EVALUATION OF ANTIOXIDANT ACTIVITIES OF SOME LOCALLY AVAILABLE EDIBLE PLANTS OF DARJEELING HIMALAYA

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By

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Guide

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DECLARATION

I declare that the thesis entitled "**EVALUATION OF ANTIOXIDANT ACTIVITIES OF SOME LOCALLY AVAILABLE EDIBLE PLANTS OF DARJEELING HIMALAYA**" has been prepared by me under the guidance of Mr. Palash Mandal, Assistant Professor of Botany Department, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Reactive oxygen and nitrogen species are the by-products of energy metabolism which are generated from the cellular redox processes. Excess free radicals accumulated during oxidative stress have been implicated in various neurodegenerative, cardiovascular, pulmonary and gastrointestinal disorders. Only antioxidants can prevent radical mediated oxidative reactions. But synthetic antioxidants, besides their beneficial impacts also have adverse side-effects like enhanced sensitivity towards other medicine, increased tumour yield from chemical carcinogen and enhanced mutagenic activity. Plants produce an incredible diversity of secondary metabolites having multiple functions throughout their life cycle. Epidemiological and *in vitro* studies on medicinal plants, fruits and vegetables strongly supported the idea that plant constituents with antioxidants are capable of exerting protective action against oxidative stress in human system and can be used to prevent diseases like postprantal diabetic complications. Natural antioxidants have attracted considerable interest due to their presumed safety and prospective therapeutic values.

Darjeeling Himalaya is one of the biodiversity hotspot where wild plants are mostly used for food and medicinal purposes. Several types of edible plants are available in divergent markets of Darjeeling Himalaya, most of which are till now underexplored but have potential ethnomedicinal values. It was recorded from market survey that these fresh and dry edible fruits were traditionally utilized for managing gastro-entities, hypertension, diabetes and hepatoprotection. In this context underexplored edible plants available in different markets of Darjeeling Himalaya were initially screened for antioxidant activity and bioactive phytochemicals. Potential free-radical scavenging properties were obtained from fruits of *Calamus erectus, Cyphomandra betacea, Capsicum annum, Solanum incanum* and *S. anguivi*, leafy vegetable *Nasturtium officinale, Dioscorea alata* underground plant parts and *Evodia fraxinifolia*, a spice yielding plant with aromatic flavour. As the phytochemicals present in herbs, fruits and spices of Darjeeling Himalaya have potential medicinal properties, their

qualitative and quantitative variation was studied. The polyphenolic components were present in significantly higher amount in different edible fruits. More precisely, total phenols and flavonoids contents were potentially accumulated in mesocarp and endocarp of *Calamus erectus*, *Pyrus communis* and *Rubus ellipticus*; epicarp of *Docynia indica* and *Machilus edulis*; pericarp of *Elaeocarpus lanceifolius*, *Persea americana* and *Prunus domestica*.

For testing *in vitro* antidiabetic properties, different plants were initially chosen for ethnomedicinal market survey records and among them, *Calamus erectus* and *Dioscorea alata* showed higher inhibition of α-amylase and α-glucosidase which are important for carbohydrate digestion in the intestinal lumen. Further studies with *C. erectus* fruit extract in streptozotocin treated rats exhibited a dose-dependent significant hypoglycaemic activity, comparable with standard drug glibenclamide. The same fruit extract also minimized oxidative stress by enhancing liver GSH, SOD and CAT along with reducing TBARS. Improvement of lipid profile by oral administration of *C. erectus* extract during diabetic condition also indicated activation of LDL receptors in mammalian system.

For determining the perfect stages of maturation, where bioactive phytonutrients are accumulated in optimal amount, two varieties of *C. betacea* and *S. anguivi* were chosen. In both fruits, free-radical scavenging potency gradually increased with ripening stages. The lower IC_{50} values were noticed for *in vitro* inhibition of enzymes participated in carbohydrate digestion during ripening stages of these fruits, indicating the accumulation of antidiabetic components through senescence programme. Bioactive molecules like phenolic compounds, lycopene and carotenoids present in fruits were all accumulated during ripening stages. So it might be stated that the pharmacological properties and phytochemical attributes enhanced successively from immature to mature transition.

It is well known that post-harvest thermal processing significantly alters chemical composition and bioavailability of compounds present in vegetables. In Darjeeling Himalaya popular post-harvest thermal processing includes half-cooking, boiling, semi-frying, deep frying and sun drying. When the ripe fruits of *C. betacea* were processed through different thermal treatments, best result was obtained from sun drying process, where free-radical

scavenging potency and bioactive phytonutrients were enhanced with the time of treatment. Sun drying is beneficial because it reduces moisture content under restricted heating, but during boiling and frying, sharp rise in temperature might partially destroy bioactive molecules. In Solanaceous fruits like *S. incanum* and *S. anguivi* thermal processing for certain time improved antioxidant quality. In contrast, both antioxidant and antidiabetic potency of different underground plant parts (taruls) was deteriorated with any aggressive thermal processing, might be due to degradation of thermo-labile components responsible for the said activity.

After establishing the bioactivity, an attempt was made for purification and identification of bioactive components from two important and unexplored edible fruits of Darjeeling Himalaya, i.e. *Solanum anguivi* and *Calamus erectus*. Methanolic extract of *S. anguivi* was fractionated through solvent partitioning and best bioactive fraction was passed through silica gel $G_{200\text{-}400}$ column chromatographic system, where nineteen fractions were obtained based on UV-visible spectral characteristics. One phyto-metabolite 1-oleoyl lysophosphatidic acid (LPA) was identified with highest abundance through High Resolution LCMS screening of fraction exhibiting best antioxidant and antidiabetic activity. LPA is an intermediate product of lipid biosynthesis in plants and display vital physiological functions in association with G-protein linked receptors. Similarly components of *C. erectus* were separated through bioassay-guided purification and ESI-mass was performed with best bioactive fraction. More than 30 peaks were recorded from ESI-mass spectrum between M_w 180-1900, from which the existence of free phenolic acids might be speculated.

As the soil nutrient profile and altitude variation ultimately influence accumulation of secondary metabolites and antioxidant activity, changes of these attributes with different soil profile of Darjeeling hills were evaluated among different members of Solanaceae. It was observed that the antioxidant activity and nutrient content of the soil enhances with the increasing altitude level. Top soil nutrient profile contributes for total phenolics accumulation in fruits of *C. betacea* and *S. anguivi*. Correlation co-efficient studies and PCA analysis indicate that the available potassium in soil might elicit accumulation of antioxidant rich

components, whereas phosphorus significantly influenced metal chelating activity. Therefore, soil macronutrients significantly influence the accumulation of bioactive phyto-metabolites in edible vegetables.

In conclusion, it might be stated that the edible plants of Darjeeling Himalaya could be potentially utilized as pharmaceutical and nutraceutical components. It is therefore required to develop appropriate conservation strategies considering their sustainability and future use.

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(MITALI GHOSAL)

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GENERAL INTRODUCTION

Human civilization is going under serious crisis. The crisis is workload and social/professional pressure due to better lifestyle and more comfortable life leading. The ultimate result of this is oxidative stress which is converted into metabolic disorders such as atherosclerosis, cataracts, cancer and osteoarthritis (Scott, 1995; Behl, 1999; Khan *et al*., 2004). Excess oxidative stress might produce many free radicals which are the main cause of oxidation of lipids, DNA and protein. There are many evidences which can prove that antioxidants might prevent the primary ageing processes, as well as many of the age-associated secondary pathological complications (Aruoma, 1993). It has been recorded that the consumption of higher amount of fruits and vegetables reduced the risk of degenerative diseases like cancer as well as atherosclerosis and the dietary flavonoids can restore a range of oxidative radical damage sustained by DNA (Eastwood, 1999; Anderson *et al.*, 2000). There is no confusion that the antioxidants present in fruits and vegetables are the best solution for oxidative stress-mediated disorders (Brand-Williams *et al*., 1994). Now-a-days, synthetic medicines were frequently consumed for rapid recovery from different disorders. But these synthetic medicines generate lots of trouble-shooting side effects and sometimes accelerate apoptosis in our body. So the question has now arisen regarding therapeutic application of different synthetic medicines for all kinds of disorders. In this context, it should be noted that disease protection is better than cure. Several recent studies have already indicated that antioxidant molecules might play an important role for disease protection, particularly when it is associated with lifestyle mediated disorders. But sufficient care should be taken before consumption of antioxidants, particularly when they are synthetic or derived from semi-synthetic procedures; regarding their toxicity, bioavailability, metabolic fate, accumulation inside body and side-effects both for shorter and longer term (Lobo *et al*., 2010). One of the main side effects of these synthetic drugs for long term use is drug addiction and resistance (Gawad *et al*., 2011). Synthetic antioxidants are now compiled with several drugs for minimizing oxidation process, as well as they are also used in food as preservatives through which adverse effects of synthetic antioxidants might be generated in biological system. These adverse biological effects occur on modulation of growth and immune response, and

interference with oxygen activation in body (Arora and Bhattacharjee, 2008). Due to their toxic effect, a few antioxidants like BHA, BHT, PG and TBHQ are currently permitted for their usage in food as preservatives. A well known degradation products of BHT i.e. *tert*-Butylhydroquinone (tBHQ) which is used for stabilization and preservation of freshness, nutritive value, flavour and colour of animal food products, is known to exert carcinogenic effect by causing oxidative damage of DNA (Chun *et al*., 2006).

Antioxidants are almost universal in normally consumed herbal food products, they are pre-existing compounds in the form of natural secondary metabolites; and sometimes are also required during processing of synthetic antioxidants. As long as they are consumed in moderate concentration, natural antioxidants have been proven to have several positive health effects as compared to their synthetic counterparts (Daniel, 1986). The food which are used by common people, serve as nutrient. Side by side if these foods would have been used for nutraceutical and antioxidant purpose then the problem of oxidative stress mediated disorders might be resolved. Recently it has been conceptualized that ideal food should have all the required nutrients as well as nutraceutical and antioxidant principles (Danesi, 2009). The bioactive food component refers to nonessential bio-molecules which are present in foods, exhibit the capacity to modulate several metabolic processes that results in the promotion of better health. In different geographic location, different tribes are using several types of ethnic foods (Choudhury *et al*., 2010). In present day, it is very essential to assess the antioxidant activity of these types of edible fruits and vegetables and also processed food (i.e. the process through which the tribal prepared their own food); because an important factor controlling bioactive content in plant-based foods is post-harvest processing, particularly cooking (Miglio *et al.*, 2008). It is also required to judge the optimal concentration, persistence and thermo-stability of antioxidants, nutraceutical fate and bio-availability antioxidants present in these foods. There are several ethnic races present in Darjeeling Himalaya and many underexplored fruits and vegetables which are available in this natural diversity zone, are used by different ethnic people (Saha *et al*., 2011). These tribal people are still living in far-flung remote villages of Darjeeling Himalaya. Chhetri *et al*., 2005 recorded that only 8 hospitals and 24

public health centers are present in Darjeeling hills. Modern medical facilities are lacking in this hill and the doctor to people ratio is 1:4892 (Chhetri *et al*., 2005). Therefore, the people depend on their traditional knowledge for the treatment of their ailments. The ethnic group of inhabitants has gathered this knowledge through trial and error, during their survival in such inhospitable environments for hundreds of years. Unfortunately, this traditional knowledge is masked under the light modern medication and therapy. Although, recently the importance of plant based traditional medicine is being realized by the masses. People of different parts of the world have started recording and evaluating such herbal knowledge through assessing antioxidants as well as different pharmacological properties (Saha *et al*., 2011). Except some towns and villages of Darjeeling, several edible fruits and vegetables of Darjeeling Himalaya are not distributed and marketed in different places and almost no awareness about these foods have been generated; also no significant records about pharmacological and antioxidant properties of these foods are available in modern knowledgebase (Chhetri *et al*., 2005). It is necessary to evaluate antioxidant activity of these types of underutilized plants but unfortunately, through literature survey it has to be known that the edible plants of Darjeeling Himalaya are almost untouched.

In this context, the study was designed for in-depth analysis of antioxidants and anti-diabetic activities along with the quantity of bioactive phytochemicals of under-explored fruits and vegetables of Darjeeling Himalaya and also the dynamic alteration of bioactive substances during thermal processing particularly existing within local inhabitants of this hill.

Objectives of Research

Following objectives would have been constituted for determining the antioxidant activities of underexplored edible plants of Darjeeling Himalaya:

- A comprehensive survey on the use of plant species from various markets of Darjeeling hills.
- Characterization the antioxidant properties of locally available edible plants.
- Phytochemical screening and quantification of bio-active components of these plants.
- Evaluation of anti-diabetics activity *in-vitro* and *in-vivo.*
- Partial purification and characterization of components having potentially bioactive antioxidant and antidiabetic activity.
- Alteration of antioxidant activity during post harvest thermal processing.
- Changes of antioxidant activity with different stages of maturation of edible fruits.
- Determining the relation between soil nutritional properties and antioxidant activity of edible plants with different altitude.

Chapter - II

LITERATURE REVIEW

2.1 CONCEPT OF FREE RADICALS

Free radicals are defined as the molecules or molecular fragments containing one or more unpaired electrons in the outer orbit. These unpaired electrons are unstable and usually gives a significant degree of reactivity to the free radical. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are expressed as free radicals and other non-radical reactive derivatives. Though radicals are less stable, reactivity of these radicals is generally stronger than non-radical species (Pham-Huy *et al*., 2008). Free radicals are formed from molecules by the homolytic division of a chemical bond and via redox reactions, and once formed these highly reactive radicals can start a chain reaction (Bahorun *et al*., 2006; Valko *et al*., 2007). ROS includes superoxide ($O^{2\bullet -}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals. Nitrogen free radicals consist of nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). Oxygen and nitrogen free radicals can be readily converted to the other non-radical reactive species which are also hazardous for health. Hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH) are not the free radicals and generally named oxidants and can easily lead to the free radical reactions in living organisms. These reactive species are generated in animals and humans under physiologic and pathologic conditions (Halliwell and Gutteridge, 1999; Fang *et al*., 2002; Valko *et al*., 2007; Pham-Huy *et al*., 2008).

Free radicals can be formed from both endogenous and exogenous substances. They are continuously forming in the cells and environment. Different sources and generation of free radicals are given in the Figure 2.1.

Free radical reactions take three different individual steps which are as follows: 1st step i.e. formation of radicals; 2^{nd} step which is known as propagation step where free-radicals are reproduced with repeated chain reaction and last step is termination step in which destruction of radicals occurs.

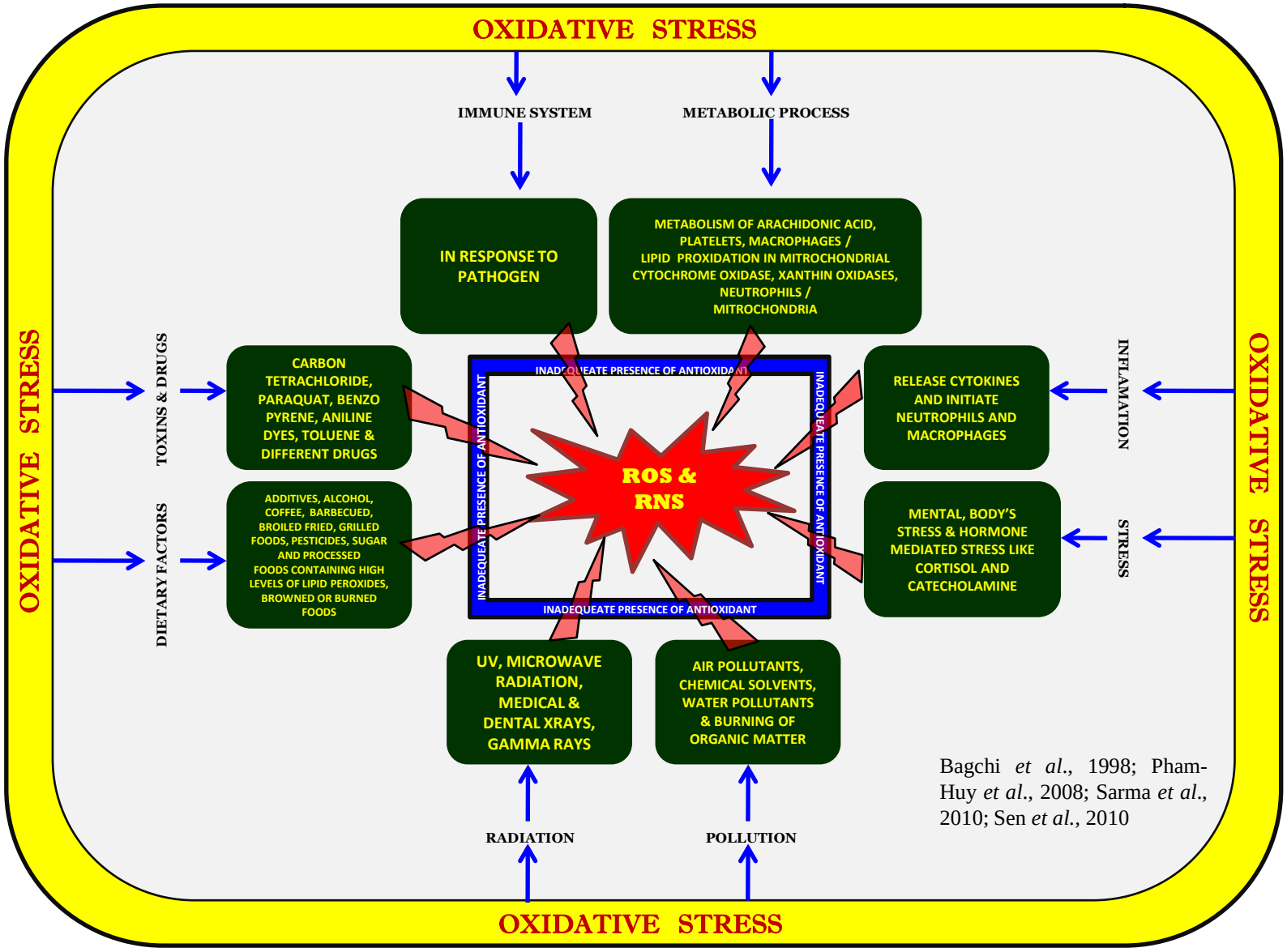


Figure 2.1 Generation of free radicals by oxidative stress

ROS and RNS play a dual role in human beings as both toxic and beneficial compounds. The insubstantial balance between their two opposite effects is undoubtedly a key aspect of the life. At low or moderate levels, these reactive species exert beneficial effects on cellular redox signaling and immune function; on the other hand at high concentration, they produce oxidative stress which is a harmful process that can damage cell function and structures (Pham-Huy *et al*., 2008; Sen *et al*., 2010).

2.2 OXIDATIVE STRESS AND THEIR IMPACT ON HUMAN HEALTH

Oxygen is a crucial element for life. Oxidative property of oxygen plays a fundamental role in various biological phenomena; being essential for life, oxygen can also increase the damage within the cell by oxidative events. Oxygen is used by the cell to generate energy and various free radicals are formed as an outcome of ATP (adenosine triphosphate) production by mitochondria. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products which are generated from the cellular redox process. One side, the oxygen is essential for the production of all ROS and RNS, in another side, molecular oxygen is absolutely essential for aerobic life. It can be toxic under certain conditions and this phenomenon has been named as oxygen paradox (Devasagayam *et al*., 2004). The term oxidative stress has coined to explain a harmful condition caused by the excess production of ROS and/or a decrease in antioxidant levels. Similarly overproduction of the RNS is named as nitrosative stress. Oxidative stress is the condition described as a shift towards the pro-oxidants in pro-oxidant/antioxidant balance that can occur as a result of an increase in oxidative metabolism. Even at a low concentration, prolonged exposure to free radicals may be responsible for the damage of cell structures, including lipids, proteins, DNA and also membranes (Valko *et al.*, 2007).

ROS and RNS have different beneficial effects at low or moderate concentrations and they are involved in several normal physiological functions against several cellular responses (Devasagayam *et al*., 2004). They also participate in different cellular signaling at low

concentration like boosting up of some cytokines and signaling growth factors, activation of non-receptor tyrosine kinases, protein tyrosine phosphatases and nuclear transcription factors as well as release of calcium from intracellular stores. ROS play an important role in gene transcription. NO which is produced by endothelial cells is essential for blood pressure regulation of vascular smooth muscle, leukocyte adhesion, platelet aggregation, angiogenesis, and thrombosis. NO is also produced by neurons and acts as neural plasticity. NO is furthermore playing a key moderator of the immune response generated by activated macrophages. Moreover, recent studies also recommended that the ROS may be harmful for life if the accumulation of these free radicals is increased (Halliwell *et al*., 1991; Fang *et al*., 2002; Valko *et al.,* 2007). Free radicals cause different diseases by membrane lipid peroxidation, protein oxidation, DNA damage and disturbance in reducing equivalents of the cell; which leads to cell destruction, altered signalling pathways. These free radicals generated oxidative stress which has been implicated in various diseases like neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, memory loss and depression), cardiovascular disease (atherosclerosis, cardiac hypertrophy, ischemic heart disease, hypertension, shock and trauma), pulmonary disorders (inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease), diseases associated with premature infants (broncho-pulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, retinopathy of prematurity and necrotizing enterocolitis), autoimmune diseases (rheumatoid arthritis), renal disorders (glomerulonephritis, tubulointerstitial nephritis, proteinuria, chronic renal failure, uremia), gastrointestinal diseases like peptic ulcer, colitis and inflammatory bowel disease), diabetes, cancers and tumors (Bagchi and Puri, 1998; Sen *et al*., 2010).

2.3 PREVENTION OF HUMAN DISEASES THROUGH DIETARY INTAKE OF ANTIOXIDANT RICH FOOD

The basic problem always arises about the ways of relieving from this oxidative stress. One of the chief solutions is dietary intake of antioxidants which are any substance that inhibit or delay oxidative damage to a target molecule. At a time one antioxidant molecule can able to react with single free radicals and have the capability to neutralize free radicals by donating one of their own electrons. Hence antioxidants prevent cell and tissue damage as they act as scavenger.

In 21st century, demands for consumption of antioxidant food or dietary antioxidant are increasing with the hope of keeping body healthy and free from diseases (Benzie 2003; Serafini, 2006). Food is a major source of exogenous antioxidants and has been estimated that a typical diet provides more than 25,000 bioactive food constituents and many of this may modify a multitude of processes that are related to different disorders. Antioxidants are abundant in vegetables and fruits and are also found in grain cereals, legumes, tea, nuts and other food products. A systematic survey has known more than 3100 antioxidant foods, spices, beverages, herbs and supplements which are commonly consumed by different cultures. Decrease in intake of nutraceutical and antioxidant rich food may enhance oxidative stress which may lead to cell damage, for that reason intake of such natural antioxidants may give defensive effect against free radical induced diseases. Recently it has been reported that the plant-based foods are usually higher in antioxidant content than animal-based as well as mixed food product. Beverages such as unprocessed tea leaves, tea powders and coffee beans have better antioxidant values from wine, beer and lemonades. The dairy products, fish and meat generally have low in antioxidant content (Carlsen *et al*., 2010). Therefore herbs, fruit, spice and food from plant sources may still be chief contributors to our antioxidant intake, especially in dietary cultures where herbs and spices are consumed regularly. Antioxidant which can be classified in different aspect is presented in the Figure 2.2.

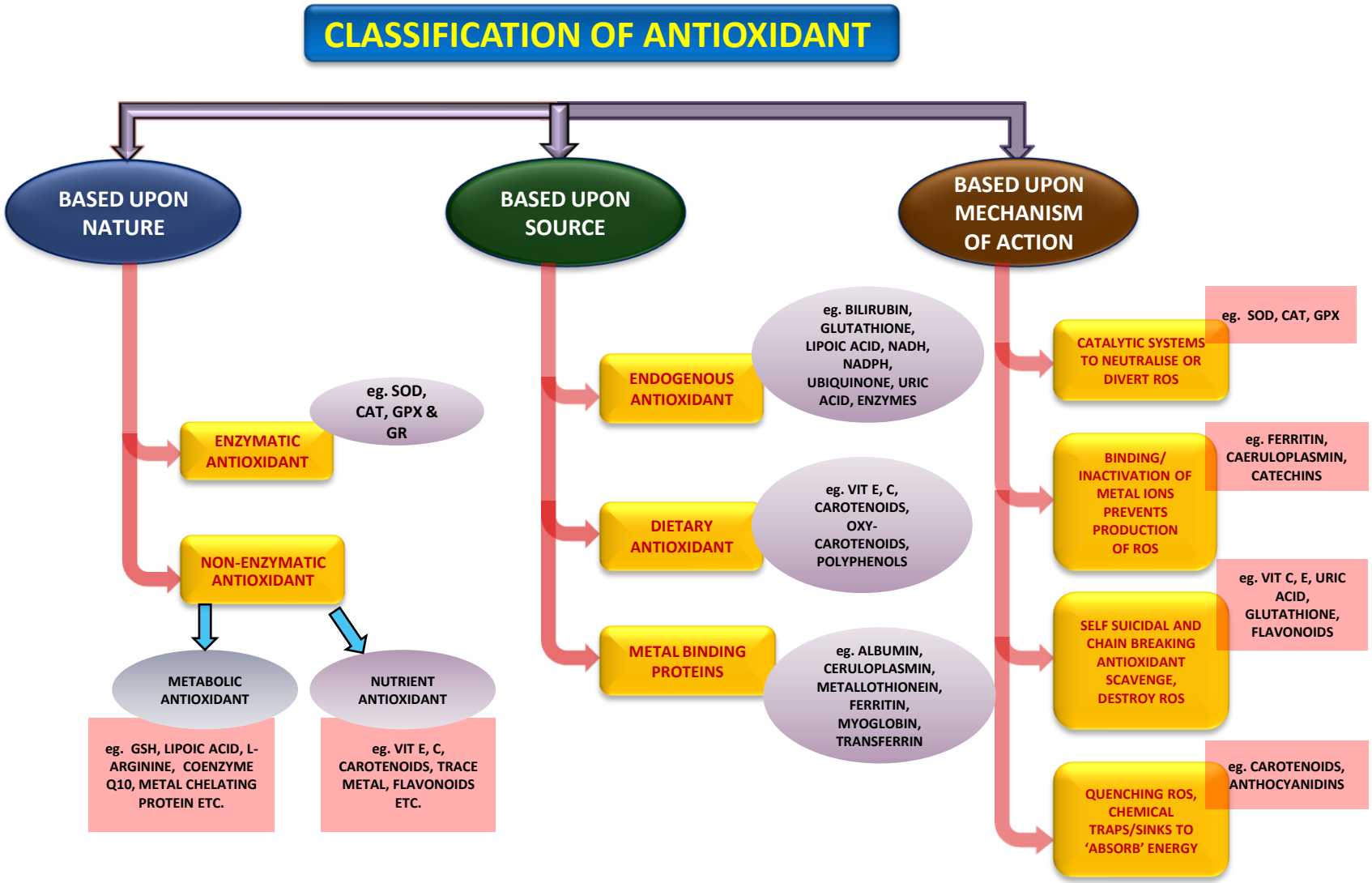


Figure 2.2 Schematic classification of antioxidant

REFERENCE: SEN & CHAKRABORTY, 2011

2.4 MECHANISM OF ANTIOXIDANT ACTION

The efficacy and biological action of antioxidant molecules vary with homogeneous nature of cellular system. So mechanism of action of antioxidants has changed with chemical atmosphere. Natural antioxidants in biological mechanisms may serve as physiological barrier to prevent generation of reactive oxygen species (ROS) access to important target sites. Sometimes antioxidants like carotenoids or anthocyanidins develop chemical trap or sinks that absorb energy and electron and quenching with ROS (Figure 2.2). Antioxidant enzymes like Catalase, glutathione reductase and superoxide dismutases neutralize or divert ROS through catalytic mechanisms (Chaudiere and Ferrarilliou, 1999). Sometimes binding or chelation of metal ions by ferritene ceruloplasmin and catachin also prevents generation of ROS. Beside these there also chain breaking antioxidants like ascorbic acids, tocopherol, uric acid, glutathione and flavonoids (Benzie and Strain, 1996) which scavenge and destroy ROS (Figure 2.2). It is also important to understand the mechanism and dynamics of antioxidant action for appropriate selection of antioxidants. The quantitative structure activity relationship (QSAR) is determined by chemical nature of antioxidants and governed by several other factors like chemical reactivity towards free radicals and their stoichiometric ratio, fate of antioxidant derived radicals interaction with other antioxidants concentration and mobility at a particular microenvironment along with absorption, distribution, retention, metabolism and fate of antioxidant molecules in a particular biological system. Some antioxidants are present in free form while others are existing as metabolic intermediate or in bound form. Hydrophilic antioxidants like ascorbic acid or uric acid scavenge the free radical primarily in aqueous phase. The biological action of lipophilic antioxidants is mainly observed within the membrane and lipoprotein. The efficacy of radical scavenging by antioxidants in the membrane and lipoprotein particles depend on physical nature like fluidity of microenvironment and relative mobility of antioxidants. For example the peroxyl radical scavenging capacity by α-tocopherol in the membrane is insignificant than that of homogeneous solution probably because of restricted mobility of α-tocopherol. It was also noticed that the side

chain of lipophilic antioxidants reduce the mobility inside the membrane and lipoprotein, thus minimizing the apparent antioxidant capacity. It was previously reported that the radical scavenging capacity of ubiquinol didn't depend on length of isoprenoid chain in homogeneous solution but in heterogeneous membranes ubiquinols having short side chain significantly inhibit lipid peroxidation than their longer side chain homolog (Niki, 2010).

Some free radical scavenging antioxidants also inhibit oxidation of bio-molecules by synergistic co-operation with other antioxidants. The examples of the synergistic antioxidant interaction are efficient combination of vitamin C and vitamin E during oxidative stress. Vitamin E also induces another chain oxidation during scavenging of active free radicals by converting into vitamin E radical through which polyunsaturated lipids may be disrupted. Vitamin E also enhances the oxidation of isolated LDL and plasma lipids by phase transfer mechanism but combination of Vitamin E and vitamin C inhibit their oxidation completely. In contrast, another hydrophilic radical scavenging antioxidant uric acid present in plasma does not reduce vitamin E radical and inhibit pro-oxidants action of vitamin E. Phenoxy radical from polyphenolic antioxidants available in different medicinal plants having hydroquinone and catechol structure reacts rapidly with active oxygen and produces corresponding quinone and hydroperoxyl radical, which may elicit new chain reaction. It was also observed that ubiquinol and tocopheroyl hydroquinone undergo auto-oxidation *in vitro*. The reactivity of these hydroquinones towards free radical is larger than the α-tocopherol (Shi *et al*., 1999). So, the fate of antioxidant derived radical ultimately determines radical scavenging capacity in biological system.

2.5 SYNTHETIC ANTIOXIDANTS

Synthetic antioxidants are not available in nature and frequently mixed with food products as preservative to prevent the lipid peroxidation. These antioxidants act mainly by the way of two different pathways and are functionally categorized as primary and secondary antioxidants. Primary antioxidants stop the generation of free radicals and they are classified into free radical

terminators, oxygen scavengers and chelating agents. The radical terminators include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ) and gallates (Ozkan and Erdogan, 2011). The important example of oxygen scavengers is glucose oxidase, sulphites and ascorbyl palmitate, while chelating agents are known as transitional heavy metal like iron, copper with incompletely filled d-orbital. Secondary antioxidants such as thiodipropionic acid and dilauryl theodipropionate act by breaking down hydroperoxides formed during lipid oxidation and produce stable end products (Sen and Chakraborty, 2011). Moreover, these antioxidants are used in processed fruits and vegetables, margarine, soft drinks, and canned shellfish. Several antioxidants found in plants and are also prepared synthetically through different chemical processing i.e gallic acid. These phenolic synthetic antioxidants have the similar common biological effect on molecular, cellular and organ levels. Several synthetic anti-diabetic drugs like metformin, glibenclamide, repaglinide also have antioxidant activity (Kahl, 1984; Venkatesh and Sood, 2011). Commonly used synthetic antioxidants like BHT, BHA, ethoxyquinone and propyl gallate can produce beneficial interactions such as antimutagenic activity and antitumorigenic action, radioprotection, protection against acute toxicity of chemicals. Besides the beneficial impacts of synthetic antioxidants, these chemicals also have some adverse side-effects, like they play a key role in radio sensitization, enhanced toxicity from other chemicals, increased tumor yield from chemical carcinogens and increased mutagenic activity (Kahl, 1984; Venkatesh and Sood, 2011).

2.6 SECONDARY METABOLITES AS A RICH SOURCE OF ANTIOXIDANT

In the 19th century it was discovered that two major classes of metabolites are present in both plants and microorganisms; among which primary metabolites are essential for cell survival and propagation (carbohydrates, proteins, amino acids, lipids). Plants produce an incredible diversity of secondary metabolites, which have multiple functions throughout the plant's life cycle. Besides their role as mediators in the interaction of the plant with its biotic and abiotic environments, such

as plant-microbe, plant-animal and plant–plant interactions, plant secondary metabolites are involved in the fertility and germination of pollen (Schijlen *et al*., 2004). Secondary metabolites are derived from primary metabolites, like all amino acids and carbohydrates are converted to secondary metabolites through methylation, hydroxylation, and glycosylation biochemical pathways. Up to date, a few thousands of different secondary metabolite structures have been identified in plants; the largest of them are the phenyl-propanoids (synonym, phenylethanoids), then isoprenoids and alkaloids are placed. By chemical structure, secondary metabolites in plants are divided in three major classes such as (Korkina, 2007): - terpenes (isoprenoids, terpenoids) - phenylpropanoids and their derivatives (flavonoids, tannins, glycosides, and lignins) - nitrogen-containing compounds (alkaloids and heterocyclic aromatics). Phenyl-propanoids belong to a large class of plant phenols produced through shikimic acid pathway (Guillet and De Luca, 2005). Many of plant-derived phenolic compounds (flavonoids, isoflavonoids, coumarines, and lignans) are secondary products of phenyl-propanoids metabolism (Dixon and Paiva, 1995; Douglas, 1996). For example, flavonoids evolve from shikimic acid pathway (Figure 2.3). First, cinnamic and then, hydroxycinnamic acids are formed, both acids belong to phenylpropanoids. Then, chalcone synthase uses 3 cinnamoil radicals to produce flavonoids (Estrov *et al*., 2003). Lignins are phenolic polymers playing an important role by reducing the permeability of the cell wall to water, by increasing the rigidity of cell wall, which is a part of the pathogen resistance mechanism. These plant polymers are products of the oxidative coupling of phenylpropanoids monomers: peroxidase catalyses the oxidation of phenylpropanoids to their phenoxyl radicals, and the subsequent non-enzymatic coupling controls the pattern and extent of polymerization that results in a vast structural diversity of natural lignins (Russell *et al*., 2006). Plants normally develop several components of the antioxidant system in response to naturally occurring stresses such as stress at high altitude, chilling, draught, and nutrient deficiencies (Jordan, 2002). More attention has been paid over the past ten years to the effects of UV-B radiation on oxidative stress as well as phenylpropanoids are paying a vital role as antioxidants in plants (Turunen and Latola,

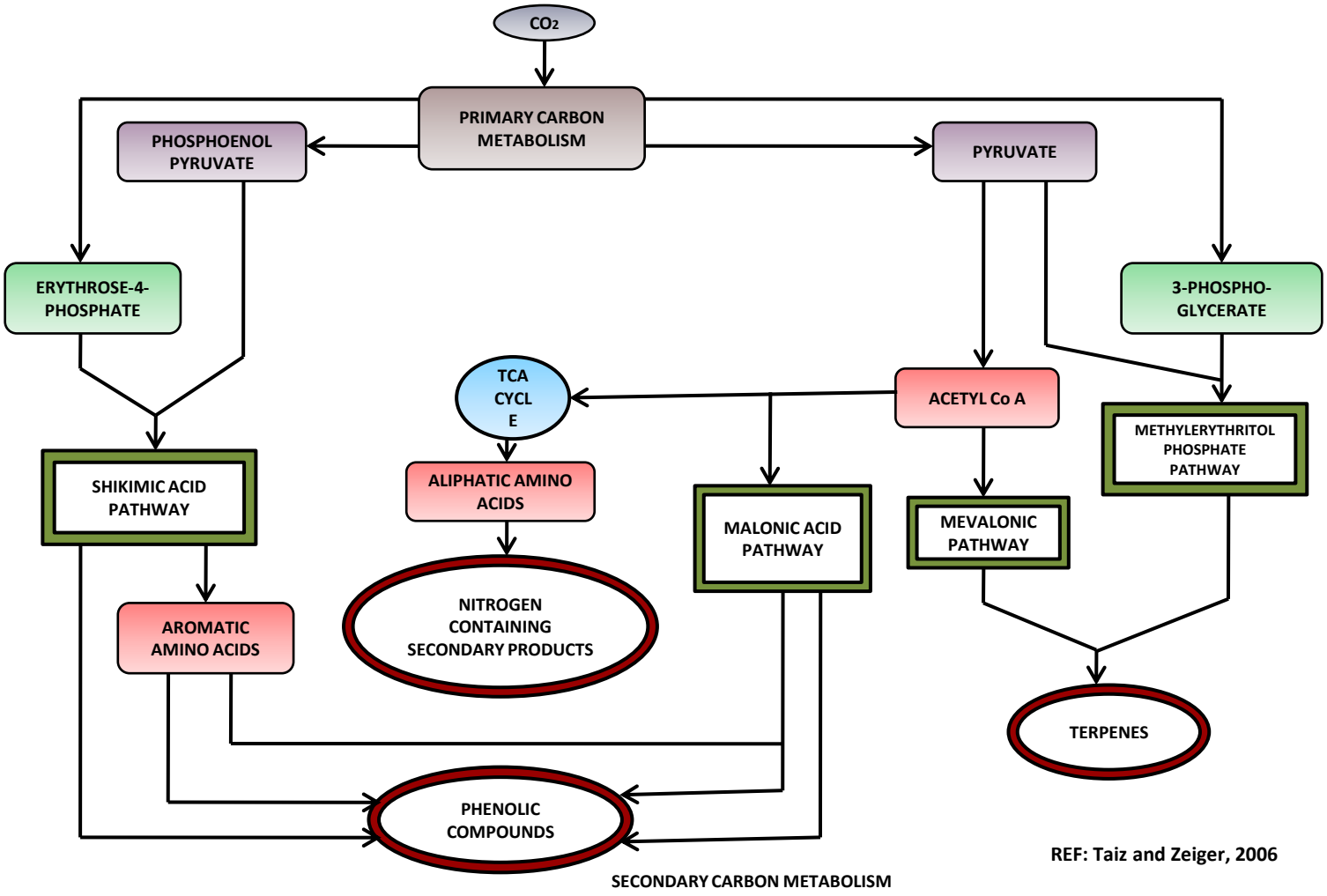


Figure 2.3 Major pathway of secondary metabolites synthesis and their interrelationship with primary metabolites

2005). In higher plants, phenylpropanoids, mainly, hydroxycinnamic acid, cinnamoyl esters, flavones, flavonols, and anthocyanins provide a UV-A and UV-B screen (Lavola *et al*., 2003). Terpens or terpenoids, constitute the largest class of secondary products, act as antioxidant. These groups are biosynthesized from primary metabolites in two different ways: mevalonic acid pathway (MAP) and methylerythritol phosphate pathway (MEP) (Figure 2.3) (Lichtenthaler, 1999). The monoterpenes limonene and perillyl alcohol may be promising substances in cancer therapy. Several investigations have studied the antioxidant activity of monoterpenes and diterpenes *in vitro*. A newly discovered antioxidant is gamma-terpinene which is a very effective antioxidant proposed by Grassmann (2005). According to Hartmann (1999) alkaloids which are a large family of nitrogen-containing secondary metabolites, are functioned as defense against predators, especially mammals, because of their toxicity and deterrent capabilities. In 2004, Rackova *et al*. discovered that three alkaloids like berberine, jatrorrhizine, and magnoflorine from *Mahonia aquifolium* have potent anti-radical as well as antioxidant capacity. The isoquinoline alkaloids like stylopine, protopine, fumaritine, fumaricine, fumarophycine, fumariline, fumarofine possess various kinds of pharmacological properties. The antimicrobial, antimalarial, cytotoxic, and anti HIV activities of the isoquinoline alkaloids have been reported and the possible chemopreventive antitumor promoters are probably related to their radical scavenging activity against DPPH radical (Cui *et al*., 2006). Phenolic alkaloids also serve as a new class of antioxidant agents of various medicinal plants. Antioxidant activities of different phenolic alkaloids (oleracein A, oleracein B and oleracein E) was also reported based on scavenging activity against DPPH radical and inhibitory effect on hydrogen peroxide-induced lipid peroxidation in rat brain homogenates (Zijuan *et al*., 2009). Recently Das *et al*., (2011) proposed that ethanol, ethyl acetate and n-hexane extracts of the whole plant *Lucus aspera* have got profound antioxidant effect and may have potential use in medicine due to the presence of superior amount of alkaloids. On the other hand, plant secondary metabolites are invaluable

resources, useful in food additives, fragrances, pigment or directly in medicines (Bourgaud *et al.*, 2001; Yazaki, 2006).

However, for the existence of chemical and pharmaceutical properties in secondary metabolites is interesting for human health (Raskin *et al*., 2002; Reddy *et al*., 2003) and the abundance of the target secondary metabolites is usually low (Bourgaud *et al*., 2001), which present a great challenge in the recovery and purification of plant secondary metabolites.

2.7 ASSESSMENT OF ANTIOXIDANT ACTIVITY

The functional properties of antioxidants are different in response to various oxidant sources. It can be stated that carotenoids are exceptional singlet scavenger but very poor quenchers of peroxyl radical when compared with phenolics (Huang *et al*., 2005). In fact various free radicals involved in the oxidative stress have different physiochemical and biological properties within the cell. Therefore no single assay accurately reflects antioxidant potential of a particular molecule against all radical sources.

The major free radicals that react predominantly with lipids, protein, carbohydrates and DNA are hydroxyl, alkoxyl, peroxyl, superoxide, nitric oxide along with sulphur and nitrogen centered radicals. On the basis of inactivation mechanisms performed by major antioxidants the reaction methods have been categorically differentiated into two groups: hydrogen atom transfer (HAT) reaction and electron transfer (ET) reaction (Karadag *et al*., 2009). HAT based methods quantify antioxidant ability to scavenge free radicals by donating hydrogen and subsequent formation of stable compound. They are considered as radical chain breaking antioxidant capacity (Prior *et al*., 2005). ET based method detect the ability of antioxidant potential through the capacity to transfer one electron by reducing any compounds like metals, carbonyls and radicals.

$$M(III) + AH \rightarrow AH^{\cdot} + M(II)$$

Several analytical strategies are available for determining reaction which include measurement at a fixed time, measurement of a reaction rate, lag phase measurement of end point change and

integrated rate measurement with different orders of reaction kinetics (Antolovich *et al*., 2002). In general, added antioxidants components with probes for the radicals are retardation of the oxidation of probe. Assays with these features include total radical antioxidant trapping assay (TRAP) and oxygen radical absorbance capacity (ORAC) assay (Huang *et al*., 2005). These assays have the following attributes:

1. Thermo-labile azo-radical initiator which produces radical ($R^{\cdot}$) that react fast with oxygen give steady flusk of $ROO^{\cdot}$ radical
2. Oxidizable molecular probe (UV/fluorescent for monitoring reaction process)
3. Antioxidants
4. Kinetic parameters of reaction (Magalhaes, 2008).

Other assays include carotenoids bleach via auto-oxidation induced by heat or peroxyl radicals (eg. oxidized lipids). The assay measures decrease in the rate of β-carotene or decay of crocin probe by herbal antioxidants. End point loss of colour was measured optically at 443 nm in phosphate buffer (pH 7.0) (Laguerre *et al*., 2007). The major advantage is that the kinetic approach in the measurement allows the determination of total inhibitory effect and provides more precise evaluation and efficiency of antioxidant defense (Roginsky and Lissi, 2005). Although β-carotene is often used as a target in this assay, its discoloration at 470 nm can accomplish by multiple pathways. Therefore interpretation of these results can be difficult. For overcoming this problem, carotenoid derivative protein which is natural compound with extremely strong absorbance in visible range has become the reagent choice recently (Prior *et al*., 2005).

The antioxidant capacity of a compound can be determined by two factors i.e. kinetics of scavenging radicals and number of radical each antioxidant molecule can scavenge. These two attributes can be determined by the reaction with reference free radical like 2,2-diphenyl-1-pyicrichydrazyl (DPPH) and cataionic radical ABTS. DPPH is long lived organic nitrogen radical with deep purple colour. Also DPPH is commercially available, stable, easy to handle and has

long visible spectrum with high molar extinction coefficient. The purple chromogenic DPPH radical is reduced by antioxidant compounds to the corresponding pale yellow hydrazine. The DPPH assay is technically simple and rapid and analyses of a large number of samples could be made by using microplate (Fukumoto and Mazza, 2000). ABTS on the other hand is a peroxidase substrate which is intensely coloured and spectrophotometrically monitored within the wavelength range of 600-750 nm. $ABTS^{\cdot +}$ can be solubilized in both organic and aqueous media and is not influenced by ionic strength; therefore the antioxidant capacity can be determined for both lipophilic and hydrophilic compounds (Arnao, 2000). In contrast DPPH can only be dissolved in organic media which is an important limitation for interpreting the role of hydrophilic antioxidants.

It is generally observed that a potent radical scavenging antioxidant can often behave like efficient reductant. The ferric reducing antioxidant power (FRAP) assay is based on the ability of antioxidant to reduce ferric tripyridyl triazine complex (FeIII-TPTZ) to blue ferrous complex (FeII-TPTZ) by the action of electron donating capacity (Benzie, 2003). Here, ferric salt is used as an oxidant and redox potential is comparable to that of ABTS. FRAP assay is rapid, inexpensive, robust and does not require specialized equipments. The major limitation of this assay is that the ultimate product is a complex of $Fe(II)^{+}$ which is also known as pro-oxidant. The ferrous ion can react with H_2O_2 to produce hydroxyl radical ($OH^{\cdot}$) which is most harmful free radical found in biological system. Ultimately not all reductants that are able to reduce $Fe(III)^{+}$ are potent antioxidants (Prior and Cao, 1999). In addition FRAP cannot detect compounds that act by radical quenching (hydrogen transfer) particularly applicable for thiols and proteins (Ou *et al*., 2002). Sometimes polyphenols may bind to metal ions and form complex, which may perform another function of polyphenolic compounds as an antioxidants.

The assay methods so far discussed are particularly applicable on hydrophilic or hydrophobic targets. But lipids like free and esterified forms of polyunsaturated fatty acids and cholesterol are sensitive target of free radical. From different investigations it was proved that

peroxidation of membrane lipid induces disturbance and dynamic alteration of membrane functions. Obviously it is also an essential task for scavenging antioxidants to suppress lipid peroxidation. One of the major advantages of lipid peroxidation of plasma is that both hydrophilic and lipophilic antioxidants and their interactions can be assayed through biologically relevant system. The capacity of antioxidant for inhibition of lipid peroxidation can be assayed by determining the extent of suppression of peroxidation by the test antioxidants. In fact in this assay the thioburbituric acid reactive substances (TBARS) or malonaldehyde generation can be determined precisely through chromogenic reactions. TBARS in biological system can also be acceptable as biomarkers of oxidative stress. Overall it can be stated that elucidation, understanding and evaluation of antioxidant action can only be possible through determining several radical scavenging species by different *in vitro* and *in vivo* assays and understanding the correlation among them.

2.8 RELATION BETWEEN ANTIOXIDANTS AND ANTIDIABETIC ACTIVITY

Natural antioxidants strengthen the resistance potency of endogenous antioxidants from ROS and restore the optimal balance by neutralizing the reactive species. They have shown their critical role in disease prevention. Diabetes mellitus is a multi-factorial disease which is characterized by hyperglycemia, raised basal metabolic rate, lipoprotein abnormalities, reactive oxygen species scavenging enzymes, defect as well as high oxidative stress induced damage to pancreatic beta cells (Shajeela *et al*., 2012). The disease is rapidly increasing in most part of the world due to dramatic changes in diet and lifestyle (Zhao *et al.,* 2006). In 2008, Shah *et al*., defined diabetic mellitus is a metabolic disorder characterized by an elevation of the blood glucose or hyperglycemia. Karpen *et al*., (1982) observed an elevated level of lipid peroxides in the plasma of streptozotocin induced diabetic rats and lipid peroxidation is one of the characteristic features of chronic diabetes.

GSH which is a major non-protein thiol in living organisms play a central role in co-ordinating antioxidant defense in body. SOD, CAT and GPx are acting as mutually supportive team of defense against ROS. SOD is a metallo-protein and is the first enzyme involved in the antioxidant defense by means of lowering the steady-state level of $O_2^{\cdot-}$. In hyperglycaemia, glucose undergoes auto-oxidation and produces superoxide like free radicals that in turn leads to lipid peroxidation in lipoproteins. CAT is peroxisomic or the micro-peroxisomic hemeprotein, which catalyses the H_2O_2 to water and oxygen and thus protect the cells from oxidative damage caused by H_2O_2. GPx catalyses the hydrogen peroxides with reduced glutathione to form glutathione disulphide (GSSG) (Sabu and Kuttan, 2004).

There is no known cure for diabetes. Therefore the best strategy is to bring out awareness for improvement of the diet and minimizing lifestyle disorder. There has been a remarkable progress and development in the therapy of diabetes mellitus through synthetic drugs. However, investigation for the anti-diabetic substances from natural sources for managing the hyperglycemia is being pursued due to their lower side effects and toxicity (Ahmad *et al.*, 2009a; Pinto and Shetty, 2010). Recent studies have found the activities of various plant materials containing anti-metabolites that help to prevent the oxidation pathway of fatty acids (Ahmad *et al*., 2009a), which has consequences for managing hypoglycemia.

For *in vitro* evaluation of antidiabetic activity, α-amylase and α-glucosidase enzymes are used through their inhibition percent. Plants are the main source of natural inhibitors of glucosidase that provides protection against various insects and microbial pathogens (Ryan, 1989; Lu *et al.,* 1999). Pancreatic and intestinal glucosidase are the key enzymes for digestion of dietary carbohydrate. The natural α-amylase and α-glucosidase inhibitors from food-grade plants provide dietary strategies to control postprandial hyperglycemia, and the natural form of these inhibitors could be used in therapies with minimum side effects (Kwon *et al.*, 2006; Shetty *et al*., 2008; Pinto and Shetty, 2010).

Recently, a large variety of animal models have been developed for better understanding of the diabetes mellitus and new herbal drugs have been established to treat this disease. Most experiments related with antidiabetic activity were carried out on rodents, although some investigations are still performed in larger animals. The classical model employed by Banting and Best was pancreatectomy in dogs (Bliss, 2000). It is also described as prone strains to diabetes mellitus that have been employed in several researches (Chen and Wang, 2005; Rees and Alcolado, 2005; Masiello, 2006). Streptozotocin (STZ, 69%) and alloxan (31%) are most frequently used drugs and this model has been used for the experimentation of multiple aspects of diabetic disease. Both drugs exert their diabetogenic action when they are administered peritoneally, intraperitoneally, intravenous, or subcutaneously. The dose of these agents varied for inducing diabetes depends on the animal species, nutritional status and route of administration (Lenzen *et al*., 1996; Mythili *et al*., 2004). Plants show the hundred percent recoveries from diabetic relevant disorders caused by alloxan or streptozotocin diabetic rats (Nagappa *et al*., 2003; Selvan *et al*., 2008; Ilango *et al*., 2009).

Therefore, food-based biochemical studies generate enormous magnitude for managing hyperglycemia and hypertension linked to diabetes mellitus through prescribing appropriate diet formulations.

2.9 INFLUENCE OF ANTIOXIDANT ACTIVITY WITH MATURATION AND SENESCENCE

Several reports are available where it has been proved that the free radical scavenging activities of the plant increased gradually during maturation and senescence (Wang *et al*., 2009; N'Dri *et al.*, 2010). However, there are many controversies related to the above argument (Cavaiuolo *et al*., 2013). Senescence requires regulation of several genes which is activated by internal and external factors. The plant hormones ethylene and abscisic acid play a significant role as internal signals for regulating senescence. In case of ethylene sensitive plant parts, this hormone strongly

regulates the senescence method (Cavaiuolo *et al*., 2013). Besides its physiological roles in different maturation stages of plant, ethylene was recognized as a stress hormone because the synthesis of ethylene is induced by a variety of stress signals, like mechanical wounding, extreme temperatures, chemicals and metals, drought and pathogen infection (Kende, 1993; Johnson and Ecker, 1998). Stress-induced ethylene generation is controlled by accelerating conversion of *S*-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC), suggesting that expression of the ACC synthase is major target of regulation. Among environmental stresses mostly ozone, UV irradiation and wounding, stimulation for ethylene synthesis has been reported to involve the generation of reactive oxygen species such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, cause damage to cellular organelles by lipid peroxidation (Surplus *et al.,* 1998; Orozco-Cardenas and Ryan, 1999; Pellinen *et al.,* 1999). In addition, ROS, in particular hydrogen peroxide, have been shown to function as signalling molecules (Levine *et al*., 1994). It was earlier reported that phenylpropanoid metabolism is enhanced by this plant hormone and certain phenolic compounds those have been associated with this metabolism can reduce certain diseases mainly diabetes (Hertog *et al*., 1992; Frankel *et al*., 1995). A lots of studies also showed that several plants exhibited high oxygen radical absorbance capacity against peroxyl radicals, superoxide radicals, hydrogen peroxide and singlet oxygen due to the presence of anthocyanins (Wang and Jiao, 2000; Wang and Lin, 2000). Anthocyanin content in raspberries is increased during maturation of berries from green to the pink stage followed by a significant increase in total phenolics from the pink to the ripe stage (Wang and Lin, 2000).

2.10 ALTERATION OF ANTIOXIDANT ACTIVITY WITH DOMESTIC THERMAL PROCESSING

As we already know that the phytochemicals present in vegetables and fruits are beneficial for the management of different human disorders which has created prime attraction for both consumers and researchers mainly due to the presence of antioxidant activity in these food substances.

Several factors like genetics and growing conditions viz. fertilization, pest, moisture, and disease burden, and so on are known to affect the concentration and bioavailability of bioactive compounds having antioxidant activity and therefore modulating their total antioxidant activity (Kalt, 2005). Processing, mainly cooking of food, is another vital factor that have certain impact on total antioxidant activity (Papas, 1996). As vegetables are the usually consumed after processing/cooking, it is particularly important for vegetables. However, positive and negative effects have been reported which is dependent on difference in processing condition as well as morphological and nutritional properties of different vegetables (Nicoli *et al*., 1999; Lee and Kader, 2000; Ou *et al*., 2002; Bernhardt and Schlich, 2006; Podsedek, 2007). Physical characteristics of vegetables are also affected by heat treatments (Waldron *et al*., 1997; Turkmen *et al*., 2006). There are several methods to cook vegetables such as boiling, frying, sun drying, steaming, baking etc. Boiling is the most usual process, where the food materials are thermally processed in water nearly at 100º C. Another interesting *de novo* process is microwave heating that has been used for cooking where the interaction of an electromagnetic field with chemical constituents of food occur (Young and Jolly, 1990). Many authors suggested that each vegetable has a preferential cooking process and that could be selected to preserve or improve its nutritional and physico-chemical properties; this selection may facilitate consumers on the choice of cooking practices to progress the nutritional quality of food (Miglio *et al*., 2008).

2.11 AWARENESS ABOUT NUTRACEUTICALS AND THEIR MARKET POTENTIAL, BASED ON MARKET SURVEY REPORTS

The concept of functional foods is susceptible to different analysis that referred to their characteristics and their active components as well as their regulatory framework (Kwak and Jukes, 2001; Griffiths *et al*., 2009; Shahidi, 2009). Functional foods provide benefits for constructive body functions and important for the maintenance of health or also can reduce disease risk (Hardy, 2000). The concept of nutraceuticals is also controversial. However, from the

view point of the consumers, nutraceuticals are functional foods which help to prevent a disease or acts for its treatment and for that reason their particular effects are recognized. In this context it is notable that a consumer use a functional food, on the other hand the food can act as a nutraceutical (Kalra, 2003). Kalra (2003) also reported that, common people are not aware of their specific components; however, functional foods are recognized because they are good for health. The functional foods and nutraceuticals are linked to the traditions of people. However, some functional foods and nutraceuticals are prevalent within the immigrant community go on sale in the markets and according to their diffusion level, spread by the media, they (functional foods and nutraceuticals) enter into the general commercial circuit.

Investigation on traditional markets have usually been addressed from the anthropology as well as the economic geography points of view as systems in which their components (actors and social networks, exchange and distribution, the products with their origin and destination) have to be explored (Cunningham, 2001). It is important to note that the markets represent valuable places for ethnobotanical researches as they condense in a reduced area and perpetuate the local knowledge and values on biological products. Markets are public spaces devoted to sell several products, as well as they are spaces for exchange and acquisition of cultural information. The nutraceutical market develops under three prime components which include functional foods, dietary supplements and herbal/natural products. Global nutraceutical market has now reached at USD 120 billion. According to recent reports, nutraceutical market in India is growing at 21% per annum, which is equivalent to INR 44 billion (Chouhan *et al*., 2013). Though this estimated is much faster than global rates fixed at 18% (estimated average of last three years), still the concept of nutraceutical and functional food is at the stage of infancy in different markets of Indian sub-continent. Among nutraceuticals, market demand for dietary supplement and natural/herbal product is 20% and 12% per year respectively. Many authors reported that, due to market demand of plants (for food as well as for medicine) plant dwellers collect plants from forests or natural habitat randomly (Sundrial and Sundrial, 2003; Chhetri *et al*., 2005; Ahmed and Javed, 2007).

They haven't any knowledge about nutraceuticals value of the plants. Anthropogenic activities are the main factors for rarity of the valuable species (Chhetri *et al*., 2005).

2.12 EDIBLE PLANTS OF DARJEELING HIMALAYA AND THEIR ETHNO-MEDICINAL IMPLICATIONS

Darjeeling district is present at the northern region of West Bengal. This district is subdivided into four Sub-Divisions viz., Darjeeling sadar, Kurseong, Kalimpong and Siliguri (Figure 1). The region lies between 26º 31' and 27º 31' north latitude and between 87º 59' and 88º 53' east longitude in the Eastern Himalayan region of India (Bhujel, 1996). It is bordered by Sikkim in the north, Bhutan in the east, Terai and Dooars in the south and Nepal in the west. The district has two different topographical features. The hill area is formed by Darjeeling, Kurseong and Kalimpong whereas Siliguri is stationed at the foothill of Darjeeling Himalaya. Darjeeling Himalaya has the richest biodiversity in the world (Copra, 1956). It is well known that the wild plants are mostly used as food and medicinal purposes in rural area of the world. Darjeeling Himalaya is a most diversified region where the local people are dependent on forests for food and medicine. In 2003, Sundriyal and Sundriyal reported about six edible plants of this hill such as *Baccaurea sapida, Diploknema butyracea, Eriolobus indica, Spondias axillaris, Machilus edulis and Elaeagnus latifolia.* They have prepared a detailed survey report on these plants and accounted that these plants are present only in natural habitat and have a significant market value. These plants are facing in extent of anthropogenic pressure and the available information about nutrient content and growth performance of the species is scanty (Sundriyal and Sundriyal, 2003). Bantawa and Rai (2007), prescribed the formulation and administration procedure of the plants species which are grown in different pockets of Darjeeling Himalaya. Yonzone *et al.* (2012a) recorded ethnomedicinal use (Yonzone *et al.,* 2012b), genetic resources, current ecological status, and altitude wise distribution of medicinal plants diversity of Darjeeling Himalaya. In 2011, Saha *et al*. reported 78 plants of this hills which are used medicinally for treating various ailments by

different tribal communities residing within the three hilly sub-divisions of Darjeeling Himalaya. They also evaluated antimicrobial activity of those plants. Recently, Sharma (2013a) investigated antibacterial activity of another 33 plants of this hill which are used to treat microbial infection in folk medicine. He also recorded the ethnomedicinal plants used against skin diseases by indigenous population of Darjeeling Himalayas (Sharma, 2013b).

2.13 ANTIOXIDANT ACTIVITY OF DIFFERENT PLANTS OF DARJEELING HIMALAYA

Higher plants generally utilised as important sources of foods, medicinal drugs as well as health products. Our ancestors have practiced the plants in their daily life as medicines. Therefore, the traditionally used medicinal knowledge could be a key factor and also could promote the development of modern drugs. Literature review investigations about plants have given up amazing discoveries and development in modern medicine. Edible plants also contain a variety of phytochemicals with different biological activities, which act in various ways and interact with different metabolic processes (Danesi, 2009). Experiments have proven the correlation between the consumed of fruits and vegetables and the mortality from degenerative diseases (Rimm *et al.,* 1996). The reason is not known which specific dietary constituents are liable for this association, but it is assumed that antioxidants are the major compounds that play an important role (Gey *et al.,* 1991). Recently several authors claimed that Darjeeling Himalaya contain several plants with huge amount of potent bioactive components. They worked on antioxidant activities of different plants of Darjeeling Himalaya. For example in 2009b Mandal *et al.* investigated free radical scavenging capacity of *Urtica dioica* which is a green vegetables of Darjeeling Hill. They found that different solvent fraction extracted from inflorescence of this plant has significant antioxidant capacity. Recently, Das and Coku (2013) noticed that petroleum ether extract of *Osbeckia stellata* leaves which is a plant of this hill had exhibited potent antioxidant as well as antimicrobial properties. Similar works have been performed by different plants like *Bambusa vulgaris* (Goyel

et al., 2010), *Daphniphyllum himalense* (Majumdar and Roy, 2012; Roy and Majumdar, 2013), *Stephania hernandifolia* (Sharma *et al*., 2010), ten clones of tea (Dasgupta *et al*., 2013), different bryophytes (Dey *et al*., 2013) and different lichens (Sharma and Kalikotay, 2012). If compared with the flora of Darjeeling, the compiled species related to above works is a small portion of the flora of this region (Das and Chanda, 1986; Das and Lahiri, 1997).

2.14 QUANTITATIVE VARIATION OF ANTIOXIDANT ACTIVITY WITH SOIL NUTRITIONAL PROPERTIES

Studies on the effects of various environmental factors on accumulation of secondary metabolites were well known and documented elsewhere in this review, but only limited studies covering the response of secondary metabolites and antioxidant activities under different soil nutrient sources (Ibrahim *et al*., 2013). Recent research has uncovered the fact that the availability of plant nutrients can be important factors for elicitations of secondary metabolites and antioxidants within plants (Stewart *et al*., 2000). Mineral nutritional status and physical properties; generally pH, EC and micro-nutritional properties of soil greatly influence the phytochemical constituent present in different plant parts (Mandal *et al*., 2010). Macronutrients of soil like nitrogen, phosphorus, potassium and sulphur interfere with the biosynthesis of phenolic compounds produced through phenylpropanoid pathway (Coria-Cayupan *et al*., 2009). Interestingly while the enrichment of nitrogen, phosphorus, potassium and calcium in soil through fertilizer application have been shown to affect secondary metabolites production in some plants (Kraus *et al*., 2004), there are other plants also where polyphenol accumulation was not significantly influenced by mineral nutrition (Mogren *et al.,* 2006). So, intensive research is required for determining the influential parameters of soil macronutrients on the biosynthesis and metabolic fate of antioxidant molecules. Among macronutrients of soil, nitrogen is one of the most important growth factors in regulating the quality and target plant parts. Nitrogen supply and accumulation in soil has both positive and negative effect on the biosynthesis of phenylpropanoids in plants. The enhancement

of total plant phenolics and flavonoid compounds under limited nitrogen fertilization was previously reported (Koricheva *et al*., 1998; Felgines *et al*., 2000). Improvement of carbon based secondary metabolites under poor nitrogen soil condition was in agreement with Carbon Nutrient Balance theory proposed by Bryant *et al.,* (1983). The increase in polyphenol based antioxidant molecules under low nitrogen condition might be attributed to excess availability of unutilized phenyl alanine due to restricted protein synthesis under nitrogen deficiency (Awad and de Jager, 2002). Reduced nitrogen fertilizer also increased glutathione which has a strong correlation with total phenolics, flavonoids, ascorbic acid and saponin content (Ibrahim *et al.,* 2013). However, some studies also indicated that the available amendment of organic nitrogen fertilizer might improve leaf antioxidant status in association with soil biota (Montalba *et al*., 2010). In *Ziziphus jujube* Mill. potassium enrichment in soil improved the accumulation of total phenolics, total flavonoids and total pro-anthocyanidin content (Wu *et al*., 2013). Potassium supplementation also helps in accumulation of phenylpropanoid components in apricot fruit (Radi *et al*., 2003). Interestingly, on onion, addition of potassium in soil enhanced total polyphenols but bears negative correlation with free radical (DPPH) scavenging properties (Bystricka *et al*., 2013). In some cases, application of lime and phosphorus improved the plant nutrition, with enhanced dry matter content and plants antioxidative system (Mora *et al.,* 2008). But phosphorus also showed negative impacts on the accumulation of phenolics, expect for protocatechuic acid (Wu *et al*., 2013). Overall, it can be stated that natural growing practices and soil nutrient profile are important attributes for simultaneous improving yields, phenolics level and antioxidant activity.

2.15 ISOLATION AND PURIFICATION OF DIFFERENT SECONDARY METABOLITES AND ANTIOXIDANTS FROM PLANT SYSTEM

Each secondary metabolites family has their specific chemical characteristics which imply that specific extraction and analysis method should be developed to study each family in details. On the other hand, using their polarity properties, which range from polar for polyhydroxylated

alkaloids or flavonoid glycosides to non polar compounds for terpenoids or lipids, and improved database systems, it is possible to explore an increasing number of compounds by using different analytical systems such as GC-MS, LC-MS and HPLC-DAD coupled to a sequential extraction. The technique described in Figure 5 is aimed at profiling a wide range of compounds from different classes of secondary metabolites. These compounds can be easily extracted from plant material using aqueous ethanol, methanol and dichloromethane allowing the extraction of a large range of compounds from water soluble to non polar compounds. Then it is possible to separate them by using different chromatographic methods and to analyze the fractions obtained by GC-MS and HPLC-DAD/MS (Respail *et al*., 2005). In 1988, Harborne proposed that after the separation of secondary metabolites by successive fractionation through non-polar to polar solvents (Figure 2.4), these components are characterized and purified by UV VIS Spectra, HPLC and lastly identified by MASS and NMR Spectroscopy. Recently Armand *et al*., (2012) proposed that ten spices like cumin, chili, pepper, nutmeg, garlic, cloves, ginger, coriander, onion and thyme have high quantum of antioxidant activity as determined through UV-VIS Spectroscopy. By chemical, biochemical and electrochemical assays, Barros *et al*., (2011) suggested that three different extracts of edible flowers like *Cytisus multiflorus* (L'Hér.), *Filipendula ulmaria* (L.) Maxim. and *Sambucus nigra* L could be suitable for incorporation into functional beverages or products with potential anti-inflammatory and other health-promoting properties. Correspondingly, Faiza *et al*., (2011) noticed that *Olea europaea* L., which is an economically important fruit trees in Mediterranean countries, is a potent source of polyphenols having antioxidant property. *O. europaea* may constitute a good source of healthy compounds, especially phenolics, in the diet, suggesting that their consumption could be useful in the prevention of diseases in which free radicals are implicated. Medoua *et al*., (2011) detected three compounds by using HPLC viz. Myricetin, morin, quercetin which are responsible for antioxidant activity of carrot and orange. Recently four phenolic compounds like gallic acid, pyrogallol, syringic acid, caffeic acid and three flavonoid compounds like rutin, naringenin and quercetin were identified

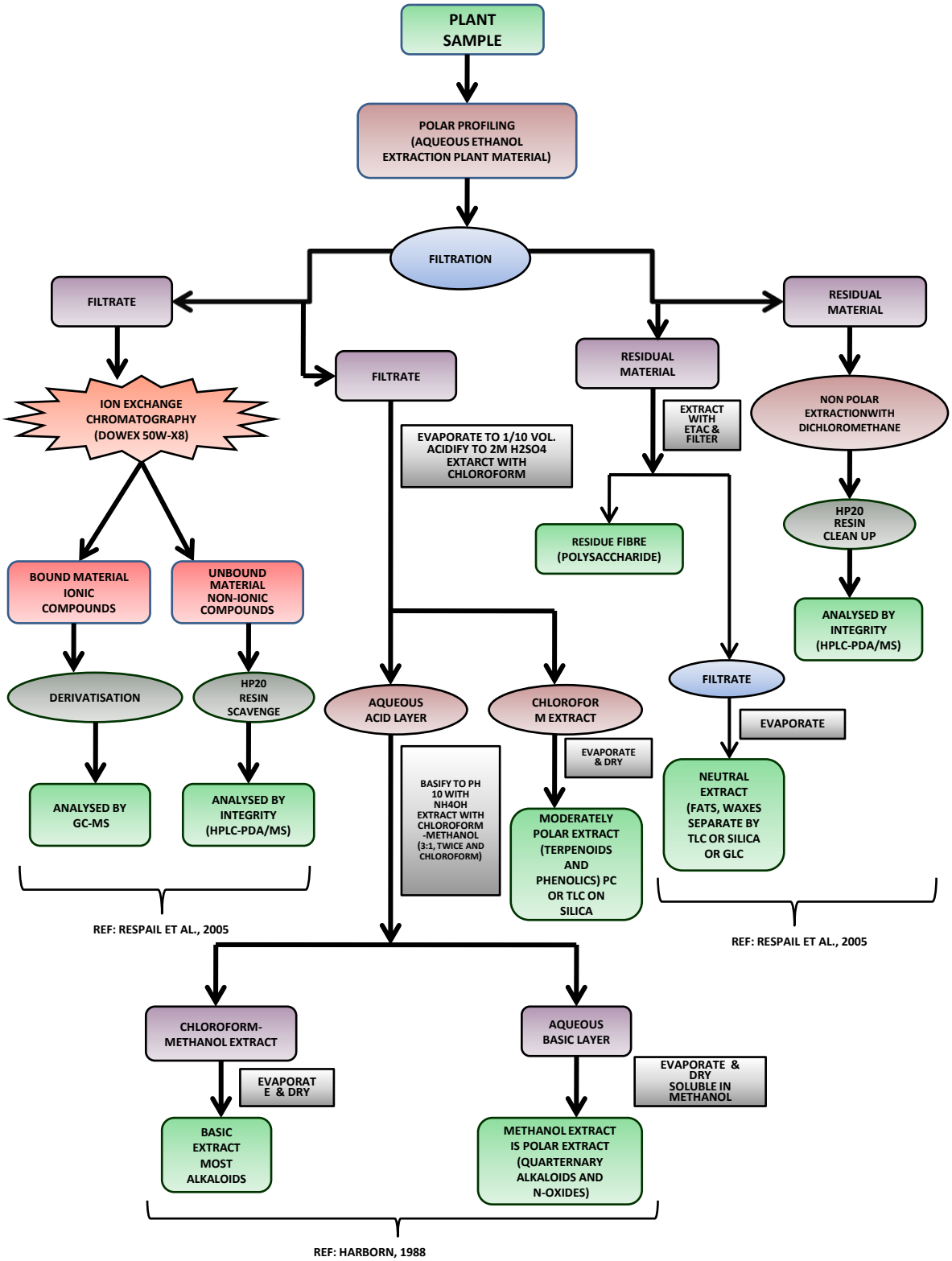


Figure 2.4 Schematic diagram of secondary metabolism profiling method

by Karimi *et al*., 2012 by RP-HPLC chromatography from *Citrus aurantium* bloom. *Euryale ferox* seeds are strongly used to treat kidney diseases including diabetic nephropathy. Song *et al*., (2011) isolated and identified the respective components which are actually responsible for the inhibition of the oxidative stress by NMR. From *Euryale ferox* seeds they have got rel-(2R,3β)-7-O-methylcedrusin, syringylglycerol-8-O-4-(sinapyl alcohol) ether and (þ)-syringaresinol which were found to be most active on DPPH assay, whereas (1R,2R,5R,6S)-2-(3,4-dimethoxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7 ioxabicyclo [3.3.0] octane, and buddlenol E could significantly inhibit high glucose-stimulated reactive oxygen species production in mesangial cells. Through GC-MS, Yang *et al*., (2012) isolated eight bioactive components like trans-anethole; anisyl aldehyde; anisyl acetone; 4-(2-propenyl)-phenol; 5-methoxy-2-methyl-benzenamine; phenol; benzenecarboxylic acid; anisyl alcohol from *Illicium verum*.

2.16 FUTURE PROSPECTS

After a long period, it is now time to illuminate the outputs of antioxidant research and their further development for mankind. Most of the degenerative diseases are related to radical damage, which requires more secure data. It can be expected that antioxidants can check and cure a number of patho-physiological problems also which require intensive attention. Many novel approaches are made in antioxidant research and significant findings have come out in last few years with improved phytotherapeutic research. The traditional Indian diet, spices as well as medicinal plants are the rich sources of natural antioxidants. Higher intake of these foods with functional attributes including high level of antioxidants is one strategy that is gaining importance in the advanced countries and also it is making formal shape in India. Coordinated investigation involving biomedical scientists, botanists, nutritionists and physicians will definitely create significant difference in phytotherapy research for overcoming critical human disorders in the coming decades. Research on the free radicals and antioxidants is one attempt for inventing the nutraceutical and pharmaceutical products.

Chapter - III

SURVEY OF UNDERUTILIZED FRUITS AND VEGETABLES AVAILABLE IN MARKETS OF DARJEELING HIMALAYA

3.1 INTRODUCTION

Markets have the power on income generation, food security, and other important developmental objectives of a particular area. Marketing is given topmost attention or credibility in the developing countries. The effectiveness of marketing for fruits and vegetables in India has been of significant concern in the recent years. Poor organization in the marketing circuits and insufficient marketing infrastructure are believed to be the cause of not only high and erratic consumer prices, but also insufficient of the consumer rupee that generally has reached to the marginal farmers (Ashturker and Deole, 1985; Kaul, 1997). Markets may provide the reason to optimize profits for the maximum participants by developing new technologies and simultaneously the methods for exploiting the natural resources. Likewise valuable plants available in nature have also been seriously over-exploited for solving market purposes, where animals live in equilibrium with these plants and using them as sources of food and phytomedicine. As a result of which surrounding ecosystem is realizing serious threats of extinction. Darjeeling Himalayan region is one of the hotspots in plant biodiversity with a large number of plant varieties (Ahmedulla and Nayar, 1999) and are also rich in varied human races too with a huge number of ethnic groups, who are sharing the common habitats. Numerous plant species of this region have been used as medicinal plants and a variety of their therapeutic properties have been recognized as folk medicine. Traditionally, main occupation of the people of Darjeeling is agriculture, agro-forestry, horticulture and animal husbandry. The farmers typically depend heavily on middlemen particularly for fruits and vegetable marketing. In 2005, Chettri *et al* reported that the Federation of Societies of Environmental Protection, an NGO is involved in the collection and marketing of local herbs along with local awareness about food quality for rural upliftment of Darjeeling Himalaya. But unfortunately, till now there are no comprehensive reports on nutraceutical based market survey of edible plants available in Darjeeling Himalaya. In light of these issues, this study seeks to examine the market environment for underexplored fruits and vegetables in different localised areas. It examines various aspects of fruits and vegetable

marketing such as market infrastructure, consumer awareness, transportation facilities etc. The study also made an attempt to determine the availability of the plants throughout the year, sources and edible part of these plants, post harvest treatment for preparation of food and awareness among local people of Darjeeling Himalaya about the quality of nutraceuticals.

3.2 MATERIALS AND METHODS

3.2.1 Data collection and investigation

This study is based on information accumulated from the market vendors and consumers of the selected fruits and vegetable markets around the selected town. The market vendors and consumers were directly interviewed and consulted using prepared questionnaires for collecting the information on the overall activities of these markets, marketing infrastructure as well as other relevant information.

During the years 2008 to 2013, we interviewed different sellers (who were also culturally and linguistically different in selected markets of Darjeeling hills during different seasons in order to collect information on plant species that were rarely found in the markets of other parts of West Bengal. Most of the sellers of Darjeeling Hills were women belonging to the Nepali culture and some of them could not speak the Hindi language also.

The questionnaire has been arranged through the following specific points

- Morphology of edible parts
- Process of collection for trees and shrubs
- Source of collection
- Local transport facilities
- Versatility of products in an area
- Collection amount and availability in local market
- Market demand for local people and foreigners

- Seasonal restriction and maturation time of edible components
- Export potential from local area
- Taste quality of the edible parts
- Local awareness about nutraceutical values
- Ethno-medicinal use of plant parts
- Post harvest processing and type of cooking practices with those vegetables

3.2.2 Plant identification

Plant specimens were collected from natural forests and cultivated fields in collaboration with farmers and suppliers of the specimens during reproductive season. Finally these plants represent our voucher specimens and are deposited in the 'NBU Herbarium' of Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal for identification. Plant specimens were identified by Prof. A.P. Das and accession number were recorded against each specimen (Table 3.1 and Figure 3.1).

3.2.3 Study area

For this study nineteen markets of Darjeeling Himalaya were visited from different town viz. Near Darjeeling Town, Ghum, Sokhiapokhri, Pasupati Market, Sonada, Near Mirik Town, Kurseong and Kalimpong. Detailed of markets are given in the Table 3.2, Figure 3.2 - 3.3.

3.2.4 Method of analysis

Data and graphical statistics were processed by MS Excel 2007.

Table 3.1 Fruits and vegetables of Darjeeling Himalaya

SL NO.	SCIENTIFIC NAME	FAMILY	LOCAL NAME	PLANT PARTS USED AS FRUITS/VEGETABLES	EHNO-MEDICINAL USE	ACCESSION NUMBER
1.	*Aconogonon molle* (D. Don) H. Hara	Polygonaceae	Thotne	Leaf/ Stem/ Inflorescence	No idea	9580; 16.08.09
2.	*Apium graveolens* L.	Apiaceae	Celery	Leaf/ Petiole	Gastric	9577; 16.08.09
3.	*Brassica juncea* (L.) Czern.	Brassicaceae	Rai Sak	Leaf/ Petiole	Stomach	9578; 16.08.09
4.	*Brassica rapa* L.	Brassicaceae	Chinese sak	Leaf/ Petiole	No idea	9614; 09.07.10
5.	*Calamus erectus* Roxb.	Arecaceae	Bedgera	Epicarp/ Mesocarp/ Endocarp	Diabetes	9585; 28.05.10
6.	*Capsicum annuum* L.	Solanaceae	Dollo Khorshani	Whole fruit	Gastric	9579; 04.03.09
7.	*Cinnamomum glaucescens* (Nees) Hand.-Mazz.	Lauraceae	Mallagiri	Fruit without seed	No idea	9594; 06.01.09
8.	*Cyclanthera pedata* (L.) Schrad.	Cucurbitaceae	Chuche korola	Epicarp & mesocarp/Endocarp	No idea	9599; 12.09.09
9.	*Cyphomandra betacea* (Cav.) Miers	Solanaceae	Tree tomato	Epicarp/ Mesocarp/ Endocarp	Diabetes	9579; 04.03.09
10.	*Dioscorea alata* L.	Dioscoreaceae	Ghar tarul	Underground part	No idea	7887; 15.01.10
11.	*Dioscorea hamiltonii* Hook. f.	Dioscoreaceae	Ban tarul	Underground part	No idea	9545; 11.03.10

Table 3.1 Fruits and vegetables of Darjeeling Himalaya Cont…….

