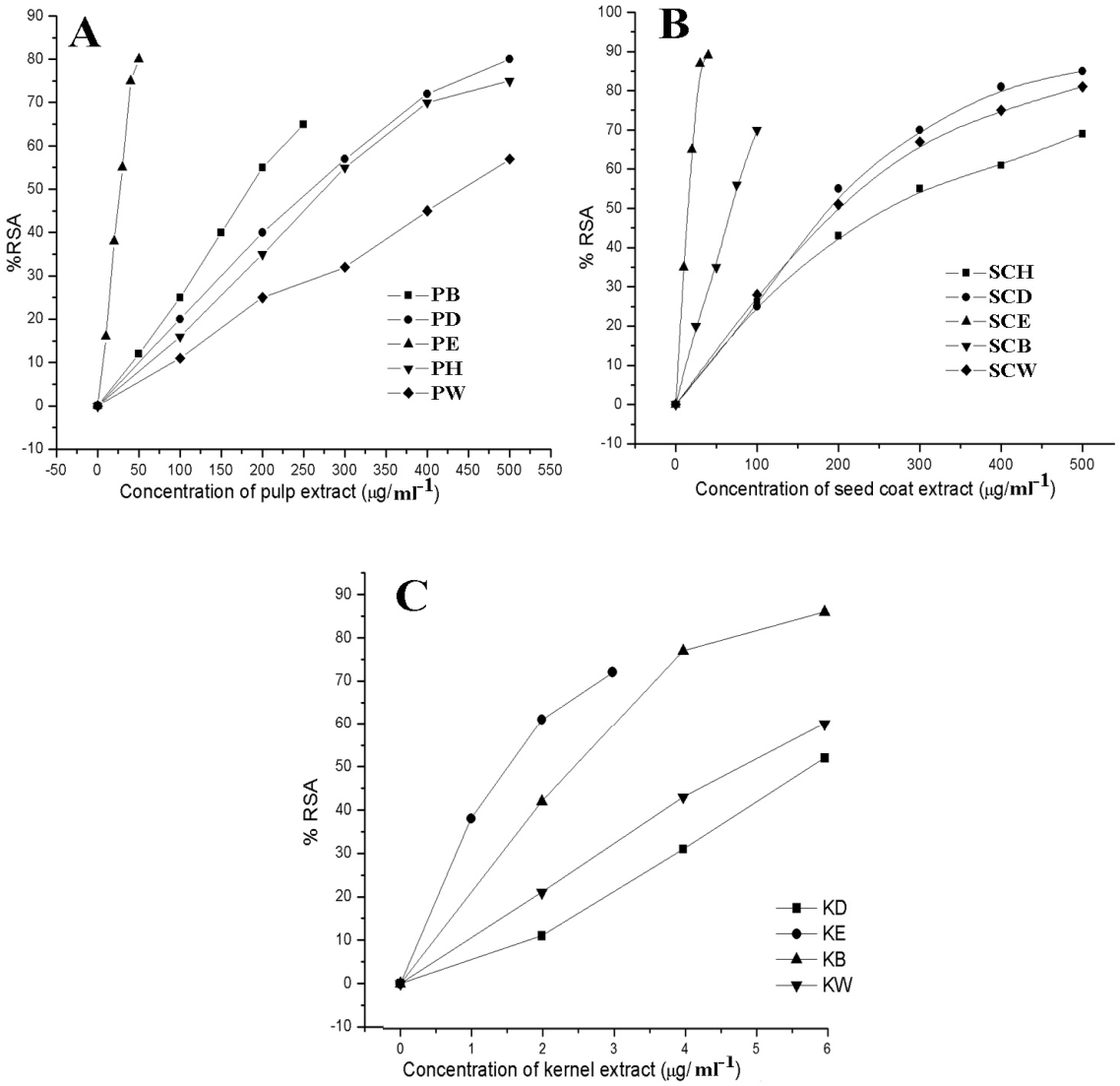

Fig. 3-15 Dose dependent DPPH radical scavenging activities of solvent fractions from KM, PM and SCM

Hexane and dichloromethane fractions were eliminated for further studies because of low yield in terms of phenolics and antioxidant activity. Butanol and water fractions showed higher yield but, the TPC was found to be significantly less with non phenolic compounds. Therefore further studies of butanol and water fractions were eliminated. Among the case of ethyl acetate fractions from all three fruit parts, KE showed highest yield in terms of phenolic content and antioxidant activity. The histogram (Fig. 3-16)

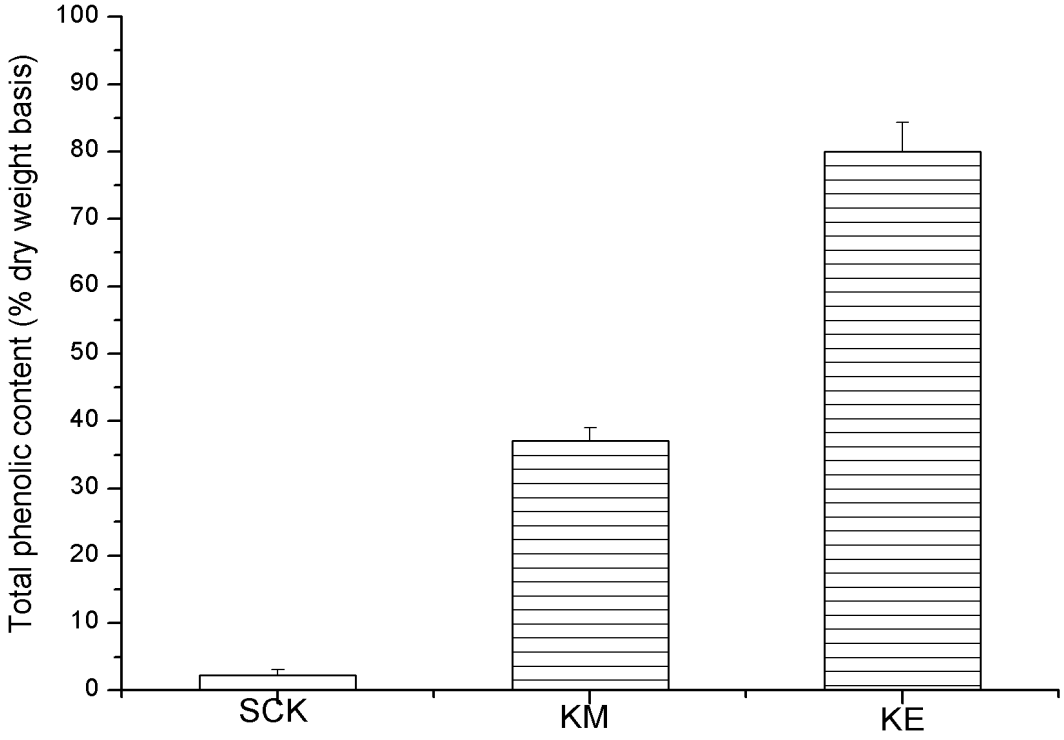

Fig. 3-16 Comparison of total phenolic content of kernel (SCK), kernel methanol extract (KM) and ethyl acetate fraction (KE) of KM

Table 3-12 Comparison of antioxidant activities of KE and KM

Sample	DPPH$^{\bullet}$	HO$^{\bullet}$	$O_2^{-\bullet}$	LOO$^{\bullet}$
	IC50 µg/ml			
Trolox	4.3 ±1.0	169 ±31	544.7 ±2.5	126 ±4
KM	8.3 ±2.5	140 ±10	80 ±3	205 ±15
KE	2.3 ±0.5	55 ±6	57 ±3.6	150 ±8.5

KM, kernel methanol extract; KE, ethyl acetate fraction of kernel methanol extract

shows the pattern of phenolic enrichment from fresh kernel to the ethyl acetate fraction. TPC of fresh seed was 5% and ethyl acetate fraction was 80%. Efficacy of phenolics enriched KE was evaluated in all 4 different antioxidant activity assay systems. The activity of KE in terms of IC50 values were derived from the dose response curve and presented in Table 3-12. IC50 values were compared with the activities of KM and Trolox standard. The increase in activities of KE over KM in different assay methods were; DPPH•, 260%; HO•, 154%; O_2^{-}•, 40%; LOO•, 36%. This comparison showed that KE is enriched with phenolic compounds that are more potent electron donors. This is evidenced from several fold increase in DPPH• and HO• radical scavenging activities of KE than that of KM and Trolox. On the basis of phenolic enrichment, antioxidant activity and yield, KE (ethyl acetate fraction of the crude methanol extract of kernel) was selected for further studies (Fig. 3-16).

3.2.3.3 Preparative HPLC fractionation of EtOAc fraction from KM

For the reasons explained before, further fractionation was performed on ethyl acetate fraction of KM using preparative HPLC to identify the marker compound(s) responsible for high antioxidant activities. The column specifications and gradient solvent systems are as follows: Column, Phenomenex C18 preparative, 21x250 mm size, particle size 15 -, Solvent A: 2% acetic acid, solvent B: methanol, binary gradient, 0-5 min A: 100%, 5-60 min B:100% linear, 60-80 min B 100%, 81 min A:100, 81-100 min A:100, total flow rate: 25 mL/min. About 100 mg of ethyl acetate fraction of *S. cumini* kernel methanol extract (JKE) was loaded into the column and the fractions were collected using the programmable fraction collector

Fig. 3-17 is the 3D photodiode array (PDA) chromatogram of ethyl acetate fraction from kernel methanol extract. Perusal of this image showed that none of the compounds separated in the HPLC was sensitive above 320 nm and most of the UV active compounds were eluted before 40 min. Z axis (wave length) of the 3D chromatogram was scanned and identified 270 nm for 2D chromatographic presentation. Based on the peak base values in X axis were analyzed for writing time program for fraction collection. The chromatogram was separated into 16

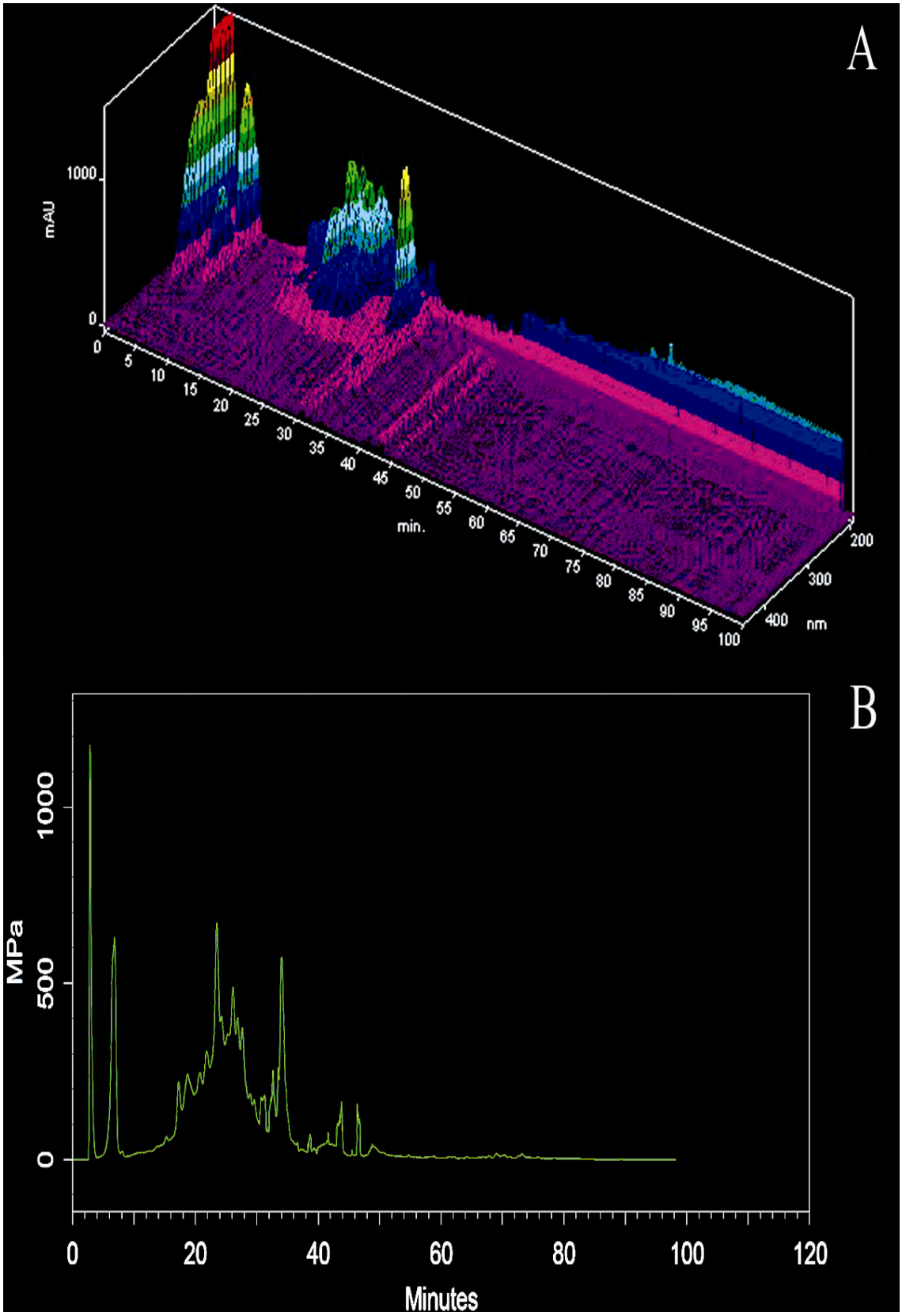

Fig. 3-17 HPLC chromatogram of s cumini kernel EtOAc fraction. A: 3D PDA detector response, B: chromatogram view at 270nm.

Table 3-13 Fractionation of chromatogram based on retention time.

Fraction	Peak	group	start Time (min)	end
F1	p1		2.4	3.8
F2	p2		5.5	7.5
F3		g1	7.6	16
F4		g2	16.2	22.2
F5	p3		22.4	23.7
F6	p4		24	27
F7		g3	27.1	32.1
F8	p5		32.1	33.5
F9	p6		33.5	35
F10	p7		43	44.2
F11		g4	45	54
F12	p8		54.3	55
F13		g5	55.2	60
F14	p9		60.2	63.2
F15		g6	65	70
F16		g7	71.2	92

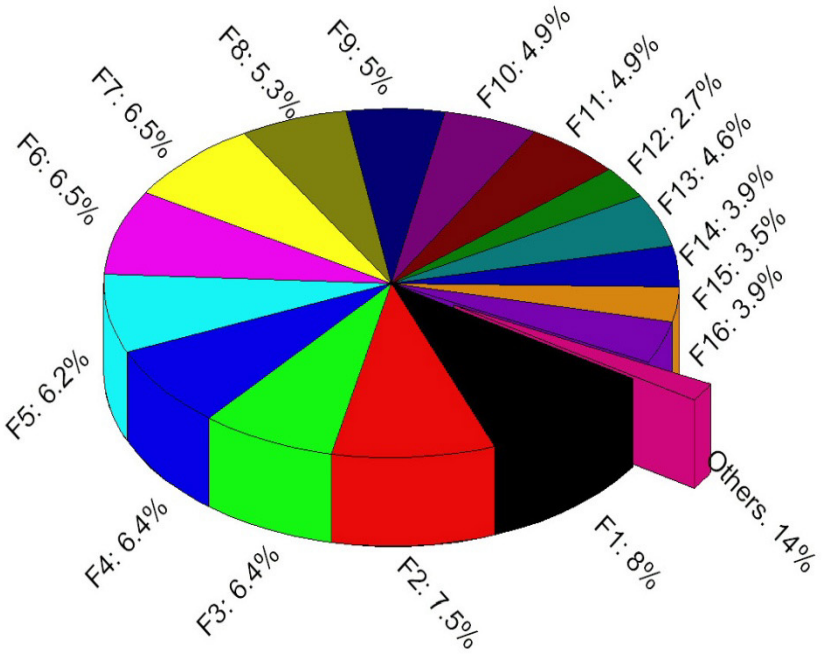

Fig. 3-18 Yield (%) of *S. cumini* Kernel ethyl acetate fractions HPLC fraction

segments (Table 3-13), of which nine were single peaks and seven were groups containing more than one peak. The reproducibility of the fractionation and collection were validated and scrutinized throughout the study. The fractions collected were concentrated using rotary evaporator under reduced temperature and pressure. The yields of fractions are presented in the pie chart.

3.2.3.4 DPPH radical scavenging activities of HPLC fraction

The preparative HPLC fractions collected were subjected to in vitro antioxidant (DPPH system) activity studies. DPPH radical scavenging activity of all 16 HPLC fractions studied here are presented in Fig. 3-19 A**.** Among the 16 fractions evaluated, fractions 1, 2, 4, 5, 6, and 7 showed more than 50% RSA between 6-10 µg/ml. Among these six active fractions, F2, F4, and F6 were more potent with greater than 90% RSA at 10 µg/ml concentration. Fig. 3-19B is the dose dependent radical scavenging activity of 6 active fractions. All fractions exhibited a linear RSA in response to sample concentration. IC50 values for the six active fractions were derived from the linear dose response curve and presented in figure Fig. 3-19C. Among these six fractions, two fractions were most active (IC50 value; F2, 1.2±0.5; F6, 1.7±0.6 µg/ml) which is comparable with that of the most powerful antioxidant; gallic acid (IC50: 1.4 µg/ml). Other fractions were 0.7 to 1.5 fold less active than the fraction two.

3.2.3.5 Significance of antioxidant activity guided fractionation

The significance of antioxidant activity guided fractionation was assessed based on the activity (IC50 values in DPPH assay) and yield of each fraction. The activity to yield ratio arbitrarily shows the total antioxidant units in each fraction. Total antioxidant units of all key fractions, KM (parent), KE (liquid-liquid partition) and F2 (prep HPLC), showed that majority of antioxidants in parent extract was partitioned in ethyl acetate fraction (Table 3-14). On the other hand, yield to activity ratio of F2 was several folds less than that of KM. Therefore it could be presumed that majority of the total antioxidant activity in KM was partitioned in its ethyl acetate fraction (KE) and on fractionation by HPLC, total

Table 3-14 Number of units of antioxidant activity (based on DPPH assay) in key fractions

Sample	Yield[a] (g%)	IC50[b] (µg/ml)	Total antioxidant units[c]
KM	15	8.3	1,807,228.92
KE	3.75	2.3	1,630,434.78
F2	0.3	1.2	250,000.00

a, Yield of KM, KME and F2 on the basis of SCK dry weight (g%); b, IC50 (µg/ml) of key fractions in DPPH radical scavenging assay; c, total antioxidant units in each fraction (assuming IC50 as one unit and number of units in each fraction)

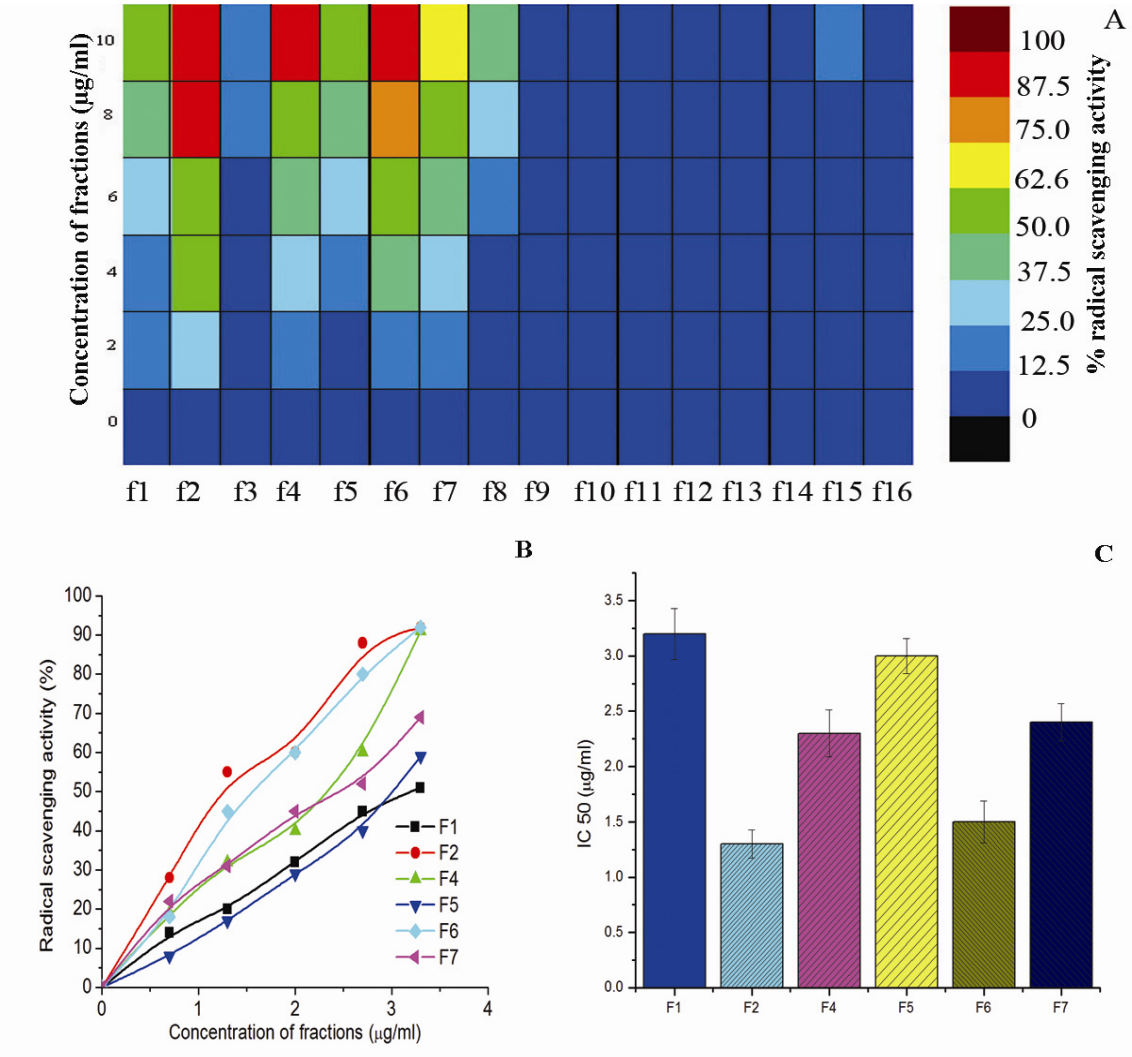

Fig. 3-19 A: DPPH radical scavenging activities of 16 preparative HPLC fractions, B: dose dependent RSA of six active fraction, C: bar chart showing the IC50 values of active fractions

antioxidant activity in KE was distributed in all 16 fractions and hence the activity to yield ration of F2 became less significant. Therefore for making antioxidants enriched extract, a high activity to yield ratio should be one criterion. However the fine fractionation by chromatographic techniques would be useful in understanding the chemical characteristics of active principles in it.

3.2.4 Effect of *S. cumini* extracts on DNA under oxidative stress.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously produced in all living beings, especially in higher organisms as a result of normal cellular metabolism, phagocytosis, and inflammation and by other exogenous factors like ionizing radiations and xenobiotics. (Beckman and Ames, 1998). The predominant radicals encountered in higher organisms are superoxide ($O_2^{\bullet-}$), peroxyl ($ROO^{\bullet}$), nitroxy ($NO^{\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals (kappus, 1985). However, $HO^{\bullet}$ is more reactive and is capable of causing damage to biomolecules such as lipids, proteins and DNA. It is generally recognized that in physiological system $HO^{\bullet}$ is produced under aerobic condition by Fenton's reaction (Chen and Schopfer, 1999) and its interaction with DNA causes oxidative damage. DNA damage such as single and double strand breakage, base modification, and cross-linking of DNA with other biomolecules particularly proteins are reported to be early events of diseases (Cadet et al., 1997). Chronic exposure of tissues to excess $HO^{\bullet}$ therefore could predispose to cancer, cardiovascular diseases, cataract and neurological disorders (Vaya and Aviram, 2001). Mitochondrial DNA (mtDNA) is presumed to be more susceptible to oxidation by Fenton's reaction and thus play a key role in cellular degeneration (Liu et al., 2000; Melov, 2004).

For DNA damage study, Fenton's reaction was selected because the hydrogen peroxide as its reactant is generated by the dismutation of superoxide, a range of oxidase enzymes and beta oxidation of fatty acids continuously. The hydrogen peroxide thus available is decomposed to $HO^{\bullet}$ by Fe^{2+} which is known as Fenton's reaction. The Fe^{2+} for the Fenton's reaction could normally be available from iron binding proteins under acute oxidative stress (Flora et al., 1997; Flora et al., 1996;

Izzotti, 1998; Izzotti, 2003). The hydroxyl radicals produced through the Fenton's reaction is most reactive free radical that can damage DNA in its proximity. Some of the dietary phytochemicals are capable of either sequestering Fe^{2+} and/or scavenging $HO^{\bullet}$ radicals thus preventing damage to DNA. However, predictability of such phytochemicals based on their structure-function is poor. The phytochemicals selected as standard were based on their commonality in the diet, their antioxidant properties and biological activities. The six standard phytochemicals (Fig. 3-21) selected for the study represent different classes, namely, gallic acid (phenolic acid), catechin (flavan-3-ols), apigenin (flavone), naringenin (flavanone), and naringin (flavanone glycoside). Vitamin C was included as the most common antioxidant and essential nutrient for human body. The plant extracts used in this study were with high antioxidant activity as observed in preliminary studies conducted..