SL NO.	SCIENTIFIC NAME	FAMILY	LOCAL NAME	PLANT PARTS USED AS FRUITS/VEGETABLES	EHNO-MEDICINAL USE	ACCESSION NUMBER
12.	*Diploknema butyracea* (Roxb.) H. J. Lam	Sapotaceae	Tiuri	Fruit without seed	Blood pressure	9545;11.03.10
13.	*Docynia indica* (Wall.) Decne.	Rosaceae	Mel	Epicarp/Mesocarp/Endocarp	No idea	9478; 12.12.08
14.	*Elaeocarpus lanceifolius* Roxb.	Elaeocarpaceae	Bhodrasi	Fruit without seed	No idea	9595; 06.01.09
15.	*Evodia fraxinifolia* (Hook.) Benth.	Rutaceae	Khanapa	Whole fruit	No idea	9546; 11.03.10
16.	*Heracleum wallichii* DC.	Apiaceae	Chimping	Seed	Skin, Blood clotting	7886; 17.07.09
17.	*Ipomoea batatas* (L.) Poir.	Convolvulaceae	Sheto sakar kanda	Underground part	No idea	7890; 15.01.10
18.	*Ipomoea batatas* (L.) Poir.	Convolvulaceae	Lal sakar kanda	Underground part	No idea	7889; 15.01.10
19.	*Lepidium sativum* L.	Brassicaceae	Chamsur	Leaf/ Stem/ Root	No idea	7885; 17.07.09
20.	*Litsea cubeba* (Lour.) Pers.	Lauraceae	Sil timbur	Whole fruit	Diarrhoea, Vomiting, Gastric, Warm killer	9573; 04.03.09
21.	*Machilus edulis* King ex Hook. f.	Lauraceae	Lopche kawlo	Epicarp/ Mesocarp	Skin	9543; 11.03.10
22.	*Manihot esculenta* Crantz	Lauraceae	Simal tarul	Underground part	No idea	7887; 15.01.10
23.	*Nasturtium officinale* R.Br.	Brassicaceae	Simrio	Leaf	Jaundice	9575; 16.08.09

Table 3.1 Fruits and vegetables of Darjeeling Himalaya Cont…….

SL NO.	SCIENTIFIC NAME	FAMILY	LOCAL NAME	PLANT PARTS USED AS FRUITS/VEGETABLES	EHNO-MEDICINAL USE	ACCESSION NUMBER
24.	*Persea americana* Mill.	Lauraceae	Ghiu kawlo	Epicarp/ Mesocarp	No idea	9544; 11.03.10
25.	*Phytolacca acinosa* Roxb.	Phytolaccaceae	Jiango	Leaf	No idea	9596; 06.01.09
26.	*Prunus domestica* L.	Rosaceae	Arucha	Fruit without seed	Blood pressure	9597; 11.09.09
27.	*Pyrus communis* L.	Rosaceae	Naspati	Mesocarp/ Endocarp	No idea	9600; 12.09.09
28.	*Rubus ellipticus* Sm.	Rosaceae	Oisilo	Whole fruit	No idea	9615; 09.07.10
29.	*Sechium edule* (Jacq.) Sw.	Cucurbitaceae	Squash jara	Underground part	No idea	9576; 16.08.09
30.	*Solanum anguivi* Lam.	Solanaceae	Bihi	Whole fruit	Blood pressure, Diabetes	9574; 24.02.09
31.	*Solanum incanum* L.	Solanaceae	Bara Bihi	Epicarp /mesocarp/ Endocarp	Diabetes	9542; 11.03.10
32.	*Spondias mombin* L.	Anacardiaceae	Lopsi	Fruit without seed	No idea	9477; 12.12.08
33.	*Xanthosoma brasiliense* (Desf.) Engl.	Araceae	Pindalu	Underground part	No idea	9598; 11.09.09
34.	*Zanthoxylum acanthopodium* DC.	Rutaceae	Boke timbur	Whole fruit	Diarrhoea, Gastric	9616; 11.03.10

***Aconogonon molle* (D. Don) H. Hara**

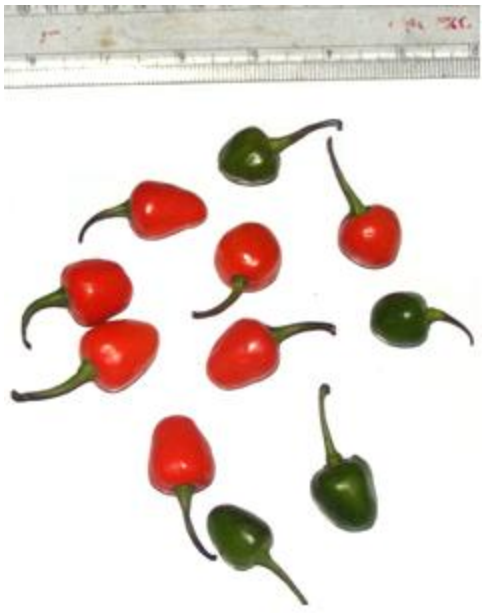
***Apium graveolens* L.**

***Brassica juncea* (L.) Czern.**

***Brassica rapa* L.**

Figure 3.1 Plants available in markets of Darjeeling Himalaya

***Calamus erectus* Roxb.**



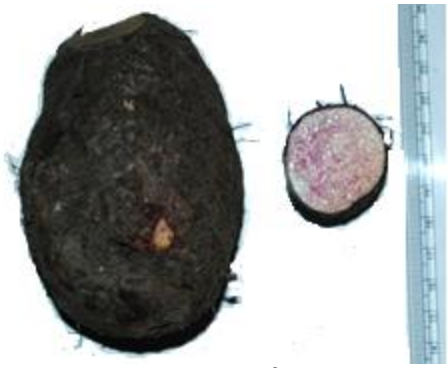
***Capsicum annuum* L.**

***Cinnamomum glaucescens* (Nees) Hand.-Mazz.**

***Cyclanthera pedata* (L.) Schrad.**

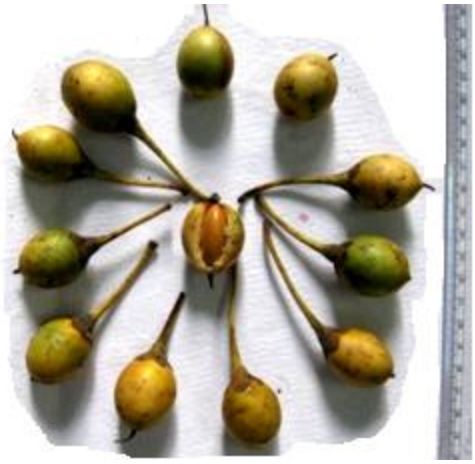
Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

***Cyphomandra betacea* (Cav.) Miers**



***Dioscorea alata* L.**

***Dioscorea hamiltonii* Hook. f.**



***Diploknema butyracea* (Roxb.) H. J. Lam**

***Docynia indica* (Wall.)Decne.**

Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

***Elaeocarpus lanceifolius* Roxb.**

***Evodia fraxinifolia* (Hook.) Benth.**

***Heracleum wallichii* DC.**

***Ipomoea batatas* (L.) Poir.[Red]**

***Ipomoea batatas* (L.) Poir. [White]**

***Lepidium sativum* L.**

Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

Litsea cubeba (Lour.) Pers.

Machilus edulis King ex Hook. f.

Manihot esculenta Crantz

Nasturtium officinale R.Br.

Persea americana Mill.

Phytolacca acinosa Roxb.

Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

***Prunus domestica* L.**

***Pyrus communis* L.**

***Rubus ellipticus* Sm.**

***Sechium edule* (Jacq.) Sw.**

***Solanum anguivi* Lam.**

***Solanum incanum* L.**

Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

***Spondias mombin* L.**

***Xanthosoma brasiliense* (Desf.) Engl.** ***Zanthoxylum acanthopodium* DC.**

Figure 3.1 Plants available in markets of Darjeeling Himalaya (Cont…)

Table 3.2 Names of fruits and vegetables markets

Name of Town	Name of Market
Near Darjeeling Town	Chowk Bazaar Market
	Pound Bazaar Market
	Mall Market
	Chowrasta market
	Singa Mari Market
	North Point Market
	Lebong Market
	Masjid Market
Ghum	Ghum Station Market
Sonada	Sonada Market
Tung	Tung Market
Kurseong	Hut Bazaar Sabji Market
	Super Market
Near Mirik Town	Mirik Town Market
	Mirik Lake Market
	Sourini Market
Sokiapokhri	Sokiapokhri Super Market
Kalimpong	Hut Bazaar Markets
	Raja Dorjee Markets

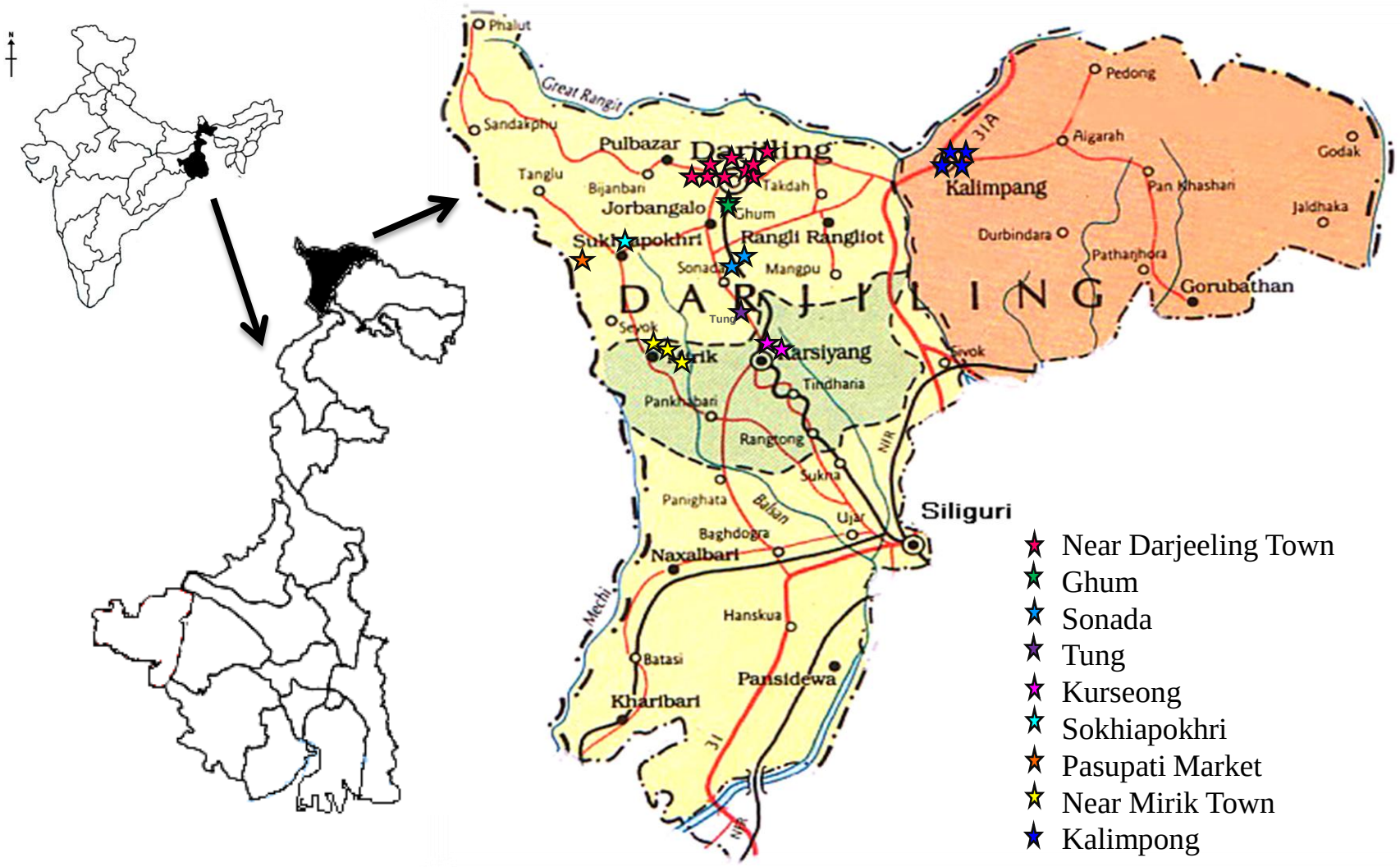


Figure 3.2 Location of fruits and vegetables markets in Darjeeling Himalaya

Figure 3.3 Fruits and vegetable markets of Darjeeling Himalaya

Figure 3.3 Fruits and vegetable markets of Darjeeling Himalaya (Cont..)

Figure 3.3 Fruits and vegetable markets of Darjeeling Himalaya (Cont..)

3.3 RESULTS AND DISCUSSIONS

For determining the structure of underexplored fruits and vegetable markets, 192 sellers were enquired on collection and cultivation process of these plants, their post harvest processing and seasonal availability and nutraceutical properties. Along with this, questions were designed to obtain information regarding the name of specimens sold at markets, the parts of the plants commercialized and the uses attributed to each of them. On that basis, the market based botanical knowledge about underexplored fruits and vegetables were evaluated as well as their dynamics of growth and yield potential and possible threats of conservation in relation to diffusion of the products. From survey reports it was realised that most of these types of fruits and vegetables were collected rampantly from wild habitat (almost 88%) (Figure 3.4) by local tribes who are also socially marginal. Our observations are in agreement with the opinion of other authors who stated that excessive collections of wild edible and medicinal plants of Darjeeling Hills have provided a great deal of vulnerability to individual species (Chhetri *et al*., 2005). Fortunately, now some of these plants are domesticated and few are also cultivated, which will reduce the dependence of wild source in future at least partially (Figure 3.4). From survey, it was also observed that sufficient care has not been taken during collection from wild. Almost 32% of wild collection of edible parts was performed by destroying branches, and 38% was gathered by cutting or felling the whole plants (Figure 3.5). These anthropogenic activities not only reduce the source of natural edible plants but also threaten the biodiversity to a great extent. When several authors have argued directly on rarity of economically important Himalayan plant specimens (Rai *et al*., 1998; Rai *et al*., 1999; Chhetri *et al*., 2005), appropriate research should be carried out to develop agro-techniques for the cultivation of these under-explored fruits and vegetables on priority basis.

Till now, a large variety of wild growing plants are used for food by the local communities of Darjeeling hills (Sundriyal and Sundriyal, 2003). As most of the markets are quite far from wild sources, these types of fruits and vegetables are transported by local poor farmers physically after taking lots of pain. However established vendors sometimes used trolleys

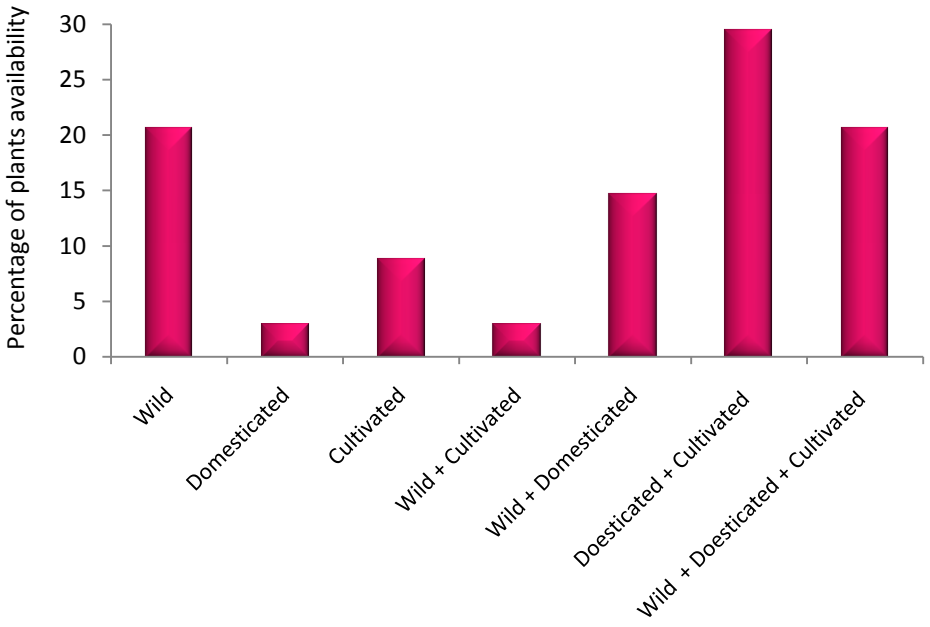


Figure 3.4 Source of collection of under-explored fruits and vegetables

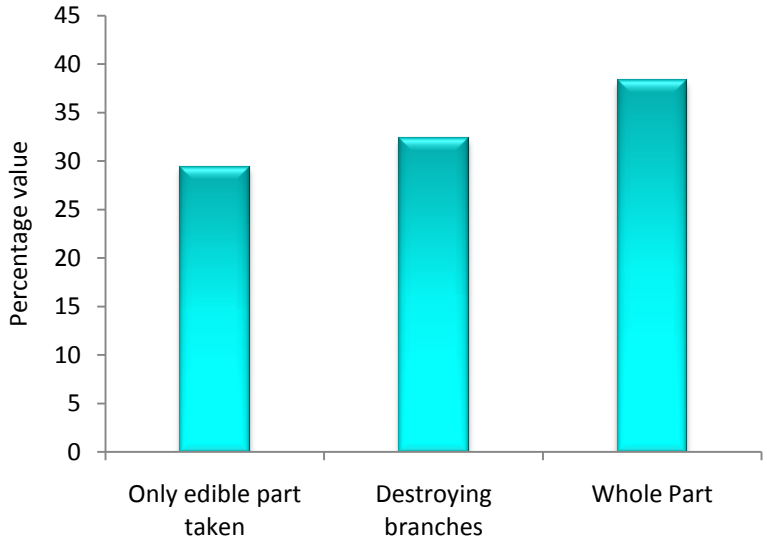


Figure 3.5 Process of collection of under-explored fruits and vegetables

for transport. Versatile groups of fruits and vegetables were mainly available in markets located at high altitude like Darjeeling town, Ghoom and Sonada. At lower altitudes like Mirik, Kalimpong or in plains of Darjeeling Himalaya, this product versatility was much reduced (Figure 3.6). Among cultivated specimens, edible leafy vegetables are collected in huge amount and their market demands are also very high, which bears stable dynamic relationship between production and consumption. But most of the prominently used fruit specimens like *Docynia indica, Eleaeocarpus lanceifolius, Evodia fraxinifolia, Prunus domestica, Pyrus communis, Rubus ellipticus* and *Spondias mombin* create maximum pressure in natural habitat due to lack of their cultivation techniques (Table 3.3 and Figure 3.4). Unfortunately in spite of high market demand, most of these fruits are available only in restricted markets in scanty amount as the wild sources are gradually depleting. In neighbouring Sikkim Himalaya, also the same problem was noticed by Sundriyal and Sundriyal (2003), where as many as 190 food plants were grown in wild habitat and were commercialized, but most of them are not yet cultivated or domesticated. Moreover, most of the fruits available in Darjeeling Himalaya were restricted in a particular season. Seasonal availability of different fruits and vegetables were shown in Figure 3.7. Some important delicious fruits like *D. indica, Machilus edulis, Persea americana, S. mombin* etc. were restricted only in some primary markets of Darjeeling hills due to their unavailability throughout the season. Valuable spices like *E. fraxinifolia, Zanthoxylum acanthopodium* and most of the underground vegetables were available only for 3-4 months in a year. So in spite of high market demands, valuable fruits, spices and vegetables were geographically restricted due to lack of appropriate cultivation techniques. The life style and economic status of marginal farmers of Darjeeling Hills might have been improved, if the production of valuable fruits and vegetables will be enhanced along with exploring their export potential which is now restricted only in Nepal and Bhutan (Table 3.3).

Table 3.3 Collection parameter of under-explored plants in markets of Darjeeling Himalaya

Plants name	Taste quality	Taste response	Availability in local market	Market demand for local people and foreigners		Export potential from local area	Cultivation techniques
				Local hilly people	Foreigners/Visitor		
Aconogonon molle	Sour	Moderate	Scanty	Moderate	Low	No idea	-
Apium graveolens	-	Good	Sufficiently high	High	Low	No idea	Organic
Brassica juncea	-	Good	Sufficiently high	High	Low	No idea	Organic
Brassica rapa	-	Good	Moderate	High	Low	Nepal	Organic
Calamus erectus	Spicy	Good	Sufficiently high	High	Moderate	Nepal/ Bhutan	-
Capsicum annuum	Chilly hot	Good	Sufficiently high	High	High	No idea	Organic
Cinnamomum glaucescens	Sour	Good	Scanty	Moderate	Low	No idea	-
Cyclanthera pedata	-	Moderate	Sufficiently high	High	Low	No idea	-
Cyphomandra betacea	Sour	Good	Sufficiently high	High	Low	No idea	-
Dioscorea alata	-	Good	Moderate	High	Low	No idea	Inorganic
Dioscorea hamiltonii	-	Good	Moderate	High	Low	No idea	Inorganic
Diploknema butyracea	Sweet	Good	Moderate	Moderate	Moderate	No idea	Organic
Docynia indica	Sour	Good	Scanty	Moderate	Moderate	No idea	-
Elaeocarpus lanceifolius	Sour	Moderate	Scanty	Moderate	Moderate	No idea	-
Evodia fraxinifolia	Spicy/Aromatic	Good	Scanty	High	Moderate	Nepal/ Bhutan	-
Heracleum wallichii	Spicy/Aromatic	Good	Sufficiently high	High	Moderate	No idea	-

Table 3.3 Collection parameter of under-explored plants in markets of Darjeeling Himalaya (Cont….)

Plants name	Taste quality	Taste response	Collection amount/availability in local market	Market demand for local people and foreigners		Export potential from local area	Cultivation techniques
				Local hilly people	Foreigners/Visitor		
Ipomoea batatas (Red)	Sweet	Good	Moderate	High	Moderate	No idea	-
Ipomoea batatas (White)	-	Good	Moderate	High	Low	No idea	-
Lepidium sativum	-	Moderate	Sufficiently high	High	Low	No idea	Organic
Litsea cubeba	Spicy/ Aromatic	Good	Sufficiently high	High	Moderate	Nepal	-
Machilus edulis	Buttery	Good	Moderate	High	Moderate	No idea	-
Manihot esculenta	-	Good	Sufficiently high	High	Low	No idea	Organic
Nasturtium officinale	-	Good	Sufficiently high	High	Low	No idea	Organic
Persea americana	Buttery	Good	Scanty	High	Moderate	No idea	-
Phytolacca acinosa	-	Good	Sufficiently high	High	Low	No idea	Inorganic
Prunus domestica	Sweet	Good	Scanty	Moderate	High	No idea	-
Pyrus communis	Sour	Good	Scanty	Moderate	High	No idea	-
Rubus ellipticus	Sour	Good	Scanty	Moderate	Low	No idea	-
Sechium edule	-	Good	Sufficiently high	High	Low	No idea	-
Solanum anguivi	Bitter	Good	Sufficiently high	High	Low	No idea	Organic
Solanum incanum	Bitter	Good	Moderate	High	Low	No idea	Organic
Spondias mombin	Sour	Good	Moderate	High	Low	No idea	Organic
Xanthosoma brasiliense	-	Moderate	Sufficiently high	High	Low	No idea	Organic
Zanthoxylum acanthopodium	Spicy/ Aromatic	Good	Moderate	High	Low	Nepal/ Bhutan	-

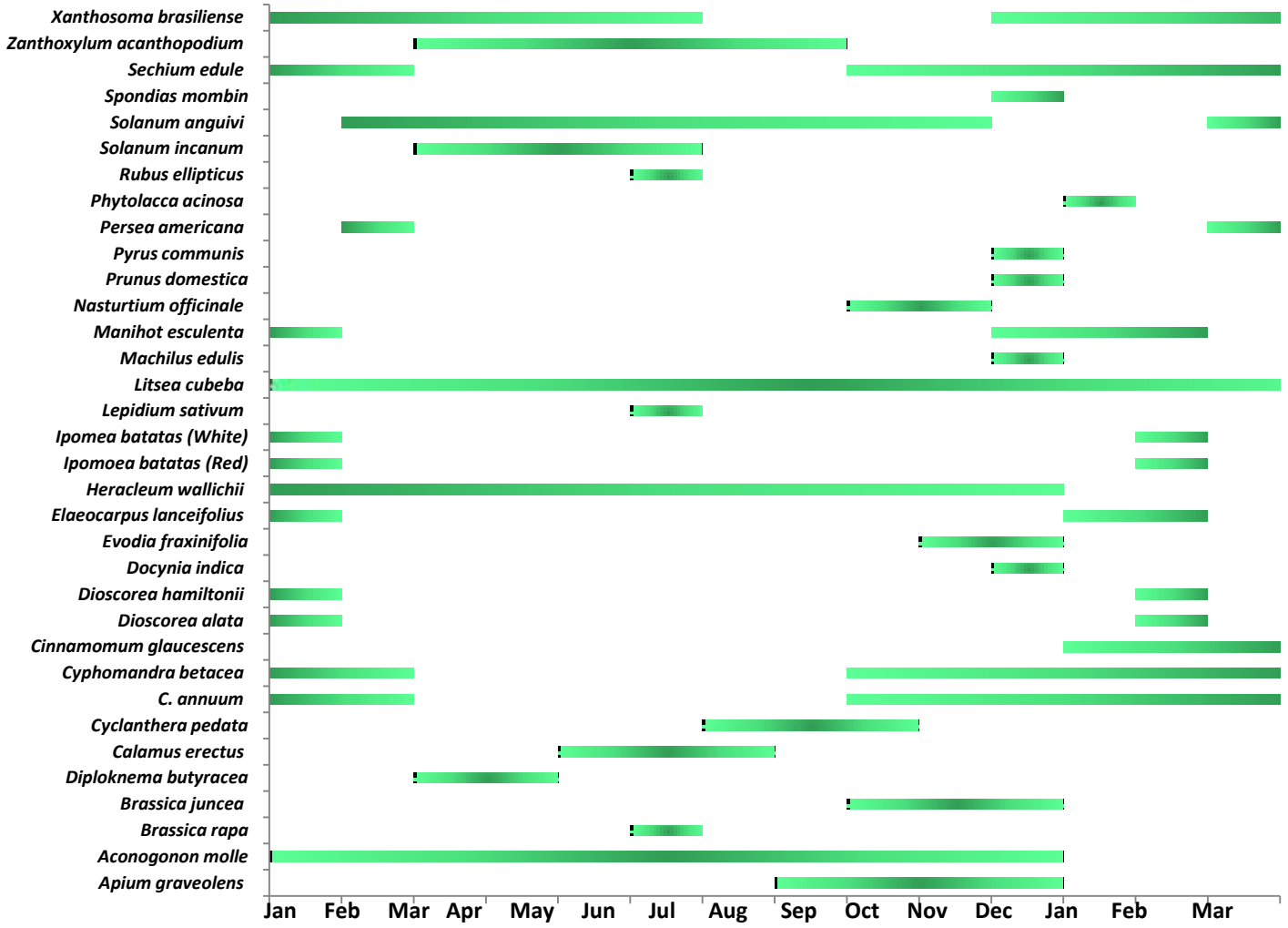


Figure 3.7 Seasonal availability of fruits and vegetables in markets

Among different edible plant parts, morphologically fruits are mostly consumed followed by underground rhizome/tubers and seeds (Figure 3.8). Underground rhizomes and tubers are popularly called 'Taruls' and are mostly available during winter season (Figure 3.7). The festival named 'Maghi Sankranti' was organized in the month of January particularly for the purpose of commercializing diversified edible underground species ('Taruls') grown in Darjeeling Hills. Considerable amount of leafy vegetables (11.76% among total edible plants) were also marketed (Figure 3.8). Taste response of most of these underexplored fruits and vegetables are more or less nice along with versatile taste quality which includes sour, sweet, spicy, aromatic, chilly hot, buttery or even having bitter principles (Table 3.3). Our survey data also focused on Himalayan plant species that are consumed as food and parallely for therapeutic purposes. During urban agglomeration, shops are the places chosen to commercialize dietary supplements, functional foods and nutraceuticals (Pochettino *et al*., 2012). The concept of functional foods is flexible to different interpretations that referred to their characteristics, their active components or their regulatory framework (Griffiths *et al*., 2009; Shahidi, 2009). Besides their conventional value as a source of nutrients, functional foods improve body function and reduce disease risk (Hardy, 2000). However common people of Darjeeling Hills are not properly aware of their specific components, even when these foods contain lots of nutraceuticals. From the view points of consumers, nutraceuticals are functional foods that help to recover from disorders or co-operate in its treatment; so their specific effects are recognized (Kalra, 2003). Awareness about nutraceutical properties among merchants and marketplace sellers indicated that in case of 41% edible plants they are unaware about the functional components while they stated that vitamin content was present in 9.4% followed by minerals in 23.52% of edible fruits and vegetables (Figure 3.9). But market place sellers were well aware about the ethno-medicinal properties of underexplored fruits and vegetables and regarding this high level of co-operation was received from their end during

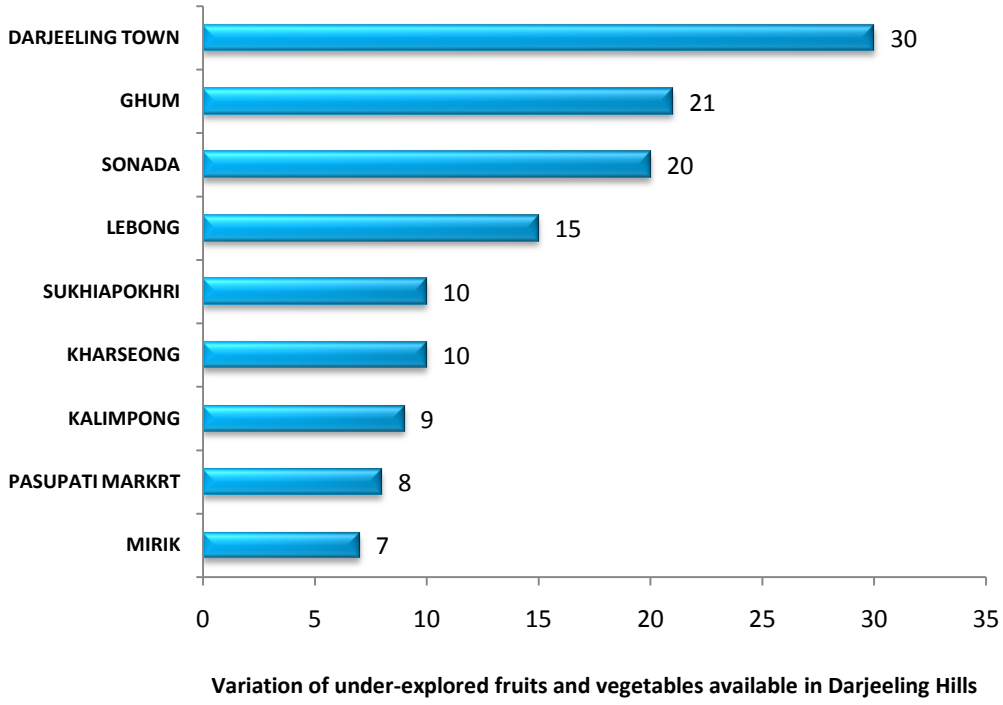


Figure 3.6 Versatility of products in different area of Darjeeling Hill

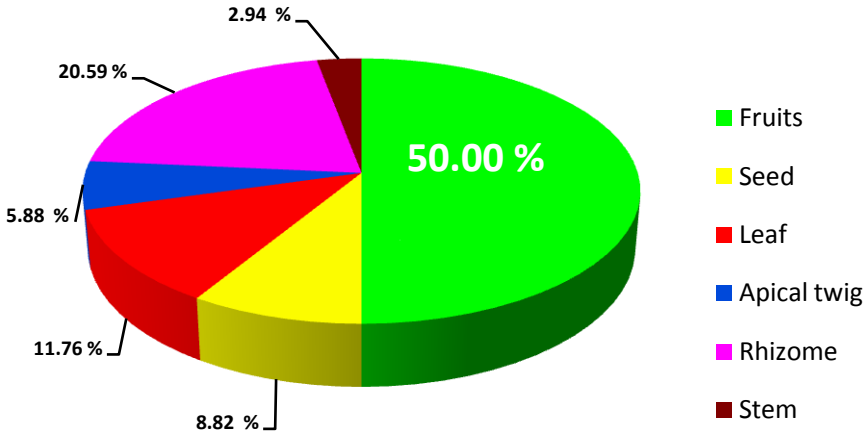


Figure 3.8 Edible parts of plants available in Darjeeling Himalaya

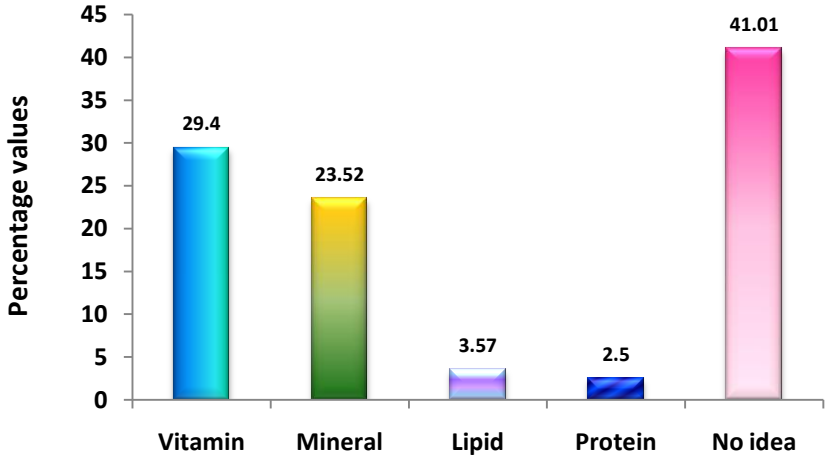


Figure 3.9 Awareness of local people of Darjeeling Himalaya about nutraceutical properties

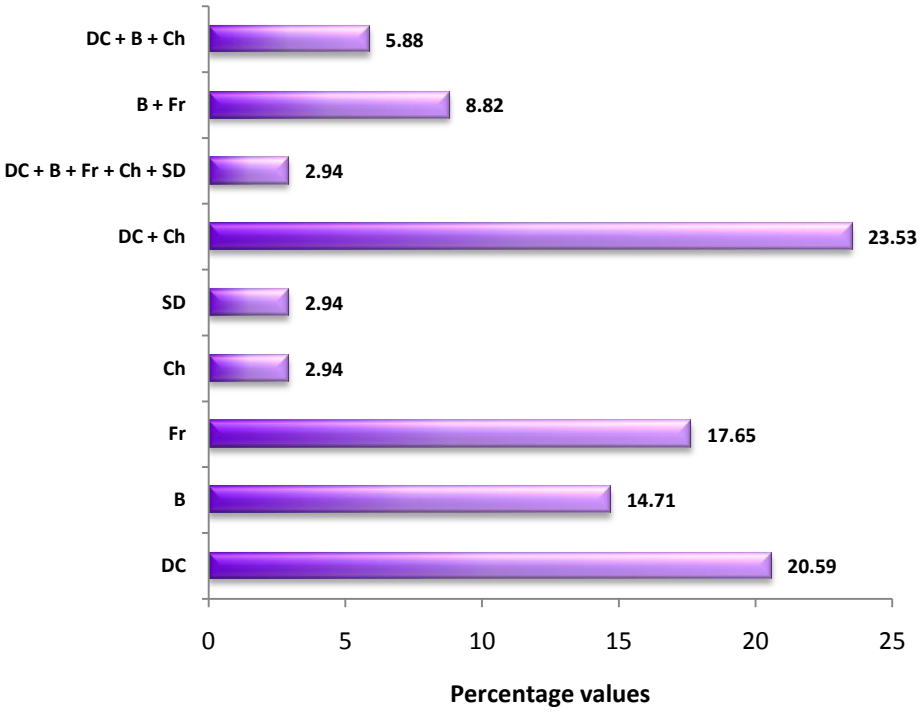


Figure 3.10 Post harvest of fruits and vegetables available in markets
Where, **DC**= Directly consumed, **B**= Boiled,
Fr= Frying, **Ch**= Chutney, **SD**= Sun Drying

our survey. Ethno-botanical data were obtained according to usual quantitative methods (Marttin, 1995), and it was recorded that these fresh or dry fruits were ethnomedically important for managing disorders like gastrositis and having important medicinal properties such as anthelmintic, hypertensive, anti-diarrheal, blood coagulation, antidiabetic and hepatoprotechtion (Table 3.3). Actually the uses of food with therapeutic end is not new, it has been the part of human knowledge since ancient time and it is still persisting in different ethnic races of Darjeeling Himalaya. Bantawa and Rai (2009) reported that in some places of Darjeeling hills the people are well aware about modern system of medicine but in rural villages, traditional medicine holds prime importance. In this sense traditional markets are relevant place to acquire functional food and nutraceuticals, and for these reasons they can satisfy the needs for regular consumers (Pochettino *et al*., 2012). Not only those market place sellers also have adequate knowledge about postharvest processing of fruits and vegetables available in markets. From Figure 3.10 it can be stated that vegetable and fruits may be directly consumed or processes through boiling, frying, sundrying etc. Boiling is the main traditional method for using these vegetables followed by frying (Figure 3.10). In some cases thermal processing may enrich the nutraceutical properties of cooked vegetables (Miglio *et al.*, 2008; Ranilla *et al.*, 2009; Kumar *et al.*, 2010) but controversial reports were obtained by different authors (Nicoli *et al*., 1999; Lee and Kader, 2000; Ou *et al.*, 2002; Bernhardt and Schlich, 2006). In this regard, the pattern of changes of antioxidants and bioactive phytochemicals during different thermal process performed by local people of Darjeeling Himalaya will be discussed in latter chapter.

Chapter – IV

EVALUATION OF ANTIOXIDANT ACTIVITY OF DIFFERENT EDIBLE FRUITS AND VEGETABLES OF DARJEELING HIMALAYA

4.1 INTRODUCTION

The term antioxidant means to demolish the oxidants which are formed through different metabolic activity in body. Oxidative process is one of the most vital routes for generating free radicals in foods, drugs and even living systems (Halliwell, 1978). The most effective path to scavenge and diminish the action of free radicals which cause oxidative stress is antioxidative defense mechanisms. Free radicals are the chemical species that are capable of existing with one or more unpaired surface shell electrons. They are very reactive, generally highly unstable and potentially damage chemical species (Martı´nez-Cayuela, 1995). The most common reactive oxygen species (ROS) are hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), peroxyl radicals ($ROO^{\cdot}$), nitric oxide ($NO^{\cdot}$), peroxynitrite anion ($ONOO^{\cdot}$) and highly reactive hydroxyl radical ($^{\cdot}OH$) (Tepe and Sokmen, 2007). They are produced continuously in all cells, as metabolic derivative by a number of intracellular systems like small cytoplasmic molecules, membrane enzymes, cytoplasmic proteins, peroxisomes, and mitochondrial electron transport systems (Martı´nez-Cayuela, 1995). Beside these the sources of free radicals are ionizing radiation, tobacco smoke, pollutants, pesticides, and some medications (Martı´nez-Cayuela, 1995). Over production of such free radicals might be able to cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), and ultimately which leads to a number of chronic diseases, such as atherosclerosis, ischemia-reperfusion injury, malaria, inflammatory joint disease, cardiovascular diseases, immune system decline, asthma, cataracts, diabetes, cancer, neurodegenerative diseases, aging, and other degenerative diseases in humans (Halliwell, 1994; Niki, 1997; Poulson *et al*., 1998 Florence, 1995; Nakagami *et al.*, 1972; Martı´nez-Cayuela, 1995; Schoneich, 1999; Young and Woodside, 2000). Lipid peroxidation is one of the major causes of foods spoilage that results in the formation of toxic compounds through prospective chain reaction. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), propylgallate (PG) etc. are widespread food additives used to protect

against oxidative stress; however, their use is restricted, due to their probable health risks and some serious side-effects. Moreover, there is an increasing awareness among consumers regarding food safety (Moure *et al*., 2001).

Plants (fruits, vegetables, medicinal herbs, etc.) as well as their downstream products may contain versatile group of free radical scavenging molecules like phenolic compounds (e.g. phenolic acids, flavonoids, coumarins, lignans, quinones, stilbenes, tannins), nitrogen compounds (alkaloids, betalains, amines), vitamins (ascorbic acid), terpenoids (including carotenoids), peptides (glutathione) and some other endogenous metabolites that are rich in antioxidant activity (Shahidi and Naczk, 1995; Cotelle *et al*., 1996; Velioglu *et al*., 1998; Zheng and Wang, 2001; Cai *et al*., 2003). Darjeeling Himalaya, with its enormous biodiversity, has a great potential for providing new nutraceutical components. Edible plants of this area belong to several botanical families, such as Apiaceae, Polygonacae, Brassicaceae, Sapotaceae, Arecaceae, Cucurbitaceae, Araceae, Solanaceae, Lauraceae, Dioscoreaceae, Rosaceae, Rutaceae, Elaeocarpaceae, Convolvulaceae, Phytolaccaceae, and Anacardiaceae (Table 3.2). In the flora of Darjeeling Himalaya, many edible plants are ethnomedicinally very important and have been used for years by the local people of this region to cure degenerative diseases, gastroenteritis, diabetes etc; but till now that has not revealed through appropriate scientific investigations. Through this study, an attempt was made to determine different free-radical scavenging potential of these under-explored fruits and vegetables for considering their therapeutic potential against versatile newly emerging degenerative and metabolic lifestyle disorders.

4.2 MATERIALS AND METHODS

4.2.1 Plant samples collection and identification

The procedure of collection and identification was specified in Chapter III Section 3.2.2.

4.2.2 Preparation of methanolic plant extracts

Different fresh fruits and vegetables were surgically separated and were separately crushed with mortar and pestle. Under a Soxhlet extractor, crushed fruits were individually extracted with methanol for 8h. The methanol was completely removed by vacuum rotary evaporator at 50°C. These crude extracts were freeze-dried. The powder was stored at 4°C and used for further investigation. The extractive value of the plant materials were calculated on dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

4.2.3 Animal material

Goat liver, used for anti-lipid peroxidation assay, were collected from slaughter house immediately after slay and experiment was conducted within one hour after collection.

4.2.4 Determination of DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined spectrophotometrically. The changes in color of DPPH free-radical (from deep-violet to light-yellow) were measured at 517 nm wavelength in presence of antioxidants. Radical scavenging activity of extracts was measured by standard method proposed by Blois (1958). Two micro-liters of each sample, prepared at various concentrations were added to 2 ml of 0.2 mM DPPH solution. The mixture was shaken and allowed to stand for 30 min at 20ºC in dark condition and then the absorbance was measured at 517 nm with UV-VIS spectrophotometer (Systronics, 2201). The percentage inhibition activity was calculated by the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample}) / A_{control} \times 100]$$

Where, $A_{control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{sample} is the absorbance of the remaining concentration of DPPH in the presence of methanol. IC_{50} values (mg/ml) were determined from a plotted graph of scavenging activity against the concentrations of the *C. betacea* fruit extracts, where IC_{50} is defined as the total amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

4.2.5 Determination of ABTS radical scavenging assay

The free radical-scavenging activity was determined by 2,2´ azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS radical cation decolorization assay described by Re *et al.* (1999). ABTS was dissolved in water to a 7 μM concentration. ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS stock solution with 2.45 μM potassium persulfate (final concentration) and kept in the dark at room temperature for 12–16h before use. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of infusion, the samples containing the $ABTS^{\cdot +}$ solution were diluted with redistilled water to an absorbance of 0.700 (±0.02) at 734 nm and equilibrated at 30°C. A reagent blank reading was taken. After addition of 3.0 ml of diluted $ABTS^{\cdot +}$ solution (734 nm) to 30 μl of plant extracts, the absorbance reading was taken exactly 6 min after initial mixing. The IC_{50} value was calculated by the same procedure mentioned above.

4.2.6 Determination of superoxide anions scavenging activity

The superoxide anions generated by phenazine methosulphate (PMS) and reduced nicotinamide-adanine dinucleotide phosphate (NADPH), were detected by the reaction with 2,2´-di-*p*-nitrophenyl-5,5´-diphenyl-(3,3´-dimethoxy-4,4´-diphenylene) di-tetrazolium chloride, nitro-blue tetrazolium (NBT) (Nishikimi *et al*., 1972). Reaction mixture contained 1 ml samples (different concentration), 1 ml of NBT solution (312 μM prepared in phosphate buffer, pH-7.4) and 1 ml of NADH solution (936 μM prepared in phosphate buffer, pH-7.4). Finally, the reaction was

accelerated by adding 100 µl PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixture was incubated at 25^{o} C for 5 min and absorbance at 560 nm was measured against methanol as control. Percentage inhibition and IC_{50} value was calculated using the same formula mentioned above.

4.2.7 Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined by using the 2-deoxyribose oxidation assay (Jung *et al*., 2008). A solution (0.2 ml) of 10 mM $FeSO_4$, $7H_2O$ and 10 mM ethylenediamine tetraacetic acid was prepared in a screw capped test tube. Then, 0.2 ml of 10 mM 2-deoxyribose solution, 0.5 ml of each sample (different concentration) and 0.1M sodium phosphate buffer (pH 7.4) were added to give a total volume of 1.8 ml. Finally, 200 µl of 10 mM H_2O_2 solution were added to this reaction mixture and incubated at 37º C for 120 min. After incubation, 1 ml each of 2.8% trichoroacetic acid and 1.0% thiobarbituric acid were added to the reaction mixture. The sample was boiled at 100º C for 10 min, cooled in ice, and then its absorbance was measured with a spectrophotometer at 515nm. The IC_{50} value was calculated by the same process mentioned above.

4.2.8 Determination of nitric oxide activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci *et al*., 1994). 320µl methanol extract, 360µl (5mM) sodium nitroprusside-PBS solution, 216µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride) was mixed and incubated at 25^{o}C for one hour. Lastly 2 ml water was added and absorbance was taken at 546 nm. The IC_{50} value was calculated by the same procedure mentioned above.

4.2.9 Metal chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al.* (1994) with slight modification. To 0.4 ml of methanol extract, 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30s, 0.8 ml ferrozine (5 mM) was added. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated using the same formula mentioned above.

4.2.10 Determination of reducing power

One milliliter of plant extract, 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 ml potassium ferricyanide (1% w/v) were incubated at 50º C for 20 minutes. The tube was cooled on ice and 2.5 ml 10% trichloroacetic acid was added. The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer of solution (2.5 ml) and mixed with distilled water (2.5 ml) and 0.25 ml of $FeCl_3$ (0.1% w/v). Finally, the absorbance was measured at 700 nm against blank sample (Aiyegoro and Okoh, 2009).

4.2.11 Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the extracts of plants was determined by the standard method followed by slight modification with the goat liver homogenate (Bauchet and Barrier, 1998). 2.8 ml of 10% goat liver homogenate, 0.1ml of 50 mM hydrated ferrous sulphate and 0.1 ml extract was mixed. This mixture was incubated for 30 minutes at 37^{o}C. 1 ml of reaction mixture was taken with 2 ml 10% trichloroacetic acid (TCA) -0.67% thiobarbituric acid (TBA) in acetic acid (50%) for blocking the reaction. Then the mixture was boiled for 1hour at 100^{o}C and centrifuged at 10,000 rpm for 5 min. Supernatant was taken for absorbance at 535 nm. ALP % was calculated by using the following formula:

$$\text{ALP percent} = \frac{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{abs. of sample}}{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{abs. of control}} \times 100.$$

4.2.12 Statistical analysis

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) for drawing the relation between different types of antioxidant attributes and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes of different edible plants of Darjeeling Himalaya. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p < 0.05$ was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals and antioxidant traits, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

4.3 RESULT AND DISCUSSIONS

Oxidative stress arises when there is an excessive formation of reactive oxygen species (ROS) or when antioxidant capacity decreases. The mitochondrial electron transport chain is mainly recognized as main cellular producers of ROS, where some electrons escape out of the chain and combine with molecular oxygen to form superoxide. The generation of ROS by mitochondria is supposed to be important in ageing process as well as in the pathogenesis of neurodegenerative diseases like Parkinson's disease (Liu *et al*., 2002). In support of the naturally occurring resistance mechanism against oxidative stress, plants are developing many antioxidants which are naturally preferred for their limited or no side effects as compared to their synthetic counterparts

(Kuo *et al*., 2005). Different types of plant parts of Darjeeling Himalaya have shown to be effective against various disorders (discussed in Chapter II), an information which has initiated interest for the study of their antioxidant, radical scavenging and iron chelating activities. According to Koleva *et al*. (2002) DPPH stable free radical method is an easy, rapid and sensitive way to assess the antioxidant activity of a specific compound or plant extract. Unlike other free radicals like hydroxyl radicals and superoxide anions, DPPH has the advantage of being impassive by certain supported reactions, such as metal ion chelating and enzyme inhibition (Katalinic *et al*., 2006; Jayasri *et al*., 2009). Another stable free-radical $ABTS^{\bullet+}$ is a blue chromophore generated by the reaction between ABTS and potassium per-sulfate which is reduced to ABTS in a concentration dependant manner upon addition of the methanolic extracts (Lissi *et al*., 1999). Perhaps the most dangerous of all the free radicals formed in the biological systems is hydroxyl radical that has the potency to damage the bio-molecules (Halliwell, 1991) enormously. According to Das *et al*., (2012), addition of plant extracts to the Fenton's reaction has revealed that they effectively scavenge the substantial hydroxyl radicals, which if executed in biological system, may block deoxyribose damage. Another potent ROS i.e. superoxide anion is leading to extensive damage of components and is also considered to be the cause of induction of lipid oxidation by singlet oxygen generation (Halliwell *et al*.,1978). Formation of highly reactive peroxynitrite ($ONOO^{-}$) in excess amounts, in inflamed tissues by the diffusion-limited reaction of $NO^{\bullet}$ with superoxide anion generated many pathological problems. Direct tissue toxicity as well as vascular collapse associated with septic shock may result from a sustained formation of the nitric oxide radical; moreover chronic expression of the radical gives many inflammatory conditions including juvenile diabetes, arthritis, multiple sclerosis and ulcerative colitis (Okuda *et al.*, 1983). The capability of iron chelating shows high perceptibility in living system as it decreases the concentration of transition metals in the catalytic processes like lipid peroxidation. Lipid peroxidation is mainly stimulated by iron during Fenton reaction and by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can radical elongate the chain reaction

(Kochevar and Redmond, 2000). Kochevar and Redmond (2000) also stated that the reducing capacity of a compound may be the indicator of its antioxidant potential. Lipid peroxidation is an oxidative modification of polyunsaturated fatty acids in cell membranes that produces a number of degradation products. The inhibition of $FeSO_4$-ascorbic acid induced TBARS formation in liver homogenate by the extracts indicated their lipid peroxidation inhibitory activities.

In Table 4.1, lists the 34 species belonging to 16 families were examined for inhibitory effects of DPPH radical, $ABTS^{\cdot +}$, superoxide radical, nitric oxide, hydroxyl radical, lipid peroxidation, metal chelating and reducing power capacity. The table also encompasses different fruits, herbs, shrubs and trees that are used as vegetables by the local people of Darjeeling. Among sixteen plant families tested in this study, except Araceae all species exhibited high levels of antioxidant activity (Figure 4.1a-4.1h). *Apium graveolens, Brassica juncea, B. rapa*, *Lepidium sativum, Nasturtium officinale* and *Phytolacca acinosa* are the six leafy vegetables predominantly used by the people of Darjeeling Himalaya. Leaves show higher scavenging potential than petiole (Table 4.1). *A. graveolens, B. juncea* and *N. officinale* showed the high DPPH scavenging capacity than other leafy vegetables. Aydemir and Becerik (2009) stated that *A. graveolens* contain more free radical scavenging capacity than *L. sativum.* Our experiments also established the same fact. The levels of antioxidant activity of different leafy vegetables were as follows: *N. officinale* > *A. graveolens* > *B. juncea* > *P. acinosa* > *B. rapa* > *L. sativum* (Table 3.1).

Along with leafy vegetables, fruits, spices, underground plant parts and other vegetables of Darjeeling hills exhibited significant role in scavenging free radicals (Table 3.1). Chimping (*Heracleum wallichii*), Sil timbur (*Litsea cubeba*), Boke timbur (*Zanthoxylum acanthopodium*) and Khanppa (*Evodia fraxinifolia*) are the wild trees and the fruits of these plants are commonly used as spices in Nepali food recipes. Many authors reported that, due to the presence of flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins, various spices like *Ocimum sanctum, Piper cubeba, Allium sativum, Terminalia bellerica, Camellia sinensis, Zingiber officinale* exhibited antioxidant activity (Khalaf *et al*., 2005; Aqil *et al*., 2006). In our

Table 4.1 Antioxidant properties of fruits and vegetables of Darjeeling Himalaya

SL NO.	SCIENTIFIC NAME	LOCAL NAME	PLANT PARTS	ASSAYS OF ANTIOXIDANT CAPACITY							
				DPPH	ABTS	SO	NO	OH	MC	ALP	RP
				mg/ml (FWT)							mg AAE /g (FWT)
1	*Aconogonon molle*	Thotne	Leaf	0.63±0.01^{abc}	0.06±0.001^{b}	83.87±0.41^{k}	17.5±0.013^{cd}	3.36±0.05^{bc}	40.02±0.15^{x}	10.32±0.56^{j}	0.10±0.01^{vexy}
			Stem	2.32±0.02^{abcd}	0.35±0.002^{g}	311.00±4.62^{C}	129.31±1.32^{l}	12.23±0.07^{de}	2.81±0.05^{e}	5.46±0.45^{g}	0.16±0.09^{uvw}
			Inflorescence	1.06±0.01^{abc}	0.12±0.004^{c}	40.02±1.23^{e}	17.5±0.09^{cd}	5.57±0.05^{c}	2.81±0.03^{e}	3.62±0.06^{ef}	0.06±0.001^{yz}
2	*Apium graveolens*	Celery	Leaf	5.49±0.09^{efg}	0.56±0.009^{j}	28.06±0.25^{d}	N/A	28.88±0.12^{h}	4.26±0.05^{f}	21.62±0.3^{p}	0.26±0.01^{rs}
			Petiole	66.69±0.13^{o}	6.23±0.02^{x}	N/A	N/A	350.12±5.14^{w}	5.38±0.01^{gh}	41.21±0.45^{u}	1.93±0.01^{g}
3	*Brassica juncea*	Rai Sak	Leaf	7.3±0.11^{gh}	0.73±0.009^{l}	239.38±5.32^{w}	143.61±1.35^{n}	39.38±0.10^{k}	20.15±0.02^{r}	12.16±0.12^{k}	0.16±0.009^{uv}
			Petiole	N/A	N/A	N/A	NA	N/A	N/A	12.45±0.21^{k}	0.29±0.01^{qr}
4	*Brassica rapa*	Chinese sak	Leaf	11.09±0.09^{i}	1.13±0.01^{o}	250.03±2.01^{x}	277.50±5.21^{u}	58.22±0.64^{m}	0.38±0.04^{a}	42.95±0.65^{v}	0.39±0.02^{nop}
			Petiole	104.86±2.21^{q}	10.32±0.09^{C}	208.85±2.21^{t}	287.98±3.29^{v}	N/A	13.37±0.6^{m}	15.95±0.21^{l}	2.08±0.01^{f}
5	*Calamus erectus*	Bedgera	Epicarp	2.87±0.01^{bcd}	0.26±0.01^{ef}	22.44±1.38^{c}	148.96±1.31^{o}	15.07±0.09^{ef}	19.02±0.15^{q}	69.42±0.91^{x}	0.09±0.01^{vxy}
			Mesocarp	0.12±0.001^{a}	0.02±0.002^{ab}	39.25±0.92^{e}	94.71±1.85^{j}	0.63±0.01^{a}	15.43±0.32^{n}	53.12±0.45^{w}	0.02±0.001^{z}
			Endocarp	0.10±0.02^{a}	0.01±0.001^{a}	23.66±0.94^{c}	31.30±0.32^{e}	0.53±0.08^{a}	16.80±0.42^{o}	32.26±0.39^{t}	0.01±0.001^{z}
6	*Capsicum annuum*	Dollo Khorshani	Whole fruit	17.05±0.12^{j}	2.12±0.01^{u}	93.61±1.32^{l}	N/A	89.51±0.41^{r}	5.06±0.56^{fg}	10.39±0.42^{j}	0.51±0.04^{lm}
7	*Cinnamomum glaucescens*	Mallagiri	Fruit without seed	3.21±0.03^{cde}	0.40±0.001^{h}	N/A	195.50±2.37^{s}	16.56±1.49^{f}	N/A	1.21±0.25^{abc}	0.10±0.01^{vwxy}
8	*Cyclanthera pedata*	Chuche korola	Epicarp & mesocarp	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
			Endocarp	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

Table 4.1 Antioxidant properties of fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	LOCAL NAME	PLANT PARTS	ASSAYS OF ANTIOXIDANT CAPACITY							
				DPPH	ABTS	SO	NO	OH	MC	ALP	RP
				mg/ml (FWT)							mg AAE /g (FWT)
9	*Cyphomandra betacea*	Tree tomato	Epicarp	206.62±4.86^{t}	0.53±0.02^{ij}	10.91±0.9^{ab}	32.32±2.63^{e}	682.88±6.6^{B}	5.98±0.82^{hi}	101.53±1.69^{y}	0.50±0.03^{lm}
			Mesocarp	378.98±11.93^{v}	1.63±0.01^{s}	11.98±0.82^{b}	9.65±1.31^{b}	534.45±1.3^{A}	29.98±0.22^{v}	19.5±0.3^{mn}	0.59±0.1^{jk}
			Endocarp	167.59±3.93^{s}	0.86±0.005^{m}	7.82±0.08^{a}	15.28±2.11^{c}	388.18±2.1^{y}	10.21±0.30^{k}	151.15±1.61^{z}	0.41±0.021^{no}
			placenta	139.28±8.26^{r}	0.98±0.003^{n}	8.55±0.8^{ab}	18.86±1.30^{d}	295.51±4.3^{v}	8.43±0.90^{j}	168.22±2.3^{A}	0.49±0.021^{lm}
			Seed	284.39±9.78^{u}	1.95±0.02^{t}	9.23±0.08^{ab}	17.5±1.11^{cd}	456.56±3.2^{z}	16.83±0.5^{o}	177.08±2.4^{B}	0.36±0.02^{op}
10	*Dioscorea alata*	Ghar tarul	Underground part	16.90±0.09^{j}	1.51±0.01^{r}	101.42±1.21^{m}	1.43±0.85^{a}	84.53±0.18^{q}	2.01±0.05^{de}	7.62±0.73^{h}	1.09±0.01^{i}
11	*Dioscorea hamiltonii*	Ban tarul	Underground part	104.24±1.21^{q}	8.49±0.09^{B}	N/A	3.73±0.76^{a}	N/A	0.81±0.01^{abc}	9.25±0.65^{i}	2.26±0.02^{e}
12	*Diploknema butyracea*	Tiuri	Fruit without seed	1.94±0.02^{abcd}	0.13±0.005^{c}	145.47±1.12^{p}	391.40±3.25^{y}	10.19±0.16^{d}	8.29±0.56^{j}	2.12±0.01^{cd}	0.24±0.01^{rst}
13	*Docynia indica*	Mel	Epicarp	2.87±0.03^{bcd}	0.24±0.002^{de}	213.59±3.21^{uv}	188.96±1.21^{q}	34.22±0.35^{j}	18.02±0.14^{p}	1.32±0.07^{bc}	0.35±0.04^{opq}
			Mesocarp	1.12±0.01^{abc}	0.12±0.001^{c}	84.35±1.65^{k}	104.71±1.29^{k}	5.88±0.09^{c}	13.43±0.02^{m}	0.93±0.09^{ab}	0.42±0.02^{no}
			Endocarp	1.10±0.01^{abc}	0.20±0.001^{d}	76.16±0.95^{j}	81.30±1.21^{i}	5.78±0.34^{c}	11.80±0.02^{l}	0.83±0.01^{ab}	0.42±0.01^{no}
14	*Elaeocarpus lanceifolius*	Bhodrasi	Fruit without seed	0.26±0.003^{ab}	0.03±0.001^{ab}	60.71±1.32^{h}	279.76±3.12^{u}	1.367±0.05^{ab}	10.49±0.04^{k}	0.12±0.01^{a}	0.37±0.01^{op}
15	*Evodia fraxinifolia*	Khanapa	Whole fruit	2.40±0.02^{abcd}	0.28±0.02^{ef}	66.28±1.21^{i}	315.96±3.28^{x}	12.6±0.95^{de}	0.71±0.01^{ab}	0.31±0.02^{ab}	0.10±0.02^{vwxy}
16	*Heracleum wallichii*	Chimping	Seed	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

Table 4.1 Antioxidant properties of fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	LOCAL NAME	PLANT PARTS	ASSAYS OF ANTIOXIDANT CAPACITY							
				DPPH	ABTS	SO	NO	OH	MC	ALP	RP
				mg/ml (FWT)							mg AAE /g (FWT)
17	*Ipomoea batatas* (Red)	Lal sakar kanda	Underground part	37.02±0.3l^{l}	3.01±0.04^{v}	277.65±3.32^{z}	2.91±0.75^{a}	350.63±3.16^{w}	1.72±0.04^{cd}	3.62±0.05^{ef}	1.10±0.21^{i}
18	*Ipomoea batatas* (White)	Sheto sakar kanda	Underground part	28.93±0.096^{k}	2.10±0.05^{u}	210.39±2.32^{tu}	2.34±0.26^{a}	128.96±1.75^{u}	0.83±0.01^{abc}	2.82±0.02^{de}	4.23±0.12^{b}
19	*Lepidium sativum*	Chamsur	Leaf	11.14±0.13^{i}	1.39±0.02^{q}	277.47±3.21^{z}	N/A	58.49±0.95^{m}	25.10±0.2^{t}	13.21±0.75^{k}	0.15±0.01^{uvwx}
			Stem	N/A	N/A	208.66±2.96^{t}	N/A	N/A	28.18±0.96^{u}	24.23±0.24^{q}	0.39±0.12^{nop}
			Root	N/A	N/A	286.30±3.21^{A}	N/A	N/A	120.21±1.21^{A}	25.12±0.62^{q}	4.27±0.03^{b}
20	*Litsea cubeba*	Sil timbur	Whole fruit	2.87±0.01^{bcd}	0.24±0.002^{de}	44.73±0.12^{f}	142.07±1.65^{n}	15.07±0.63^{ef}	0.62±0.01^{ab}	2.16±0.04^{cd}	0.20±0.01^{stu}
21	*Machilus edulis*	Lopche kawlo	Epicarp	0.09±0.001^{a}	0.01±0.0003^{a}	118.84±1.21^{o}	74.21±0.21^{h}	0.47±0.29^{a}	8.65±0.02^{j}	0.9±0.06^{ab}	0.01±0.001^{z}
			Mesocarp	12.80±0.01^{i}	1.29±0.002^{p}	415.73±4.21^{F}	105.37±1.21^{k}	67.23±0.79^{o}	5.78±0.95^{ghi}	1.32±0.05^{bc}	1.59±0.01^{h}
22	*Manihot esculenta*	Simal tarul	Underground part	48.60±0.32^{m}	6.99±0.08^{z}	364.53±3.21^{E}	53.25±0.92^{g}	103.27±1.38^{t}	1.58±0.45^{bcd}	43.21±0.75^{v}	3.59±0.02^{c}
23	*Nasturtium officinale*	Simrio	Leaf	12.73±0.03^{i}	1.26±0.02^{p}	360.19±5.23^{D}	206.73±2.18^{t}	66.83±0.89^{o}	37.76±0.62^{w}	19.21±0.25^{m}	0.18±0.01^{tu}
			Immature stem	3.85±0.01^{def}	0.37±0.005^{gh}	N/A	N/A	20.21±0.31^{g}	41.31±0.52^{y}	3.52±0.43^{ef}	0.40±0.01^{nop}
			Mature stem	N/A	N/A	275.40±3.28^{z}	N/A	N/A	194.97±2.25^{B}	16.21±0.05^{l}	0.45±0.01^{mn}
24	*Persea americana*	Ghiu kawlo	Epicarp	68.74±0.1^{o}	6.95±0.04^{y}	267.14±4.29^{y}	434.39±2.95^{A}	358.05±4.21^{x}	12.81±0.02^{m}	16.85±0.75^{l}	0.54±0.02^{kl}
			Mesocarp	1.15±0.01^{abc}	0.12±0.002^{c}	172.90±2.52^{r}	297.08±1.56^{w}	6.04±0.15^{c}	0.64±0.01^{ab}	1.32±0.09^{bc}	0.06±0.001^{yz}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

Table 4.1 Antioxidant properties of fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	LOCAL NAME	PLANT PARTS	ASSAYS OF ANTIOXIDANT CAPACITY							
				DPPH	ABTS	SO	NO	OH	MC	ALP	RP
				mg/ml (FWT)							mg AAE /g (FWT)
25	*Phytolacca acinosa*	Jiango	Leaf	12.86±0.11^{i}	0.12±0.001^{c}	305.88±4.32^{B}	159.00±1.34^{p}	67.52±0.97^{o}	0.71±0.01^{ab}	25.21±0.52^{q}	0.33±0.008^{pq}
26	*Prunus domestica*	Arucha	Fruit without seed	6.11±0.03^{fgh}	0.64±0.004^{k}	N/A	N/A	32.08±0.24^{ij}	N/A	21.21±0.24^{op}	0.18±0.01^{tu}
27	*Pyrus communis*	Naspati	Mesocarp	5.83±0.04^{fg}	0.51±0.004^{i}	57.05±0.27^{g}	N/A	30.6±0.17^{hi}	N/A	12.36±0.49^{k}	0.20±0.01^{stu}
			Endocarp	61.46±0.09^{n}	6.23±0.05^{x}	N/A	N/A	61.35±1.92^{n}	17.71±0.42^{p}	26.27±0.73^{r}	0.22±0.06^{stu}
28	*Rubus ellipticus*	Oisilo	Whole fruit	1.94±0.006^{abcd}	0.96±0.009^{n}	114.11±1.82^{n}	49.54±1.94^{f}	10.19±0.85^{d}	0.92±0.04^{abc}	1.39±0.08^{bc}	0.05±0.007^{yz}
29	*Sechium edule*	Squash jara	Underground part	101.03±2.2^{p}	3.65±0.03^{w}	N/A	399.98±2.71^{z}	62.44±1.85^{n}	0.79±0.01^{abc}	20.32±0.52^{no}	3.05±0.04^{d}
30	*Solanum anguivi*	Bihi	Whole fruit	13.38±0.09^{i}	1.38±0.02^{q}	100.62±1.86^{m}	629.26±6.21^{B}	70.15±1.25^{p}	22.68±1.05^{s}	6.32±0.45^{g}	0.62±0.01^{j}
31	*Solanum incanum*	Bara Bihi	Epicarp & mesocarp	18.07±0.09^{j}	1.93±0.04^{t}	158.69±2.16^{q}	N/A	94.87±1.03^{s}	23.47±1.08^{s}	4.29±0.92^{f}	0.58±0.04^{jk}
			Endocarp	8.37±0.03^{h}	0.84±0.007^{m}	213.92±2.31^{v}	N/A	43.94±0.94^{l}	19.01±0.85^{q}	3.25±0.87^{ef}	0.64±0.01^{j}
32	*Spondias mombin*	Lopsi	Fruit without seed	1.09±0.05^{abc}	0.14±0.001^{c}	81.75±1.85^{k}	139.47±1.76^{m}	5.72±0.61^{c}	91.17±1.16^{z}	1.08±0.07^{abc}	0.04±0.001^{yz}
33	*Xanthosoma brasiliense*	Pindalu	Underground part	60.70±1.43^{n}	7.76±0.02^{A}	N/A	191.65±1.13^{r}	128.760±3.16^{u}	1.59±0.02^{bcd}	29.31±0.68^{s}	5.00±0.06^{a}
34	*Zanthoxylum acanthopodium*	Boke timbur	Whole fruit	2.87±0.01^{bcd}	0.30±0.001^{f}	203.45±9.21^{s}	10.72±1.92^{b}	15.07±0.21^{ef}	6.56±0.04^{i}	10.95±0.05^{j}	0.24±0.01^{rst}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

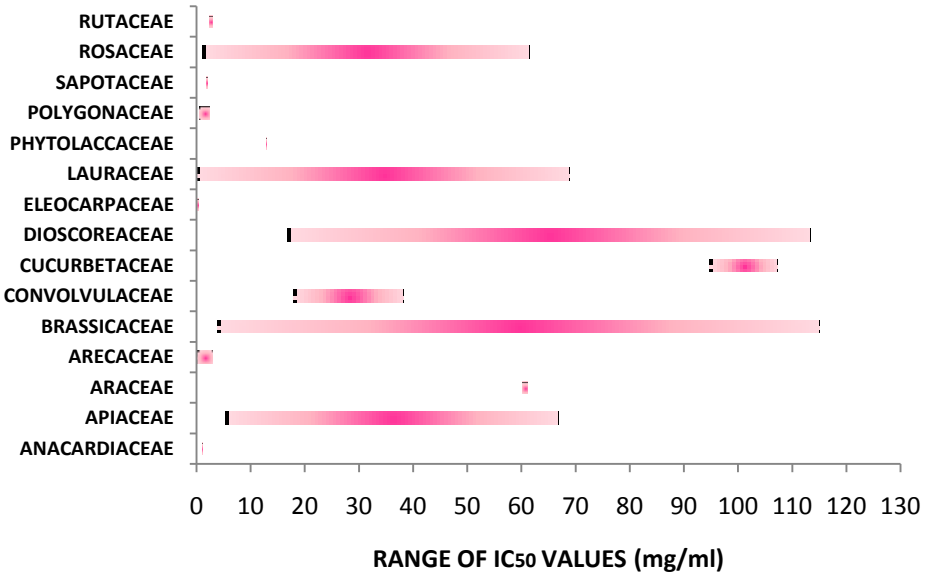


Figure 4.1a Range of DPPH scavenging activity of different family available in Darjeeling Himalaya

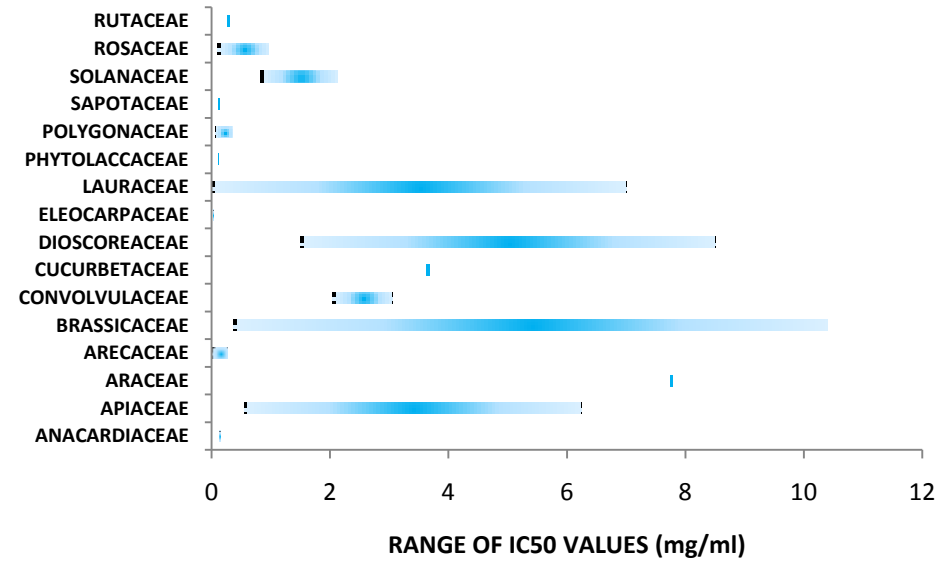


Figure 4.1b Range of ABTS scavenging activity of different family available in Darjeeling Himalaya

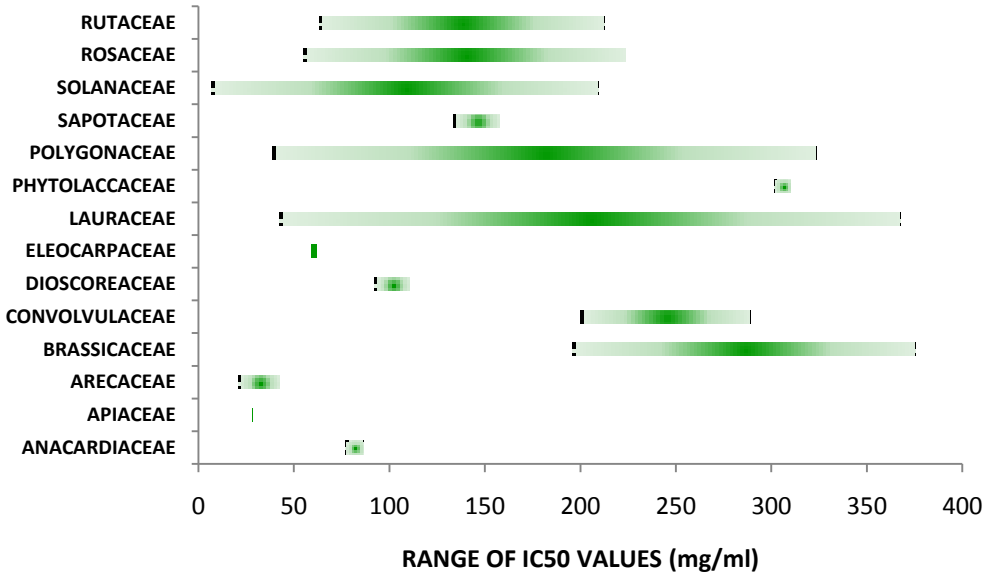


Figure 4.1c Range of superoxide radical scavenging activity of different family available in Darjeeling Himalaya

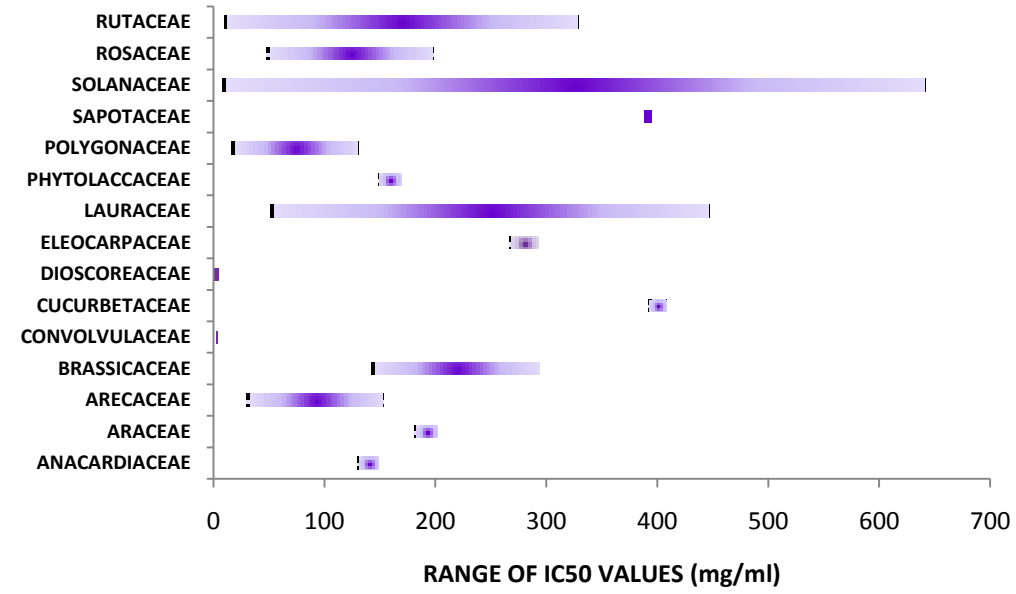


Figure 4.1d Range of nitric oxide scavenging activity of different family available in Darjeeling Himalaya

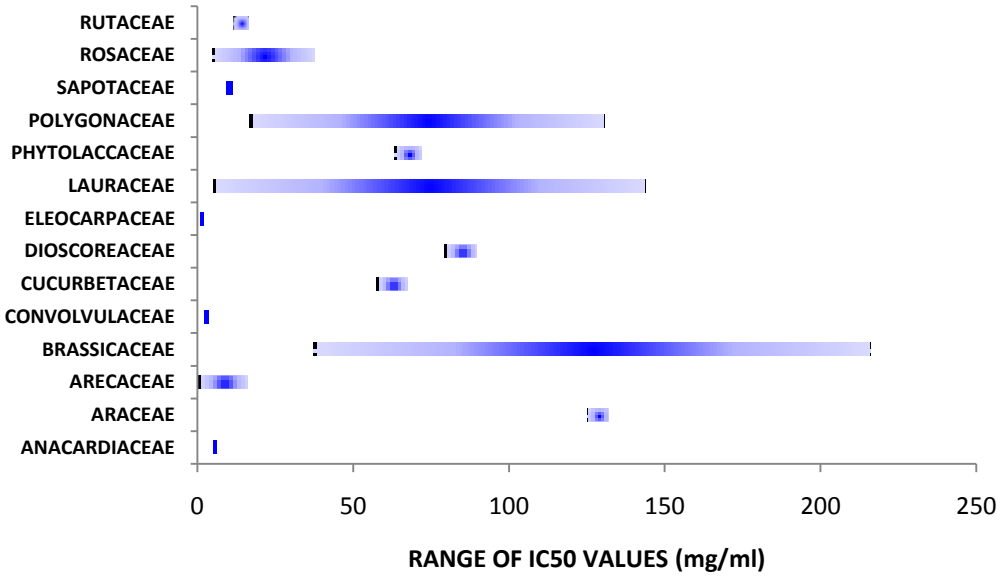


Figure 4.1e Range of hydroxyl radical scavenging activity of different family available in Darjeeling Himalaya

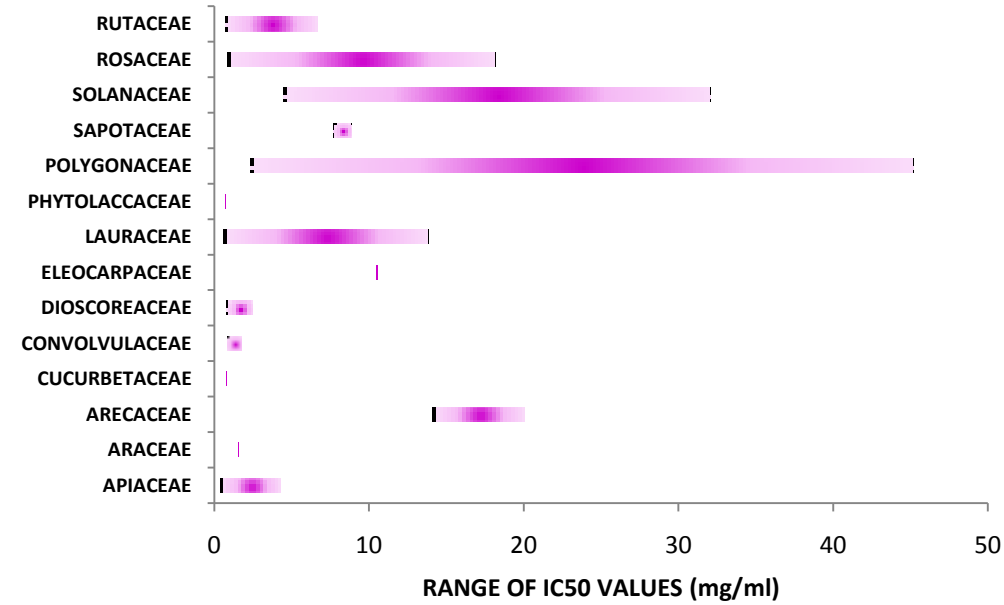


Figure 4.1f Range of metal chelating activity of different family available in Darjeeling Himalaya

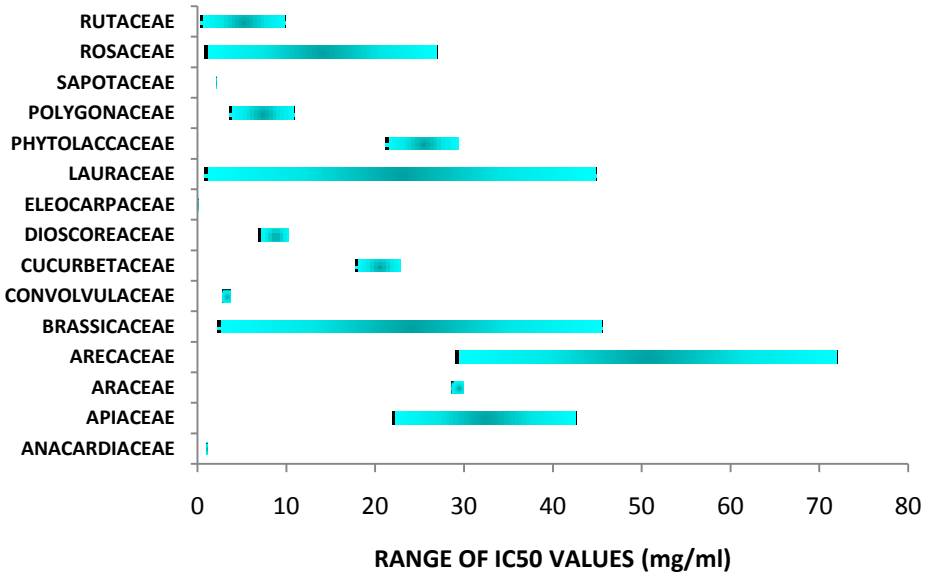


Figure 4.1g Range of anti-lipid peroxidation activity of different family available in Darjeeling Himalaya

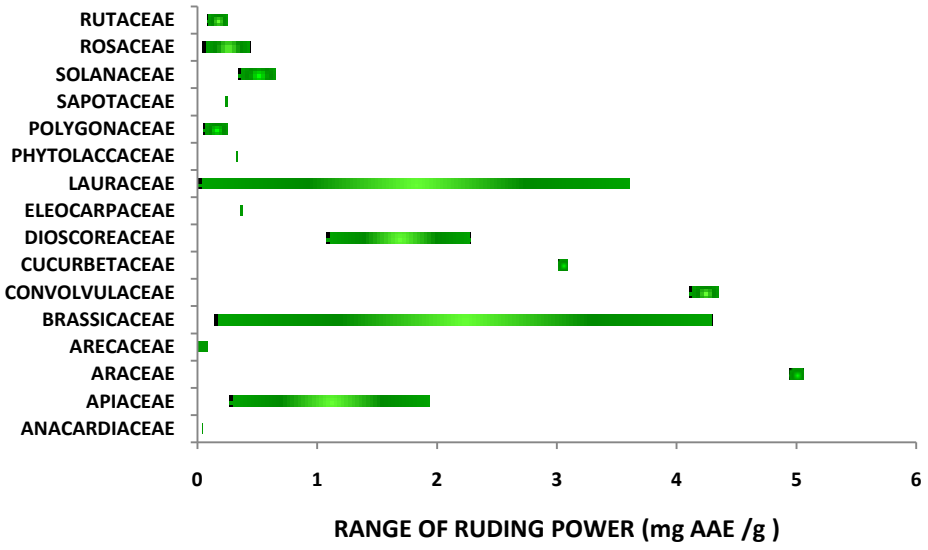


Figure 4.1h Range of reducing power of different family available in Darjeeling Himalaya

experiments except chimping, all other spices showed potent inhibition of free radical scavenging activity and among these, *E. fraxinifolia* was considered to be most potent (Table 4.1).

It is well known that the nutraceutical quality of a fruit tissue is associated with function of carbohydrate metabolism, pigment, color, flavour and antioxidative capacity. These antioxidants provide chemical protection for biological systems against harmful effects of reaction or processes that cause excessive oxidation, DNA and protein damage and cell death (Papas, 1999, Arnao *et al*., 2001). During our initial literature survey, we got only few references related with antioxidant activity of fruits available predominantly in Darjeeling Himalaya like *Brassica juncea* (Singh and Malik, 2011), *Capsicum annuum* (Ganiyu and Batista, 2007), *Cyphomandra betacea* Vasco *et al*. (2009), *Dioscorea alata* (Das *et al*., 2012), *Docynia indica* (Khomdram and Devi, 2010), *Machilus edulis* (Prakash *et al*., 2012), *Persea americana* (Vinha *et al*., 2013), *Prunus domestica* (Kayano *et al*., 2002), *Pyrus communis* (Kaur and Arya, 2012), and *Rubus ellipticus* (Vadivelan *et al*., 2009). Through our study, antioxidant activities of different edible parts of these fruits were established but their scavenging potential against different free radical species was versatile (Table 4.1). Endocarp showed higher activity than other part of fruits like mesocarp, epicarp, placenta and seed. Table 4.1 also demonstrated that the intensity of free-radical scavenging capacity of different parts of fruit were as follows: endocarp > (endocarp + mesocarp + epicarp) > mesocarp > placenta > seed.

Similar to fruits, underground parts of the plant are very important factor for restoration of nutritional property of plant (Acipa *et al*., 2013). Different types of underground plant parts which are commonly known as 'Taruls', are available in the markets of Darjeeling Himalaya at the time of January. Several authors proved that tubers and rhizomes have the potency to prevent various types of metabolic disorders and they can act as anti-diabetic (Undie and Akubue, 1986; Iwu *et al*., 1990), anti-neoplastic (Hu *et al*., 1996; Hu and Yao, 2002; Yu *et al*., 2004), hypocholesterolaemic (Ma *et al*., 2002; Yin *et al*., 2004), anti-osteoporotic, antimicrobial (Atindehou *et al*., 2002), anti-hepatonephrotoxic against acetaminophen drug (Lee *et al*., 2002),

anti-obese (Kwon *et al*., 2003) and immune cell stimulating activity (Choi *et al*., 2004). In this study, an attempt was made to evaluate methanolic extracts of seven underground tubers and rhizomes of edible plants of Darjeeling Himalaya for their antioxidant potential, radical scavenging and iron chelating activity. Among them, *Dioscorea alata* executed significant scavenging capacity than others (Table 4.1). In 2001 Afoakwa and Sefa-Dedeh stated that usually one small piece of *D. alata* tuber is equivalent to several kilogram of ordinary vegetables belonging to other plant families, and when this tuber is consumed, may be effective in preventing metabolic disorders in a comparatively shorter period of time. It has been noticed in our experiment, that red variety of *Ipomoea batatas* and *Manihot esculenta* which are the two popular underground edible plants of Darjeeling Hills exhibited high amount of antioxidant activity (Table 4.1).

The results achieved with Pearson's correlation have shown that the IC_{50} values of DPPH and $ABTS^{+}$ radicals of different fruits and vegetables of Darjeeling Himalaya are closely associated, indicating that the mechanism of action of these two radical scavenging activity are similar. These two radicals again allow high cohesiveness with hydroxyl radical scavenging property. $ABTS^{+}$, on the other hand, is related with superoxide and nitric oxide scavenging activity. Conversely, metal chelating activity exhibits strong correlation with almost all other scavenging species, acting specifically in hydrophilic system. This is in contrast with the opinion of other authors like Wong *et al*., (2006) and Zhao *et al*., (2008) who reported the negative correlation between DPPH free-radical scavenging activity, ferric ion reducing ability and cupric ion chelating properties. Our results obtained from correlation matrix (Table 4.2) suggested that antioxidant activity of edible fruits and vegetables determined by several methods might have been integrated.

To understand more precise relation between variables and clustering group, PCA was applied with antioxidant attributes (Figure 4.2). PCA on these attributes explained 61.06% of the variability in the data for the first two dimensions. The first two principle components accounted

Table 4.2 Correlation matrix of different antioxidant activity

	DPPH	**ABTS**	**SO**	**NO**	**OH**	**MC**	**ALP**
ABTS	**0.752****						
SO	0.061	**0.425****					
NO	0.219	**0.399****	**0.315***				
OH	**0.864****	**0.803****	0.144	0.165			
MC	**0.347***	**0.343***	**0.280***	**0.440****	0.244		
ALP	**0.481****	0.162	-0.150	-0.107	**0.401****	0.076	
RP	0.190	**0.435****	**0.398****	-0.074	**0.275***	-0.123	-0.050

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.3 Explained variability of the first five principal components (PCs) on the basis of factor loading from the Principal Component Analysis (PCA)

Parameters		Factor loadings				
		PC 1	PC 2	PC 3	PC 4	PC 5
DPPH	IC_{50} values	**0.863**	0.375	-0.186	-0.079	-0.062
ABTS		**0.951**	0.050	0.066	-0.182	-0.022
SO		0.454	**-0.581**	0.404	0.395	0.065
NO		0.486	**-0.553**	-0.327	-0.183	0.548
OH		**0.860**	0.374	-0.016	-0.117	-0.120
MC		0.467	-0.438	**-0.488**	0.430	-0.320
ALP		0.033	**0.755**	-0.171	0.522	0.354
RP	Ascorbic acid equivalence	0.381	0.050	**0.840**	0.089	0.053

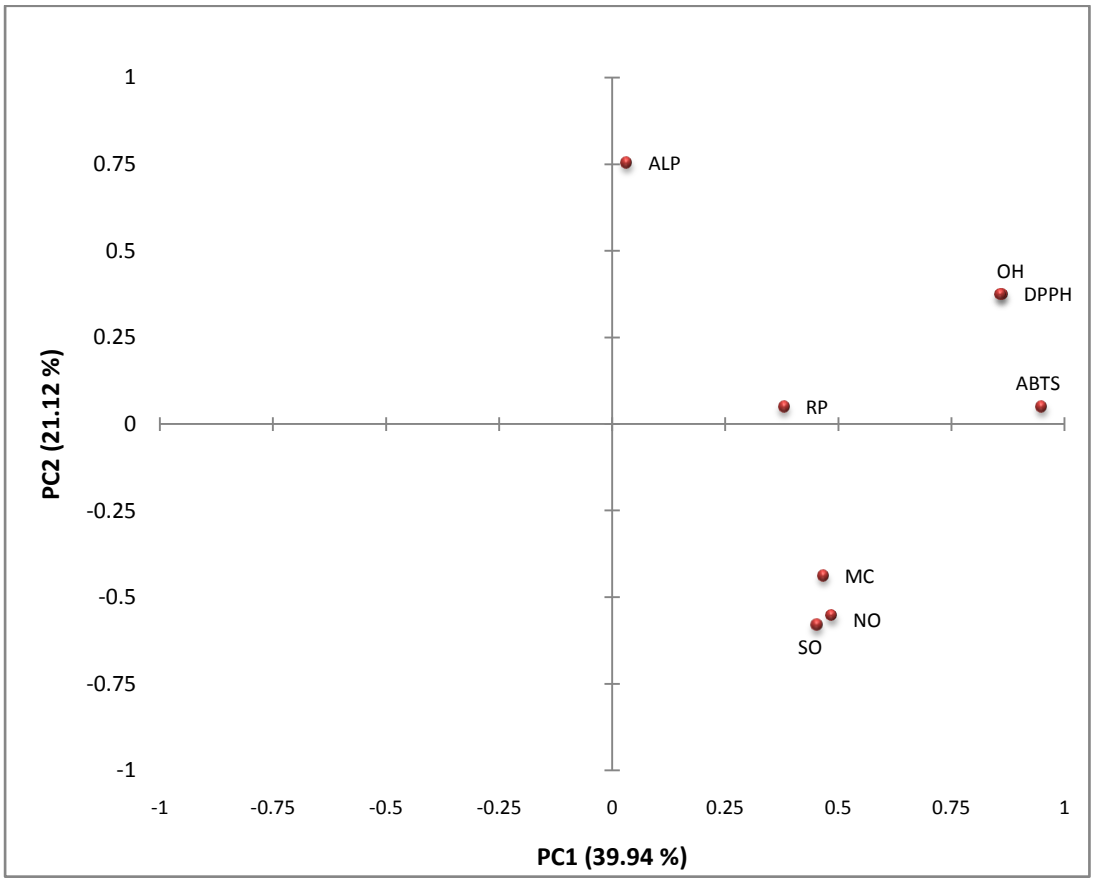


Figure 4.2 Principal component analysis factor loading plot of antioxidant activity of different edible fruits and vegetables

for 39.94% and 21.12% of the data variance. Further components explained 16.02%, 8.85% and 6.92% of total data variance, respectively. The loading of PC1 had a strong positive correlation with DPPH, $ABTS^{+}$ and hydroxyl radicals (Table 4.2), indicating that the mechanism of action of the extracts for these antioxidant activity may be identical. The second principal component (PC2) was related to superoxide and nitric oxide scavenging activity, and in factor loading plot (Figure 4.2) they are closely clustered and are located in opposite direction with anti-lipid peroxidation capacity. Superoxide and nitric oxide radicals are reactive oxygen and nitrogen species, mainly acting in hydrophilic system, whereas lipid peroxidation is principally achieved in hydrophobic system; so their scavenging components might be different and were accumulated in inverse way in plant system. Again, the inverse relation between metal chelating and reducing power activity was significant on PC3, but actually they were similarly loaded and the apparent negative relation is due to their units of measurement (IC_{50} and AAE for metal chelating and reducing power respectively). The relation between free-radical scavenging property and reducing power was also established in edible mushrooms, where phenolic compounds but not the ascorbic acid made a significant contribution to antioxidant activity (Keles *et al*., 2011). PCA score plot was also performed to gain an overview of the similarities and differences among different morphological parts of edible fruits and vegetables of Darjeeling Himalaya in relation with their contribution towards antioxidant activity. The results of PCA are shown in Figure 4.3. The origin of graph represents the mean of free radical scavenging property of all the samples. The distance between the locations of any two samples on the score plot is directly related with the degree of difference between them. From PCA score plot, it was observed that the underground parts were mainly clustered in one direction with positive score on PC1, whereas the majority of fruits, spices and leafy vegetables were accumulated in other direction with negative score on same component. This is because underground parts exhibited significantly higher nitric oxide scavenging, reducing power and metal chelating activity in respect to others that were responsible for different grouping of morphological parts in score plot. Similar results were also

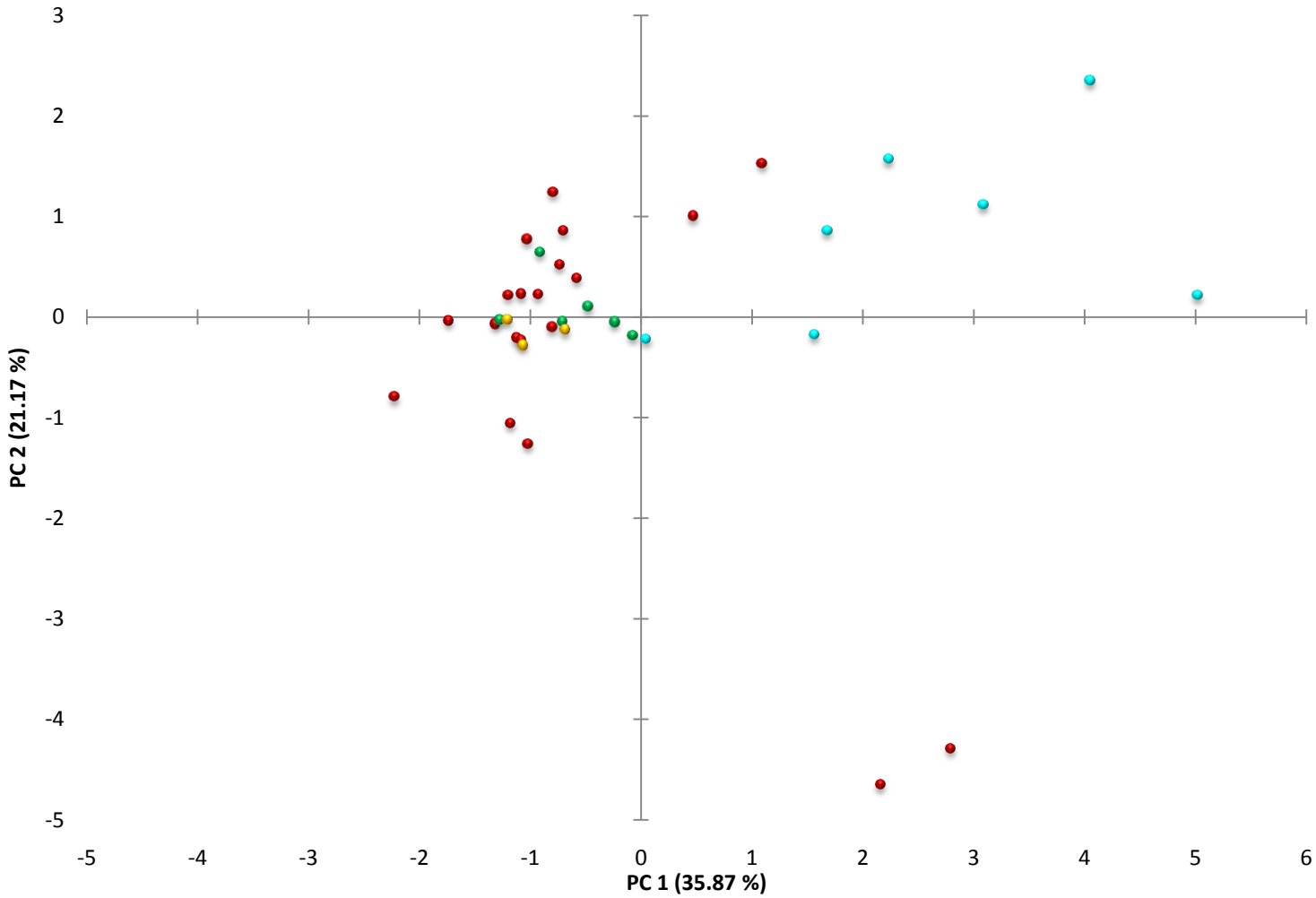


Figure 4.3 Principal Component Analysis score plot of different edible morphological parts of fruits and vegetables contributing antioxidant activity

[**Indicators:** Red dot: Fruit, Blue dot: Underground part, Green dot: Leaf, Yellow dot: Spice]

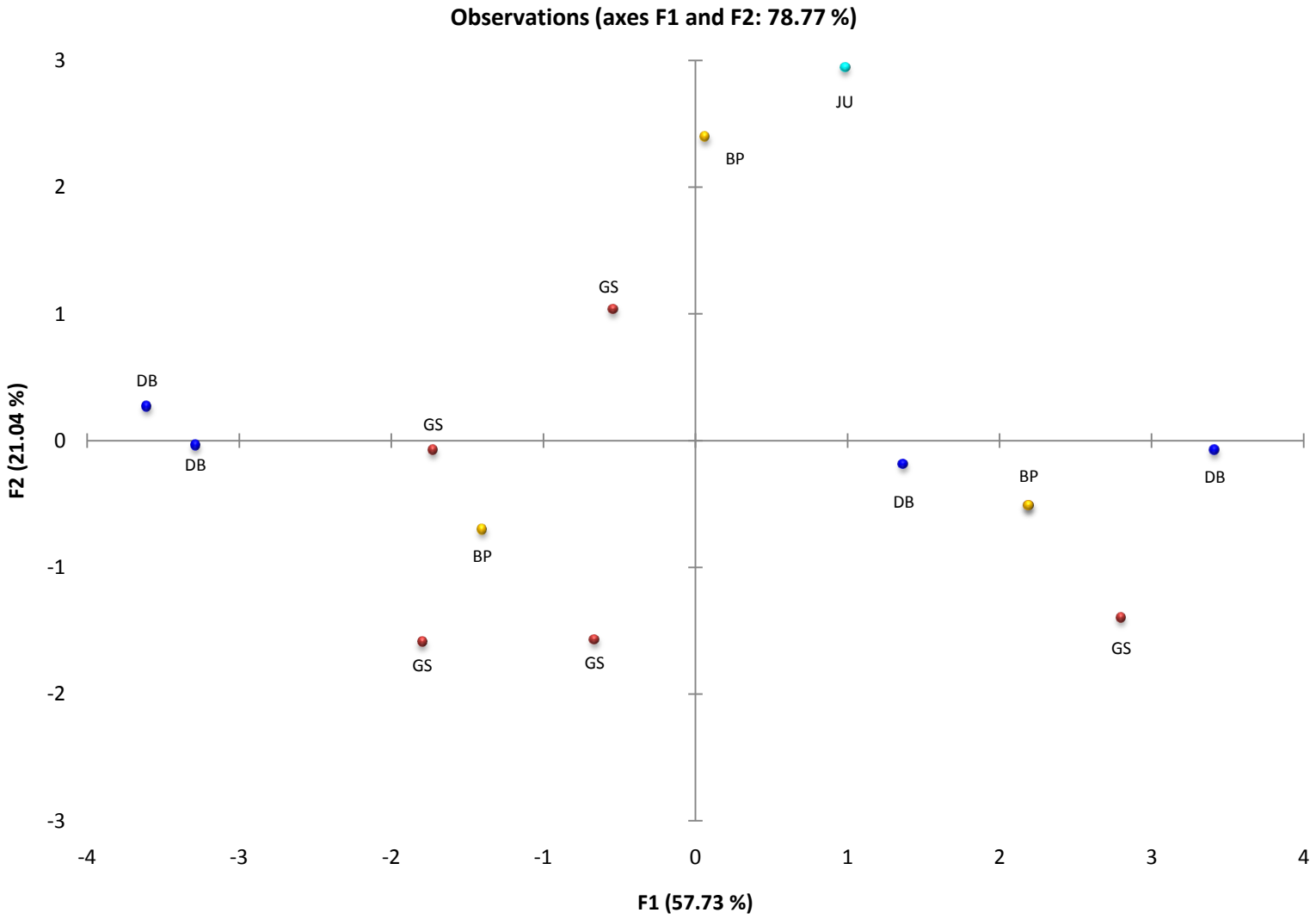


Figure 4.4 Principal Component Analysis score plot of different ethnomedicinally important fruits and vegetables in relation to antioxidant activity

[**Indicators:** Red dot: Gastric (GS), Blue dot: Diabetes (DB), Yellow dot: Blood pressure (BP)]

obtained by Zhao *et al*., (2008) where they classified and discriminated barley cultivars on PCA score plot mainly on the basis of variation in metal chelating activity. PCA score plot was again performed for analyzing the similarities and differences among different edible fruits and vegetables which are used as ethnomedicine on the basis of their free radical scavenging capacity. The results of this PCA analysis were shown in Figure 4.4. Here also, the origin represents the mean antioxidant properties of all the samples which are ethnomedicinally important. The scattering of points from origin indicated that no specific antioxidant mechanism is involved for the treatment of overall disorders. Rather highly efficient scavenging of specific free radical species might be responsible for using plant as phytomedicine. As for example, antidiabetic plant like *C. erectus* efficiently scavenged DPPH, ABTS and superoxide radical, whereas another antidiabetic plant *S. incanum* exhibited strong anti-lipid peroxidation activity. Likewise the plants which are ethnomedicinally used for the treatment of gastritis, stomach problem, indigestion and vomiting are highly capable for iron induced metal chelating.

The study of these edible plants may lead to the discovery of new, functional antioxidant sources; therefore consuming these wild edible vegetables may supplement dietary as well as nutraceutical requirement for conservation and sustainable utilization of these plant species for presentation of their extinction in future and providing a supportive on planned development of this region through appropriate cultivation technique.

Chapter – V

PHYTOCHEMICAL SCREENING AND QUANTIFICATION OF SECONDARY METABOLITES PRESENT IN EDIBLE PLANTS OF DARJEELING HIMALAYA

5.1 INTRODUCTION

In Greek, 'Phyto' means plant which could be described as a wonderful chemical cabinet filled with attractive chemicals i.e. phytochemicals. Plants produce these chemicals to protect themselves but recent research confirmed that many phytochemicals could protect human against diseases. There are many families of phytochemicals and they are able to protect the human body in a variety of ways. Polyphenolic compounds are generally found in both edible as well as non-edible plants and they have multiple biological effects, including antioxidant property (Kahkonen *et al*., 1999). Herbs, fruits and spices are used in many domains, including medicine, nutrition, beverages, flavouring and dyeing agents, fragrances, repellents and cosmetics. At present there is an upsurge of attention about phytochemicals as potential source of natural antioxidants. The target is to use them in foods as well as pharmaceutical preparations to replace synthetic antioxidants (Cai *et al*., 2004; Katalinic *et al*., 2006; Wong *et al*., 2006). Most of the antioxidants isolated from higher plants are polyphenols. In vascular plants approximately more than 4000 phenolic and flavonoid compounds have been documented (e.g. phenolic acids, flavonoids, coumarins, tannins, anthraquinones) (Trease and Evans, 1989; Middleton and Kandaswami, 1994). Among herbal antioxidants, a wide range of molecular weight (low and high) plant polyphenols have been documented (Hagerman *et al*., 1998). The antioxidant activity of plant phenolic compounds is mainly due to their redox properties, which allow them to perform as hydrogen donors, reducing agents and singlet oxygen quenchers. They also have the metal chelating property (Rice-Evans *et al*., 1995). In addition, some of them have potential medicinal properties and are beneficial to health, e.g. digestive stimulation action, anti-inflammatory, antimutagenic, antimicrobial, anti-viral, anti-allergic, estrogenic, hypo-lipidemic and anti-carcinogenic effect (Larson, 1988; Aaby *et al*., 2004; Djeridane *et al*., 2006). Crude extracts of herbs, fruits and spices, and other plant materials loaded with phenolics are of increasing interest in the food industry as they retard oxidative degradation of lipids and thus improve the quality and nutritional value of food. As in Chapter IV, we already established that different types of

underexplored fruits and vegetables of Darjeeling Himalaya have significant amount of antioxidant activity but in particular, quantitative and qualitative data on phytochemicals in different edible specimens are still missing. Consequently, in this Chapter we have established total phenol and flavonoids content quantitatively as it is well known that polyphenolic compounds are potential antioxidants; and along with this, detection and qualitative profiling of different types of secondary metabolites present in edible plants.

5.2 MATERIALS AND METHODS

5.2.1 Plant samples collection and identification

Plants collection and identification were specified in Chapter III Section 3.2.2.

5.2.2 Preparation of methanolic plant extracts

Different fresh fruits and vegetables were surgically separated and were separately crushed with mortar and pestle. Under a Soxhlet extractor, crushed fruits were individually extracted with methanol for 8h. The methanol was completely removed by vacuum rotary evaporator at 50°C. These crude extracts were freeze-dried. The powder was stored at 4°C and used for further investigation. The extractive value of the plant materials were calculated on dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

5.2.3 Total phenol estimation

Total phenolic compounds of plant extracts were determined by Folin-Ciocalteu method (Folin and Ciocalteu, 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-

Ciocalteu reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hr. at 20º C and the calibration curve was drawn. 1 ml methanolic fruit extracts (50 mg/ml FWT) was mixed to the same reagent and the mixture was incubated for one hour in room temperature. After 1 hour the absorbance was measured at 765nm.

5.2.4 Total flavonoids determination

Spectrophotometric aluminum chloride method was used for flavonoids determination (Sultana *et al*., 2009). Each methanolic fruit extracts (0.5 ml of 100mg/ml) were separately diluted with 4 ml double distilled water. Then the diluted fruits extracts were mixed with 5% (0.3 ml) $NaNO_2$ and 10% aluminum chloride were then added with reaction mixture. After 6 min 2ml (1.0 M) NaOH and 2.4 ml double distilled water was added and mixed well. There after absorbance was measured at 510 nm in spectrophotometer. Standard solution of quercetin (0-500 mg L^{-1}) was used as calibration curve.

5.2.5 Phytochemicals evaluation of the crude extracts

The methanolic crude extract (200 mg/ml) of the plant was subjected to various chemical tests in order to determine the secondary metabolites present by employing the use of various methods as follows:

5.2.5a Test for reducing sugars

To 0.5ml of the extracts, 2ml of a mixture (1:1) of Fehling's solution I (A) and Fehling's solution II (B) were added and the mixture were boiled in a water bath for five minutes. A brick-red precipitate indicated the presence of free reducing sugars (Brain and Turner, 1975).

5.2.5b Test for resins

0.5ml of extract was evaporated and dissolved in 2ml of petroleum ether, 2ml of 2% cupper acetate solution was then added and the mixture was shaken vigorously and allowed to separate, a green colour indicated the presence of resin (Trease and Evans, 1983).

5.2.5c Test for amino acid

0.5 ml methanolic plant extracts were treated with few drops of ninhydrin reagent, heated in water bath, a purple colour indicated the presence of amino acids (Kumar *et al*., 2009).

5.2.5d Test for anthraquinones

1ml methanolic plant extracts were evaporated and dissolved in 2ml chloroform. 2ml of ammonia was added. Occurrence of red colour suggested the presence of anthraquinones (Kumar *et al*., 2009).

5.2.5e Test for tannin

0.5 ml methanolic extract of each plant part was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al*., 2009).

5.2.5f Test for triterpenoids

0.5 ml of methanolic plant extracts were evaporated and dissolved in 1ml chloroform. 1ml acetic anhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If reddish violate colour appeared, the existence of triterpenoids was confirmed (Kumar *et al*., 2009).

5.2.5g Test for alkaloids

0.5 ml of each plant extract was added with 0.2ml of 36.5% hydrochloric acid and 0.2 ml Dragendroff's reagent. Production of orange precipitation denoted the presence of alkaloids (Kumar *et al*., 2009).

5.2.5h Test for glycosides

0.5 ml methanolic extracts of plant were added with 2ml of 50% hydrochloric acid. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1 ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides (Kumar *et al*., 2009).

5.2.5i Test for steroid

0.5 ml methanolic extracts were evaporated and dissolved in 2ml chloroform. 2ml of concentrated H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al*., 2009).

5.2.5j Test for saponins

2 ml of double distilled water was added with 1 ml of each methanolic extract. Few drops of olive oil were added and agitated. Formation of soluble emulsion indicated the presence of saponin (Ngbede *et al*., 2008).

5.2.5k Test for cardiac glycoside

0.5 ml of methanolic plant extracts were evaporated and dissolved in 1 ml glacial acetic acid. One drop of 10% ferric chloride was then added. 1 ml of conc.H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al*., 2008).

5.2.6 Statistical analysis

The data were pooled in triplicate and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the phytochemicals of different edible plants of Darjeeling Himalaya. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p < 0.05$ was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the values of phytochemicals and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

5.3 RESULTS AND DISCUSSIONS

The extractive yields of different parts of fruits and vegetables of Darjeeling Himalaya were presented in Table 5.1. Relatively higher extraction yields were obtained from mesocarp and epicarp than other parts of fruits like endocarp, seeds and placenta. The higher extraction yield was found in the methanol extract from the leaves, while the petiole and underground plant parts had the lower extraction yields (Table 5.1). These results showed that the extraction yield varied with methanol, indicating that each part of these plants consist of different components. Several studies reported the increase of extractive yield by the action of pectinases, cellulases and hemicellulases; reduction of particle size increases the polyphenols extraction rate and the extraction yield (Furhman *et al.,* 2001; Landbo and Meyer, 2001). The range of total phenol, flavonoids and extraction yield of different types of plant families of Darjeeling Himalaya were shown in Figure 5.1a-5.1c. The analysis of methanolic extracts of the underexplored edible plants of Darjeeling Himalaya indicated the presence of phenolics, flavonoids, glycosides, cardiac glycosides, phytosterol, triterpenoids, tannins, saponins, alkaloids and amino acids (Table 5.2). A

Table 5.1 Extractive values of selected plant parts of Darjeeling Himalaya

Sl no	Scientific name	Plant parts	Extractive values (%)
1	*Aconogonon molle*	Leaf	5.75±0.002^{r}
		Stem	3±0.001^{G}
		Inflorescence	9.25±0.01^{j}
2	*Apium graveolens*	Leaf	19.8±0.01^{a}
		Petiole	14.95±0.01^{d}
3	*Brassica juncea*	Leaf	2.5±0.001^{I}
		Petiole	3.5±0.001^{E}
4	*Brassica rapa*	Leaf	7.8±0.002^{n}
		Petiole	5.35±0.001^{s}
5	*Calamus erectus*	Epicarp	8.75±0.002^{l}
		Mesocarp	5±0.001^{x}
		Endocarp	5.25±0.001^{u}
6	*Capsicum annuum*	Whole fruit	2.5±0.001^{I}
7	*Cinnamomum glaucescens*	Fruit without seed	2.8±0.001^{H}
8	*Cyclanthera pedata*	Epicarp & mesocarp	2±0.001^{M}
		Endocarp	7.75±0.002^{o}
9	*Cyphomandra betacea*	Epicarp	4.5±0.001^{z}
		Mesocarp	1.25±0.001^{Q}
		Endocarp	1.6±0.001^{O}
		placenta	2.1±0.001^{L}
		Seed	0.6±0.0001^{R}
10	*Dioscorea alata*	Underground part	2.3±0.001^{K}
11	*Dioscorea hamiltonii*	Underground part	3.1±0.001^{F}
12	*Diploknema butyracea*	Fruit without seed	5.29±0.003^{t}
13	*Docynia indica*	Epicarp	15.5±0.006^{c}
		Mesocarp	4.25±0.002^{A}
		Endocarp	4.75±0.002^{y}
14	*Elaeocarpus lanceifolius*	Fruit without seed	6.8±0.001^{p}
15	*Evodia fraxinifolia*	Whole fruit	5.05±0.002^{w}
16	*Heracleum wallichii*	Seed	3.5±0.001^{E}
17	*Ipomoea batatas* (Red)	Underground part	1.6±0.001^{O}
18	*Ipomoea batatas* (White)	Underground part	1.8±0.001^{N}
19	*Lepidium sativum*	Leaf	3±0.002^{G}
		Stem	4.25±0.002^{A}
		Root	4.75±0.001^{y}
20	*Litsea cubeba*	Whole fruit	8.4±0.001^{m}
21	*Machilus edulis*	Epicarp	4.01±0.002^{B}
		Mesocarp	5.21±0.002^{v}
22	*Manihot esculenta*	Underground part	2.8±0.001^{H}
23	*Nasturtium officinale*	Leaf	1.5±0.001^{P}
		Immature stem	0.5±0.0001^{S}
		Mature stem	4±0.002^{C}
24	*Persea americana*	Epicarp	13.3±0.004^{f}
		Mesocarp	9.4±0.003^{i}
25	*Phytolacca acinosa*	Leaf	17.05±0.006^{b}
26	*Prunus domestica*	Fruit without seed	3.9±0.001^{D}
27	*Pyrus communis*	Mesocarp	9.1±0.005^{k}
		Endocarp	12±0.006^{g}
28	*Rubus ellipticus*	Whole fruit	14.2±0.003^{e}
29	*Sechium edule*	Underground part	2.3±0.002^{K}
30	*Solanum anguivi*	Whole fruit	2.4±0.001^{J}
31	*Solanum incanum*	Epicarp & mesocarp	9.6±0.002^{h}
		Endocarp	6.2±0.001^{q}
32	*Spondias mombin*	Fruit without seed	0.5±0.0001^{S}
33	*Xanthosoma brasiliense*	Underground part	4.5±0.001^{z}
34	*Zanthoxylum acanthopodium*	Whole fruit	8.4±0.003^{m}

Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

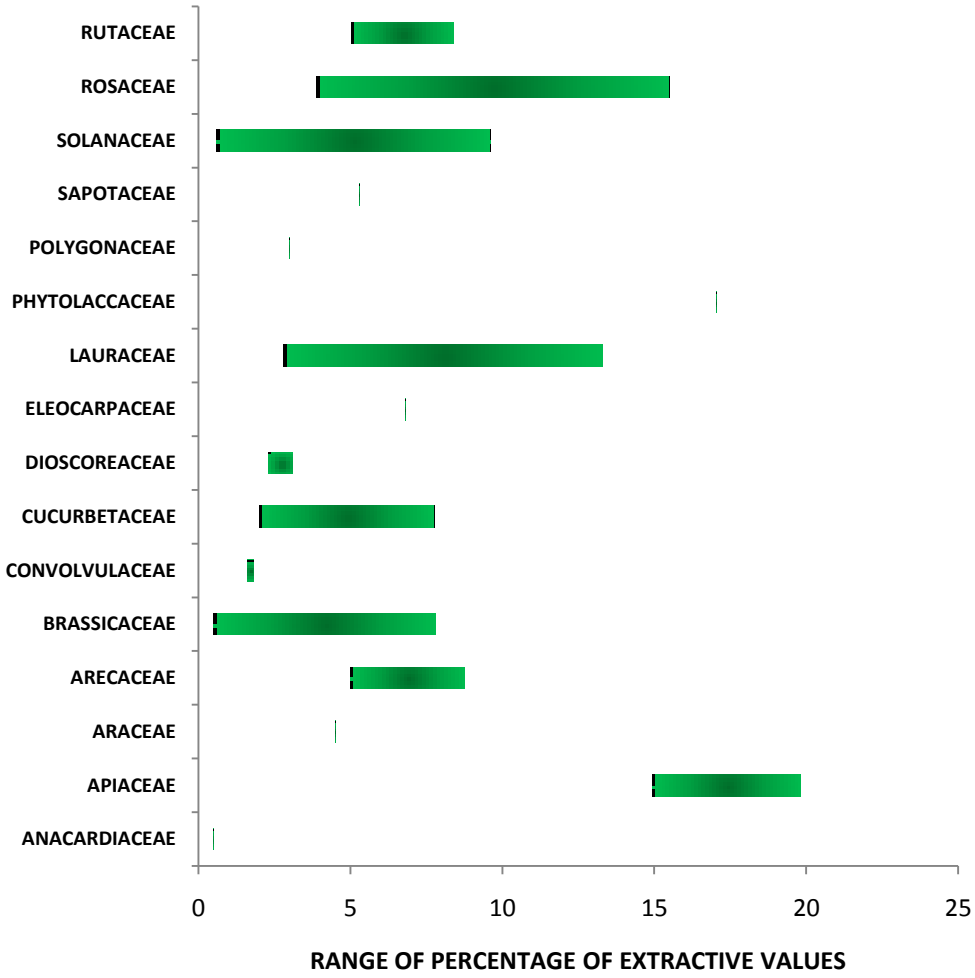


Figure 5.1a Range of extractive values of different family available in Darjeeling Himalaya

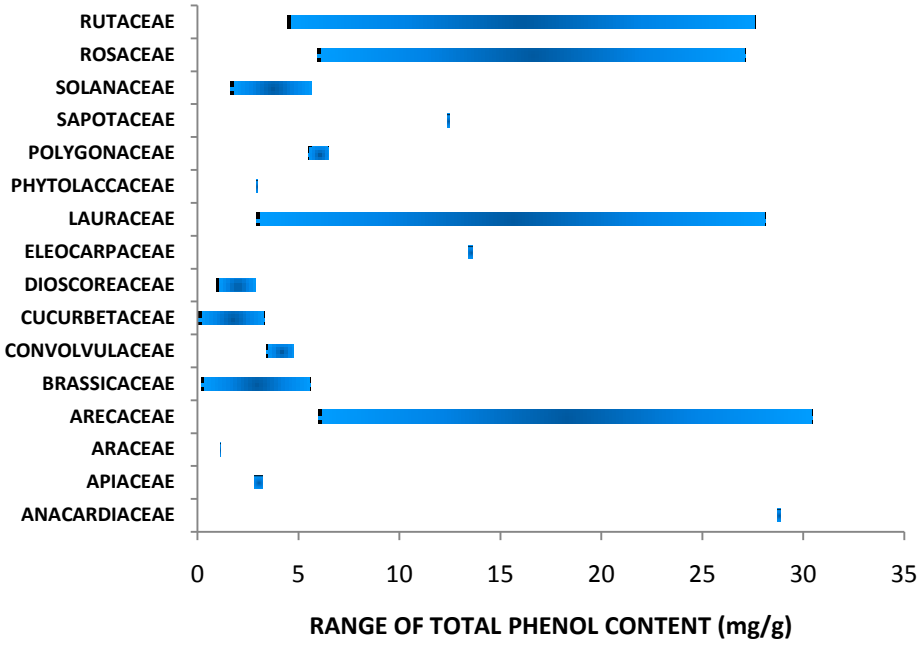


Figure 5.1b Range of total phenol content of different family available in Darjeeling Himalaya

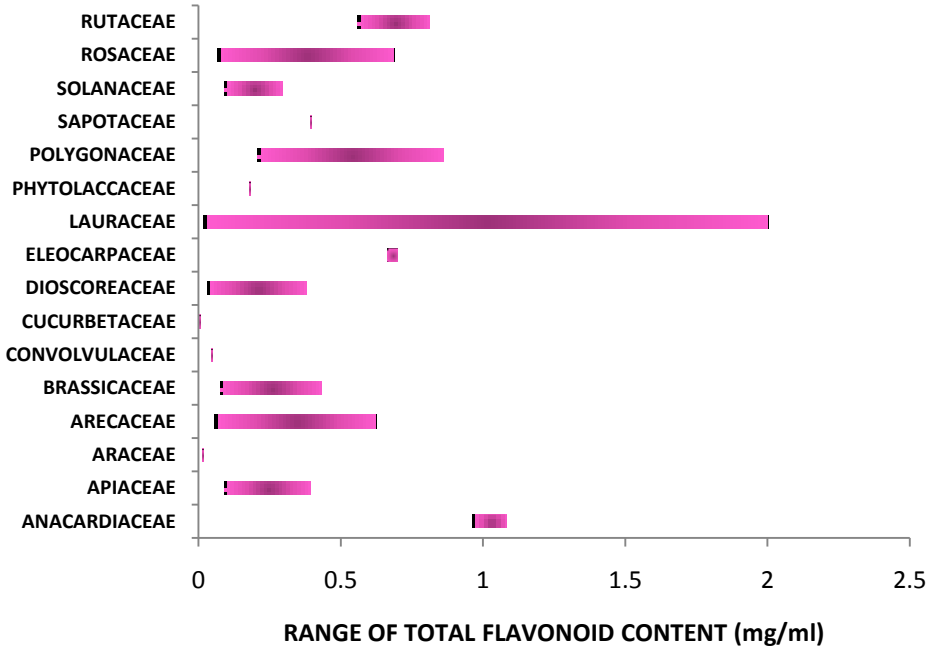


Figure 5.1c Range of total flavonoid content of different family available in Darjeeling Himalaya

Table 5.2 Phytochemical profile of edible fruits and vegetables of Darjeeling Himalaya

SL NO.	SCIENTIFIC NAME	PLANT PARTS	ALKALOID	AMINO ACID	ANTRAQUINONES	STEROIDS	GLYCOSIDES	SAPONINS	TANNINS	REDUCING SUGAR	TRITERPEN	CARDIAC GLYCOSIDES	RESIN
1	*Aconogonon molle*	Leaf	+++	++++	-	+++	+	++	++++	++++	+	+++	+++
		Stem	++	++	-	+++	+	+++	++	+++	+	-	+
		Inflorescence	+++	++++	-	+++	++	+	+++	++++	++	+++	+++
2	*Apium graveolens*	Leaf	++	++++	-	+++	-	+	++	+++	-	-	++
		Petiole	+	++++	-	+	-	+	++	+++	-	-	-
3	*Brassica juncea*	Leaf	++	++++	-	+++	-	++	+++	++	+	+	++
		Petiole	++	+++	-	++	-	+	+	++	-	-	-
4	*Brassica rapa*	Leaf	++	++++	-	++	-	+	++	+	-	-	++
		Petiole	+	++++	-	+	-	+	+	+++	-	-	-
5	*Calamus erectus*	Epicarp	-	++	++++	++++	++	++	++	++++	++++	+++	+++
		Mesocarp	-	++	++++	++++	+++	+++	++++	++++	++++	++++	++++
		Endocarp	-	++++	++++	++++	++++	+	++++	++++	+	++	+
6	*Capsicum annuum*	Whole fruit	++	++++	-	++++	+	+++	++	++++	+	+	++++
7	*Cinnamomum glaucescens*	Fruit without seed	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
8	*Cyclanthera pedata*	Epicarp & mesocarp	-	++	-	+	-	++++	+	++	-	-	+
		Endocarp	-	++	-	+	-	++	+	+++	-	-	+

(Number of '+' indicates the intensity of phytochemical)

Table 5.2 Phytochemical profile of edible fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	PLANT PARTS	ALKALOID	AMINO ACID	ANTRAQUINONES	STEROIDS	GLYCOSIDES	SAPONINS	TANNINS	REDUCING SUGAR	TRITERPENE	CARDIAC GLYCOSIDES	RESIN
9	*Cyphomandra betacea*	Epicarp	+++	++++	+	++	+	+	+++	++++	+	++	+
		Mesocarp	++	++++	-	+++	+	+	++	++++	+	+	-
		Endocarp	++	++++	-	+++	+	+	+++	++++	+	++	++
		placenta	++++	++++	-	++++	+	++++	++++	++++	+	+	++
		Seed	++	++	-	++	+	+	++	++	+	+	++
10	*Dioscorea alata*	Underground part	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
11	*Dioscorea hamiltonii*	Underground part	++++	++	++	++	+	++++	+	+	++	+	-
12	*Diploknema butyracea*	Fruit without seed	-	+++	-	++++	+++	-	+++	+++	-	++++	++
13	*Docynia indica*	Epicarp	-	-	-	-	+++	++	+++	++++	++++	+++	++++
		Mesocarp	-	-	-	++++	+	++	++	++++	++++	++	++
		Endocarp	-	+	-	++++	+	++	++	++++	+++	+	-
14	*Elaeocarpus lanceifolius*	Fruit without seed	-	-	+++	++++	++++	+++	-	+++	+++	+++	++++
15	*Evodia fraxinifolia*	Whole fruit	+++	-	++++	+	++	-	+	+	++++	++	++
16	*Heracleum wallichii*	Seed	-	++	-	+	-	-	++	+	-	-	++
17	*Ipomoea batatas* (Red)	Underground part	+++	++++	++	++++	++++	++	++++	++++	++++	+++	++
18	*Ipomoea batatas* (White)	Underground part	-	++++	++	++++	+	++	++++	+++	+	+	++

(Number of '+' indicates the intensity of phytochemical)

Table 5.2 Phytochemical profile of edible fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	PLANT PARTS	ALKALOID	AMINO ACID	ANTRAQUINONES	STEROIDS	GLYCOSIDES	SAPONINS	TANNINS	REDUCING SUGAR	TRITERPENE	CARDIAC GLYCOSIDES	RESIN
19	*Lepidium sativum*	Leaf	++	++++	-	+++	-	+	+++	++	+	-	+++
		Stem	++	++++	-	++	-	+	++	+++	-	-	-
		Root	++	++++	-	+	-	+	+	+++	-	-	-
20	*Litsea cubeba*		++++	-	++++	+++	+	-	+	+	++++	-	+
21	*Machilus edulis*	Epicarp	++	+	++++	+++	+++	+	++++	++++	+++	++++	++++
		Mesocarp	+++	++++	-	+++	+++	++	+	+++	++	+++	-
22	*Manihot esculenta*	Underground part	-	++++	+	++	+++	+++	++	++	+++	++++	-
23	*Nasturtium officinale*	Leaf	+++	++++	-	+++	-	++	+++	+++	-	-	+++
		Immature stem	++	+++	-	++	-	+	+	+++	-	-	-
		Mature stem	++	+++	-	++	-	+	+	++	-	-	-
24	*Persea americana*	Endocarp	+	-	-	+	-	-	-	++	++	-	-
		Epicarp	+++	-	-	+	+	+	-	+	++++	+	-
		Mesocarp	++++	-	++++	++++	+++	+++	++	++	++++	++	++++

(Number of '+' indicates the intensity of phytochemical)

Table 5.2 Phytochemical profile of edible fruits and vegetables of Darjeeling Himalaya (Cont…..)

SL NO.	SCIENTIFIC NAME	PLANT PARTS	ALKALOID	AMINO ACID	ANTRAQUINONES	STEROIDS	GLYCOSIDES	SAPONINS	TANNINS	REDUCING SUGAR	TRITERPENE	CARDIAC GLYCOSIDES	RESIN
25	*Phytolacca acinosa*	Leaf	+++	++++	-	++	-	+	++	+	-	-	-
26	*Prunus domestica*	Fruit without seed	-	-	+++	++	++++	+	++	+++	++	++++	+++
27	*Pyrus communis*	Mesocarp	++	-	++++	++	+++	-	-	++	++++	+++	+
28	*Rubus ellipticus*	Whole fruit	+	-	-	+	-	+	+	++	+++	-	+
29	*Sechium edule*	Underground part	-	+++	++	+	++	++	+	+++	++++	-	+
30	*Solanum anguivi*	Whole fruit	++	++++	-	+++	+	+	++	++++	++	+	-
31	*Solanum incanum*	Epicarp & mesocarp	++	++++	-	+++	+	+	+	++++	+	-	-
		Endocarp	++	++++	-	+++	+	+	+	++++	+	+	+
32	*Spondias mombin*	Fruit without seed	+	+	++++	+++	+++	+	+++	++++	++	++	++++
33	*Xanthosoma brasiliense*	Underground part	-	++++	+	+++	+	++++	+	+	+	-	-
34	*Zanthoxylum acanthopodium*	Whole fruit	+++	+	++	+++	++	+++	+++	+++	+++	+++	+++

(Number of '+' indicates the intensity of phytochemical)

variety of phytochemicals have been found to possess an extensive range of actions, which may help in protection against chronic disorders. It was reported that the phenolic compounds constitute a major group of compounds that act as primary antioxidants (Hatano *et al.,* 1989). It was also stated that flavonoids showed anti-allergic, anti-inflammatory and antimicrobial activity. This compound may reduce the risk of a variety of cancers and also prevent menopausal symptoms (Hodek *et al*., 2002). Epidemiological studies suggest that the use of flavonoids is effective in preventing the risk of coronary heart diseases (Fergusion, 2001). In our experiments, Table 5.3 showed that leafy vegetables, fruits, spices as well as edible underground parts of plant of Darjeeling Himalaya had higher amount of total phenol and flavonoids content. Dharmananda (2003) claimed that plants containing tannins are astringent and the tannins are used for treating intestinal disorders like diarrhoea and dysentery. The presence of tannins in *Litsea cubeba* and *Zanthoxylum acanthopodium* supports the traditional medicinal use of these plants in the treatment of different diseases. Morta *et al*. (1985) discovered that tannins are used for the treatment of inflamed or ulcerated tissues. Trease and Evans (1989) stated that tannins are potent antimicrobial, anticancer as well as antioxidants activities. The observations (Table 5.2) support the use of most of the edible plants which are available in the markets of Darjeeling Himalaya like *Calamus erectus*, *Cyphomandra betacea*, *Cinnamomum glaucescens*, *Dioscorea alata*, *Diploknema butyracea*, *Docynia indica*, *Ipomoea batatas*, *Machilus edulis*, *Nasturtium officinale*, *Spondias mombin* and *Zanthoxylum acanthopodium* in herbal cure remedies. Another compound *i.e*. steroids, abundant in most of the plants have hypercholesterolemic effects (Kapil *et al*., 1994). Plant steroids are important for their cardiotonic activities; they possess anticancer, anti-viral, insecticidal agents and antimicrobial properties (Minocha and Tiwari, 1981; Kokpol *et al*., 1984). They are also used as herbal medicine, nutraceuticals and cosmetics (Callow, 1936). Table 5.2 showed that almost all plants contain steroids and saponins. Saponins are responsible for many of the attributed biological effects like inhibition of inflammation (Liu *et al*., 2002). Therefore, therapeutic effects of the edible plants of Darjeeling Himalaya can be attributed to the antioxidant

Table 5.3 Quantification of total phenol and flavonoid contents of fruits and vegetables of Darjeeling Himalaya

SL NO.	SCIENTIFIC NAME	PLANT PARTS	PHENOL CONTENT	FLAVONOID CONTENT
1	*Aconogonon molle*	Leaf	5.871 ± 0.024^{no}	0.778 ± 0.021^{d}
		Stem	5.495 ± 0.021^{q}	0.219 ± 0.011^{pq}
		Inflorescence	6.458 ± 0.031^{m}	0.821 ± 0.042^{c}
2	*Apium graveolens*	Leaf	3.177 ± 0.023^{x}	0.363 ± 0.031^{l}
		Petiole	2.836 ± 0.016^{z}	0.091 ± 0.001^{xyz}
4	*Brassica juncea*	Leaf	5.544 ± 0.032^{pq}	0.424 ± 0.011^{j}
		Petiole	2.767 ± 0.012^{z}	0.231 ± 0.011^{p}
3	*Brassica rapa*	Leaf	2.836 ± 0.021^{z}	0.227 ± 0.011^{p}
		Petiole	2.780 ± 0.023^{z}	0.078 ± 0.001^{yzA}
5	*Calamus erectus*	Epicarp	6.053 ± 0.036^{n}	0.058 ± 0.001^{ABC}
		Mesocarp	23.39 ± 0.090^{g}	0.124 ± 0.011^{vw}
		Endocarp	30.375 ± 0.089^{b}	0.605 ± 0.021^{fg}
6	*Capsicum annuum*	Whole fruit	5.492 ± 0.016^{q}	0.163 ± 0.002^{tu}
7	*Cinnamomum glaucescens*	Fruit without seed	6.076 ± 0.024^{n}	0.350 ± 0.03^{lm}
8	*Cyclanthera pedata*	Epicarp & mesocarp	0.080 ± 0.001^{F}	0.001 ± 0.0001
		Endocarp	0.070 ± 0.002^{F}	0.002 ± 0.0001^{F}
9	*Cyphomandra betacea*	Epicarp	2.7 ± 0.35^{z}	0.186 ± 0.021^{rst}
		Mesocarp	1.53 ± 0.13^{C}	0.147 ± 0.022^{uv}
		Endocarp	3.88 ± 0.6^{u}	0.192 ± 0.023^{rs}
		placenta	4.11 ± 0.3^{t}	0.199 ± 0.031^{qrs}
		Seed	3.66 ± 0.55^{v}	0.14 ± 0.024^{uv}
10	*Dioscorea alata*	Underground part	2.850 ± 0.021^{z}	0.098 ± 0.001^{xy}
11	*Dioscorea hamiltonii*	Underground part	0.970 ± 0.004^{E}	0.032 ± 0.001^{D}
12	*Diploknema butyracea*	Fruit without seed	12.424 ± 0.05^{i}	0.396 ± 0.001

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

Table 5.3 Quantification of total phenol and flavonoid contents of fruits and vegetables of Darjeeling Himalaya (Cont….)

SL NO.	SCIENTIFIC NAME	PLANT PARTS	PHENOL CONTENT	FLAVONOID CONTENT
13	*Docynia indica*	Epicarp	7.172 ± 0.027^{k}	0.226 ± 0.021^{p}
		Mesocarp	5.981 ± 0.024^{n}	0.100 ± 0.006^{xy}
		Endocarp	6.303 ± 0.012^{m}	0.068 ± 0.001^{zAB}
14	*Elaeocarpus lanceifolius*	Fruit without seed	13.536 ± 0.092^{h}	0.682 ± 0.016^{e}
15	*Evodia fraxinifolia*	Whole fruit	27.584 ± 0.068^{e}	0.798 ± 0.014^{d}
16	*Heracleum wallichii*	Seed	2.920 ± 0.014^{yz}	0.492 ± 0.012^{h}
17	*Ipomoea batatas* (Red)	Underground part	4.745 ± 0.016^{r}	0.045 ± 0.001^{CD}
18	*Ipomoea batatas* (White)	Underground part	3.415 ± 0.013^{w}	0.048 ± 0.001^{BCD}
19	*Lepidium sativum*	Leaf	2.742 ± 0.015^{z}	0.338 ± 0.027^{mn}
		Stem	2.690 ± 0.013^{z}	0.127 ± 0.021^{v}
		Root	0.198 ± 0.021^{F}	0.192 ± 0.011^{rs}
20	*Litsea cubeba*	Whole fruit	6.670 ± 0.28	0.504 ± 0.016^{h}
21	*Machilus edulis*	Epicarp	30.935 ± 0.087^{a}	1.988 ± 0.016^{a}
		Mesocarp	3.102 ± 0.013^{xy}	0.499 ± 0.017^{h}
22	*Manihot esculenta*	Underground part	2.900 ± 0.025^{yz}	0.018 ± 0.001^{EF}
23	*Nasturtium officinale*	Leaf	5.741 ± 0.019^{op}	0.278 ± 0.016^{o}
		Immature stem	2.692 ± 0.015^{z}	0.103 ± 0.001^{wx}
		Mature stem	2.764 ± 0.024^{z}	0.142 ± 0.001^{uv}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

Table 5.3 Quantification of total phenol and flavonoid contents of fruits and vegetables of Darjeeling Himalaya (Cont….)

SL NO.	SCIENTIFIC NAME	PLANT PARTS	PHENOL CONTENT	FLAVONOID CONTENT
24	*Persea americana*	Epicarp	26.840 ± 0.098^{f}	0.318 ± 0.002^{n}
		Mesocarp	28.024 ± 0.097^{d}	0.627 ± 0.006^{f}
25	*Phytolacca acinosa*	Leaf	2.935 ± 0.014^{yz}	0.182 ± 0.001^{st}
26	*Prunus domestica*	Fruit without seed	11.685 ± 0.072^{j}	0.675 ± 0.013^{e}
27	*Pyrus communis*	Mesocarp	27.031 ± 0.091^{f}	0.447 ± 0.011^{i}
		Endocarp	26.972 ± 0.099^{f}	0.181 ± 0.001^{st}
28	*Rubus ellipticus*	Whole fruit	13.565 ± 0.016^{h}	0.451 ± 0.021^{i}
29	*Sechium edule*	Underground part	3.278 ± 0.021^{wx}	0.008 ± 0.0001^{EF}
30	*Solanum anguivi*	Whole fruit	2.306 ± 0.098^{A}	0.101 ± 0.01^{wxy}
31	*Solanum incanum*	Epicarp & mesocarp	1.749 ± 0.014^{B}	0.128 ± 0.08^{v}
		Endocarp	1.606 ± 0.009^{BC}	0.207 ± 0.09^{pqr}
32	*Spondias mombin*	Fruit without seed	28.781 ± 0.076^{c}	1.024 ± 0.06^{b}
33	*Xanthosoma brasiliense*	Underground part	1.140 ± 0.009^{DE}	0.016 ± 0.001^{EF}
34	*Zanthoxylum acanthopodium*	Whole fruit	4.481 ± 0.013^{s}	0.584 ± 0.024^{g}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c… A, B etc) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT)

properties of their constituents. The extracts of different types of plants of Darjeeling Himalaya like *C. erectus, C. betacea, C. glaucescens, D. alata, D. hamiltonii, Evodia fraxinifolia, Elaeocarpus lanceifolius, I. batatas, L. cubeba, M. edulis, Manihot esculenta, Prunus domestica, Pyrus communis, Persea americana, S. mombin, Sechium edule, Z. acanthopodium* and *Xanthosoma brasiliense* may be used as dyes due to the presence of anthraquinones (Table 5.2). Glycoside and tri-terpenoids decreased blood sugar level (Cherian and Augusti, 1995; Luo *et al*., 1999). *C. erectus, Solanum anguivi* and *S. incanum* fruits contained these two antidiabetic compounds (Table 5.2). The presence of these two secondary metabolites in these fruits supports the traditional medicinal use of these plants in the treatment of hypoglycemic disease. Alkaloid is another type of antidiabetic agent (Oliver, 1980) which is present in two *Solanum* fruits (Table 5.2). Due to this characteristic feature, it is confirmed that *Solanum* fruits have potency to prevent hyperglycemia.

For determining the precise relationship among different existing phytochemicals of various edible plant parts, principal component analysis (PCA) was performed. The results of PCA are shown in Figure 5.2. Two principal components explaining the 57.46% of data variance have been chosen on the basis of their eigenvalues (>1). The first principal component (PC1 36.50%) correlates well with median polar components like anthraquinones, steroids, glycosides, triterpene etc. having factor loadings ranging between 0.526 to 0.880 (Table 5.4). This correlation indicates that the accumulations of median polar components are clustered together and might be utilized by plant system for mitigation of emergent stress through some common mechanism. The second principal component (PC2 20.95%) was loaded with amino acids and total phenols but interestingly they are negatively correlated with each other, indicating that aromatic amino acids are mainly allocated for biosynthesis of polyphenols through phenylpropanoid pathway mainly by enzyme Phenylalanine Ammonia Lyase (PAL). Similarly Factor-3 was related with amphipathic compounds like saponin and flavonoids; whereas the polar charged compound alkaloids is separately coordinated on 4th principal component. Antioxidants and phytochemical concentration

Table 5.4 Explained variability of the first five principal components (PCs) on the basis of factor loading from the Principal Component Analysis (PCA)

	F1	F2	F3	F4	F5
ALKALOID	0.108	0.258	0.096	**0.898**	-0.196
AMINO ACID	-0.160	**0.845**	0.226	0.009	-0.279
ANTRAQUINONES	**0.754**	-0.321	-0.164	0.146	-0.185
STEROIDS	**0.601**	0.533	0.028	-0.028	-0.135
GLYCOSIDES	**0.880**	-0.110	-0.160	-0.124	-0.241
SAPONINS	0.405	0.489	**-0.557**	0.181	0.247
TANNINS	**0.596**	0.548	0.311	-0.037	0.209
REDUCING SUGAR	**0.526**	0.508	0.209	-0.379	-0.116
TRITERPEN	**0.653**	-0.398	-0.475	0.091	-0.042
CARDIAC GLYCOSIDES	**0.870**	-0.022	-0.025	-0.133	-0.110
RESIN	**0.795**	0.098	0.098	0.075	0.467
TOTAL PHENOL CONTENT	0.470	**-0.658**	0.375	-0.086	-0.124
TOTAL FLAVONOID CONTENT	0.413	-0.379	**0.680**	0.255	0.135

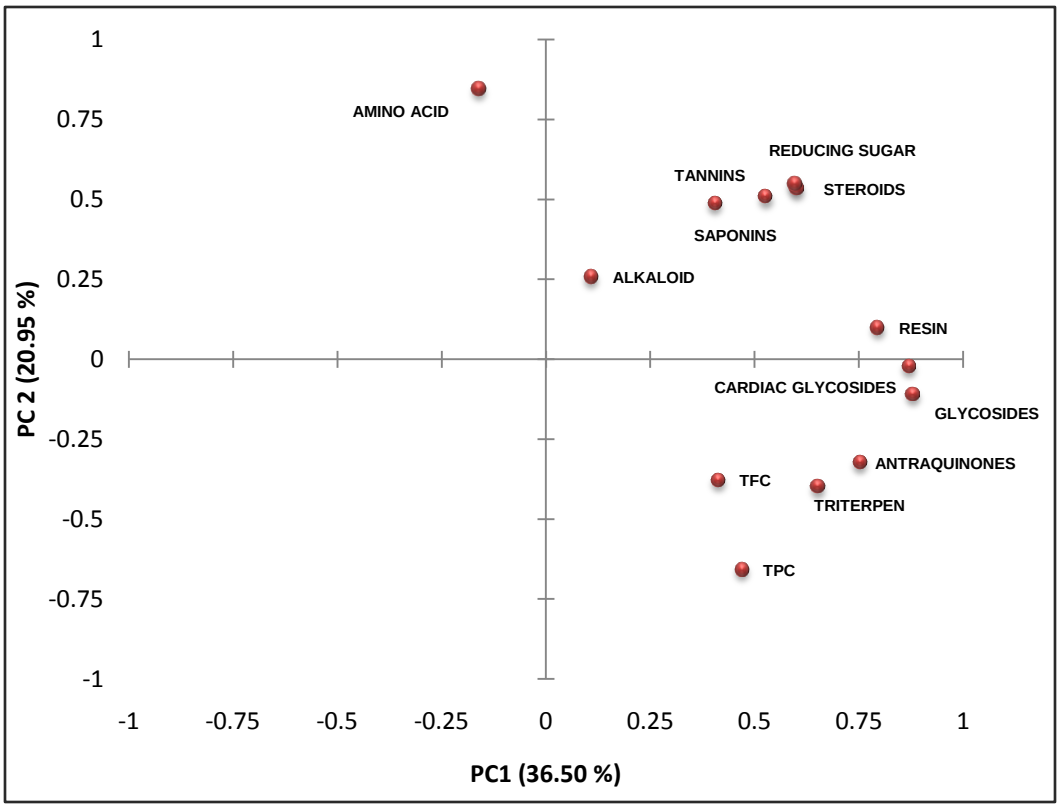


Figure 5.2 Principal component analysis factor loading plot of different secondary metabolites of different edible fruits and vegetables

are proved to be very important for nutritional classification of edible fruits and vegetables and PCA helps for clustering these nutritional attributes (Mditshwa *et al*., 2013).

This study therefore provided groundwork to the folkloric use of these plants as the remedy for diabetes, blood pressure, diarrhea, gastric, and other infections caused by the pathogens. It also justifies the ethno-medicinal uses and claims about the therapeutic values of these plants by Local people of Darjeeling Hills as curative agent and we therefore, have proceeded for the purification and characterization of the phytochemicals in later chapter. We believe that these compounds could be harnessed for their utilization in industrial as well as medicinal purposes.

Chapter – VI

PHARMACOLOGICAL EVALUATION OF ETHNO-MEDICINALLY IMPORTANT ANTIDIABETIC PLANTS OF DARJEELING HIMALAYA

6.1 INTRODUCTION

Medicinal plants are the backbone of the traditional system of therapy (Ramya *et al*., 2008). About 80% of the world population relies on traditional plant based medicines and till now various rural and tribal communities of India depend solely on Indian System of Herbal Medicine. Ethno-botanical information indicates that more than 400 plants are used as traditional therapies for the treatment of diabetes (Kumar *et al*., 2012). The term diabetes mellitus is the most prevalent metabolic disorder characterized with increased blood sugar level and improper primary metabolism (Patel *et al*., 2012). It is the most common disease in the world affecting 25% of population and troubles 150 million people and is set to rise to 300 million by 2025 (Vats *et al*., 2005). Diabetes also gives rise to various secondary problems such as retinopathy, peripheral vascular insufficiencies and neuropathy. These secondary problems take place due to the oxidative stress and DNA damage caused by the generation of free radicals in the cells (Carvalho *et al*., 2012). Diabetes is still not completely curable by the present antidiabetic agents. Insulin therapy is the only satisfactory approach in diabetes mellitus, even though it has several drawbacks like insulin resistance, anorexia, brain atrophy and fatty liver in chronic treatment (Piedrola *et al*., 2001). Diabetes mellitus is associated with increased oxidative stress. Free radicals are continuously produced in the body as the result of normal metabolic processes and interaction with environmental stimuli. The level of lipid peroxidation in the cell is controlled by various cellular defense mechanisms consisting of enzymatic and non-enzymatic scavenging systems during oxidative stress mediated propagation of reactive oxygen and nitrogen species (RONS), which is always related with diabetes (Peng *et al*., 2011; D'Orazio *et al*., 2012). But disturbances of innate antioxidant defense mechanism in prolonged diabetic condition showed alteration in antioxidant enzyme levels such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) along with impaired glutathione (GSH) metabolism (Lennan *et al*., 1991). Hence it is necessary to bring out the antidiabetic components which may also overcome the oxidative stress and hyper-lipidemic problems. The

hypoglycemic activity of a large number of these plants have been evaluated and confirmed in different animal models (Eidi *et al*., 2005). But unfortunately therapeutic potential of most of the established antidiabetic herbal products are lesser than standard synthetic drugs. Again the hypoglycemic plants which are non-edible are of limited use in therapeutics in ultra-high dose as intensive toxicity assessment for most of them are not yet completed. So the primary objective of herbal drug research should be to identify such kind of edible antidiabetic plant which has very less amount of toxicity and can be consumed in any required quantity for managing hyperglycemic condition. As we now know that many edible fruits and vegetables of Darjeeling Himalaya (discussed in Chapter – III) are traditionally used as antidiabetic agent by local people of this region, we would like to confirm the truthfulness of this information in this chapter.

6.2 MATERIALS AND METHODS

6.2.1 Plant samples collection and identification

Plants collection and identification were specified in Chapter III Section 3.2.2.

6.2.2 Preparation of methanolic plant extracts

Different types of traditionally used antidiabetic edible plant extracts of Darjeeling Himalaya were prepared by methanol. The procedure of extraction was specified in Chapter III Section 3.2.2.

6.2.3 Animal materials

Male Wister albino rats having weights of 100-140 g were purchased from Ghosh Enterprise, Kolkata. For experimentation, the animals were grouped, housed in polypropylene cages and were kept in quarantine for 10 days under standard husbandry conditions (25 ± 2°C, relative humidity, 65 ± 10%) for 12 h dark and light cycle respectively. All animals were fed with

standard laboratory diet (Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. The study was permitted by the Institutional Animal Ethical Committee.

6.2.4 *In vitro* methods employed in antidiabetic studies

6.2.4a Inhibition of *α*-amylase enzyme

α-Amylase was premixed with the aqueous extract at various concentrations (50-250 µg/ml) and starch (0.5% w/v) as a substrate was added to start the reaction. This was carried out at 37°C for 5 min and terminated by addition of 2 ml of DNS (3,5-dinitrosalicylic acid) reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath (Heidari *et al.,* 2005). *α*-Amylase activity was determined by measuring concentration of α-amylase inhibitor to inhibit 50% of its activity under the assay conditions.

$$\% \text{ Inhibition} = (A_{540\ control} - A_{540\ sample})/(A_{540\ control}) \times 100,$$

Where $A_{540\ control}$ = Absorbance of control at 540 nm and $A_{540\ sample}$ = Absorbance of sample at 540 nm.

6.2.4b Inhibition of α-glucosidase enzyme

α-Glucosidase inhibitory activities were assayed according to Oki *et al.,* (1999) with slight modifications. The reaction was initiated with 0.05 ml each of the samples at different concentrations in 0.2 Mm phosphate buffer (pH 6.8), followed by incubation at 37°C for 15 min, after which 0.1 ml of enzyme solution was immediately added to the mixture before mixing and incubation at 37°C. Then, 3 Mm *p*-nitrophenylglucopyranoside (pNPG) (0.25 ml) was added, after which the reaction was stopped by the addition of 4 ml of 0.1 molar Na_2CO_3. α-Glucosidase inhibitory activity was determined by measuring the release of pNPG at 405 nm. The control contained all reagents without the tested sample. The reactions were conducted in triplicate. The α-glucosidase inhibitory activity was calculated as follows: Inhibitory ratio % = [1- (As- Ab)/ Ac] × 100

where Ac, As, and Ab represent the absorbance levels of the control, sample, and blank, respectively. The concentration of α-glucosidase inhibitor required to inhibit 50% of α-glucosidase activity under the assay conditions is defined as the IC_{50} value.

6.2.5 *In vivo* methods employed in antidiabetic studies

6.2.5a Experimental induction of diabetes

After fasting for 18 h, 35 rats (average 120gm each) (Figure 6.1A) were injected intra-peritonealy with a single dose of 55 mg/kg streptozotocin after dissolving it in freshly prepared ice cold 0.1 M citrate buffer (pH 4.5) (Siddique *et al*., 1987). After the injection they had given free access to feed and water and were given 5% glucose solution to drink over night to counter the hypoglycemic shock. The development of diabetes was confirmed after 48 h of the streptozotocin injection Figure 6.1B. Blood glucose content was measured by using Accu-Chek$^{(R)}$ Active glucometer (made in India) (Figure 6.1C, Figure 6.1 D, and Figure 6.1 E). The animals having fasting blood glucose levels more than 200 mg/dl were selected for the experimentation. Out of 35 animals three were died before grouping and two was omitted from the study because of mild hyperglycemia (below 150 mg/dl).

6.2.5b Experimental design

The 30 diabetic animals were divided into 6 groups each having 6 rats and extra 5 non-diabetic rats are taken for control group (Brosky and Logothetopoulos, 1969). The standard used was glibenclamide and was given to one group. The remaining groups were administrated orally with four different doses viz. 50, 100, 200 and 400 mg/kg body weight of powered drug of *C. erectus* dissolved/suspended in water by tween80 (1% v/v) (Figure 6.1F).

- Group I Normal saline treated rats
- Group II STZ treated rats

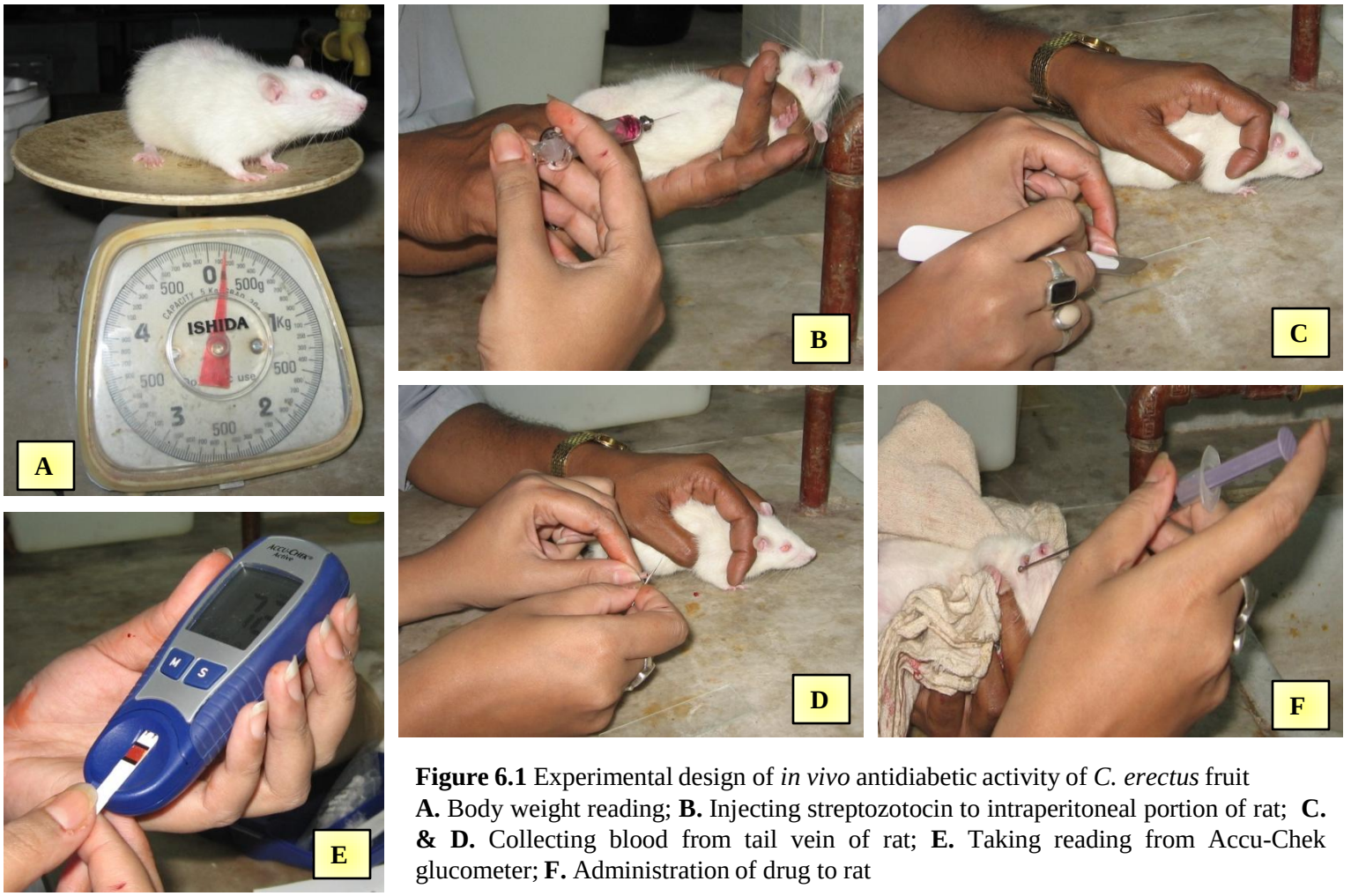


Figure 6.1 Experimental design of *in vivo* antidiabetic activity of *C. erectus* fruit
A. Body weight reading; **B.** Injecting streptozotocin to intraperitoneal portion of rat; **C. & D.** Collecting blood from tail vein of rat; **E.** Taking reading from Accu-Chek glucometer; **F.** Administration of drug to rat

- Group III Diabetic rats given aqueous solution of glibenclamide 10 mg/kg per day for 14 days
- Group IV Diabetic rats given suspension of *C. erectus* (50 mg/kg, per day p.o for 14 days)
- Group V Diabetic rats given suspension of *C. erectus* (100 mg/kg, per day p.o for 14 days)
- Group VI Diabetic rats given suspension of *C. erectus* (200 mg/kg, per day p.o for 14 days)
- Group VII Diabetic rats given suspension of *C. erectus* (400 mg/kg, per day p.o for 14 days)

On day 14th, blood was collected by cardiac puncture (Figure 6.2) under mild ether anesthesia from overnight fasted rats and fasting blood sugar (Giordano *et al*., 1989) was estimated. Serum was separated and analyzed for serum cholesterol, serum triglycerides, serum HDL and LDL. Total cholesterol, triacylglycerol, and HDL-c levels in plasma were determined by enzymatic kits that were procured from Bayer's Diagnostics Pvt. Ltd., Baroda, India. LDL-c levels were calculated by using Friedewald's equation.

The rat liver was weighted and 10% liver homogenate was prepared with 0.1 M phosphate buffer (pH 7.0) after centrifugation at 1000 rpm for 15 min. The supernatant was used to measure Lipid peroxidation (LPO), SOD and CAT.

6.2.5c Lipid peroxidation

Lipid peroxidation was estimated by the method of Ohkawa *et al*. (1979). Liver homogenate was mixed (1 ml) with 100 µl of 8.1% sodium dodecyl sulfate (SDS), and 600 µl of 20% acetic acid solution was kept for 2 min at room temperature, then 600 µl of 0.8% solution of TBA was added, heated at 95ºC for 60 min in water bath and cooled with ice cold water at 4ºC. The mixture of n-butanol and pyridine (15:1, v/v) were added, shaken vigorously and centrifuged at 10000 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm.

Figure 6.2 Dissected rat to collect liver and cardiac puncture for *in vivo* study of diabetes

6.2.5d Superoxide dismutase

The SOD was estimated by the method of Beauchamp and Fridvich (1971) and Chidambara *et al.* (2002), based on the reduction of NBT. 0.5 ml of liver homogenate, 1 ml of 50 mM sodium carbonate, 0.4 ml of 24 µm NBT, and 0.2 ml of 0.1 mM ethylenediaminetetraacetic acid (EDTA) were added. The reaction was started by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by 5 min at 25º C. The control was determined without liver homogenate.

6.2.5e Catalase

Catalase was estimated by using the method of Sinha (1972). 0.1 ml of liver homogenate, 1 ml of 0.01 M phosphate buffer (pH 7.0) and 0.4 ml of 2 M hydrogen peroxide was mixed. The reaction was stopped by the addition of 2 ml dichromatic acetic acid reagent. The control was carried out without addition of hydrogen peroxide. Absorbance was measured at 620 nm.

6.2.6 Toxicity testing against brine shrimp

Brine shrimp eggs, *Artemia salina* were hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 liter of distilled water. After 24 h incubation at room temperature (22-29º C), the hatching shrimp larvae was attracted to one side of the vessel with a light source and collected by pipette (Figure 6.3A). Larvae (Figure 6.3B) were separated from eggs by aliquoting them three times in small beakers containing seawater.

6.2.6a Brine shrimp assay

Toxicity of the extract was monitored by the brine shrimp lethality test (Meyer *et al*., 1982). Two ml of seawater was placed in all the bijoux bottles. A two-fold dilution of methanol extract was carried out with the artificial seawater to obtain the concentrations ranging from 50 mg/ml to 0.098 mg/ml. Potassium dichromate was used as a positive control and was prepared by dissolving it in artificial seawater to obtain concentration ranging from 0.1 to 0.9 mg/ml

Figure 6.3A Hatching of Brine Shrimp egg to larvae

Figure 6.3B Brine Shrimp larvae

(Colegate and Molyneux, 1993). The last bottle was filled with sea salt water only to serve as a drug-free control or negative control. 100 µl of suspension of larvae containing about 10-15 larvae was added into each bottle and incubated for 24 h. The bottles were then examined and the number of dead shrimp in each bottle was counted. The total number of shrimp in each bottle was counted and recorded. Cytotoxic drug Etoposide was taken as standard (positive control).

6.2.7 Statistical analysis

Statistical analysis of the biochemical estimates were performed using SPSS 12.0 (SPSS Inc. Chicago, USA). The results were expressed as mean ± SEM. Significant differences ($p < 0.05$) between means were determined using Duncan's Multiple Range Test (DMRT) with the help of DSAASTAT ver. 1.022. Principal component analysis (PCA) of biochemical estimates were calculated by using Multivariate Statistical Package (MVSP 3.1) and dendrogram preparation were done through XLSTAT 2009 Version 1.02.