Standardization of Method for DNA Damage in Fenton's system Using S. cumini extract: Fenton's reaction refers to iron-catalyzed decomposition of hydrogen peroxide and generation of highly reactive hydroxyl radicals. DNA damage caused in response to various levels of oxidative stress by Fenton's reaction is shown in the electrophoresis pattern (Fig. 3-20: A and B). Ethidium bromide was the fluorescent probe used in this study to quantify the amount of detectable DNA in agarose gel with respect to blank. Ethidium bromide intercalates with double stranded DNA and produces bright fluorescence on irradiation at 254 nm. The fluorescence intensity is proportional to the amount of double stranded DNA which intercalates the dye. The high resolution images of the ethidium bromide stained gel illuminated at 254 nm was captured using a digital camera and quantified the fluorescence intensity of each lane by an image processing software. The fluorescent region was selected and quantified as average intensity of pixels. Since the image was 8-bit gray-scale format, the intensity of each pixel, which ranges from black to white, was in the scale of 0 to 255 respectively. The average signal strength, as integers, obtained was proportional to the amount of double stranded DNA. Lane 1 is the blank in which the DNA was treated with ferrous chloride (0.25 mM) alone and therefore no

oxidative damage was expected. No difference in total fluorescence was observed between DNA alone with buffer and DNA with ferrous chloride (data not shown). Therefore, the total fluorescence in lane 1 was considered as nil damage and used as blank for calculating relative DNA damage in presence of both ferrous chloride and hydrogen peroxide.

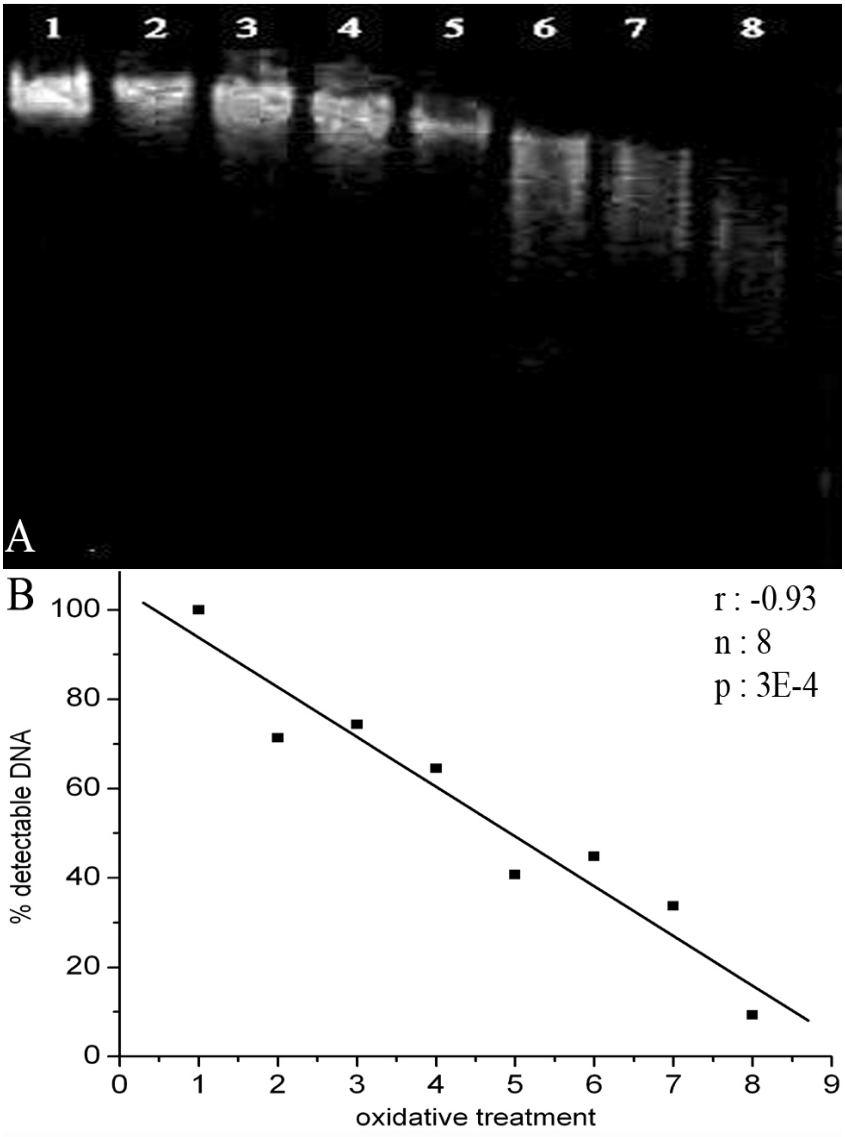

Fig. 3-20 Electrophoresis pattern of dose dependent DNA damage (A). Scatter plot showing correlation between oxidative treatments versus amount of detectable DNA (B). Lane 1, DNA + $FeCl_2$; Lane 2 to 8, DNA+ $FeCl_2$ + 0.05 to 0.35 mM of H_2O_2 (step 0.05).

Lane 2 to 8 are the electrophoresis pattern of DNA treated with 0.05 to 0.35 mM (step: 0.05) H_2O_2 and 0.25 mM ferrous chloride. A linear relation was observed

between DNA damage and concentration of hydrogen peroxide used here (r -0.93, N 12, P <0.01) (Fig. 3-20 B). The assay conditions of lane 6 (0.25 mM $FeCl_2$ and 0.25 mM H_2O_2) was considered as optimum and used as control for the subsequent evaluation of standard compounds and samples. This intermediate oxidative state of DNA was selected so that antioxidant or pro-oxidant action of test samples could be measured quantitatively in terms of reduced or increased DNA damage.

Effect of standards and samples: To understand the effect of the selected standard phytochemicals, Fenton's system containing DNA and phytochemicals was evaluated under equimolar conditions. Fig. 3-22 is the results of λ DNA treated with naringenin, naringin, apigenin, catechin, vitamin C, and gallic acid at 0.25 mM concentration in optimized Fenton's system. The blank (lane-1, λ DNA alone in buffer) was assumed as 0.0 % damage. Lane 2 shows the DNA damage in control (λ DNA and Fenton's reagent). With respect to blank, the mean DNA damage in control was 51.0 ± 6.0 %. Lane 3 displays the pattern of DNA treated with Fenton's reagent and naringenin and percentage of DNA damage observed was 52.0 ± 7.0, which was similar to that of control.

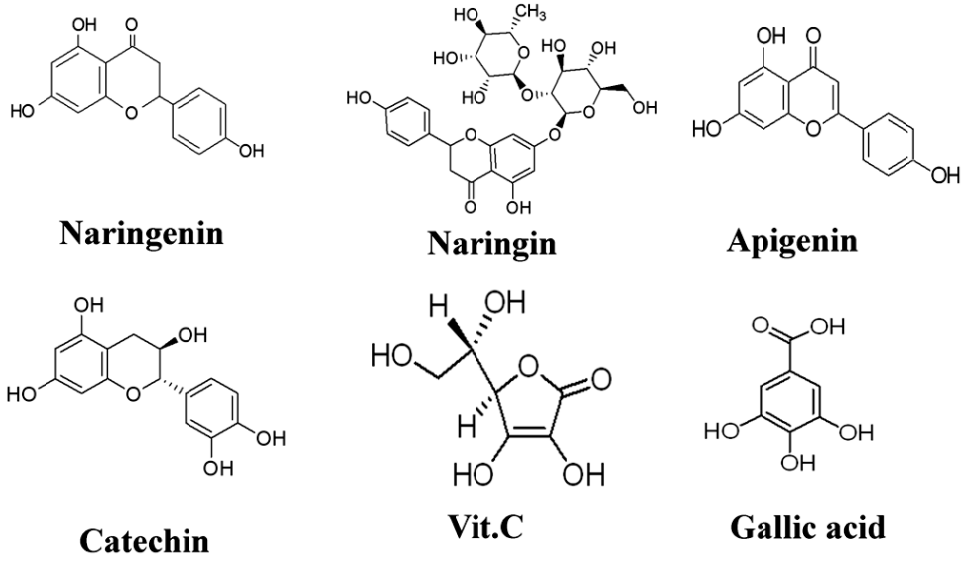

Fig. 3-21 Structures of the six phytochemicals evaluated for their effect on the integrity of double stranded DNA in Fenton's system.

The mean DNA damage observed for naringin (lane 4) was 19.0 ± 5.0 suggesting

that naringin afforded 32 percentage protection to DNA as compared to that of control. The mean DNA damage observed in the assay treated with apigenin and catechin was 52.0 ± 7.0 % and 54.0 ± 8 % respectively indicating that they had no protective effect against the oxidation of DNA. Interestingly, vitamin C and gallic acid, the two well-known antioxidants caused mean DNA damage of 68.0 ± 4.0 % and 72.0 ± 9.0 % respectively, the values being significantly greater than that of control.

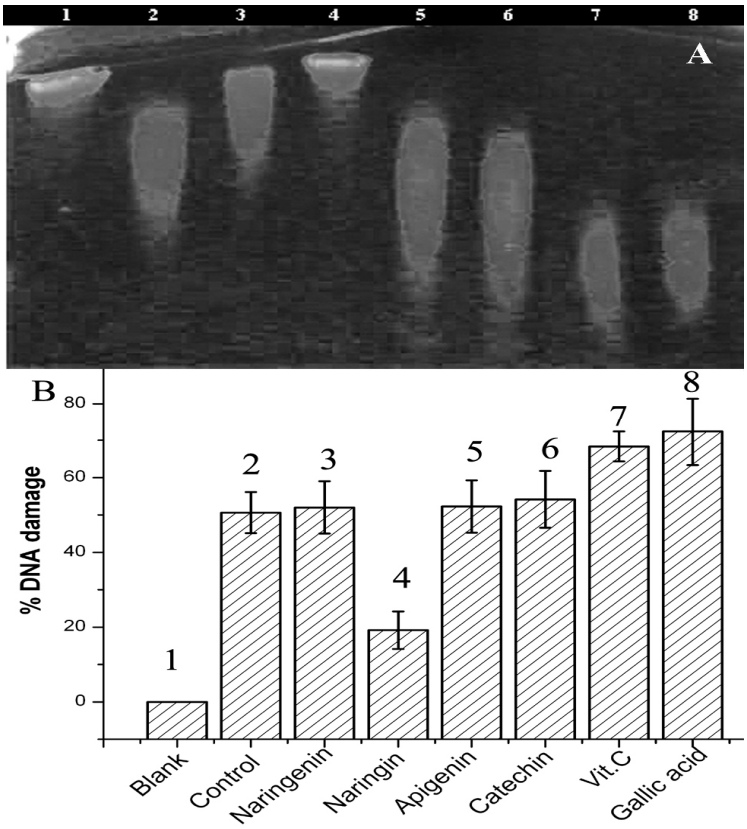

Fig. 3-22 Electrophoresis pattern of DNA in Fenton's system treated with six standard phytochemicals (A). Bar chart representing the gel pattern assuming blank as 0% damage (B). Final concentration of all phytochemicals were 2.5 mM; lane-1, blank; lane-2, DNA+Fenton's reactants; lane-3, DNA+Fenton's reactants + naringenin; lane-4, DNA+Fenton's reactants + naringin; lane-5, DNA+Fenton's reactants + apigenin, lane-6, DNA+Fenton's reactants + catechin; lane-7, DNA+Fenton's reactants + vitamin C; lane-8, DNA+Fenton's reactants + gallic acid.

Among the plant extracts tested (**Fig.** 3-23 A, lane 4 to 5), both SCK and HRK were found to damage DNA (SCK, 79.0 ± 3.0 %; HRK, 68.0 ± 5.0%). The electrophoresis pattern and corresponding histogram (Fig. 4B) showed that the total DNA damage observed for SCK and HRK were greater than that of control by 25.0 % and 14.0 % respectively. Among the plant extracts tested, only PGP showed protective effect with 18.0 ± 3.0 % DNA damage compared to the control value of 51.0 ± 6.0 %.

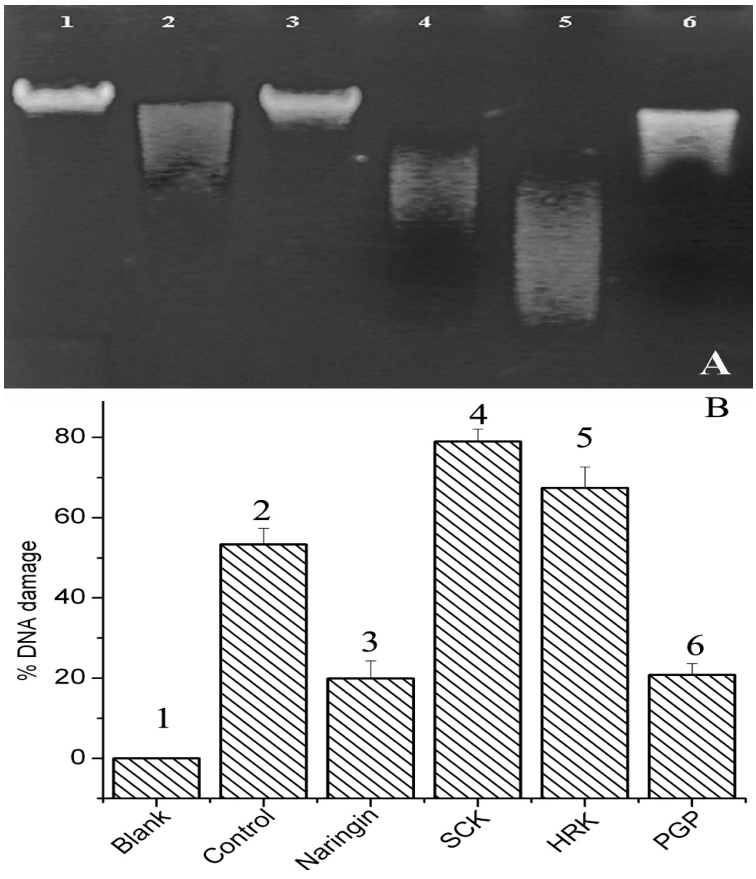

Fig. 3-23 Electrophoresis pattern of DNA treated with Fenton's reagents and plants' methanol extracts (A).Bar chart representing the gel pattern assuming blank as 0% damage (B). Final concentrations of all extracts were 2.5 mM phenolic equivalent; naringin, 2.5 mM. SCK, *S. cumini* Kernel; HRK, *H. rhamnoides* kernel; PGP, *P. granatum* pericarp. Lane-1, blank; lane-2, DNA+Fenton's reactants; lane-3, DNA+Fenton's reactants + naringin; lane-4, DNA+Fenton's reactants + SCK; lane-5, DNA+Fenton's reactants + HRK; lane-6, DNA+Fenton's reactants + PGP

Iron chelation property: Bivalent transition metal ions play a very important role in the process of Fenton's reaction that leads to generation of highly reactive hydroxyl radicals (Halliwell, 1997). The agents that can attenuate the action of these bivalent metal ions have been classified as secondary antioxidants which retard the rate of radical initiation reaction by means of eliminating initiators (Vaya and Aviram, 2001). Iron chelation properties of the standard molecules and plant extracts were evaluated and the results are presented at 10.0 mg/mL concentration (Table 1). Among the six standard compounds evaluated here, naringin showed more than 80% iron chelation. Activities of naringenin, apigenin and catechin were; 20.83 ± 1.39%, 13.47 ± 1.36% and 11.17 ± 1.6% respectively. No measurable iron chelation was observed for vitamin C and gallic acid. Among the plant extracts, PGP was found to be more active in chelating iron (68.67 ± 2.27%) followed by SCK (7.3 ± 1.27%) and HRK (6.03 ± 1.87%).

Reducing potential: Total reducing potential (TRP) of the standard compounds and samples were evaluated at 25 µg/mL (**Table 3-15**). The reducing potential was proportional to color developed and expressed in absorbance unit. The Iron (III) reduction method employed here is often used to indicate electron transfer potential of antioxidant molecules (Meir et al., 1995), though there are reports that this mode of action is not happening always (Yildirim et al., 2000). Among the standard compounds studied, gallic acid was found to be the most potent (1.32 ± 0.1) followed by vitamin C (0.97 ± 0.04) and catechin (0.74 ± 0.04). Reducing capacity of naringin, naringenin and apigenin were relatively very less. In the case of plant extracts, the results were comparable with those of reference standards. Extracts such as SCK and PGP showed high reducing activity 0.89 ± 0.07 and 0.81 ± 0.07 respectively followed by HRK (0.71 ± 0.04).

Antioxidant activities: Antioxidant activity of the standard compounds and extracts were studied using the stable free radical DPPH. Conversion of DPPH into non-radical form is characterized by decrease in absorption at 520 nm. Hydrogen atom donation capacity, the characteristic feature of primary antioxidants (Esterbauer, 1995; Gordon, 1990; Pryor, 1994), of the standard compounds and plant extracts were evaluated here.

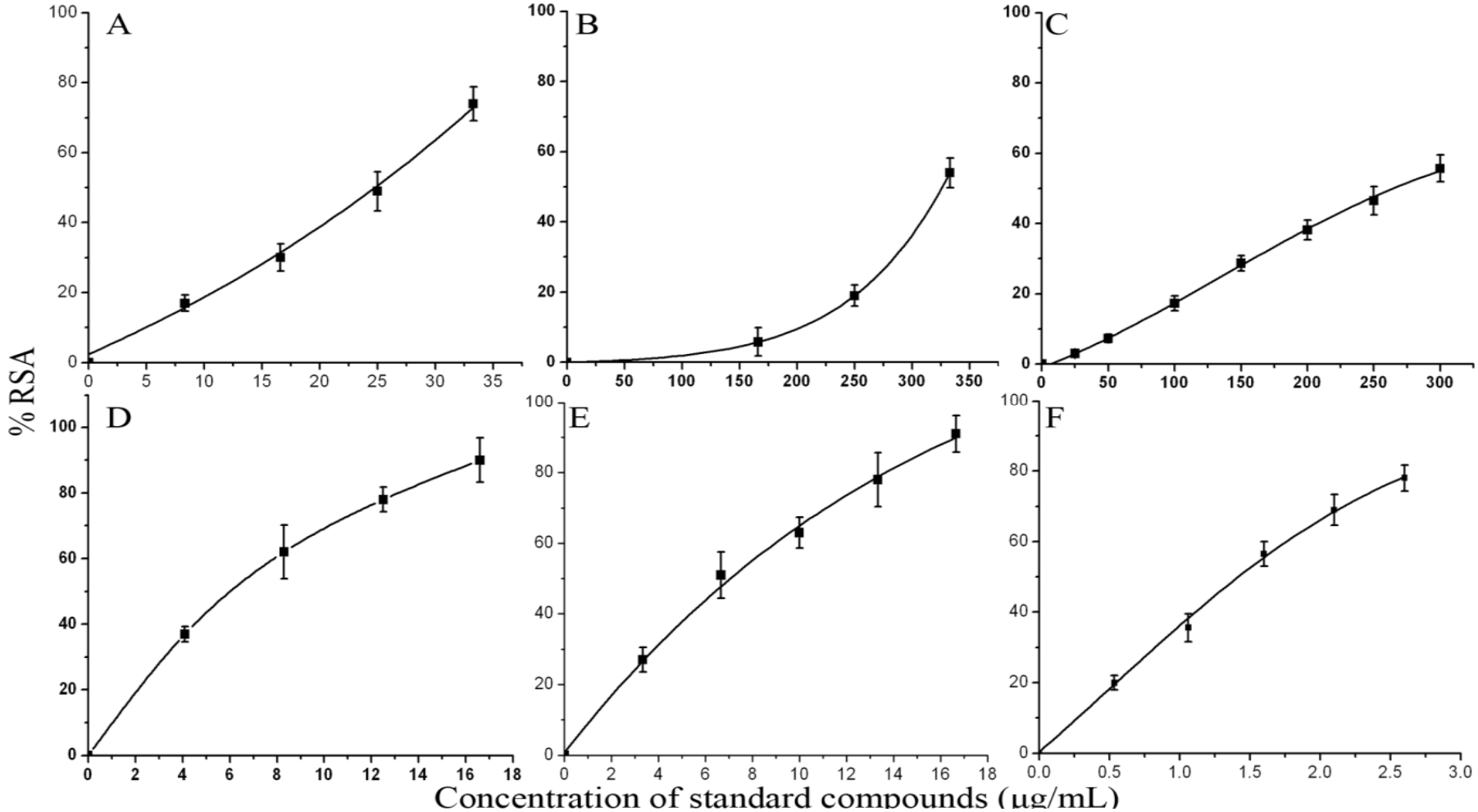

Fig. 3-24 Dose dependent DPPH radical scavenging activity of standard phytochemicals. A, naringenin; B, naringin; C, apigenin; D, catechin; E, vitamin C; F, gallic acid.

Fig. 3-24 is the dose dependent radical scavenging activity (RSA) of the above-mentioned standard compounds. Among the six standard compounds studied here, gallic acid was found to be most active with IC50 value of 1.4 ± 0.3 µg/mL. Catechin, vitamin C and naringenin showed the next highest activity with IC50 values of 6.1 ± 1.3 µg/mL, 7.0 ± 0.7 µg/mL and 25.0 ± 4.0 µg/mL respectively (**Table 3-15**). Apigenin was relatively less active in DPPH$^{\bullet}$ scavenging (267.0 ± 7.7 µg/mL) and the lowest activity were observed for naringin (327.0 ± 3.0 µg/mL).

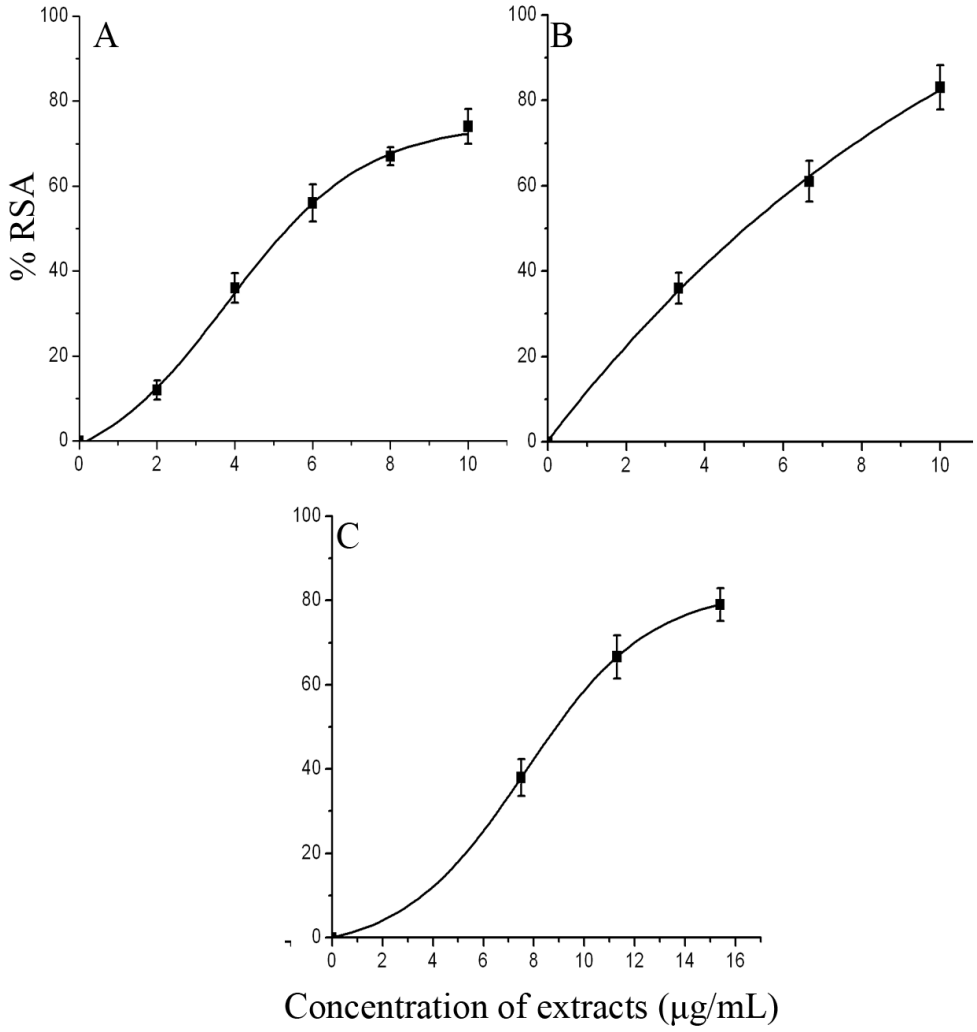

Fig. 3-25 Dose dependent DPPH radical scavenging activity of different plants' methanol extracts. A, *H. rhamnoides* kernel; B, *P. granatum* pericarp; C, *S. cumini* Kernel

Table 3-15 DPPH radical scavenging activity, total reducing power, iron chelation and DNA protective/damaging effects of a few standard compounds and plants' methanol extracts.