6.3 RESULTS AND DISCUSSIONS

Oxidative stress in diabetes has been shown to co-exist with a reduction in the antioxidant status (Boynes, 1991). Oxidative stress may have significant effect in the glucose transporter protein as well as at insulin receptor (Jacqueline *et al*., 1997). Scavengers of oxidative stress may have an effect in reducing the increased serum glucose level in diabetes and may alleviate the diabetes as well as retarding its secondary complications. This information is parallel to our study; since we have found that plant extracts like *Calamus erectus, Solanum incanum, S. anguivi, Cyphomandra betacea*, and different types of Taruls like *Dioscorea alata, D. hamiltonii, Ipomoea batatas* (red and white), *Manihot esculenta, Sechium edule* and *Xanthosoma brasiliense* which are being used in the traditional medicine to reduce the serum glucose level, have significant antioxidant (already discussed in Chapter – IV as well as antidiabetic activity *in vitro*. Lack of insulin influence the metabolism of carbohydrates, proteins, fat and take part in water and electrolyte homeostasis

disturbance (Frier and Fisher, 2006). α-Amylase and α-Glucosidase which are important in carbohydrate digestion as well as glucose absorption lead to the development of newer pharmacological agents. α-Glucosidase enzymes play cardinal roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed in the intestinal lumen and in brush border membrane. It was recorded that suppression of the action of such digestive enzymes would interrupt the degradation of starch and oligosaccharides, which would reduce the absorption of glucose and consequently decrease of postprandial (PP) elevation of blood glucose level (Puls *et al*., 1997). α-Glucosidase inhibitor delays the digestion of carbohydrates and retards the absorption. Hence one of the therapeutic approaches for reducing PP blood glucose levels in diabetes mellitus patient is to prevent absorption of the carbohydrate after food intake (Davis and Granner, 2001). Inhibition of α-amylase and α-glucosidases reduced the high PP blood glucose peaks in diabetes (Conforti *et al*., 2005). The α-amylase inhibitors like acarbose and miglitol act as an anti-nutritie factor that obstructs the digestion. Among them acarbose is complex oligosaccharides that cause hindrance in digestion of carbohydrates. These oligosaccharides slow down the activity of pancreatic amylase in breakdown of starch. Synthetic inhibitor also causes side effect such as diarrhea, abdominal pain and soft feces in the colon. The data obtained from Figure 6.4 and Figure 6.5 state that the antidiabetic action of these plants may also be attributed to the intestinal α-glucosidase and α-amylase inhibitory activity. The above observations support the idea of traditional use (as discussed in Chapter–III) of these plants to cure diabetes and its patho-physiological complications through herbal mode. Among these fruits and vegetables, *C. erectus* and *D. alata* showed higher *in vitro* antidiabetic activity (Figure 6.4 and Figure 6.5). Cluster analysis with these plant parts was performed for determining their cohesiveness on the basis of antidiabetic activity (Figure 6.6). This clustered dendrogram was based on dissimilarity matrix among traditionally used antidiabetic plants with their inhibitory potential of two important digestive enzymes of glucose metabolism; i.e. α-amylase and α-glucosidase. On the basis of these characters, plants were classified with three main groups:

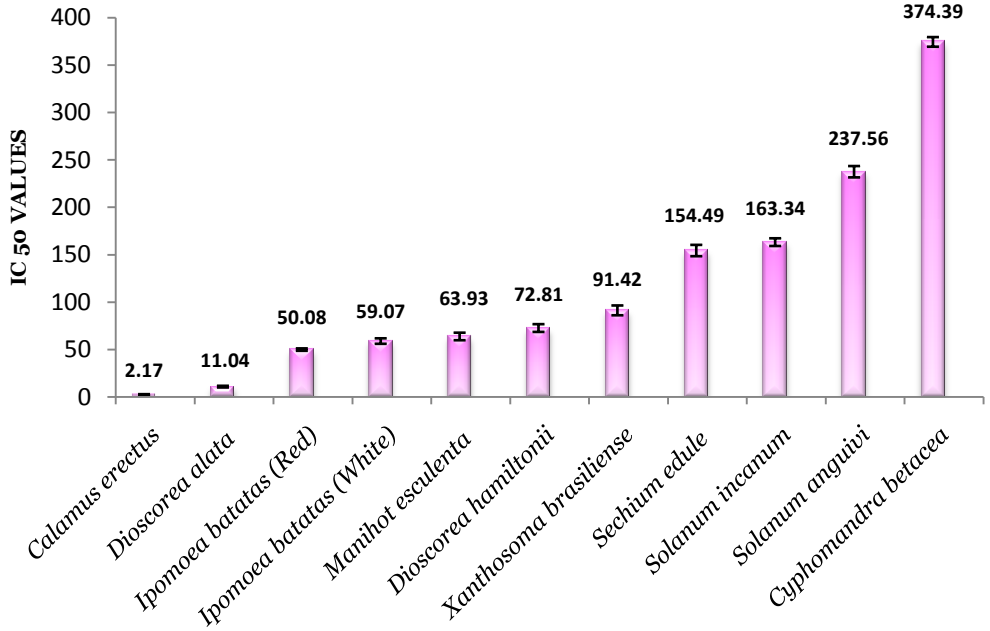


Figure 6.4 α-Glucosidase scavenging (IC_{50}) activity of different plants of Darjeeling Himalaya

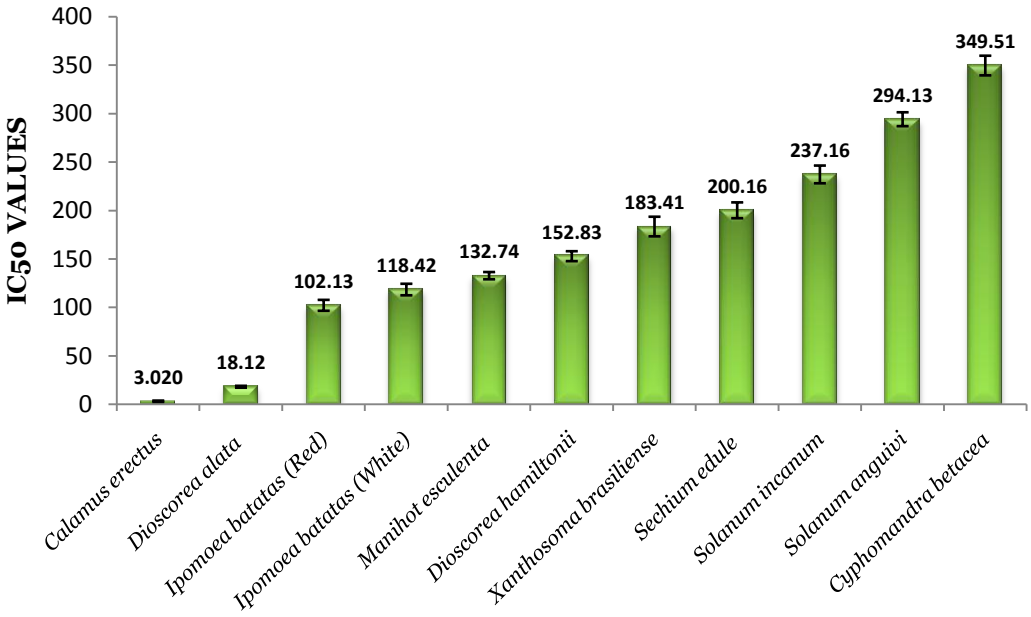


Figure 6.5 α-Amylase scavenging (IC_{50}) activity of different plants of Darjeeling Himalaya

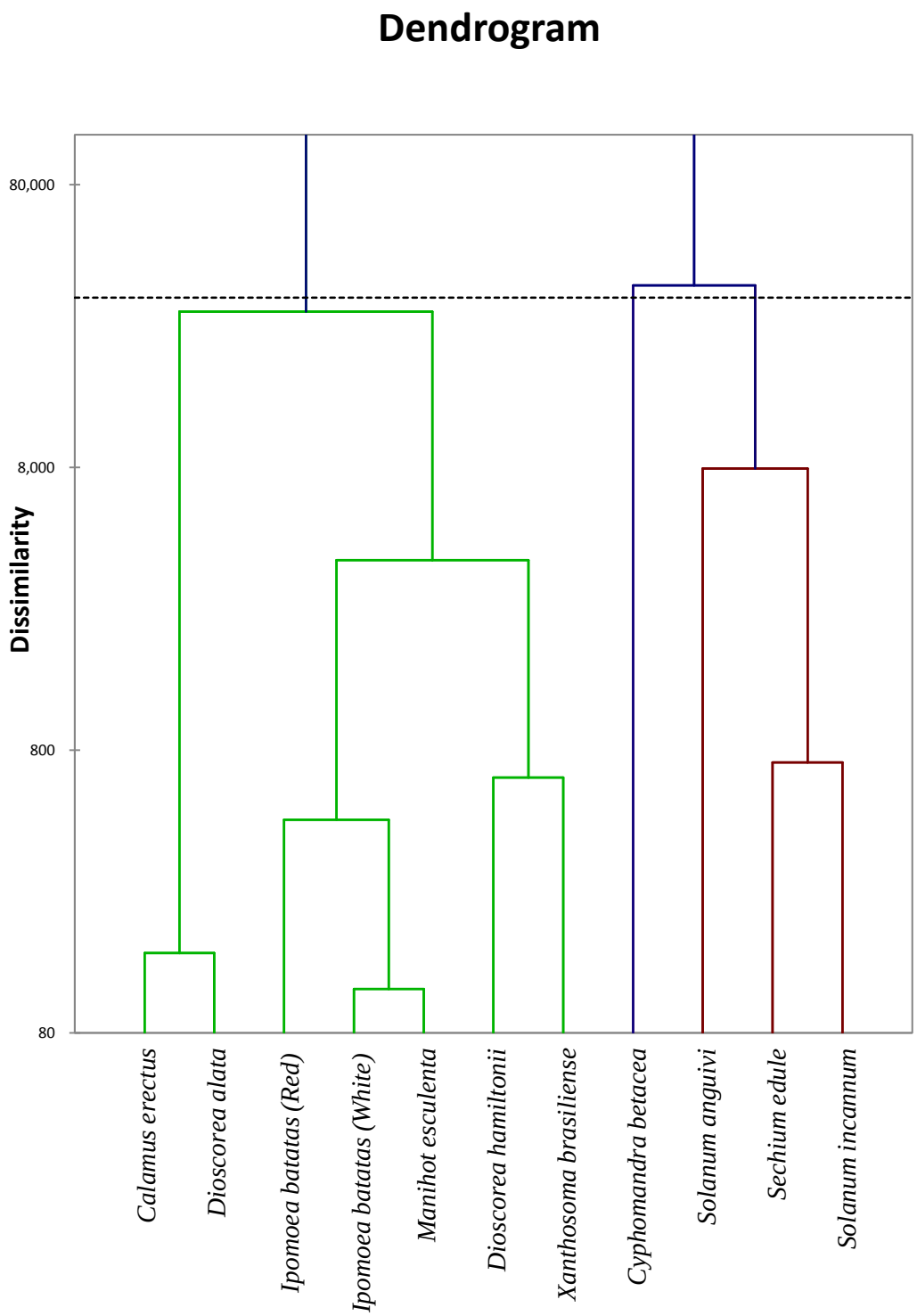


Figure 6.6 Dendrogram showing relationships among the edible plants of Darjeeling Himalaya based on antidiabetic activity

Group I contains *C. erectus* and *D. alata* with excellent *in vitro* antidiabetic potential, Group-II belongs to underground parts and Group-III mainly represents the members of Solanaceae.

Many authors reported that *Dioscorea* plant has significant hypolipidemic as well as hypoglycemic activity (Ahmed *et al*., 2009b; Ashajyothi *et al*., 2012). Though ethno-medicinally well recognized, until now there is no published scientific reporting, where pharmacological investigations related to antidiabetic activity of *C. erectus* plant have shown. Hence after *in vitro* study the fruit of *C. erectus* was investigated for its hyperglycemic and hypo-lipidemic activity *in vivo* in streptozotocin induced diabetic rat model and to compare the same with glibenclamide, a standard hypoglycemic drug. Administration of STZ selectively destroys the β-cells of the islets of Langerhans (Elsner *et al*., 2000). The destruction of β-cells cause the marked decrease in insulin levels (Gilman *et al*., 2001). In this study the results indicate that fruit drug of *C. erectus* can able to decrease the level of blood glucose in STZ induced diabetic rats. A marked rise in fasting blood glucose level was observed (Figure 6.7) in diabetic control group as compared with normal control rats. Methanolic extract of *C. erectus* fruits (at 50, 100, 200 and 500 mg/kg) exhibited a dose dependent significant antihyperglycemic activity on 0, 7^{th} and 14^{th} day post treatment. The antihyperglycemic effect of methanol extract was found to be more effective than the reference standard, glibenclamide which produced a significant reduction in blood glucose compared to diabetic control (Figure 6.7). The hypoglycemic action of this drug in diabetic rats may be possible through the insulinomimetic action or by other mechanisms such as stimulation of glucose uptake by peripheral tissues, inhibition of endogenous glucose production, or activation of gluconeogenesis in liver and muscles, as similar mechanisms have been reported for plant drug with antidiabetic activity (Burcelain *et al*., 1995). Normal control animals were found to be stable in their body weight but diabetic rats showed significant increase in body weight during 14 days. Streptozotocin mediated body weight incrassation was significantly reversed by the methanol extract in dose dependent fashion (at 200 and 400 mg/kg p.o). Results are shown in Figure 6.8. Consequently, the level of GSH, SOD and CAT were significantly improved by the

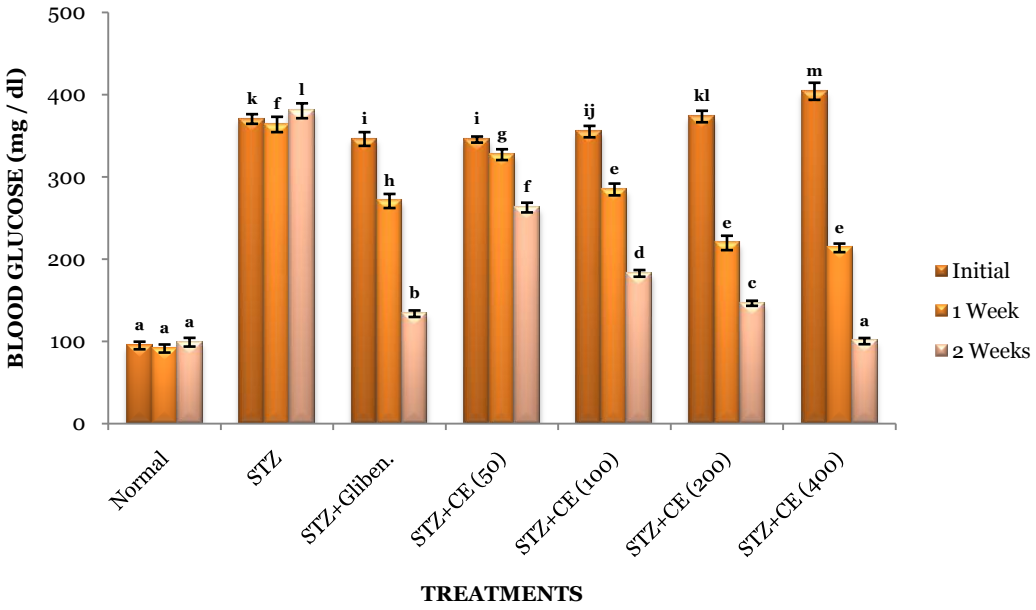


Figure 6.7 Effect of *C. erectus* on fasting blood glucose levels after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean blood glucose levels (±S.E.M.) of six animals

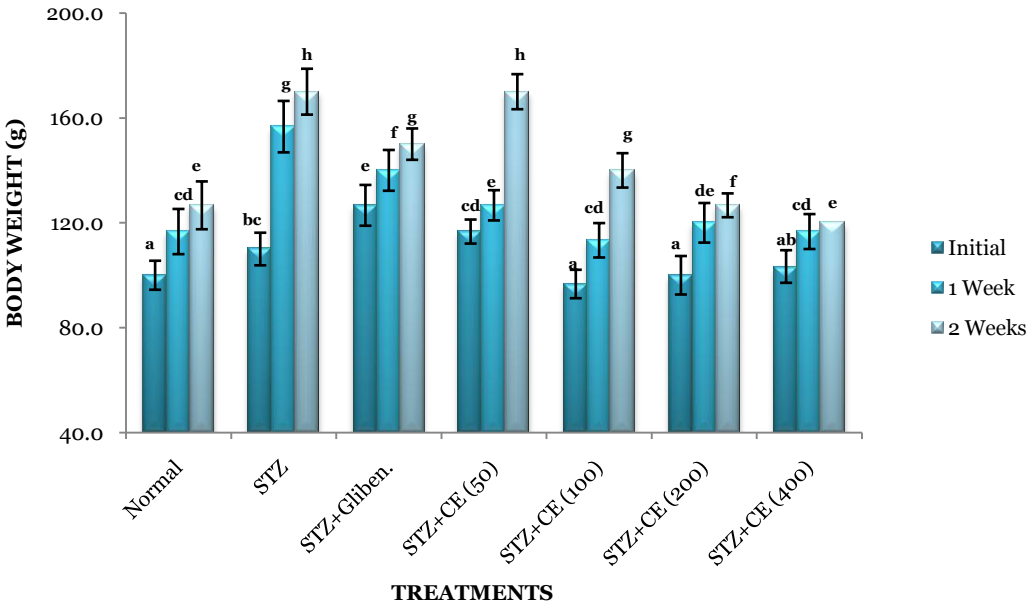


Figure 6.8 Effect of *C. erectus* on body weight after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean body weight (±S.E.M.) of six animals

application of this fruit extract on experimental diabetic rat. Earlier reports indicated that STZ-induced diabetic animals may exhibit most of the diabetic problem mediated through oxidative stress (Sathisheskar and Subramanian, 2005). The GSH, SOD and CAT are the three effective scavenging enzymes that remove free radicals *in vivo* (Jin *et al*,. 2008) as well as they play an important role in restoring antioxidant activities in the tissue of diabetic animals (Tuzum *et al*., 1999; Elmali *et al*., 2004; Rahimi *et al*., 2005). In our study, GSH, SOD and CAT enzymes were significantly decreased in STZ-induced diabetic control rats, may be due to inactivation caused by free radicals. STZ induced diabetic rats were found to decrease SOD, GSH and CAT enzyme level in liver as compared to control. Administration of *C. erectus* fruit drug to the diabetic rats resulted in significant increase in the activities of SOD, GSH and CAT (Table 6.1). STZ diabetic rats were found to exhibit significant increase in TBARS level in lever as compared to control. Treatment with *C. erectus* fruit extract produced significant decrease in TBARS (Table 6.1). The above observation may clearly suggest that increased level of these antioxidants enzymes with *C. erectus* fruit extract may be due to free radical scavenging activity with that extract, which may exert a beneficial effect against pathological alterations caused by reactive oxygen species. Lipid plays an important role in the pathogenesis of complications concerned with diabetes mellitus. The elevated level of serum cholesterol and reduced level of serum HDL cholesterol in diabetic condition may generate versatile factors for developing microvascular complication leading to atherosclerosis, which further culminates into different fatal cardiovascular disorders (Daisy *et al*,. 2008). In the present study, there was a significant reduction in the levels of total cholesterol, triglycerides and LDL-c. The change in the cholesterol, HDL-c and triglyceride level was measured and observed on potent reduction in serum cholesterol and triglycerides and effective elevation in HDL-c level over diabetic control when the rats were fed with aqueous reconstituted methanolic *C. erectus* fruit extract. The level of serum cholesterol was maintained at lower limit in normal rats that were not treated with streptozotocin and enhancement of the same was found in diabetic control (Figure 6.9) whereas maximum reduction was seen (58.33 ± 1.45 mg/dl) after

Table 6.1 Effect of *C. erectus* on *in-vivo* antioxidant parameters from liver homogenate in STZ-induced diabetic rats

Treatment Parameters	**TBARS [mmol/100 g FWT]**	**GSH [mmol/100 g FWT]**	**SOD [unit/min/g tissue]**	**CAT [µmol H_2O_2/min/g tissue]**
Vehicle	4.82 ± 0.060 a	60.82 ± 0.89 a	3.75 ± 0.018 a	16.48 ± 0.62 a
STZ	6.91 ± 0.011 c	41.11 ± 0.91 c	1.98 ± 0.009 b	7.57 ± 0.52 b
STZ + Glibenclamide	4.55 ± 0.049 a	57.29 ± 1.06 ab	3.62 ± 0.015 a	12.59 ± 0.46 ab
(100 mg / kg BW) *C. erectus* **+ STZ**	6.01 ± 0.038 b	49.81 ± 1.18 bc	2.04 ± 0.007 b	10.02 ± 1.05 ab
(200 mg / kg BW) *C. erectus* **+ STZ**	5.63 ± 0.091 b	52.92 ± 0.95 ab	2.88 ± 0.240 ab	13.11 ± 0.89 ab
(400 mg / kg BW) *C. erectus* **+ STZ**	4.41 ± 0.065 a	61.78 ± 0.29 a	3.91 ± 0.052 a	15.93 ± 0.21 a

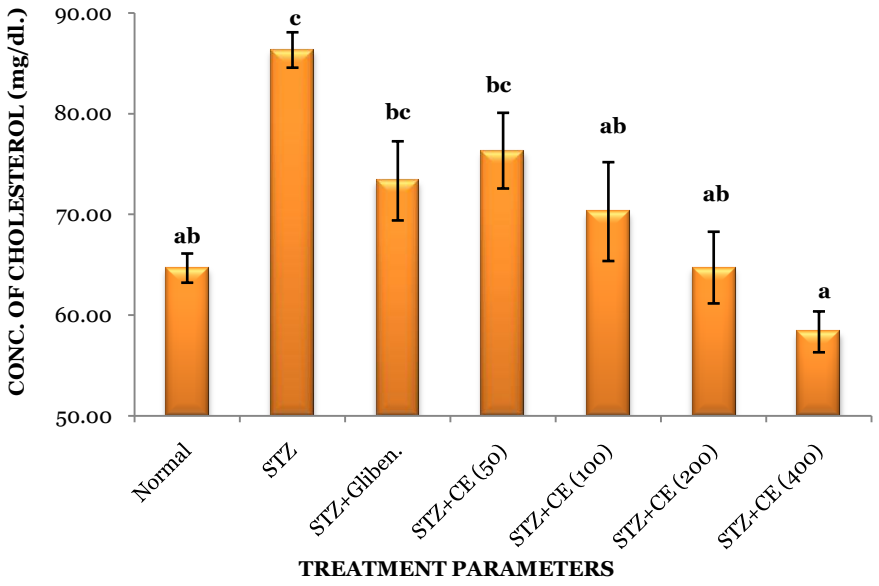


Figure 6.9 Effect of *C. erectus* on serum cholesterol levels after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean cholesterol levels (±S.E.M.) of six animals

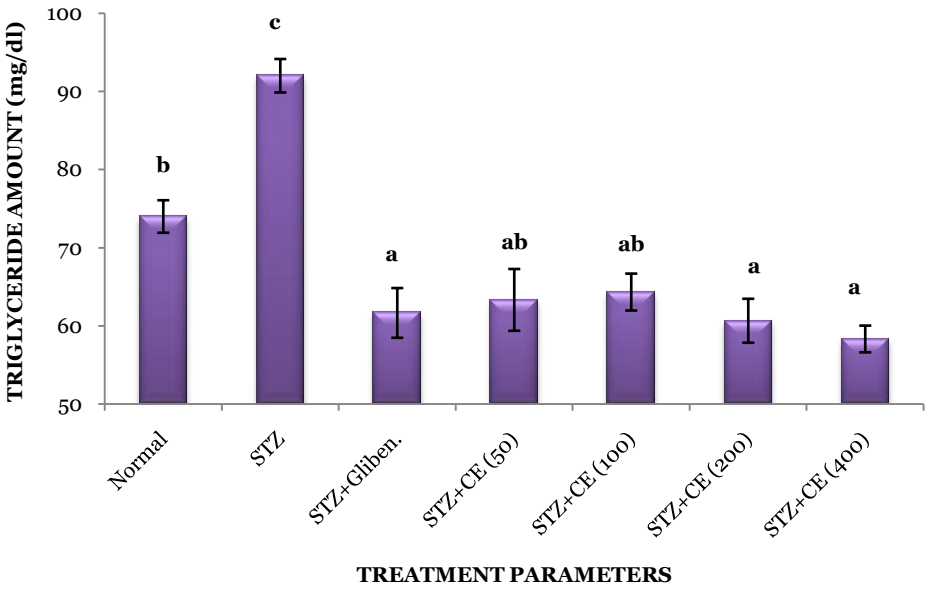


Figure 6.10 Effect of *C. erectus* on serum triglyceride levels after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean triglyceride levels (±S.E.M.) of six animals

the administration of 400 mg / kg body weight treatment of *C. erectus*. When HDL-c was considered, it showed normal range of 42.67 ± 3.18 mg/dl in control rats but maximum elevation were recorded (46.33 ± 4.67 mg/dl) in *C. erectus* treated group VII rats (Figure 6.11). Remarkable reduction of serum triglyceride was noted on feeding at 50, 100, 200, 400 mg fresh weight of extract / kg body weight (Figure 6.10) over control. In the same way LDL level of *C. erectus* treated groups was also significantly decreased from diabetic control on 14th day (Figure 6.12), whereas, HDL/LDL ratio was also increased after feeding of *C. erectus* fruit drug, as shown in (Figure 6.13). Also the observation from our experiments determines that administration of these fruit extracts could enhance the level of HDL in blood through which the progression of atherosclerosis may be declined along with cardio protection (Figure 6.11). The process of harmful cholesterol reduction in association with enhanced HDL/LDL ratio with herbal drug treatment may be due to activation of LDL receptors in hepatocytes which is responsible for taking up LDL into the liver (Khosla *et al*., 1995).

To understand more about the relationship among different biochemical attributes, PCA was applied. PCA on these attributes explained 85.45% of the variability among the data in the first two dimensions and the first PCs' accounted for 73.04% and 12.43% of the data variance (Figure 6.14). The loading of PC1 had a strong positive correlation with body weight, blood sugar level, thiobarbituric acid reactive substances, LDL, cholesterol and triglycerides (Table 6.2). The same principal component also bears strong negative correlation between biochemical attributes mentioned above and enzymological parameter like peroxidase, catalase, superoxide dismutase along with glutathione and high density lipoprotein. Actually the attributes persisting on left coordinate on the plot attenuated body physiology towards betterment by mitigating oxidative stress through endogenous system. If this oxidative stress is not properly ameliorated excess free radical in the system will create metabolic disorder as a result of which biochemical attributes located in right coordinate of PCA will be enhanced (Figure 6.14). This crisis of endogenous antioxidant system might be improved by the application of herbal drugs fortified with adequate

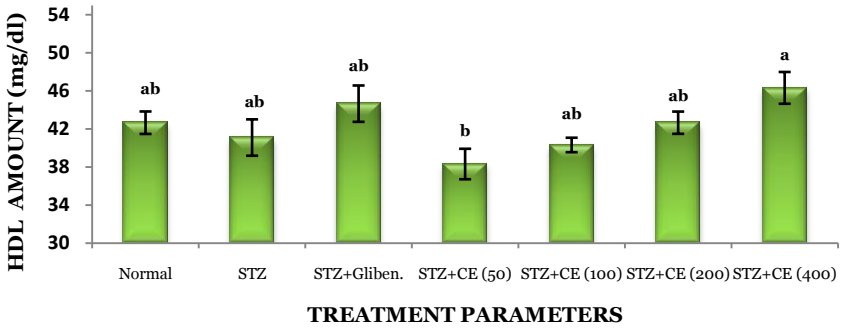


Figure 6.11 Effect of *C. erectus* on HDL levels after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean HDL levels (±S.E.M.) of six animals

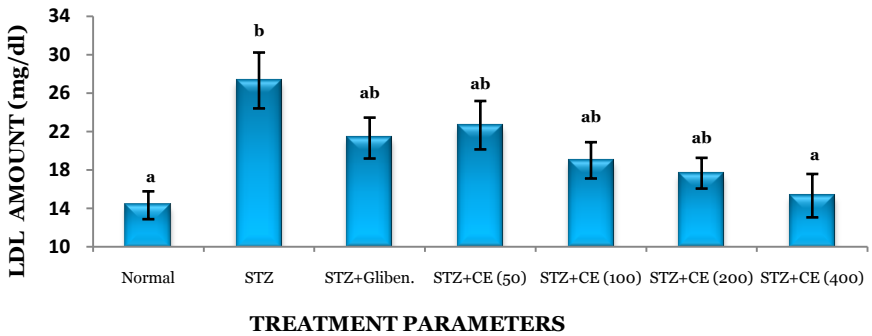


Figure 6.12 Effect of *C. erectus* on LDL levels after interperitonial administration in streptozocin-induced diabetic rats. Each bar indicates the mean LDL levels (±S.E.M.) of six animals

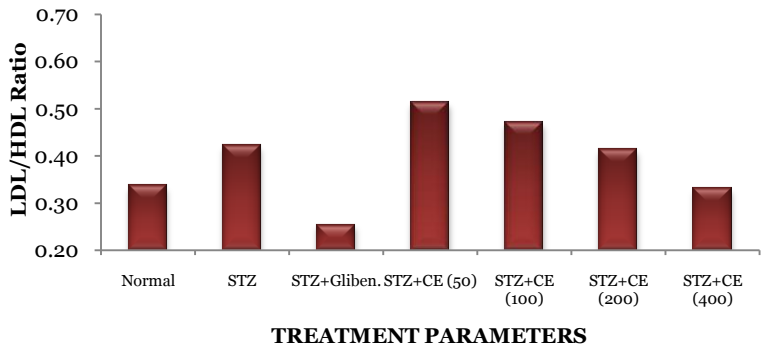


Figure 6.13 Effect of *C. erectus* on LDL/HDL ratio after interperitonial administration in streptozocin-induced diabetic rats

Table 6.2 Explained variability of the first five principal components (PCs) on the basis of factor loading from the Principal Component Analysis (PCA)

PARAMETERS	PC1	PC2	PC3	PC4	PC5
BW	**0.835**	-0.186	0.516	-0.030	-0.016
BS	**0.960**	-0.114	0.091	0.087	0.221
COLES	**0.918**	-0.307	0.222	0.011	-0.115
TGLY	**0.688**	-0.413	-0.265	0.532	-0.027
HDL	**-0.723**	-0.540	-0.319	-0.203	0.203
LDL	**0.896**	-0.318	0.230	-0.185	0.088
HDL/LDL	0.620	**0.751**	0.112	0.085	0.172
TBARS	**0.919**	0.139	-0.356	0.058	0.027
GSH	**-0.955**	0.023	0.268	0.104	-0.023
SOD	**-0.880**	-0.265	0.342	0.158	0.099
CAT	**-0.926**	0.087	0.175	0.306	0.078

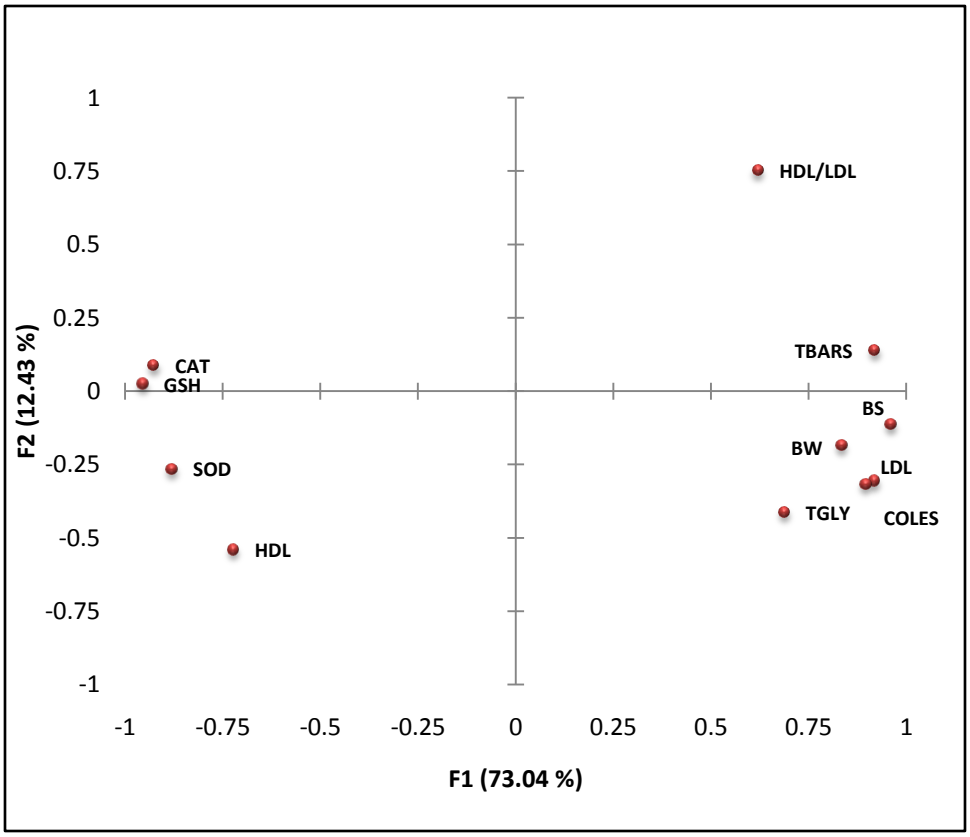


Figure 6.14 Principal Component Analysis factor loading plot of ethnomedicinally important fruits in relation to antidiabetic activity

Table 6.3 Toxic activity of the crude extract of *Calamus erectus* fruit using Brine shrimp lethality test

Plant Specimen / Standard Phytochemical	Extract	*Treatment*	*Petri dish number*	*Total insects evaluated*	*Survival insects*	*Dead insects*	*Percentage of Death*	*Average (%)*	*SD*	$Log_{10} X$	LC_{50} *(ppm)*
			I	12	11	1	8.33				
			II	12	11	1	8.33				
		CONTROL	III	12	11	1	8.33	**11.67**	4.57	-	-
			IV	12	10	2	16.67				
			V	12	10	2	16.67				
			I	12	9	3	25.00				
			II	12	10	1	8.33				
		10 PPM	III	12	10	2	16.67	**15.00**	6.97		
			IV	12	9	1	8.33				
			V	12	10	2	16.67				
			I	12	10	2	16.67				
			II	12	9	3	25.00				
Calamus erectus	Methanolic, aqueous reconstituted	100 PPM	III	12	9	1	8.33	**16.67**	5.89		
			IV	12	8	2	16.67				
			V	12	9	2	16.67			**15.958**	**Not detected (>10,000)**
			I	12	11	1	8.33				
			II	12	9	3	25.00				
		500 PPM	III	12	10	2	16.67	**18.34**	6.98		
			IV	12	10	2	16.67				
			V	12	9	3	25.00				
			I	12	9	3	25.00				
			II	12	10	2	16.67				
		1000 PPM	III	12	10	2	16.67	**20.00**	4.56		
			IV	12	9	3	25.00				
			V	12	10	2	16.67				
			I	12	10	2	16.67				
			II	12	10	2	16.67				
		1 PPM	III	12	11	1	8.33	**13.33**	4.57		
			IV	12	10	2	16.67				
			V	12	11	1	8.33				
			I	12	9	3	25				
			II	12	8	4	33.33				
		5 PPM	III	12	7	5	41.67	**33.33**	5.89		
			IV	12	8	4	33.33				
			V	12	8	4	33.33				
			I	12	6	6	50.00			**0.929**	**8.48**
			II	12	5	7	58.33				
Etoposide	Methanolic, aqueous reconstituted with DMSO	10 PPM	III	12	4	8	66.67	**56.67**	6.97		
			IV	12	5	7	58.33				
			V	12	6	6	50.00				
			I	12	2	10	83.33				
			II	12	3	9	75.00				
		50 PPM	III	12	1	11	91.66	**83.33**	5.89		
			IV	12	2	10	83.33				
			V	12	2	10	83.33				

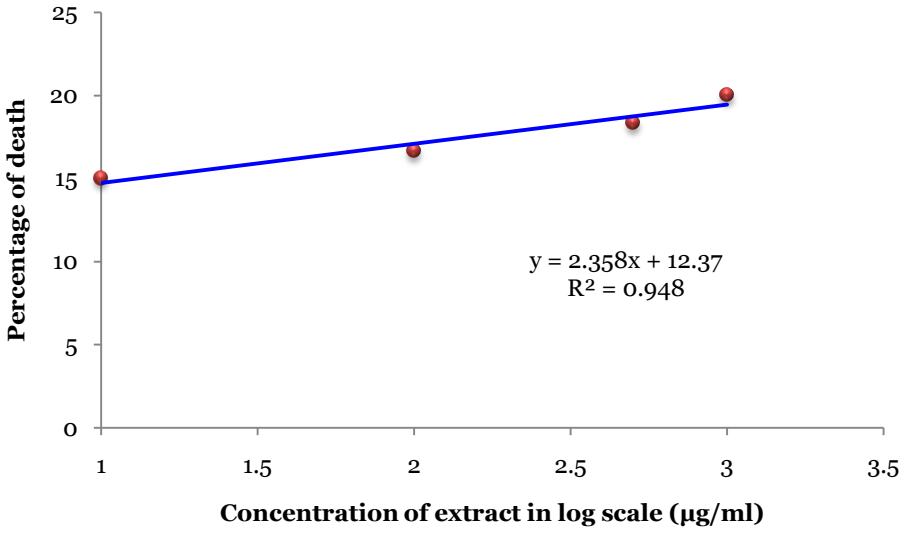


Figure 6.15A Death of Brine shrimp after administration of *C. erectus* extract

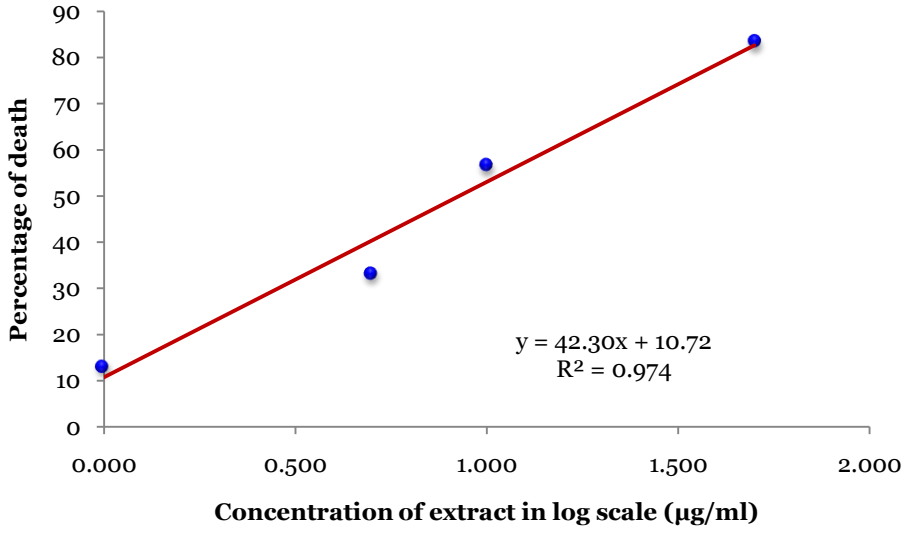


Figure 6.15B Death of Brine shrimp after administration of Etoposide (standard drug)

quantity of polyphenols which might also improve diabetes and other stress mediated metabolic disorders.

According to the standards of American National Cancer Institute, ED_{50} values of ≤ 20 mg/ml for crude phytocomponents are considered active, on that basis we can take the level for median lethal concentration (LD_{50}) up to 200 g/ml (Cordell, 1993). The cytotoxic activities of different traditionally used medicinal plants were investigated by different researchers against brine shrimp (*Artemia salina*) *in vitro*. Different members of Arecaceae plant family were active against brine shrimp. Syahmi *et al*., (2010) reported that methanolic crude extract of *Elaeis guineensis* which belongs to Arecaceae family, was showed positive results against Brine shrimp lethality test. Another Arecaceous plant i.e. *Nypa fruticans* has anti-hyperglycemic activity and has no mortality in the extract (Reza *et al*., 2011). Like *N. fruticans* no significant mortality was recorded after application of fruit extract of *C. erectus* at tested doses (10 µg/ml to 1000 µg/ml) till 24 hrs of observation, when compared with Etoposide standard (8.48 µg/ml) (Table 6.3, Figure 6.15A- 6.15B).

In conclusion, the results of this investigation revealed that methanol extracts of fruits and vegetables possess significant antidiabetic activity and among them *C. erectus* fruits have higher hypolipidemic as well as hypoglycemic potency in a dose-dependent manner, as established in STZ-induced diabetic rat model. This *in vivo* experimental result also showed that the extract is able to improve the oxidative state associated with diabetes along with upgradation of bioactive cholesterol and lipid profile.

Chapter – VII

CHANGES IN ANTIOXIDANT AND ANTI-DIABETIC ACTIVITY OF TWO DIFFERENT FRUITS OF SOLANACEAE DURING MATURATION AND SENESCENCE

7.1 INTRODUCTION

The two different fruits of Solanaceae of Darjeeling Himalaya namely *Cyphomandra betacea* (Cav.) Miers and *Solanum anguivi* Lam. are commonly known among Nepalese as 'Rukh tamatar' and 'Bihi' respectively. The fruits of *C. betaceae* are eaten fresh, cooked in stews and sauces, prepared as chutney, pickles as well as directly consumed with salads and *S. anguivi* is consumed as vegetables. Traditionally these plants are also used as herbal remedy for severe disease like diabetes (Discussed in Chapter – III). Many authors reported that *C. betacea* fruit is typically acidic, is recommended for its nutritional qualities, as a good source of provitamin A, vitamin C, B_6 and E, and iron (Wills *et al*., 1986; Romero-Rodriguez *et al*., 1994; Vera de Rosso *et al*., 2007) and *S. anguivi* fruit contains vital enzymes like maltase, melibiase and saccharase (Rai *et al*., 2000). Harvest time is important to achieve a high quality fruit with storage potential. It is usually considered that different parameters like variety, stages of maturity, season and climatic conditions influence phytochemical composition of fruits (Cordenunsi *et al*., 2002). These secondary metabolites are the main contributors for the presence of antioxidant property as well as antidiabetic activity of plants (Dongre *et al*., 2008; Stangel and *et al*., 2009; Manikandan *et al*., 2013). Since the accumulation of secondary metabolites depend on maturation stages and agro-climatic condition, the pharmacological activity of a plant part could be varied with different developmental changes. So far our knowledge goes; there are no available data on this phase-specific alternation of antioxidants and antidiabetic activity during different developmental stages of these two fruits of Solanaceae. Hence, the present study was aimed to investigate the antioxidant and anti-diabetic potential of fruits of *C. betacea* and *S. anguivi* from Darjeeling hills and the dynamic shifting of these pharmacological properties associated with the stages of maturation.

Through this investigation, we have determined the free-radical scavenging efficacy and *in vitro* antidiabetic capacity of theses fruits from immature to mature stages as well as the quantitative evaluation of phytonutrients like total carotene, lycopene, total phenolics and total

flavonoid content. To our opinion, these types of investigations are required for determining the exact stages of maturation where bioactive phytonutrients are accumulated in maximum amount and those maturation stages of fruits could be utilized as functional food or nutraceutical source for minimizing the use of synthetic drugs.

7.2 MATERIALS AND METHODS

7.2.1 Plant samples collection and identification

Golden-yellow (Figure 7.1a) and Purple-red (Figure 7.1b) variety of *C. betacea* fruits and *Solanum anguivi* (Figure 7.2) fruits were collected at different stages of their maturity from Takdha Basti, Darjeeling, West Bengal, India. Taxonomic position was authenticated by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

7.2.2 Preparation of fruit extracts

The procedure of extraction was specified in Chapter IV Section 4.2.2.

7.2.3 Animal material

For the assay of antilipid peroxidation animal material collection procedure was mentioned in Chapter IV Section 4.2.3.

7.2.4 Determination of *in vitro* antioxidant activity

7.2.4a Determination of DPPH radical scavenging assay

The assay was performed as prescribed by Blois (1958) and specified in details in Chapter IV Section 4.2.4.

Figure 7.1a Different stages of maturity of Golden-Yellow variety of *C. betacea* fruit

Figure 7.1b Different stages of maturity of Purple-Red variety of *C. betacea* fruit

Figure 7.2 Different stages of maturity of *S. anguivi* fruit

7.2.4b Determination of superoxide anions scavenging activity

The assay was performed as prescribed by Nishikimi *et al*., (1972) and specified in details in Chapter IV Section 4.2.6.

7.2.4c Determination of hydroxyl radical scavenging activity

The assay was performed as prescribed by Jung *et al*., (2008) and specified in details in Chapter IV Section 4.2.7.

7.2.4d Determination of reducing power

The assay was performed as prescribed by Aiyegoro and Okoh, (2009) and specified in details in Chapter IV Section 4.2.10.

7.2.4e Anti-lipid peroxidation (ALP) assay

The assay was performed as prescribed by Bauchet and Barrier, (1998) and specified in details in Chapter IV Section 4.2.11.

7.2.4f Total phenol estimation

The assay was performed as prescribed by Folin and Ciocalteu, (1927) and specified in details in Chapter V Section 5.2.3.

7.2.4g Total flavonoids determination

The assay was performed as prescribed by Sultana *et al*., (2009) and specified in details in Chapter V Section 5.2.4.

7.2.4h Estimation of lycopene content

Lycopene content was measured spectrophotometrically at one of its absorption maxima Thimmaiah (2004). 5 ml of methanolic fruit extract was dissolved with 15 ml of acetone. The extract was transferred to a separating funnel containing about 20 ml of petroleum ether and mixed adequately. To it, about 20 ml of 5% Na_2SO_4 solution was added and gently shaken. 20 ml of petroleum ether was added again to the funnel for clear separation of the two layers. The upper layer was mostly coloured. The two phases were separated and the lower aqueous phase was re-extracted with 20 ml of petroleum ether until the aqueous phase become colourless. The petroleum ether extract was pooled and washed once with a little distilled water. The petroleum ether extracts enriched with lycopene was transferred into a brown bottle containing about 10 gm anhydrous Na_2SO_4 and kept aside for 30 minute. Petroleum ether extract was decanted into a 100 ml volumetric flask through a funnel containing cotton wool. The Na_2SO_4 slurry was washed with petroleum ether until it became colourless and was transferred to the volumetric flask. Finally the volume was made up and the absorbance was measured in a spectrophotometer at 530 nm using petroleum ether as blank. The lycopene content was calculated by the formula:-

$$\text{Lycopene Content} = \frac{3.1206 \times \text{final volume} \times \text{Mean value}}{\text{Initial weight of the sample}}$$

Where, 1 O.D = 3.1206 µg/gm

7.2.4i Estimation of the total carotene content

Total carotenoids were estimated spectrophotometrically through solubility based solvent partitioning followed by hydrolysis of bound carotenoid esters with concentrated KOH solution (Vasco *et al*., 2009). 1 ml of methanolic fruit extract was mixed with 2 ml of petroleum ether in separating funnel. Two layer of aqueous phase was observed. The upper phase was collected

whereas the lower phase was decanted, this procedure was repeated thrice. The collected upper phase containing carotenoids was evaporated at 37°C and the residue was dissolved in 2 ml of ethanol. 2 ml of aqueous 60% KOH was added to the residue mixture and boiled for 5 to 10 min. Equal volume of water i.e. 2 ml was added and partitioned thrice with petroleum ether and evaporated. To the reaction mixture 2.5ml of ethanol was added and the absorbance was taken at 450 nm. The carotene content was calculated by the formula:-

$$\text{Carotene content} = \frac{D \times V \times F \times 10}{2500} \text{ mg}$$

Where, D = Absorbance value

V = Volume of original extract in ml

F = Dilution factor

2500= Average extinction coefficient of the pigment

7.2.5 Determination of *in vitro* antidiabetic activity

7.2.5a Inhibition of *α*-amylase enzyme

The assay was performed as prescribed by Heidari *et al.*, (2005) and specified in details in Chapter VI Section 6.2.4a.

7.2.5b Inhibition of α-glucosidase enzyme

The assay was performed as prescribed Oki *et al.*, (1999) and specified in details in Chapter VI Section 6.2.4b.

7.2.6 Statistical analysis

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between phytochemicals and antioxidant as well as antidiabetic attributes and MS Excel of Microsoft Office, 2007 was used for comparing

the antioxidant attributes of different maturation stages of these fruits. The data were analyzed by different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software ver. 1.022; $p<0.05$ was considered significant in all cases. Smith's Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidants, antidiabetic activity and their standard error of estimates (SEE).

7.3 RESULTS AND DISCUSSIONS

Most of the plants under the family Solanaceae contain anthocyanins, which is one of the important group of compounds associated with antioxidant activity and is generally present conjugated with carbohydrates (Cuyckens and Claeys, 2005). Synthetic free radical DPPH can be efficiently used for determining antioxidant activity of several natural defense molecules like cysteine, glutathione, ascorbic acid, tocopherol and other polyhydroxy aromatic phenylpropanoids (Blois, 1958). According to Vasco *et al*. (2009) the peels of both purple-red and golden-yellow variety and the seed-jelly of the purple-red *C. betacea* have high absorbance at 520 nm due to their anthocyanin content. Figure 7.3a shows that DPPH free-radical scavenging efficacy is significantly higher in the purple-red variety than in golden-yellow variety. In both varieties of *C. betacea* fruits and *S. anguivi*, the IC_{50} values are gradually decreased towards immature to mature fruits, which indicate that the accumulation of antioxidant compounds are associated with ripening (Figure 7.3a and 7.4a). Methanol extractive values were also exhibited similar pattern as revealed from Figure 7.3b and 7.4b, and were attained optimally during fruit senescence in both cases.

Carotenoid biosynthesis and its regulation during development and ripening of fruits of Solanaceae is a complex process that occurs during transformation from chloroplasts into chromoplasts and associated changes of the organoleptic properties of the fruit (Bramley, 2002). Figure 7.3c and Figure 7.4c explains that total carotenoid content is present in significant quantity in the mature stages of both fruits (1.92 mg in *S. anguivi*, 3.42 mg and 3.48 mg per kg in golden

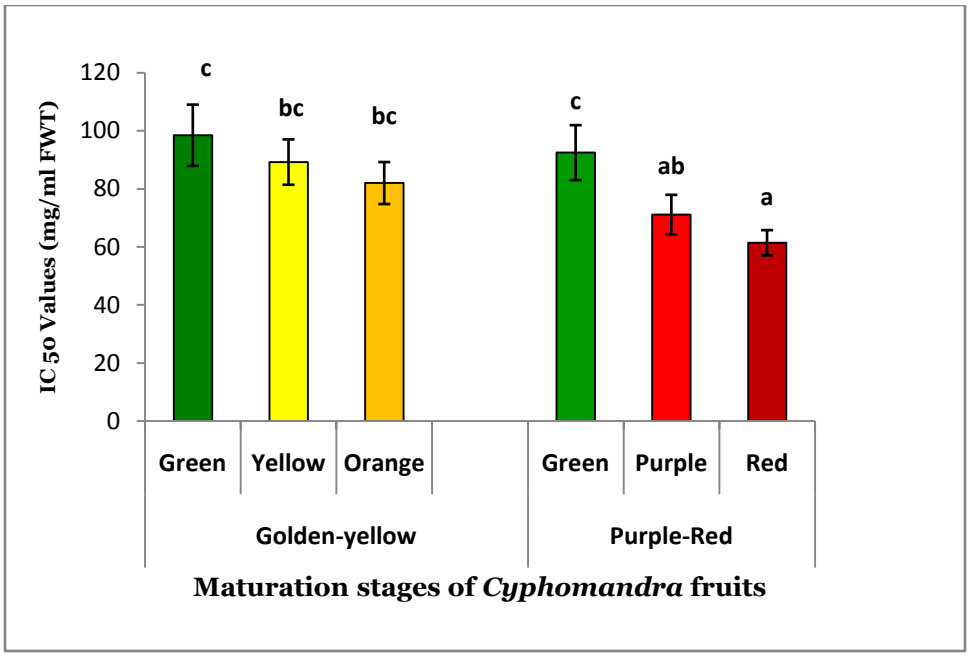


Figure 7.3a DPPH radical scavenging (IC_{50}) activity of different maturation stages of *C. betacea* fruits

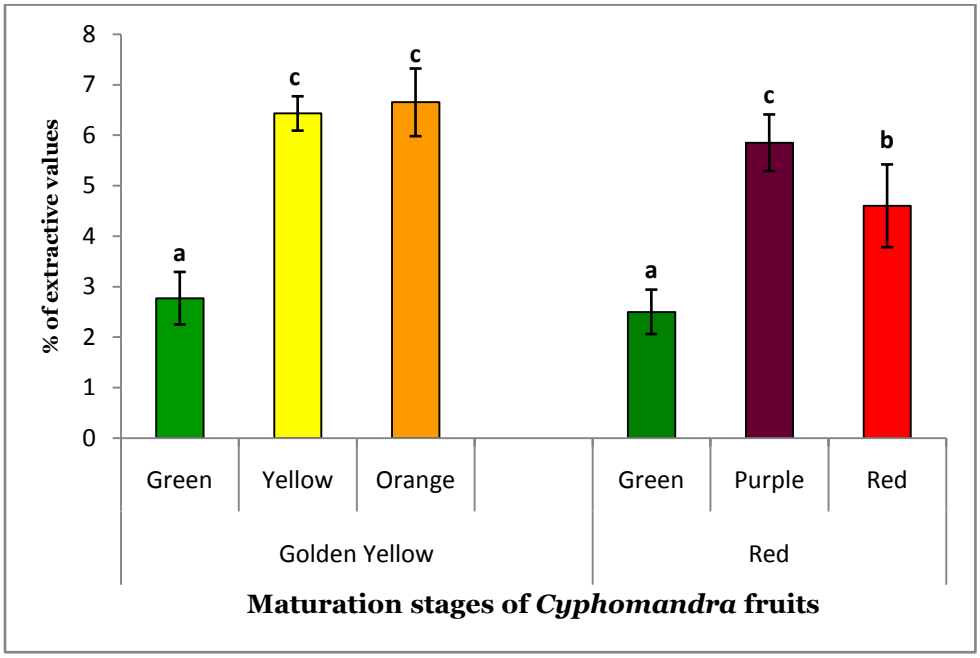


Figure 7.3b Determination of extractive values of different maturation stages of *C. betacea* fruits

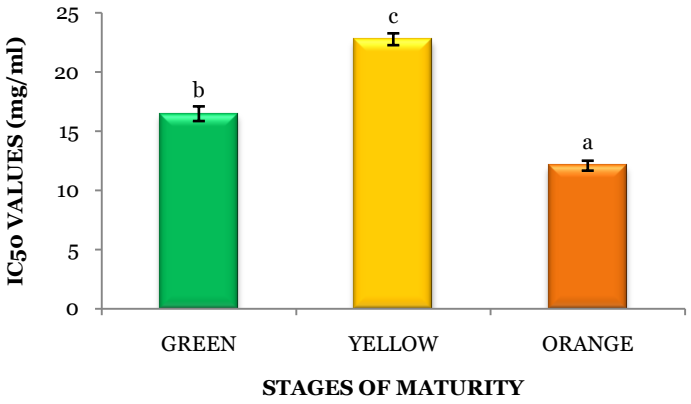


Figure 7.4a DPPH radical scavenging (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

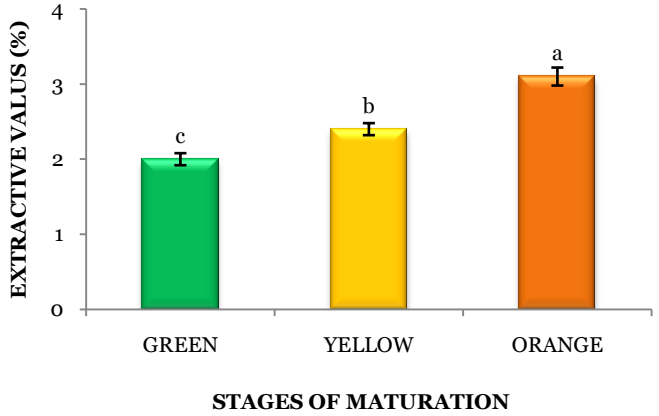


Figure 7.4b Determination of extractive values of different maturation stages of *S. anguivi* fruits

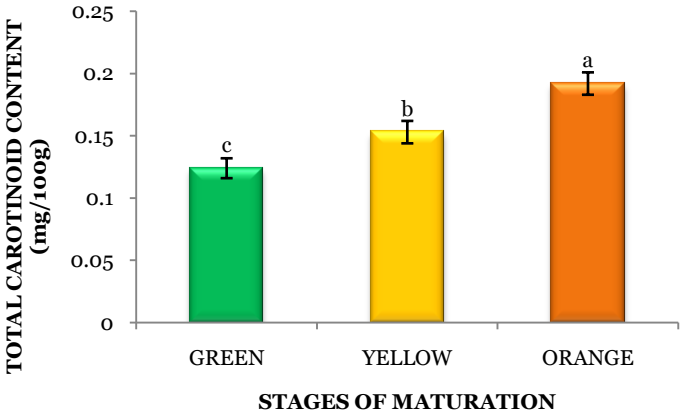


Figure 7.4c Total carotenoid content (mg/100g) of different maturation stages of *S. anguivi* fruits

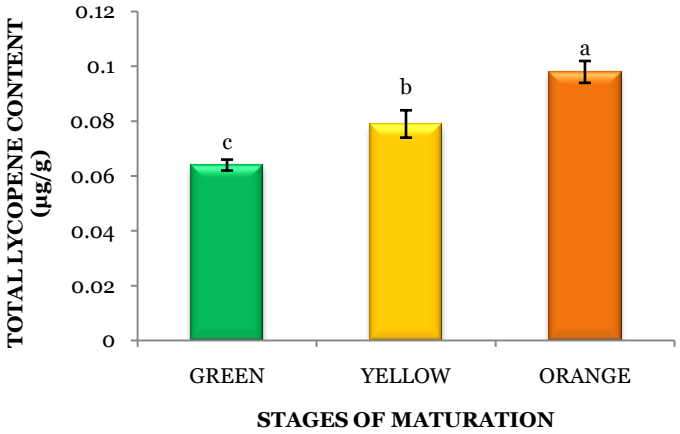


Figure 7.4d Lycopene content (µg/g) of different maturation stages of *S. anguivi* fruits

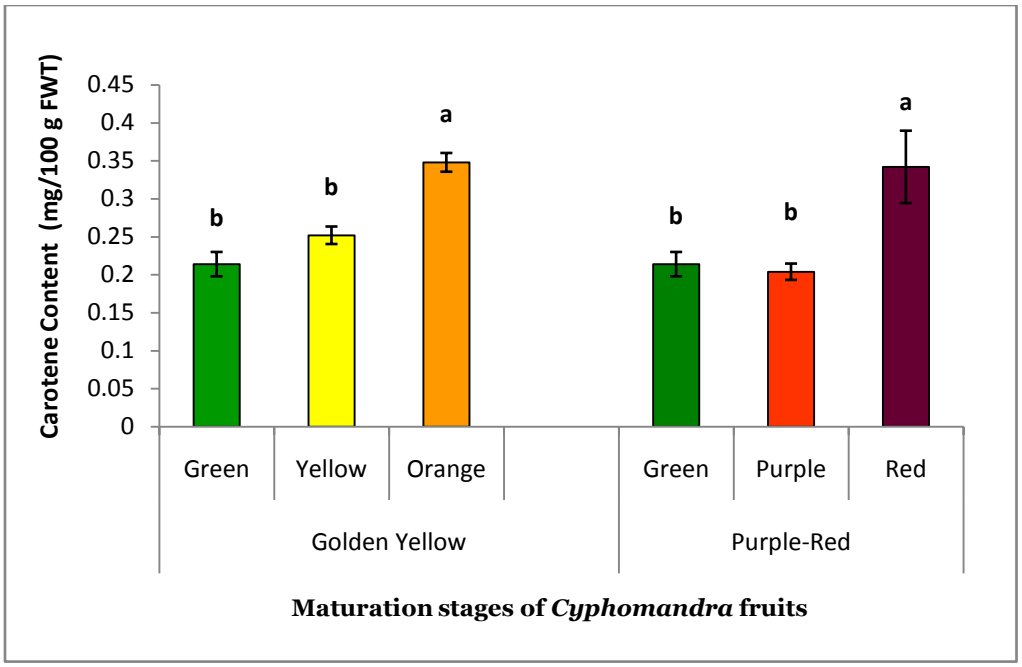


Figure 7.3c Carotene content (mg/100g) of different maturation stages of *C. betacea* fruits

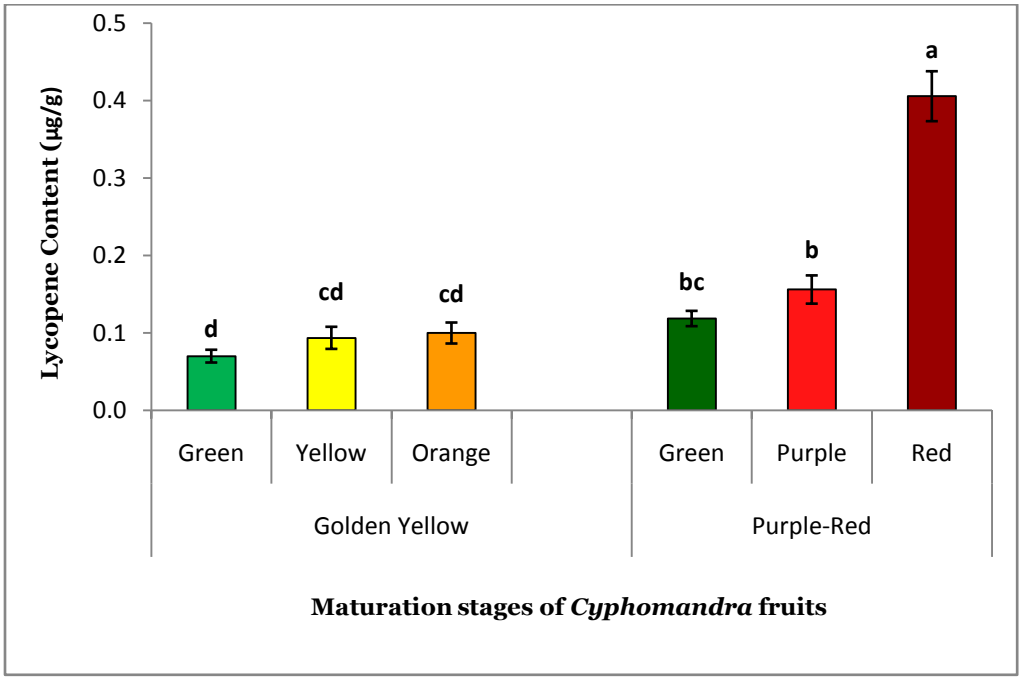


Figure 7.3d Lycopene content (µg/g) of different maturation stages of *C. betacea* fruits

Table 7.1 Correlation matrix

	EV	DPPH	ABTS	MC	SO	OH	ALP	RP	α- GLU	α- AMY	TPC	TFC	TCC
DPPH	**0.916****												
ABTS	-0.354	-0.202											
MC	0.294	0.415	0.557										
SO	-0.227	-0.032	**0.773***	**0.793***									
OH	**-0.963****	**-0.907****	0.459	-0.229	0.344								
ALP	**-0.829****	**-0.754***	0.420	-0.040	0.514	**0.888****							
RP	0.336	0.082	-0.543	-0.507	**-0.777***	-0.366	-0.596						
α- GLU	**-0.822****	-0.638	**0.671***	0.223	**0.733***	**0.872****	**0.893****	**-0.698***					
α- AMY	**-0.681***	-0.502	**0.758***	0.409	**0.848****	**0.757***	**0.812****	**-0.699***	**0.955****				
TPC	**0.773***	0.551	-0.534	-0.106	-0.597	**-0.781***	**-0.825****	**0.840****	**-0.897****	**-0.801****			
TFC	**0.964****	**0.868****	-0.273	0.425	-0.113	**-0.925****	**-0.782***	0.348	**-0.747***	-0.558	**0.794***		
TCC	-0.562	**-0.697***	-0.190	-0.764*	-0.513	0.520	0.145	0.482	0.083	-0.078	-0.053	-0.599	
TLC	-0.547	-0.593	-0.211	-0.691*	-0.442	0.445	0.316	0.394	0.127	0.028	-0.009	-0.507	0.596

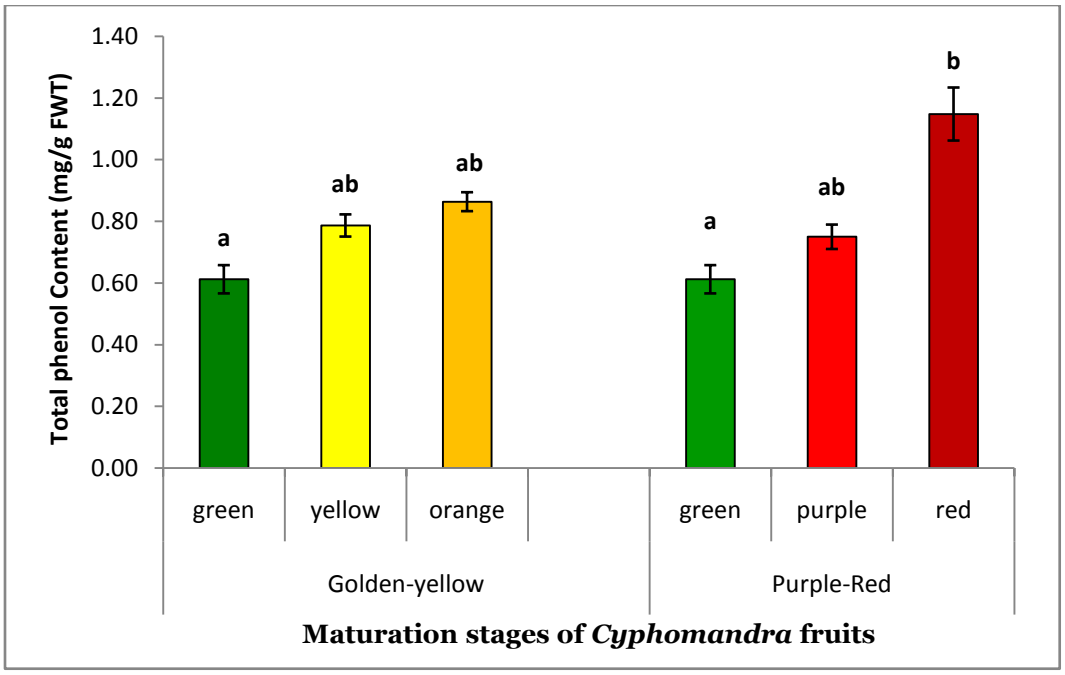


Figure 7.3e Total phenol content (mg/g) of different maturation stages of *C. betacea* fruits

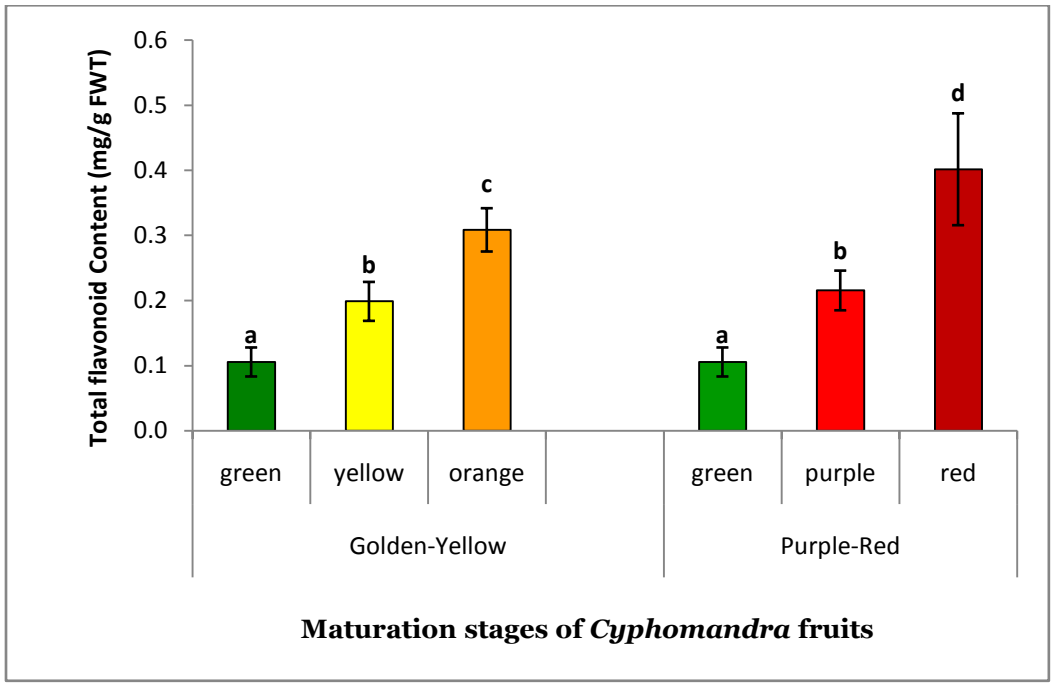


Figure 7.3f Total flavonoids content (mg/g) of different maturation stages of *C. betacea* fruit

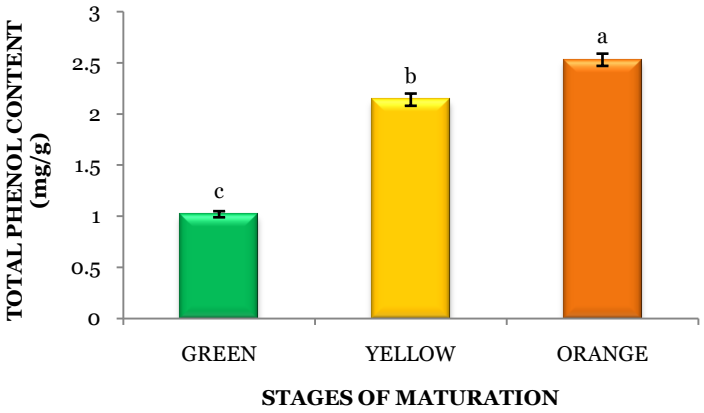


Figure 7.4e Total phenol content (mg/g) of different maturation stages of *S. anguivi* fruits

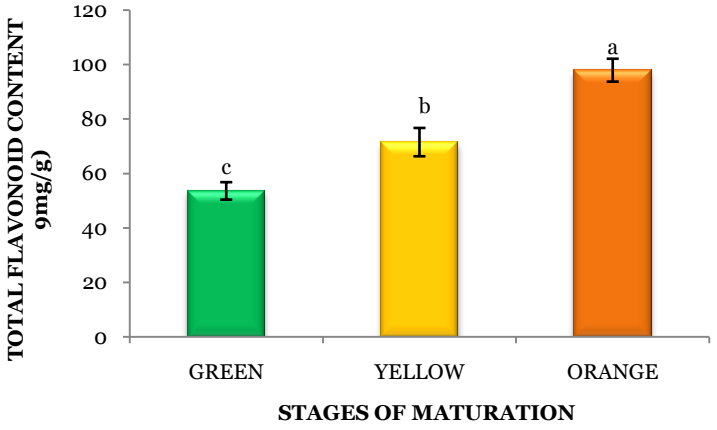


Figure 7.4f Total flavonoids content (mg/g) of different maturation stages of *S. anguivi* fruits

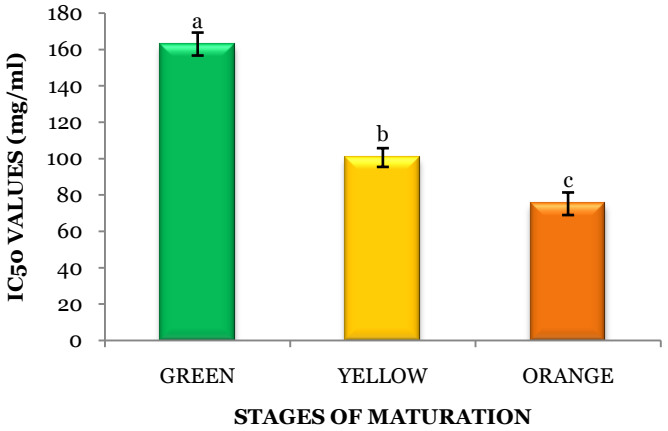


Figure 7.4g Superoxide scavenging (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

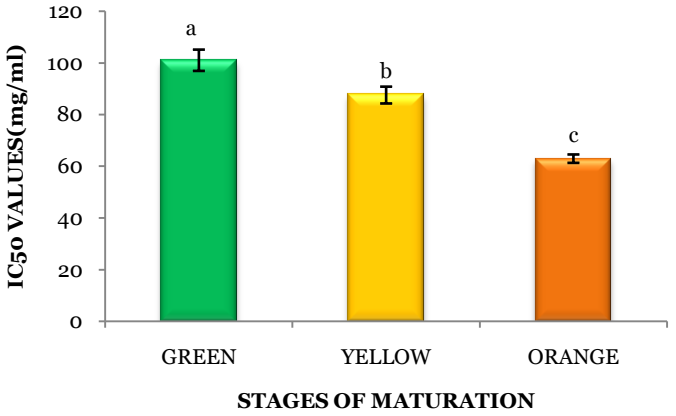


Figure 7.3h Hydroxyl scavenging (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

yellow and purple-red variety of *C. betacea* respectively). Of all the carotenoid pigments, lycopene is the most efficient singlet oxygen quencher (Elbe and Schwartz, 1996). *cis*-Lycopene isomers were found to have higher antioxidant potential with an estimated twice the activity of *all-trans-α*-carotene (Elbe and Schwartz, 1996). The ripe fruits of the representatives of Solanaceae family have the tendency to accumulate large amount of lycopene, which is also revealed from the pattern of gene expression found during fruit ripening (Vasco *et al*., 2009). Our study also confirms that the purple-red variety of *C. betacea* mature fruits contain highest amount (0.406 µg/g) of lycopene when compared with all fruits of different maturation stages (Figure 7.3d, Figure 7.4d and Table 7.1).

Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids (Bohm *et al*., 2002). Phenolic acids have been implicated as natural antioxidants in fruits, vegetables and other plants. For example, caffeic acid, ferulic acid, and vanillic acid are widely distributed in the plant kingdom (Larson, 1988). The total soluble phenolic contents for golden-yellow and purple-red *C. betacea* and *S. anguivi* are presented in Figure 7.3e and Figure 7.4e. Higher amount of total phenols and total flavonoids were present in purple-red than golden-yellow fruit variety (Figure 7.3f and Figure 7.4f). The mature orange fruit of *S. anguivi* contained 2.14 mg/g and 0.096 mg/g of total phenol and flavonoids content respectively. The amount of total phenol content of this fruit was greater than other *Solanum* fruits which were estimated by different authors (Aberoumand and Deokule, 2008; N'Dri *et al*., 2010).

An wide range of naturally occurring phenyl propanoid derivatives, including flavonoids, isoflavones, flavones and catechins can prevent or reduce oxidative stress by scavenging free radicals (Velioglu *et al*., 1998; Kahkonen *et al*., 1999) During oxidative stress, large quantities of reactive oxygen species (ROS) like hydrogen peroxide, superoxide, hydroxyl radical, singlet oxygen and nitrogen species are generated. These ROS have a role in degenerative disease and early apoptosis in animals (Melov, 2002). Although the superoxide anions are relatively weak

oxidant, it can combine with nitric oxide, to give more reactive species (Ardestani *et al*., 2007; Duan *et al*., 2007). In this study, the superoxide anion scavenging effects of different stages of *S. anguivi* fruit and two variety of *C. betacea* were analyzed by the PMS-NADH superoxide generating system. Among them, methanolic extracts of these Solanaceae fruits, mainly mature red one of *C. betacea*, exhibited outstanding superoxide anion radical scavenging activity (IC_{50} value11.98 mg/ml) (Figure 7.3g and Figure 7.4g).

Among the oxygen radicals, the hydroxyl radical and lipid peroxidation induce severe damage to adjacent bio-molecules in cells and causing cell death. Thus, the removal of these radicals is very important for the protection of living system (Yang *et al*., 2008). Like superoxide, the scavenging activities against hydroxyl (Figure 7.3h and Figure 7.4h) and lipid peroxidation (Figure 7.3i and Figure 7.4i) were optimal in the mature red fruit of purple-red variety of *C. betacea*. The antioxidant activities were enhanced successively during immature to mature transition.

In the reducing power assay, the presence of antioxidant in the two types of Solanaceae fruit extracts catalyze the reduction of oxidized Fe^{3+}/ferricyanide complex to ferrous (Fe^{2+}) form. Therefore, the amount of Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Enhancement of absorbance indicates an increase in reducing ability. From the results, represented in Figure 7.3j and Figure 7.4j, reducing potential values of fruit extracts of two different mature fruits of Solanaceae were expressed as milligram of ascorbic acid equivalents per gram (mg AAE /g) of sample.