Samples / Standards	DPPH radical scavenging activity ((IC50 (µg/mL))	Total reducing power at 25.0 µg/mL concentration (Absorbance)	% Iron chelation at 1.0 mg/mL	Effect of standards and samples on the integrity of double stranded DNA	
				% DNA damage	CI level (95%)
Control	NA	NA	NA	50.63	5.88425
Naringenin	25.0 ± 4.0	0.10 ± 0.01	20.83 ± 1.39	52.01	7.9211
Naringin	327.0 ± 13.0	0.01 ± 0	83.67 ± 3.51	19.19	5.65793
Apigenin	267.0 ± 7.7	0.05 ± 0.01	13.47 ± 1.36	52.27	7.9211
Catechin	6.1 ± 1.3	0.74 ± 0.04	11.17 ± 1.65	54.17	8.60005
Vitamin. C	7.0 ± 0.7	0.97 ± 0.04	nd	68.36	4.52634
Gallic acid	1.4 ± 0.3	1.32 ± 0.1	nd	72.36	10.18427
SCK	9.0 ± 1.0	0.89 ± 0.07	7.33 ± 1.27	78.97	3.55544
HRK	5.5 ± 0.3	0.71 ± 0.04	6.03 ± 1.85	67.44	5.91027
PGP	5.0 ± 0.8	0.81 ± 0.07	68.67 ± 2.27	20.79	3.21936

SCK, S cumini Kernel; HRK, H rhamnoides kernel; PGP, P granatum pericarp. a, control (DNA and Fenton's reagents) with a mean DNA damage of 51.0 ± 6.0 %. ndc, not significantly different than control; nd, not detected.

The plant extracts evaluated here showed that HRK and PGP scavenged more than 75.0 % radicals at 10.0 µg/mL concentration. SCK showed slightly less activity as compared to those of other extracts (**Fig. 3-25**). IC50 values (µg/mL) for $DPPH^{\bullet}$ scavenging activity of HRK, PGP and KM were 9.0 ± 1.0, 5.5 ± 0.3 and 5.0 ± 0.8 respectively (**Table 3-15**).

The effect of phytochemicals and plant extracts in Fenton-DNA system was correlated with their corresponding $DPPH^{\bullet}$ scavenging activity (IC50 values). A weak inverse correlation was observed between the IC50 values and percentage DNA damage as shown in Fig. 3-26 (r,-0.806; n, 8; *p*, <0.01). PGP (grated in the figure) was excluded from the correlation analysis, as it exhibited high antioxidant as well as high iron chelation property. The iron chelation property of PGP was dominant over its reducing potential and hence showed less DNA damage, by retarding Fenton's reaction. With inclusion of PGP, the correlation between reducing power of phytochemicals/extracts and DNA damage was further weakened (r,-0.5; n, 8; *p*, 0.12) suggesting that reducing power of PGP did not have direct influence on DNA damage in Fenton's system.

In physiological system, redox regulation is a vital process for homeostasis and presently studies are focused to understand its detailed mechanism. Normally a balance in the level of ROS/RNS by endogenous and exogenous antioxidant defense system is maintained (Halliwell and Gutteridge, 1989). The optimum levels of these ROS/RNS is known to facilitate several signal transduction and inter cellular communications (Valko et al., 2004). However, excessive production of ROS/RNS is deleterious to tissues. Among the various ROS in physiological system, $HO^{\bullet}$ is the most reactive and it can inflict damage to biomolecules in its vicinity. Hydroxyl radicals are known to be produced by Fenton's reaction and combinations of several factors are warranted for the Fenton's reaction to take place. Availability of H_2O_2 and free iron (Fe^{2+}) is the most critical elements for initiation of Fenton's reaction. Hydrogen peroxide is generated *in vivo* by dismutation of superoxide radicals by a range of oxidase enzymes including glycollate and monoamine oxidases as well as by the beta-oxidation of fatty acids.

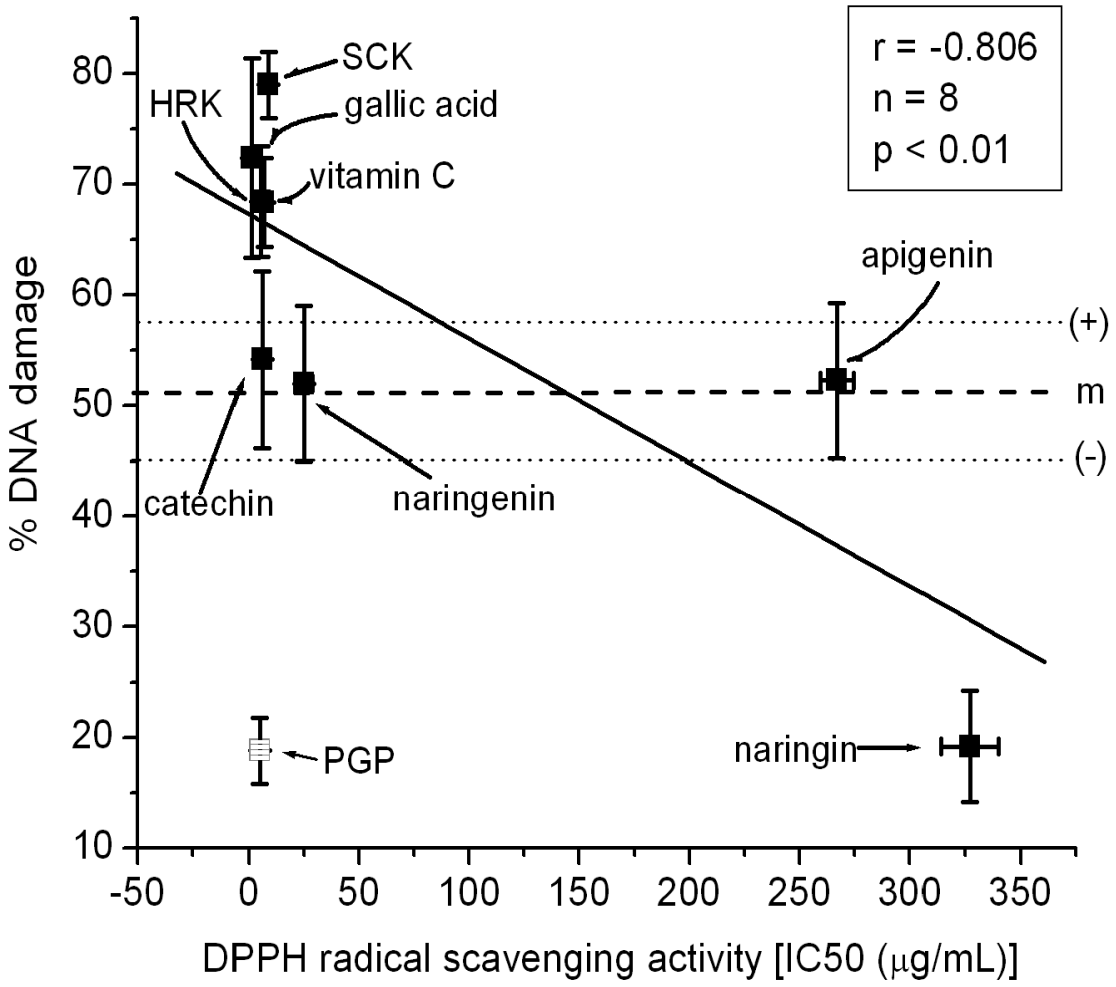

Fig. 3-26 Correlation between DPPH$^{\bullet}$ scavenging activity of standard compounds and plant extracts (excluding PGP) versus DNA damage. m, mean DNA damage in control; +/-, deviation limits of DNA damage observed in control. (r = -0.83, n = 8, p = 0.01)

The free iron required for the Fenton's reaction could be generated by radiation, xenobiotics or excessive oxidative stress (Leonard et al., 2004; Liochev and Fridovich, 1994; Reelfs et al., 2004). The free iron thus available under such conditions promotes Fenton's reaction by liberating hydroxyl radicals spontaneously from H_2O_2. Hydroxyl radicals produced in close proximity to DNA cause strand break, base modification and DNA protein cross linking (Poli et al., 2004). Under oxidative stress, this mechanism may be dominating and the protective mechanism could be by scavenging the $HO^{\bullet}$ at very faster rate either by antioxidants or by chelation of the free iron.

In the present study, six standard compounds and three plant extracts were evaluated for their effect on the integrity of double stranded DNA in Fenton's system. The results obtained were then correlated with their iron chelation property, total reducing power and DPPH radical scavenging activity to understand the mode of action (primary or secondary) as antioxidants and their possible role in the DNA-Fenton's system. Among the six phytochemicals and three plant extracts studied, 2 were protecting DNA (naringin and PGP), 4 were promoting DNA damage (gallic acid vitamin C, HRK and KM) and 3 of them were neutral (catechin, apigenin and naringenin). Naringin and PGP exhibited high iron chelation activity while the other test materials showed poor iron chelation under the assay conditions used here. The iron chelation property of the test materials could also be attributed to their secondary antioxidant activity which is their ability to retard or block upstream event of electron transfer from Fe (II) to hydrogen peroxide that triggers hydroxyl radical generation. To be an efficient inhibitor of Fenton's reaction, the compound in the assay system should either chelate iron and prevent the electron transfer or should accept the electron from Fe (II). It is obvious from the results of this study that in the control assay system (Fenton's reactants and DNA alone) the DNA damage was caused due to electron transfer from Fe II to hydrogen peroxide as it did not contain any chemical agent to chelate iron. However, in the presence of naringin and PGP, the percentage DNA damage was significantly less than that of control suggesting that the Fenton's reaction was retarded by naringin and PGP with their higher efficiency to chelate iron at 83.67 ± 35 and 68.69 ± 2.27% respectively . Naringin is a major flavonoid glycoside of many citrus fruits reported to have high iron chelation capacity (Mira et al., 2002). DNA protective activity of naringin in cell lines are also reported due to its ability to chelate iron (J.Hynes and Mairtın O Coinceanainn, 2004). The protective activity exhibited here by PGP could be attributed to its high tannins (Gil et al., 2000; Lopes et al., 1999) that are known to chelate iron thereby limiting the availability of free iron for Fenton's reaction. Though naringin and PGP had high iron chelation capacity they behaved differently for their TRP and $DPPH^{\bullet}$ scavenging activity. While PGP showed powerful antioxidant and iron chelation property, naringin exhibited poor activity

for TRP and DPPH$^{\bullet}$ and strong iron chelation suggesting that iron chelation property should be dominant to retard Fenton's reaction.

Vitamin C, gallic acid, KM and HRK were excellent antioxidants but they were found to promote DNA damage probably by regenerating Fe^{2+} from the product of Fenton's reaction (Fe^{3+}) (Ahmad et al., 2002; Kobayashi et al., 2004; Ng et al., 2005; Santra et al., 2002). This may not happen under normal physiological condition except in the event of much higher concentration of iron with other factors being favorable for Fenton's reaction. Vitamin C, gallic acid, KM and HRK were very poor iron chelators but all of them had high reducing potential. The DNA damaging effect observed in the presence of gallic acid, vitamin C, KM and HRK therefore were due to the lack of their iron chelation ability.

Naringenin, apigenin and catechin showed neutral effect in Fenton-DNA system that they neither promoted nor protected DNA damage though they had poor iron chelation properties. In the case of catechin, its high reducing potential comparable with that of vitamin C did not promote DNA damage like vitamin C, gallic acid, HRK and KM. The mechanism for promotion of DNA damage by phytochemicals with high reducing power therefore is unclear and studies related to their kinetics may throw some light on this. Neutral or DNA damaging effect of phytochemicals did not have correlation with their chemical structure or antioxidant potential. To confirm this, the results were further subjected to statistical analysis. A weak negative correlation ($r = -0.83$, $n = 8$, $p = 0.01$) between antioxidant activity (IC50) and percentage DNA damage (Fig. 7) was observed when PGP were excluded. With inclusion of PGP, the correlation further weakened($r = -0.5$, $n = 9$, $p = 0.12$) indicating that phytochemicals with very high reducing power without iron chelation ability may damage DNA in Fenton's system.

Thus, chronic exposure to oxidative stress, particularly HO$^{\bullet}$ may lead to cellular and tissue degeneration, in the context of factors favorable for Fenton's reaction. The HO$^{\bullet}$ produced by Fenton's reaction under the conditions described elsewhere can not only damage DNA, but it can also react with proteins, lipids and carbohydrates. Prolonged exposure to such oxidation is presumed to be early

events of cancer, CVD, diabetes, cataract, and neurological disorders. Phytochemicals of dietary and non-dietary origin can intercept at various levels and retard the progress of tissue degeneration. However, factors such as bioavailability, stability, access to the site of oxidation, blood brain barrier, and effective concentration, may limit efficacy of phytochemicals.

The method employed here for measuring DNA damage may have deficiencies like low sensitivity and accuracy. However, the results are convincing due to the large difference between the protective and damaging effects of the phytochemicals and extracts used. The results were also analyzed using appropriate statistical tool to further establish the confidence level at 95% and above. The fact that we selected only six phytochemicals is also another limitation of the study as numerous other phytochemicals of dietary and non-dietary origins that we consume may exert synergistic and antagonistic effects as far as DNA damage is concerned. It may be worthwhile therefore to conduct studies using more number of phytochemicals that we consume and their interactions to further establish the findings reported here.

3.3 Anticancer studies on *S. cumini* fruit (SCF)

Plants, being a rich source of diverse phytochemicals, are extensively studied for anticancer. According to Schwartzman, plants have been considered as valuable source for anticancer drugs other than microorganisms and marines living beings due to their diversity (Schwartsmann et al., 2002). Several studies have demonstrated that extracts from herbs have anticancer potentials under *in vitro* as well as *in vivo* conditions (Bonham et al., 2002; Hu et al., 2002; Kao et al., 2001; Lee et al., 2002). Most of the studies related to evaluation of anticancer activity of plants showed that polar extracts are more potent in retarding cancer cell growth than that of nonpolar extracts (Hu et al., 2002; Kao et al., 2001; Lee et al., 2002). A previous study on *S. cumini* fruit (SCF) pulp also showed that the polar extract retarded the cancer cells effectively under in vitro conditions (Nazif, 2007). However no comprehensive study was reported to evaluate anticancer potency of SCF. Therefore in the present preliminary anticancer activity evaluation, polar extracts from anatomically distinct parts of SCF were used and the results are presented in the following sections.

3.3.1 Preliminary evaluation of SCF for cytotoxicity

Cytotoxic agents can be of chemical substances, immune cells, proteins etc. On exposure to a cytotoxic agent, the cells may lose its membrane integrity (necrosis), stop dividing, or undergo apoptosis (Promega, 2006). There are several cytotoxic assay methods suitable for screening anticancer potency of substances. Here we used MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and LDH (lactate dehydrogenase) methods to measure the cytotoxicity of extracts and standard cytotoxic agents in terms of metabolic inactivity and necrotic death respectively.

Cytotoxic activity evaluation using MTT Method: SCF extract's cytotoxic activity was studied by MTT assay. MTT is a tetrazolium salt which is yellow in color in oxidized form. In metabolically competent cells the yellow colored MTT is reduced to purple colored soluble formazan dye by mitochondrial reductases.

The color developed formazan dye can be quantified colorimetrically. Since the reduction of MTT and color development is proportional to mitochondrial reductase enzymes, this assay is a measure of live and metabolically active cells (Mosmann, 1983). In the preliminary study the anticancer potency of methanol extracts from anatomically distinct parts of SCF against cervical cancer cells (HeLa-S3) were studied by this method and the details of method is described in sections 2.6.4 and 2.6.5. HeLa-S3 cells were seeded at a concentration of 10 X 10^4 in a 24 well plate containing RPMI 1640 culture media supplemented with 10% heat inactivated foetal bovine serum. Methanol extract were prepared from pulp (PM), seed coat (SCM) and kernel (KM) and introduced into cell culture system at different concentrations as follows; PM, 0-500 µg/ml (step 100); KM and SCM, 0-125 µg/ml (step 25). After sample introduction, the assay plates were incubated for 24 hrs. and the percentage cell viability was estimated colorimetrically by measuring the amount of reduced MTT. Among the three methanol extracts studied here, KM and SCM exhibited significant cytotoxic activity against the HeLa-S3 cells in a dose dependent manner (Fig. 3-27). Cytotoxic activity of KM was found to be more because, it inhibited up to 80% cell growth at 80µg/ml concentration. Cytotoxic activity of SCM also showed a trend similar to that of KM but, the rate of cell death in the range of 20-60µg/ml concentration was significantly less than that of KM. Therefore even at 120 µg/ml concentration, the maximum cytotoxicity observed for SCM was 90%. The cytotoxic potential of PM stood several folds less than that of the activity of KM and SCM. Because of the low cytotoxic activity of PM, the concentration of PM was increased several fold (0-500 µg/ml) than that of KM and SCM to understand the cytotoxic potential It was observed that in cells treated with PM, a maximum of 70% cell death occurred at 500µg/ml concentration. We also studied cytotoxic activity of a standard anticancer drug (methotrexate) and other cytotoxic agents viz. curcumin and camptothecin under identical conditions simultaneously as that of samples. Fig. 3-28 shows the dose dependent cytotoxic activity of curcumin, camptothecin and methotrexate.

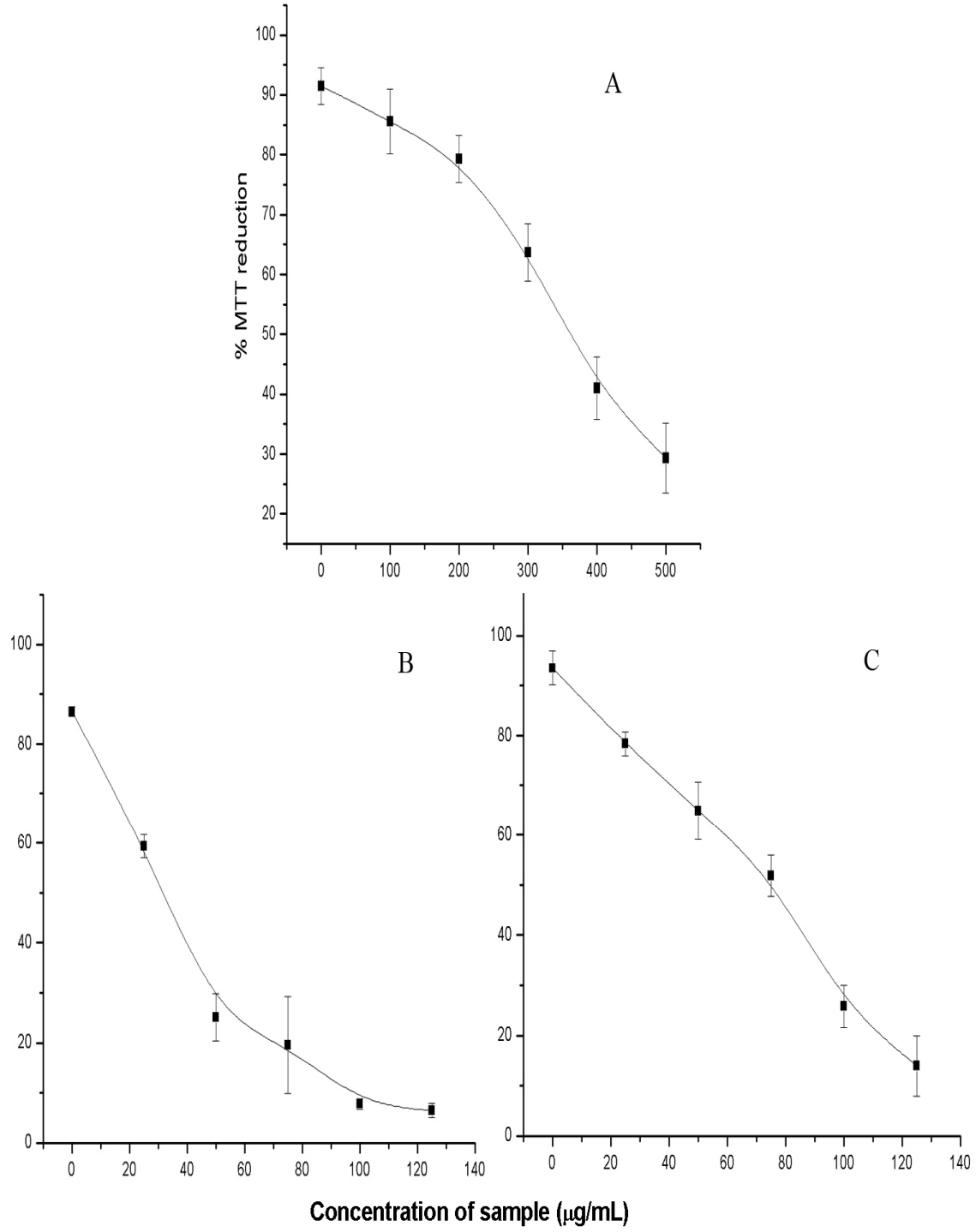

Fig. 3-27 Dose dependent cytotoxic activity of methanol extracts of pulp, A; kernel, B; and seed coat, C; extract against HeLa-S3.

Among the three different standards studied here, cytotoxic activity of camptothecin and methotrexate was found to be several folds higher than that of samples. Cytotoxic effect of camptothecin studied at different concentration (0.001, 0.005, 0.025, 0.5, 0.25, and 1.25 μg/ml) showed more than 90% cell death

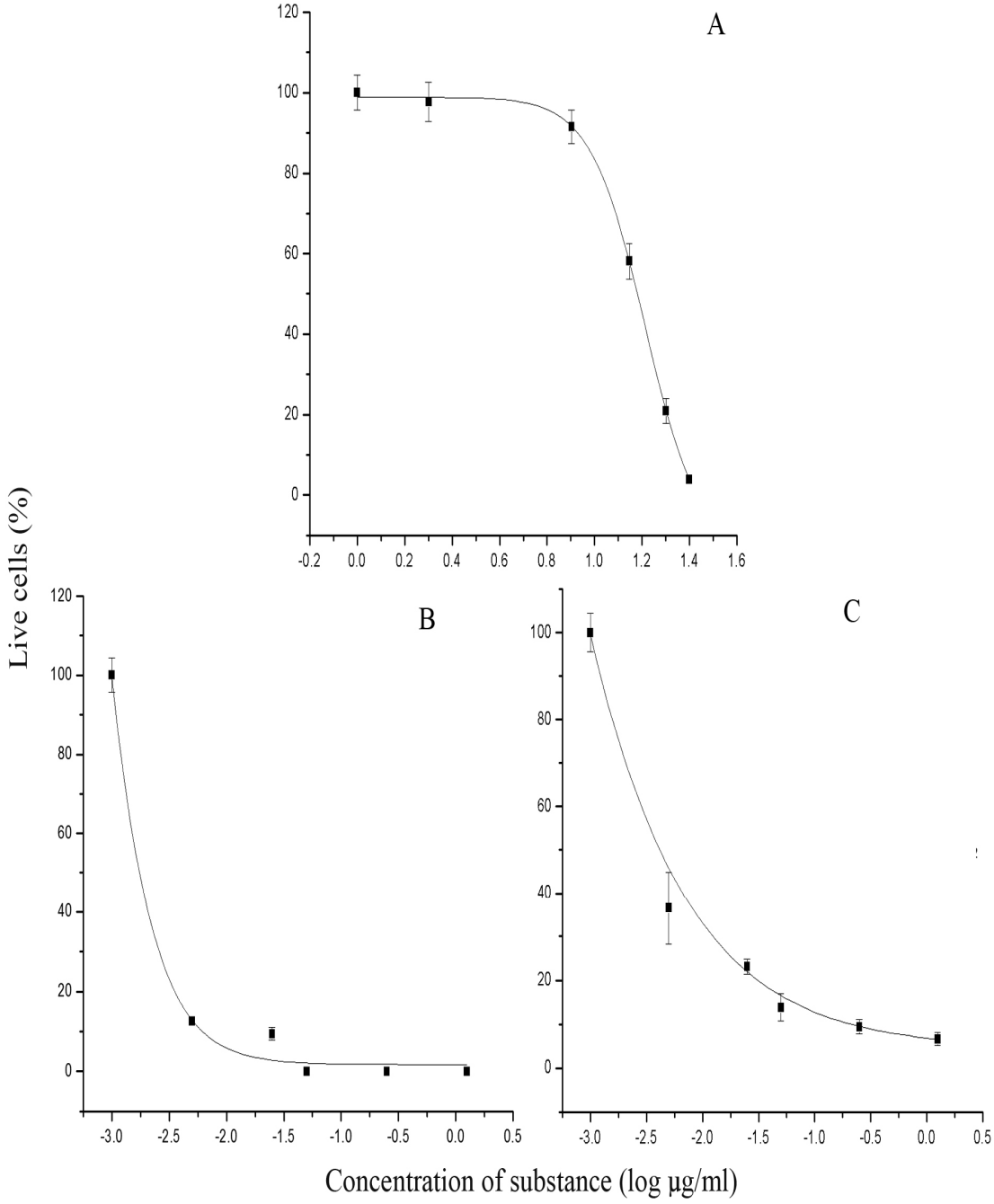

Fig. 3-28 Dose dependent cytotoxic activities of standard cytotoxic agents against HeLa-S3 cells. A, Curcumin; B, Camptothecin; C, Methotrexate.

above 0.005 µg/ml and 100% cell death above 0.025 µg/ml concentration (Fig. 3-28-B). Dose dependent cytotoxic activity of methotrexate on HeLa-S3 cells is shown in Fig. 3-28-C. More than 80% cytotoxicity was observed at 0.025 µg/ml and further reached 90% cytotoxicity at 1.25 µg/ml. In contrast to camptothecin and methotrexate curcumin showed a different concentration dependent

cytotoxicity profile that was comparable with that of the samples analyzed here. Fig. 3-28-A shows the concentration dependent cytotoxic activity of curcumin. The cells treated with 1, 2 and 8 µg/ml of curcumin did not show significant cytotoxicity. However at 14, 20 and 25 µg/ml of concentration, the cytotoxicity was found to be proportional to the concentration. More than 80% cytotoxicity was observed above 20µg/ml.

To simplify the analysis and to find out the relative positions of samples in the standard's cytotoxic activity scale, IC50 values were derived from the dose response curves of samples and standards. IC50 is the amount of substance required to induce 50% toxicity in cells under the standard conditions. Table 3-16 shows the IC50 values of samples and standards derived from the dose dependent cytotoxic activity graph. The highest cytotoxicity was exerted by camptothecin and methotrexate followed by curcumin. IC50 value of curcumin (14.8 µg/ml) was lower than that of samples. (PM, 361 µg/ml; SCM, 74 µg/ml and KM, 31 µg/ml). The highest cytotoxic activity was found in KM (31µg/ml) which was close to the IC50 value of a pure reference standard and therefore significant. It could be presumed that the KM may contain phytochemicals whose activity may be equivalent or superior to that of curcumin standard.

Table 3-16 Cytotoxic activity (IC50) of SCF extracts

Sl. No.	Sample	IC50 (µg/mL)
1	PM	361±16
2	KM	31±2.3
3	SCM	74±4.1
4	curcumin	14.8±1.2
5	camptothecin	$1.8x10^{-3}±3x10^{-4}$
6	methotrexate	$4.15x10^{-3}±8x10^{-4}$

KM; S. cumini kernel methanol extract, PM: S. cumini pulp methanol extract, SCM; S. cumini seed coat methanol extract. All values are expressed in dry weight of extract.

Cytotoxic studies (LDH method): LDH assay is another method for cytotoxicity analysis. When a cell population is exposed to cytotoxic agents, they can induce cytotoxicity by necrosis. Widely used cytoplasmic marker enzyme membrane damage study is LDH and its activity under the optimized condition can provide indication about whether necrotic cell death has happened or not.

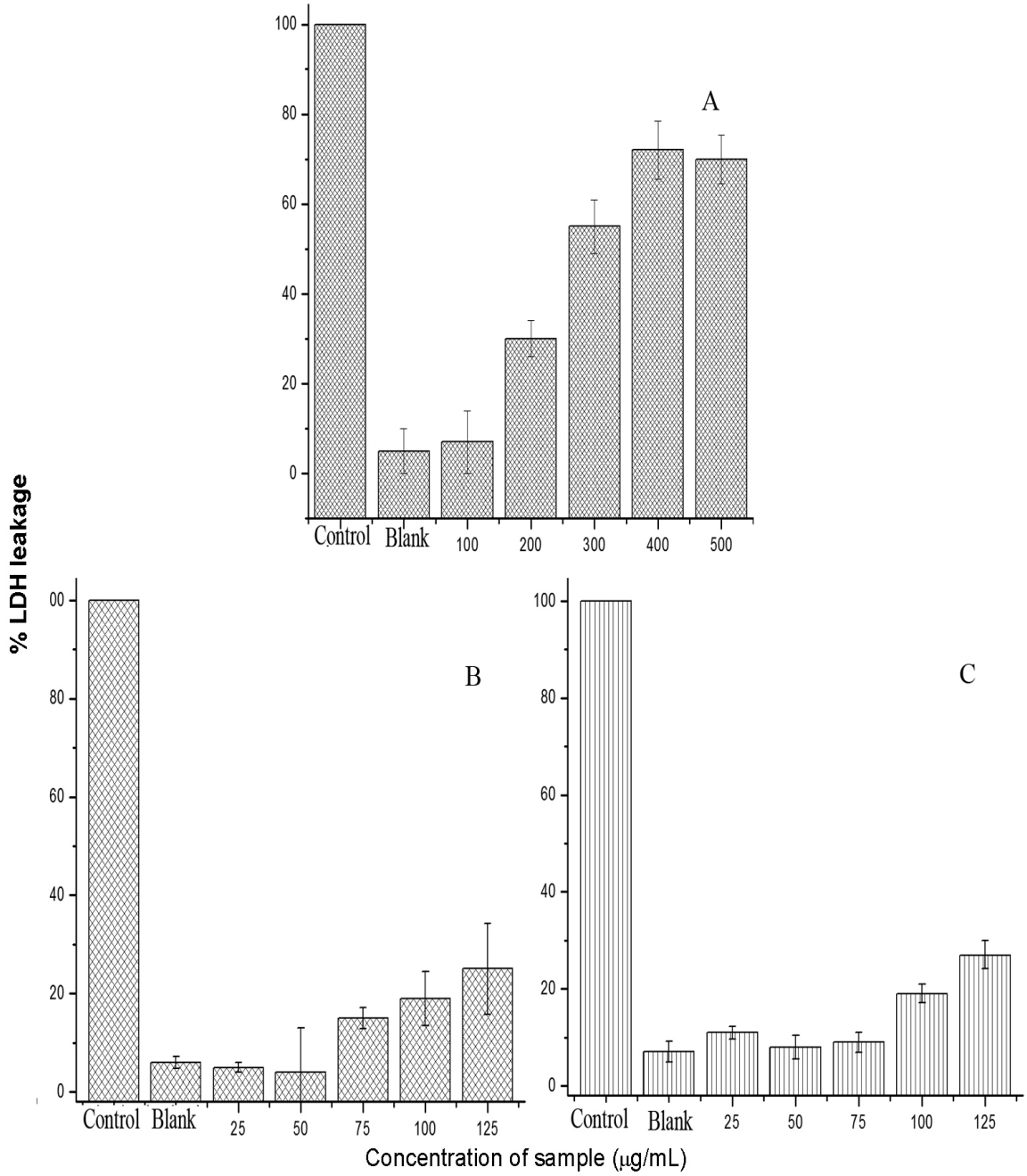

Fig. 3-29 Cytotoxic activity of methanol extracts from anatomically distinct parts of S cumini fruit (A, PM; B, KM; C, SCM) using LDH leakage assay (Incubation period 24 hrs)

To study the potential of PM, KM, and SCM to cause necrotic cell death, HeLa-S3 cell were used and the method is described in section 2.6.6. The cells were seeded at a density of 10 X 10^4 in 24 well plate containing RPMI1640 culture media supplemented with 10% heat inactivated foetal bovine serum. The cells were treated with sterile methanol extract prepared from anatomically distinct part of SCF at different concentrations as follows; PM, 0-500 µg/ml (step 100); KM and SCM, 0-125 µg/ml (step 25).

The plates were incubated for 24 hrs and the percentage necrotic cell death was calculated by measuring the released LDH enzyme with reference to a positive control (cells treated with detergent) and blank (cells without any treatment). Since the assay was conducted under similar conditions as that of the conditions set up for MTT assay, a better picture about the relation between necrotic and non necrotic cell death induced by any of the sample can be obtained. Fig. 3-29-A shows the dose dependent cell death caused by PM quantified by LDH method. The cells treated with samples were compared with that of control and blank. The cells treated with 100µg/ml of PM did not show significant LDH leakage compared to blank but, at higher concentration, LDH leakage was observed proportional to concentration. Comparison of the MTT assay and LDH assay (Fig. 3-30-A) showed similar trend in cell death and LDH leakage under identical condition respect to sample concentration. Therefore the cell death as observed in the MTT assay may be because of necrotic cell death caused by the PM at 200 to 500 µg/ml. LDH leakage in cells treated with pulp may be because of cell membrane damage caused by hypertonic condition in the assay system by concentrated PM in the range of 200-500µg/ml. Composition analysis also showed that >50% of PM is soluble sugars (Table 3-3). In the case of KM and SCM, comparison of dose dependent LDH release and MTT reduction showed a different profile. Fig. 3-30-B and C show that the percentage cell death seen in MTT assay is not proportional to dose dependent LDH release. Therefore it is assumed that the cell death as seen by MTT assay was not purely of necrosis and hence further evaluations need to be carried out to know non necrotic mode of cell death induced by KM and SCM. In this preliminary cytotoxicity evaluation,

among the three extracts studied, KM was found to be the most cytotoxic fraction of SCF with low LDH leakage. SCM also showed similar trend as that of KM in both MTT and LDH assay however its yield was less. In the case of PM, its cytotoxic activity was found to be several fold less than that of KM as well as curcumin standard and therefore for further studies, SCM and PM were not included.

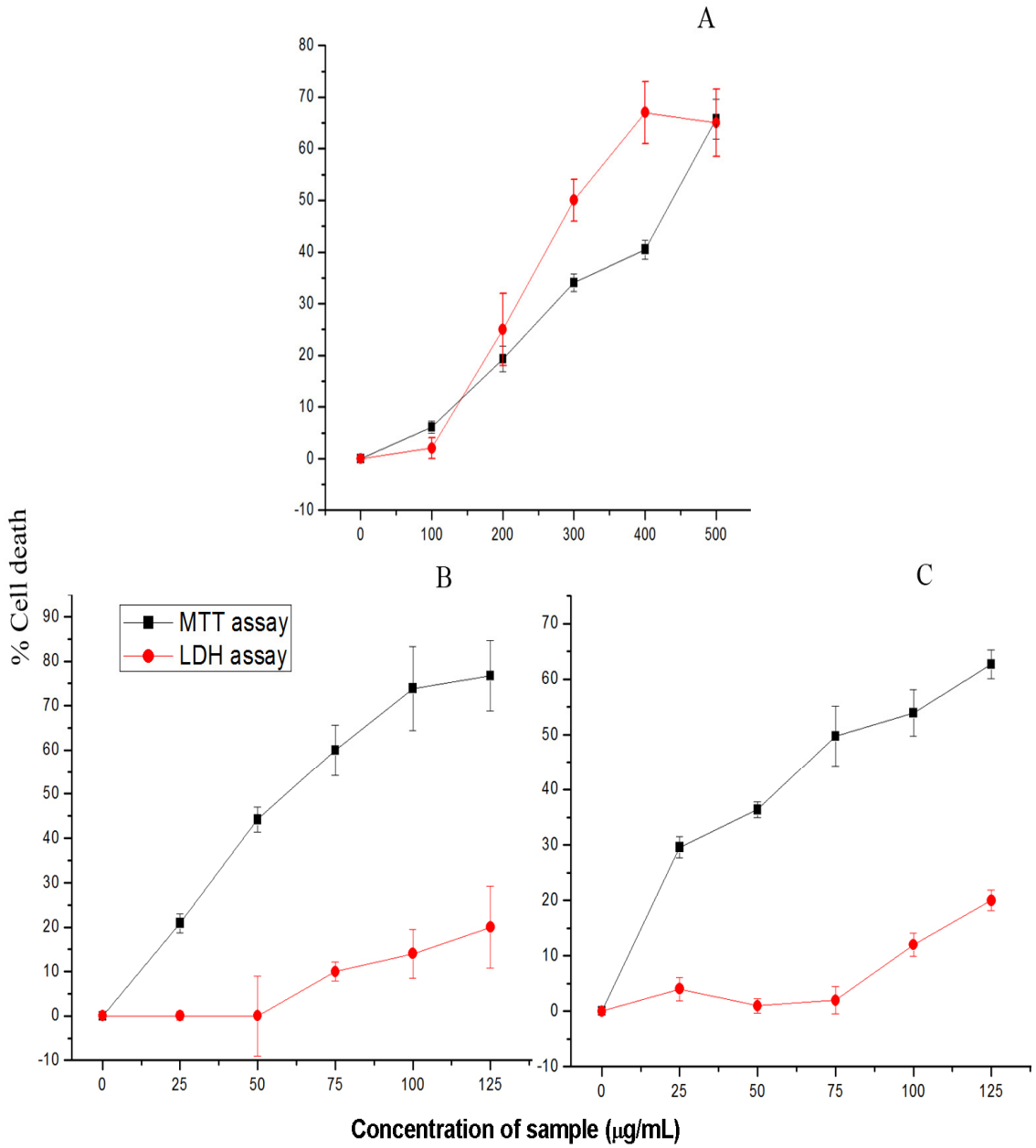

Fig. 3-30 Comparison of cytotoxic activity Profiles of PM (A), KM (B), and SCM (C) by two assay method viz. MTT and LDH

3.3.2 Cytotoxic activity guided fractionation of KM

Cytotoxic activity of CME from three anatomically distinct parts of SCF was discussed in section 3.3.1. Though both KM and SCM showed similar cytotoxicity, the yield of SCM was very low and hence not investigated further. However KM was selected for further fractionation due to its high cytotoxicity and higher yield and the results are discussed in the following sections.

Solvent-solvent fractionation: To identify the active fraction/principle from KM responsible for the cytotoxicity, it was further fractionated. KM was fractionated into five components based on their polarity by liquid-liquid partition. Thus methanol fraction of KM was desolventized and fractionated using solvents of increasing polarity as described (Sec. 2.6.3).

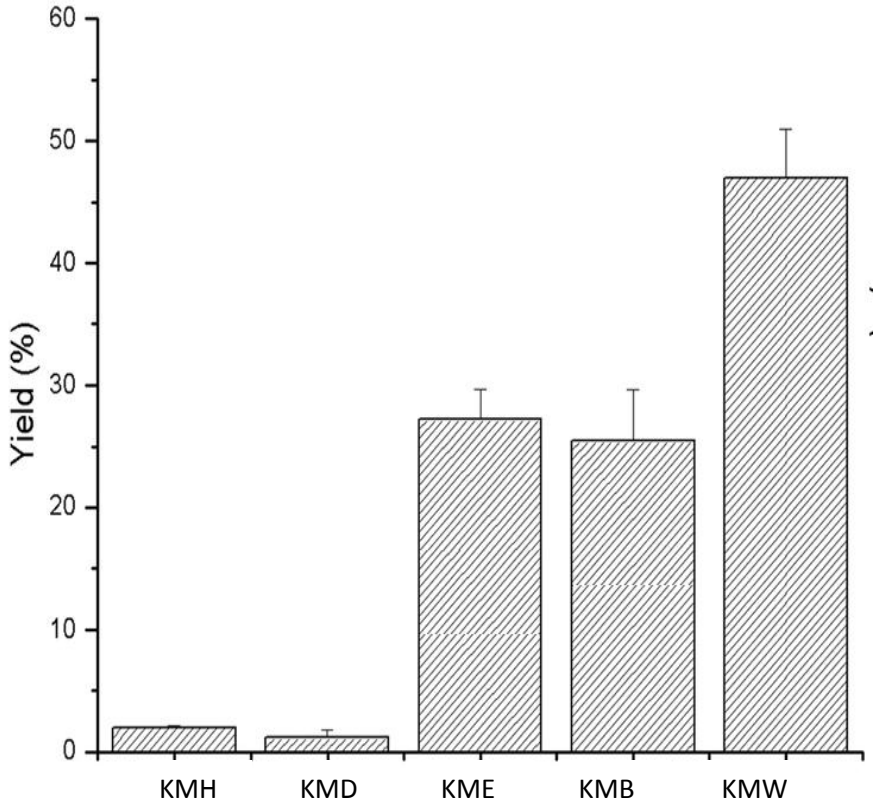

Fig. 3-31 yield of fractions prepared from KM by liquid-liquid partition. Hexane (KMH), dichloromethane (KMD), ethyl acetate (KME), butanol (KMB) and water (KMW) fractions of kernel methanol extract.

Table 3-17 Cytotoxic activity (IC50) of curcumin, KM, KMH, KMD, KME, KMB and KMW by MTT method

Sl. No.	Sample	IC50 (µg/mL)
1	**curcumin**	**14.8±1.2**
2	**KM**	**33±2.7**
3	KMH	85±3.5
4	KMD	82±4.3
5	**KME**	**18±3.4**
6	KMB	61±5.2
7	KMW	98±4.8

KM, S. cumini kernel methanol extract; KMH, hexane fraction of S. cumini kernel methanol extract; KMD, dichloromethane fraction of S. cumini kernel methanol extract; KME, ethyl acetate fraction of S. cumini kernel methanol extract; KMB, butanol fraction of S. cumini kernel methanol extract; KMW, water fraction of S. cumini kernel methanol extract. All values are expressed in dry weight of extracts.

Fig. 3-31 shows the yield of fractions prepared from KM. Among the five fraction prepared, yield of hexane fraction of KM (KMH) and dichloromethane fraction (KMD) was found to be less than 2.5% of KM. In the case of ethyl acetate (KME) and butanol fraction (KMB) yield was found to be > 25% of KM. Yield of water fraction was the highest (45%). All fractions were evaluated for cytotoxicity by MTT method in the concentration range 10-120 µg/ml and the results of dose dependent cytotoxic activity are presented in Fig. 3-32. Compared to parent extract (KM) cytotoxic activity of KMH and KMD was less, as indicated by the IC50 value besides their low yield (Table 3-17).

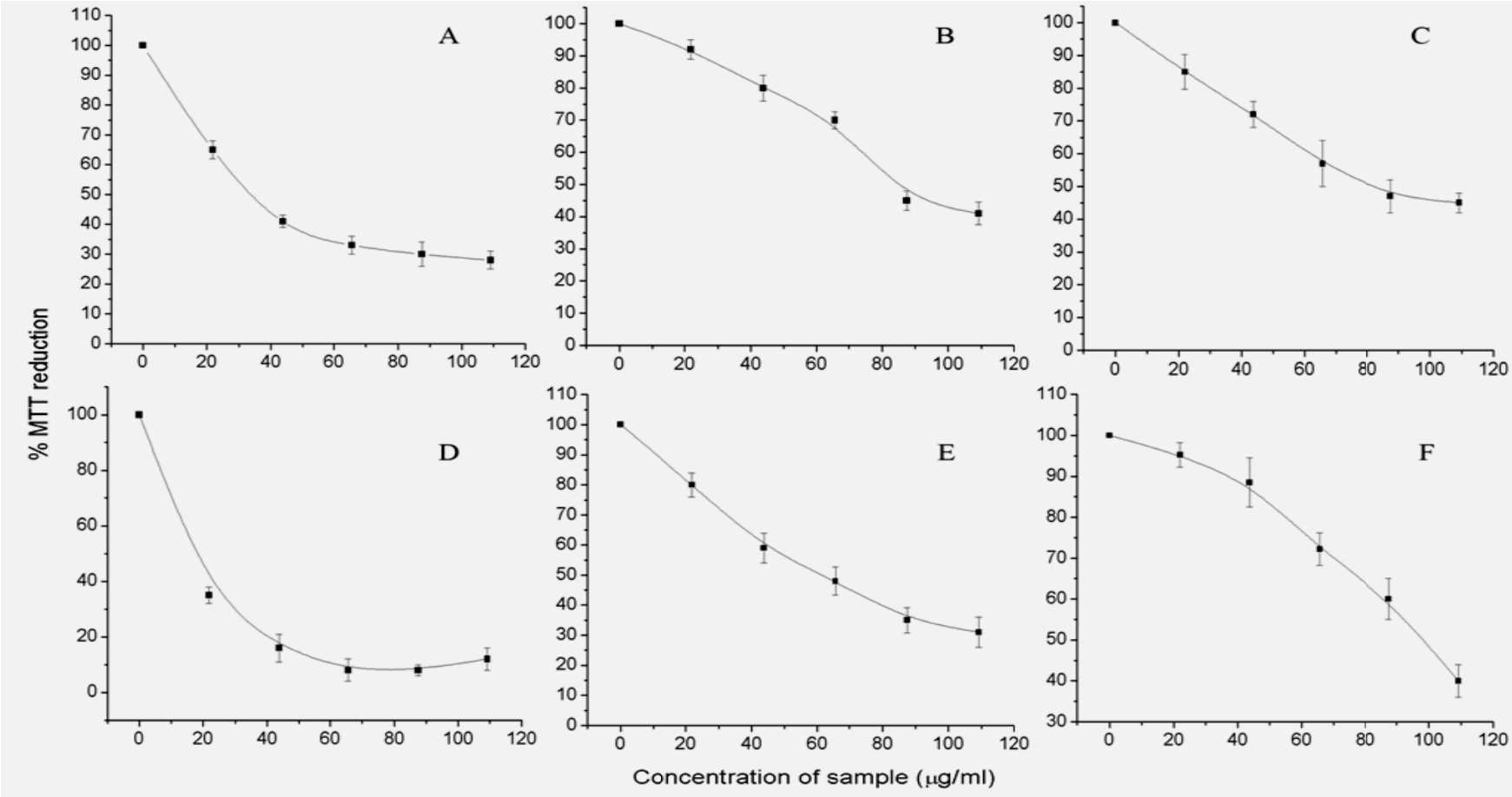

Fig. 3-32 Dose dependent cytotoxic activity of; A, kernel methanol extract (KM);B, hexane fraction of KM (KMH); C, dichloromethane fraction of KM (KMD); D, ethyl acetate fraction of KM (KME); E, butanol fraction of KM (KMB); water fraction of KM (KMW) by MTT method

KMH and KMD therefore were eliminated from further studies. In the case of KMW and KMB, the activity was not very significant. Subsequently only KME was selected for detailed investigation because of its high cytotoxic activity (18μg/ml) and high yield.

Preparative HPLC fractionation of KME: Preparative HPLC (Shimadzu) system integrated with photodiode array (PDA) detector and programmable fraction collector was employed for activity guided fractionation of KME (Sec. 2.6.3.). Fig. 3-17 A presents the 3D PDA chromatogram of KME. The chromatogram show that no compound/compounds separated by the HPLC had absorbance above 320 nm and most of the UV active (having absorption between 180-320 nm) compounds were eluted before 50 min. Z axis (wave length; 180-450 nm) of the 3D chromatogram was scanned and found 270 nm as λmax for the chromatogram and for programming fraction collector. The 2D chromatogram at 270 nm (Fig. 3-17 B) showed more than 30 peaks. The peaks grouped into 16 fractions, with nine as single peak fraction. Based on the peaks and groups, the fraction collection start time and stop time was recorded (Table 3-13) for the programmable fraction collector.

The reproducibility of the preparative chromatogram, fractionation, and collection were validated by conducting several trials and monitor the resolution of peaks. The fractions collected by the fraction collector were concentrated to appropriate strength using vacuum rotary evaporator under reduced temperature and pressure. The yield of preparative HPLC fractions were quantified gravimetrically and presented in Fig. 3-18. Among the fractions collected, yield of F1 and F2 were found to be highest with 8% and 7.5% respectively of KME. Yield of F3-F9 were; F3, 6.4%; F4, 6.4%; F5, 6.2%; F6, 6.5%; F8, 5.3% and F9, 5%. Other each of the fractions yielded less than 5% of KME.

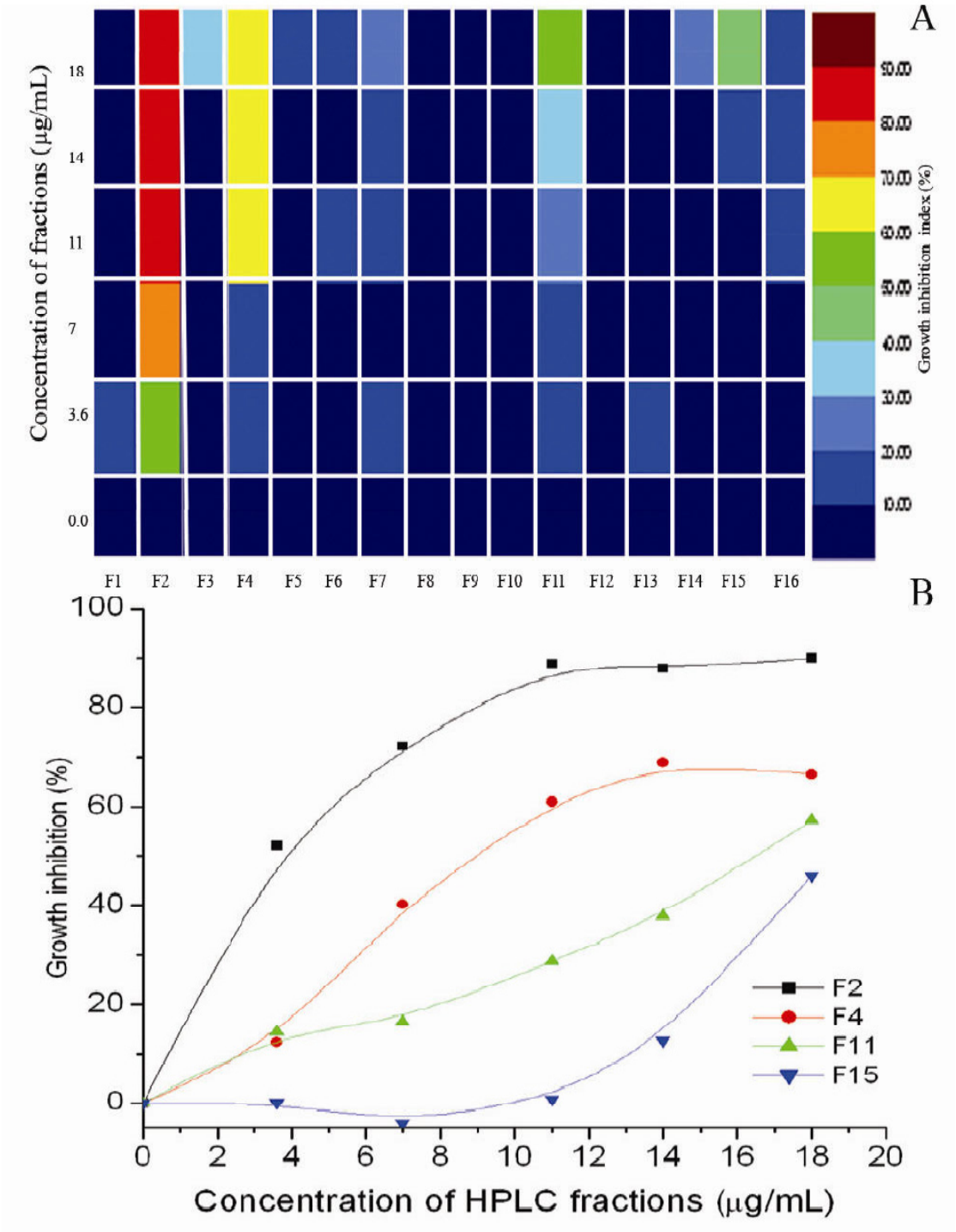

Fig. 3-33 Cytotoxic activity (MTT) of KME's preparative HPLC fractions. A, Contour graph showing the cytotoxic potential of all 16 fractions (F1-F16), color index: blue (0-10%) to brown (90-100%) cell death; B, dose dependent cytotoxic activity of selected potent fractions.