In previous chapter we already discussed that *C. betacea* and *S. anguivi* have *in vitro* antidiabetic activity. In present chapter we have noticed that the IC_{50} values of antidiabetic activity are gradually decreased towards immature to mature fruits and like antioxidant property and the results also indicate that the accumulation of antidiabetic components are related with ripening (Figure 7.3k-7.3l and Figure 7.4k-7.4l). The correlation between total phenol content,

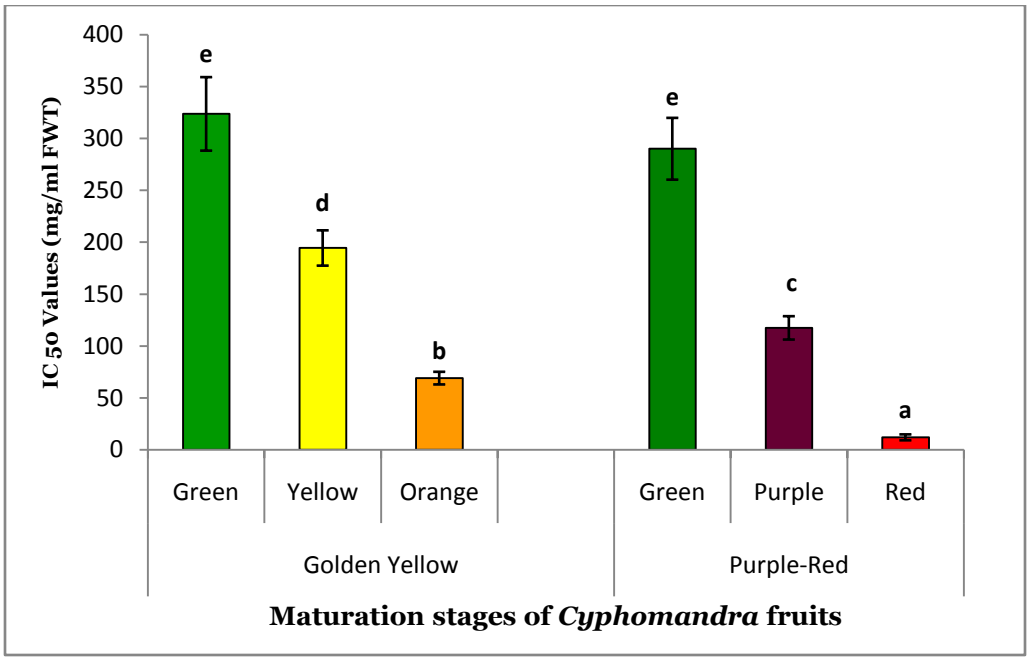


Figure 7.3g Superoxide scavenging (IC_{50}) activity of different maturation stages of *C. betacea* fruits

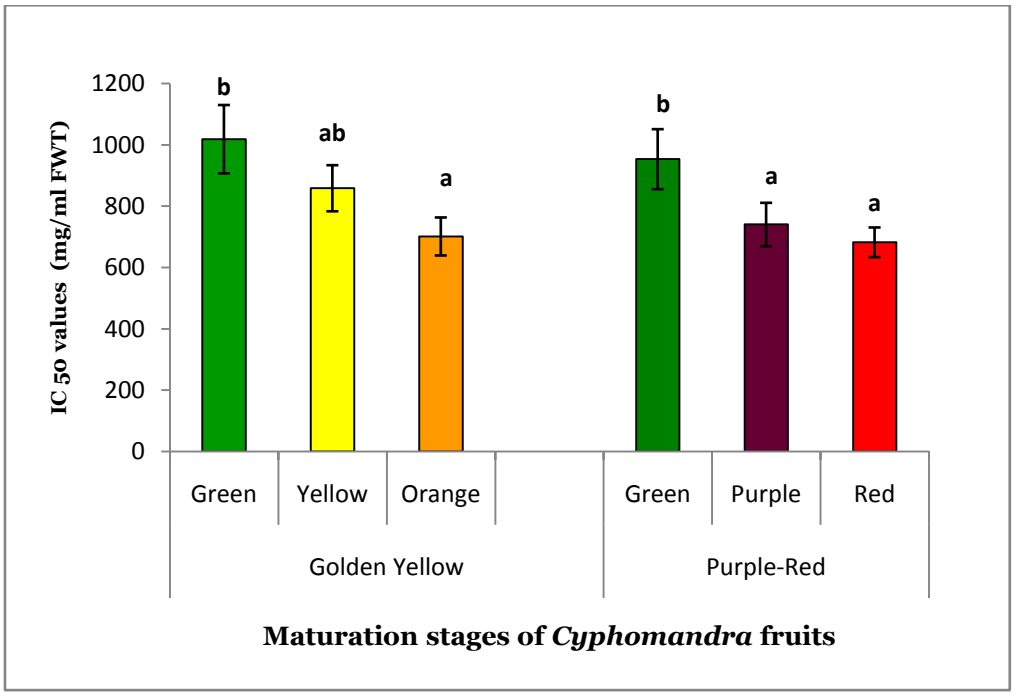


Figure 7.3h Hydroxyl scavenging (IC_{50}) activity of different maturation stages of *C. betacea* fruits

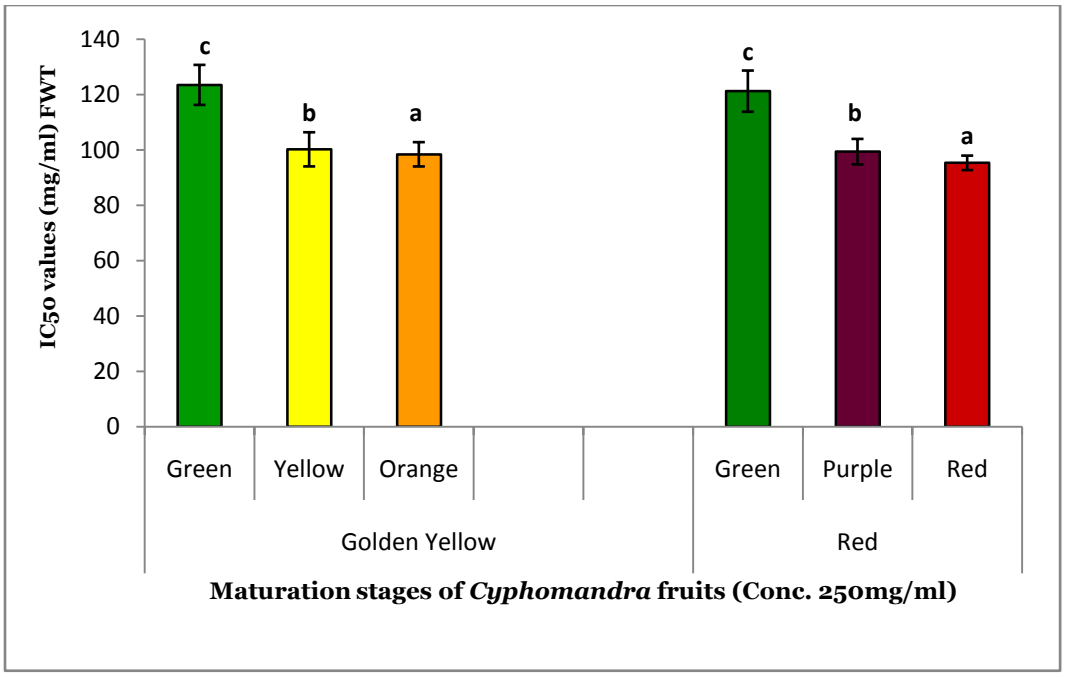


Figure 7.3i Anti-lipid peroxidation (IC_{50}) activity of different maturation stages of *C. betacea* fruits

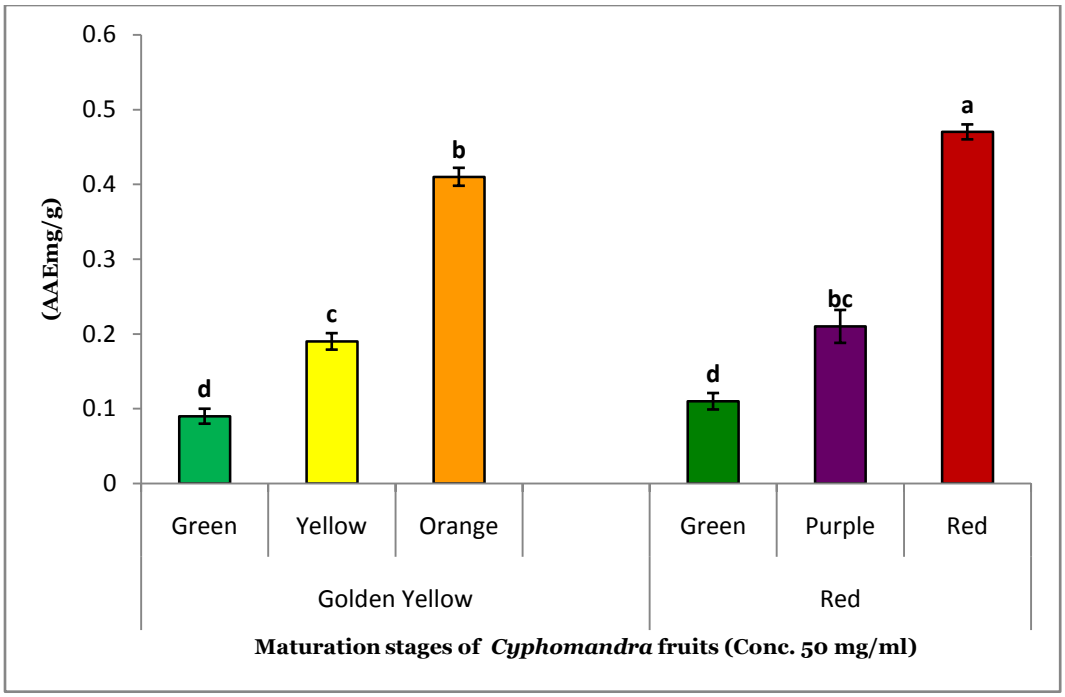


Figure 7.3j Determination of reducing power of different maturation stages of *C. betacea* fruits

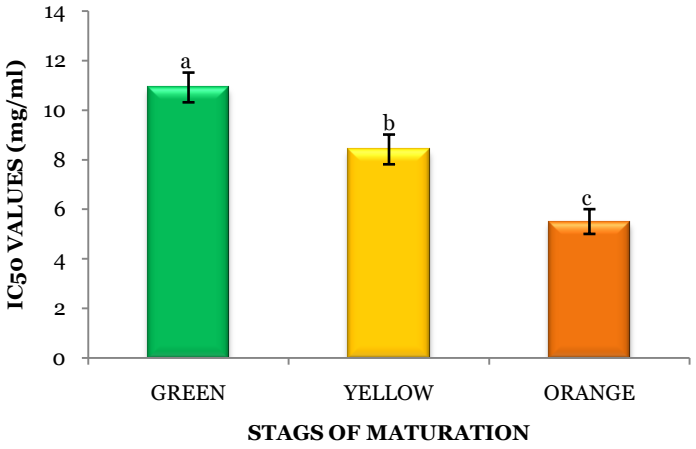


Figure 7.4i Anti-lipid peroxidation (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

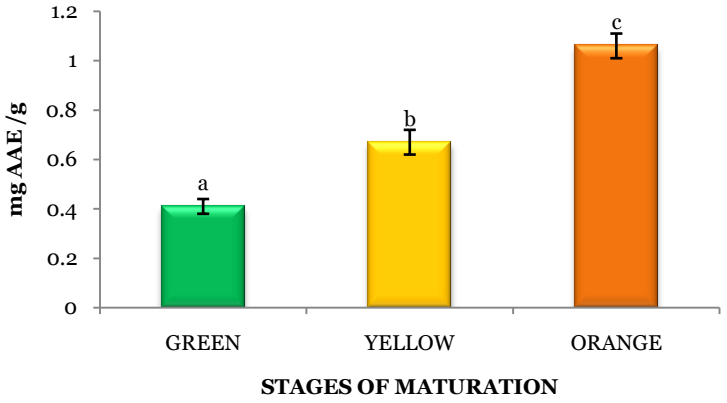


Figure 7.4j Determination of reducing power of different maturation stages of *S. anguivi* fruits

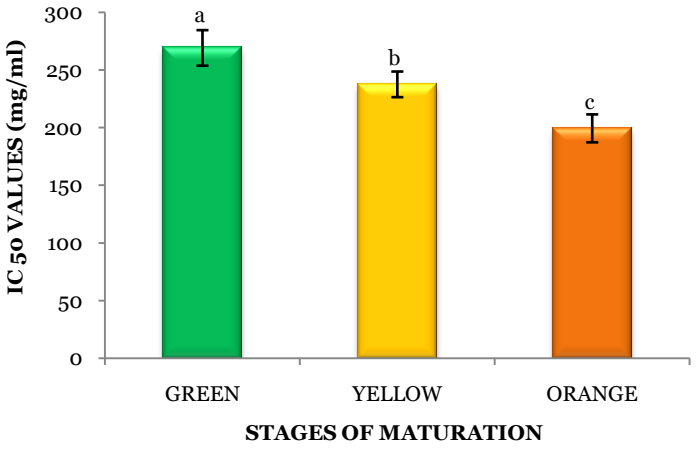


Figure 7.4k α-Glucosidase inhibition (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

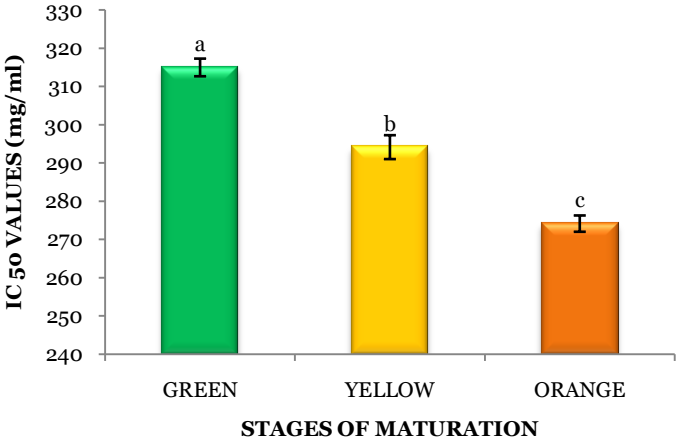


Figure 7.4l α-Amylase inhibition (IC_{50}) activity of different maturation stages of *S. anguivi* fruits

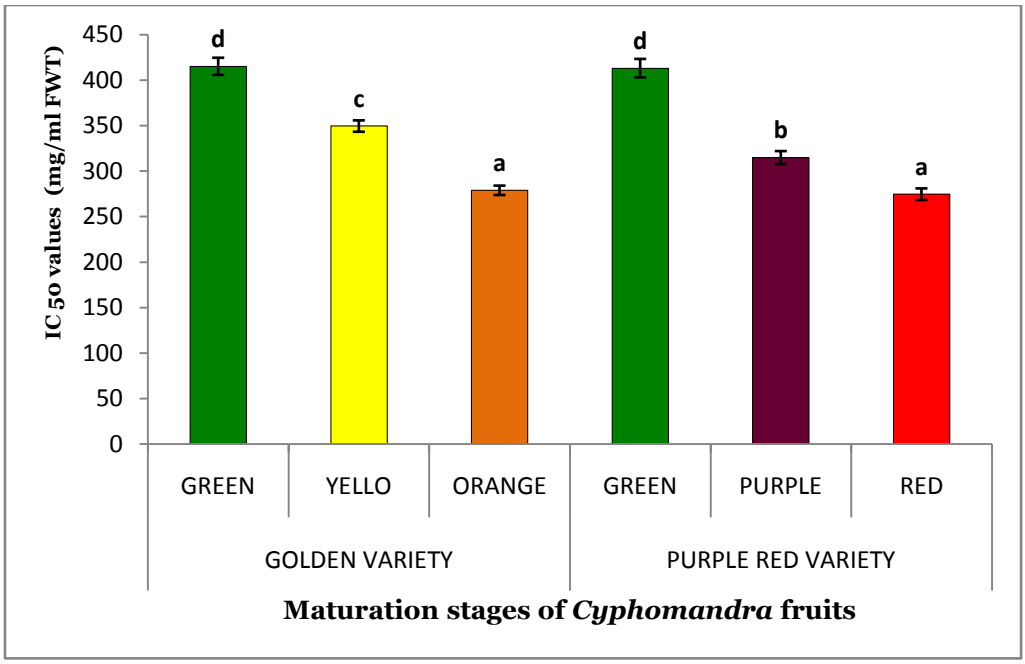


Figure 7.3k α-Glucosidase inhibition (IC_{50}) activity of different maturation stages of *C. betacea* fruits

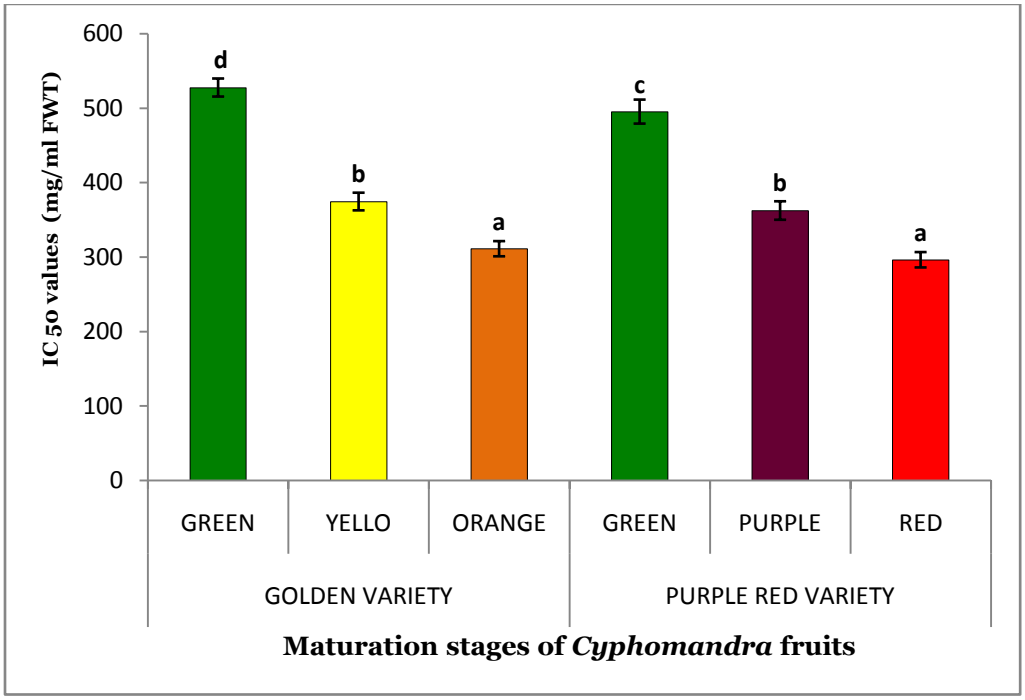


Figure 7.3l α-Amylase inhibition (IC_{50}) activity of different maturation stages of *C. betacea* fruits

antioxidant activity and *in vitro* antidiabetic activity has been widely studied. Some reports suggested that phenolic components are highly correlated with *in vitro* inhibition of enzymes responsible for diabetes and glucose release and also radical scavenging activity in different fruits and vegetables (Kedage *et al*., 2007; Klimczak *et al*., 2007; Yang *et al*., 2008; Saleem, 2010). In our study, significant correlations were obtained between free-radical, α-glucosidase, α-amylase scavenging activity and total phenol content of the sample. As prescribed in Table 7.1, total phenolics are genuinely correlated with DPPH, superoxide, hydroxyl and nitric oxide radicals along with reducing power of the samples. In case of flavonoids, wide significant correlations were observed (Table 7.1). However, good correlations were not established between free-radical scavenging and total carotenoids of *C. betacea*. Interestingly, a superior correlation was registered between lipophilic lycopene content, which is a specific type of carotenoid pigment, and partially hydrophilic DPPH radical scavenging but poorly correlated with other radical scavengers, which are purely hydrophilic in nature.

To understand more about the relation between pharmacological properties and functional phytocomponents PCA was applied separately on *C. betacea* and *S. anguivi*. The first two components of PCA on these attributes accounted for 78.41% and 10.38% of the data variance for *C. betacea* whereas 86.46% and 13.54% for PC1 and PC2 respectively in case of *S. anguivi* (Figure 7.5-7.6). In both cases PC1 is heavily loaded with DPPH, $ABTS^{+}$, hydroxyl radical, metal chelating and superoxide scavenging activity along with α-glucosidase and α-amylase inhibition properties, all of which are present in one cluster with positive factor loadings (Table 7.2-7.3). These attributes which clearly demonstrated antioxidant and antidiabetic activity of these plants was placed in opposite co-ordinates with the phyto-components of fruits like total phenol, flavonoids, total carotenoids and lycopene content along with reducing power activity in terms of ascorbic acid equivalence (Figure 7.5 and 7.6). The results from PCA analysis indicated that the phenolic components and carotenoids might be contributed for persistent free radical

Table 7.2 Explained variability of the five principal components (PCs) on the basis of factor loading from the Principal Component Analysis (PCA)

	PC1	PC2	PC3	PC4	PC5
EV	-0.669	0.673	-0.212	0.221	0.074
DPPH	0.932	-0.330	-0.130	-0.064	-0.044
ABTS	0.759	-0.251	-0.382	0.459	-0.075
MC	0.977	-0.101	-0.176	0.000	0.072
SO	0.993	-0.035	-0.082	-0.028	0.068
OH	0.970	-0.169	-0.130	0.015	0.118
ALP	0.822	0.287	0.474	0.054	0.122
RP	-0.900	-0.390	-0.122	0.012	0.148
α- GLU	0.982	-0.128	0.018	-0.068	0.120
α- AMY	0.977	-0.196	0.015	-0.048	-0.063
TPC	-0.923	-0.326	-0.006	0.102	0.174
TFC	-0.980	-0.187	-0.057	0.038	0.002
TCC	-0.831	-0.228	-0.463	-0.207	-0.011
TLC	-0.546	-0.553	0.607	0.161	-0.049

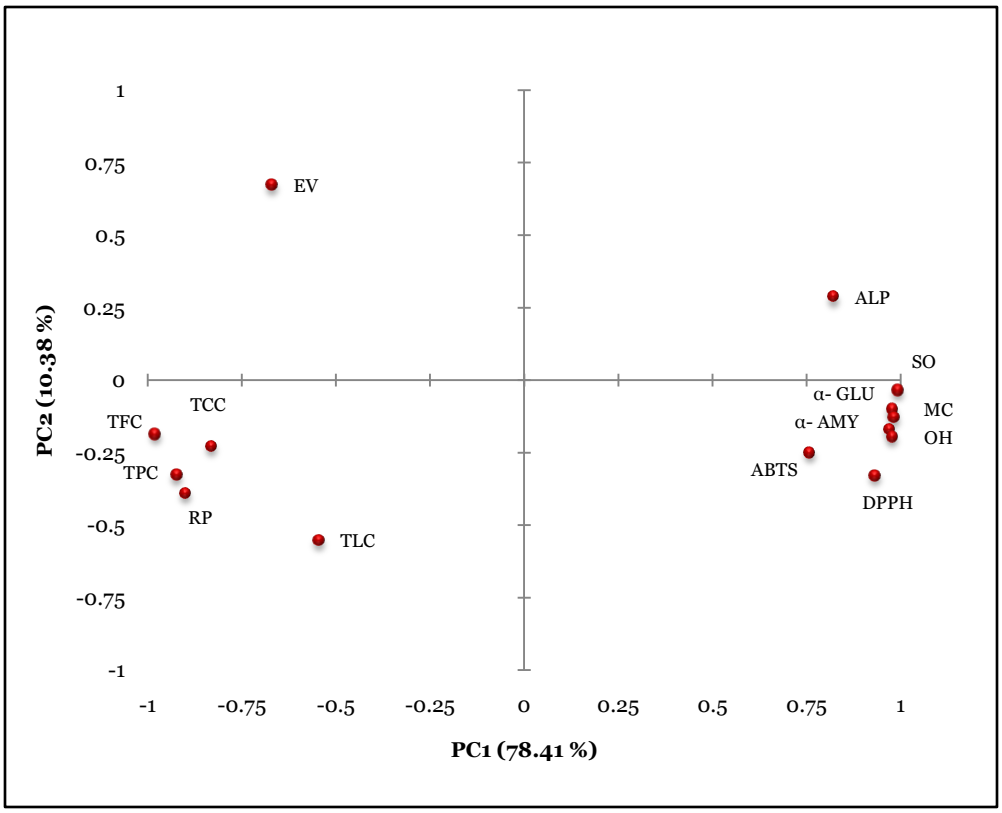


Figure 7.5 Principal component analysis factor loading plot of antioxidant, antidiabetic activity and phytochemicals of *C. betacea* fruits

Table 7.3 Explained variability of the two principal components (PCs) on the basis of factor loading from the Principal Component Analysis (PCA)

	PC1	PC2
EV	-0.848	0.530
DPPH	0.442	0.897
ABTS	1.000	0.008
MC	0.490	-0.872
SO	1.000	0.013
OH	0.991	0.131
ALP	1.000	0.007
RP	-0.997	-0.078
α- GLU	1.000	0.018
α- AMY	0.999	-0.048
TPC	-0.992	-0.123
TFC	-0.997	-0.074
TCC	-0.999	-0.048
TLC	-1.000	-0.031

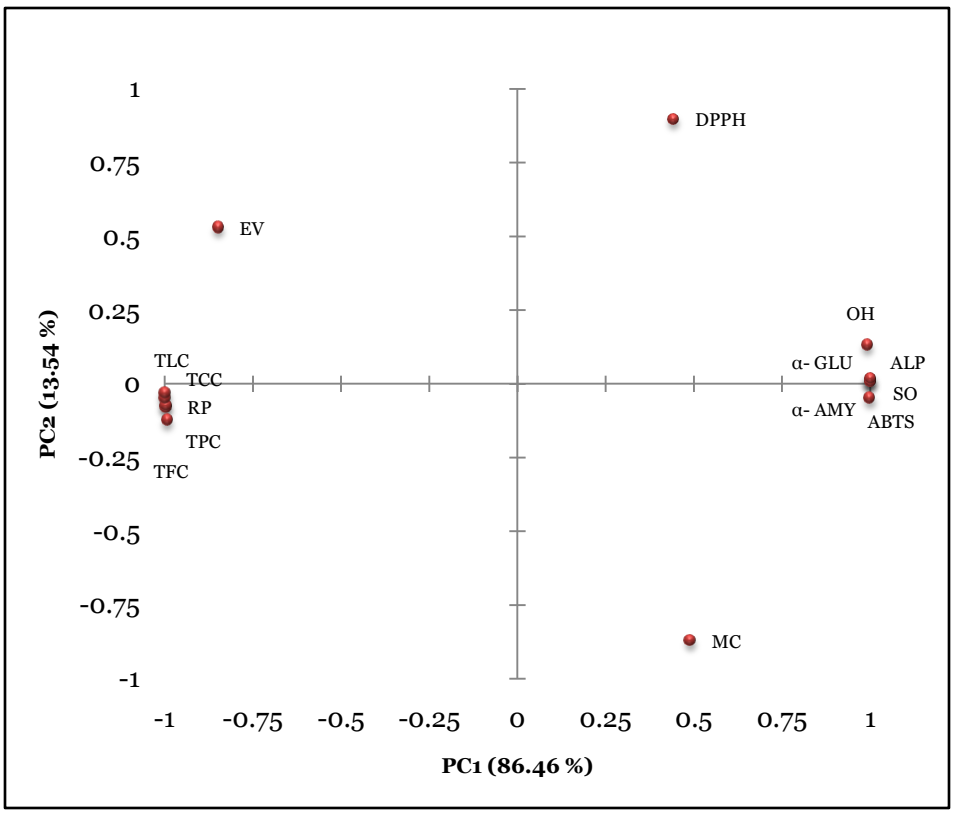


Figure 7.6 Principal component analysis factor loading plot of antioxidant, antidiabetic activity and phytochemicals of *S. anguivi* fruits

scavenging and antidiabetic attributes with maturation in these two plant species. Similar opinion was published by Dantas *et al*., (2013) who showed through PCA analysis that the variability of antioxidants in fruits of strawberry guava during maturation stages was strongly associated to the ascorbic acid and carotenoid components.

In conclusion it can be stated that the fruits of *C. betacea* and *S. anguivi* are most acceptable for their pharmacological properties at maturity stages. Based on the present results, these fruits should be consumed at full ripeness in order to take benefit from the bioactive molecules such as phenolic compounds, lycopene and carotenoids present in these fruits. Considering their higher content of antioxidant and antidiabetic properties, they might be utilized as effective nutraceuticals in food and pharmaceutical industry.

Chapter – VIII

EFFECT OF DOMESTIC COOKING METHODS ON THE ANTIOXIDANT ACTIVITY OF SOME SELECTED VEGETABLES OF DARJEELING HIMALAYA

8.1 INTRODUCTION

Naturally occurring secondary plant metabolites which have attracted large attention from the scientific community for their antioxidant attributes and their implications in a variety of biological mechanism to prevent degenerative processes are also responsible for plant food colour, flavor and taste (Kaur and Kapoor, 2001). Fruits and vegetables are a good source of these secondary metabolites. Several epidemiological studies have supported the protective effects of fruits and vegetables consumption, against the risk of numerous age related diseases due to the presence of secondary metabolites (Siddhuraju and Backer, 2003). Most vegetables are normally cooked before being consumed. It is well known that cooking stimulates significant changes in chemical composition, influencing the concentration as well as bioavailability of bioactive compounds from vegetables. However, both positive and negative effects have been reported depending in morphological and nutritional characteristics of vegetables species (Makris and Rossiter, 2001; Dewanto *et al*., 2002; Ismail *et al*., 2004; Jiratannan and Liu, 2004; Zang and Hamauzu, 2004). In addition, length of heating time may have special effect on the antioxidant properties of vegetables (Hunter and Fletcher, 2002; Zang and Hamauzu, 2004). During vegetable processing, qualitative changes of phytochemicals and their leaching into surrounding water may influence the antioxidant property of the vegetables (Podsedek, 2007). Though, flavonoids and some phenolics are quite stable at high temperature and remain unaltered over long periods of storage (Vallejo *et al*., 2003), many antioxidant compounds like ascorbic acids and carotenoids are very sensitive to heat and lost their properties during different vegetable processing steps (Zang and Hamauzu, 2004). In 2002, Hunter and Fletcher found that antioxidant activity of vegetables was influenced by storage under different conditions like freezing, canning and jarring etc. According to Lin and Chang (2005), antioxidant property of broccoli increased under different cooking treatments. In another study Turkmen (2005), examined the antioxidant activity of different selected green vegetables and found that antioxidant activity increased or remain unchanged after different cooking treatments (i.e. steaming, boiling and microwave cooking). In

the same year, Oboh (2005) noticed that antioxidant property is decreased by blanching of vegetables.

In Darjeeling Himalaya vegetables are consumed as raw, half-cooked or boiled, after semi-frying or deep frying and sun drying. These treatments may influence the efficacy and concentration of constituents of the underexplored vegetables found in Darjeeling Himalaya. However, literature data on the effect of cooking on nutritional properties of vegetables of this region are still missing. Therefore, the aim of the present study was to evaluate the effect of the major domestic cooking practices (i.e. boiling, frying and sun drying), selected on the basis of the eating habits recorded by survey (already discussed in Chapter–III) on phytochemical constituents and antioxidant activity of ten vegetables, chosen on the basis of their popularities (already discussed in Chapter–III), nutritional profiles and antioxidant capacities (already discussed in Chapter–IV).

8.2 MATERIALS AND METHODS

8.2.1 Plant samples collection and identification

Plants collection and identification were specified in Chapter III Section 3.2.2.

8.2.2 Preparation of samples

Samples of ten vegetables, i.e. bihi (*Solanum anguivi*), bara bihi (*S. incanum*), tree tomato (*Cyphomandra betacea*), ghar trul (*Dioscorea alata*), ban tarul (*D. hamiltonii*), lal and seto sakarkanda (*Ipomoea batatas*, red and white cultivars), simal tarul (*Manihot esculenta*), squash jara (*Sechium edule*) and pindalu (*Xanthosoma brasiliense*) were collected from Takdah Basti. Vegetables were washed with tap water after removing manually the non-edible parts. Vegetables were dried with paper towel and were cut into almost equal small pieces or slices, and mixed well. These processed samples were subjected to different cooking methods followed by measurement of different phytochemicals and antioxidant activity, keeping one portion as control

(uncooked). Cooking conditions were determined with a preliminary experiment for each vegetable.

8.2.3 Cooking treatments

Three of most common cooking methods used by the Nepali population, i.e., boiling, frying and sun drying, were used. For all cooking treatments, minimum cooking time to achieve a similar tenderness for an adequate palatability as well as taste was used.

- Boiling: 100g each sample was added to 500ml boiling tap water in a covered stainless steel pot and was cooked on moderate flame. Then the sample was drained off for 30 seconds and cooled rapidly on ice.
- Frying: 2.2 liter peanut oil was warmed up in a stainless steel pan on a moderate flame. After 2 minutes, 100 gm vegetable was added and cooked. Then, the vegetable was drained off, dabbed briefly with blotting paper and cooled rapidly on ice.
- Sun drying: 400 gm of sample was kept in blotting paper under sunlight for variable day time.

According to Nepali food recipes, all the vegetables were boiled (5, 10, 15 and 20 minutes respectively for all vegetables; except for taruls where 15 minutes boiling was performed). *C. betacea* was only pickle item after sun drying (1, 3, 5 and 7 days respectively), whereas frying (1, 2, 4 and 6 minutes respectively) was used both for *C. betacea* and two *Solanum* vegetables except taruls.

8.2.4 Preparation of methanolic extracts

Samples of raw and cooked vegetables were extracted with methanol. The extraction procedure was specified in Chapter IV Section 4.2.2.

8.2.5 Animal material

For the assay of antilipid peroxidation animal material collection procedure was mentioned in Chapter IV Section 4.2.3.

8.2.6 Determination of *in vitro* antioxidant activity

8.2.6a Determination of DPPH radical scavenging assay

The assay was performed as prescribed by Blois (1958) and specified in details in Chapter IV Section 4.2.4.

8.2.6b Determination of superoxide anions scavenging activity

The assay was performed as prescribed by Nishikimi *et al*., (1972) and specified in details in Chapter IV Section 4.2.6.

8.2.6c Determination of hydroxyl radical scavenging activity

The assay was performed as prescribed by Jung *et al*., (2004) and specified in details in Chapter IV Section 4.2.7.

8.2.6d Determination of reducing power

The assay was performed as prescribed by Aiyegoro and Okoh, (2009) and specified in details in Chapter IV Section 4.2.10.

8.2.6e Anti-lipid peroxidation (ALP) assay

The assay was performed as prescribed by Bauchet and Barrier, (1998) and specified in details in Chapter IV Section 4.2.11.

8.2.6f Total phenol estimation

The assay was performed as prescribed by Folin and Ciocalteu, (1927) and specified in details in Chapter V Section 5.2.3.

8.2.6g Total flavonoid determination

The assay was performed as prescribed by Sultana *et al*., (2009) and specified in details in Chapter V Section 5.2.4.

8.2.6h Total lycopene content

The assay was performed as prescribed by Thimmaiah (2004) and specified in details in Chapter VII Section 7.2.11.

8.2.6i Total carotene content

The assay was performed as prescribed by Vasco *et al*. (2009) and specified in details in Chapter VII Section 7.2.12.

8.2.7 Determination of *in vitro* antidiabetic activity

8.2.7a Inhibition of *α*-amylase enzyme

The assay was performed as prescribed by Heidari *et al.*, (2005) and specified in details in Chapter VI Section 6.2.4a.

8.2.7b Inhibition of α-glucosidase enzyme

The assay was performed as prescribed Oki *et al.*, (1999) and specified in details in Chapter VI Section 6.2.4b.

8.2.8 Statistical analysis

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between phytochemicals and antioxidant as well as antidiabetic attributes and MS Excel of Microsoft Office, 2007 was used for comparing the antioxidant attributes of different maturation stages of these fruits. The data were analyzed by different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software ver. 1.022; $p<0.05$ was considered significant in all cases. Smith's Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidants, antidiabetic activity and their standard error of estimates (SEE).

8.3 RESULTS

8.3.1 Thermal treatment of *C. betacea* fruit

The principle for reduction of the DPPH free radical is that the antioxidant reacts with the stable free radical DPPH and converts it to 2, 2-diphenyl-1-picryl hydrazl. The free radical scavenging activity of post-harvest thermal processing was evaluated by means of a DPPH stable radical assay and expressed as the IC_{50}. Another free radical ABTS scavenging was also determined for evaluation of antioxidant activity. Results were given in Figure 8.1 and Figure 8.2 showed that in all cases of post harvest thermal treatment *in vitro* antioxidant activity was enhanced significantly as revealed from their lower IC_{50} values. The outcome of superoxide anion scavenging activities of *C. betacea* fruits during post-harvest thermal processing measured by the PMS-NADH superoxide generating system are shown in Figure 8.3. The methanolic fruits extracts exhibited superior *in vitro* superoxide anion radical scavenging activity during post-harvest processing. The superoxide anion derived from dissolved oxygen by Phenazine methosulfate/NADH coupled reaction reduces nitro blue tetrazolium. The decrease in the absorbance at 560 nm with the methanolic fruits extract thus indicates the consumption of superoxide anion in the reaction mixture. The hydroxyl radical scavenging activity of this fruits extract by deoxyribose assay is

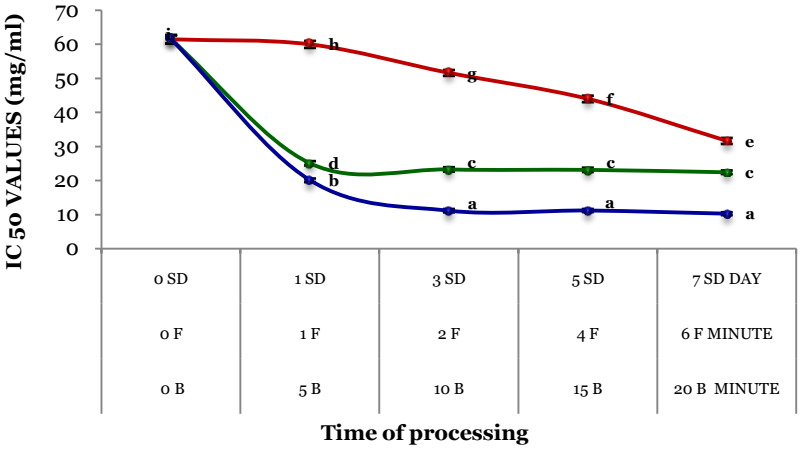


Figure 8.1 DPPH radical scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

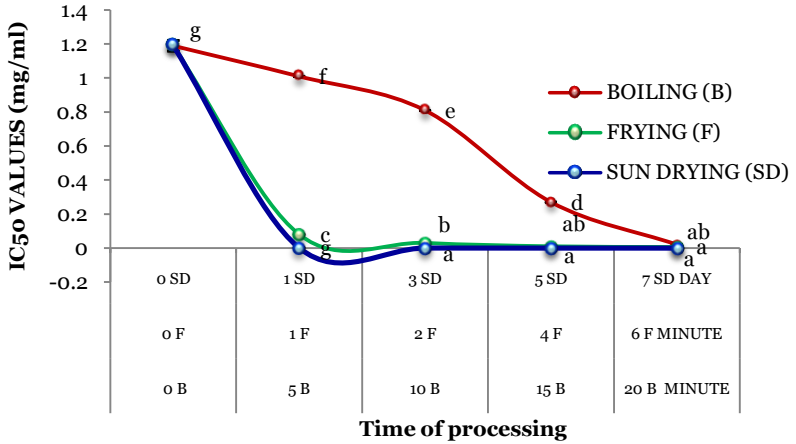


Figure 8.2 ABTS scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

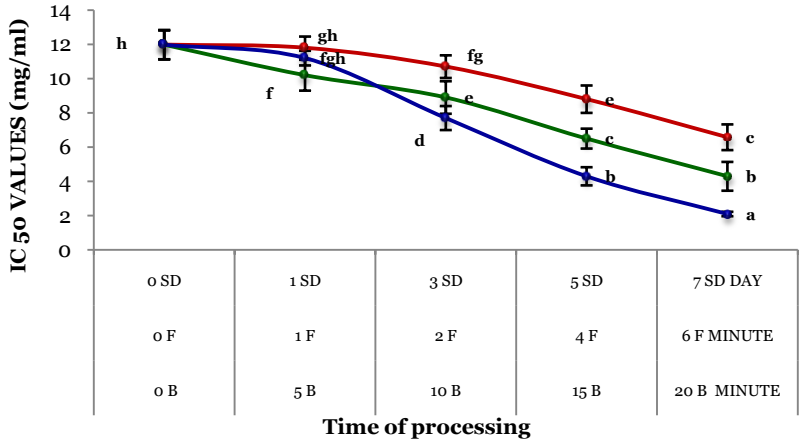


Figure 8.3 Superoxide scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

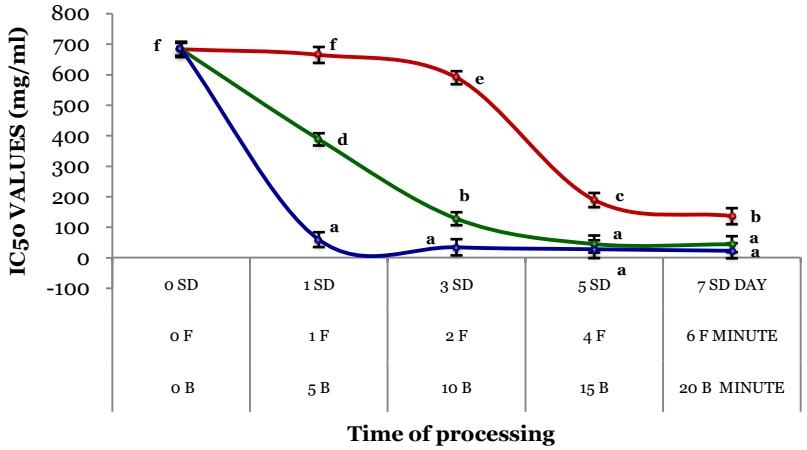


Figure 8.4 Hydroxyl scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

presented in Figure 8.4. In case of different types of thermal processing, sun drying treatment would accumulate components which can effectively scavenge hydroxyl radicals as it is apparent from their lower IC_{50} values. As shown in Figure 8.5, the reducing power of the extracts increases with the increase in concentration. In case of of thermal processing higher absorbance value indicates greater reducing power. The reducing power activity increased in order of thermal processing is as follows: boiling<frying<sundry. The results for anti-lipid peroxidation assay are given in Figure 8.6. The anti-lipid peroxidation activity was found to enhance gradually with increase in time period for all thermal processing but slightly more activity was found in case of sundry. Heat treated i.e. boiled, fried as well as sun dried fruits of *C. betacea* were the good sources of total phenol and flavonoids contents (Figure 8.7 and Figure 8.8). These phytochemical contents of cooked vegetables were increased in this order: boiling<frying<sundry. Similar approaches were also observed in case of other total two phytochemicals like carotenoids and lycopene content. From the results it is clear that these compounds were significantly enhanced in case of sundry with gradual increase of processing time when compared with boiling and frying (Figure 8.9 and Figure 8.10). Yield extractive of *C. betacea* fruit was more in sundry treatment than boiling and frying as revealed from Figure 8.11. More interesting results have noticed in case of *in vitro* antidiabetic activity of *C. betacea* with its cooking period. Both α-glucosidase and α-amylase activity were decreased with boiling and frying; whereas at the time of sun drying the scavenging activities were significantly increased with time (Figure 8.12 and Figure 8.13).

Correlation coefficient matrix analysis in Table 8.1 showed that the antioxidant properties, antidiabetic activity and phytochemicals constituents were significantly correlated with each other at $p< 0.01$ and $p< 0.05$ level.

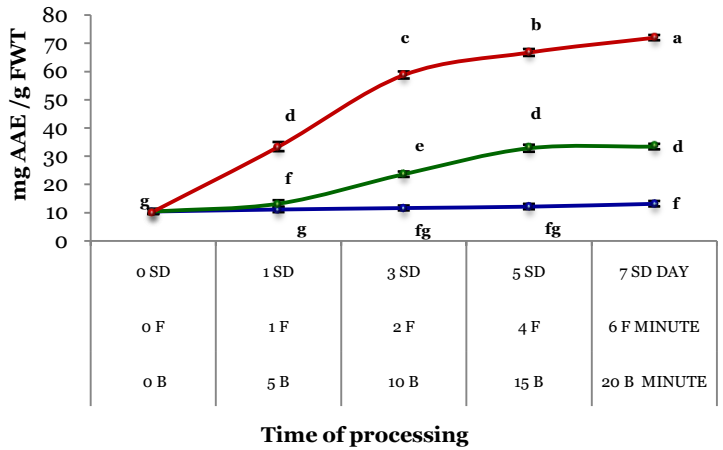


Figure 8.5 Reducing power of *C. betacea* fruits during different thermal processing

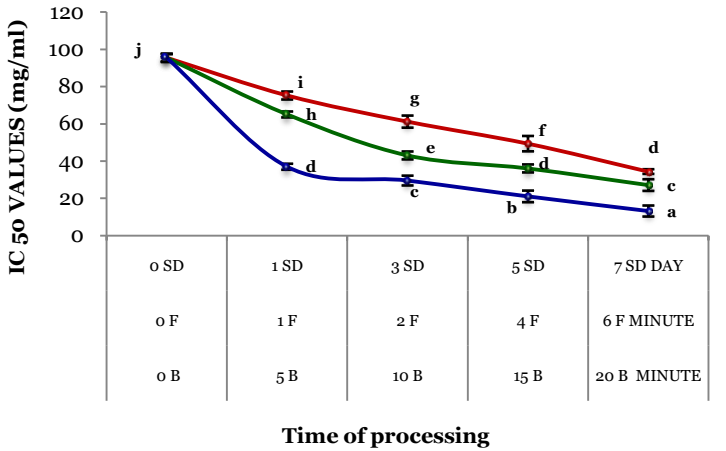


Figure 8.6 Lipid peroxidation (IC_{50}) activity of *C. betacea* fruits during different thermal processing

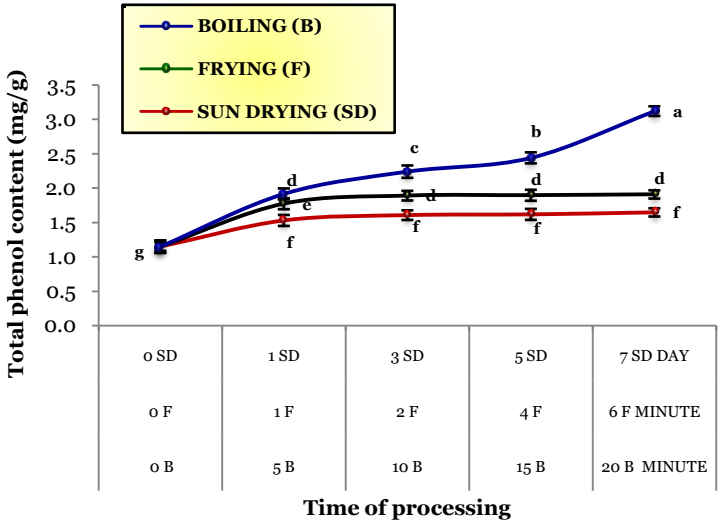


Figure 8.7 Total phenol content of *C. betacea* fruits during different thermal processing

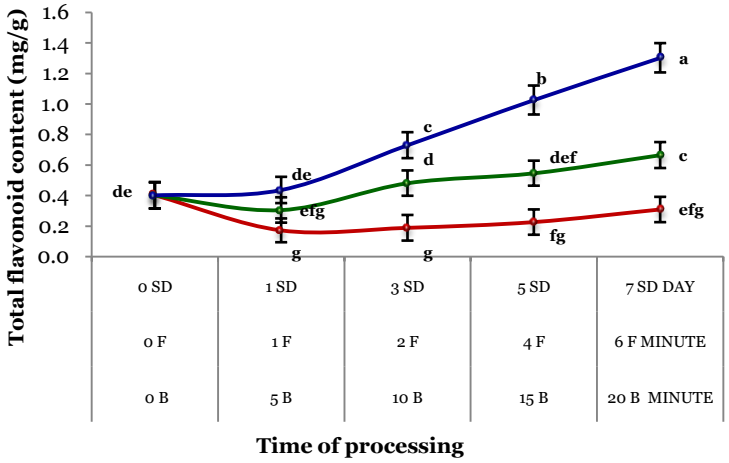


Figure 8.8 Total flavonoids content of *C. betacea* fruits during different thermal processing

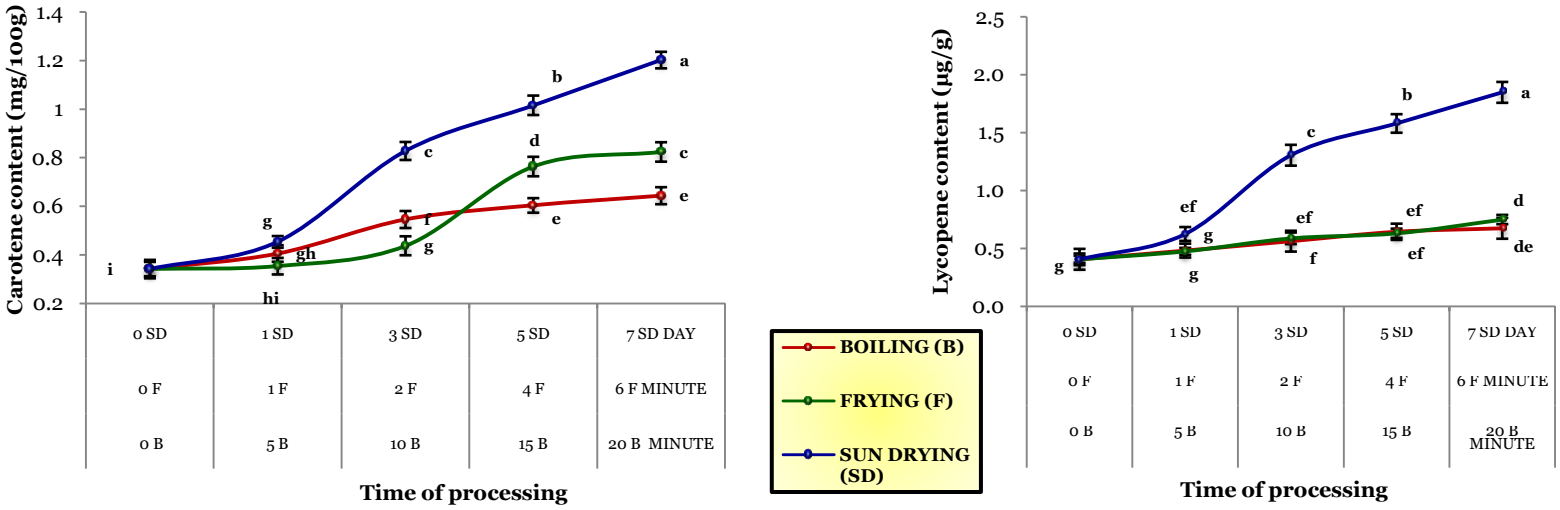


Figure 8.9 Total carotene content of *C. betacea* fruits during different thermal processing

Figure 8.10 Total lycopene content of *C. betacea* fruits during different thermal processing

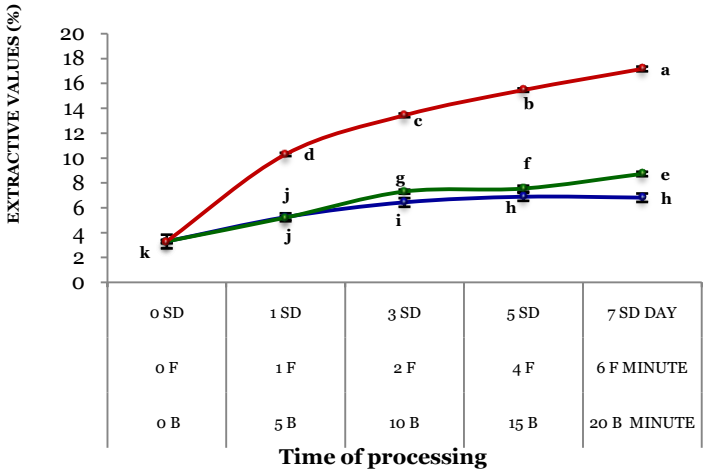


Figure 8.11 Extractive values of *C. betacea* fruits during different thermal processing

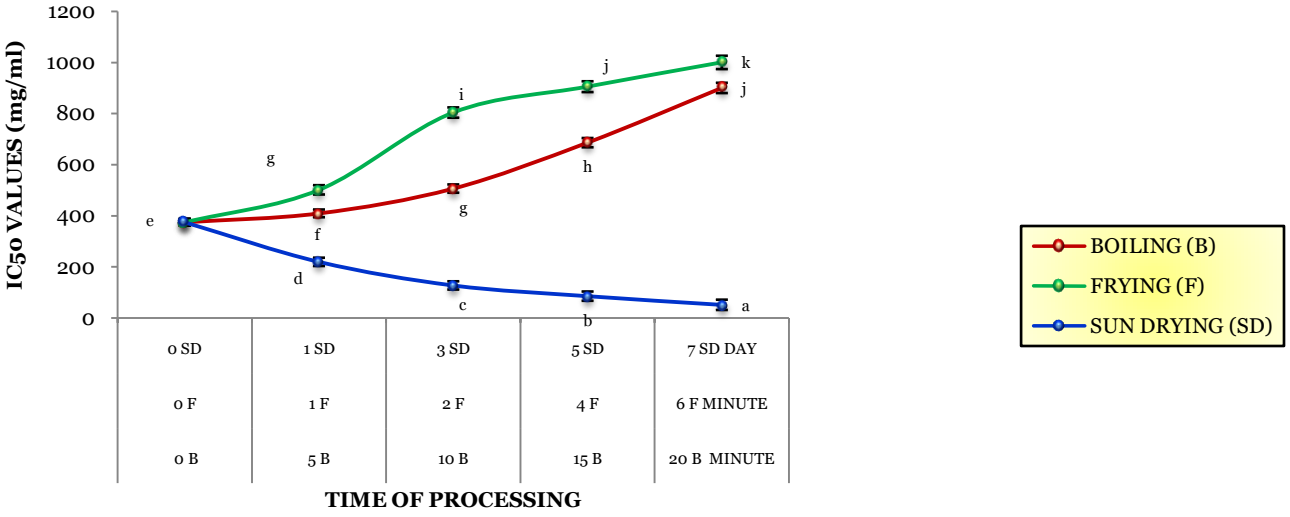


Figure 8.12 α-Glucosidase scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

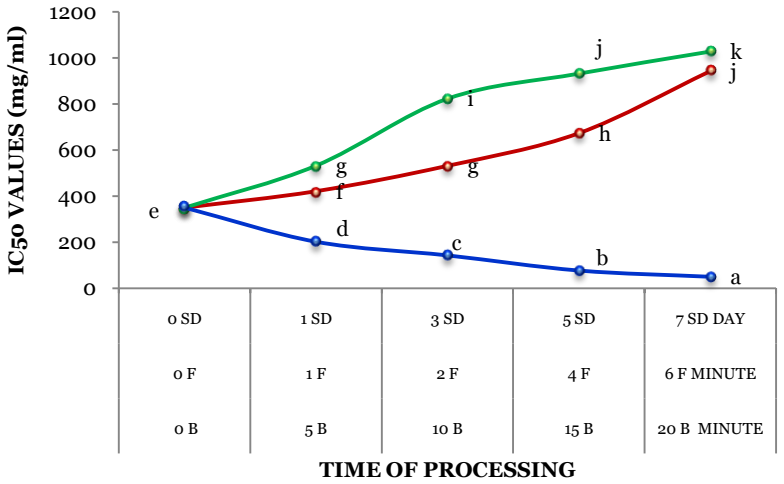


Figure 8.13 α-Amylase scavenging (IC_{50}) activity of *C. betacea* fruits during different thermal processing

Table 8.1 Correlation among phytochemical content of two verities of *C. betacea* fruit and various measurements of antioxidant activity

	EV	DPPH	ABTS	SO	OH	ALP	RP	TPC	TFC	TLC
EV										
DPPH	-0.788**									
ABTS	-0.585*	0.921**								
SO	-0.741**	0.709**	0.655*							
OH	-0.700**	0.902**	0.939**	0.741**						
ALP	-0.831**	0.878**	0.864**	0.850**	0.929**					
RP	0.968**	-0.800**	-0.554*	-0.743**	-0.682*	-0.776**				
TPC	0.942**	-0.811**	-0.633*	-0.790**	-0.684**	-0.823**	0.917**			
TFC	0.902**	-0.712**	-0.465	-0.790**	-0.584*	-0.691**	0.932**	0.895**		
TLC	0.960**	-0.683*	-0.460	-0.782**	-0.577*	-0.736**	0.942**	0.922**	0.933**	
TCC	0.877**	-0.669*	-0.529	-0.929**	-0.666*	-0.827**	0.870**	0.866**	0.848**	0.914**

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8.2 Correlation among phytochemical content of *Solanum* fruits and various measurements of antioxidant activity

	DPPH	SO	MC	ALP	RP	OH	TPC	TFC	TLC	TCC	GLU
DPPH											
SO	**0.627****										
MC	**0.904****	**0.804****									
ALP	**0.706****	0.186	**0.617****								
RP	**-0.661****	**-0.477***	**-0.604****	**-0.555***							
OH	**0.893****	**0.587***	**0.755****	**0.521***	**-0.554***						
TPC	**-0.530***	-0.437	**-0.542***	-0.133	0.420	-.0354					
TFC	**-0.562***	**-0.644****	**-0.721****	-0.359	**0.627****	-0.402	**0.673****				
TLC	**-0.744****	-0.418	**-0.648****	**-0.646****	**0.692****	**-0.593****	**0.710****	**0.646****			
TCC	**-0.700****	-0.285	**-0.542***	**-0.698****	**0.626****	**-0.596****	**0.559***	0.415	**0.931****		
GLU	**-0.620****	-0.419	**-0.536***	-0.375	**0.880****	**-0.521***	**0.679****	**0.683****	**0.724****	**0.622****	
AMY	**-0.543***	**-0.618****	**-0.569***	-0.218	**0.849****	**-0.505***	0.367	**0.607****	0.402	0.267	**0.792****

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8.3 Correlation among phytochemical content of underground parts and various measurements of antioxidant activity

	DPPH	ABTS	OH	MC	RP	EV	TPC	TFC	ODP	GLU
DPPH										
ABTS	0.412									
OH	0.076	**0.562***								
MC	-0.288	0.008	-0.047							
RP	0.093	0.371	0.329	-0.067						
EV	0.079	-0.071	0.188	-0.106	-0.398					
TPC	**-0.584***	-0.519	-0.371	0.422	-0.475	-0.139				
TFC	-0.498	**-0.552***	**-0.543***	0.344	-0.526	-0.180	**0.963****			
ODP	**-0.897****	**-0.629***	-0.322	0.305	-0.349	-0.056	**0.636***	**0.609***		
GLU	0.186	0.442	0.285	0.305	-0.019	0.409	-0.520	-0.519	-0.159	
AMY	0.233	0.245	0.213	0.291	-0.021	0.366	**-0.552***	**-0.552***	-0.128	**0.933****

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

8.3.2 Thermal treatment of two *Solanum* fruits

Antioxidant activities of raw and cooked *S. anguivi* and *S. incanum*, as determined by the DPPH radical scavenging method, are shown in Figure 8.14. Cooking method and duration affected the antioxidant activity of both species of *Solanum*. Significant differences in DPPH radical scavenging activity were established between raw *S. anguivi* and *S. incanum* after boiling and frying, but these differences became more significant with longer cooking times. Raw *S. incanum* showed dramatically higher DPPH scavenging capacity, compared with raw *S. anguivi*, interestingly cooking act as favourable effect on the antioxidant content of both vegetables (Figure 8.14). After boiling for 15 min and frying for 4 min, there was significant increase in DPPH radical scavenging activity of the two fruits. Similar results have noticed in case of superoxide ion (Figure 8.15), hydroxyl radical scavenging (Figure 8.16), lipid peroxidation (Figure 8.17), metal chelating activity (Figure 8.18) as well as reducing power capability (Figure 8.19).

Extractive yield of these vegetables were more in boiling treatment than frying as it is detectable from Figure 8.20. The effects of boiling on total phenolics, flavonoids, carotenoids and lycopene content of *S. anguivi* and *S. incanum* are presented in Figure 8.21A, Figure 8.22A, Figure 8.23A and Figure 8.24A and the effect of frying is presented in Figure 8.21B, Figure 8.22B, Figure 8.23B and Figure 8.24B. Both cooking methods affected the phytochemical content of *Solanum* spp. The four phytochemicals of raw *Solanum* were both dramatically higher in *S. incanum*, compared with *S. anguivi*; however, both cooking methods had a positive (for a particular time) effect on the total phenolic, flavonoids, lycopene and carotenoid content of both species.

Like *C. betacea* antidiabetic activity like α-glucosidase and α-amylase inhibition activity also decreased with boiling and frying treatment of these vegetables (Figure 8.25 and Figure 8.26). As represented in Table 8.2, the strong positive correlation between tasted phytochemicals and *in vitro* anti-diabetic activity deserves special attention. Reducing power (ascorbic acid

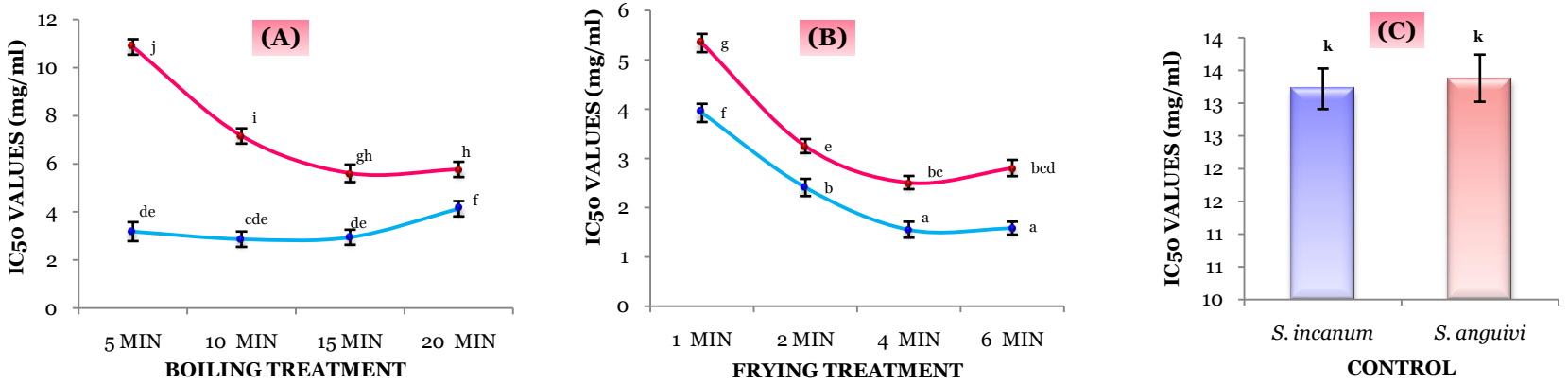


Figure 8.14 DPPH radical scavenging (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

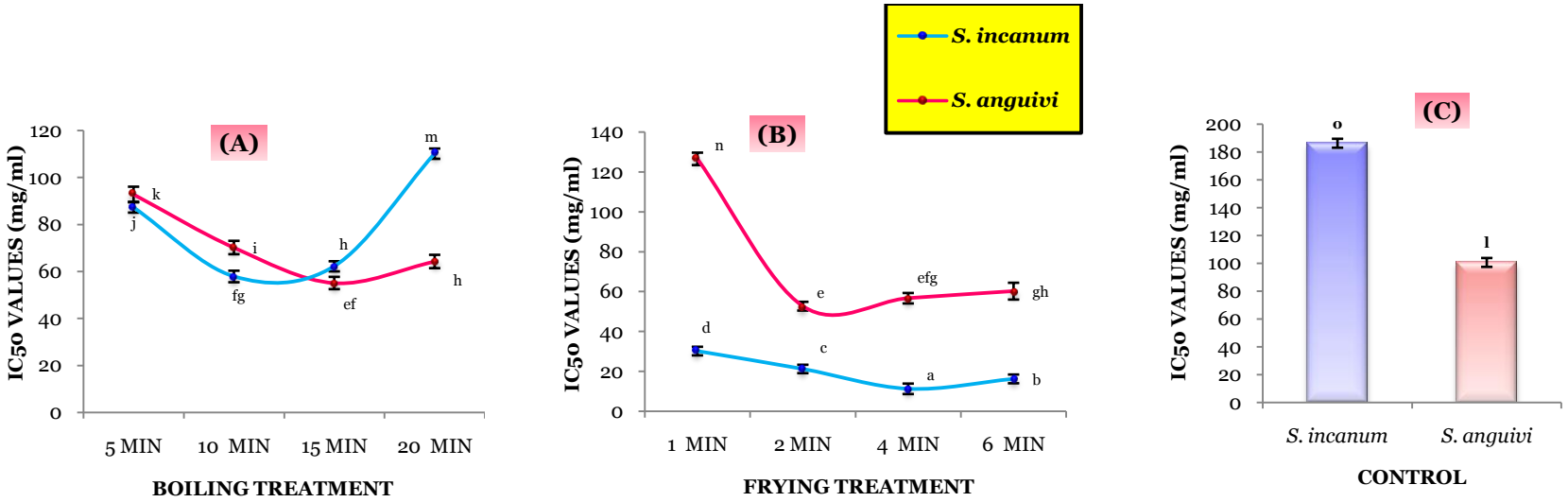


Figure 8.15 Superoxide radical scavenging (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

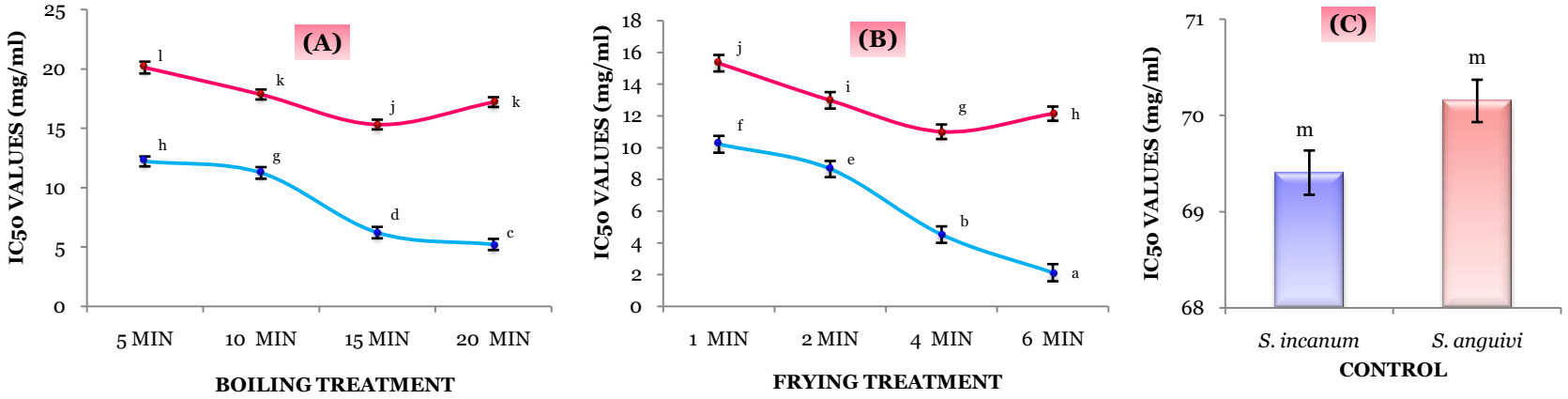


Figure 8.16 Hydroxyl radical scavenging (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

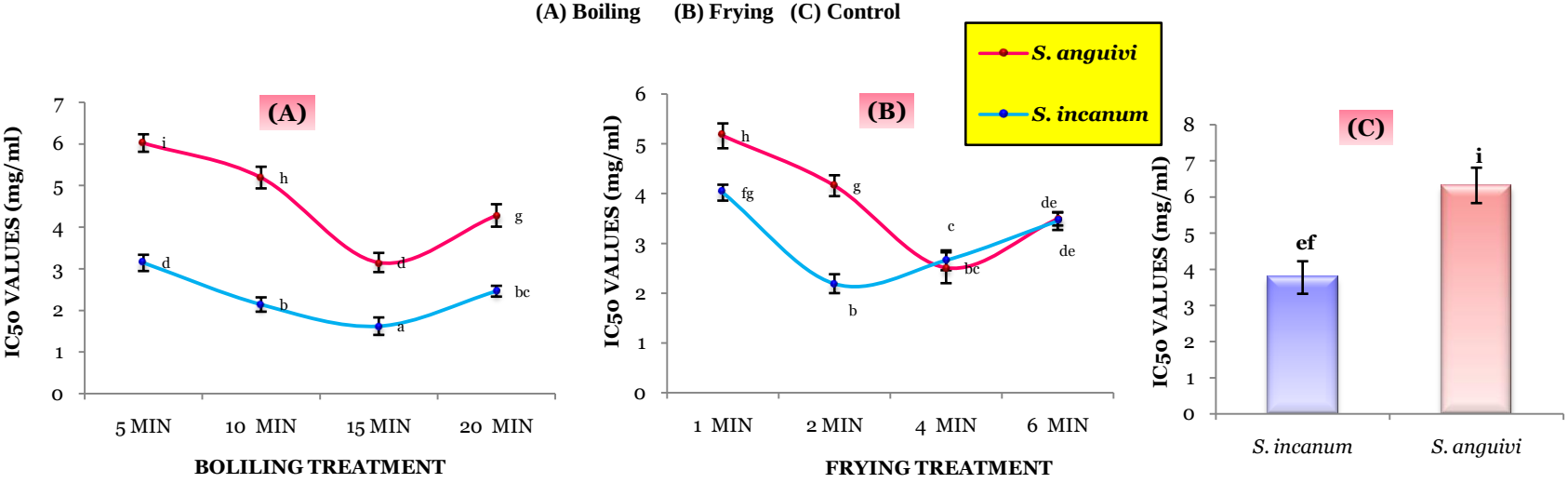


Figure 8.17 Lipid peroxidation (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

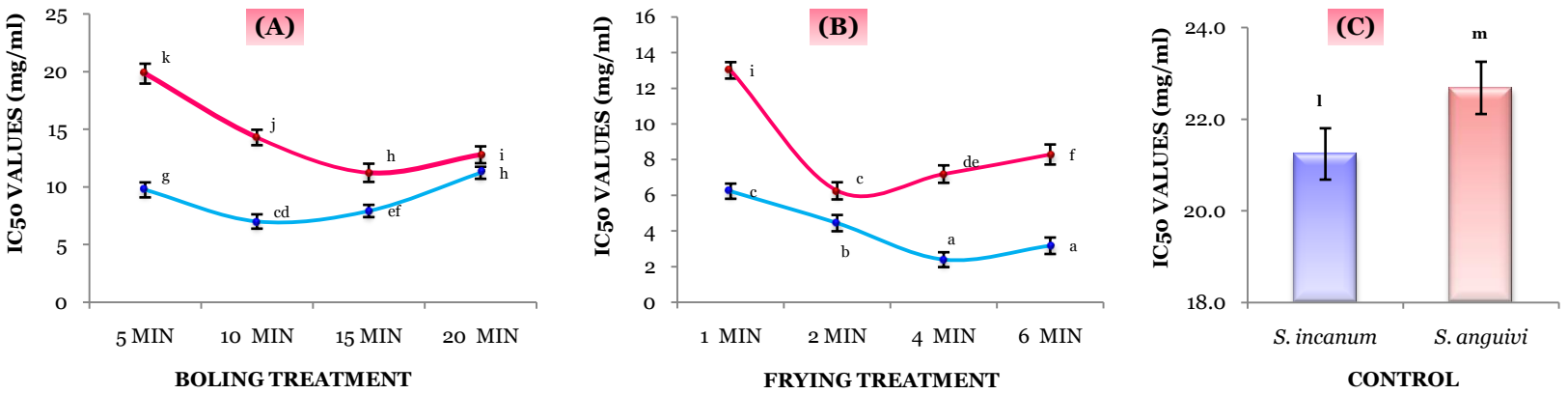


Figure 8.18 Metal chelating (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

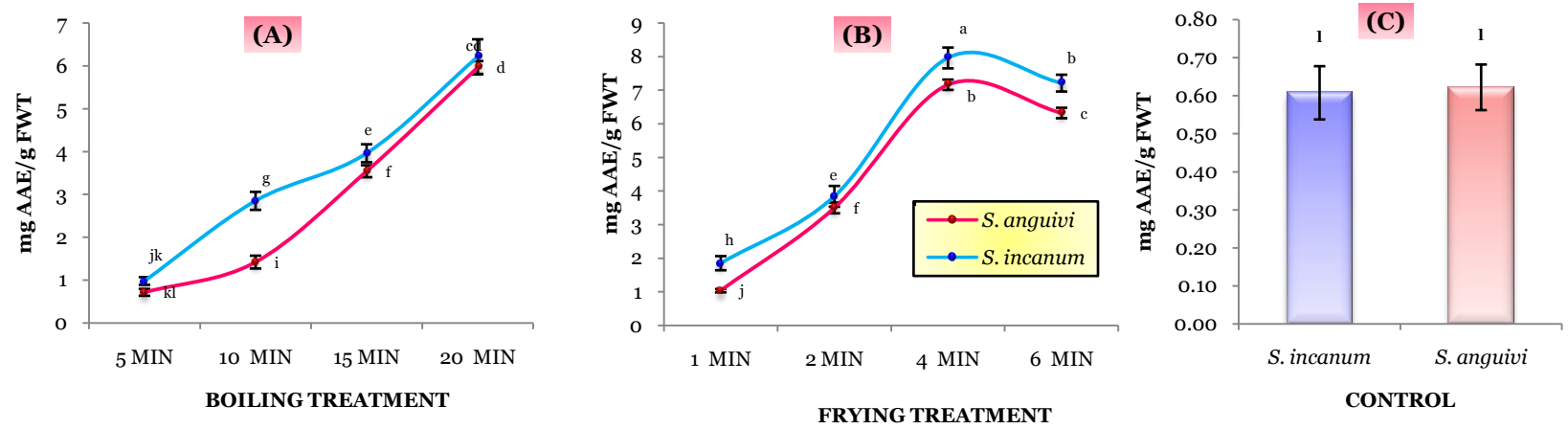


Figure 8.19 Reducing power of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

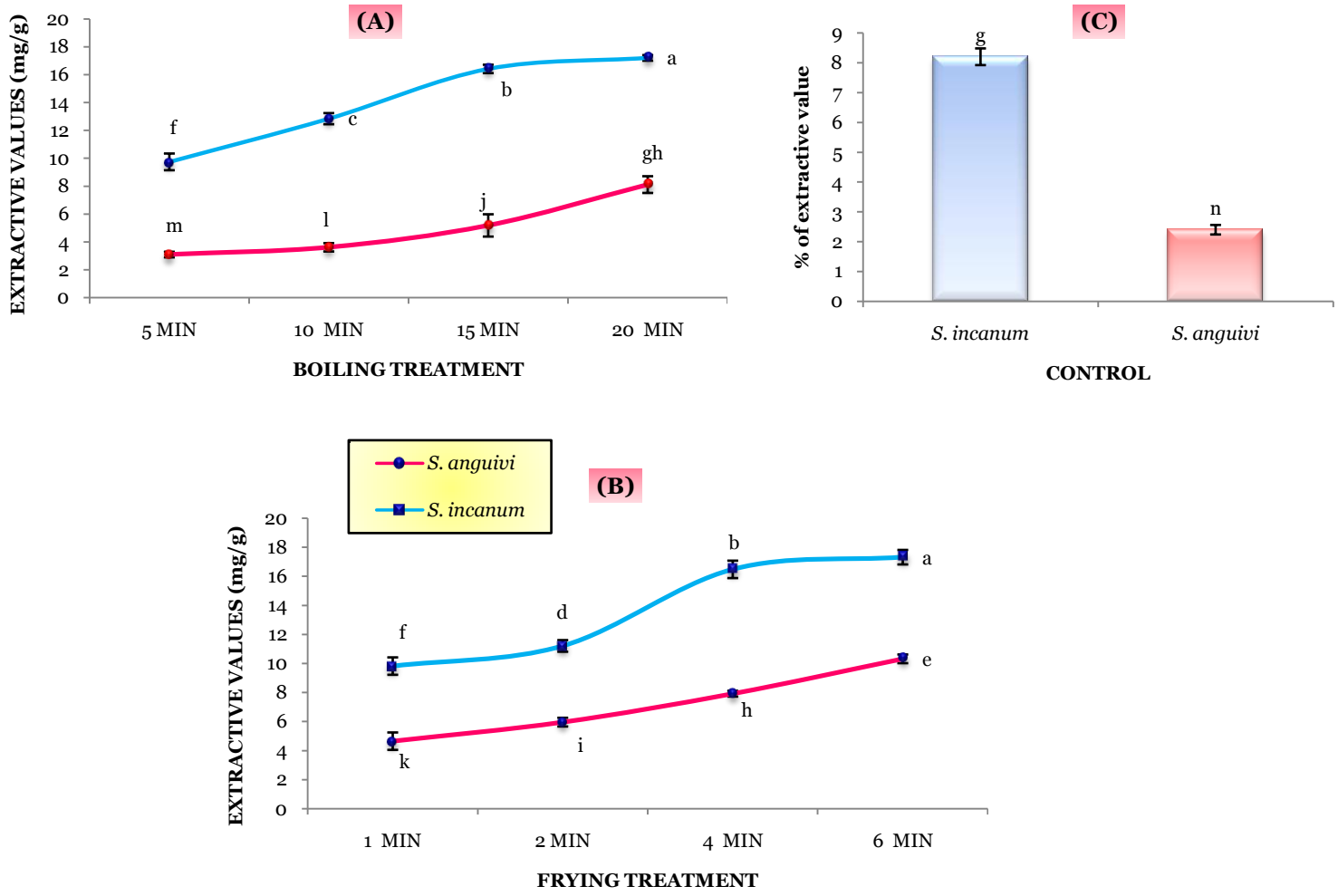


Figure 8.20 Extractive values of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

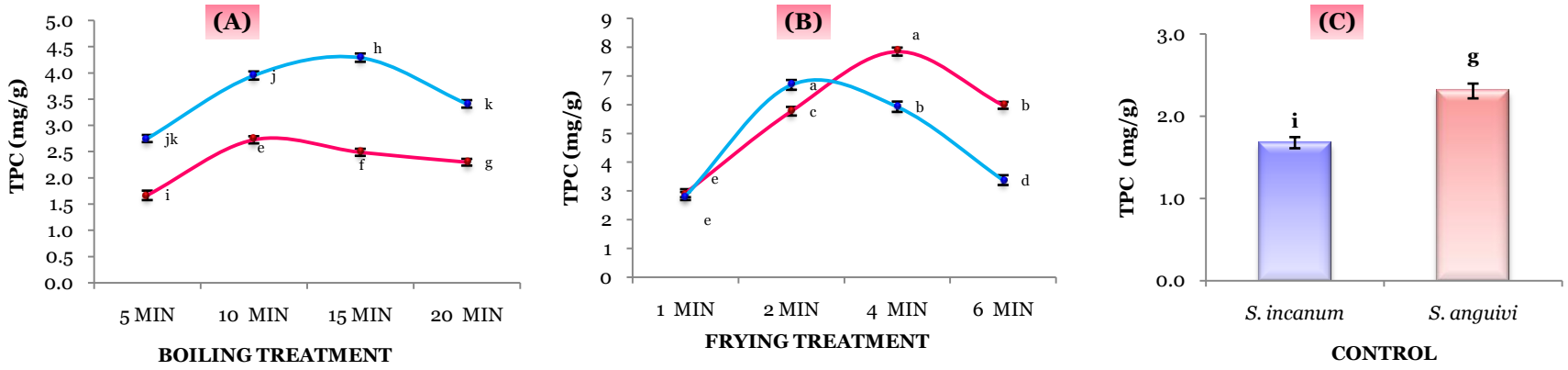


Figure 8.21 Total phenol content (TPC) of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

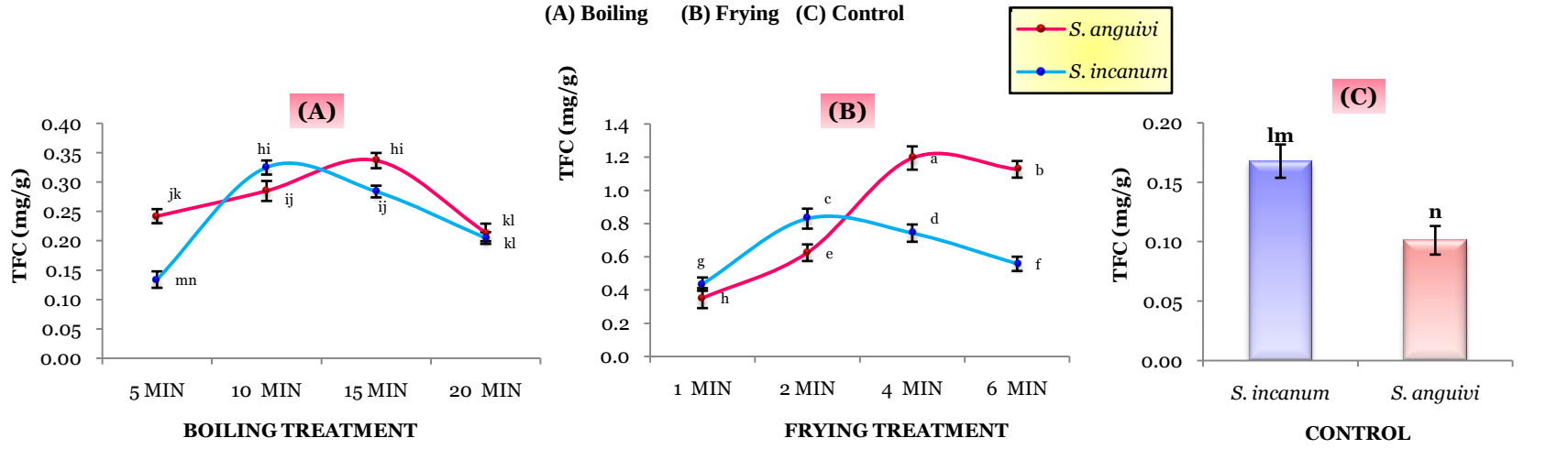


Figure 8.22 Total flavonoids content (TFC) of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

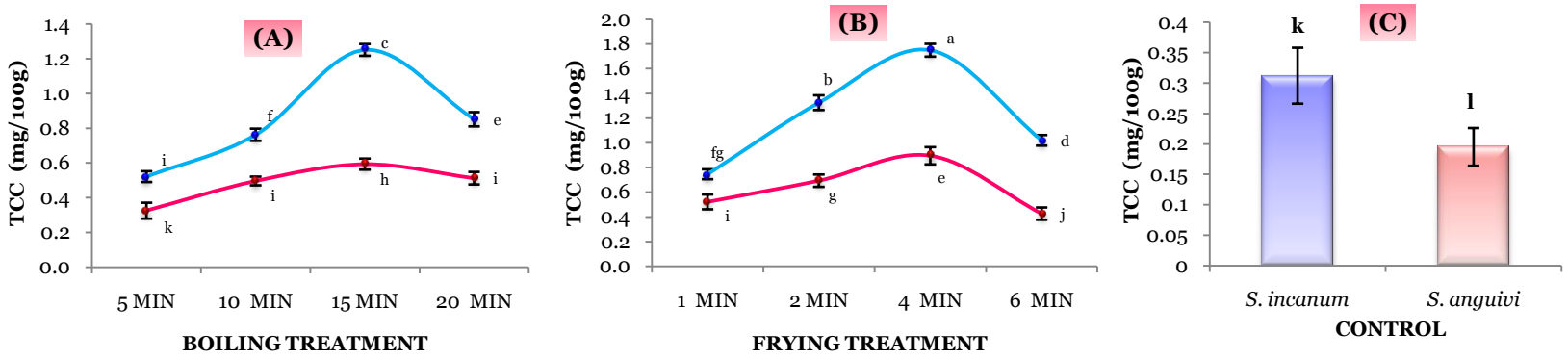


Figure 8.23 Total carotene content (TCC) of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

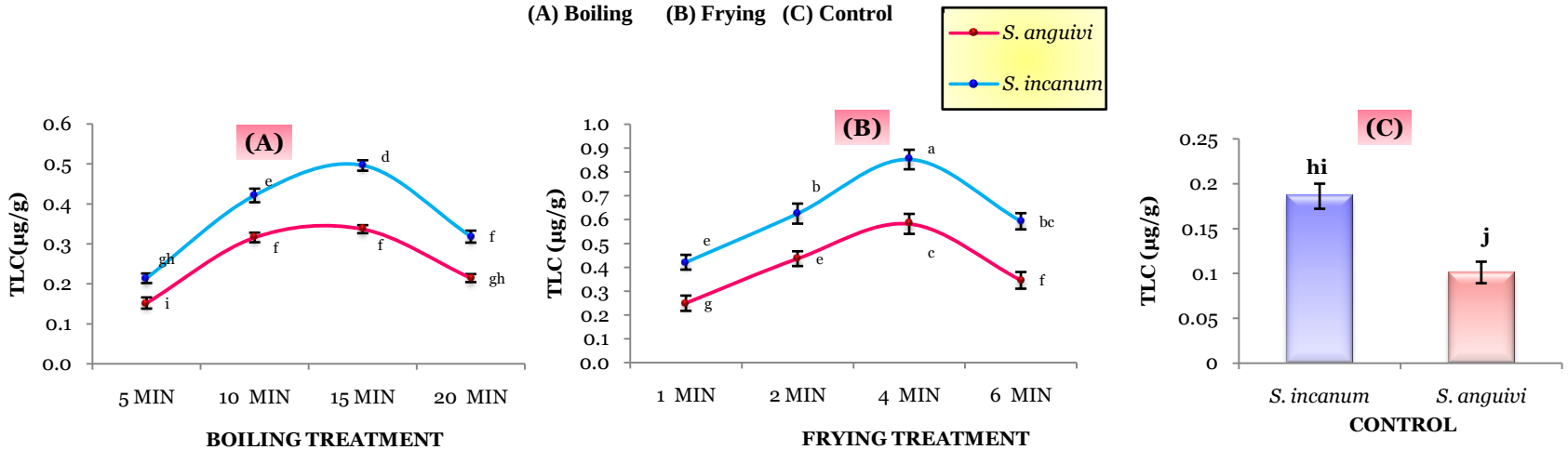


Figure 8.24 Total lycopene content (TLC) of *Solanum* fruits during different thermal processing
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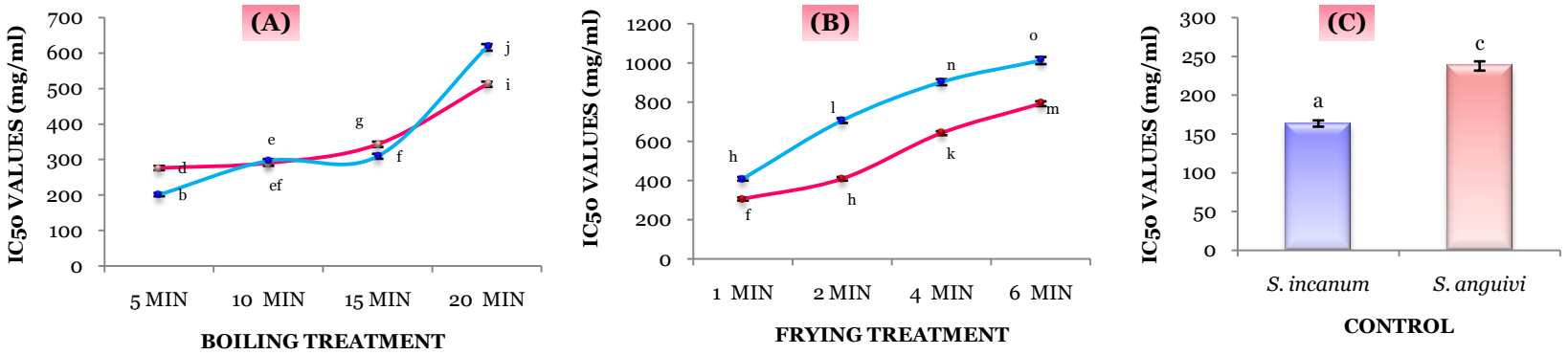


Figure 8.25 α-Glucosidase (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

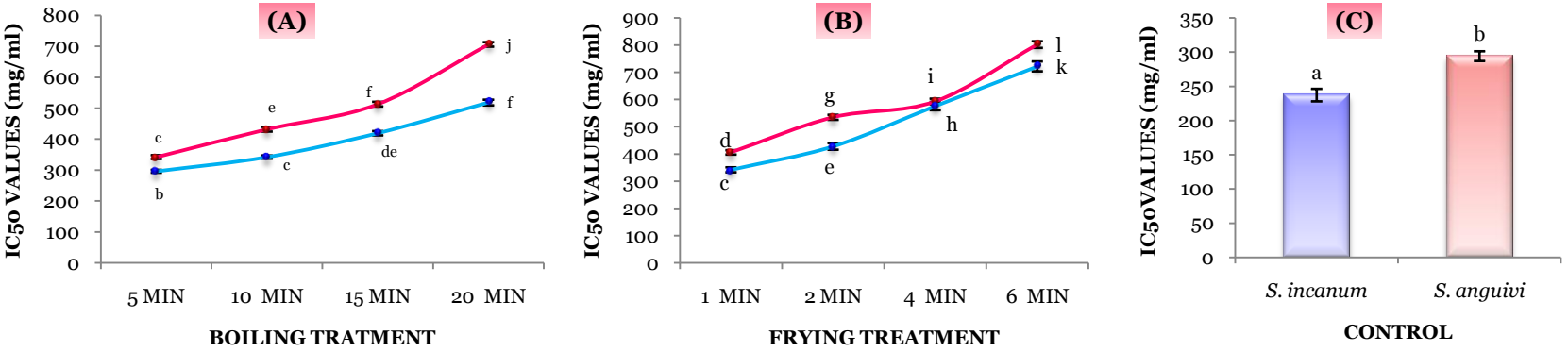


Figure 8.26 α-Amylase (IC_{50}) activity of *Solanum* fruits during different thermal processing
(A) Boiling (B) Frying (C) Control

equivalence) is also significantly correlated to α-amylase as well as α-glucosidase activity (Table 8.2).