For cytotoxic activity evaluation, 2 ml of each fraction from concentrated stock was desolventized by vacuum evaporation and redissolved, first in minimum

amount of methanol and then resuspended in a standard volume of mammalian cell culture media. Amount of methanol in each sample was noted and sample blanks were prepared accordingly to nullify the effect of solvents in the cell culture assay system. Cytotoxic activity of all 16 fractions collected from preparative HPLC were evaluated against HeLa-S3 cells by MTT assay. Fig. 3-33 A is the contour graph showing dose dependent cytotoxic activity of all 16 fractions. Among the sixteen fractions studied here, F2, F4, F11 and F15 were found to be relatively more cytotoxic compared to other fractions. Fig. 3-33 B shows the dose dependent line graph of selected active fractions viz. F2, F4, F11 and F5. Concentration dependent cytotoxicity of these fractions in the descending order were; F2> F4> F11>F5. Among these active fractions, except F 15, all other three fractions induced more than 50% cytotoxicity within the sample concentration used in the study.

IC50 values of these fractions derived from the dose response curve are presented in Table 3-19. Among these three fractions, F2 was found to most active with an IC50 value of 2.5±0.5 µg/ml followed by F4 (IC50: 9µg/ml). Compared to the cytotoxic activity of curcumin, the activity of F2 was found to be six fold more. Chromatographic analysis of F4 showed that it was a mixture of several peaks (data not shown). Further studies were focused on the most active and relatively pure F2 fraction

Table 3-18 MTT based cytotoxicity activity (IC50) of potent HPLC fractions from KME

Sl. No.	Sample	IC50 (µg/mL)
1	curcumin	14.8±1.2
2	F2	2.5±0.5
3	KME	18.0±3.4
4	F4	9.0±0.5
5	F11	17.0±1.3
6	F15	19.0±0.4

F2, F4, F11 and F15, Preparative HPLC fractions from KME (ethyl acetate fraction of KM)

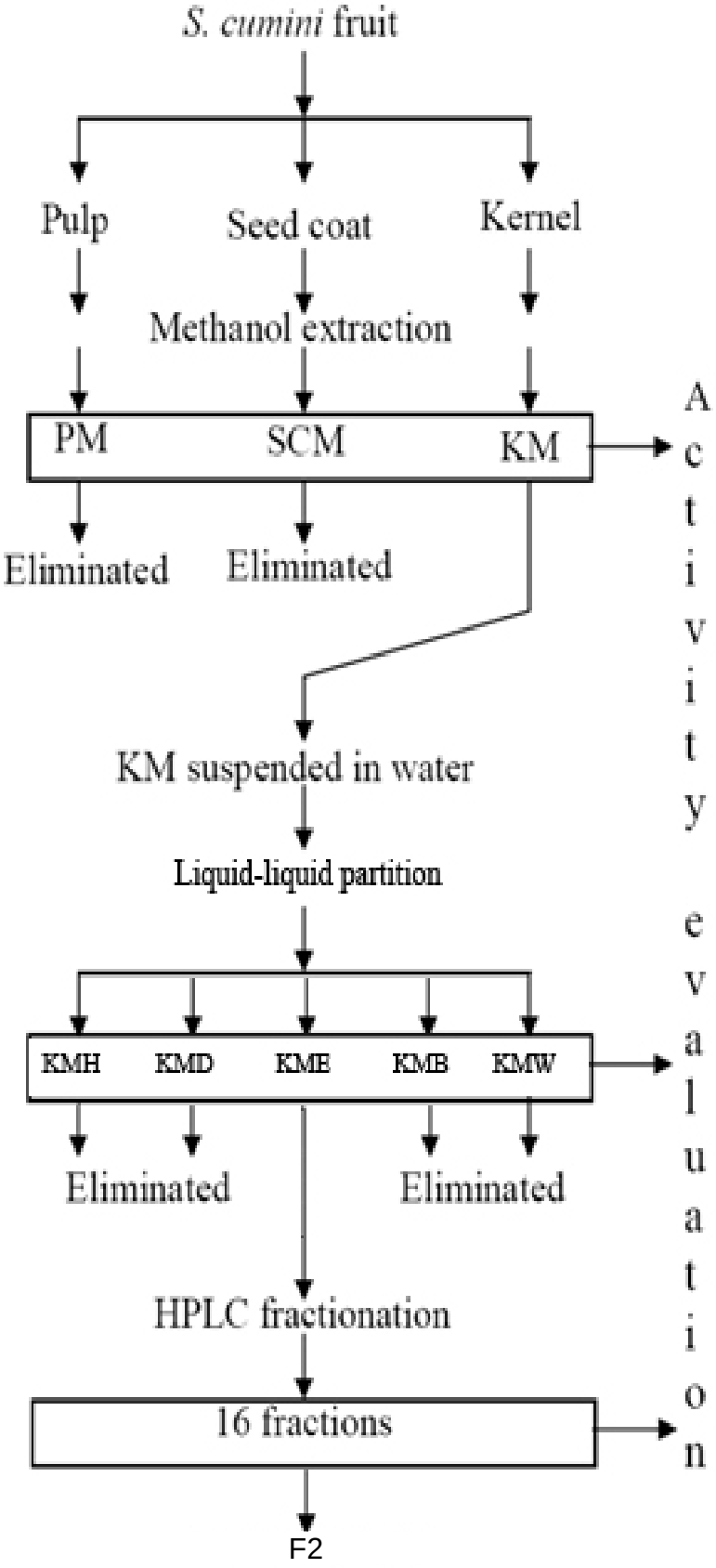

Fig. 3-34 Overall scheme for the isolation of cytotoxic entity F2 from SCF by bioassay guided fractionation

Fig. 3-34 show overall bioassay guided fractionation of SCF. There were three levels of fractionations viz. based on anatomical parts (level 1), liquid-liquid partition (level 2) and by preparative HPLC (level 3). From these three levels, a total of 24 fractions were prepared as follows; level 1, 3 fractions; level 2, 5 fractions; and level 3, 16 fractions Based on cytotoxicity and yield, each fraction were selected from each level for further evaluation. From the starting material, most active fraction was isolated by the activity guided fractionation which is evidenced from Fig. 3-35

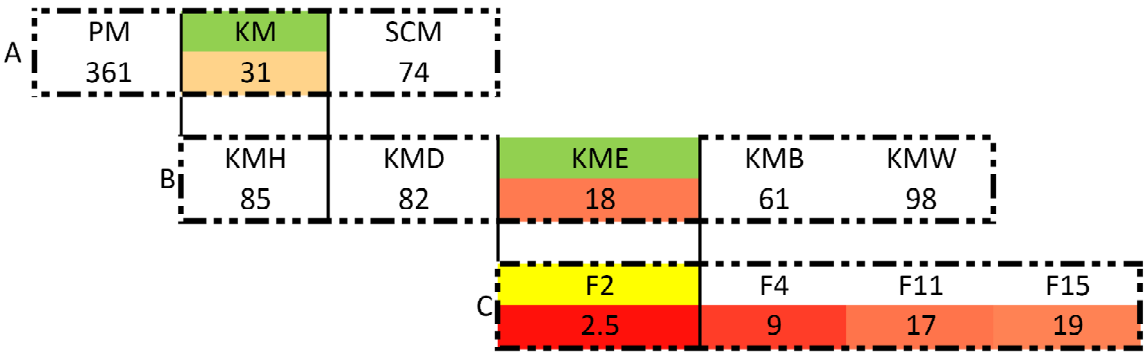

Fig. 3-35 Increase in cytotoxic potential (decrease in IC50 values (µg/ml)) of SCF on bioassay guided fractionation. A, crude methanol extracts from anatomically distinct parts of fruit; B, fractions prepared by liquid-liquid partition KM; C, selected fractions from KME by preparative HPLC.

3.3.3 Cytotoxic activity evaluation of F2 on other cancer cells

In HeLa-S3, the cytotoxic activity of F2 (IC50 2.5±0.5 µg/ml) was manifold compared to curcumin reference standard (IC50 14.8±1.2 µg/ml). A six fold higher cytotoxic activity of a substance compared to that of a standard cytotoxic agent is considered as very important and hence cytotoxic action on other cancer cells were investigated using COLO-205 (human colon cancer cell), HL-60 (Human leukemia cell) and MCF-7 (Human breast cancer cells). All these cells were cultured in 24 well plate (Sec. 2.6.4) and treated with different concentrations (0, 2, 8, 14, 20, 25µg/ml) of F2 and standard curcumin.

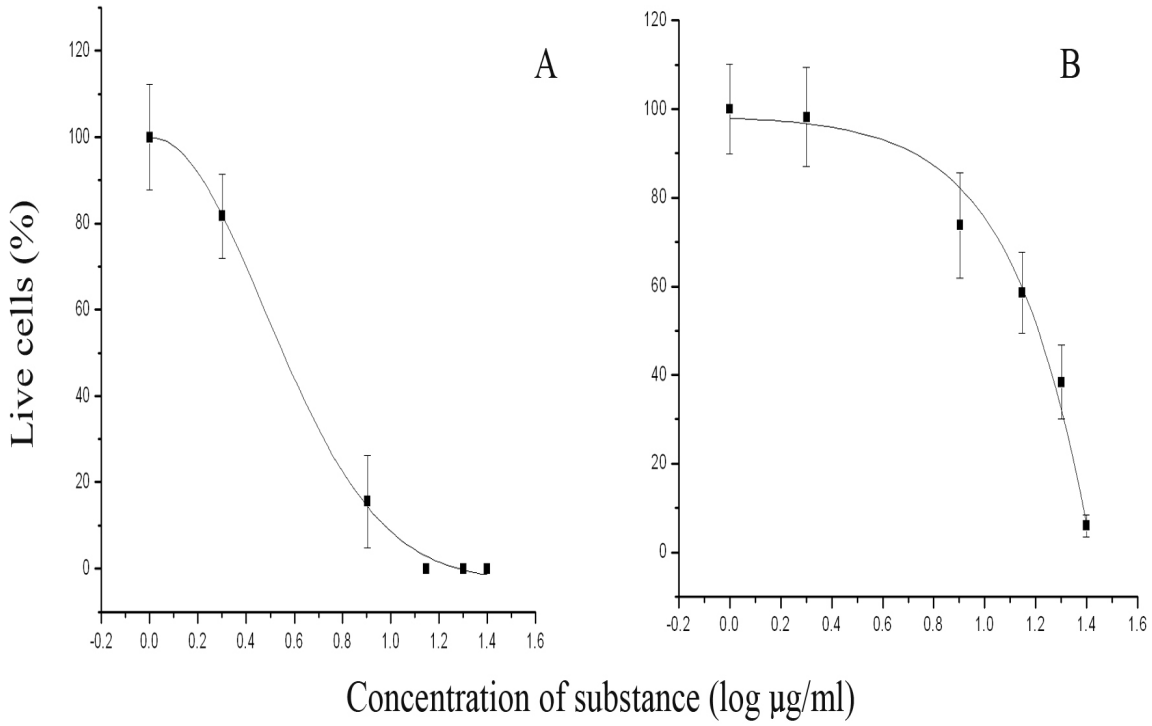

Fig. 3-36 Dose dependent cytotoxic activity of F2 (A) and curcumin (B), in MCF-7 cells by MTT assay

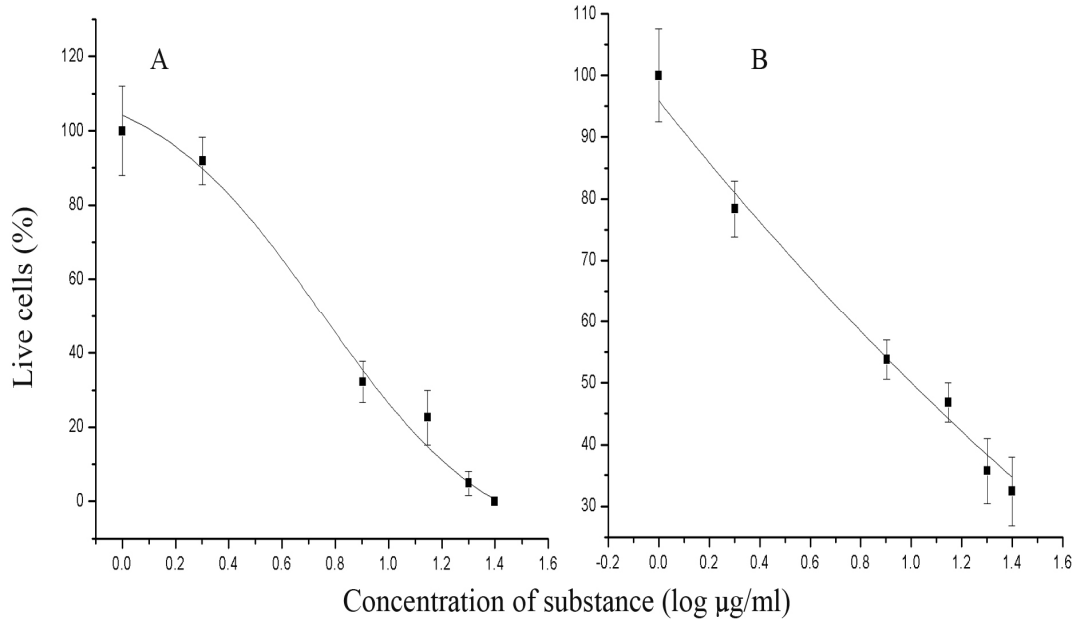

Fig. 3-37 Dose dependent cytotoxic activity of F2 (A) and curcumin (B) in COLO-205 cells by MTT assay

F2's cytotoxic activity was very strong on MCF-7 and the dose response curve fitted a Gaussian model and the response dose dependent. Greater than 80% cell death was observed at 8µg/ml concentration itself and 100% death was achieved at 14 µg/ml. Dose dependent cytotoxicity activity of curcumin on MCF-7 cells also followed a Boltzmann model curve. For curcumin, the curve exhibited a low slope till 8µg/ml and above the cytotoxicity was proportional to concentration and 100% mortality was achieved at 25µg/ml. Fig. 3-37 shows the dose dependent cytotoxic activity of F2 (A) and curcumin (B) on colon cancer cells. Both samples and reference followed a Gaussian type of dose response curve. The figure show that 100% cytotoxicity in COLO-205 cells was achieved at 25µg/ml concentration by F2 but, at the same concentration, curcumin induced only 70% cytotoxicity which indicated that cytotoxic activity of F2 stronger than that of curcumin as shown for HeLa-s3 cells. IC50 values were drawn from the dose response curves (Fig. 3-37) and were found to be; F2, 5.7µg/ml and curcumin, 9.8µg/ml.

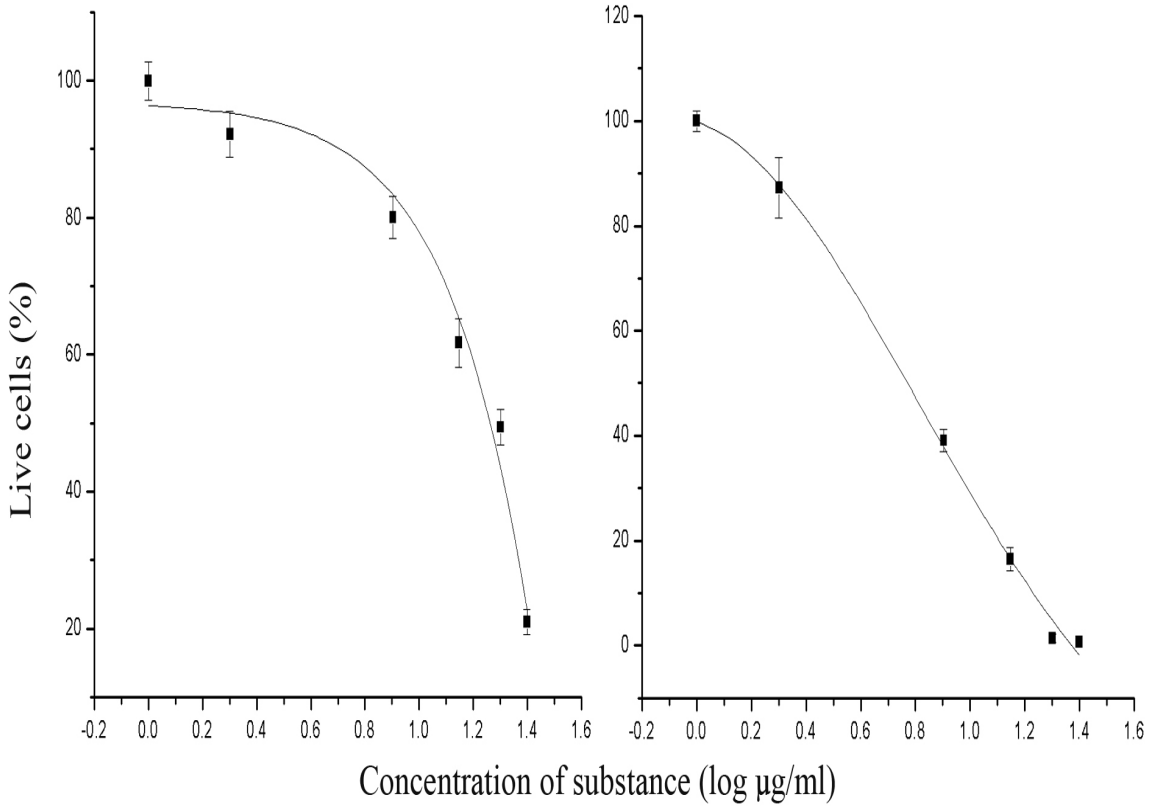

Fig. 3-38 Dose dependent cytotoxic activity of F2 (A), curcumin (B) in HL-60 cells by MTT assay

Comparison of cytotoxic activity of F2 and Curcumin on HeLa-S3 and COLO-205 thus showed that the cytotoxicity for F2 over HeLa-S3 cells was higher than that of its activity on COLO-205 cells. However comparison of the activity of curcumin showed a different trend that its cytotoxic potential on COLO-205 cells was more than that of HeLa-S3 cells (Table 3-19). Cytotoxic potential of F2 and curcumin on HL-60 cells is presented in Fig. 3-38 A. The dose dependent cytotoxic activity of F2 over this cell type was found to follow Boltzmann model. The curve showed that up to 8 µg/ml, cytotoxic induction potential of F2 was observed to be very low (20% cell death at 8 µg/ml) that can be visualized from the low slope of the curve in X axis region of 0-8µg/ml. After 8µg/ml, a strong cytotoxic activity, evidenced by a steep reduction in the number of live cells, was shown up to 25 µg/ml. However a complete cell death was not observed even at the highest concentration of sample used in the study with maximum being 80% at the highest concentration. The effect of curcumin in this cell type showed a near linear dose dependent cytotoxicity as indicated by the Gaussian curve (Fig. 3-38 B). At 8 µg/ml, almost 60% cells were killed but, in the case of F2 at this concentration only 20% cells were dead. This shows that the potential of F2 to induce toxicity on HL-60 cells at lower concentration was low compared to curcumin. The IC50 values as drawn from the dose response curve were; F2, 18; and curcumin, 5.6 µg/ml (Table 3-19). Dose dependent cytotoxic activity of F2 and standard curcumin over HeLa-S3, MCF-7, COLO-205 and MCF-7 were analyzed and the IC50 values were calculated and presented in Table 3-19 for comparison. The results show that among the cells studied for the evaluation of F2 and curcumin, He-La-S3 was found to be most sensitive. In HeLa- S3 cells, cytotoxic activity of F2 was more than 6 times more than that of curcumin standard. The same pattern was observed for other cell lines such as MCF-7 and COLO-205 cancer cells. In the case of HL-60, the trend was different, as the activity of curcumin was more than that of F2. HL-60 is leukemia cell and the cell morphology, nature of growth etc. are different from other three cell types that grow by adhering to the plate surface.

Table 3-19 Cytotoxic activity (IC50) of F2 and curcumin in HeLa-s3, COLO-205, HL-60 and MCF-7 cells by MTT assay

Cell line	sample	IC50 (μg/ml)
HeLa-S3	F2	2.5 ± 0.72
	curcumin	15 ± 1.15
MCF-7	F2	3.56 ± 0.59
	curcumin	16.22 ± 1.03
COLO-205	F2	5.68 ± 0.73
	curcumin	9.77 ± 1
HL-60	F2	18.16 ± 1
	curcumin	5.8 ± 1.15

F2, fraction 2 prepared by HPLC from ethyl acetate fraction of kernel methanol extract

Further studies are required to understand the molecular mechanism behind the differences in sensitivity observed. Studies related to the mode of transport, of F2 and curcumin into cell cytoplasm, knowledge about the mechanism of action of induction of cytotoxicity etc. are essential to associate at the mechanistic aspects of cytotoxic potential. The observations presented from this study warrant more investigations on the mechanistic of F2 fraction including its chemical profiling.

3.3.4 Evaluation of apoptotic induction potential of F2

Search for suitable chemotherapeutic agents to treat various cancers has been the area of focus for more than a century. A chemical entity capable of inducing apoptosis is considered as a potent candidate in the cancer drug discovery process. At present several standardized methods are available for the evaluation of apoptosis induction potential of chemical entities. In the present study, apoptotic induction potential of F2 on HeLa-S3 cells were conducted by following two methods viz. morphological evaluation for cell shrinkage and membrane blebbing and immunochemical method to evaluate the expression abundance of pro-apoptotic and anti-apoptotic protein.

Morphological evaluation: For morphological evaluation of apoptosis, HeLa-S3 cells were cultured in 24 well plates and treated with different concentrations of F2. After incubation, the plates were examined under inverted bright field microscope. Fig. 3-41 A shows photographs of blank well containing growing cancer cells. Compared to blank, the well treated with 2µg/ml of F2 showed cells with irregular boundaries and a few detached cells and in Fig. 3-39 B at 1 'O' clock position shrunken cells can also be seen. In fig C, more than 80% of the cells were shrunken as evidenced by irregular cell boundaries

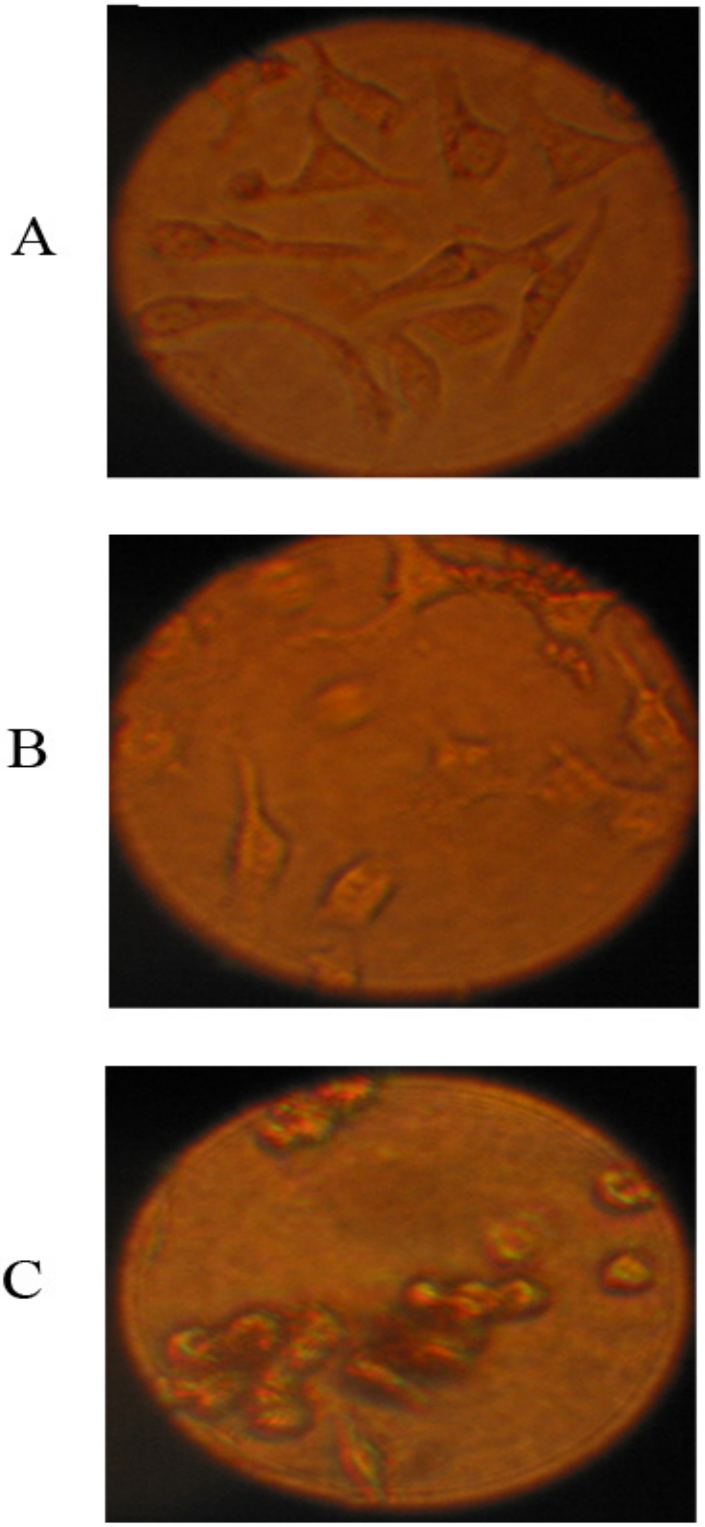

Fig. 3-39 Microphotograph (400X) of HeLa-S3 treated with F2. A, blank; B, 2μg/ml of F2; C, 8μg/ml of F2. Cell shrinkage and membrane blebbing indicate the induction of apoptosis

Fig. 3-40 is a1000X magnified image of cell treated with 2 µg/ml of F2. The morphology shows that the cell was shrunken with several membrane blebs indicating formation of apoptotic bodies. From this morphological analysis, it could be presumed that fraction 2 prepared from *S. cumini* kernel by BAGF, was capable of inducing apoptosis under in vitro conditions. Induction of apoptosis by a chemical entity is considered important in cancer treatment.

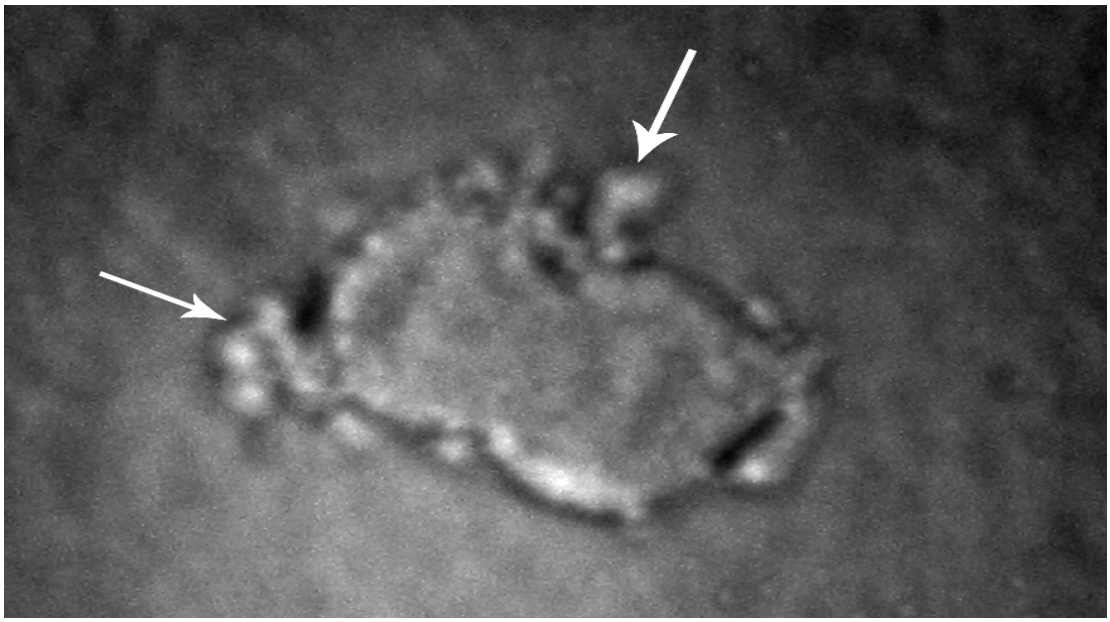

Fig. 3-40 Microphotograph (1000X) of HeLa-S3 treated with 2µg/ml of F2. Membrane blebbing shows the induction of apoptosis

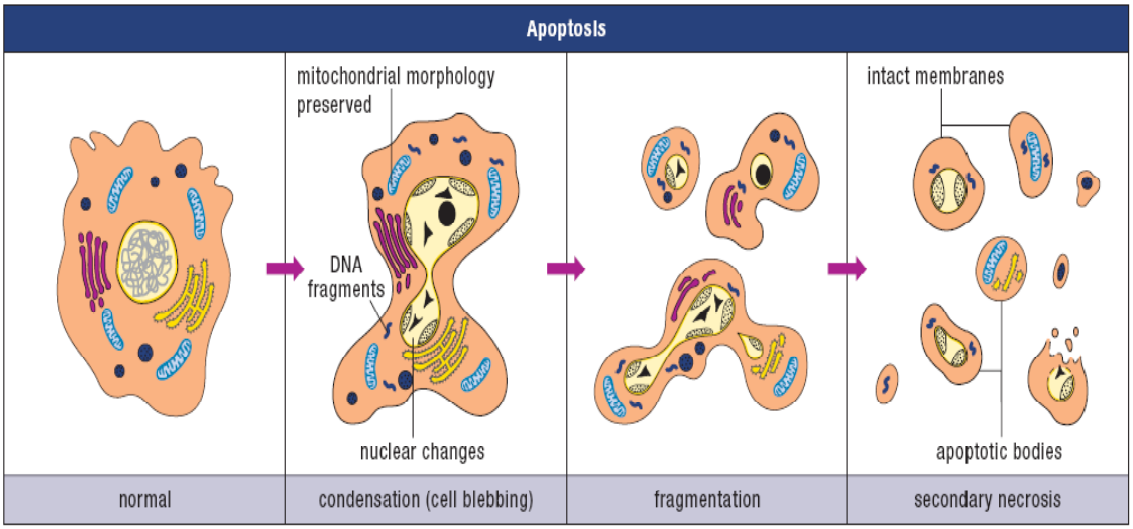

Fig. 3-41 Morphological changes of cells that undergo apoptosis. (Courtesy: Boehringer Mannheim)

Membrane blebbing is frequently found after exposure of cells to stressful stimuli (Gores et al., 1990). Membrane blebbing has been associated with various biochemical alterations (including a decrease in ATP levels, increase in cellular calcium, activation of nonlysosomal proteolytic system, and activation of phospholipase A 2) as well as with cytoskeletal alterations affecting the dynamics of actin and myosin and the formation of focal adhesion complexes (Cunningham, 1995; Fishkind et al., 1991; Martin et al., 1994; Mills et al., 1998; Miyoshi et al., 1996). Membrane blebbing is an early manifestation of toxicity followed by apoptosis (Gores et al., 1990). Apoptosis is highly regulated at the morphological level, characterized by membrane blebbing, cell shrinkage, chromatin condensation (Fig. 3-41), nuclear/cytoplasmic fragmentation, and formation of dense bodies that are quickly removed via phagocytosis by macrophages. Apoptosis can be induced through receptor-mediated mechanisms (Frisch and Francis, 1994; Xia et al., 1995) or exposures to cytotoxic drugs (Hale et al., 1996; Muschel et al., 1998). Though exact mechanism of membrane blebbing is not clear, it is widely accepted that mitogen-activated protein (MAP) kinase ERK1/2 (extracellular signal-regulated kinase) and the stress-activated protein kinases-1 (SAPK1/JNK) and -2 (SAPK2/p38) are central elements of homologous pathways used by mammalian cells to coordinate the signals generated by external stimuli (Cohen, 1997; Waskiewicz and Cooper, 1995). Therefore from the present study the observation that the F2 induced apoptosis in HeLa-S3 cells as characterized by membrane blebbing could be by activation of protein kinases. However further mechanistic studies are required.

Evaluation pro and anti-apoptotic protein markers: For the confirmation of apoptosis by F2, a few marker protein analyses were also conducted by dot blot technique. The detailed methods of cell culture, treatment, cell lysate preparation, blotting and immunochemical detection etc. are described elsewhere (Sec. 2.6.8). For molecular analysis of apoptosis, two pro-apoptotic marker proteins, and one anti-apoptotic marker protein and for reference, actin was evaluated by dot blot. Fig. 3-42 is a combined image displaying abundance of various marker proteins in HeLa-S3 cells on treatment with different concentration of F2.

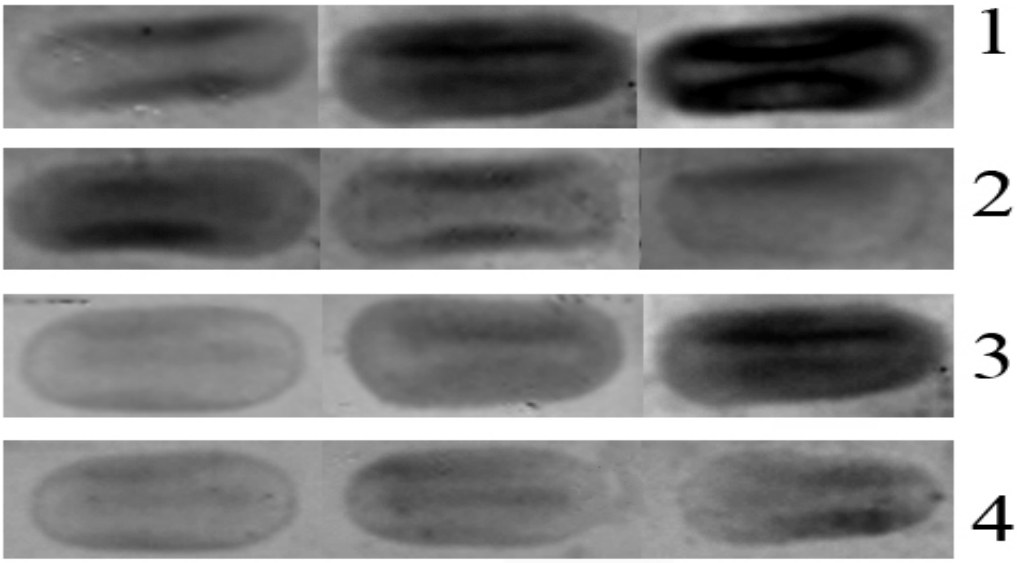

Fig. 3-42 Dot blot image shows the upregulation and down regulation of pro-apoptotic (Bax and Caspase-3)and anti-apoptotic (BdXL) proteins. The rows display the abundance of Bax (1), BdXL (2), caspase-3(3) and actin (4) in HeLa-S3 cells treated with 0.0, 2.0 and 4.0 µg/ml of F2

Each row shows the abundance of each targeted marker protein from HeLa-S3 cell treated with 0, 2, and 8 µg/ml of F2. Row 1 shows the abundance of a pro-apoptotic factor Bax. The Bax level increased with the degree of apoptosis that in turn related to the concentration of F2. The Bax protein seen in blank well was considered as background level.

Compared to blank, the cell treated with different concentration of F2, Bax level was also found to increase that confirmed the molecular events leading to programmed cell death. Thus, in the apoptotic induction study, on the basis of morphology and protein marker, F2 was found to induce apoptosis in heLa-S3 cells. Induction of apoptosis in cancer cells are considered as important and the molecule that induce apoptosis could be considered a drug candidate (Dixon et al., 1997).

Apoptosis is modulated by anti-apoptotic and pro-apoptotic effectors, which involve a large number of proteins. The coordination and highly regulated activities of pro-apoptotic and anti-apoptotic proteins regulate the apoptosis process. Therefore many cancer therapy protocols, targets these proteins (Baell

and Huang, 2002; Goodsell, 2002). The ratio of death antagonists (Bcl2, Bcl-xL, BdXL) to agonists (Bax, Bcl-xs, Bad, Bid) determines whether cells respond to an apoptotic stimulus. Down-regulation of the death suppressor Bcl-2 could repress tumor growth via promoting programmed cell death (Kluck et al., 1997; Zhivotovsky et al., 1998). In our study also the balance between pro-apoptotic proteins and anti-apoptotic proteins have been found disturbed. Fig. 3-42 shows a high level of pro-apoptotic proteins viz. Bax and caspase and low level of the anti-apoptotic protein (BdXL) in HeLa-S3 cells treated with different concentration of F2 favoring induction of apoptosis. The results further suggest that apoptosis induction could be through caspase-dependent mechanism. Further studies are required to establish the mechanistic of F2 fraction mediated apoptosis as demonstrated in this study.

3.3.5 Cytotoxic significance of SCF

The possibility of SCF as a source for anticancer drug candidate has not been comprehensively studied except one preliminary study that evaluated the cytotoxic potential of SCF pulp that exhibited measurable cytotoxicity against various cancer cells viz. MCF-7, HeLa, Hep G2, H460, and U251 (Nazif, 2007). No detailed investigations have been done further to identify active principle/fraction from pulp or to study the cytotoxic potential of non-edible portions of SCF. In contrast to previous cytotoxic study on SCF pulp, in the present study, pulp did not show significant cytotoxicity comparable with that of any standards cytotoxic agents used here. Maximum cytotoxicity observed here was 30% under the highest concentration of PM (500µg/ml). The 30% cytotoxicity observed at this concentration was considered as insignificant and the induced cytotoxicity could be because of the hypertonic as well as hyper glucose condition of assay medium because of high soluble sugars (Table 3-3) in PM. Hyper glucose as well as hypertonic conditions have been reported to induce cytotoxicity in a variety of cells in vitro (Horio et al., 2000; Pal et al., 2007; Rossetti et al., 1990). In the case of SCM, though it's cytotoxic activity was several fold higher than that of PM, because of low yield, least importance was given. On the other hand, being a crude extract, KM's cytotoxicity was considered

as significant because of the comparable IC50 value with that of curcumin standard. A strong correlation was observed between TPC and cytotoxic potential (IC50) of fractions viz. KMH, KMD, KME, and KMB (Fig. 3-43) prepared from KM by liquid-liquid partition as second level fractionation of SCF but, the cytotoxicity of KMW was not proportional to phenolic content. Since the cytotoxicity is proportional to the TPC (Slope:-1), it is presumed that the active principle(s) responsible for the observed cytotoxicity could be phenolic compound(s); therefore the possibility of redox reactions as a cause of cytotoxicity need to be investigated. The poor correlation between cytotoxicity and TPC in KMW warrants the need of investigations in different class of phenolic compounds and their relation to cytotoxicity. The capacity of F2, prepared by prep.HPLC in third level fraction, to induce cytotoxicity as well as apoptosis as seen by membrane blebbing and immunochemical study is considered as important and further studies are required to chemically characterize it.

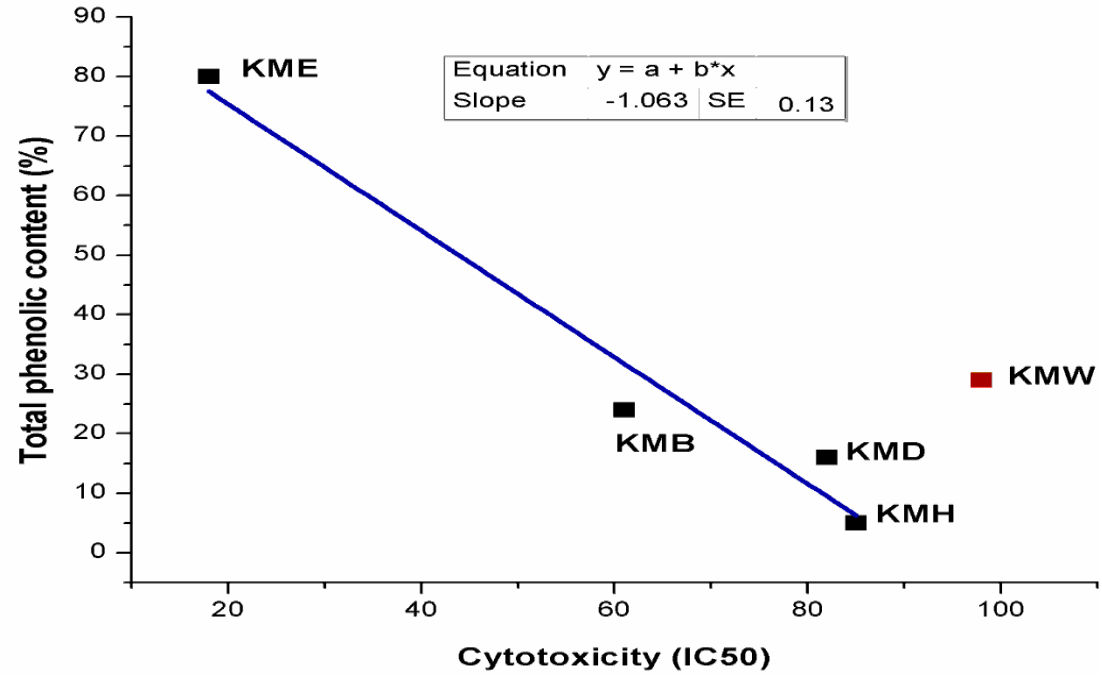

Fig. 3-43 Correlation between cytotoxicity and TPC of fraction (by liquid-liquid partition) of KM

3.4 Antidiabetic studies on *S. cumini* fruit (SCF)

Syzygium cumini fruit and seed have been used in Indian traditional medicine to treat diabetes mellitus for centuries. Though seed kernel of the fruit was mainly used; uses of pulp, bark, and leaves have also been reported in several ancient and modern literatures. However scientific validation to establish their efficacy has not been conducted as followed in drug discovery. During past three decades many scientific works have been reported to validate the therapeutic efficacy of fractions of traditional medicines. As a result active principles in plants used traditional medicine have been reported. *S. cumini* has also been evaluated scientifically in limited way for its antidiabetic property. The present study was designed to prepare active fractions from crude extracts of SCF by BAGF and further to identify active principles responsible for the antidiabetic activity using cell lines with particular reference to glucose transport across cell membrane. The scheme of BAGF of SCF is presented in Fig. 3-44. Briefly; KM, PM and SCM prepared from SCF parts were evaluated first and selected KM as active fraction for further fractionation. KM was fractionated to yield KMEd and KMWd by liquid-liquid partition. KMEd was then selected based on high activity for third level fractionated by preparative HPLC

3.4.1 GLUT-4 receptor as indicator of antidiabetic activity.

Enhancing insulin action on glucose uptake and metabolism in skeletal muscle, a major insulin target tissue regulating whole-body metabolism, constitutes one possible strategy for normalizing glycaemia and glucose homeostasis in insulin-resistant humans. Defects in whole-body glucose uptake are closely linked to impaired insulin stimulated glucose transport activity (Zierath et al., 2000). GLUT-4 is known to mediate glucose transport across the cell membrane and insufficient expression of this receptor protein is implicated in type II diabetes mellitus. Any agent that stimulates the expression of GLUT-4 is expected to facilitate glucose transport in skeletal muscle cells.

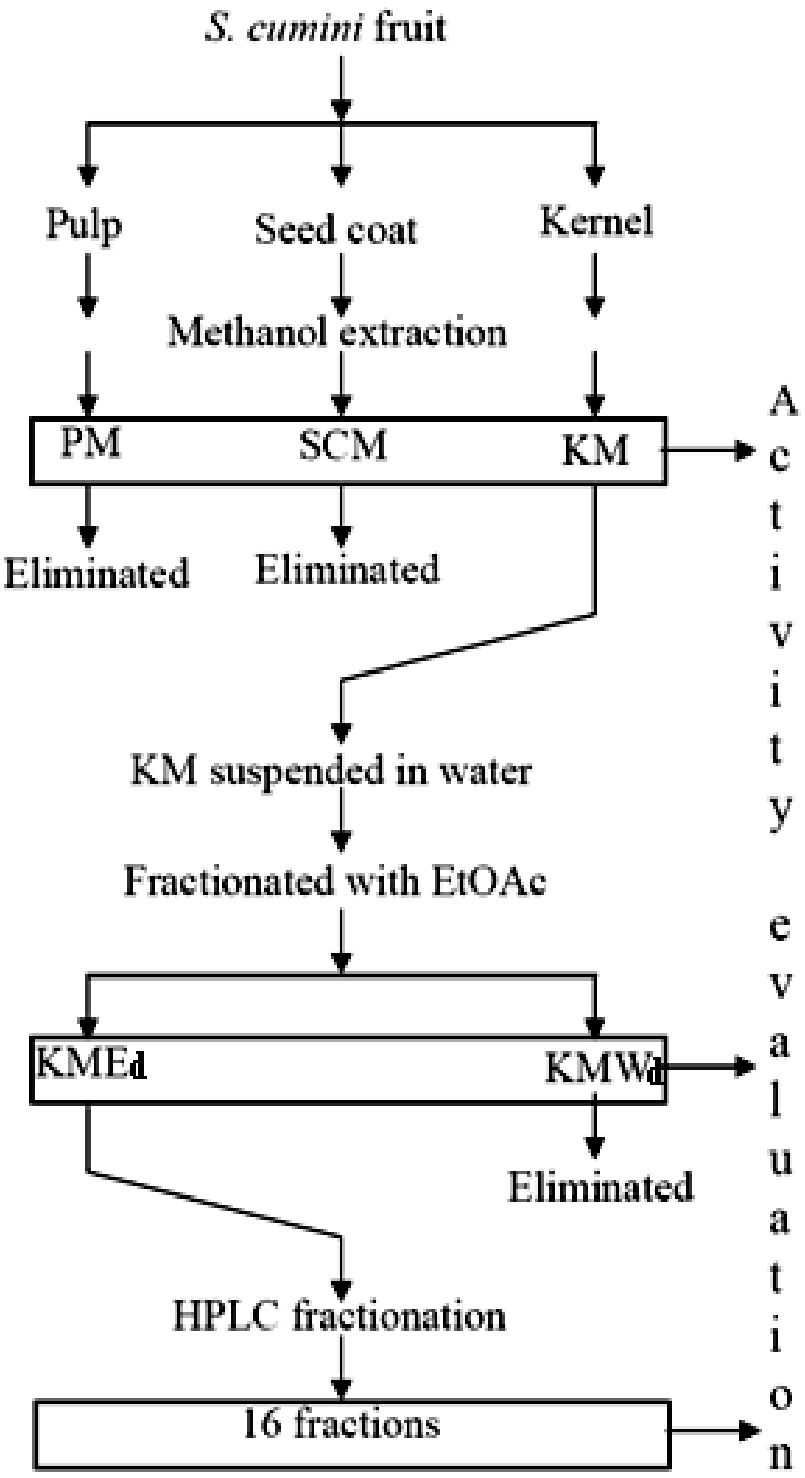

Fig. 3-44. Fractionation scheme of SCF for in vitro antidiabetic activity. PM, KM, and SCM; methanol extracts of fruit parts; KMEd and KMWd; ethyl acetate and water fractions of kernel methanol extract

The methanol extracts of pulp (KM), seed coat (SCM) and kernel (KM) were subject to in vitro antidiabetic assay. The preliminary evaluation of in vitro antidiabetic potency of SCF fractions to promote expression of GLUT-4 was done in differentiated 3T3-L1 cells. The culture conditions as well as assay details are described elsewhere (Sec. 2.7.4). The differentiated 3T3-L1 (D3T3-L1) cells treated with solvent alone (Blank), samples (PM, SCM and KM), and positive control (pioglitazone) were harvested and the cell lysate were prepared and subjected to dot blot assay to measure the abundance of GLUT-4 and actin under standard conditions. Fig. 3-45 shows the image of dot blot that displays the abundance of GLUT-4 receptors on D3T3-L1cells treated with PM (A), SCM (B) and KM (D) at 0 (1), 5 (2) and 10 (3) µg/ml concentration.