8.3.3 Thermal treatment of seven different underground plant parts

Data for the percentage yield of methanolic extracts of raw and boiled taruls are shown in Figure 8.27. Percentage yield of extracts of vegetables increased during thermal processing in *Ipomoea* spp. and decreased in most *Dioscorea* spp., *Manihot esculenta*, *Sechium edule* and *Xanthosoma brasiliense*. Figure 8.28 demonstrated the DPPH scavenging activities of different taruls of Darjeeling Himalaya. The IC_{50} values of DPPH scavenging activity of different taruls cooked by boiling showed significant differences. For all cases, heat treatment significantly decreased DPPH scavenging activities. Similar trends were also observed in case of scavenging capacity of other free radicals like ABTS (Figure 8.29), metal chelating (Figure 8.30) reducing power ability (Figure 8.31), as well as hydroxyl radicals (Figure 8.32) after boiling treatment. Like antioxidant activity, an *in vitro* antidiabetic property which was parametrically assessed through α-glucosidase and α-amylase enzyme inhibition capacity was decreased with heat treatment Figure 8.33 and Figure 8.34).

Total phenol content was highly correlated with DPPH scavenging activity whereas total flavonoids content was associated with ABTS and hydroxyl radical scavenging property. On the other hand, both total phenol and flavonoid contents were accountable for α-amylase enzyme inhibition activity of taruls during heat treatment (Table 8.3), as revealed from high cohesiveness of both of the data variables. However, the quantity of bioactive phytochemicals responsible for antioxidant as well as antidiabetic activity namely total phenol (Figure 8.35), flavonoids (Figure 8.36) and ortho-dihydric phenol (Figure 8.37) are also reduced after boiling.

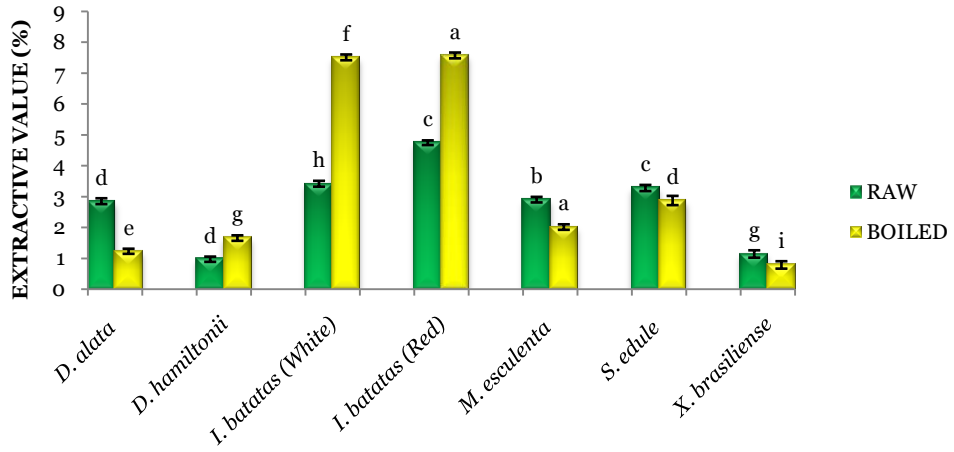


Figure 8.27 Extractive value (%) of different taruls

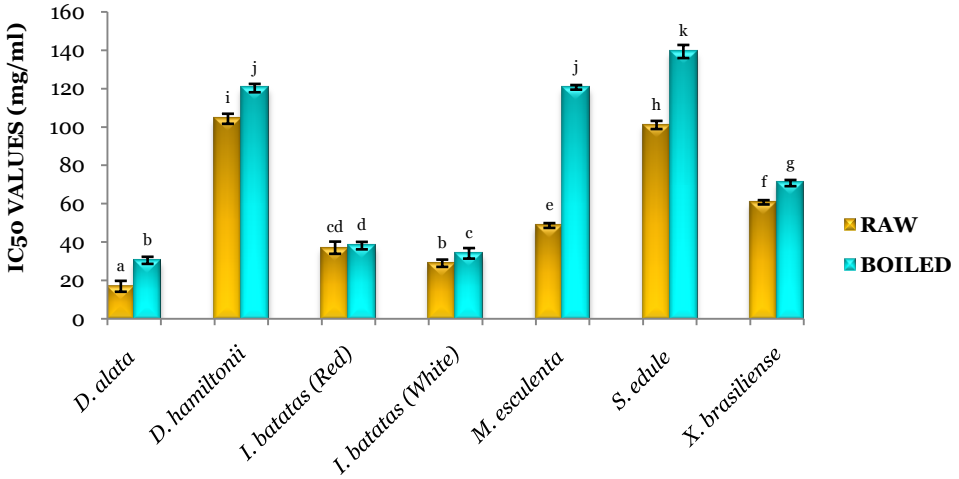


Figure 8.28 DPPH free radical scavenging activity (IC_{50} values mg/ml) of different taruls

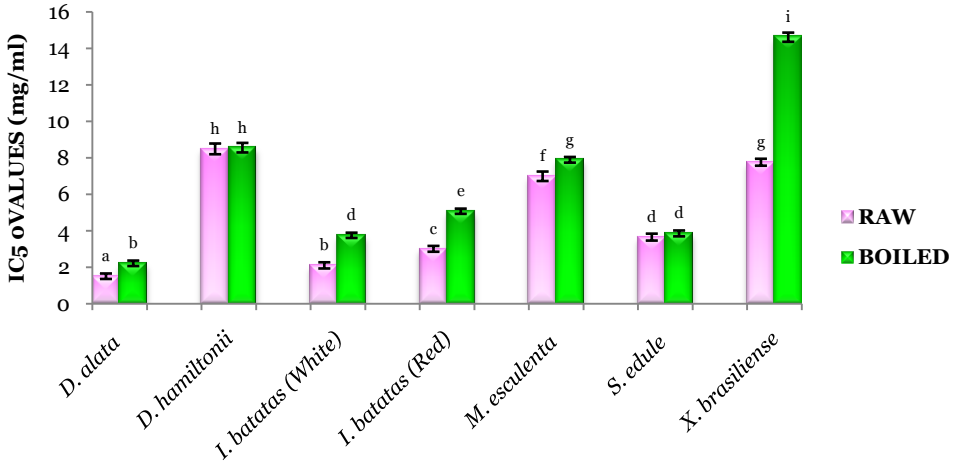


Figure 8.29 ABTS radical scavenging activity (IC_{50} values mg/ml) of different taruls

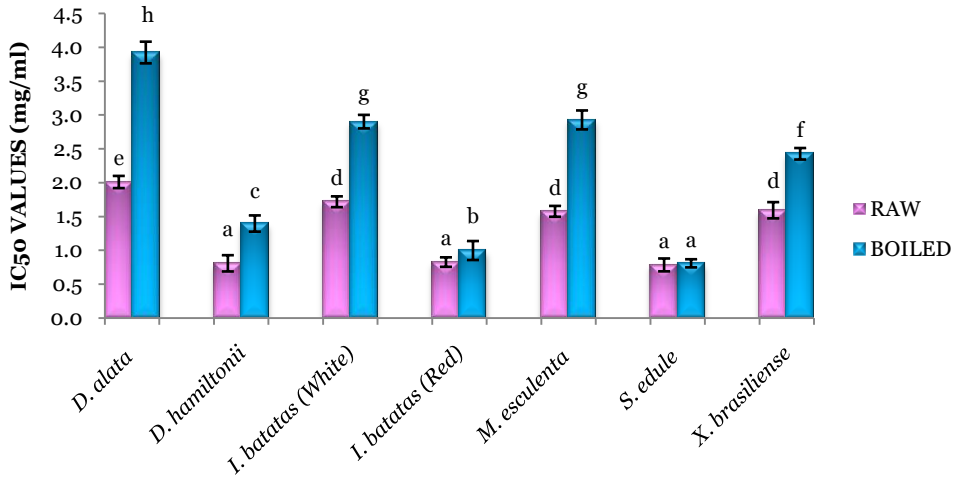


Figure 8.30 Metal chelating activity (IC_{50} values mg/ml) of different taruls

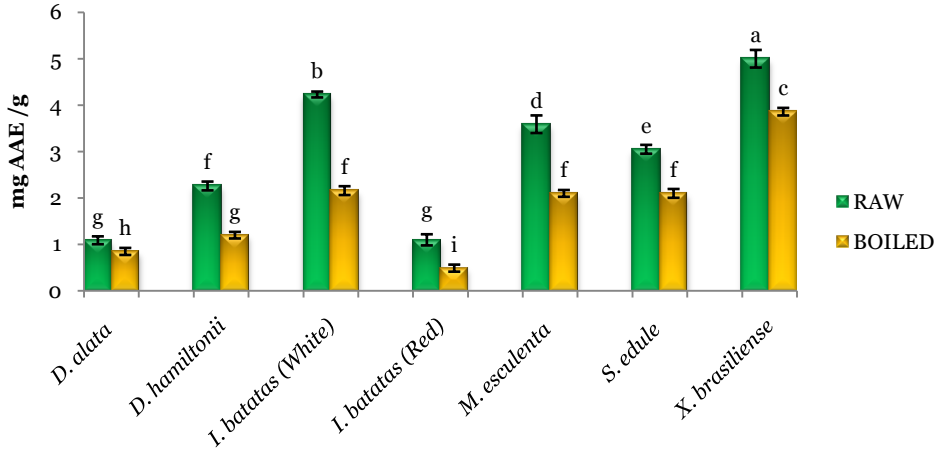


Figure 8.31 Reducing power capacity (mg AAE/g) of different taruls

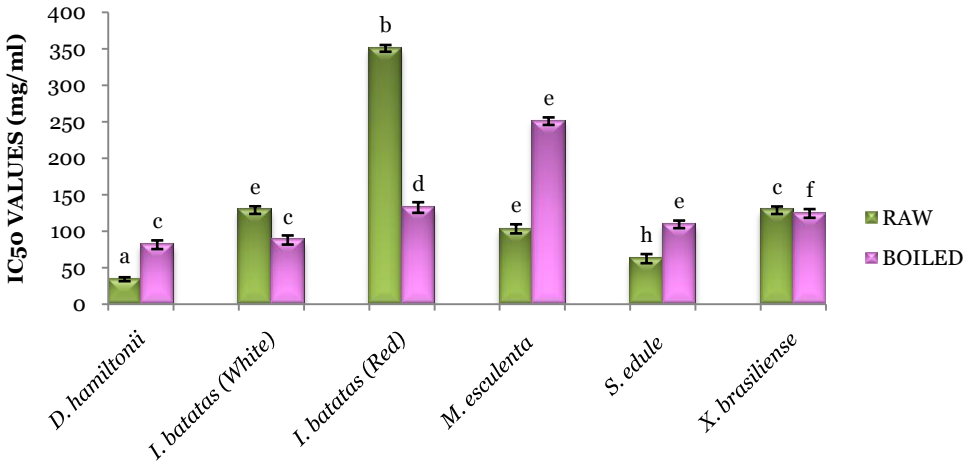


Figure 8.32 Hydroxyl radical scavenging activity (IC_{50} values mg/ml) of different taruls

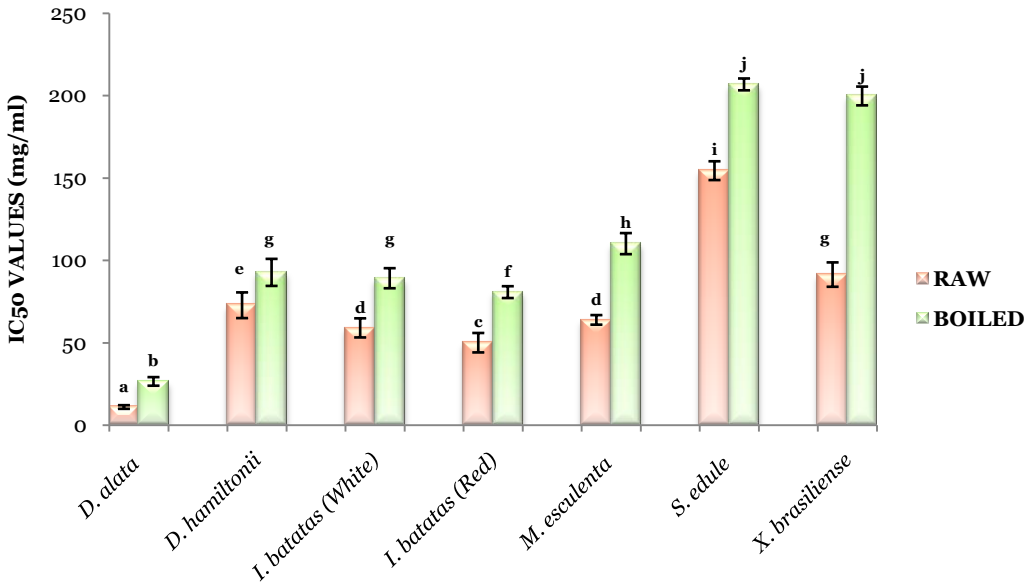


Figure 8.33 α-Glucosidase scavenging activity (IC_{50} values mg/ml) of different taruls

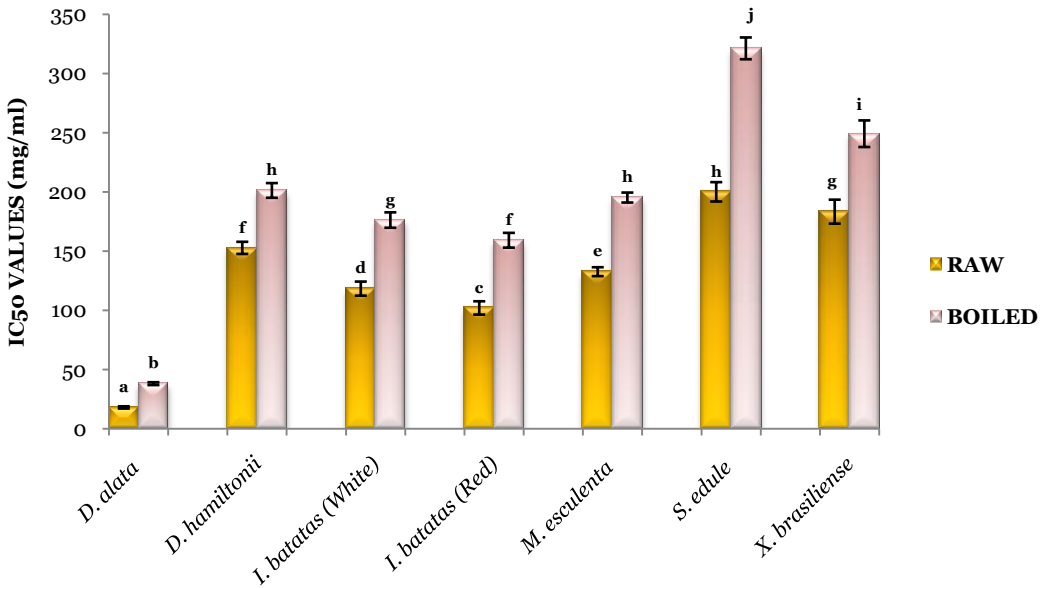


Figure 8.34 α-Amylase scavenging activity (IC_{50} values mg/ml) of different taruls

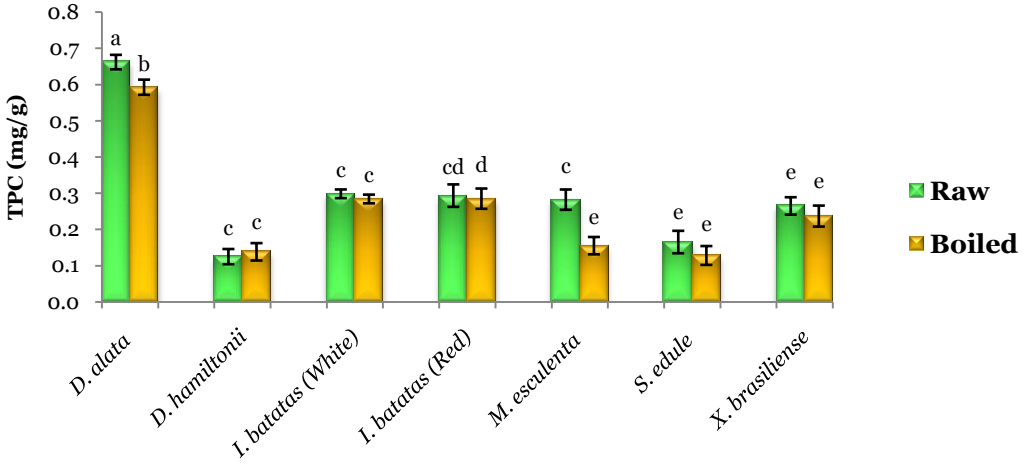


Figure 8.35 Total phenol content (TPC) (mg/g) of different taruls

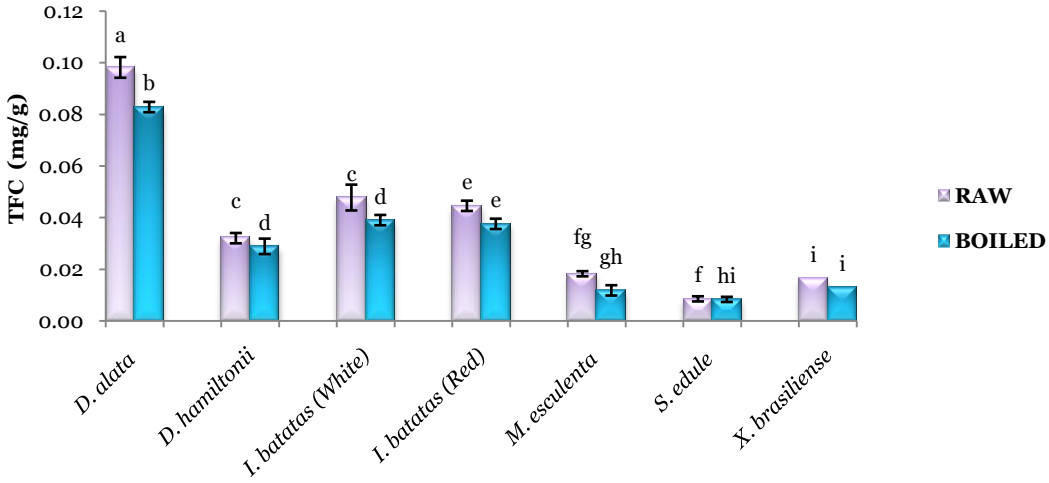


Figure 8.36 Total flavonoids content (TFC) (mg/g) of different taruls

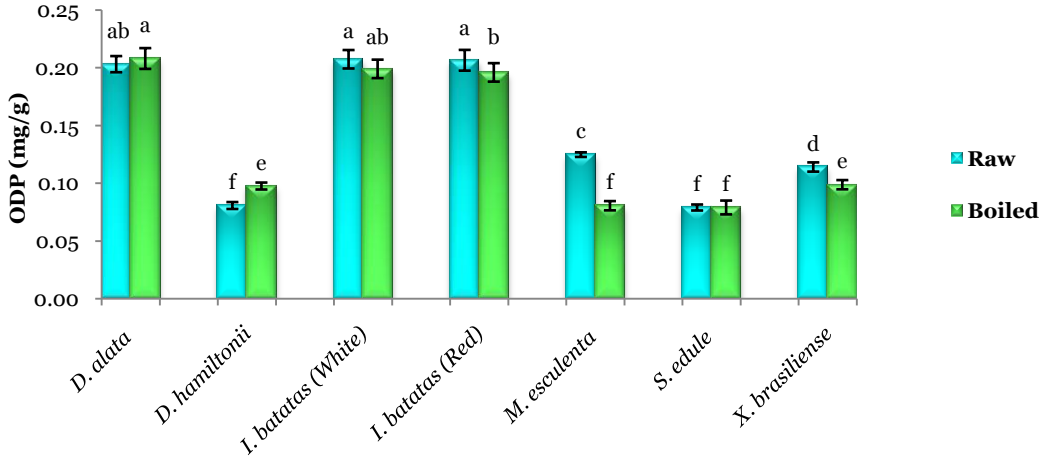


Figure 8.37 Ortho dihydric phenol content (ODP) (mg/g) of different taruls

8.4 DISCUSSIONS

The amount of the yield materials that can be extracted from a particular plant depends on strength of the extraction procedure as well as possibility exists between sample-to-sample differences in extracted materials. In our findings we noticed that the percentage yield of methanolic extracts of different plants enhanced 3 to 4 times after heat treatment. The increase in percentage yield of the extracts might be due to the fact that the heat treatment converts insoluble phenolic compounds into the soluble extractable forms (Jeong *et al*., 2004). Phenolics are the main contributors to the total antioxidant capacity of fruits and vegetables (Heo *et al*., 2007). In our study, compared with raw vegetables, boiling and frying treatments showed significant alteration of total phenolic content. For Solanaceae plants, the effect of frying treatment on total phenolic content was significantly higher than boiling methods and the phenol content was remarkably changed with time. Soler-Rivas *et al*., (2009) proved the effects of heat treatment on the duration of cooking. But in case of underground parts cooking treatments significantly decreased phenolic contents. The reason might be that heating sometimes destroyed the structure of phenolics and decrease their contents (Barros *et al*., 2007). Like total phenolics, flavonoids also reduced in boiled vegetables. Flavonoid contents leached in cooking water during boiling (Makris & Rossiter, 2001). Many parallel reports are available like Dietrych-Szostak (2006) noticed that heat treatment of buckwheat significantly decreased the concentration of flavonoids. Similar losses in onions were also reported (Price *et al*., 1997; Lombard *et al*., 2005; Lee *et al*., 2008). In the context of heat treatments, contradictory findings were also reported. Lombard *et al*. (2005) examined an increase of total flavonoids of onions. In 2008, Lee *et al*. noticed that steaming and baking didn't affect the flavonoids content significantly. Conversely, baking is found to increase quercetin conjugate and total flavonol content as these compounds were concentrated in the tissues (Lombard *et al*., 2005). Similar results were also noticed in our experiments. In case of *C. betacea* and *S. anguivi* cooking was favourable thermal processing for increase of flavonoid contents (Figure 8.8 and Figure 8.22). According to Hachett *et al.* (2002)

the loss of β-carotene during processing is due to alteration of *trans* to *cis* form. Other investigations have also demonstrated that processing of carotenoid-rich vegetables with heat and the addition of oil increases carotenoid (lycopene and β- carotene) concentrations (Gartner *et al*., 1997; Boileau *et al*., 1999). These contradictory results might be due to the diversity of food products used and the lack of the standardization of domestic processes. Carotenoids and lycopene contents of each cooked vegetable prepared by different methods was significantly higher than raw ones (except taruls). Effect of cooking methods and time on carotenoids and lycopene contents in the studied vegetables were varied. Frying and sun drying have increased the retention of carotenoids and lycopene in most of the selected vegetables 2-3 times than raw ones. Because of water loss, frying and sun drying process may concentrate these two compounds, giving higher amounts per unit weight of vegetables (Rodriguez-Amaya and de Sa, 2003). Dewanto *et al*., (2002) reported that at 88°C cooking temperature, lycopene was increased during 30 min heating. This suggested that mild thermal processing could simultaneously enhance lycopene concentration of vegetables by increasing the free and bioaccessible form where as degrading lycopene through high temperature. Along with changing temperature, the time might often play an important role in lycopene and carotenoid concentrations of different vegetables.

Several previous studies have evaluated the relationship between free-radical scavenging capacity and bioactive secondary metabolites (Aires *et al*., 2011; Ninfali *et al*., 2005). The antioxidant activity of these vegetables of Darjeeling Himalaya is increased by boiling (except taruls). Many authors suggested the cause of this occurrence that the pro-oxidant capacity which might be due to peroxidases, were inactivated at high temperatures (Gazzani *et al*., 1998). Some authors claimed another clarification that the antioxidant activity enhanced due to improvement of antioxidant properties of naturally occurring components or formation of novel compounds like Maillard reaction products having antioxidant capacity (Nicoli *et al*., 1999; Manzocco *et al*., 2001). In 1998, Manzocco *et al*., in their study reported that by pasteurization of tea extracts increased its antioxidant activity. They also reported that during heat treatment by formation of

new compounds, antioxidant property was enhanced. Oboh *et al*., (2013) noticed that heat treatment reduced the α-amylase as well as α-glucosidase inhibition activity of *Amaranthus cruentus* plant. Similarly, boiling and frying treatment were also destroyed these inhibition property of plants of our study (Figure 8.12-8.13 and Figure 8.25-8.26); whereas higher condensed products of sun drying material enriched the inhibition property of *C. betacea* (here mild heat treatment was not affected the assets).

From the Tables 8.1 it is clear that good correlations were established between free-radical scavenging and different phytochemicals of *C. betacea*. Interestingly, a superior correlation was registered between antidiabetic activities with these bioactive secondary metabolites. Recent research demonstrated that phenolic rich ethyl acetate fraction inhibited TBARS formation and scavenge DPPH and ABTS+ radical more effectively than *n*-butanol and aqueous fractions of *C. betacea* (Gomez-Romero *et al*., 2007). These authors also concluded that *C. betacea* phenolics are potent antioxidants, which can inhibit LDL oxidation *in vitro* and ROS production in a rat adrenal pheochromocytoma cell line. As represented in Table 8.1, the strong negative correlation between IC_{50} values of free radical scavengers and different bioactive polyphenols deserves detailed attention. Basically antioxidants are reducing agents and are capable of donating a single electron or hydrogen atom for detoxifying free radicals (Wong *et al*., 2006). In this study, the cohesiveness of free radicals with phytochemicals indicated that free hydroxyl groups present in phenolics and other phytochemicals play important role as reducing agent. The ferric reducing power of different cooked vegetables of *Solanum* fruits were also highly correlated with all tasted antioxidant capacity. Arnous *et al*. reported a strong correlation between DPPH free radical scavenging ability and ferric ion reducing capacity of wines (Arnous *et al*., 2002). Pulido *et al*. also suggested that ferric ion reducing capacity correlates with the results from different methods used to estimate antioxidant activity (Pulido *et al*., 2000). Interestingly, a superior correlation was recorded in case of Taruls between α-amylase inhibition with total phenol and flavonoids content (Table 8.3).

In conclusion, the present investigation clearly indicates that physicochemical as well as nutritional qualities of vegetables are extremely modified by domestic cooking and that the modifications of the evaluated parameters are strongly dependent upon the vegetable species. These cooking conditions would have promoted the release of antioxidant compounds from *C. betacea* and *Solanum* spp.; and would determine the formation of novel antioxidant compounds. Conversely, the overall decrease of antioxidant and antidiabetic values observed in case of taruls is in agreement with the concept that processed vegetables have lower nutritional quality than the raw ones. Moreover, our results suggest that for each vegetable a special cooking method could be selected to improve or preserve its nutritional and physicochemical qualities. This choice may help consumers on the selection of the cooking practices to improve nutritional quality of the foods.

Chapter – IX

BIOASSAY GUIDED PARTIAL PURIFICATION OF TWO EDIBLE PLANTS OF DARJEELING HIMALAYA

9.1 INTRODUCTION

Analysis of different bio-active components occurring in different plant parts has attracted considerable attention due to the recent discovery of pharmacological properties of different phytochemicals for human healthcare (Hertog *et al*., 1992; Hertog, 1993; Tsuchiya *et al.*, 1997; Dalluge *et al.*, 1998). Various techniques have been used for the determination of natural antioxidants and antidiabetic agents, including high performance UV spectrophotometric (McMurrough *et al*., 1983; Lunte, 1987; Amakura *et al*., 2000; Rodrguez-Delgado *et al*., 2001; Escarpa *et al*., 2002) and fluorometric (Rodrguez-Delgado *et al.*, 2001; Rodrguez-Delgado *et al*., 2002) detection, thin-layer chromatography (Simonovska *et al.*, 2003), capillary zone electrophoresis (Minussi *et al*., 2003), liquid chromatography (HPLC) with electrochemical detection (Guo *et al.*, 1997; Peyrat-Maillard *et al.*, 2000; Nardini and Ghiselli, 2004), HPLC/MS (Justesen, 2000; Robards, 2003; Schez-Rabaneda *et al*., 2003; Simonovska *et al*., 2003; Gil *et al*., 2003; Shui and Leong, 2004) or nuclear magnetic resonance spectrometry (Gil *et al*., 2003). Isolation and purification is essential for these techniques. Bioassay-guided purification is a procedure where the extract is initially fractionated and re-fractionated chromatographically, until a pure biologically active compound is isolated (Weller, 2012). This type of purification is commonly employed in drug discovery research for enhancing its effectiveness; to analyze extract and targeted compounds that followed with certain biological activity. There are three focal reasons for screening this study i.e. to confirm the ethnomedicinal use of plants; to find new lead compounds for developing pharmaceuticals or to prepare phytomedicines designed for use as plant-based healthcare. Apparently, little effort has established for developing cheaper technology-based strategies for the use of medicinal plants in rural communities in the whole world. There are still limited publications on the biological activities of edible plants of Darjeeling Himalaya. Hence, we have tried to purify and identify the bioactive components of two edible plants like *Solanum anguivi* and *Calamus erectus* of Darjeeling Himalaya used by local people of this region through chromatographic and spectroscopic techniques.

9.2 MATERIALS AND METHODS

9.2.1 *Solanum anguivi*

9.2.1a Extraction procedure

S. anguivi fruits were collected and the calyx and stalk was removed. The mature fruits were separated. Initial extraction was started with 1 kg plant material and processed through soxhlet apparatus with methanol for 3-4 days. The sample extract was dried and their total extractive values were calculated. The sample was then kept in freeze for further use.

9.2.1b Solvent partitioning of the crude extracts

The crude methanolic extract of the ripe fruits (i.e. the orange fruits) was found to be highly rich in antioxidants (Chapter IV) and antidiabetic agents (Chapter VII) which prompted us to carry out further investigation. Solvent-solvent extraction is one of the most popular methods for partial purification, i.e. group separation according to polarity of crude extracts. The crude extracts of ripe fruits of *S. anguivi* was reconstituted with distilled water and successively partitioned with solvents on the basis of increasing dielectric constants i.e. increasing polarity with solvents like hexane, chloroform, ethyl-acetate, dichloromethane, butanol and water in successive order using a separating funnel. Consequently, ten partition fractions were obtained from the crude fruits' extract (Figure 9.1 and Table 9.1). The solvents from different fractions were evaporated and reconstituted with water.

Following free-radical scavenging activities were performed with each successive partitioned extracts from the ripe fruits:

9.2.1c DPPH free radical scavenging assay

The assay was performed as prescribed by Blois (1958) and specified in details in Chapter IV Section 4.2.4.

9.2.1d $ABTS^{+}$ radical cation(s) decolourization assay

The assay was performed as prescribed by Re *et al.* (1999) and specified in details in Chapter IV Section 4.2.5.

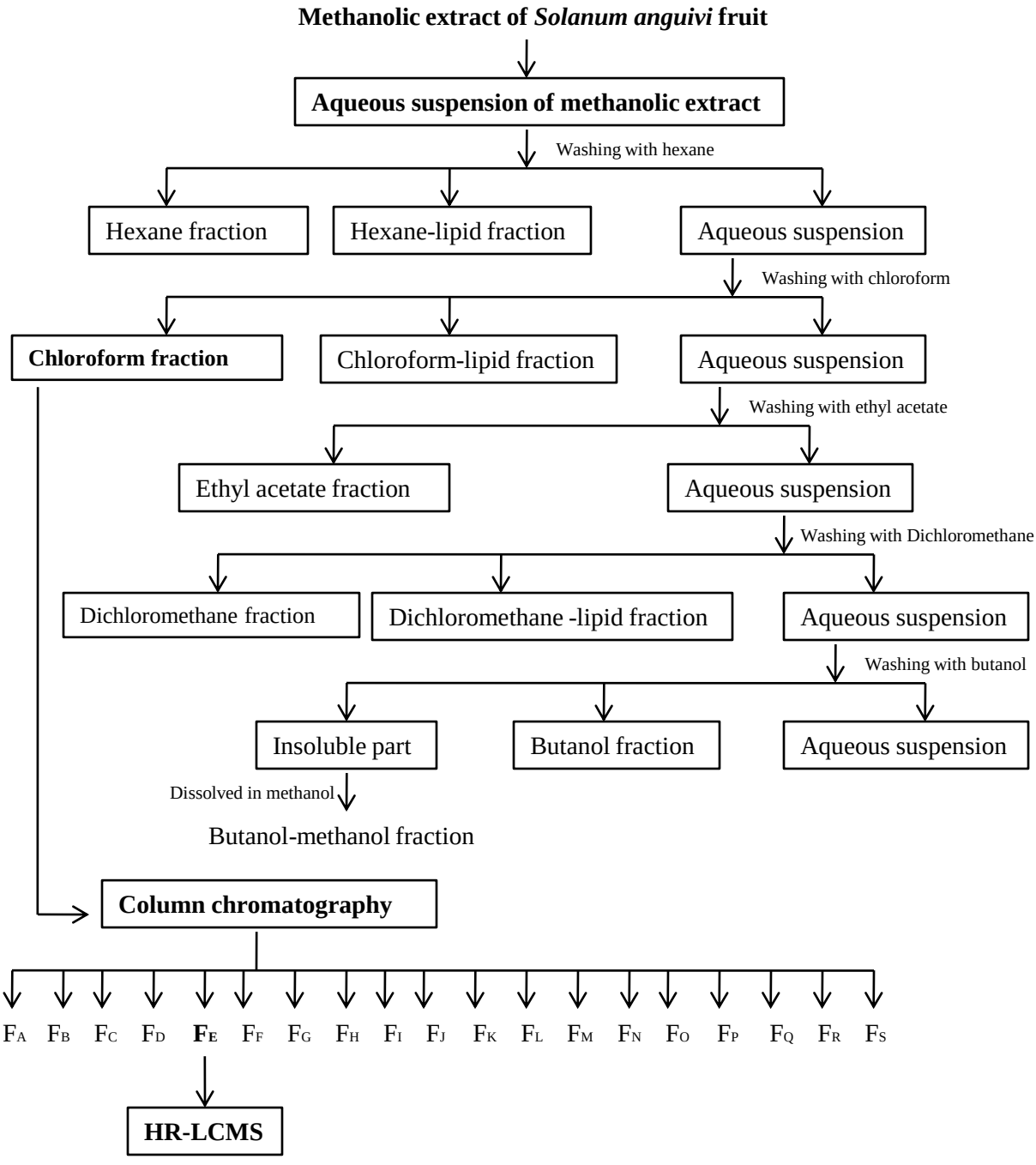


Figure 9.1 Scheme of fractions preparation of *S. anguivi* fruit

Table 9.1 Solvents used for column chromatography

FRACTIONS	SOLVENTS	AMOUNT (%)
1-5	Benzene : chloroform	75:25
6-10	Benzene : chloroform	50:50
11-18	Benzene : ethyl acetate	50:50
19-25	Ethyl acetate	1
26-31	Mithyl isobutyle kitone : benzene	50:50
32-36	Acetone : benzene	50:50
37-42	Acetone	1
43-50	Benzene : methanol	50:50
51-57	Water : methanol	50:50
58	Water	1

Table 9.4 Merged fractions according to similar peak characteristics under UV-VIS spectrum

SIMILAR PEAK CONTAINING FRACTION	NUMBER OF PEAKS	PEAK VALUES	MERGED FRACTIONS NAME
1-3	1	271	A
4	3	271, 407, 664	B
5-6	1	271	C
7-9	1	275	D
10-18	1	282	E
19	1	261	F
20-25	1	282	G
26-30	2	205, 247	H
31	2	235, 355	I
32	1	264	J
33	3	221, 259, 320	K
34-36	2	225, 264	L
37-40	3	264, 336, 572	M
41-42	3	225, 235, 573	N
43	1	309	O
44	1	279	P
45-47	3	247, 264, 335	Q
48-53	2	248, 355	R
54-58	0	0	S

9.2.1e Metal chelating activity

The assay was performed as prescribed by Dinis *et al.* (1994) and specified in details in Chapter IV Section 4.2.9.

9.2.1f Total phenol estimation

The assay was performed as prescribed by Folin and Ciocalteu, (1927) and specified in details in Chapter V Section 5.2.3.

9.2.1g Total flavonoids determination

The assay was performed as prescribed by Sultana *et al*., (2009) and specified in details in Chapter V Section 5.2.4.

Antidiabetic activity was performed by the following methods:

9.2.1h Inhibition of *α*-amylase enzyme

The assay was performed as prescribed by Heidari *et al.,* (2005) and specified in details in Chapter VI Section 6.2.4a.

9.2.1i Inhibition of α-glucosidase enzyme

The assay was performed as prescribed by Oki *et al.,* (1999) and specified in details in Chapter VI Section 6.2.4b.

9.2.1j UV-Visible Spectroscopy

Purified butanolic fraction 1-58 was scanned with UV-VIS Spectrophotometer ($\lambda_{199\text{-}700\ nm}$).

9.2.1k HR-LC/MS

Fraction E was selected for High Resolution Liquid Chromatography followed by Mass Spectrometry which was performed at the laboratory of Sophisticated Analytical Instrumental

Facilities (SAIF), Indian Institute of Technology (IIT), Mumbai for identification and characterization of fragment masses of bio-active components.

9.2.2 *Calamus erectus*

9.2.2a Extraction procedure

Calamus erectus fruits were collected and the epicarp was removed and the obtained mesocarp and endocarp were weighed 1kg and processed through soxhlet apparatus with methanol for 3-4 days. The sample extract was dried and their total extractive values were calculated. The sample was then kept in freeze for further use.

9.2.2b Solvent partitioning of the crude extracts

The crude methanolic extract was found to be highly rich in antioxidants which prompted us to carry out further investigation. Solvent-solvent extraction is one of the most popular methods for partial purification, i.e. group separation according to polarity of crude extracts. The dried crude extract of *C. erectus* fruit was reconstituted with distilled water and successively partitioned with chloroform, ethyl acetate and butanol in successive order using a separating funnel. Consequently, four partitioned fractions were obtained from the crude fruits extract (Figure 9.2 and Table 9.2-9.3). The solvents from fractions were evaporated and reconstituted with water.

Following free-radical scavenging activity was performed with each successive partitioned extracts:

9.2.2c DPPH free radical scavenging assay

The assay was performed as prescribed by Blois (1958) and specified in details in Chapter IV Section 4.2.4.

9.2.2d Determination of reducing power

The assay was performed as prescribed by Aiyegoro and Okoh, (2009) and specified in details in Chapter IV Section 4.2.10.

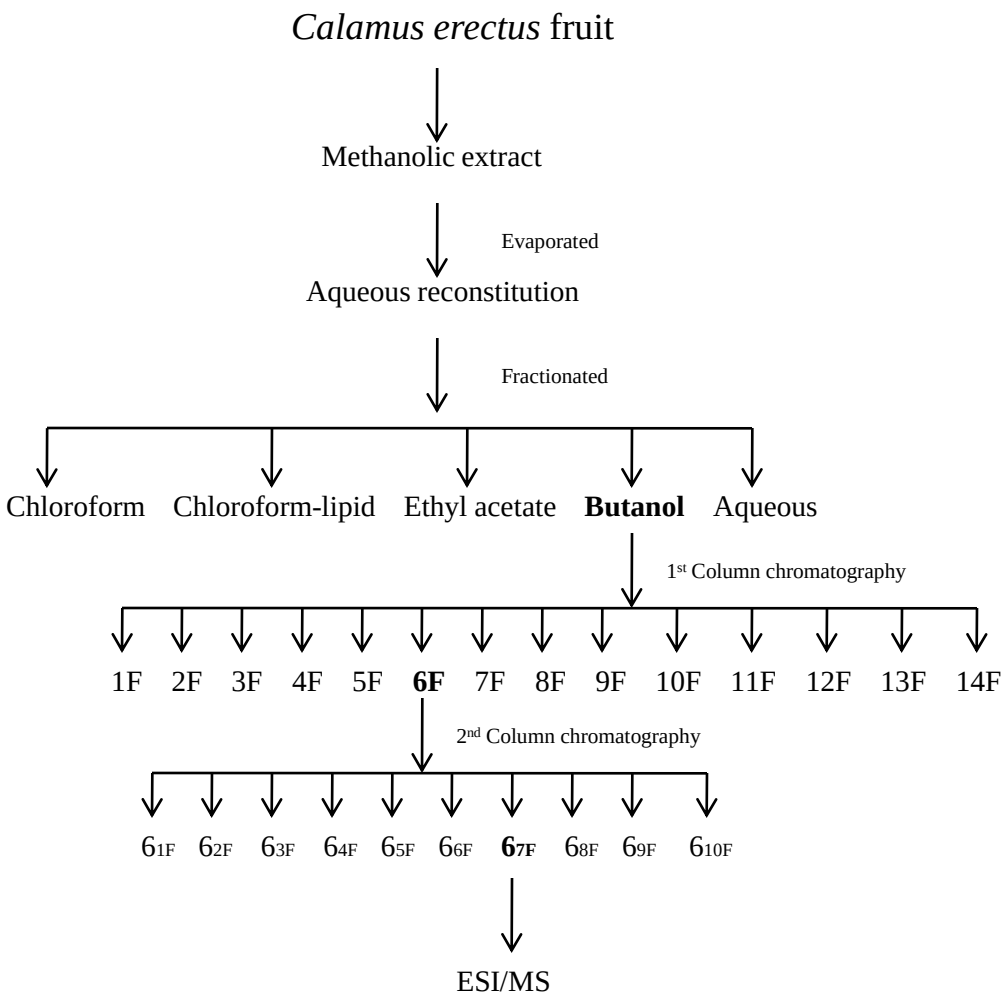


Figure 9.2 Scheme of fractions preparation of *C. erectus* fruit

Table 9.2 Solvents used for first column partitioning of butanolic fraction

Fraction no.	Mobile phase	Ratio
1	PET ETHER:CHLO	50:50
2	PET ETHER:CHLO	25:75
3	CHLOROFORM	1
4	CHLO:METH	90:10
5	CHLO:METH	80:20
6	CHLO:METH	70:30
7	CHLO:METH	60:40
8	CHLO:METH	50:50
9	CHLO:METH:H_2O	45:45:10
10	CHLO:METH:H_2O	30:60:10
11	CHLO:METH:H_2O	15:75:10
12	CHLO:METH:10%ACE	15:75:10
13	CHLO:METH:20%ACE	15:75:10
14	CHLO:METH:30%ACE	15:75:10

Table 9.3 Solvents used for second column partitioning of butanolic fraction

Fraction no.	Mobile phase	Ratio
1	CHLO:ACETONE:METHANOL	9:1:1
2	CHLO:ACETONE:METHANOL	7:3:1
3	CHLO:ACETONE:METHANOL	5:5:1
4	CHLO:ACETONE:METHANOL	3:7:1
5	CHLO:ACETONE:METHANOL	1:9:1
6	CHLO:ACETONE:METHANOL	1:7:3
7	CHLO:ACETONE:METHANOL	1:5:5
8	CHLO:ACETONE:METHANOL	1:3:7
9	CHLO:ACETONE:METHANOL	1:1:9
10	CHLO:ACETONE:METHANOL:WATER	0:1:7:3

Abbreviations: F = fraction; CHLO = Chloroform; PET ETHER = Petroleum ether; METH = Methanol; ACE = Acetone

9.2.2f Total phenol estimation

The assay was performed as prescribed by Folin and Ciocalteu, (1927) and specified in details in Chapter V Section 5.2.3.

9.2.2g Total flavonoids determination

The assay was performed as prescribed by Sultana *et al*., (2009) and specified in details in Chapter V Section 5.2.4.

9.2.2h Inhibition of *α*-amylase enzyme

The assay was performed as prescribed by Heidari *et al.,* (2005) and specified in details in Chapter VI Section 6.2.4a.

9.2.2i Inhibition of *α*-glucosidase enzyme

The assay was performed as prescribed by Oki *et al.,* (1999) and specified in details in Chapter VI Section 6.2.4b.

9.2.2j Silica gel Column chromatography

Column chromatography using silica gel of mesh size 200-400, as stationary phase was used to fractionate the bioactive compounds present in the butanolic extract by bioassay-guided fractionation (1^{st} column). From 1^{st} column purification, fraction 6 is selected and processed for 2^{nd} column purification.

9.2.2k HPTLC screening for phytochemical analysis and antioxidant activity

Qualitative screening of the constituents for phytochemicals and antioxidant activity in the butanol extract was performed by High Performance Thin Layer Chromatography (HPTLC) analysis. About 10µl of each sample was spotted on pre-coated silica gel plate 60 F-254 with Camag Linomat HPTLC System. The chromatograms were developed using the mobile phase composed of Ethyl Acetate : Formic Acid : Glacial Acetic Acid : H_2O :: 100:11:11:26. For the

detection of phytochemicals and antioxidants in each extracts, the reagents used for spraying on the developed chromatogram were: 1% ethanolic vanillin soln. & 10% ethanolic H_2SO_4.

9.2.2i ESI/MS

Fraction 7 from 2nd column ($B6_{F7}$) was selected for ESI/MS and sent to SAIF, CDRI (Lucknow) for mass characterization of bio-active components.

9.3 RESULT AND DISCUSSIONS

9.3.1 *Solanum anguivi*

Fruits of *S. anguivi* are widely used as vegetable in Darjeeling Himalayas. Traditionally, it has gained importance for its antidiabetic, digestive, laxative and antibacterial properties (Rajith and Ramachandran, 2010). The plant possesses numerous biologically active compounds which could serve as potential source of vegetable drugs in herbal medicine. The ripe (orange) fruit extracts were found to be most suitable and bioactive enough for further investigations (Chapter VII). Plants have wide array of phytochemicals ranging from non-polar to polar compounds. Thus, the orange fruits of *S. anguivi* were sequentially extracted to ensure complete extraction of all non-polar as well as polar components and thereby inclusion of all components in the screening studies. All ten successively partitioned fruit extracts were screened for their antioxidant activity, phytochemical content and antidiabetic properties described elsewhere in earlier chapters.

The wide range of effects of free radicals in the biological system have aroused a significant interests among the researchers and scientists in the past few decades and have provided a phenomenal scope for scientific investigations and experimental works. Several reports have documented the use of the antioxidant supplementation in reducing the level of oxidative stress and preventing the development of complications associated with various disorders. A huge number of members of plant kingdoms have been shown to possess bioactive components with a significant potential of free radical scavenging or antioxidant activity.

The stable free radical DPPH has been widely used to test the free radical scavenging ability of various dietary antioxidants (Brand-Williams *et al*., 2005). Our present study demonstrated that, the fractions of chloroform, butanol-methanol, butanol-butanol and aqueous phase of the ripe fruit extracts exhibited considerably stronger free radical scavenging potency in comparison to other partitioned fruit extracts of hexane, hexane/lipid, chloroform/lipid, ethyl acetate, dichloromethane (DCM), and DCM-lipid fractions as determined from their respective IC_{50} values shown in Figure 9.3.

$ABTS^+$ radical assay, can be used in both organic and aqueous solvent systems (Wu *et al*., 2006), therefore, is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Katalinic *et al*., 2006; Huang *et al*., 2008). In this experiment, among different partitioned extracts, the chloroform, butanol-methanol and butanol-butanol fractions exhibited highest $ABTS^+$ radical scavenging activity (Figure 9.4).

Phenols are very important plant products because of their scavenging capability due to their hydroxyl groups. The phenolic compounds may contribute directly to antioxidative damage (Dah *et al.,* 1999). Figure 9.5 shows that the phenol content which was found to be higher in the chloroform and butanol partitioned fruit extracts which could be partly responsible for the beneficial effects.

Total flavonoids are widely distributed in plants, both as co-pigments with anthocyanins in petals and also in leaves and fruits of higher plants. Like the anthocyanins, flavonoids also occur most frequently with the glycosides through covalent chemical attachment. In many plants studied, the participation of flavonoids in disease resistance mechanism has been demonstrated extensively. Figure 9.5 represents the quantitative profile of flavonoids which is comparatively higher in the ethyl acetate and butanolic partitioned fruit extracts than other fractions.

Herbal extracts and herbal formulations, used in the Ayurvedic literature, have recently been reviewed and have gained importance for the control of type 2 diabetes mellitus. They are being used directly or indirectly for the preparation of many modern drugs

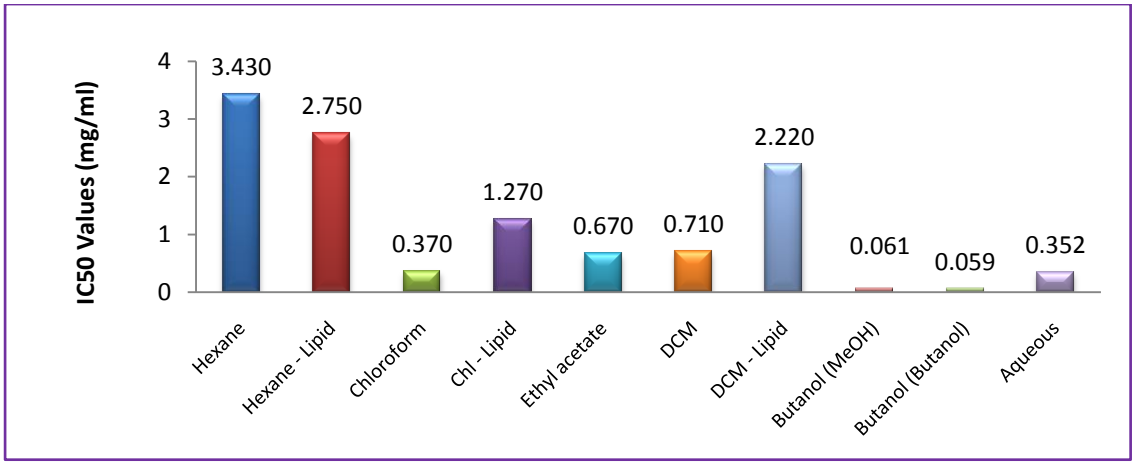


Figure 9.3 DPPH radical scavenging assay of different fractions

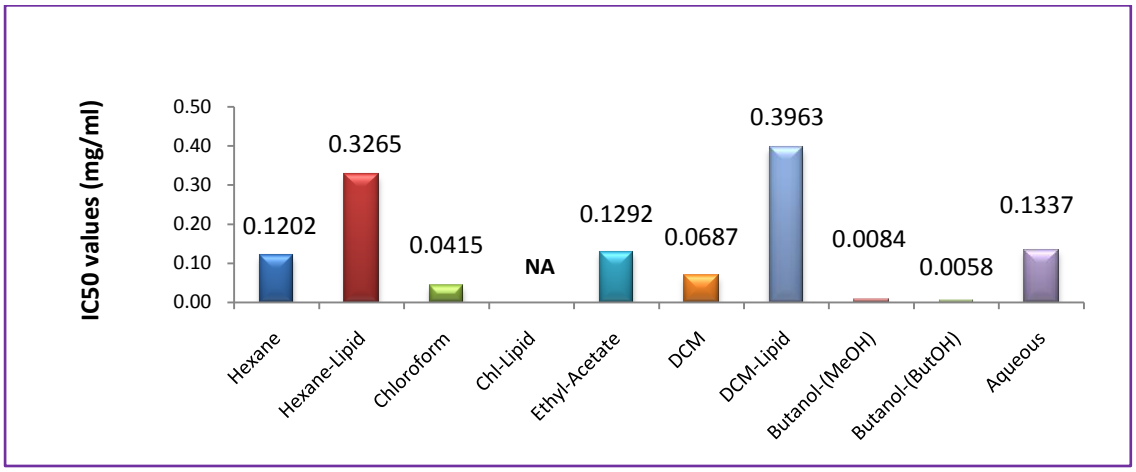


Figure 9.4 ABTS radical scavenging assay of different fractions

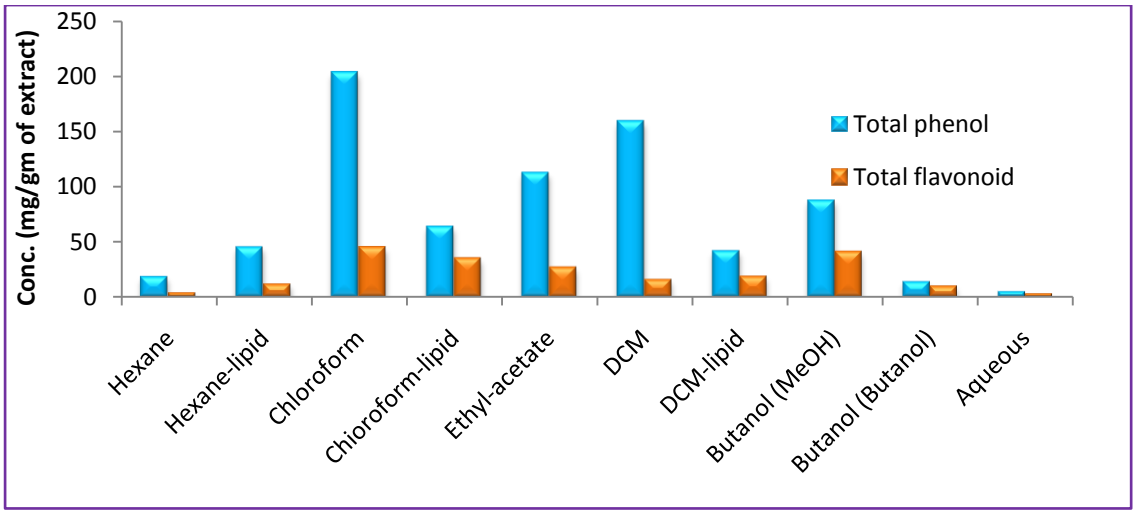


Figure 9.5 Total phenol and flavonoid content assay of different fraction

(Rasheed *et al*., 2012). Plants are known to produce a large variety of glucosidase inhibitors that provides protection against insects and microbial pathogens (Ryan, 1989 and Lu, 1999). Pancreatic and intestinal glucosidases are the key enzymes of dietary carbohydrate digestion and inhibitors of these enzymes may be effective in retarding glucose absorption to suppress postprantal hyperglycemia. Our investigation provides the first line of evidence of antidiabetic property of extracts of *S. anguivi* (Chapter VI). As no scientific evidence was recorded previously, therefore our investigation is a novel approach to identify the target molecule/s involved in lowering the glycemic index and the control of post-prandial hyperglycemia. In our study, α-glucosidase inhibitory properties were shown only by the chloroform partitioned extract (Figure 9.6).

Plant extracts are known to be potent *α*-amylase inhibitors due to their rich phenolic content that bind to the reactive sites of enzymes, thus altering its catalytic activity (Payan, 2004). It has been suggested that the inhibition of *α*-amylase might be achieved through the direct blockage of the active centre at several subsites of the enzyme which are applicable for suggested for other inhibitors also (McCue and Shetty, 2004). In our experiment only the chloroform part of the orange fruit extract possesses the *α*-amylase inhibiting property, indicating that all the bioactive compounds responsible for *α*-amylase inhibition is pulled out by chloroform (Figure 9.7).

9.3.1a Antioxidant and antidiabetic activity of chloroform fraction after column chromatography

On the basis of antioxidant and antidiabetic activity as well as extractive yield (Figure 9.8) we selected chloroform fraction for further purification and processed through silica gel column chromatography. After column purification, we got 58 fractions, each fraction was scanned under UV-Visible spectrum (190-700 nm) (Figure 9.9i-9.9lviii) for determining the existence of any chromogenic substances and the fractions having similar spectral profile was united together (Table 9.4). After mixing the fractions, we acquired 19 fractions (F_{A-S}). Thereafter we have screened for free-radical scavenging properties (DPPH and $ABTS^{+}$),

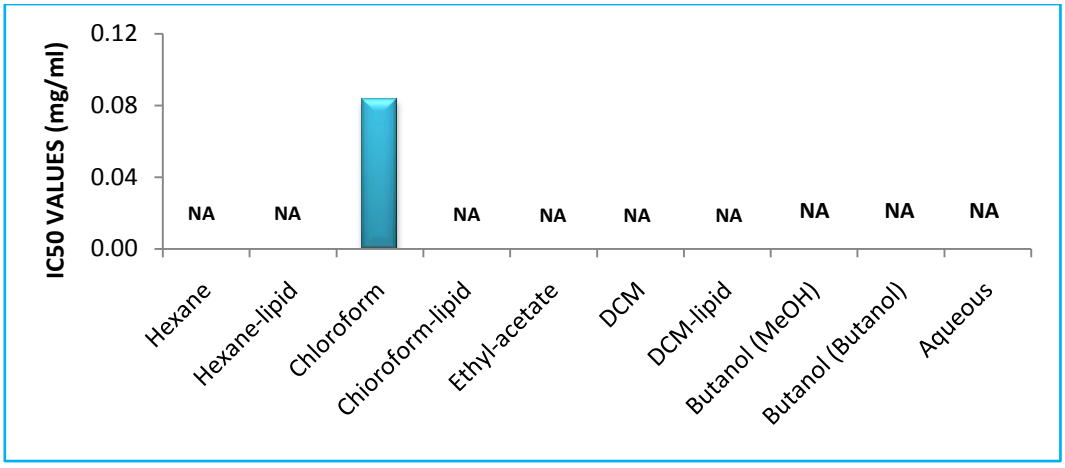


Figure 9.6 α-glucosidase inhibition assay of different fractions

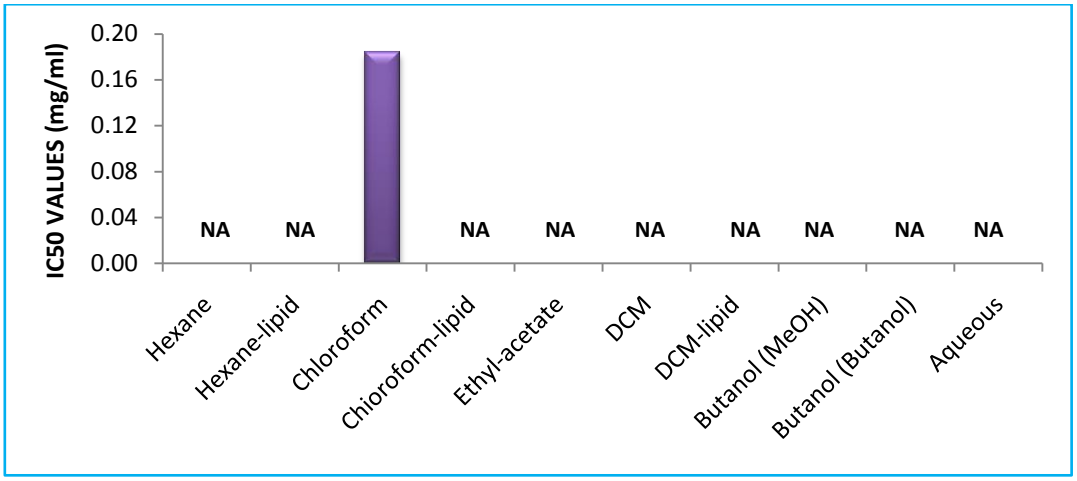


Figure 9.7 α-amylase inhibition assay of different fractions

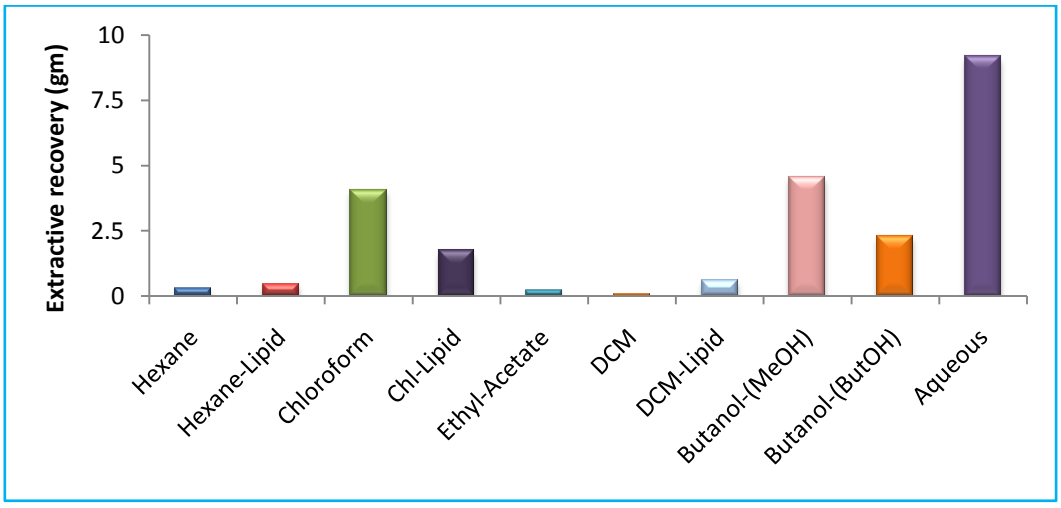


Figure 9.8 Extractive values of different fractions

Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi*

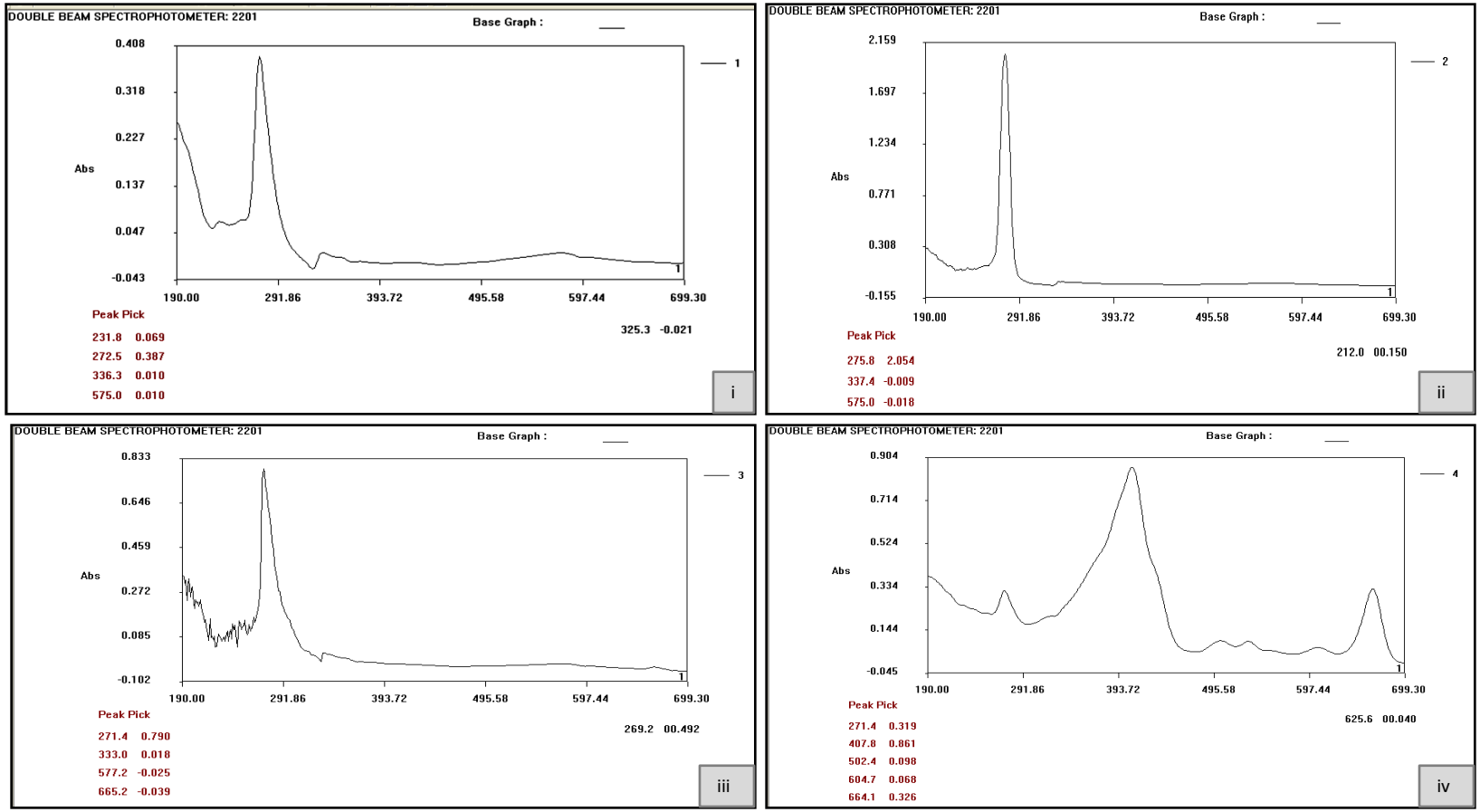


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

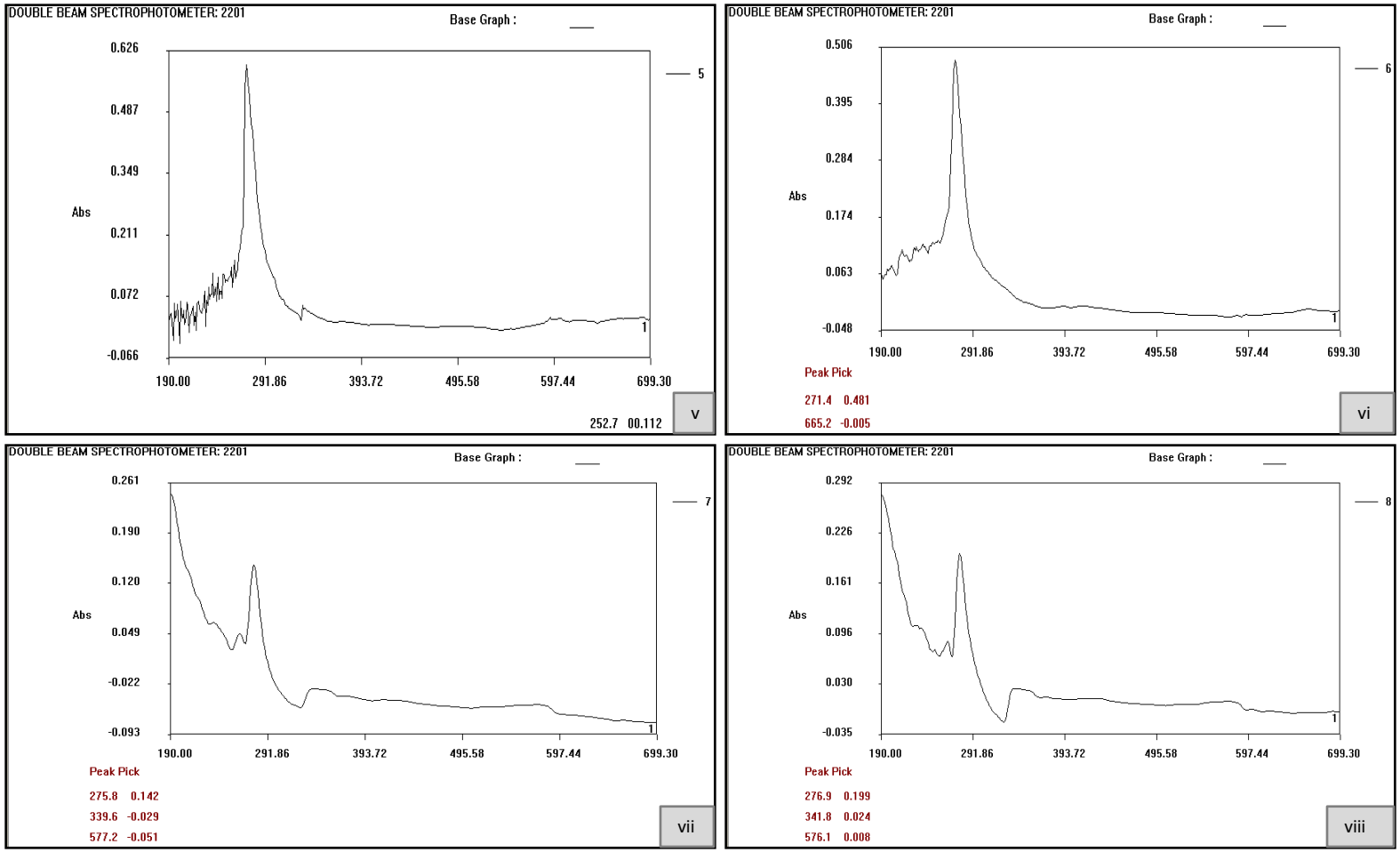


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

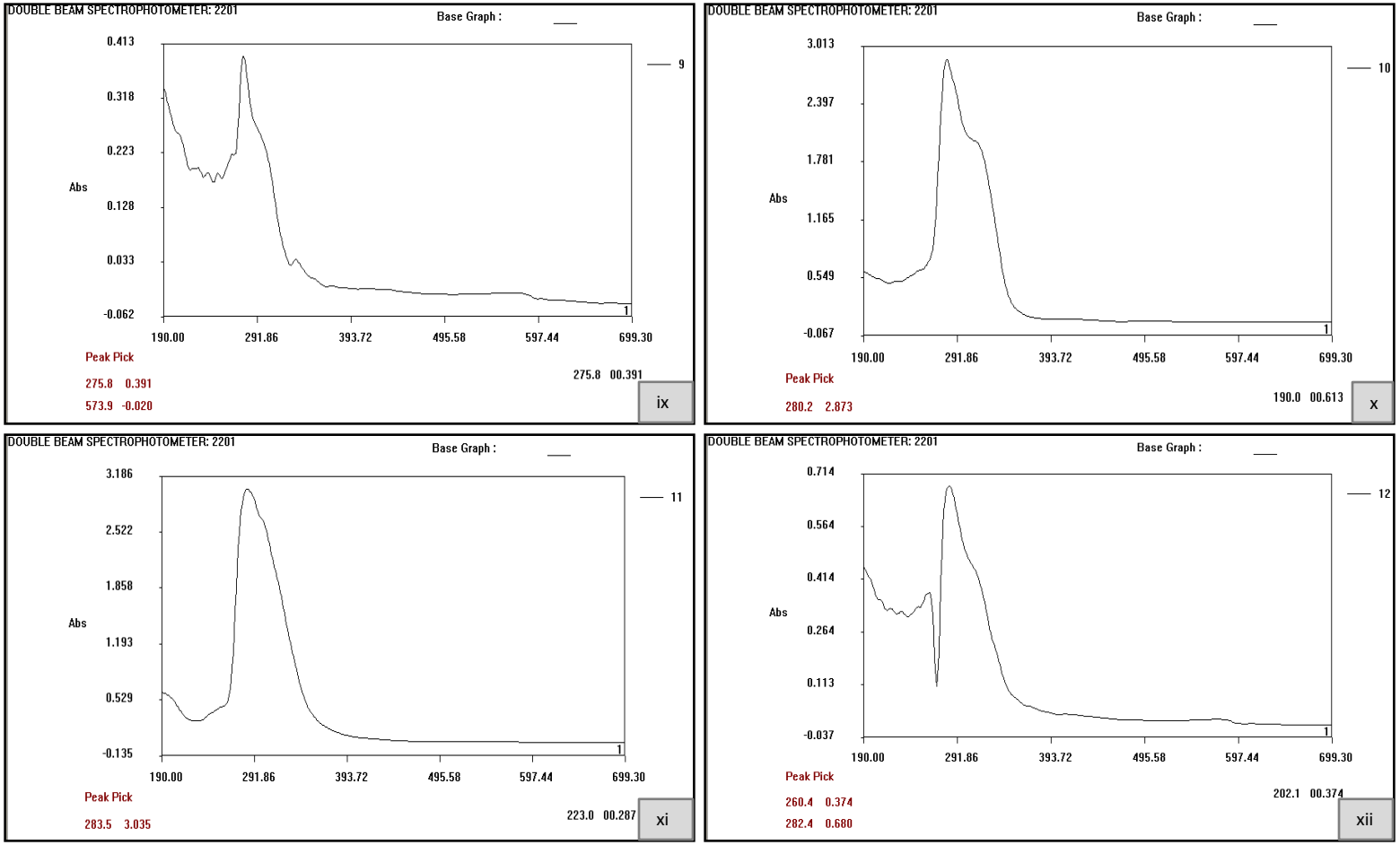


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

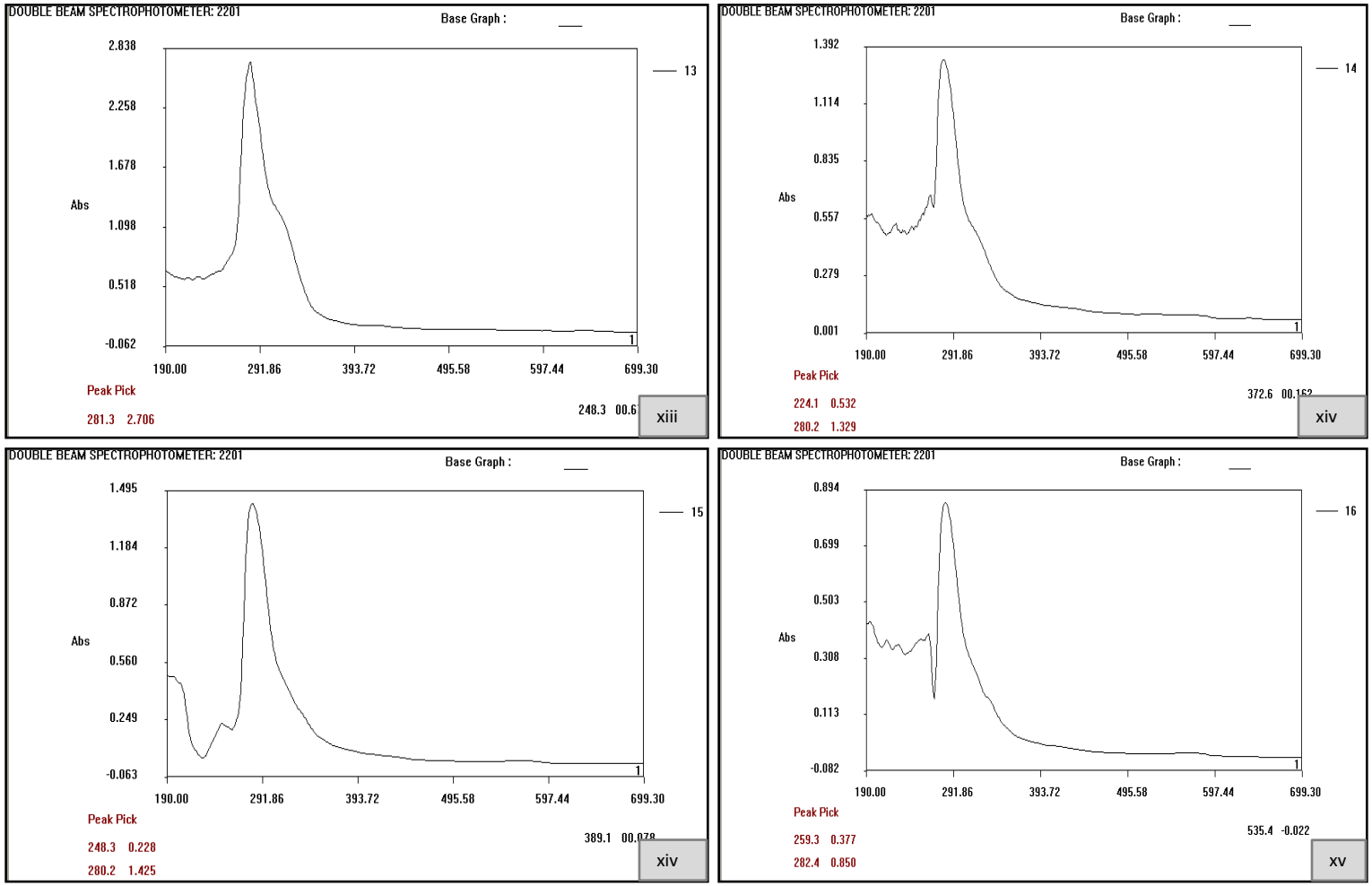


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

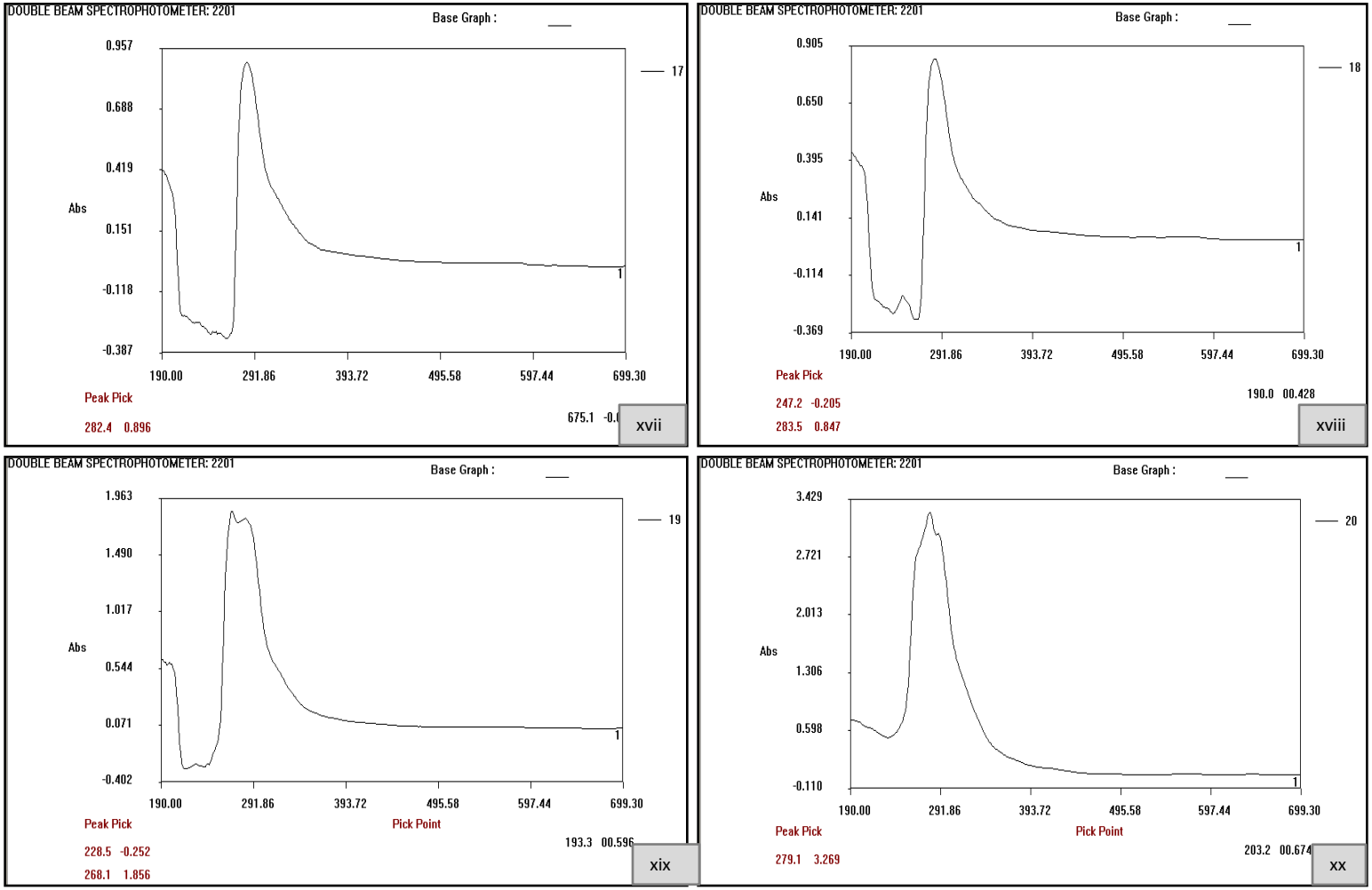
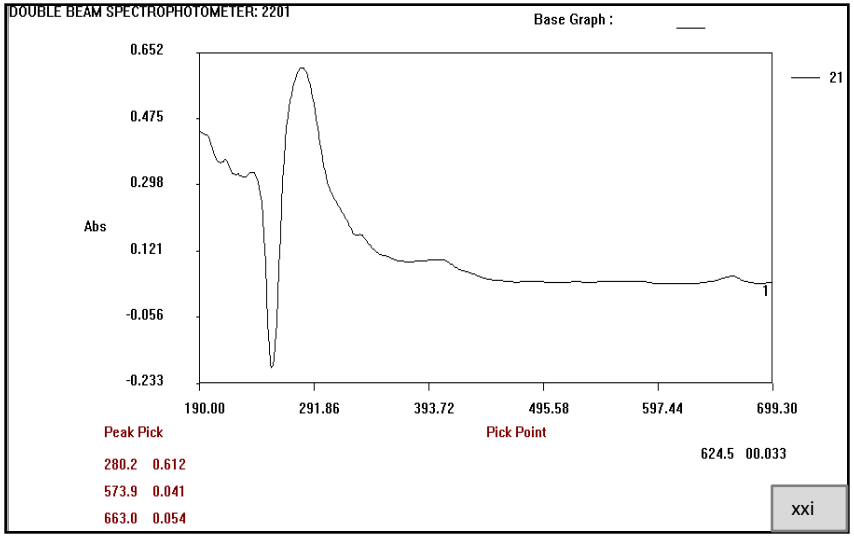
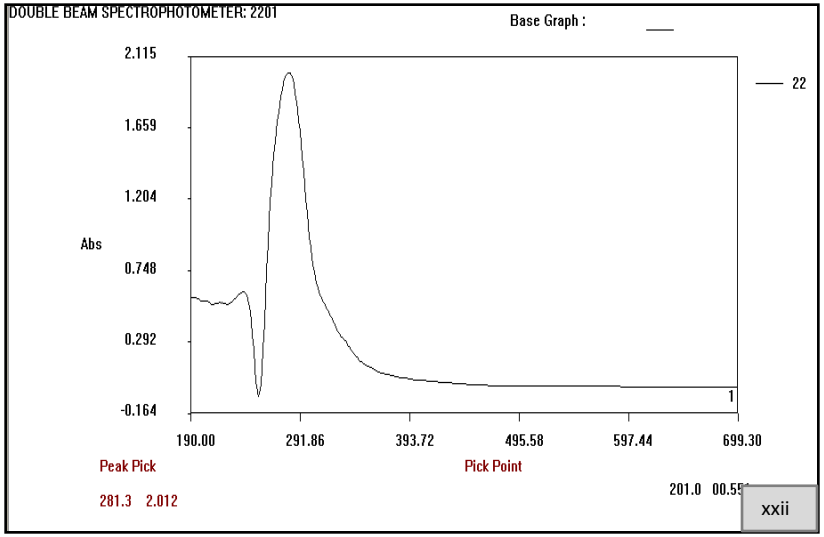
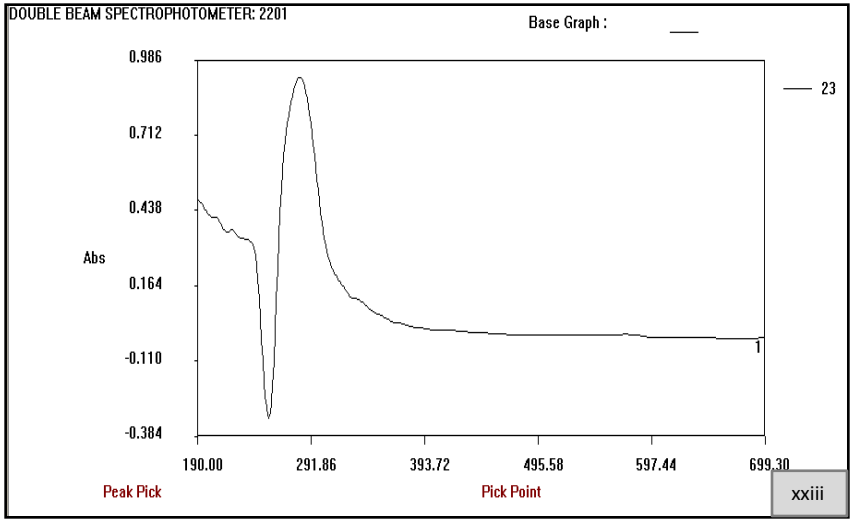


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....