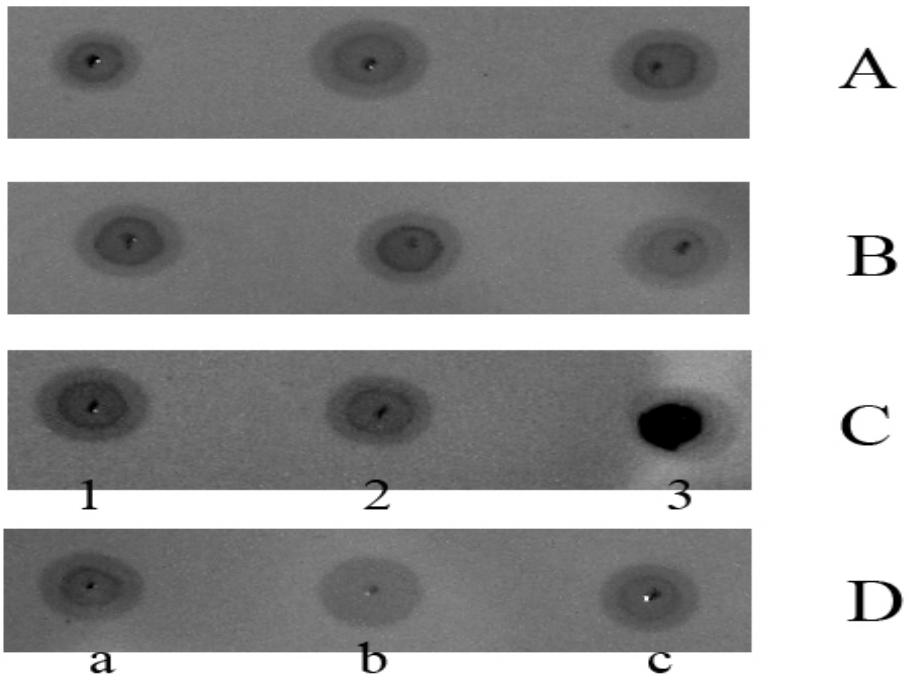

Fig. 3-45 GLUT-4 receptor expression assay using methanol extracts of; A, pulp (PM); B, seed coat (SCM); C, kernel (KM); at different concentration viz. 0 (1), 5 (2) and 10 (3) µg/ml. D; abundance of actin in cells treated with vehicle (a), 5 µg/ml KM (b) and 10 µg/ml KM (d)

The sub lethal concentration of all samples in D3T3 cell culture system for analyses was optimized by conducting a preliminary cytotoxic analysis. The

concentration of all extracts used throughout this study was below the standardized sub lethal concentration on D3T3-L1 cells. Fig. 3-45 A shows the relative abundance of GLUT-4 expressed in cells treated with 0 (1), 5 (2) and 10 (3) µg/ml of PM. There was no significant difference in the abundance of GLUT-4 between the blank and 5 & 10 µg/ml PM treated cells. Therefore it is inferred that PM could not modulate the expression of GLUT-4 under the used assay conditions and sample concentration. Similarly Fig. B shows the abundance of GLUT-4 in D3T3 cells treated with SCM in the sub lethal concentration. In this result also no significant difference between the amount of GLUT-4 expressed in blank and the cells treated with SCM at a sub lethal concentration was observed. In contrast to Fig. A and B, C showed a different profile. Fig. C shows the abundance of GLUT-4 expressed in D3T3-L1 cells treated with KM at sub lethal level. Comparison of spot 3 and 1(blank) showed that on treatment with KM at 10 µg/ml concentration, the GLUT-4 expression abundance was increased several folds. Further to ensure the reliability of protein expression, actin level was also evaluated in the cells treated with 10µg/ml of PM, SCM and KM. The results demonstrated that (Fig. D) there was no significant difference in actin level between the blank (a) and samples at the highest concentration in the expression of actin (b, 5µg/ml KM and c; 10 µg/ml KM). Even at highest concentrations of sample studied here, normal cellular translation processes were not affected. In this preliminary study among the methanol extracts from three anatomical parts evaluated at sub lethal concentration, KM was found to increase the expression of GLUT-4 receptor significantly. Therefore the results indicated that the active principle responsible for enhancing the expression of GLUT-4 could be in KM. For further evaluation fractionation of KM by (liquid-liquid fractionation technique) was conducted and the fractions were evaluated.

3.4.2 GLUT-4 receptor expression using fractions from KM.

KM was further fractionated by liquid-liquid partition on the basis of GLUT-4 receptor expression modulatory property observed in the parent extract (KM) as described before. The methanol extract of KM was desolventized and redissolved in water and fractionated with ethyl acetate. This fractionation yielded two

portions from KM that was intermediary polar ethyl acetate fraction (KMEd) and highly polar water fraction (KMWd). These two fractions were appropriately concentrated and used in differentiated 3T3-L1 assay system. A first level in vitro toxicological analysis was also done using these fractions to fix the sub lethal concentration suitable for applying in cell culture for expression assay. After treatment, the cells were harvested and prepared for GLUT-4 analysis following dot blot technique. Fig. 3-46 shows the abundance of GLUT-4 in D3T3-L1 cells treated with KM (A), KMEd (B) and KMWd at different concentrations (0 (1), 2.5 (2), 7.5 (3) µg/ml).

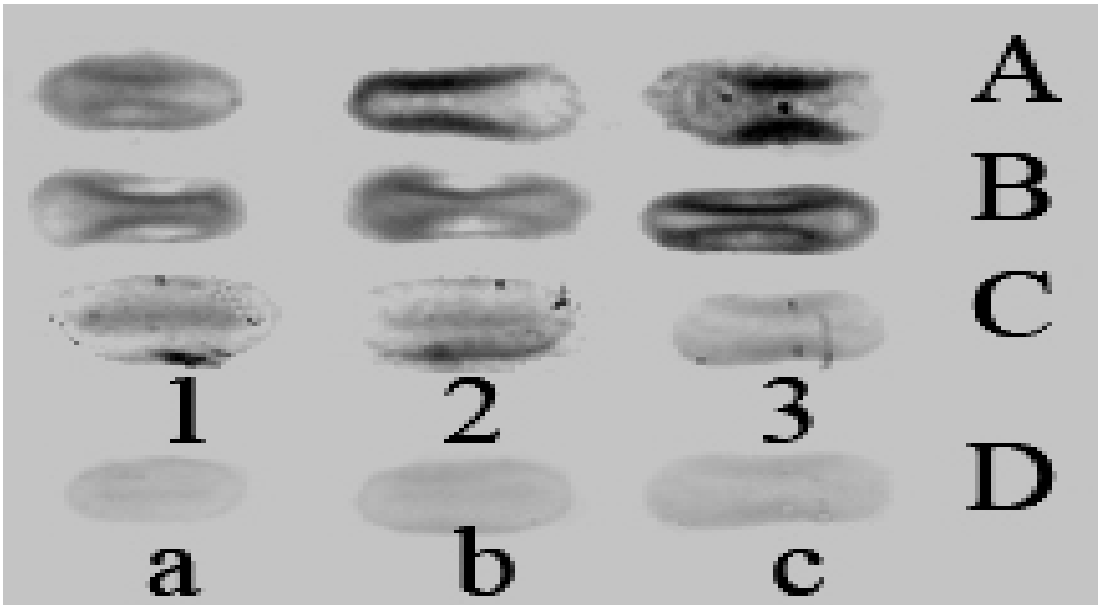

Fig. 3-46 GLUT-4 receptor expression dot blot assay on differentiated 3T3 cells treated with KM solvent fractions. A, KM; B, KMEd; C, KMWd at sample concentration: (0 (1), 2.5 (2), and 7.5 (3) µg/ml. D, actin level in cells treated with vehicle(a), 2.5 µg/ml of KMEd (b), and 7.5 µg/ml of KMEd (c).

The cells treated with KM enhanced the expression level of GLUT-4 compared to blank as seen in previous section. Among the D3T3-L1 cell population treated with fractions prepared from KM; KMEd enhanced the expression level of GLUT-4 in a dose dependent manner. Row B shows the band density at 2nd and 3rd positions was found to be more than that of the blank at 1st position. This shows that the ethyl acetate fraction prepared from KM was enhancing the

expression of GLUT-4 in D3T3-L1cells. However the water fraction of KM did not show any significant enhancement in the expression of GLUT-4 level compared to that of blank. Therefore it was inferred that the active principle that was found in KM responsible for enhancing expression of GLUT-4 was selectively extracted by ethyl acetate. Attempt was made to identify the active principle in KMEd for which, fractionation of KMEd by preparative HPLC was conducted.

3.4.3 Fractionations of KMEd employing preparative HPLC and evaluation of fractions for GLUT-4 expression and PPARγ activation

Ethyl acetate fraction prepared from SCK methanol extract was further fractionated using preparative HPLC (Sec. 2.7.2). A well resolved preparative HPLC chromatogram of KMEd was developed and divided into 16 fractions as discussed before. KMEd fraction (20 mg on dry weight) was injected into the preparative HPLC system and the fractions were collected as shown in Fig. 3-47. The corresponding fractions from different batches were pooled separately and concentrated to an appropriate concentration and sterilized. It was then diluted to a strength suitable to introduce in cell culture system. Before evaluating the antidiabetic potential of the HPLC fractions, the dose was optimized in a cytotoxic assay system using the D3T3-L1cells.

In the dose optimization analysis, a dose below 5.0 µg/ml was found to be non toxic to the cells and designated as sub lethal concentration. After the initial cytotoxic analysis, D3T3-L1 cells were treated with 16 preparative HPLC fractions from KMEd separately at sub lethal concentration level. After treatment, the cells were harvested and prepared for dot blot assay. Fig. 3-48 is an arranged image of dot blot developed from cells treated with different HPLC fractions and standards. Among the 16 fractions evaluated, cells treated with fraction 2 expressed slightly more GLUT-4 compared to that of blank at 0.5µg/ml. However at higher concentration (5µg/ml), F2 induced cytotoxicity that can be seen in the dot blot image as faded band.

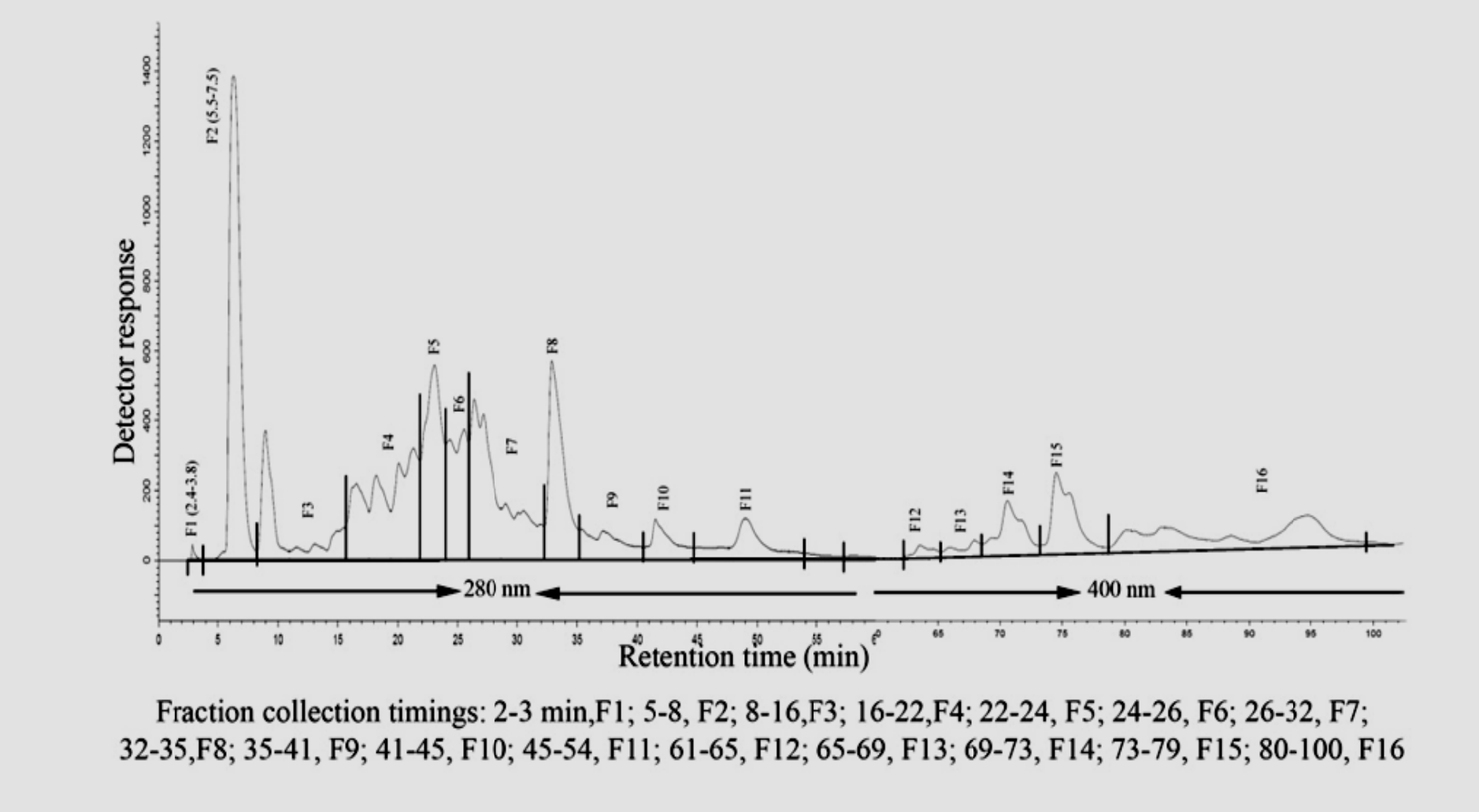

Fig. 3-47 Preparative HPLC chromatogram of KMEd and fraction collection timings

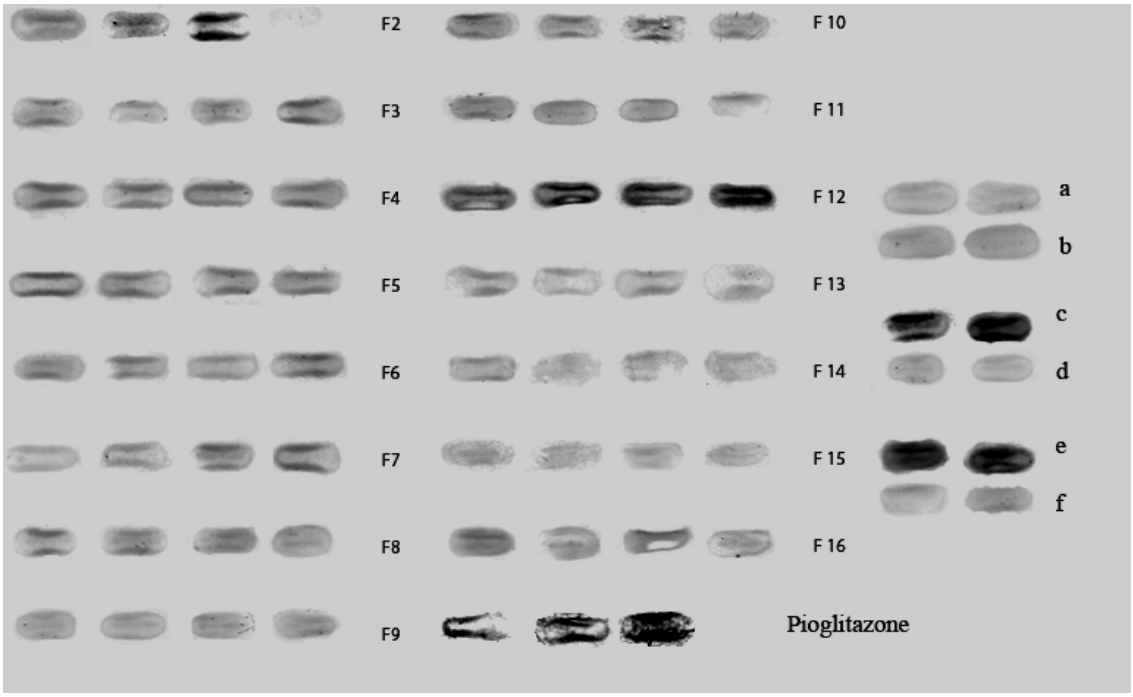

Fig. 3-48 GLUT-4 receptor expression enhancement potential of HPLC fractions of KMEd and standards in differentiated 3T3-L1 cells. F2-16, cells treated with HPLC fractions prepared in the concentration range of 0, 100, 500 and 5000 ng/ml. Reference standard: pioglitazone (25, 50 and 100 µM). a, GLUT-4 level in blank; b, actin level in blank; c, GLUT-4 level in 100 µM pioglitazone treated cells; d, actin level in 100 µM pioglitazone treated cells; e, GLUT-4 level in 5µg/ml F12 treated cells; f, actin level in 5µg/ml F12 treated cells.

However fraction 12 treated cells expressed more GLUT-4 receptor compared to that of blank. The GLUT-4 receptor expression potential of HPLC fractions were compared with a standard insulin sensitizer Pioglitazone and found GLUT-4 receptor expression enhancement potential of F12 was comparable with that of the standard insulin sensitizer; pioglitazone From this result it could be said that the active principle responsible for enhancing the GLUT-4 level in D3T3-L1cells as observed in KM was fractionated first to ethyl acetate fraction and then to fraction 12 of preparative HPLC.

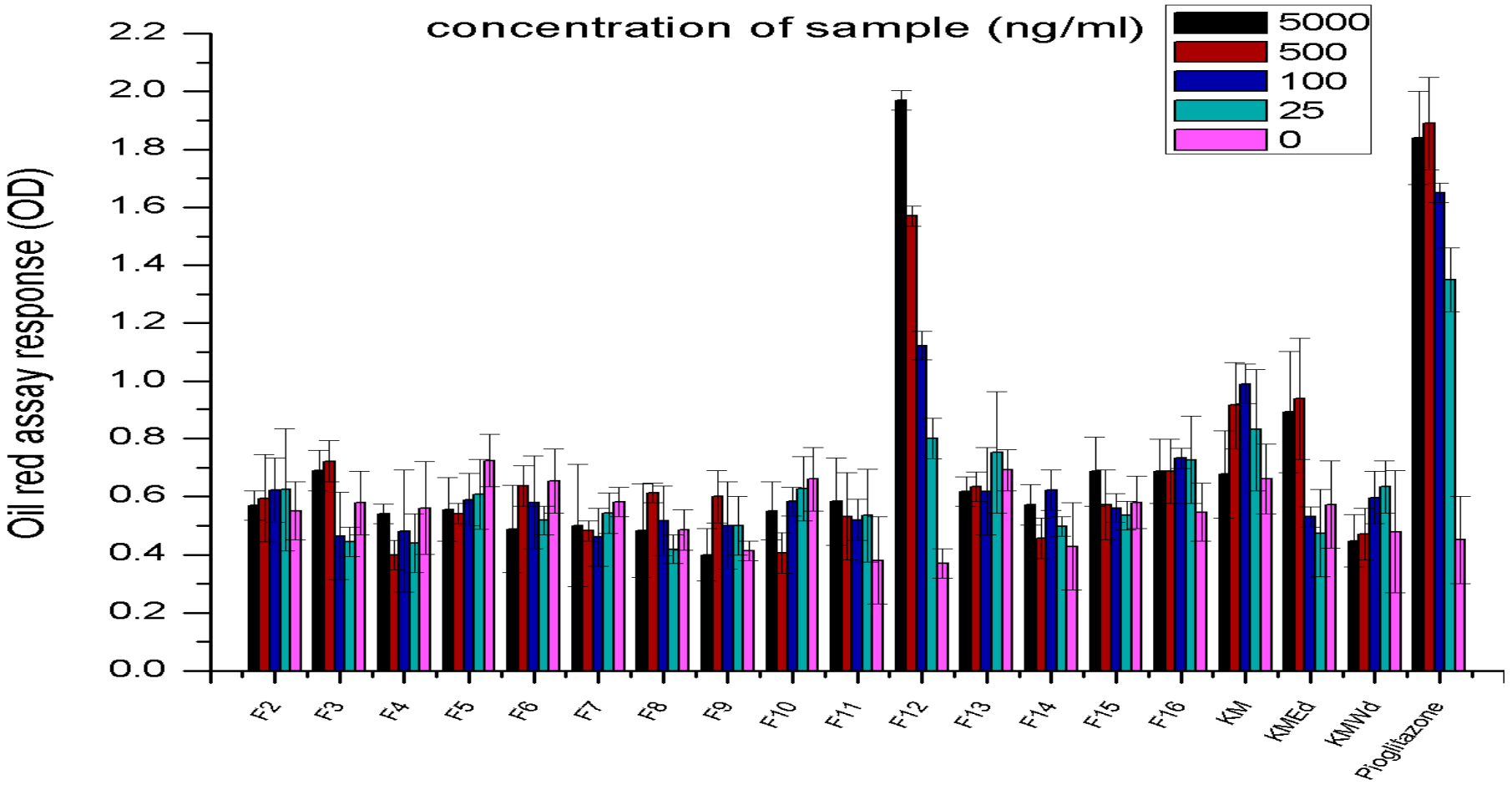

Fig. 3-49 Adipocyte differentiation assay (indirect method to measure PPAR γ activation) by oil red method in 3T3 cells treated with varying concentration of Prep HPLC fractions, KM, KMEd,

Since GLUT-4 expression is closely related to PPARγ, its activation and potential of HPLC fraction to modulate it were also studied by an indirect method (Sec.2.7.6). Using this method, differentiation of pre-adipocytes to adipocytes and accumulation of fat droplets in them was analyzed by oil red assay. Oil red is a lipophilic dye and it interacts with the lipids in the cells. The amounts of dye absorbed by the cells are directly proportional to the amount of lipid in it and hence it is proportional to the degree of differentiation as a result of PPARγ activation. The pre-adipocytes treated with 0, 25, 100, 500 and 5000 ng/ml of HPLC fractions from KMEd showed different

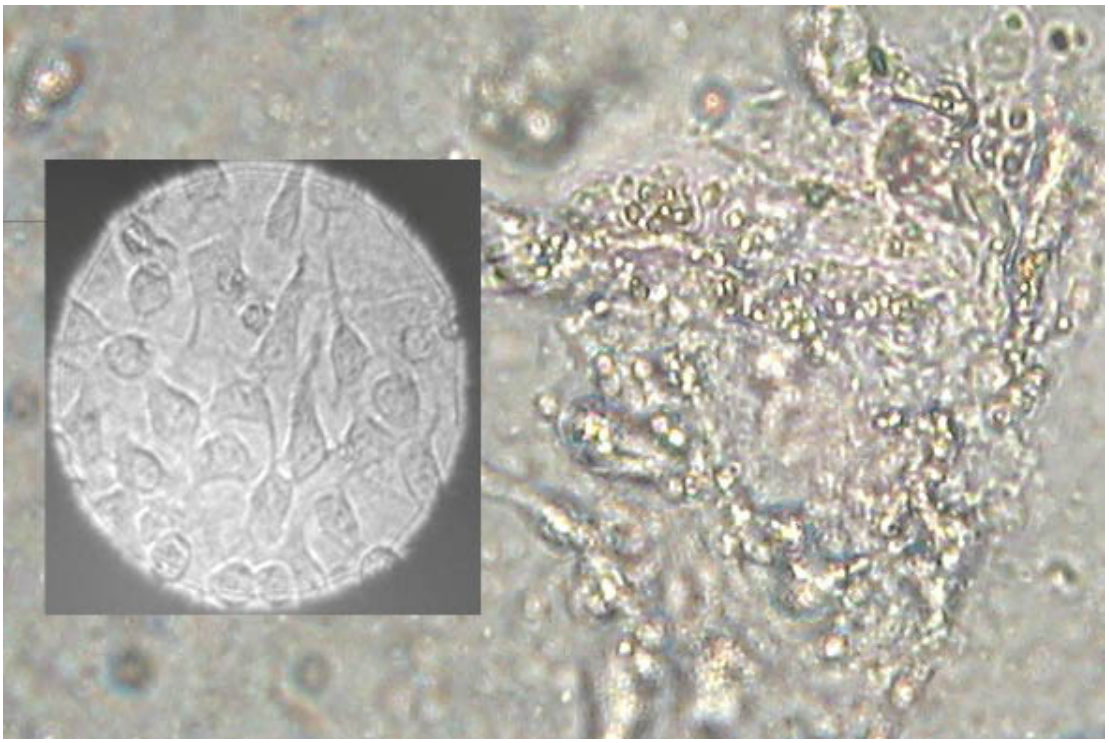

Fig. 3-50 PPAR γ activation by F12 indicated by differentiation of 3T3 pre-adipocytes to adipocytes by accumulation of fat globules in the cells. (inset: Cells treated with solvent alone)

capacities to activate PPARγ. Interestingly among the 16 HPLC fractions evaluated, only F12 was found to enhance the cell differentiation indicated by relatively more oil red uptake. In the case of other HPLC fractions, the ability to

induce differentiation of pre-adipocytes was less or nil. No statistically significant difference was observed between the blank and cells treated with different concentrations of HPLC fractions other than fraction 12. Fig. 3-49 show that KM and KMEd increased total lipid content as compared to that of blank. In the case of F12, a dose dependent increase in total Lipid content was observed as evidenced by oil red dye uptake. The differentiation of pre-adipocytes to adipocytes by F12 by accumulation of fat droplets can be seen in Fig. 3-50.