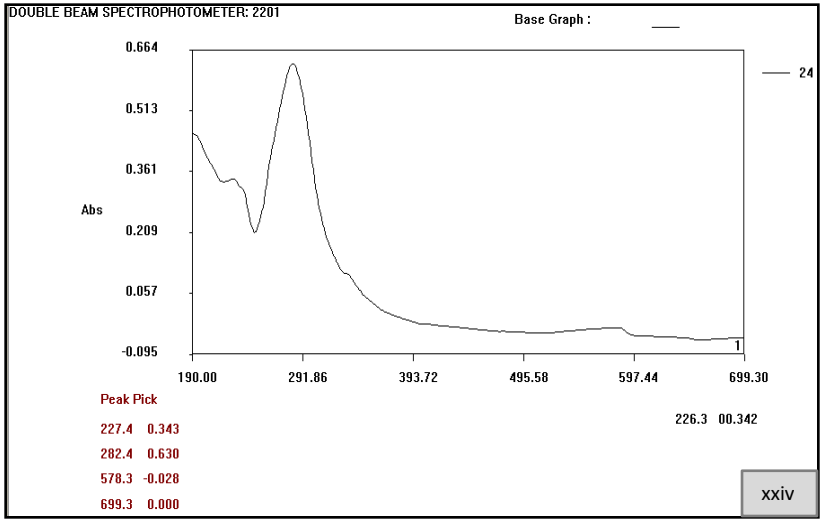


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

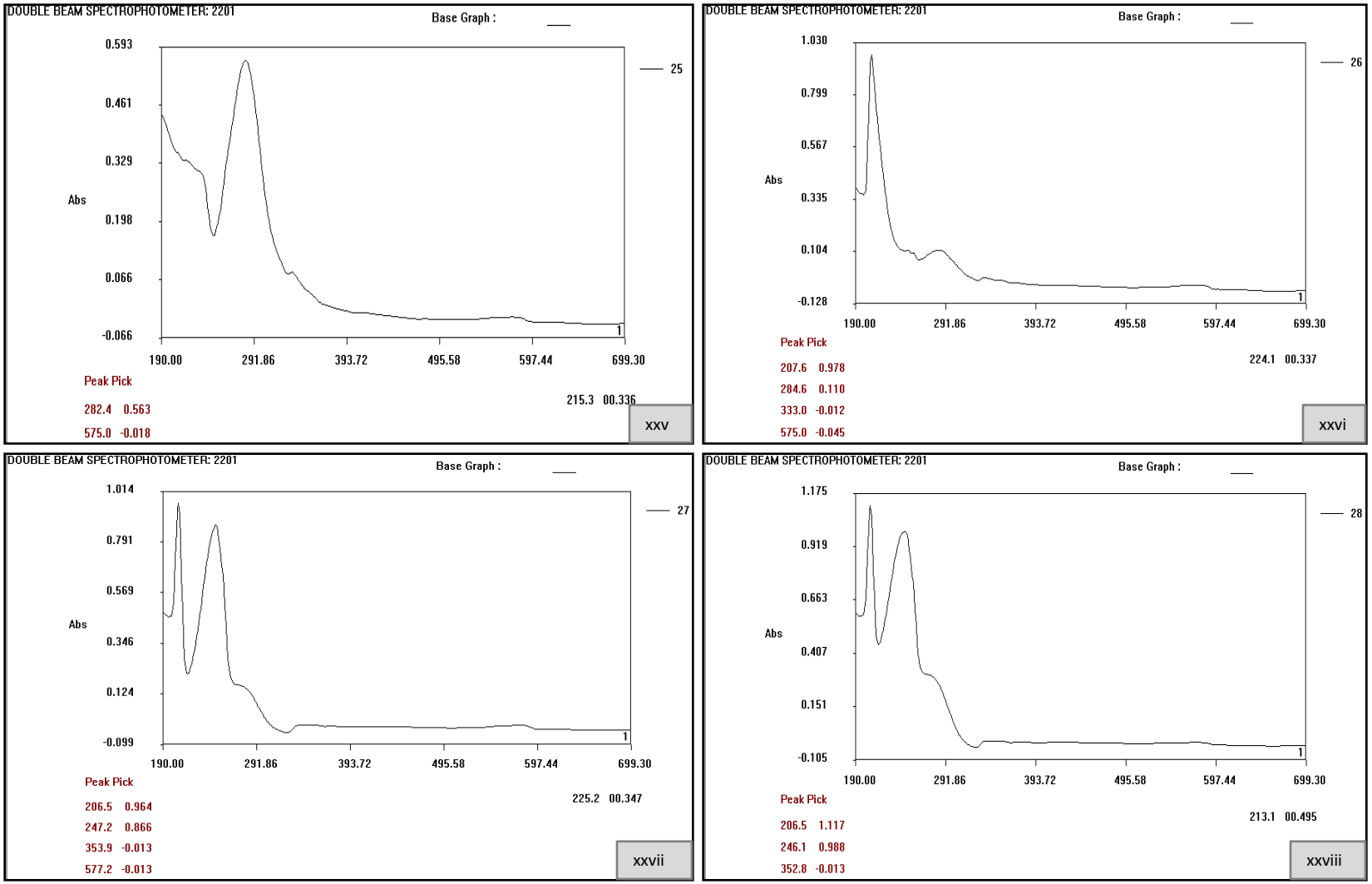
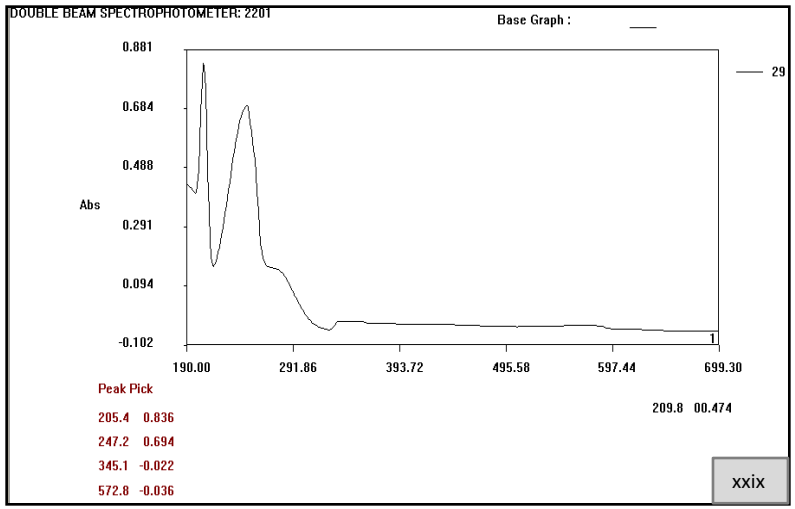
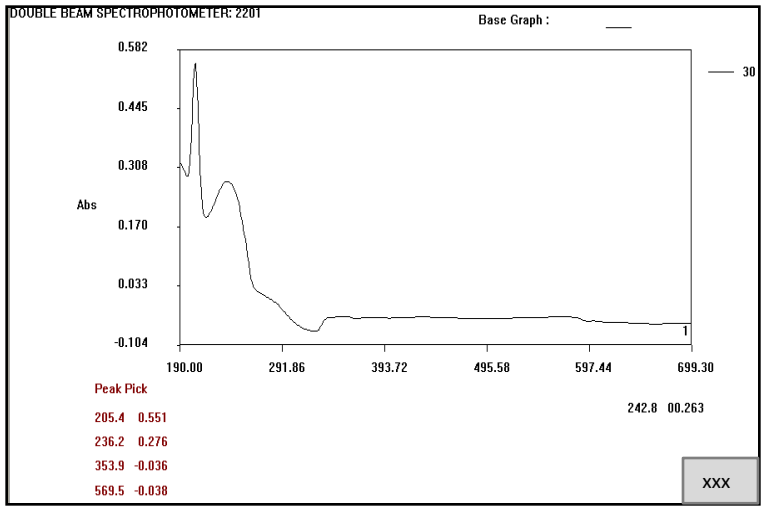
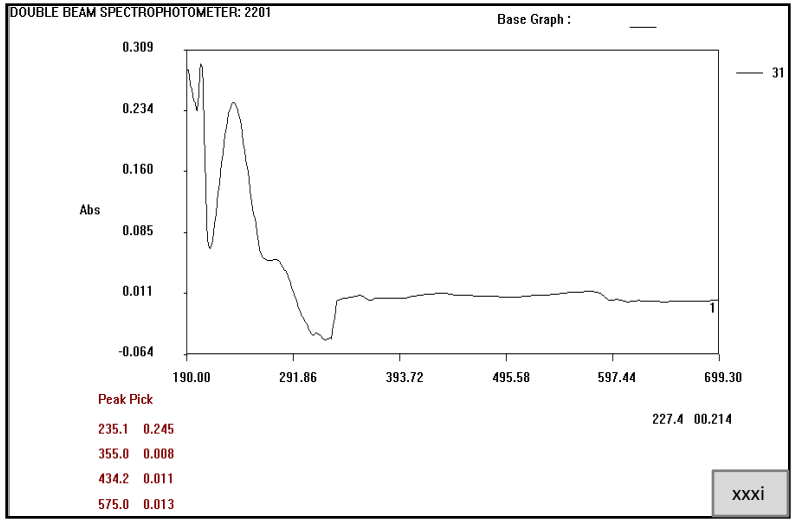


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....







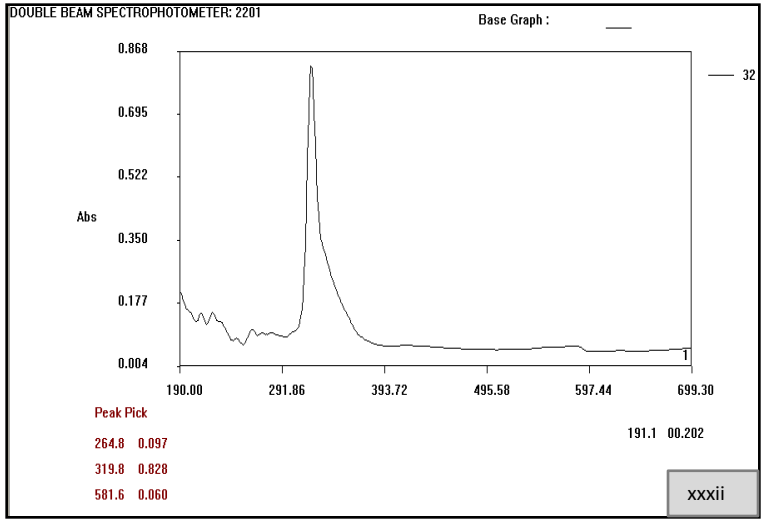


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

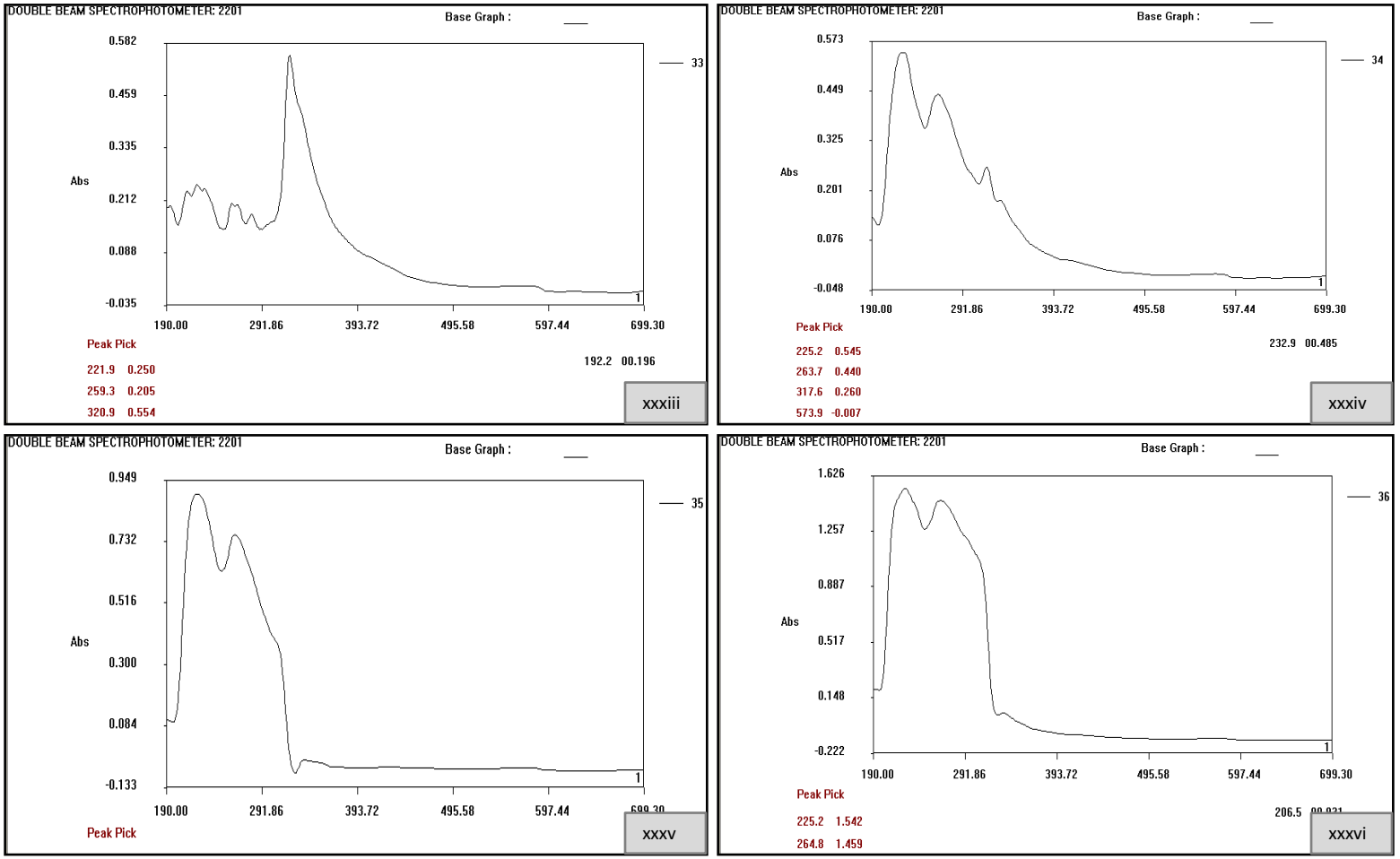
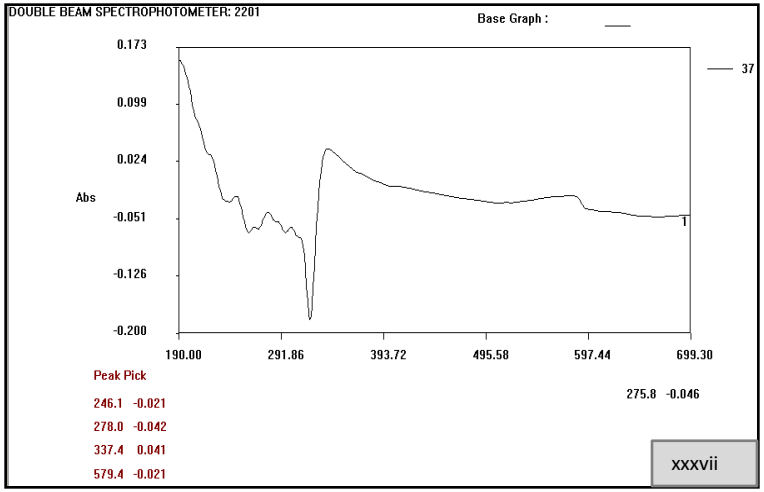
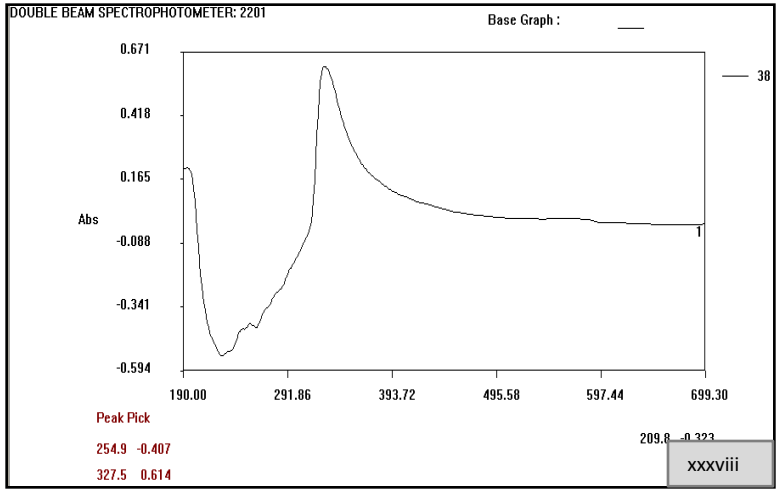
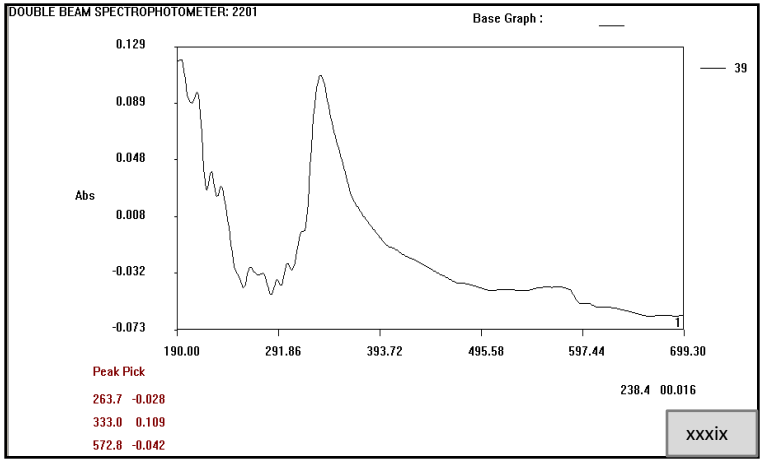


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....







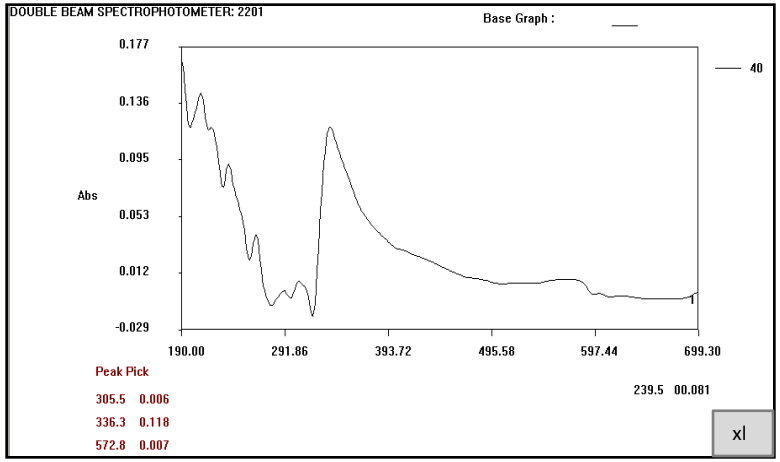


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

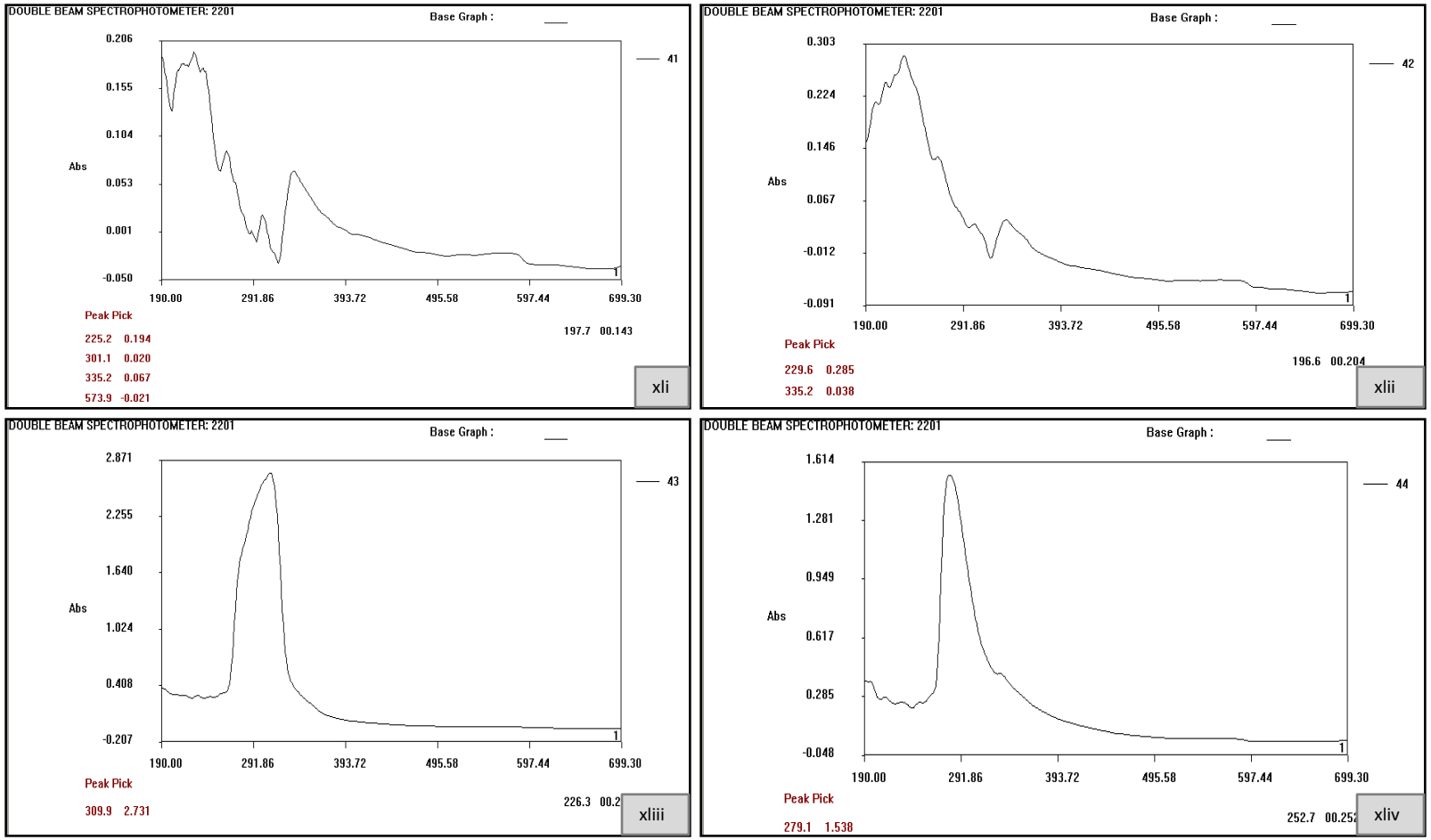


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

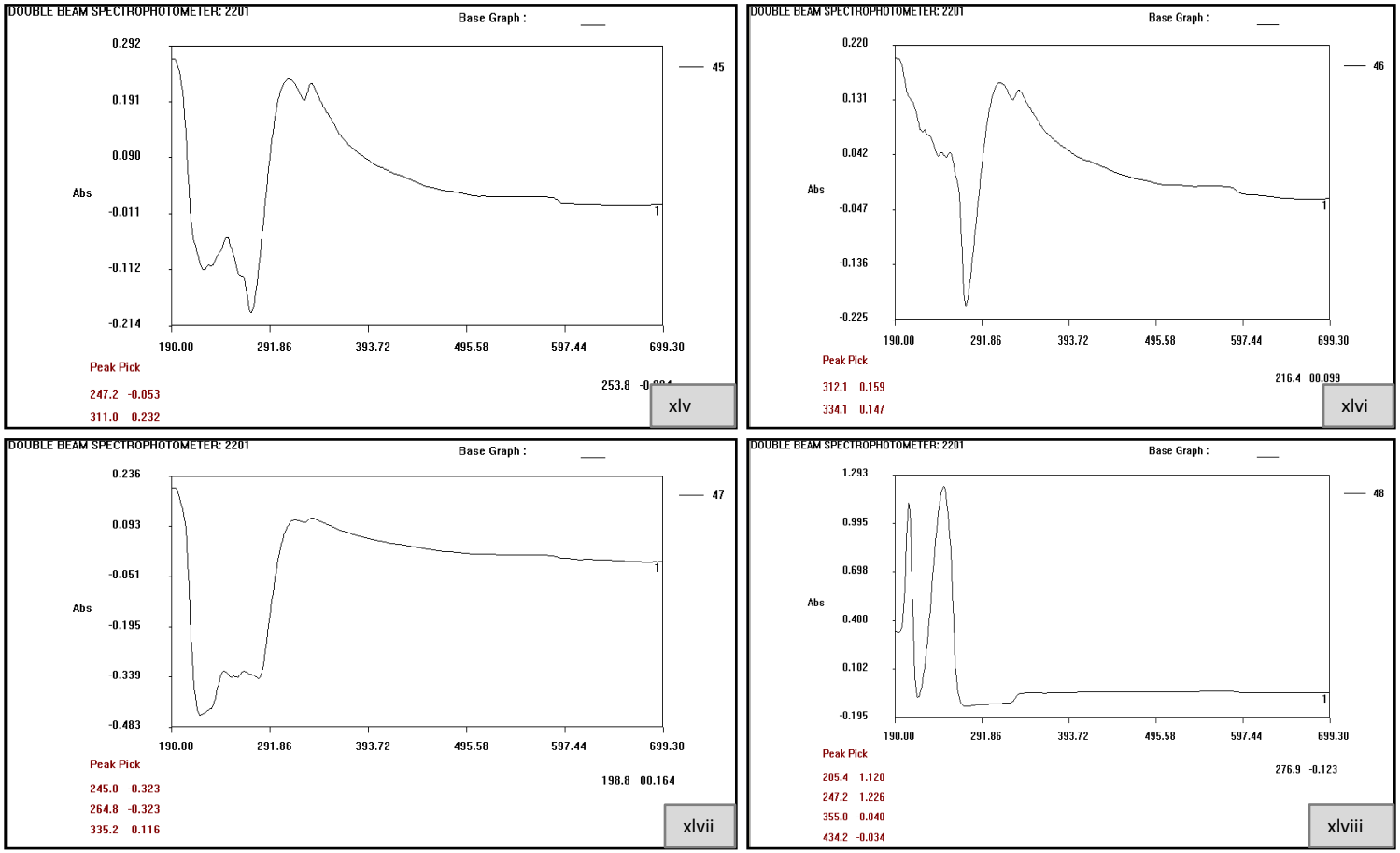


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

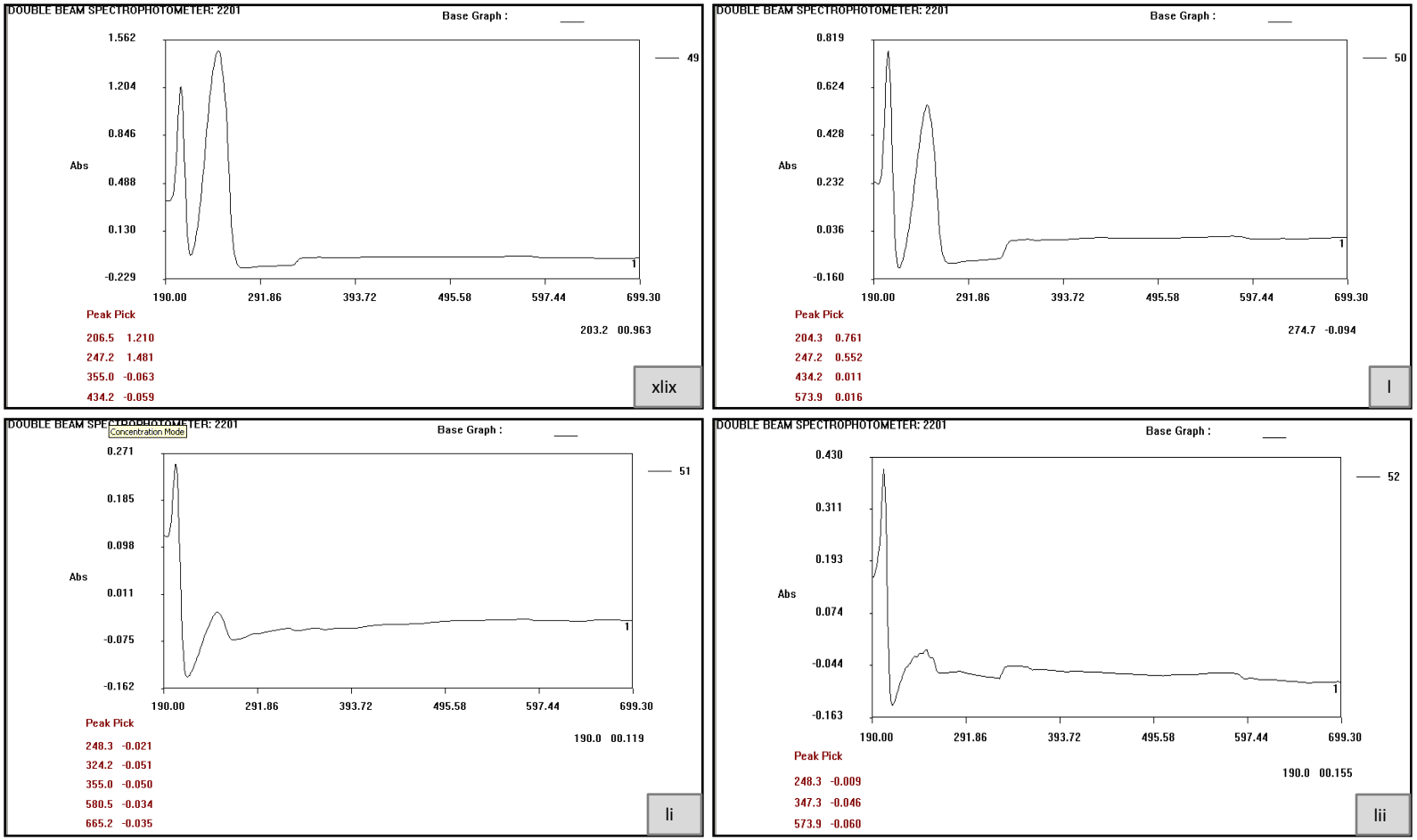


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....

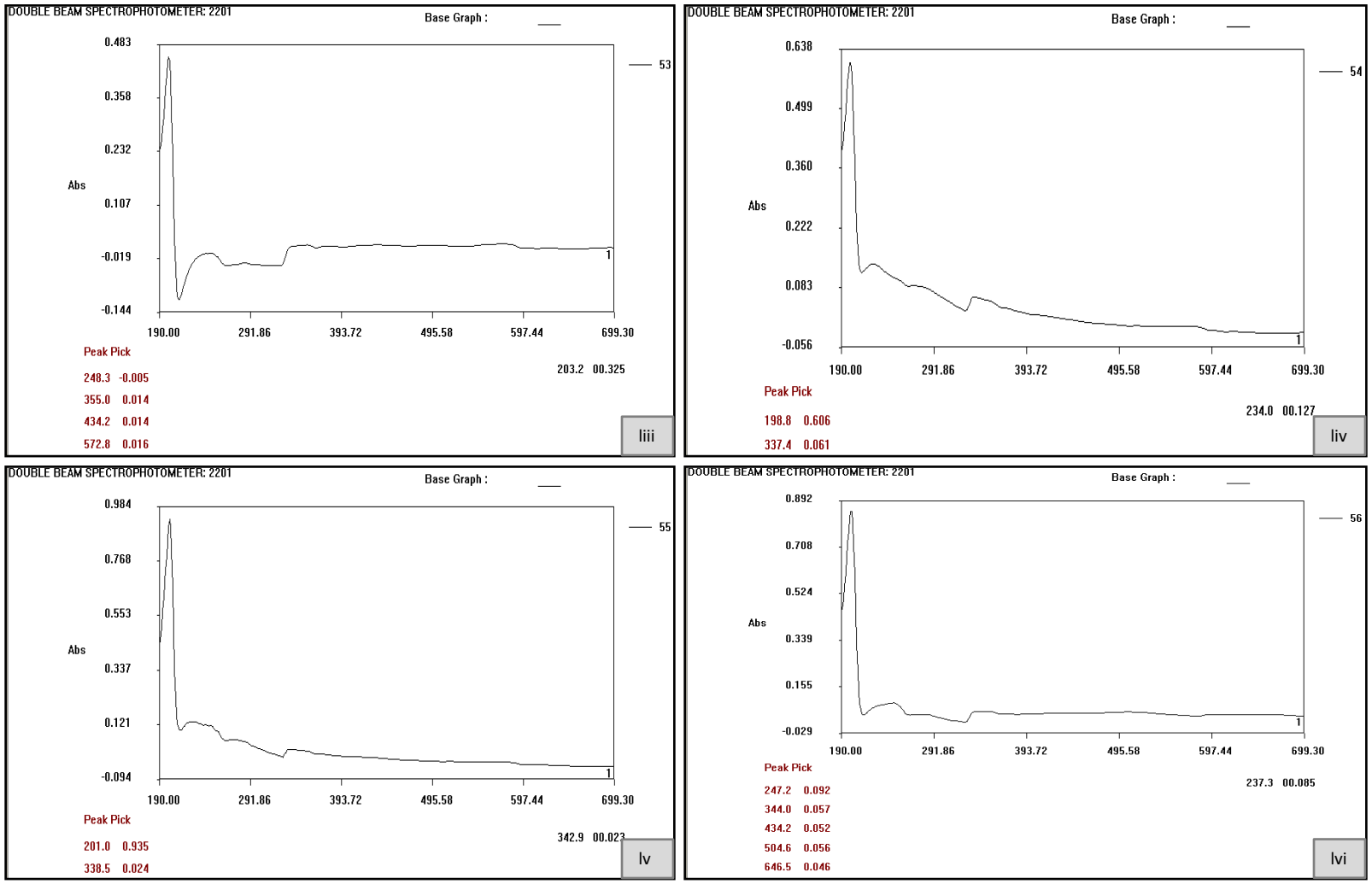
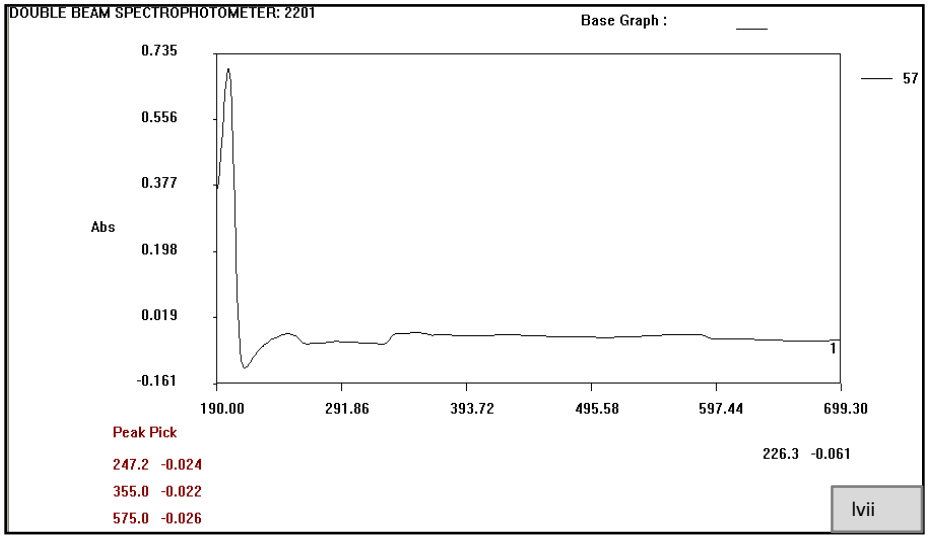
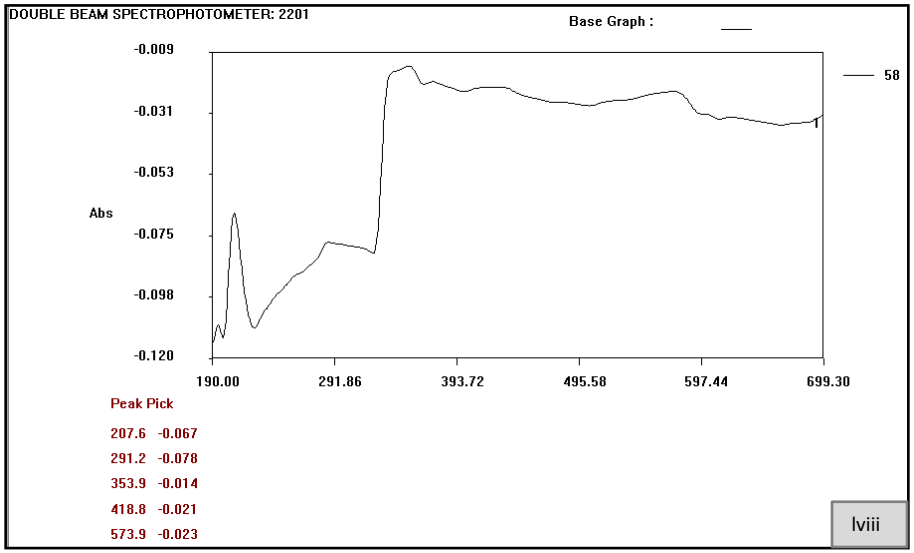


Figure 9.9 Characteristic UV-visible spectrum of bioactive compound from *Solanum anguivi* Cont.....





antidiabetic activity and phytochemical constituents (total phenol and flavonoid content) of these fractions. Fraction E (F_E) exhibited most potential activity both for *in vitro* antioxidant and antidiabetic models as well as elevated level of polyphenols (Table 9.5). The bioactive fraction was processed through liquid chromatography mass spectrometry and the scanning of HR-LCMS was shown in Figure 9.10. The library matching of mass peaks showed the high abundance of 1-oleoyl-lysophosphatidic acid in the fraction E (Figure 9.11) along with the existence of other compounds in minor quantity. So, it can be concluded that 1-oleoyl-lysophosphatidic acid might be responsible for *in vitro* antioxidant and antidiabetic activity. 1-Oleoyl lysophosphatidic acid is a species of lysophosphatidic acid (LPA) having oleic acid at 1-position. In plant system, LPA is produced as an intermediate of sequential esterification during biosynthesis of triacylglycerol in plastidial stromal site (Thelen and Ohlrogge, 2002). During lipid biosynthesis, fatty acids might enter the prokaryotic pathway by transferring oleoyl group from Acyl Carrier Protein (ACP) to glycerol-3-phosphate and 1-oleoyl-lysophosphatidic acid might accumulate in plastids (Bourgis *et al*., 1999). In animal system, LPA mediates a variety of biological responses like platelet aggregation, cell proliferation, smooth muscle contraction, neuronal retraction and cell motility by combining with one of the five different G-protein linked receptors. 1-Oleoyl-lysophosphatidic acid is a potential analogue of LPA which accelerates calcium mobilization for growth stimulation of a variety of cell lines (Montero-Moran *et al*., 2010).

9.3.2 *Calamus erectus*

Our present study demonstrated that, the ethyl acetate and butanol partitioned *C. erectus* fruit extracts have considerably stronger free radical scavenging potency as well as antidiabetic capacity when compared with other solvent partitioned extracts like water, chloroform and chloroform/lipid, as determined from their respective IC_{50} values. In respect to the concentration of bioactive molecules present, this study revealed significant scavenging potency of α-amylase, α-glucosidase, high reducing power as well as extractive yield, total

Table 9.5 Antioxidant and antidiabetic activity of different fractions of chloroform fraction acquired from column chromatography

Fraction Name	Extractive Recovery (mg)	IC50 (mg/ml)				Conc. (mg/g)	
		DPPH	ABTS	α-Glucosidase	α-Amylase	Total Phenol	Total Flavonoid
A	77	0.681	0.0126	3.543	0.0588	43.56	29.91
B	98	0.932	0.0223	5.876	0.0621	32.29	18.33
C	112	0.491	0.0113	6.822	0.0984	23.78	15.78
D	178	0.371	0.0085	5.926	0.0835	20.09	11.37
E	335	0.117	0.0051	4.829	0.0643	19.17	9.83
F	154	0.778	0.0098	7.921	0.1145	16.89	10.45
G	112	1.232	0.0175	9.453	0.1387	11.62	5.23
H	133	1.541	0.0788	13.452	0.1982	17.84	4.73
I	150	1.239	0.0681	NA	0.2295	24.46	8.91
J	168	1.347	0.1021	11.282	0.3584	56.11	27.83
K	269	1.882	0.1306	8.664	0.3431	103.92	48.91
L	377	2.241	0.2562	NA	0.3479	101.54	36.72
M	290	2.751	0.3429	7.322	0.2175	77.69	28.37
N	143	3.428	0.2118	8.931	0.2272	65.31	41.88
O	136	2.106	0.1573	10.223	0.2936	32.19	22.71
P	87	1.052	0.1435	15.684	0.3873	17.54	7.28
Q	77	0.873	0.1371	NA	0.3946	8.61	3.11
R	63	0.981	0.1661	NA	0.4081	2.34	1.04
S	35	0.944	0.2297	NA	NA	1.09	0.22

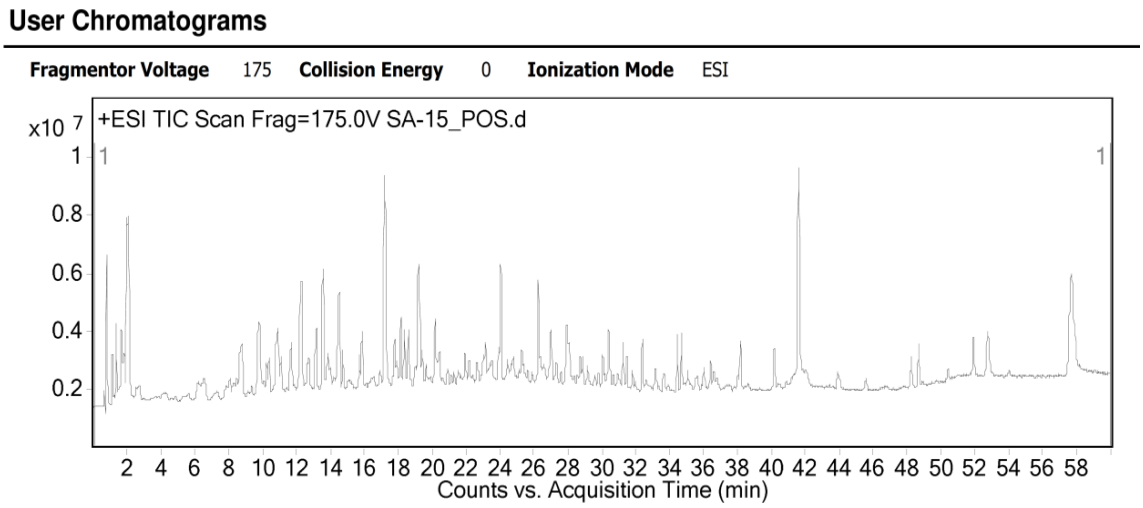


Figure 9.10 HR-LCMS scanning chromatogram of Fraction-E of butanolic fraction

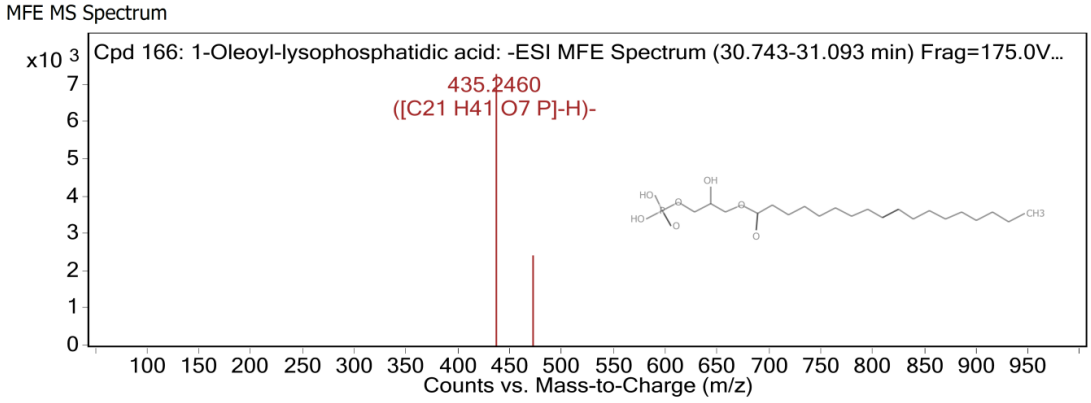


Figure 9.11 Structure of 1-Oleoyl-lypophosphatidic acid

phenol and flavonoid content in the ethyl acetate and butanolic partitioned fruit extracts (Figure 9.12-9.22).

An analysis was made for the approaches to be followed towards selecting the successive partitioned fruit extracts for further bioassay guided purification and investigations of antioxidants as well as antidiabetic agents. The ethyl acetate and butanol partitioned extracts were found to be most suitable for further investigations. As the extractive yield of the ethyl acetate fractions was not sufficient enough for approaching towards further purification steps; butanol-partitioned extract was only selected for further bioassay guided fractionation and partial purification of antioxidant and antidiabetic compounds using silica gel column chromatography, due to the existence of higher concentrations of bioactive components in them.

After bioassay-guided fractionation and partial purification using silica gel column chromatography, we managed to obtain 14 fractions from butanolic extract respectively. These obtained fractions from butanolic extracts were further subjected to phytochemical estimation and antioxidant as well as antidiabetic activity determination for performing the respective assays described above. The results obtained after performing these assays, have been graphically represented in Figure 9.13, which shows that DPPH activity was significantly higher in fraction F5, F6 and F10 along with greater amount of total phenol and flavonoid content (Figure 9.19).

HPTLC screening was performed with different fractionated extract (Figure 9.23) as well as with the 14 fractions obtained from butanolic extract (Figure 9.24) and developed through vanillin-sulphuric acid spray system. The HPTLC screening was done for the identification of flavonoids present in a particular fraction, which appeared as coloured bands on the chromatograms on spraying of vanillin-sulphuric acid solvent. The coloured bands that appeared in chromatograms were displayed in Figure 9.23 and Figure 9.24 which revealed that the butanolic fractions contain more concentrated flavonoids and fraction F5, F6 and F7 of butanolic fruit extract showed this bioactive compound. The R_f values of flavonoid bands of those respective fractions were also enlisted in Table 9.6-9.7.

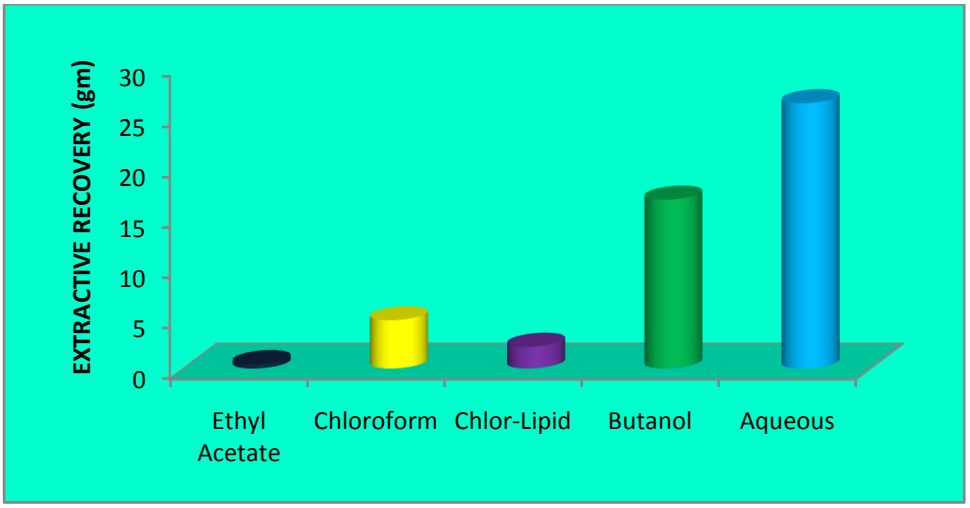


Figure 9.12 Extractive values of *Calamus erectus* fruits of each successive partitioned extracts

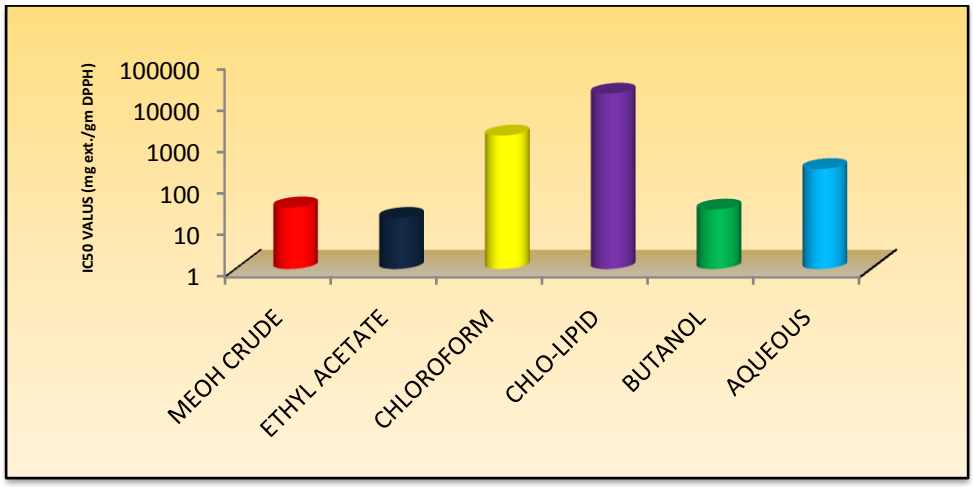


Figure 9.13 DPPH radical scavenging assay of *Calamus erectus* fruits of each successive partitioned extracts

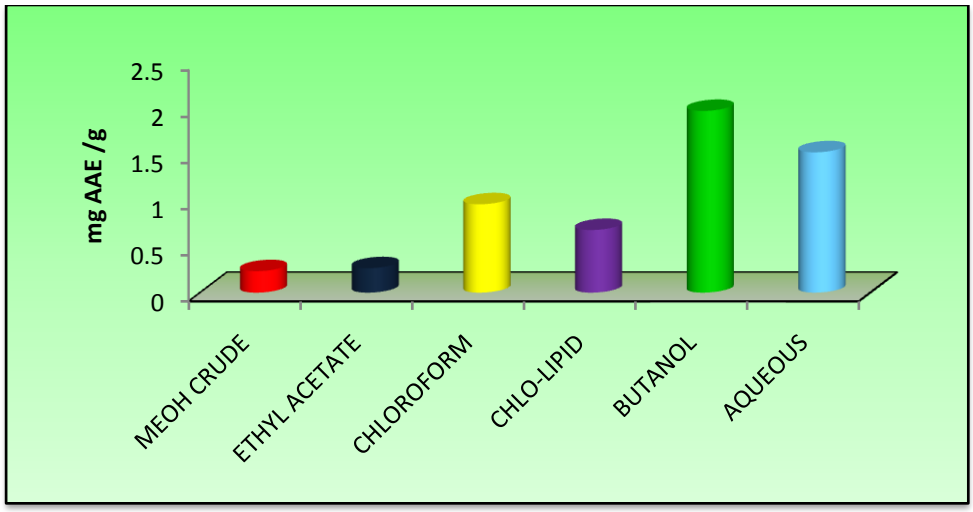


Figure 9.14 Reducing power assay of *Calamus erectus* fruits of each successive partitioned extracts

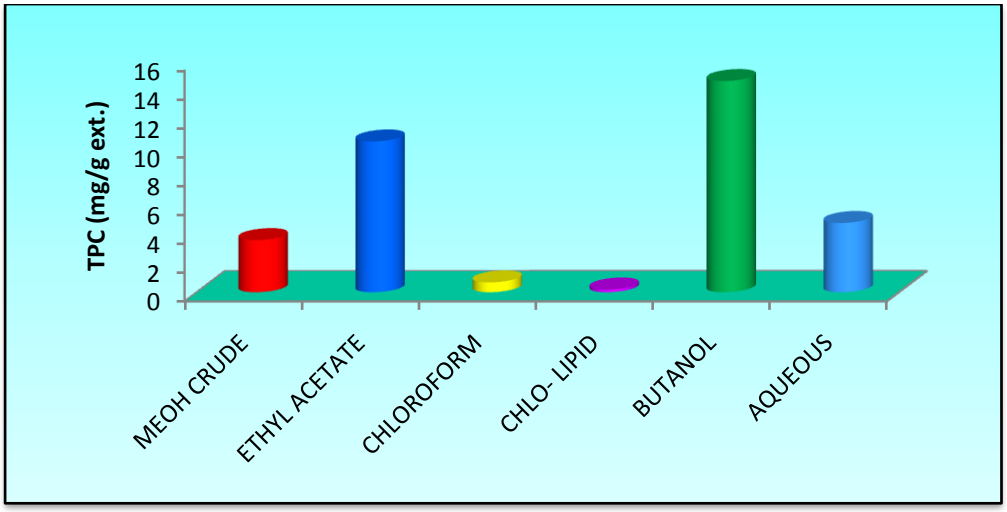


Figure 9.15 Estimation of total phenol content in *Calamus erectus* fruits of each successive partitioned extracts

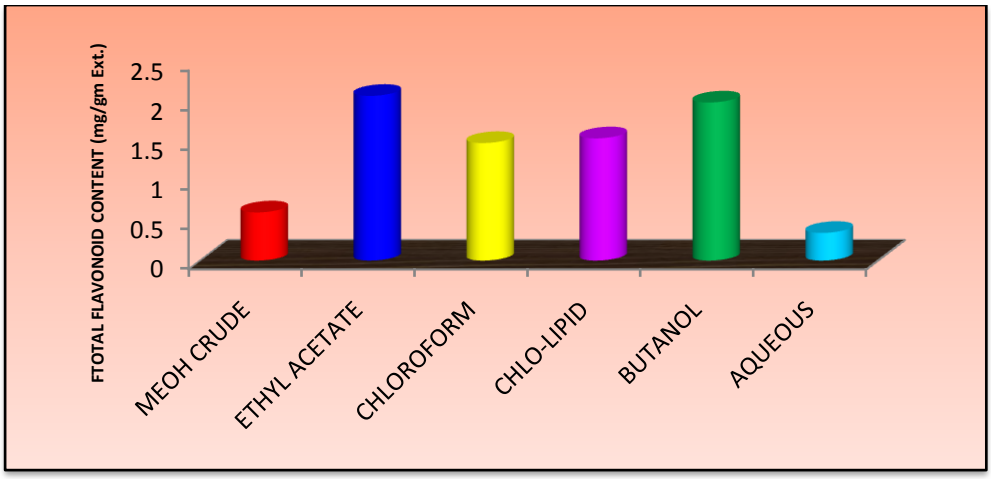


Figure 9.16 Estimation of flavonoid content in *Calamus erectus* fruits of each successive partitioned extracts

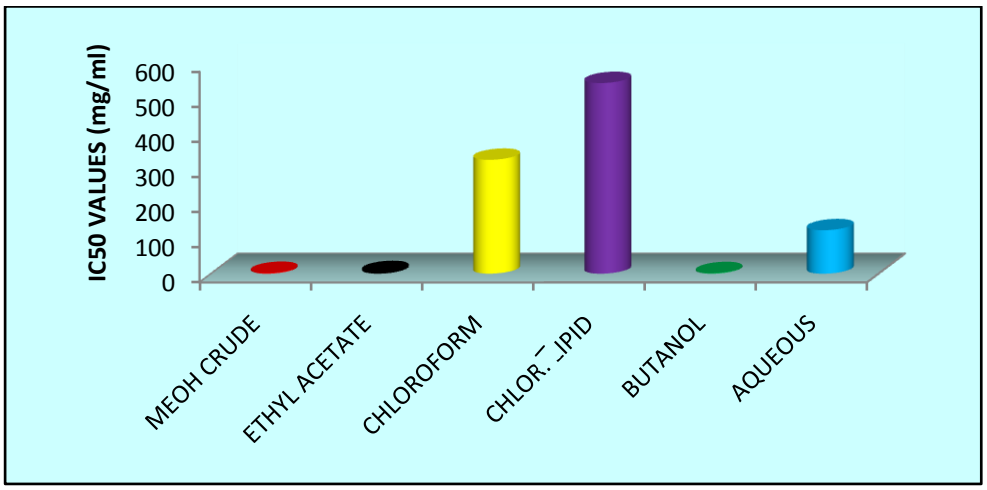


Figure 9.17 α-Glucosidase assay of *Calamus erectus* fruits of each successive partitioned extracts

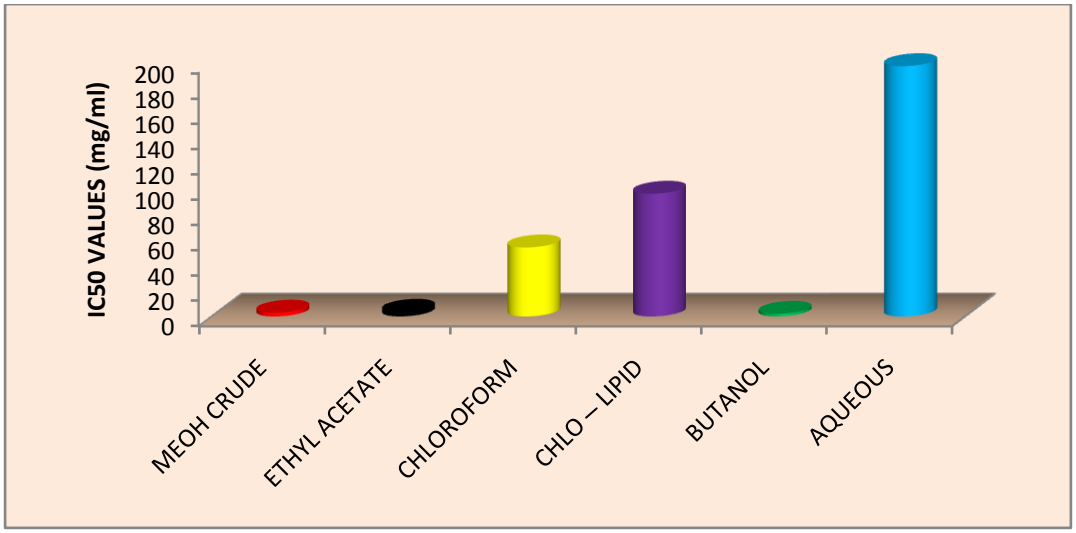


Figure 9.18 α-Amylase assay of *Calamus erectus* fruits of each successive partitioned extracts

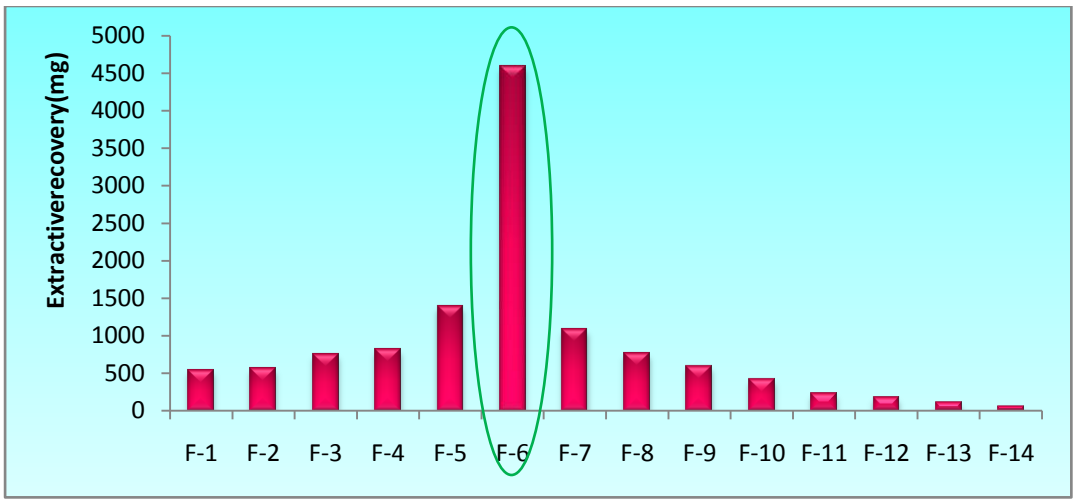


Figure 9.19 Extractive values of each obtained fractions from butanolic extracts

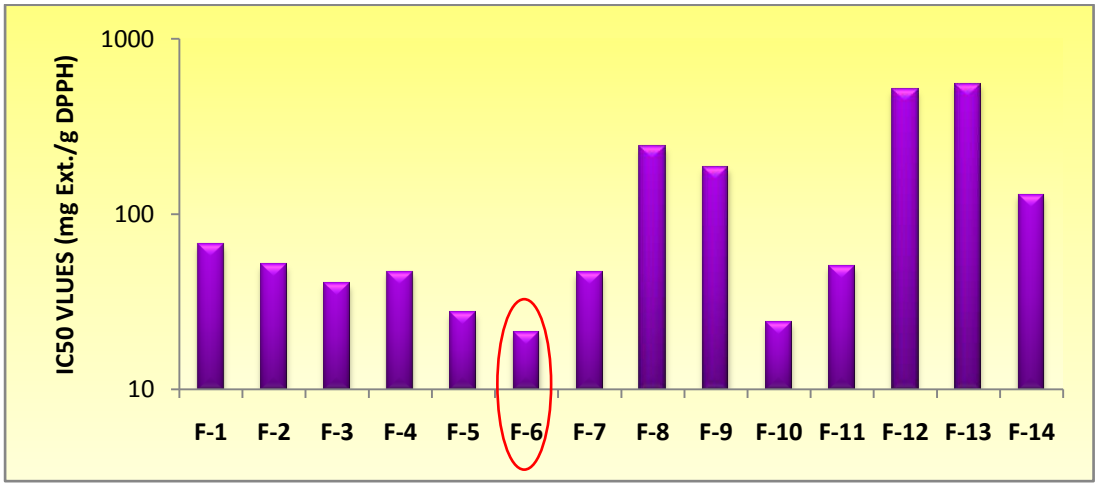


Figure 9.20 DPPH radical scavenging assay of each obtained fractions from butanolic extracts

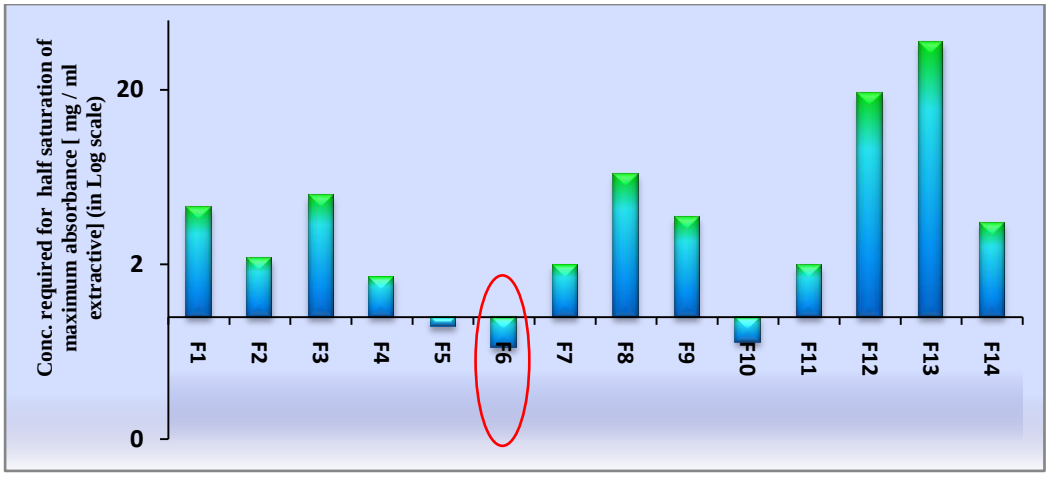


Figure 9.21 Reducing power of each obtained fractions from butanolic extracts

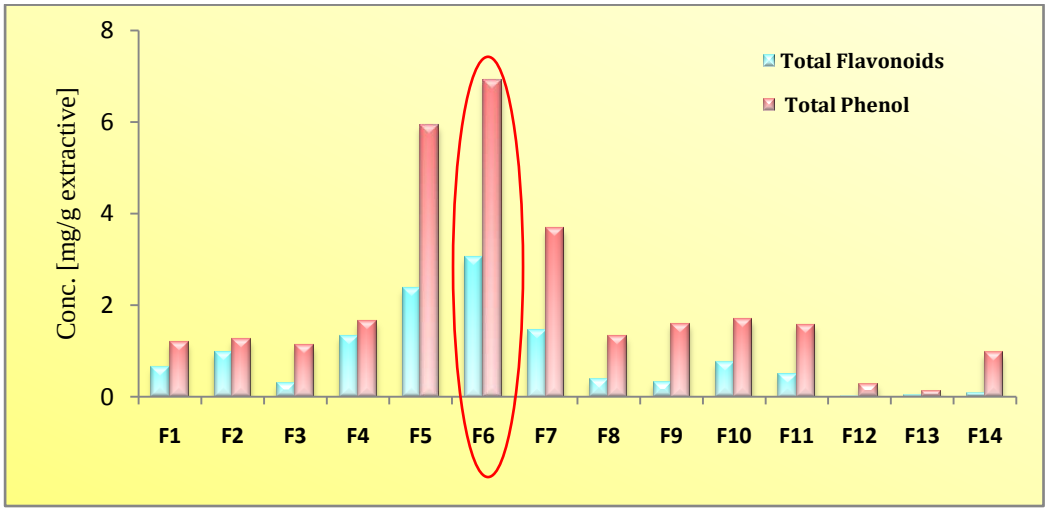


Figure 9.22 Estimation of total phenol and flavonoid content in each obtained fractions from butanolic extracts

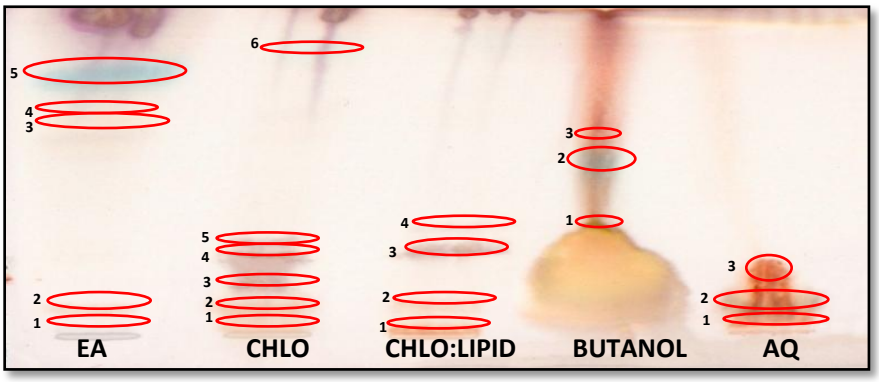


Figure 9.23 HPTLC chromatogram of successive fractions

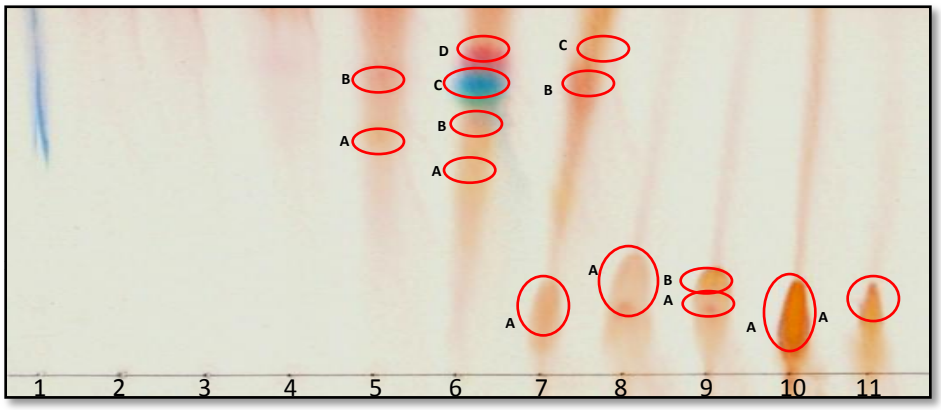


Figure 9.24 HPTLC chromatogram of butanolic fractions

Table 9.6 Retardation factors of different analytes present in bioactive fraction of *C. erectus* plant

Solvent Fractions	*Identity of Bands*	*Mobile Phase migration*	*Analyte Migration*	hRf
Ethyl Acetate	1	4.4	0.3	6.82
	2	4.4	0.4	9.09
	3	4.4	2.6	59.09
	4	4.4	2.8	63.64
	5	4.4	3.5	79.55
Chloroform	1	4.4	0.2	4.55
	2	4.4	0.4	9.09
	3	4.4	0.6	13.64
	4	4.4	1	22.73
	5	4.4	1.1	25.00
	6	4.4	3.0	68.18
	7	4.4	3.6	81.82
Choloform:Lipid	1	4.4	0.1	2.27
	2	4.4	0.4	9.09
	3	4.4	1.1	25.00
	4	4.4	1.4	31.82
Butanol	1	4.4	1.3	29.55
	2	4.4	2.3	52.27
	3	4.4	2.5	56.82
Aqueous	1	4.4	0.2	4.55
	2	4.4	0.3	6.82
	3	4.4	0.5	11.36

Table 9.7 Retardation factors of different analytes present in bioactive fraction of *C. erectus* plant

Solvent Fractions	Identity of Bands	Mobile Phase migration	Analyte Migration	hRf
F5	A	4.9	3.2	65.31
	B	4.9	4	81.63
F6	A	4.9	2.5	51.02
	B	4.9	3.6	73.47
	C	4.9	3.9	79.59
	D	4.9	4.2	85.71
F7	A	4.9	0.8	16.33
	B	4.9	2.4	48.98
	C	4.9	3.9	79.59
F8	A	4.9	0.8	16.33
F9	A	4.9	0.9	18.37
	B	4.9	1.3	26.53
F10	A	4.9	0.7	14.29
F11	A	4.9	0.8	16.33

Table 9.8 Antioxidant and antidiabetic activity of *C. erectus*

Fractions	Extractive recovery (mg)	DPPH IC_{50} (mg Ext./g DPPH)	RP (µg AAE/mg FWT)	TPC (mg/g)	TFC (mg/g)	α-amylase inhibition (IC_{50} mg/ml)	α-glucosidase inhibition (IC_{50} mg/ml)
$F6_1$	45.72	1.21	242	9.10	6.59	45.76	19.43
$F6_2$	99.06	10.83	84	4.93	0.61	39.46	234.76
$F6_3$	181.61	13.00	36	1.21	0.60	123.54	152.98
$F6_4$	149.86	16.70	42	5.54	0.46	185.08	123.86
$F6_5$	222.24	12.93	24	7.05	0.36	134.76	109.56
$F6_6$	255.26	3.30	102	3.06	1.98	23.79	23.24
$F6_7$	**353.05**	**1.41**	**206**	**15.23**	**4.23**	**2.76**	**1.45**
$F6_8$	185.42	2.25	93	5.06	3.34	147.63	165.54
$F6_9$	73.66	5.55	28	5.51	2.34	231.48	231.19
$F6_{10}$	24.13	283.94	34	4.23	2.33	342.23	124.20

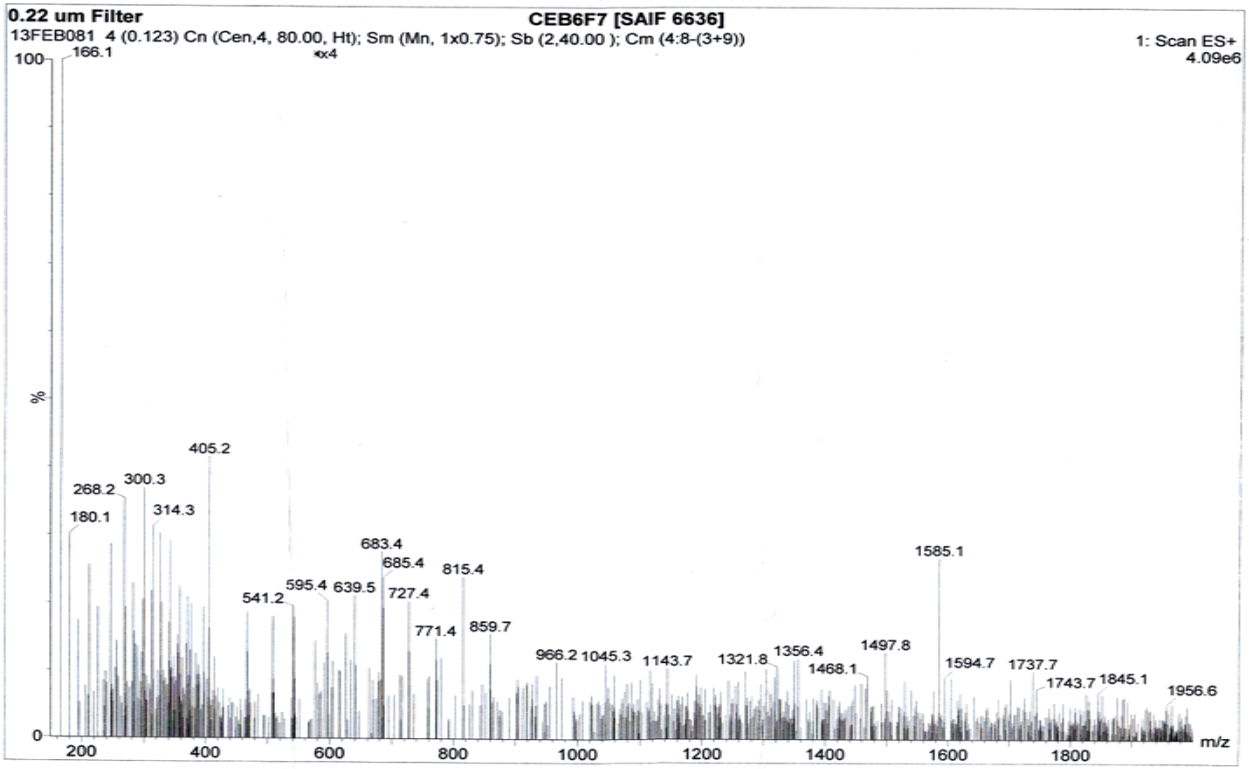


Figure 9.25 ESI mass spectroscopy of butanolic fraction of *C. erectus*

As F6 showed maximum bioactivity, it was selected for 2nd silica gel column chromatography and 10 sub-fractions were obtained. Among these 10 sub-fractions, $F6_7$ showed higher antioxidant as well as antidiabetic capacity as compared to other fractions (Table 9.8). This fraction ($F6_7$) was chosen for analyzing through ESI-mass spectroscopy (Figure 9.25). Mass spectrum generated after electro-spray ionization of the bioactive fraction indicates maximum abundance (100%) of peak having molecular weight 166.1. Other major peaks with relative abundance of each is less than 50%, are located within the molecular weight ranging between 180.1 to 1956.6, which might indicate either compound mass or their fragments. Exact molecular mass of more than 30 peaks were recorded from this spectrum among which at least 15 were located below molecular weight of 1000D (Figure 9.25); through which the abundance of free phenolic acids in this bioactive fraction might be speculated, as most of the phenolic acids isolated from plant system lies within these molecular weight range. However we were unable to process this bioactive fraction further for exact identification of bioactive compounds through bioassay-guided purification due to shortage of time and unavailability of plant material (*C. erectus*).