3.4.4 SCK as insulin sensitizer

Insulin-stimulated glucose transport is essential for the maintenance of glucose homeostasis and energy metabolism in mammals. Transport of glucose across biological membranes requires specific transport proteins. There are several glucose transport proteins and each of them (isoforms) that plays a specific role in glucose metabolism. To date, 13 such proteins have been identified (Joost and Thorens, 2001) and GLUT-4 is widely distributed and the most studied glucose transporter that regulate glucose transport across cell membrane. One of the reason for hyperglycemia is attributed to under expression of this protein. Insulin regulated GLUT-4 glucose transport operate in liver, adipocytes and myocytes essential for the maintenance of glucose homeostasis in mammals (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; Fukumoto et al., 1989; James et al., 1989). In the absence of insulin, GLUT-4 resides primarily in intracellular vesicles. The binding of insulin to its receptor initiates a cascade of tyrosine phosphorylation events that promote translocation, docking, and fusion of GLUT-4-containing vesicles with the plasma membrane, promoting glucose uptake by the cell (White and Yenush, 1998). This process plays a key role in normal physiology, and deregulation of GLUT-4 expression or trafficking is a major pathogenic factor in the insulin resistance seen in obesity and type 2 diabetes (Shepherd et al., 2000; Shepherd and Kahn, 1999). In human cells and tissues there are several isoforms of these transporters: GLUT1 to 12 and HMIT1, encoded by distinct genes of the SLC2A family (Joost and Thorens, 2001; Longo and Elsas, 1998; Wood and Trayhurn, 2003). The expression of particular genes of the SLC2A family, including the newest member of the GLUT family,

GLUT14, is specific to

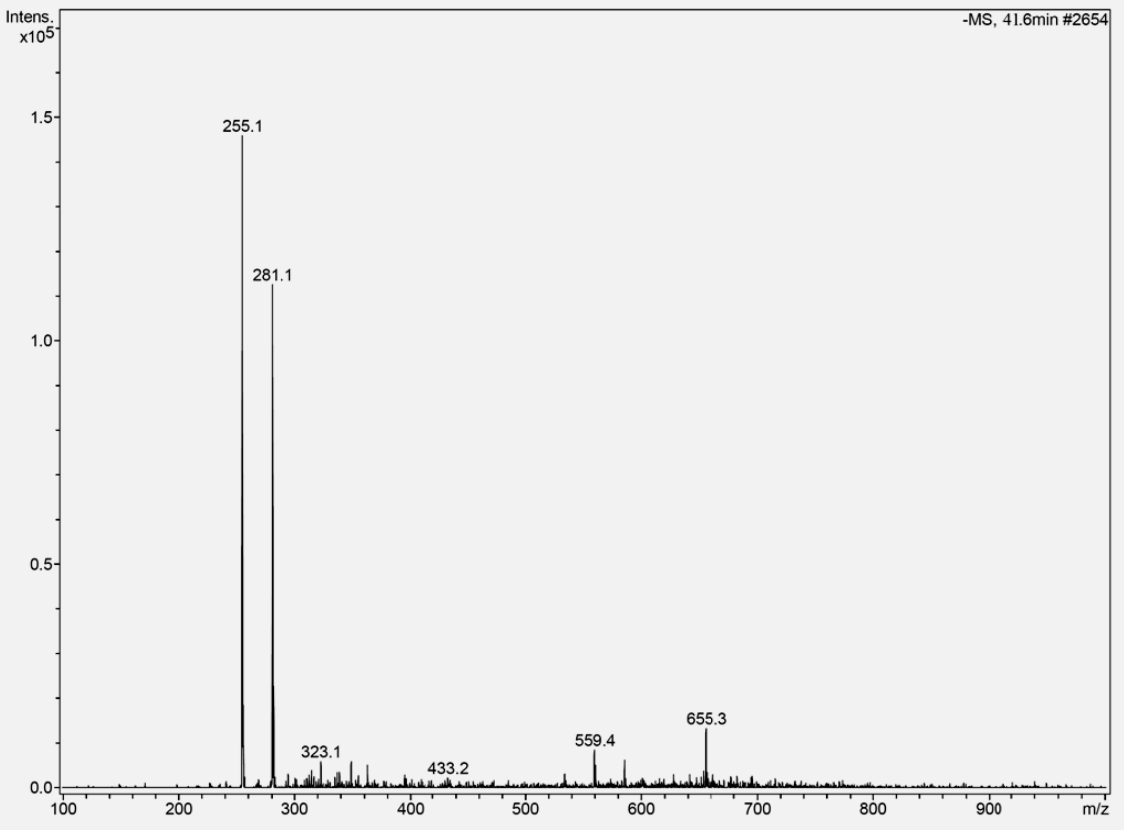

Fig. 3-51 Ion trap mass spectrum of F12

tissues and cells (Carayannopoulos et al., 2000; Doege et al., 2000; McVie-Wylie et al., 2001). Several compounds in the family of thiazolidinedions have been reported to have the potential of enhancing the expression of genes in SLC2A family and thereby enhancing insulin sensitivity. The previous report related to the in vitro antidiabetic study of *S. cumini* plant showed that its methanol extract enhanced the expression of GLUT-4 in L6 myotubes but, no further study was conducted to further fractionation and identification of active fraction. In our study, based on the result that KM enhanced the expression of GLUT-4 gene expression at translational level, we further followed bioassay guided fractionation and identified F12 as the active fraction. Since GLUT-4 gene expression is closely related to the activation of the nuclear faction PPAR γ, its activation by the fractions prepared from SCF were also studied because, PPARγ agonists (insulin sensitisers) are currently being used in the treatment of insulin resistance associated with type 2 diabetes (Nolan et al., 1994). In our study, PPAR

γ activation evidenced by cell differentiation by F12 correlated with enhanced Glut-4 translation. So the current observations confirms the increased Glut-4 expression are due to the activation of PPARγ by PPARγ agonists (Shimaya et al., 1998) in the F12. Therefore further studies are required to characterize F12 responsible for the observed PPAR γ activation and induction of GLUT-4 receptor gene expression.

An attempt was made to profile the compounds in F112 using LCMS using nondestructive soft ionization (ESI-ion trap-MS). The prefiltered F12 was directly injected into ESI module of ion trap MS using a programmable external injection module at the rate of 100µl/min. The instrument was at first set at positive polarity and scanned for ions in a broad m/z scale. At this mode, the auto scale threshold monitor window did not show any prominent peaks. On the other hand, in negative polarity mode, two prominent peaks were observed in the regions 255.5 and 281.1 of the m/z scale and the peak intensities were found between 1.0 and 1.5 x 10^5 arbitrary threshold level (Fig. 3-51). ESI MS result of F12 showed that F12 contained two compounds. Since separation and characterization of the two co-eluted compounds were not done, at this stage the antidiabetic potential of SCF was attributed to the fraction 12 containing marker compounds with molecular mass 255.1+1 and 281.1+1 (Actual molecular mass of compounds in negative polarity of ESIMS would be MW+1). The observations are preliminary and further large scale purification of KM and KMEd followed by identification and characterization of active principles using other spectrometric methods such as NMR, FTIR, etc., to establish the chemical nature of the active compounds responsible for their antidiabetic properties observed here.

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CHAPTER 4

SUMMARY AND CONCLUSION

4.1 Plant and phytochemicals for health care

The evidences accumulated clearly shows that plants are rich source of bioactive phytochemicals. Many phytochemicals are capable of modulating several biochemical pathways of higher animals. The modulatory effects of phytochemicals in biochemical system can be beneficial or harmful. While some plants are toxic, some others have preventive and therapeutic properties. However, from the ancient time, many plants have been used as primary source of healthcare that continued till the advent of modern medicine. Plants have also contributed to the development of modern drugs either directly or indirectly. However, traditional Indian medicines remained empirical as standardization and validation have not been applied to establish their efficacy. However, there have been attempts at the national level to develop evidence based traditional medicines using appropriate scientific protocols.

The present study was undertaken to evaluate *Syzygium cumini* fruit's (SCF) efficacy to modulate diabetes as claimed in Indian traditional medicine through systematic approach. *S.cumini* is a tropical plant belongs to the family of myrtacea and is endemic to India, Pakistan, Srilanka and Malaysia. In India, It is widely distributed and normally fruits in April and May. The objectives of this study were; detailed chemical profiling, activity guided fractionation, evaluation of antioxidant property, modulation of diabetes and cancer and to establish association, if any, of antioxidant properties and ability to modulate diabetes and cancer using in vitro methods.

4.2 Phytochemical profiling of SCF

Composition (pulp, seed coat and kernel) of anatomical parts of fruit were subject to chemical analysis (total moisture, total fat, total nitrogen, total soluble carbohydrates, crude fiber, total inorganic composition etc.). Minor components (total phenolic content, total flavonoids, minerals, etc.) were also estimated using

standard protocols. Phenolic compositions of SCF were analyzed and quantified using HPLC-DAD with reference standards.

The fresh fruit constituted 66.0 ± 11% pulp, 29.0 ± 4% kernel and 5.0 ± 1.5 % seed coat. Moisture content of pulp, kernel and seed coat were, 85, 47 and 10% respectively. Fresh pulp was rich in carbohydrates (free sugars 40.0 ± 3.3%, starch 35.0 ± 2.0%, and crude fiber 0.7 ± 0.13% of dry matter), protein and minerals. Kernel contained 60.0 ± 3.3% starch, 12.0 ± 0.8% soluble sugars, 2.9 ± 0.2% crude fiber and 6.8 ± 0.04% crude protein. Seed coat was composed of 12.0% crude fiber, 3.0 % starch and 6.0 ± 0.5% protein. Total fatty matter content was not significant in all three fruit parts. Detailed mineral analysis showed calcium was abundant in all fruit parts followed by potassium. Total phenolics, anthocyanins and flavonoid content (mg/100g) of pulp were; 390.0 ± 50, 134.0 ± 20.0, and 7.0 ± 4.0 respectively on dry weight basis. Kernel and seed coat did not contain significant amount of anthocyanins, but was rich in gallic acid equivalent phenolics (900 ± 70 and 810 ± 80 mg/100g of dry matter respectively).

Methanol extracts were prepared from the fruit parts for some of the above-mentioned composition analysis and for biological activity studies. Jamun pulp methanol extract (PM), kernel methanol extract (KM) and seed coat methanol extract (SCM) showed a high degree of phenolic enrichment during extraction (PM, 34±0.17; KM, 37±0.78; SCM, 27±0.34 g/100g of dry weight of extract). Pulp, seed coat and kernel were also evaluated and quantified for a few common phenolic phytochemicals such as quinic acid, gallic acid, dihydroxybenzoic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, catechin, resveratrol, quercetin, naringenin, kaempherol etc. using HPLC-PDA.

4.3 Evaluation of SCF for antioxidant properties:

Prophylactic potentials of the fruit parts and extracts were evaluated for antioxidant activity as free radicals damage to tissues is reported to be one of the early events in the onset and progression of diseases including cancer and

diabetes. The antioxidant potentials of methanol extracts from anatomical parts were evaluated using *in vitro* assays such as DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging, peroxyl radical scavenging, total reducing potential etc.

Out of three extracts evaluated for DPPH radical scavenging activity, KM was found to be more active than that of PM and SCM and values were comparable with those of reference standards such as vitamin C, Trolox and catechin, but less than that of gallic acid. Superoxide radical scavenging activity of KM was also superior to that of PM and SCM but, compared to the reference standard gallic acid, the activity was several folds more. Among the crude extracts from three anatomically distinct parts of SCF, kernel extract showed better hydroxyl radical scavenging activity as well as peroxyl radical scavenging activity. Total reducing potential of KM was also found to be more than that of PM and SCM. From the first level of antioxidant activity evaluation, it could be concluded that, crude methanol extract of kernel (KM) with higher or similar antioxidant activity in comparison with that of reference standards under the assay conditions carried out in this study.

Having established the high potency of antioxidant property for KM, further studies were focused on KM for activity guided fractionation and to obtain active fraction with high purity. The anatomical parts of SCF were freeze-dried and extracted sequentially using different solvents in the order of increasing polarity (hexane, dichloromethane, ethyl acetate, methanol and water). Among these extracts the yield of methanol extract was more than that of all other extracts. (57, 15 and 21 % respectively for pulp, kernel and seed coat). The extracts obtained by using solvents of various polarities were subjected to antioxidant activity evaluation. It was generally observed that the non-polar (hexane, dichloromethane and ethyl acetate) extracts showed less or nil antioxidant activity for all antioxidant activity assays carried out here. Methanol extract from the fruit parts showed the highest antioxidant activity. It could be concluded that the content of non-polar components in the fruit parts were less

compared to the polar components and the antioxidant activity of the fruit is mainly because of the polar components, largely composed of polyphenols.

Methanol extract thus was subjected to further fractionation. The dried methanol extracts were thus suspended in water and fractionated with different immiscible solvent of increasing polarity sequentially (hexane, dichloromethane, ethyl acetate, butanol, and water). Ethyl acetate fraction showed more phenolic enrichment with proportionally higher antioxidant activity. Pulp ethyl acetate fraction with 62% phenolic enrichment and 4% yield; seed coat with 55% phenolics and 12% yield; and kernel with 79% phenolics and 13% yield were obtained. Ethyl acetate fraction of kernel methanol extract (KE) was further subjected to preparative HPLC to obtain active fraction with high purity and the fractions were quantified for yield. Subsequently the HPLC fractions were evaluated for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Out of 16 fractions collected, F2, F4, F5, F6 and F7, but F2 and F6 registered for high activity for scavenging free radicals.

4.4 Evaluation of SCF for anticancer potency.

In vitro cell culture methods were employed to evaluate the anticancer potential of SCF. The fruit extracts were primarily evaluated against cervical cancer cell (HeLa S3). Preliminary screening is done using basic cytotoxicity evaluation protocols such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assay. The most active fraction identified from SCF against the HeLa S3 cells were further evaluated against breast, liver, colon and myeloma cancer cells. The apoptotic induction potential of the active fraction was also evaluated in HeLa cells by morphological and immunochemical analysis.

As a first level evaluation, methanol extracts of freeze-dried SCF parts were used. The samples were prepared suitable for in vitro cell culture analyses and subjected to cytotoxic evaluation by following MTT assay. Among the extracts of fruit parts studied, KM showed relatively higher cytotoxicity compared

to pulp and seed coat. KM therefore was further fractionated with different solvents (hexane, dichloromethane, ethyl acetate, butanol, and water) by liquid-liquid fractionation technique and evaluated for cytotoxic activity. Among the 5 fractions from KM, ethyl acetate fraction (KME) showed highest cytotoxic activity. The IC50 of ethyl acetate fraction was 18µg.mL compared to the crude KME, which was 33 µg/mL.

KME was then fractionated into 16 fractions by preparative HPLC. Among the 16 fractions evaluated, F2, F4, F11 and F15 showed a measurable cytotoxic activity against HeLa-S3 cancer cells. Of the active fractions, IC50 of F2 was 2.5 µg/mL followed by F4 (9 µg/mL). Activity of F11 and F15 were lesser than that of the parent fraction (KME). Activity evaluation of F2 thus was extended to other cancer cells lines such as MCF-7, HL-60, and COLO-205. Among the different cancer cells studied here, cytotoxicity of F2 were; MCF-7, 3.56; COLO-205, 5.68; and HL-60 18.6 µg/ml. The cytotoxic activity of F2 was compared with other standard cytotoxic agents such as curcumin, camptothecin, and methotrexate and found that the cytotoxicity of F2 on HeLa-S3 (IC50 2.5 µg/ml) was more than that of curcumin (14.8µg/ml).

Apoptotic induction potential of F2 was also evaluated in HeLa-S3 cell lines. As a preliminary step, changes in the morphology of cell treated with F2 for shrinkage as an indication of apoptosis was observed. Detailed microscopic analysis showed membrane blebbing as indication of induction of apoptosis. The important molecular activations that happen during the process of apoptosis were also evaluated by dot-enzyme immuno assay. The levels of pro-apoptotic proteins such as Bax and Caspase-3 were found to increase and, anti-apoptotic protein, Bcl-2 was found to be under expressed in a dose dependent manner. By following bioassay guided fractionation, the most active fraction with potent cytotoxic and apoptotic induction potential was identified as F2. Further spectroscopic studies are required to isolate and characterize the active principle in fraction F2. There appears to be strong association between phenolic content and antioxidant activity on one hand and phenolic content and cytotoxic activity on the other.

4.5 Evaluation of SCF for antidiabetic activity.

Antidiabetic activity of SCF was evaluated using in vitro mammalian cell culture method suitable for type II diabetes mellitus following glucose transport protein-4 (GLUT-4) receptor expression assay and Peroxisome proliferator-activated receptor γ (PPARγ) activation assay. Both the abundance of GLUT-4 receptor and activation of PPARγ are related to glycemic control. Differentiated adipocytes and differentiation of preadipocytes into adipocytes were used to evaluate the GLUT-4 receptor expression assay and PPARγ activation assay respectively. GLUT-4 receptor expression evaluation was performed in differentiated 3T3 adipocytes by dot enzyme immuno assay. Methanol extracts of freeze-dried SCF parts were used first at sub-lethal doses suitable for the cells under in vitro culture for optimization and analyzed for the degree of GLUT-4 receptor expression. Among the three crude methanol extracts evaluated, kernel methanol extract (KM) increased the level of GLUT-4 receptor expression. However pulp and seed coat extracts did not show any significant change in GLUT-4 receptor expression.

Based on this promising result, KM was further fractionated based on polarity and found ethyl acetate fraction (KME) to be selectively extract and enrich the active components. The ethyl acetate fraction was further fractionated using preparative HPLC as mentioned before and evaluated for GLUT-4 receptor expression induction. Dot enzyme immuno assay showed that F12 induced the expression of GLUT-4 receptor in a dose dependent manner.

PPAR γ activation potential was also evaluated to understand the antidiabetic activity of HPLC fractions prepared for GLUT-4 receptor expression analysis. PPAR γ was indirectly measured in terms of degree of differentiation of pre-adipocytes to adipocytes. The differentiation was characterized by the accumulation of fat globules in the cell matrix and the amounts of fat globules were quantified by oil red assay. Among the 16 fractions evaluated, F12 was found to induce activation of PPAR γ.

4.6 Association between antioxidant activity and anticancer/antidiabetic activity:

This study was designed to characterize SCF in terms of phytochemical profile, antioxidant activity including purification of active fraction, and to evaluate the anticancer and antidiabetic properties. The results demonstrated that an active fraction (F2) with high degree of purity enriched with high antioxidant activity that was higher than that of vitamin C could be obtained. F2 fraction was also found to possess the most cytotoxicity (anticancer) and the ability to induce apoptosis that were higher than that of curcumin that is known to have anticancer property. Thus there appears to be a strong positive correlation between antioxidant and anticancer properties. However the mechanistic aspects of this association could be subject of future studies. On the contrary, the results also showed a negative correlation between antioxidant and antidiabetic potency as demonstrated by the F12 fraction with its negligible antioxidant activity but with most active as antidiabetic.

4.7 Requirement for evidence based traditional medicine as complementary and alternative medicine (CAM).

Currently, healthcare dependent on chemical drugs and medical technology is increasingly becoming unaffordable for large section of population, besides associated side effects. It is in this context complementary and alternative medicines (CAM) advocated by WHO based on traditional knowledge with its disease preventive and management approach is relevant. However, traditional medicines are by and large empirical and lack evidence for their efficacy. Marker based standardization and validation of plants and plant products that are known to have bioactive phytochemicals have large potential to deliver cheaper healthcare to the mass. Resurgence of natural medicine in the recent past is indication of their acceptability. The studies on SCF presented here is such n

approach, though much more work is remaining to be carried out to make it effective formulation for prevention and management of diabetes. This work further supports the claim for using SCF in traditional medicine for treatment of diabetes. Further work in characterization of active/purified fraction and studies in animal model could further substantiate these findings. The methodologies employed here could be used for screening of plants and their fractions for target diseases such as diabetes.

ABBREVIATIONS

2,4-DNPH	--	2,4-Dinitrophenylhydrazine
3T3-L1	--	3T3 mouse pre adipocyte
8-OHdG	--	8-Hydroxyguanine
$AlCl_3$	--	Aluminium chloride
BAGF	--	Bio assay guided fractionation
bFGF	--	Basic fibroblast growth factor
BHT	--	Butylated hydroxytoluene
CA	--	Coumaric acid
CAM	--	Complementary and alternative medicine
Cat	--	Catechin
ClA	--	Chlorogenic acid
CME	--	Crude methanol extract
COLO-205	--	Human colon cancer cell
CVD	--	Cardio vascular disease

D3T3-L1	--	Differentiated 3T3 cells
DBA	--	Dihydrobenzoic acid
DHLA :	--	dihydrolipoic acid
DM	--	Diabetes mellitus
DPPH	--	1,1-diphenyl-2-picrylhydrazyl
EGF	--	Epidermal growth factor
ERK1/2	--	extracellular signal-regulated kinase
ESI-IT-MS-MS	--	electrospray ionization-ion trap-tandem mass spectrometer
F2	--	Fraction 2 prepared (by prep HPLC) from ethyl acetate fraction of kernel methanol extract
FA	--	Ferulic acid
GA	--	Gallic acid
GAPDH	--	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-4	--	Glucose transport protein-4
GPCR	--	G Protein-coupled receptor
GPX	--	Glutathione peroxidase
GPX-1	--	Glutathione peroxidase 1
GPX-2	--	Glutathione peroxidase 2
GR	--	Glutathione reductase
GSSG	--	Glutathione disulfide
HeLa-S3	--	Human Cervical Cancer cells
Hep G2	--	Human Liver Cancer Cell
HL-60	--	Human Leukaemia cells
HOCl	--	Hypochlorous acid
HPLC	--	High-performance liquid chromatography
HTS	--	High-throughput screening
IHD	--	ischemic heart diseases

IL	--	Interleukin
IL-1	--	Interleukin-1
KB_S	--	Kernel butanol extract (by sequential extraction)
KD_S	--	Kernel dichloromethane extract (by sequential extraction)
KE_S	--	Kernel ethylacetate extract (by sequential extraction)
KH_S	--	Kernel hexane extract (by sequential extraction)
KM	--	Kernel methanol extract
KB	--	Butanol fraction of kernel methanol extract
KD	--	Dichloromethane fraction of kernel methanol extract
KE	--	Ethyl acetate fraction of kernel methanol extract
KEd	--	Ethyl acetate fraction from kernel methanol extract for diabetes study
KH	--	Hexane fraction of kernel methanol extract
Kmp	--	Kaempherol
KM_S	--	Kernel methanol extract (by sequential extraction)
KW	--	Water fraction of kernel methanol extract
KWd	--	Water fraction from kernel methanol extract for diabetes study
KW_S	--	Kernel water extract (by sequential extraction)
LA	--	lipoic acid
LDH	--	Lactate dehydrogenase
LDL	--	Low-density lipoprotein
MAP	--	Mitogen-activated protein
MCF-7	--	Human breast cancer cell
MDR	--	multidrug resistant
mtDNA	--	Mitochondrial DNA
MTT	--	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

NAC	--	N-acetyl-L-cysteine
NADH	--	Nicotinamide adenine dinucleotide (reduced)
NAD	--	New and Approved Drugs
NADPH	--	Nicotinamide adenine dinucleotide phosphate (reduced)
NCI	--	National Cancer Institute
ND	--	Not detected
nDNA	--	Nuclear DNA
NFkB	--	Nuclear factor kappa B
Ngnn	--	Naringenin
NO	--	Nitric oxide
NOAEL	--	non-observable-adverse-effect-level
NVSR	--	National Vital Statistics Report
ONOO	--	peroxy nitrites
oxLDL	--	Oxidized low density lipoprotein
PB_S	--	Pulp butanol extract (by sequential extraction)
PCA	--	Paracoumaric acid
PDA	--	Photo diode array
PDGF	--	platelet-derived growth factor
PD_S	--	Pulp dichloromethane extract (by sequential extraction)
PDTC	--	pyrrolidinedithiocarbamate
PE_S	--	Pulp ethylacetate extract (by sequential extraction)
PHBA	--	Parahydroxybenzoic acid
PH_S	--	Pulp hexane extract (by sequential extraction)
PM	--	Pulp methanol extract
PM_S	--	Pulp methanol extract (by sequential extraction)
PPARγ	--	Peroxisome proliferator-activated receptor
PSI	--	Pounds per square inch

PTFE	--	Polytetrafluoroethelene
PW_S	--	Pulp water extract (by sequential extraction)
QA	--	Quinic acid
Qtn	--	Quercetin
RBC	--	Red blood corpuscles
Res	--	Resveratrol
RK	--	Residual kernel
RNS	--	Reactive nitrogen species
ROS	--	Reactive oxygen species
RP	--	Residual pulp
RSC	--	Residual seed coat
SA	--	Sinapic acid
SAPK1	--	Stress- Activated Protein Kinase 1
SAPK2	--	Stress- Activated Protein Kinase 2
SCB_S	--	Seed coat butanol extract (by sequential extraction)
SCD_S	--	Seed coat dichloromethane extract (by sequential extraction)
SCE_S	--	Seed coat ethylacetate extract (by sequential extraction)
SCF	--	*Syzygium cumini* fruit
SCF_S	--	*Syzygium cumini* fruit (freeze-dried)
SCH_S	--	Seed coat hexane extract (by sequential extraction)
SCK	--	*Syzygium cumini* kernel
SCM	--	Seed coat methanol extract
SCM_S	--	Seed coat methanol extract (by sequential extraction)
SCP	--	*Syzygium cumini* pulp
SCSC	--	*Syzygium cumini* seed coat
SCW_S	--	Seed coat water extract (by sequential extraction)
SH	--	thiole group

SOD	--	Superoxide dismutase
TDS	--	Total dissolved substances
TESC	--	Total 80% ethanol soluble carbohydrate
TFM	--	Total fatty matter
TGF-B1	--	Transforming growth factor, beta 1
TM	--	Traditional medicine
TNF-α	--	Tumour necrosis factor-alpha
Tr	--	Trace
U251	--	Human glioma cells
VEGF	--	Vascular endothelial growth factor
WHO	--	World health organizatio

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