Till now there are no available reports on purified bioactive molecules of the fruit of *C. erectus*; however some information are available on the purified phytochemicals isolated from some members of family Arecaceae in which this plant belongs. In 2010, Xing *et al.* isolated 10 known phenolic compounds from ethyl acetate extract of *Areca catechu* L. and they first time isolated and identified catechin from the genus of *Areca*. Similar compounds might have been expected in bioactive fractions of *C. erectus*, which possess antioxidant as well as antidiabetic activity.

Chapter – X

VARIATION OF ANTIOXIDANT ACTIVITY WITH SOIL NUTRITIONAL PROPERTIES

10.1 INTRODUCTION

In previous chapters we already seen the edible plants of Darjeeling Himalaya contained a significant amounts of polyphenols. Many authors suggested that the quantity and composition of phenolics and flavonoids in plants changes according to genotype, climate factors such as seasonal variation, relative humidity and temperature, light intensity, environment stimuli and agronomical practices (Perkins-Veazie and Kalt, 2002; Watson *et al*., 2002; Reyes-Carmona *et al*., 2005; Thomas *et al*., 2005). Since, cultivation factors like soil type, compost, mulching and fertilization also can affect the plant secondary metabolites and antioxidant activity of plant (Prange, 1997; Strik, 2008). Several factors including genotype, growing region, climate and cultural practices also affect the external and internal quality of fruit (Mirdehgham and Rahemi 2007, Caleb *et al*., 2012). Ozgen *et al*. (2008) reported that the variation of factors such as total soluble solids (TSS), acidity and pH has been changed by the region in which they are grown. Regarding this information, it has been noticed that a number of geographical and environmental factors influence the concentration of bioactive phytochemicals in the same plants collected from different region (Houghton and Raman, 1998; Marcus and Grollman, 2002). Potassium is a macronutrient which is most important nutrients in controlling yield and quality of plants (Cassman *et al*., 1990; Pettigrew, 2008; Cakmak, 2010). With enzyme activation, the levels and composition of primary and secondary compounds are also determined with K supplementation to the plant. Several authors noticed that with carbohydrate, many secondary metabolites like phenolics, scopolin, oxylipins, galanthamine contents reduced when K levels decreased. These results suggested the importance of K in regulating the production of secondary metabolites in plants (Lehman and Rice, 1972; Troufflard *et al*., 2010; Lubbe *et al*., 2011; Liaqat *et al*., 2012;). In 2011, Ibrahim and Jaafar reported that high concentration of nitrogen can reduce the production of secondary metabolites in this herb because of reduction of phenyl alanine ammonia-lyase (PAL) activity that was correlated with low C/N ratio. Fertilizer influences the productivity and nutrient quality of most of the crops. In presence of inadequate levels of the primary nutrients viz. Nitrogen, Phosphorus and Potassium are the cause of weak vegetative growth,

poor fruit setting, undesirable fruit quality and low nutritional quality (Shukla *et al.*, 1980; Alwan, 1986; Martinetti and Paganini, 2006; Liu *et al*., 2010). Oloyede (2012) reported that NPK fertilizer progressively increased the vegetative growth and the yield of pumpkin leafy vegetable as well as enhanced the antioxidant activity and total phenolics of this plant. However, no documentation of the antioxidant and phytochemical responses of the edible plants of Darjeeling Himalaya to other nutrients, especially soil nutrient profile have been reported. This information is important and will be useful in the cultivation of those plants. Usually, plants fertilized with high soil physico-chemical levels tend to increase their bioactive compound levels. The objective of this study was to examine the changes antioxidants and secondary (total flavonoids and total phenolics) metabolites with different soil profile and relationships among the parameters of different altitude of Darjeeling Hill.

10.2 MATERIALS AND METHODS

10.2.1 Plant collection and identification

Plant specimens were collected from three different attitudes of Darjeeling Himalaya. The names of the selected places are Sukhiapokhri (Alt. 2231), Lebong (Alt. 1817) and Kerseong (Alt. 1516). Finally these plants represent our voucher specimens and are deposited in the 'NBU Herbarium' of Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal for identification.

10.2.2 Soil sampling and determination of physicochemical properties

Soil samples were collected from three different cultivated plots of four plants mentioned above (top and sub soil with 0-15 m and 15-30 cm depth respectively) and composite soil were prepared as per the method of Misra and Saha, 2009. The samples were processed for physicochemical analysis viz. pH, electrical conductivity, moisture contents, organic carbon, available form of nitrogen, potash as K_2O, phosphorus as P_2O_5 and sulphur as SO_4^- (Jackson, 1973).

10.2.3 Preparation of methanolic plant extracts

Four Solanaceae fresh fruits (*Capsicum annum, Cyphomandra betacea, Solanum anguivi* and *S. incanum*) were separately crushed with mortar and pestle. Under a Soxhlet extractor, crushed fruits were individually extracted with methanol for 8h. The methanol was completely removed by vacuum rotary evaporator at 50°C. These crude extracts were freeze-dried. The powder was stored at 4°C and used for further investigation.

10.2.4 Animal material

For the assay of anti-lipid peroxidation, animal material collection procedure was mentioned in Chapter IV Section 4.2.3.

10.2.5 Determination of DPPH radical scavenging assay

The assay was performed as prescribed by Blois (1958) and specified in details in Chapter IV Section 4.2.4.

10.2.6 Determination of superoxide anions scavenging activity

The assay was performed as prescribed by Nishikimi *et al*., (1972) and specified in details in Chapter IV Section 4.2.6.

10.2.7 Determination of hydroxyl radical scavenging activity

The assay was performed as prescribed by Jung *et al*., (2008) and specified in details in Chapter IV Section 4.2.7.

10.2.8 Determination of reducing power

The assay was performed as prescribed by Aiyegoro and Okoh, (2009) and specified in details in Chapter IV Section 4.2.10.

10.2.9 Anti-lipid peroxidation (ALP) assay

The assay was performed as prescribed by Bauchet and Barrier, (1998) and specified in details in Chapter IV Section 4.2.11.

10.2.10 Total phenol estimation

The assay was performed as prescribed by Folin and Ciocalteu, (1927) and specified in details in Chapter V Section 5.2.3.

10.2.11 Total flavonoids determination

The assay was performed as prescribed by Sultana *et al*., (2009) and specified in details in Chapter V Section 5.2.4.

10.2.12 Statistical analysis

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between soil physicochemical properties and antioxidant attributes and MS Excel was used for comparing the antioxidant attributes of the fruits collected from three different altitudes. Smith' Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidant and their standard error of estimates (SEE). Principal component analysis (PCA) of soil physicochemical properties and biochemical estimates of plants were calculated by using Multivariate Statistical Package (MVSP 3.1).

10.3 RESULT AND DISCUSSIONS

Table 10.1-10.8 shows the antioxidant activity of four Solanaceous plants of Darjeeling Himalaya and soil nutrient profile of three places of this hill with different altitudes. In 2013, Ibrahim *et al*. stated that fertilizer could influence the biosynthesis of total phenolics and flavonoids. In our experiments total phenol contents of *Cyphomandra betacea* and *Solanum anguivi* fruits were highly correlated with nutrient profile of top soil in which they were

Table 10.1 Free radical scavenging capacity and phytochemical contents of *Capsicum annuum* fruits

	ALTITUDE	DPPH	ABTS	SO	NO	OH	MC	RP	BCB	ALP	TPC	TFC
Sukhiapokhri	2231	17.05±0.05	2.12±0.01	93.61±0.22	0.0001	89.51±0.86	5.06±0.05	0.82±0.01	3.12±0.03	2.14±0.008	5.49±0.01	0.163±0.001
Lebong	1817	17.09±0.06	2.46±0.02	93.29±0.31	0.0001	89.21±0.76	5.12±0.03	0.95±0.009	3.19±0.05	2.15±0.009	5.5±0.009	0.167±0.001
Kerseong	1516	18.42±0.04	2.14±0.009	94.15±0.41	0.0001	88.13±0.75	5.06±0.04	0.82±0.008	3.02±0.04	1.19±0.007	5.46±0.01	0.164±0.002

Table 10.2 Soil profile of *C. annuum* fruits with different altitudes of Darjeeling Himalaya

	MOC(T)	Ph(T)	OC(T)	N2(T)	K(T)	P(T)	S(T)	MOC(S)	pH(S)	OC(S)	N2(S)	K(S)	P(S)	S(S)
Sukhiapokhri	5.03	5.39	1.58	0.14	92.5	25	45	6.68	5.08	1.49	0.13	87.8	20	38
Lebong	5.7	5.38	1.61	0.14	89.8	25	45	6.45	5.28	1.48	0.14	82.5	28	39
Kerseong	0.08	5.09	1.39	0.12	72.5	23	40	3.22	5.33	1.50	0.13	74.0	21	40

Table 10.3 Free radical scavenging capacity and phytochemical contents of *Cyphomandra betacea* fruits

	ALTITUDE	DPPH	ABTS	SO	NO	OH	MC	RP	BCB	ALP	TPC	TFC
Sukhiapokhri	2231	167.59±1.22	0.86±0.02	7.82±0.51	15.28±0.19	388.18±4.12	10.21±0.21	0.85±0.01	44.37±0.32	151.15±0.52	5.61±0.03	0.186±0.02
Lebong	1817	167.96±1.39	0.89±0.01	7.13±0.62	15.2±0.28	388.19±5.12	10.3±0.24	0.95±0.05	44.31±0.12	151.2±0.62	5.52±0.02	0.183±0.01
Kerseong	1516	167.29±2.16	0.78±0.03	7.91±0.71	15.1±0.31	388.18±7.12	11.13±0.23	0.83±0.01	44.32±0.15	152.32±0.48	5.19±0.02	0.184±0.01

Table 10.4 Soil profile of *C. betacea* fruits with different altitudes of Darjeeling Himalaya

	MOC(T)	Ph(T)	OC(T)	N2(T)	K(T)	P(T)	S(T)	MOC(S)	pH(S)	OC(S)	N2(S)	K(S)	P(S)	S(S)
Sukhiapokhri	4.71	5.51	1.48	0.13	97.5	24	44	6.85	5.44	1.45	0.17	85.9	27	37
Lebong	5.83	5.21	1.83	0.18	84.4	29	49	6.52	5.25	1.54	0.15	81.4	31	39
Kerseong	0.15	4.86	1.05	0.11	71.7	23	39	3.72	5.13	1.63	0.12	71.3	20	40

Table 10.5 Free radical scavenging capacity and phytochemical contents of *Solanum incanum* fruits

	ALTITUDE	DPPH	ABTS	SO	OH	MC	RP	BCB	ALP	TPC	TFC
Sukhiapokhri	2231	8.37±0.04	0.84±0.001	213.92±3.12	43.94±0.12	19.01±0.05	0.63±0.003	32.37±0.09	3.25±0.02	2.87±0.03	0.151±0.001
Lebong	1817	8.36±0.03	0.84±0.005	213.93±3.45	46.32±0.21	18.23±0.03	0.69±0.002	32.19±0.06	3.62±0.01	2.82±0.05	0.152±0.002
Kerseong	1516	8.39±0.07	0.86±0.004	213.31±4.52	43.21±0.34	19.21±0.04	0.62±0.003	36.21±0.07	4.32±0.03	2.48±0.06	0.153±0.001

Table 10.6 Soil profile of *S. incanum* fruits with different altitudes of Darjeeling Himalaya

	MOC(T)	Ph(T)	OC(T)	N2(T)	K(T)	P(T)	S(T)	MOC(S)	pH(S)	OC(S)	N2(S)	K(S)	P(S)	S(S)
Sukhiapokhri	5.82	5.69	1.88	0.19	95.2	29	56	7.02	5.01	1.99	0.11	87.5	26	40
Lebong	5.65	5.48	1.73	0.11	88.3	24	51	6.85	5.19	1.78	0.13	82.6	31	37
Kerseong	0.92	5.19	1.29	0.09	71.6	20	49	3.86	4.79	1.45	0.12	75.5	19	44

Table 10.7 Free radical scavenging capacity and phytochemical contents of *Solanum anguivi* fruits

	ALTITUDE	DPPH	ABTS	SO	NO	OH	MC	RP	BCB	ALP	TPC	TFC
Sukhiapokhri	2231	13.38±0.05	1.38±0.01	100.62±3.25	629.26±7.31	70.15±3.62	22.68±1.21	0.73±0.01	49.42±2.16	6.32±0.16	2.77±0.12	0.13±0.005
Lebong	1817	13.31±0.06	1.39±0.02	99.23±4.65	639.24±9.75	70.15±4.25	22.64±1.32	0.74±0.02	49.43±2.42	6.36±0.15	2.71±0.16	0.13±0.004
Kerseong	1516	13.21±0.04	1.37±0.01	100.32±3.61	622.25±8.25	70.16±5.16	22.65±1.35	0.73±0.01	49.42±2.34	6.32±0.21	2.78±0.14	0.16±0.006

Table 10.8 Soil profile of *S. anguivi* fruits with different altitudes of Darjeeling Himalaya

	MOC(T)	Ph(T)	OC(T)	N2(T)	K(T)	P(T)	S(T)	MOC(S)	pH(S)	OC(S)	N2(S)	K(S)	P(S)	S(S)
Sukhiapokhri	6.11	5.99	1.76	0.15	98.5	26	51	6.98	4.91	1.81	0.18	85.8	23	35
Lebong	5.25	5.18	1.88	0.13	85.4	24	44	6.05	5.05	1.58	0.14	81.5	29	39
Kerseong	1.17	4.88	1.42	0.11	76.5	21	38	4.52	5.68	1.42	0.11	76.2	20	42

grown-up (Table 10.9-10.10), whereas significant correlation was also obtained between total phenol content of *S. incanum* fruits and soil physicochemical profile of three places of Darjeeling Himalaya with different altitudes (Table 10.11). In 2012, Oloyede also observed that phenolic antioxidant contents of pumpkin leaf were increased with the application of NPK fertilizer. Our findings suggested that DPPH, metal chelating, beta carotene bleaching and anti-lipid peroxidation have negative correlation with nitrogen content available in soil, which indicates that nitrogen content of soil is highly responsible for antioxidant capacity of these fruits of Darjeeling Himalaya (Table 10.9-10.12). This result is comparable with some other findings in fruits and vegetables by Skwarylo-Bednarz and Krzepilko (2008) who accounted that the use of mineral fertilizers, particularly nitrogen increases the vitamin C content which is frequently used as an indicator of the antioxidant properties of fruits and vegetables. Liu *et al*., (2010) reported that nitrogen had major effects on the concentration of total flavonoids of *Chrysanthemum morifolium* flowers. They also reported that DPPH, superoxide and hydroxyl radicals were dependent on nitrogen content of soil. Flavonoid is polyphenolic compounds derived from shikimic acid pathway and phenylpropanoid metabolism (Stafford, 1990). The transformation of *L*-phenylalanine to *trans*-cinnamic acid by the phenylalanine ammonia-lyase is the initial step in the biosynthesis of flavonoids and other phenylpropanoid derivatives (Jones, 1984; Margna, 1977). On the other hand, *L*-phenylalanine is also the precursor of proteins. Therefore, there is a competition between phenolics and proteins for the common precursor. At high supply rates, nitrogen may reduce the synthesis of flavonoids through encouraging the channelization of *L*-phenylalanine towards proteins (Margna *et al.,* 1989). They also proved that during nitrogen deficiency, the deamination of *L*-phenylalanine promted to synthesize flavonoids in order to recycle the nitrogen from the deaminated *L*-phenylalanine. Reversal of the effect of the soil nutrient was also noticed in these plants where decline of the antioxidant activity and phenolic constituents were observed (Table 10.9). This is in agreement with the study conducted by Juan *et al.*, (2008) on effects of nitrogen and sulphur on total phenol content and antioxidant activity of mustard leaf. They stated that increasing nitrogen supply considerably decreased total

Table 10.9 Correlation matrix (Pearson co-efficient) of soil properties and antioxidant traits of *Cyphomandra betacea*

	ALTITUDE	DPPH	ABTS	SO	NO	OH	MC	RP	BCB	ALP	TPC	TFC
MOC(T)	**0.755***	0.142	**0.955****	-0.531	0.182	0.420	**-0.788***	**0.709***	-0.010	-0.034	**0.762***	-0.100
Ph(T)	0.656	0.590	**0.917****	-0.210	0.638	**0.742***	-0.391	**0.776***	0.504	0.433	**0.953****	0.416
OC(T)	**0.692***	0.526	**0.954****	-0.305	0.524	**0.713***	-0.531	**0.800****	0.361	0.355	**0.912****	0.256
N2(T)	**0.689***	0.234	**0.921****	-0.504	0.281	0.499	**-0.710***	**0.735***	0.066	0.076	**0.789***	-0.046
K(T)	**0.887****	0.350	**0.912****	-0.242	0.300	0.495	**-0.740***	0.586	0.142	0.139	**0.857****	0.114
P(T)	**0.768***	0.351	**0.963****	-0.366	0.357	0.574	-0.650	**0.738***	0.204	0.207	**0.897****	0.121
S(T)	**0.770***	0.405	**0.958****	-0.340	0.389	0.604	-0.646	**0.742***	0.228	0.254	**0.911****	0.143
MOC(S)	**0.855****	0.224	**0.932****	-0.374	0.219	0.432	**-0.793***	0.618	0.043	0.021	**0.808****	-0.004
pH(S)	**-0.694***	0.360	-0.095	-0.032	0.606	0.461	**0.809****	0.368	0.642	0.445	-0.087	0.481
OC(T)	-0.156	**0.893****	0.029	0.490	**0.817****	**0.745***	0.594	0.260	**0.864****	**0.946****	0.385	**0.795***
N2(S)	0.118	0.389	**0.762***	-0.430	0.644	**0.738***	0.017	**0.884****	0.549	0.309	0.585	0.360
K(S)	**0.926****	0.441	**0.827****	-0.049	0.345	0.516	**-0.682***	0.462	0.206	0.223	**0.859****	0.215
P(S)	-0.113	-0.041	0.628	**-0.879****	0.171	0.383	-0.219	**0.887****	0.008	-0.006	0.304	-0.255
S(S)	-0.450	**0.722***	-0.285	0.560	0.644	0.503	**0.835****	0.034	**0.777***	**0.801****	0.025	**0.750***

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 10.10 Correlation matrix (Pearson co-efficient) of soil properties and antioxidant traits of *Solanum anguivi*

	ALTITUDE	DPPH	ABTS	SO	OH	MC	RP	BCB	ALP	TPC	TFC
MOC(T)	**0.742***	-0.121	-0.489	0.081	0.576	-0.633	**0.699***	**-0.910****	**-.836****	**0.919****	-0.299
Ph(T)	0.589	0.439	0.150	0.659	**0.715***	-0.133	**0.754***	-0.419	-.515	**0.923****	0.345
OC(T)	**0.729***	0.128	-0.270	0.335	**0.704***	-0.478	**0.750***	**-0.783***	**-.757***	**0.961****	-0.154
N2(T)	**0.709***	-0.081	-0.431	0.147	0.627	-0.609	**0.737***	**-0.823****	**-.777***	**0.934****	-0.132
K(T)	**0.872****	0.042	-0.303	0.219	0.508	-0.419	0.576	**-0.837****	**-.892****	**0.962****	-0.194
P(T)	**0.702***	0.241	-0.100	0.472	**0.706***	-0.332	**0.771***	-0.666	**-.692***	**0.989****	0.069
S(T)	**0.784***	0.068	-0.280	0.276	0.592	-0.448	**0.708***	**-0.809****	**-.819****	**0.985****	-0.105
MOC(S)	**0.852****	-0.044	-0.398	0.131	0.488	-0.499	0.596	**-0.891****	**-.905****	**0.947****	-0.270
pH(S)	-0.556	0.269	0.274	0.490	**0.704***	-0.098	0.615	0.366	.580	0.023	0.546
OC(T)	0.070	**0.825****	**0.908****	**0.805****	0.156	0.727*	-0.086	0.534	.206	0.087	**0.673***
N2(S)	-0.094	-0.286	-0.512	-0.037	**0.748***	**-0.838****	**0.877****	-0.502	-.106	0.412	-0.139
K(S)	**0.932****	0.194	-0.093	0.333	0.394	-0.167	0.398	**-0.690***	**-.873****	**0.923****	-0.073
P(S)	-0.117	-0.132	-0.349	0.140	**0.849****	**-0.742***	**0.932****	-0.392	-.035	0.448	0.035
S(S)	**-0.688***	0.626	**0.710***	0.567	0.204	0.524	-0.044	**0.877****	**0.849****	-0.465	0.631

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 10.11 Correlation matrix (Pearson co-efficient) of soil properties and antioxidant traits of *Solanum incanum*

	ALTITUDE	DPPH	ABTS	SO	NO	OH	MC	RP	BCB	ALP	TPC	TFC
MOC(T)	**0.741***	0.199	0.420	-0.242	0.292	0.060	0.165	-0.193	-0.003	0.288	-0.168	**-0.993****
Ph(T)	0.657	**0.712***	**0.675***	0.243	0.381	0.545	0.608	0.290	0.288	0.523	0.270	**-0.794***
OC(T)	**0.750***	0.428	0.621	-0.037	0.340	0.248	0.404	0.083	0.268	0.494	0.124	**-0.946****
N2(T)	0.618	0.343	**0.685***	0.102	0.461	0.376	0.422	0.208	0.395	0.605	0.099	**-0.839****
K(T)	**0.859****	0.428	0.526	0.014	0.205	0.246	0.313	-0.007	0.245	0.405	0.100	**-0.967****
P(T)	**0.740***	0.439	0.620	-0.088	0.203	0.240	0.441	-0.010	0.198	0.434	0.142	**-0.940****
S(T)	**0.740***	0.414	0.615	-0.063	0.261	0.267	0.410	-0.005	0.183	0.461	0.075	**-0.957****
MOC(S)	**0.850****	0.300	0.430	-0.116	0.218	0.116	0.189	-0.127	0.117	0.318	-0.041	**-0.993****
pH(S)	-0.466	0.514	0.610	0.622	0.374	**0.767***	**0.712***	**0.839****	0.607	0.646	0.529	0.354
OC(T)	0.096	**0.816****	0.456	**0.922****	0.097	**0.789***	0.615	0.870**	**0.816****	0.538	**0.814****	0.206
N2(S)	0.398	0.647	**0.824****	0.280	0.383	0.613	**0.692***	0.459	0.563	**0.704***	0.393	-0.625
K(S)	**0.921****	0.428	0.473	0.077	0.130	0.224	0.273	0.001	0.294	0.375	0.162	**-0.926****
P(S)	-0.135	-0.106	0.498	-0.389	0.627	0.082	0.290	0.003	-0.144	0.396	-0.337	-0.459
S(S)	**-0.765***	0.154	0.231	0.475	0.262	0.441	0.429	**0.689***	0.355	0.303	0.393	**0.759***

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 10.12 Correlation matrix (Pearson co-efficient) of soil properties and antioxidant traits of *Capsicum annuum*

	ALTITUDE	DPPH	ABTS	SO	OH	MC	RP	BCB	ALP	TPC	TFC
MOC(T)	**0.748***	**-0.770***	0.541	-0.291	0.166	0.210	0.529	**0.731***	**0.996****	0.153	0.297
Ph(T)	0.636	-0.291	0.398	0.190	**0.775***	0.492	0.423	0.658	**0.687***	**0.709***	0.415
OC(T)	**0.681***	-0.574	0.634	-0.034	0.411	0.455	0.643	**0.824****	**0.945****	0.435	0.465
N2(T)	**0.858****	**-0.724***	0.369	-0.208	0.310	0.144	0.344	**0.691***	**0.961****	0.202	0.306
K(T)	**0.876****	**-0.762***	0.375	-0.225	0.287	0.182	0.372	0.659	**0.981****	0.218	0.247
P(T)	**0.830****	-0.620	0.426	-0.034	0.482	0.332	0.427	**0.709***	**0.930****	0.409	0.384
S(T)	**0.839****	-0.666	0.435	-0.082	0.423	0.298	0.432	**0.718***	**0.958****	0.349	0.363
MOC(S)	**0.848****	-0.784*	0.414	-0.271	0.228	0.159	0.404	**0.681***	**0.995****	0.165	0.253
pH(S)	**-0.720***	0.646	0.586	0.613	0.138	0.641	0.610	0.248	-0.321	0.403	0.628
OC(T)	0.028	0.539	0.200	**0.895****	**0.840****	**0.712***	0.227	0.446	-0.008	**0.849****	0.636
N2(S)	-0.054	0.043	**0.789***	0.152	0.364	**0.714***	**0.825****	**0.721***	0.392	0.606	0.367
K(S)	**0.964****	**-0.744***	0.142	-0.200	0.351	0.057	0.140	0.508	**0.902****	0.198	0.161
P(S)	-0.115	-0.162	**0.973****	-0.005	0.045	0.551	**0.964****	**0.756***	0.507	0.272	0.576
S(S)	-0.544	**0.843****	0.033	**0.772***	0.499	0.531	0.079	-.084	-0.585	0.611	0.311

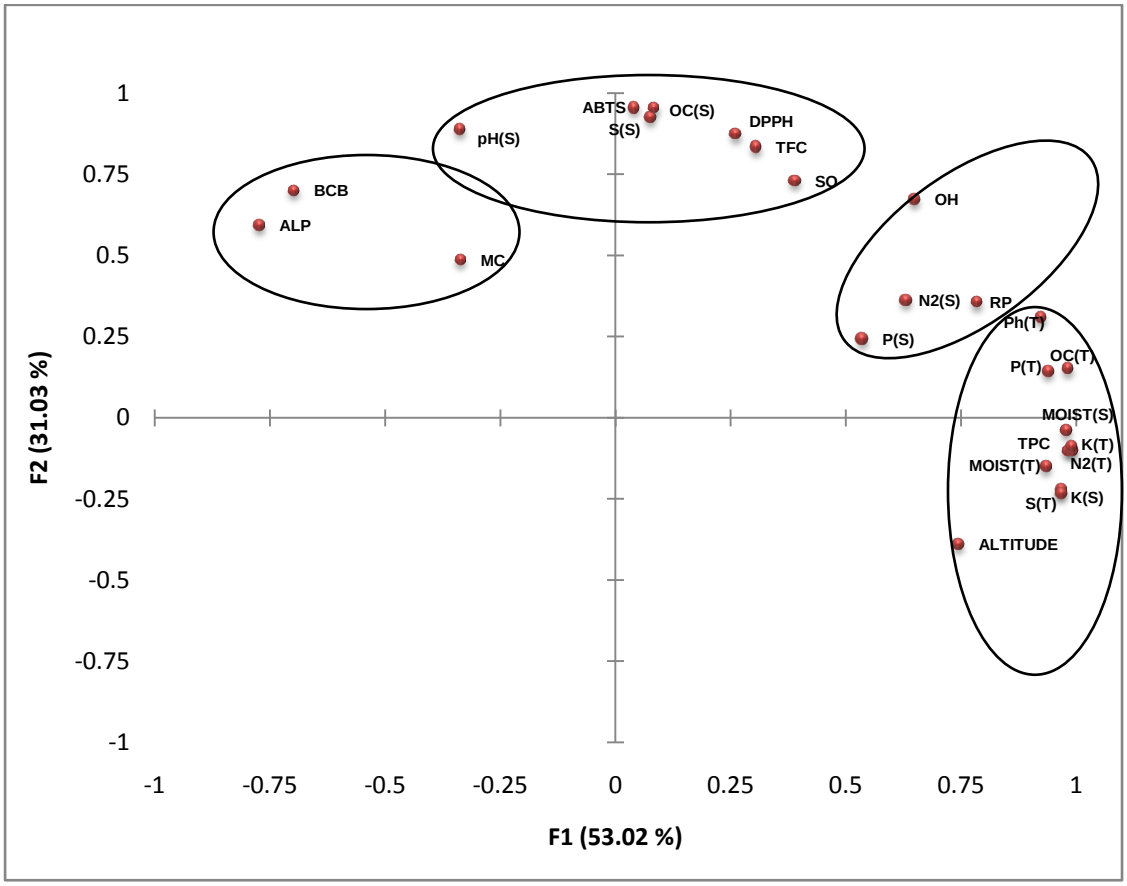
*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

phenolic concentrations. Nitrogen amendment and enhanced soil fertility sometimes reduce stress signals in root and inhibit PAL activity, which might be the cause of decreased accumulation of polyphenols and low molecular weight antioxidants. Soundy *et al*. (2007) found that the potassium fertilizers increased total polyphenols quantitatively. Similarly in 2008**,** Kamal *et al*. noticed a significant increase in the total polyphenols content of onion through potassium treatments. DPPH, metal chelating, beta-carotene bleaching and anti-lipid peroxidation of *C. betacea* and *S. anguivi* fruits are responsible for total phenol content which is highly correlated with potassium available in the soil (Table 10.9-10.10). In 2006 Engel *et al.* referred to the positive effect of using potassium-based fertilizers with the total phenol contents and antioxidant activity. Similar findings were noticed on the soil type and subsequent accumulation of nutrients by cultivated plants (Kadar, 1988; Kadar, 2002; Lester, 2007). Phosphorous is also a vital soil nutrient which is used as fertilizer for optimizing the accumulation of antioxidant molecules in plants (Dayang *et al*., 2012). Available phosphorous in soil significantly influenced metal chelating activity of *C. betacea* and *S. anguivi* fruits (Table 10.9-10.10). Physical properties generally pH and moisture content of soil greatly influence the phytochemical constituents present in the different parts of *Drymaria* plants (Mandal *et al*., 2009b). Similar results were also established as noticed in Table 10.9-10.10 and 10.12. Mandal *et al*., (2009b) also reported that phytochemicals constituents and physico-chemical properties of soil collected from different places of North Bengal (India) significantly correlated with each other. From Table 10.9-10.12, it is clear that altitude is also a vital factor for mobilizing soil nutrients and it is highly correlated with antioxidant attributes of fruits and vegetables.

To understand more about the relationship between antioxidant attributes and soil fertility profile, PCA was performed with each plant. The first two principal components was chosen on the basis of their eigenvalues >1, explaining 84.05%, 79.86%, 82.03% and 82.10% of the total data variance for *S. incanum, S. anguivi, C. betacea* and *C. annuum*. respectively. In almost all cases top soil fertility attributes were heavily loaded on the first principle component (PC1) and strongly clustered as evidenced through dendrogram (Figure 10.1-10.4). In case of *S. incanum* metal chelating activity, beta carotene bleaching

Figure 10.1 The Principal Components Analysis based on soil nutrient profile, antioxidant activity and phytochemical attributes of *S. incanum* fruit of Darjeeling Himalaya



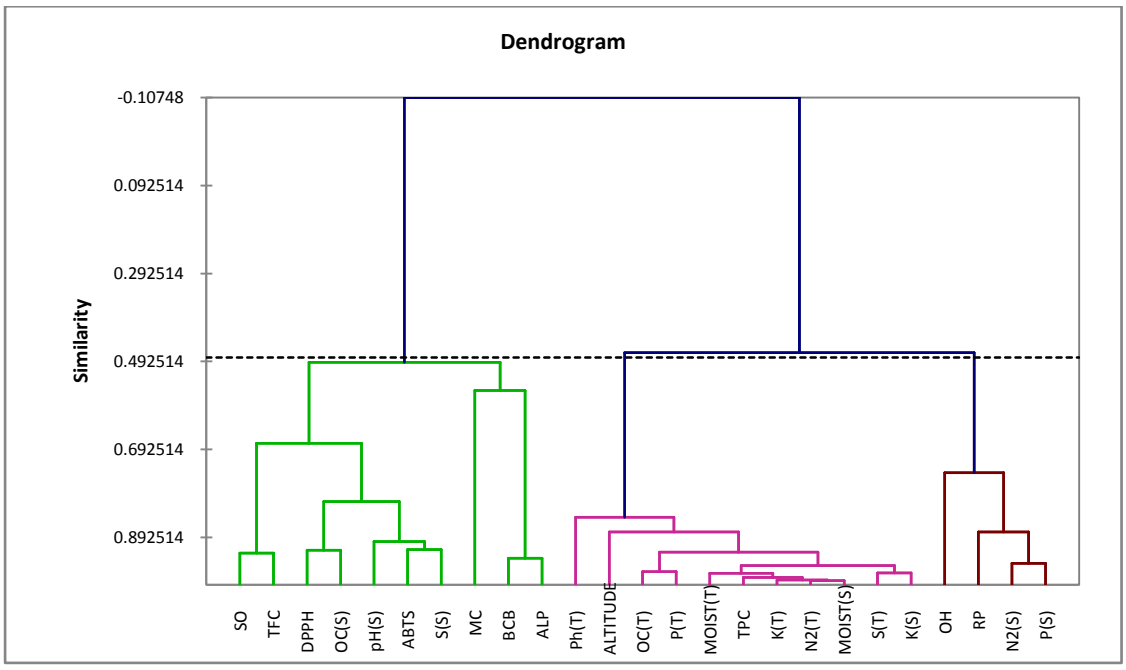
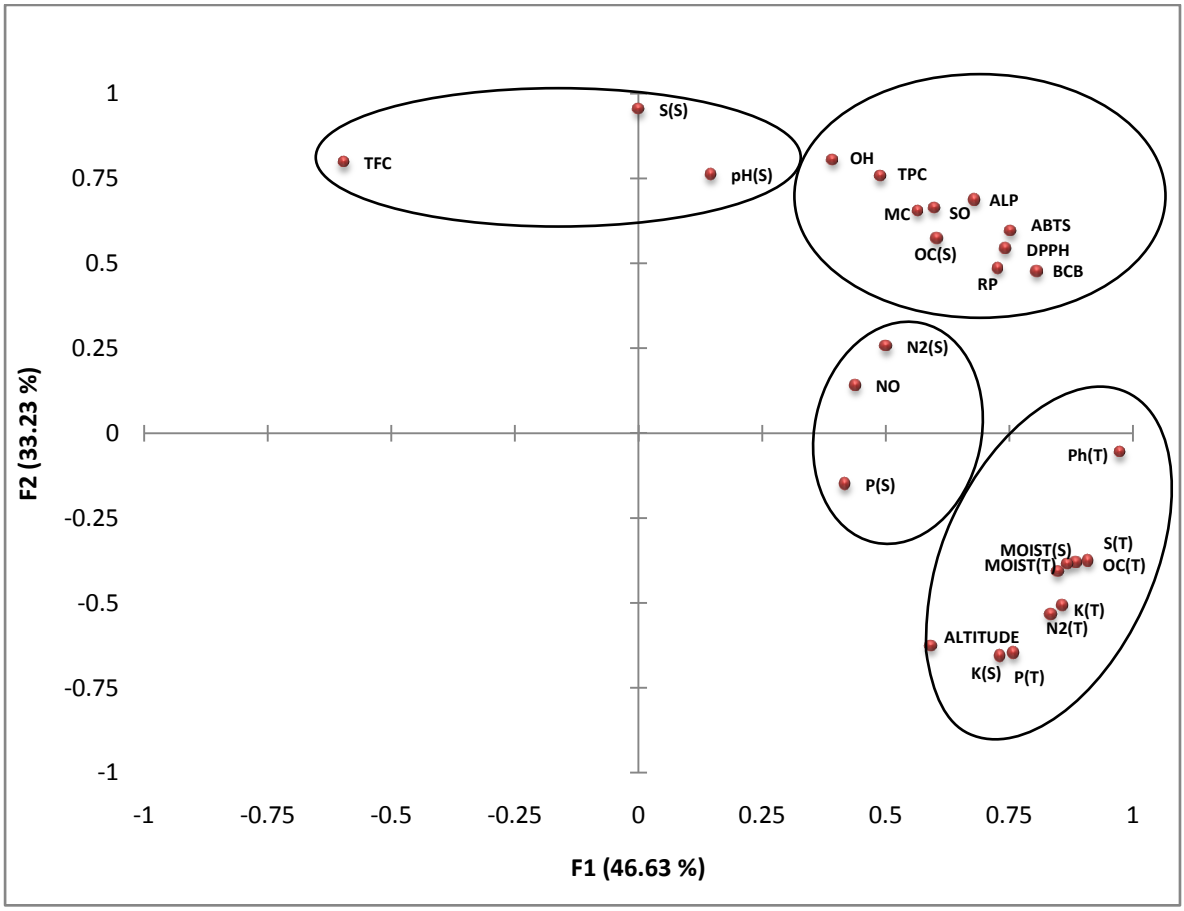


Figure 10.2 The Principal Components Analysis based on soil nutrient profile, antioxidant activity and phytochemical attributes of *S. anguivi* fruit of Darjeeling Himalaya



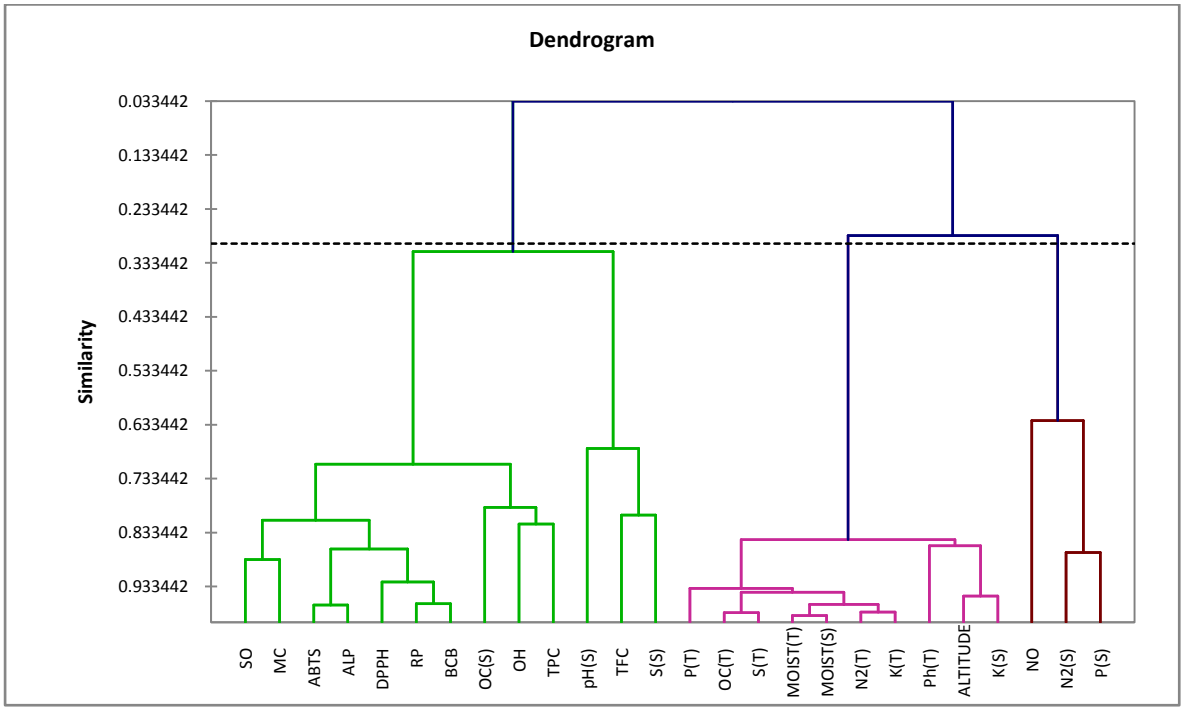
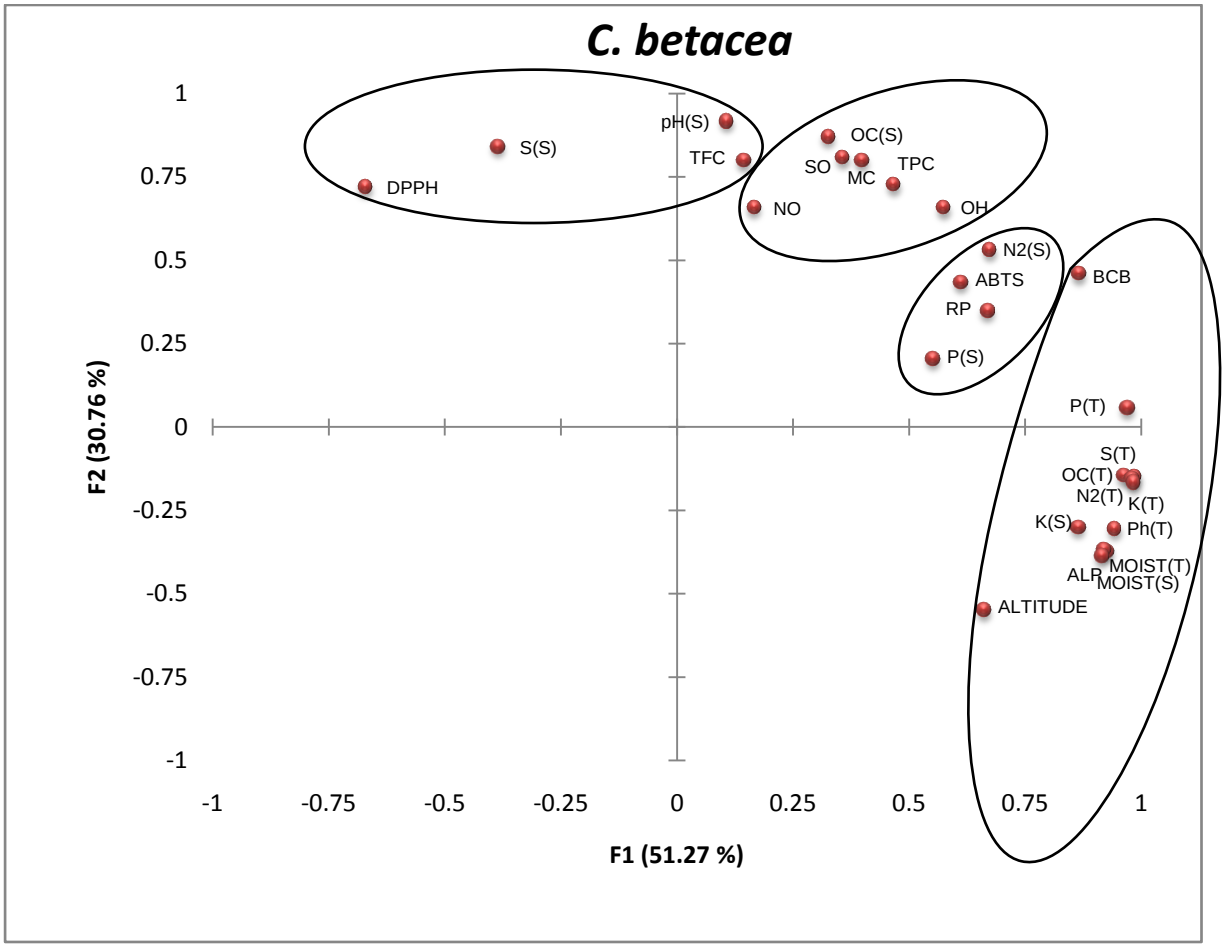


Figure 10.3 The Principal Components Analysis based on soil nutrient profile, antioxidant activity and phytochemical attributes of *C. betacea* fruit of Darjeeling Himalaya



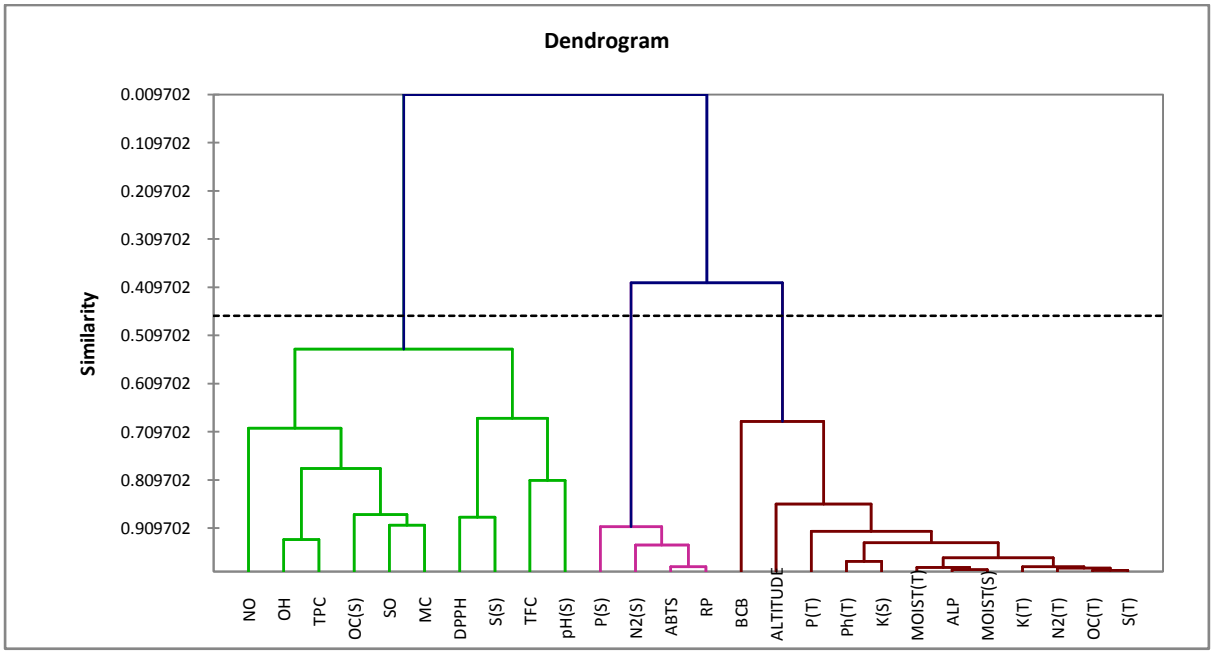
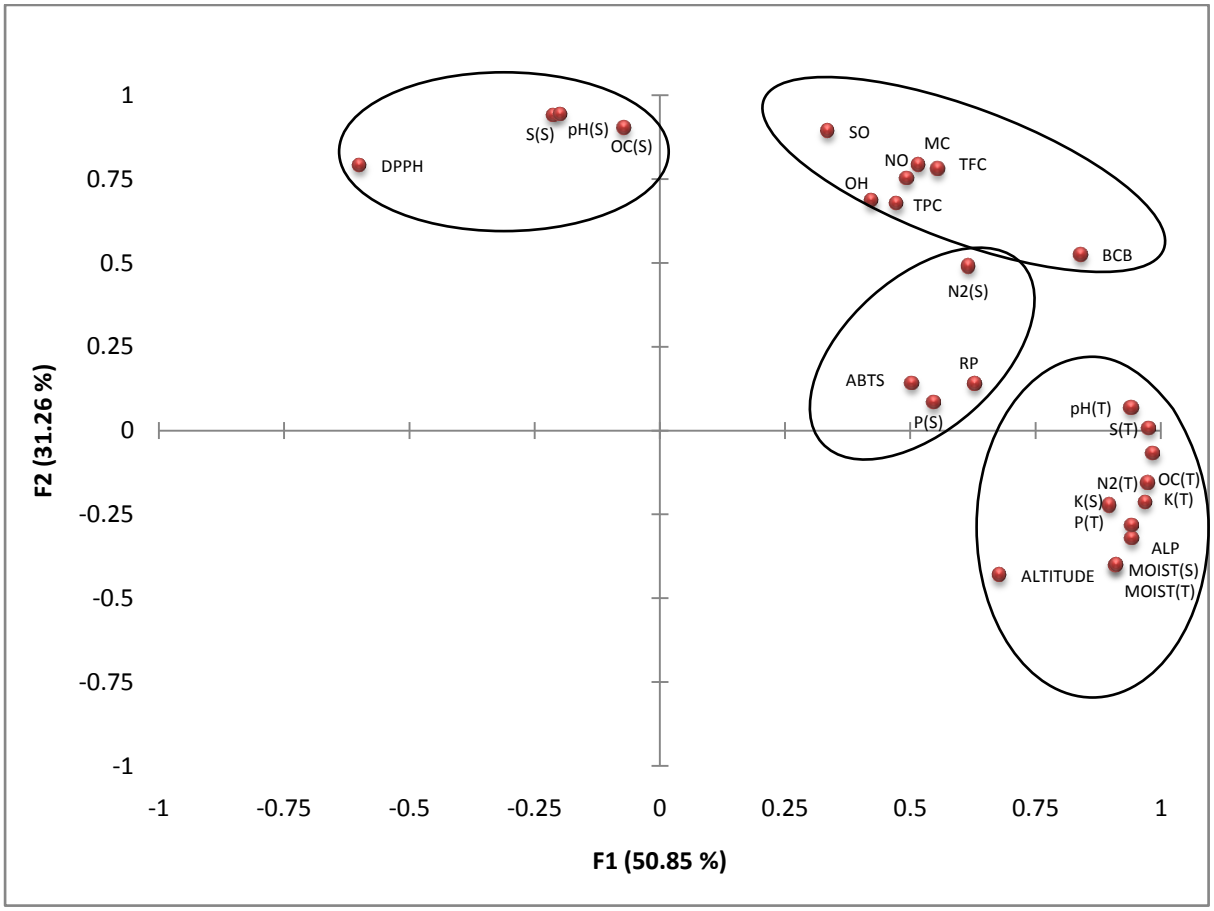
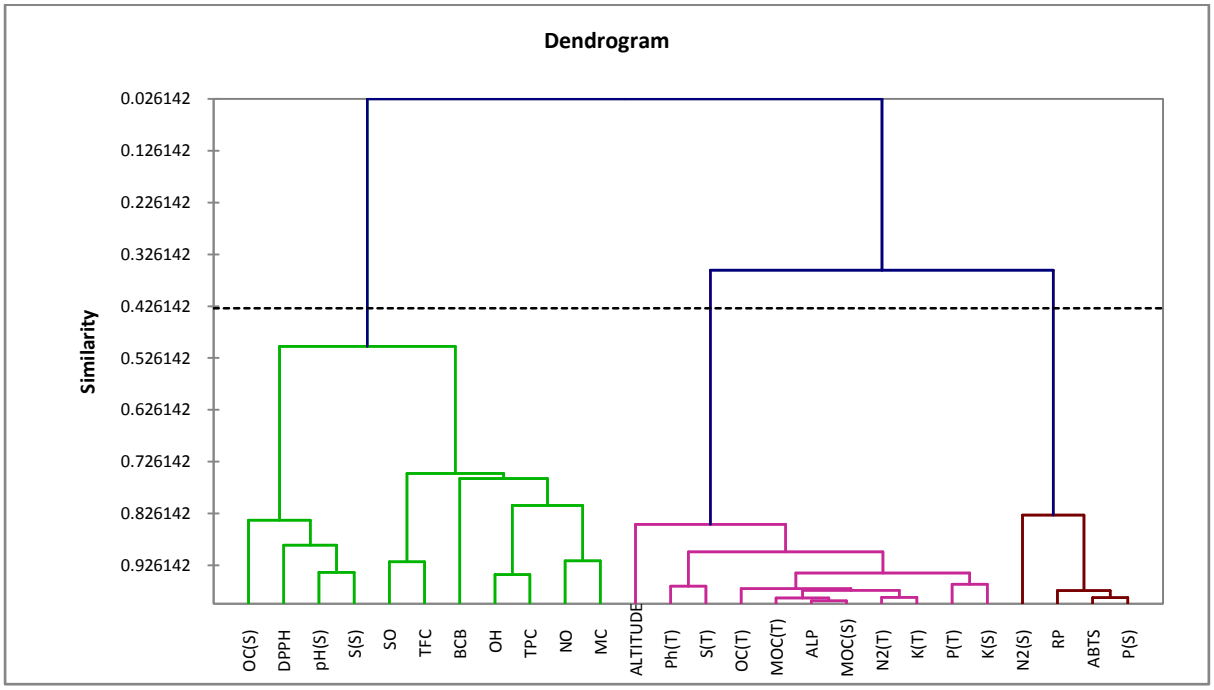


Figure 10.4 The Principal Components Analysis based on soil nutrient profile, antioxidant activity and phytochemical attributes of *C. annuum* fruit of Darjeeling Himalaya





and anti-lipid peroxidation were clustered in opposite co-ordinate which indicated that these antioxidant attributes were controlled by top soil nutrients, but for *S. anguivi* sub-soil potassium level and free radical scavenging attributes were loaded on PC2 and were clustered in opposite domain indicating that enhancement of sub-soil potassium level is important for reducing IC_{50} values of these scavenging components. Similar observations were also found by Wu *et al*., 2013 in *Ziziphus jujube* who stated that potassium supplementation improved the accumulation of bioactive phenolics and strengthened antioxidant activity. In case of all plants the PCA graphs shows that sub-soil sulphur level was placed very nearly to DPPH and ABTS, both of which were loaded on PC2, indicating that sub-soil sulphur actually increased IC_{50} values or otherwise interfere the activity of scavenging DPPH and ABTS free radical. Subsoil nitrogen and phosphorus were clustered together in most of the dendrogram, but these attributes are not significant contributors of antioxidant activity as revealed from PCA. Actually different opinions were generated regarding soil fertility status and their relationship with free radical scavenging capacity of plants. It was revealed that productivity and antioxidant based pigments in lettuce were higher in all fertilized soils than the untreated control. Agro-industrial organic wastes and vermicomposts significantly enhanced growth and productivity of lettuce but unfortunately remarkably decreased nutraceutical quality antioxidant values and bioactive phenolics level (Coria-Cayupan *et al*., 2009). In contrast, amendment of organic fertilizer in soil increased leaf antioxidant contents relative to conventional nitrogen source in blueberry (Montalba *et al*., 2010). In some cases significant organic input in soil might enhance humic acid containing stable organic free radicals (Steelink and Tollin, 1962). These free radicals accumulation in root periphery might elicit small bioactive radical scavenging molecules. In *Lebisia pumila*, enhancement of nitrate content through organic fertilizer application in soil improved secondary metabolites production and antioxidant activity (Ibrahim *et al*., 2013).

In conclusion it may be stated that soil nutrient profile particularly NPK and sulphur has changed with altitude variation which ultimately influence the accumulation of bioactive secondary metabolites and antioxidants in natural vegetation. So, for elicitation of bioactive

metabolites and high quality nutraceuticals in fruits and vegetables of Darjeeling Himalaya judicious applications of NPK is required for improving cultivation techniques.

Summary

The present study deals with the "Evaluation of antioxidant activities of some locally available edible plants of Darjeeling Himalaya". The thesis was configured with nine chapters, which excludes 'General Introduction' and 'Summary'. At the onset of the work, brief review of literature in the line of investigation has been presented. The review has started with the conceptual background of oxidative stress mediated free radicals generation and their impact on human health. The beneficial roles of ROS and RNS at their optimized concentrations and their cellular signals governing essential physiology were initially highlighted in the review. But the excess free radicals accumulated during oxidative stress have been implicated in various neurodegenerative, cardiovascular, pulmonary and gastrointestinal disorders including malfunctioning of metabolic circuits and cell cycle regulation. As the intake of antioxidants are the only solution for relieving from oxidative stress situation, dietary source of antioxidants were briefly elucidated. In this context, different classes of antioxidants were also schematically presented. While the quantitative structure-function relationship is directed by chemical nature of antioxidants and the stoichiometric ratio of free radicals existing in a particular chemical atmosphere, the different mechanism of antioxidant action towards different free radicals were analyzed with various classes of natural antioxidants. But the biological action of synthetic antioxidants and their adverse toxicity were also pointed out. Plant based secondary metabolites are rich source of natural antioxidants. Among metabolites, plant derived phenolic compounds and other phenylpropanoids play important role in free radical scavenging mechanism. The elicitation process for accumulation of secondary metabolites and their therapeutic properties were mentioned thereafter. In this connection, the relationship between natural antioxidants and anti-diabetic activity with associated physiological properties were specifically discussed. Along with this, different ways of assessment of plant derived antioxidants, their pattern of accumulation with maturation and senescence were reported as observed and inferred by different authors. Awareness about nutraceuticals, their market potential and alteration with domestic thermal processing were elucidated. For emphasising the importance of this research in local area, antioxidant capacity of different plants of Darjeeling Himalaya; their

ethno-medicinal implications and quantitative correlation with soil nutritional properties were specifically highlighted. Lastly the review was concluded by comparing different modern methods for isolation and bioassay guided purification of secondary metabolites and antioxidants through sophisticated instrumental techniques.

The rest of the chapters were written on the basis of work performed for fulfilling the objectives of the present work. Chapter-III mainly deals with market survey report of underutilized fruits and vegetables of Darjeeling Himalaya. The market based botanical knowledge about underexplored fruits and vegetables were analyzed in this chapter on the basis of mode of collection, yield potential and possible threats of products. From survey reports it was realized that 88% of these fruits and vegetables were collected rampantly from wild habitat. Among underexplored plants most of the prominently used fruit specimens create maximum pressure in natural habitat due to lack of cultivation techniques. Most of these fresh and dry fruits were ethnomedicinally important for managing disorders which claimed their further uses as functional food with therapeutic end. Along with this, different processing variations were also recorded in market survey.

As the initial market survey reports indicate potential ethnomedicinal impact of underexplored fruits and vegetables, free radical scavenging activities were performed with the extracts of those edible plant parts for determining their therapeutic potency. In next subsequent chapters the free radical scavenging activity and phytochemical constituents were analysed and discussed. Initially 34 plants of 16 families were selected for screening free radical scavenging potential through DPPH, $ABTS^{+}$, superoxide, hydroxyl radical, lipid peroxidation, metal chelation and ferric reducing method. Potential antioxidant activities were obtained from fruits of *Calamus erectus*, *Cyphomandra betacea, Capsicum annum, Solanum incanum* and *S. anguivi*, leafy vegetable *Nasturtium officinale, Dioscorea alata* underground plant parts and *Evodia fraxinifolia*, a spice yielding plant with aromatic flavour. Among edible plant parts, free radical scavenging capacity was best observed in different edible fruits followed by spice yielding plants and underground plant parts. On the basis of antioxidant activities fruits, leaves and spices were clustered together on PCA plots whereas the

underground plant parts were placed in separate coordinate, indicating that the mechanism of action of oxidative stress mitigation by underground parts were distinct from others.

The existence of secondary metabolites present in edible plants of Darjeeling Himalaya was recorded in Chapter-V. As the phytochemicals present in herbs, fruits and spices of Darjeeling Himalaya have potential medicinal properties, their qualitative and quantitative variation was studied. The polyphenolic components were present in significantly higher amount in different edible fruits. More precisely, total phenols and flavonoids contents were potentially accumulated in mesocarp and endocarp of *Calamus erectus*, *Pyrus communis* and *Rubus ellipticus*; epicarp of *Docynia indica* and *Machilus edulis*; pericarp of *Elaeocarpus lanceifolius*, *Persea americana* and *Prunus domestica*. On PCA factor loadings plot, median polar secondary metabolites were clustered together except amino acids and alkaloids, from which their functional co-ordination might be speculated. It was also observed that the plants like *Calamus erectus*, *Elaeocarpus lanceifolius*, *Machilus edulis*, *Persea americana* and *Spondius mombin* exhibited remarkable antioxidant activity as well as significant amount of phenolics and flavonoids in their edible parts indicating the apparent correlation between concentration of phenyl propanoids and free-radical scavenging properties. Recent literature survey also highlighted that these phytocomponents are pharmacologically very active, pointing towards the contribution of phenyl propanoid metabolites for the said purposes.

As these plants carry numerous secondary metabolites having potential antioxidants, it is quite natural that some pharmacologically active phytocomponents might be present in these edible fruits and vegetables. In fact ethnomedicinal market survey indicates that some edible plant parts are potentially useful for diabetic treatment which stimulates us for determining the therapeutic potential with those plant extract. Diabetes is a particular metabolic disorder where the magnitude of complications increase manifold with oxidative stress. *In vitro* assessment of antidiabetic activity was performed with α-amylase and α-glucosidase enzyme, which is important for carbohydrate digestion in the intestinal lumen. Among these fruits and vegetables, *Calamus erectus* and *Dioscorea alata* showed higher *in vitro* antidiabetic activity as revealed from inhibition of the said enzymes. Oral administration

of *C. erectus* fruit extract in streptozotocine treated rats exhibited a dose dependent significant hypoglycaemic activity, which was comparable with reference standard glybenclamide. Not only that *C. erectus* also tackled oxidative stress by improving liver GSH, SOD and CAT and reducing TBARS. Significant reduction in the progression of total serum cholesterol, triglycerides and LDL-c in association with enhanced HDL/LDL ratio might also indicate activation of LDL receptors by *C. erectus* extract. PCA analysis also indicated that oxidative stress migration is essential for managing diabetic situation which might be improved by the application of antioxidant rich herbal drugs.

It is generally considered that the phytochemical composition and antioxidant quality of fruits are strongly influenced by the stages of maturation. Considering the fact *in vitro* free radical scavenging efficiency and antidiabetic capacity of these underexplored fruits were evaluated from immature to mature stages along with quantitative variation of phytonutrients like carotene, lycopene and phenyl propanoids. In both *C. batacea* and *S. anguivi*, free radical scavenging potency gradually increased with ripening stages. The ripe fruits of the representative of Solanaceae family have the tendency to accumulate large amount of carotenoids as revealed from significantly higher accumulation of carotene and lycopene pigments in the mature and senescence stages of both fruits. The lower IC_{50} values were also noticed for *in vitro* inhibition of enzymes participated in carbohydrate digestion during ripening stages of these fruits, indicating the accumulation of antidiabetic components through senescence programme. Not only had that, phenyl propanoid derivatives also exhibited same tendency of accumulation associated with fruit development. So the antioxidant and antidiabetic property along with bioactive phytochemical attributes enhanced successively from immature to mature transition. The relationship between pharmacological properties and functional phytocomponents of *C. batacea* and *S. anguivi* was also established through principal component analysis (PCA). The results from PCA clearly indicated that the phenolics and carotenoids might be contributed for upregulated free-radical scavenging and antidiabetic attributes associated with maturation. From correlation matrix, superior association was registered between lipophilic carotenoids and DPPH radical scavenging

properties but not nicely linked with other purely hydrophilic radical scavengers. Therefore, these two fruits of Darjeeling Himalaya are most acceptable for their pharmacological properties at maturity stages.

It is well known that cooking stimulates significant changes in chemical composition, influencing the concentration as well as bioavailability of bioactive compounds from vegetables. In Darjeeling Himalaya, post-harvest vegetable processing was performed through half-cooking, boiling, semi-frying, deep frying and sun drying. Considering this fact, thermal processing of selected vegetables was performed for evaluating the alteration of phytochemical constituents and antioxidant activity during major cooking practices. When the edible fruit parts of different members of Solanaceae were considered, free-radical scavenging potency along with bioactive phytochemicals were enhanced through sun drying of *C. betacea* and frying treatments of the fruits of two *Solanum* spp. which might be revealed through lowering of IC_{50} values during evaluation. Thermal treatment also improved antioxidant quality of processed foods which might be due to release of bound phenyl propanoid derivatives in case of Solanaceae. However, for underground plant parts (taruls), boiling significantly reduced free-radical scavenging properties as compared with their unprocessed counter parts. In contrast, antidiabetic activity of different solanaceous members as well as taruls was deteriorated seriously with any aggressive thermal processing, might be due to existence of thermo-labile components mainly responsible for the said activity.

One of the major challenges in the area of phytomedicine is to isolate, purify and identify bioactive compounds from crude extracts through cheaper technology-based strategies. In Chapter-IX, an attempt was made to purify and identify bioactive from two edible fruits of Darjeeling Himalaya viz. *Solanum anguivi* and *Calamus erectus*, which are ethnomedicinally very important. After aqueous reconstitution of methanolic extract of *S. anguivi*, the extract was processed through successive solvent partitioning system according to polarity gradient and bioassays were performed. Chloroform fraction was chosen on the basis of antioxidant and antidiabetic activity and that was further passed through silica gel column chromatography of mesh size 200-400. Fifty eight fractions were obtained from

column, which was clustered into nineteen fractions on the basis of spectral characteristics. High resolution LCMS analysis of best bioactive fraction indicated the abundance of 1-oleoyl lysophosphatidic acid, which is important intermediate phyto-metabolite of lipid biosynthesis and performs vital physiological function in association with G-Protein coupled receptors. Methanolic extract of *C. erectus* was fractionated similarly and butanol extract having potential bioactivity was processed through two successive columns and fraction $F6_7$ exhibited potential bioactivity. More than 30 peaks were decoded from ESI-MS signal generated from $F6_7$, which point towards the existence of low molecular weight phyto-metabolites having significant antioxidant and antidiabetic activity. Previous records of identified phenolic compounds from mass spectral data of ethyl acetate extract of *Areca catechu* L. (Arecaceae, same family in which *C. erectus* belongs) within same molecular weight range indicates that similar compounds might have been expected in bioactive fractions of *C. erectus*.

Soil nutrient profile and cultivation factors like compost, mulching and fertilizer application also affect the secondary metabolites and antioxidant activity of different fruits and vegetables. So changes of antioxidants and secondary metabolites with different soil profile present in different altitudes of Darjeeling Hill were evaluated further with fruits and vegetables of the members of Solanaceae. Within these family, total phenolics of *C. betacea* and *S. anguivi* fruits were highly correlated with nutrient profile of top soil in which they were grown up. But fruit antioxidants exhibited negative association with nitrogen content available in soil. In contrast, enhancement of available potassium in soil might elicit accumulation of antioxidant rich components as established from their positive correlation. Also available phosphorous in soil significantly influenced metal chelating activity of *C. betacea* and *S. anguivi* fruits. PCA diagram of *S. incanum* pointed out that sub-soil potassium level is one of the important contributors for reducing IC_{50} values of different free-radical scavenging components. In a nutshell, it can be stated that macronutrients of soil with altitude variation ultimately influence the accumulation of bioactive secondary metabolites and antioxidants in natural vegetation.

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INDEX

Appendix –A

CHEMICALS USED

A

α-Amylase enzyme

α-Glucosidase

2,2′ Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS

Acetic acid

Acetic anhydride

Acetone

Aluminum chloride

Ammonia

B

Butanol

C

Chloroform

Citrate buffer (ph 4.5)

Conc.h_2so_4

Cupper acetate

D

2-deoxyribose solution

Dichloromethane

Dichromatic acetic acid

2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-(3,3′-dimethoxy-4,4′-diphenylene)di-tetrazolium chloride

DNS (3,5-dinitrosalicylic acid) reagent

DPPH (2,2-diphenyl-1- picrylhydrazyl)

Dragendroff's reagent

E

Ethylenediaminetetraacetic acid

Ethanol

Ethyl Acetate

Ethylenediamine tetraacetic acid

Etoposide

F

$FeCl_2$

$FeCl_3$

Fehling's solution I (A)

Fehling's solution II (B)

Ferric chloride

Ferrozine

$FeSO_4$, $7H_2O$

Folin-Ciocalteu reagent

Formic Acid

G

Gallic acid

Glacial acetic acid

Glibenclamide

Glucose

H

Hexane

Hydrated ferrous sulphate

Hydrochloric acid

Hydrogen peroxide

Hydroxylamine hydrochloride

L

Lead acetate

M

Methanol

N

Na_2CO_3

Na_2SO_4

NaOH

Napthylethylenediamine dihydrochloride

n-Butanol

Nicotinamide-adanine dinucleotide phosphate (NADPH)

Ninhydrin reagent

Nitro-blue tetrazolium (NBT)

O

Olive oil

P

Peanut oil

Petroleum ether

Phenazine methosulphate (PMS)

p-nitrophenol-a-D-glucopyranoside

Potassium dichromate

Potassium ferricyanide

Potassium hydroxide

Potassium persulfate

Pyridine

S

Sodium carbonate

Sodium dodecyl sulfate (sds)

Sodium hydroxide

Sodium nitroprusside

Starch

Streptozotocin

Sulfanilamide

T

Thiobarbituric acid (TBA)

Trichloroacetic acid (TCA)

V

Vanillin

Appendix - B

ABBREVIATION AND SYMBOLS USED

°C	Degree centigrade
$^{\bullet}OH$	Sydroxyl
$^{1}O_2$	Singlet oxygen
abs.	Absorbance
ABTS	2,2′ azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
$ABTS^{\cdot +}$	ABTS radical cation
ACC	1-aminocyclopropane-1-carboxylic acid
ACE	Acetone
ACP	Acyl Carrier Protein
AEE	Ascorbic acid equivalence
ALP	Anti-lipid peroxidation
AQ	Aqueous
ATP	Adenosine triphosphate
B	Boiling
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BP	Blood pressure
CAT	Catalase
Chl	Chloroform
CHLO	Chloroform
DB	Diabetes
DCM	Dichloromethane
DMRT	Duncan's Multiple Range Test
DPPH	2,2-diphenyl-1-pyicrichydrazyl
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
ET	Electron transfer
EV	Extractive value
F	Frying
F	Fraction
Fe^{2+}	Ferrous ions
FRAP	Ferric reducing antioxidant power
FWT	Fresh weight tissue
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Gastric
GSH	Glutathione
GSSG	Glutathione disulphide
h	Hour
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HDL	High-density lipoproteins
HDL-c	High-density lipoproteins cholesterol

HNO_2	Nitrous acid	$NO^{\bullet}$	Nitric oxide
HOCl	Hypochlorous acid	$NO_2^{\bullet}$	Nitrogen dioxide
HPTLC	High performance thin layer chromatography	$O^{2\bullet-}$	Superoxide
		O_3	Ozone
HR-LCMS	High resolution liquid chromatography/mass	ODP	Ortho dihydric phenol content
IC50	50% Inhibition concentration	OH	Hydroxyl radical
LD_{50}	Lethal concentration	$ONOO^-$	Peroxynitrite
LDL	Low-density lipoproteins	ORAC	Oxygen radical absorbance capacity
LDL-c	Low-density lipoproteins cholesterol		
$LOO^{\bullet}$	Lipid peroxyl	PAL	Phenylalanine Ammonia Lyase
LOOH	Lipid peroxide	PC	Principal component
LPA	Lysophosphatidic acid	PCA	principal component analysis
LPO	Lipid peroxidation	PET ETHER	Petroleum ether
M	Molar	PG	Propylgallate
MC	Metal chelating	PMS	Phenazine methosulphate
METH	Methanol	pNPG	p-nitrophenylglucopyranoside
mg	Milligram		
min	Minute	RNS	Reactive nitrogen species
ml	Millilitre	$RO^{\bullet}$	Alkoxyl
MVSP	Multivariate Statistical Package	$ROO^{\bullet}$	Peroxyl
		ROS	Reactive oxygen species
N_2O_3	Dinitrogen trioxide	RP	Reducing power
NA	Not detected/ not applicable	rpm	Revolutions per minute
NADPH	Nicotinamide-adanine dinucleotide phosphate	SD	Sun drying
		SDS	Sodium dodecyl sulfate
NBT	Nitro-blue tetrazolium	SEE	Standard error of estimates
nm	Nano meter		

SO	Superoxide
SOD	Superoxide dismutase
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thioburbituric acid reactive substances
TBHQ	Tertiary butyl hydroquinone
tBHQ	*tert*-Butylhydroquinone
TCA	Trichloroacetic acid
TCC	Total carotene content
TFC	Total flavonoid content
TLC	Total lycopene content
TPC	Total phenol content
TRAP	Total radical antioxidant trapping
VIT	Vitamin
α- AMY	α- Amylase
α- GLU	α- Glucosidase
µl	Microliter
µM	Micro molecule

Appendix – C

LIST OF PUBLICATIONS

1. **Mitali Ghosal,** Prashan Kumar Chhetri, Manas Kanti Ghosh and Palash Mandal. 2013. Changes in antioxidant activity of *Cyphomandra betacea* (Cav.) Sendtn. fruits during maturation and senescence. *International Journal of Food Properties*. 16:1552–1564.

2. **Mitali Ghosal** and Palash Mandal. 2013. *In-vitro* antidiabetic and antioxidant activity of *Calamus erectus* Roxb. Fruit: a wild plant of Darjeeling Himalaya. *International Journal of Pharma and Bio Sciences.* 4(2): 671-684**.**

3. **Mitali Ghosal** and Palash Mandal. 2013. Evaluation of antidiabetic activity of *Calamus erectus* in streptozotocin induced diabetic rats. *Asian Journal of Plant Science and Research.* 3(1): 47-53.

4. **Mitali Ghosal** and Palash Mandal. 2012. Phytochemical screening and antioxidant activities of two selected 'bihi' fruits used as vegetables in Darjeeling Himalaya. *International Journal of Pharmacy and Pharmaceutical Sciences*. 4(2): 567-574.

5. Mandal Palash and **Ghosal Mitali**. 2012. Antioxidant activities of different parts of tree tomato fruit. *International Journal of Pharmaceutical Sciences Review and Research.* 13(2): 39-47.

6. **Mitali Ghosal** and Palash Mandal. 2010. *In Vitro* Antioxidant Activity of Edible Timbur Fruits of Darjeeling Himalaya. *NBU Journal of Plant Sciences*. 2: 47-52.

Appendix -D

International Journal of Food Properties, 16:1552–1564, 2013

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CHANGES IN ANTIOXIDANT ACTIVITY OF *CYPHOMANDRA BETACEA* (CAV.) SENDTN. FRUITS DURING MATURATION AND SENESCENCE

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Antioxidants are extremely important substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress. Antioxidants are derived from dietary sources, such as fruits, vegetables, and beverages. In this study, the antioxidant activity of different maturity stages of two varieties of* Cyphomandra betacea *fruits of Darjeeling was evaluated* in vitro*. The radical scavenging properties on 2,2-diphenyl-1-picrylhydrazyl, superoxide anion, hydroxyl radical, lipid peroxidation, nitric oxide, and reducing power as well as the flavonoids, phenolics, lycopene, and total carotene contents of methanolic extracts of the fruits were determined. All fruit extracts, mainly the mature red fruit of purple-red variety exhibited strong scavenging activity towards all radicals tested due to the presence of relatively high total phenol, flavonoids, and lycopene as well as total carotene contents. The findings suggest that purple red variety of* C. betacea *fruit is endowed with antioxidant phytochemicals, which could provide protection against oxidative stress induced diseases.

Keywords: **Cyphomandra betacea***, Antioxidant, DPPH, Superoxide, Phytochemicals.*

INTRODUCTION

Oxidative stress is the major causal factor for the induction of many chronic and degenerative diseases, including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, malignancy, immunosuppression, neurodegenerative diseases, and others.[1–6] Free radicals, which are the major sources of oxidative stress, can only be removed by antioxidants of natural and synthetic origin.[7–9] In recent times, natural antioxidants have attracted considerable interest among nutritionists, food manufacturers, and consumers, due to their presumed safety and potential therapeutic values. Plants contain a wide variety of free radical scavenging molecules, such as phenols, flavonoids, vitamins, and terpenoids, that are rich in antioxidant activity.[10] Natural phytochemicals with potential antioxidant values are found in crops, beverages, oilseeds, beans, fruits, and vegetables.[11] Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chili pepper, ginger, and several Chinese medicinal plants.[12–14]

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Research Article **Pharmacology**

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IN-VITRO ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF *CALAMUS ERECTUS* ROXB. FRUIT: A WILD PLANT OF DARJEELING HIMALAYA

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ABSTRACT

Calamus erectus Roxb. (CE) fruit under the family Arecaceae is traditionally used by the local people of Darjeeling Himalaya as an antidiabetic agent but no scientific reports are available yet. Hence this study was designed to determine the *in vitro* antioxidant and antidiabetic activities of two different parts viz. mesocarp and endocarp of *Calamus erectus* fruit. The extracts were screened for their possible *in vitro* antioxidant potentials by 2,2-diphenyl-1-picrylhydrazyl, reducing power, metal chelating, nitric oxide, superoxide, hydroxyl radical scavenging capacity and anti-lipid peroxidation assays and *in vitro* antidiabetic activity by α-glucosidase and α-amylase inhibition. The DPPH radical scavenging of methanolic extract of endocarp and mesocarp has IC_{50} values of 0.10 and 0.12 mg/ml fresh weight tissue respectively. All antioxidant assays of the extract were enhanced with the increasing amount of the concentration. The methanolic extract of different fruit parts (endocarp and mesocarp) showed concentration dependent α-glucosidase (IC_{50}-1.69 and 2.00 mg/ml) and α-amylase (IC_{50}-2.74 and 3.30 mg/ml) inhibitory activity. Hence α-glucosidase and α-amylase enzyme inhibition may be the possible mechanism for diabetic therapy and considered as a potential herbal drug for the management of type-II diabetes mellitus. The bioactivity of the plant extracts may be due to the presence of high total phenol content as determined through Pearson's correlation and Principal Component Analysis. The *in vitro* studies clearly indicate that methanolic extract of endocarp of this fruit has more antioxidant and antidiabetic capability, also with extraordinary potential for their use in pharmaceuticals.

KEYWORDS: **Free radicals, α-amylase, α-glucosidase, flavonoids, phenol**

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Evaluation of antidiabetic activity of *Calamus erectus* in streptozotocin induced diabetic rats

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ABSTRACT

The present study was designed to evaluate the hypoglycemic, hypolipidemic and antioxidant activity of Calamus erectus (CE) fruit in streptozotocin (STZ) induced diabetic wistar rat. The fruit extracts of 100, 200, 300 and 400 mg/kg body weight (bw) were administrated orally to normal and STZ induced (55 mg/kg bw) diabetic (>200 mg/dl) rats. Glibenclamide (10 mg/kg) were used as a reference drug. Antioxidant effects were assayed in diabetic rats by estimating thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels. Daily oral treatment with 400 mg/kg fruit extract for 14 days resulted in 73.68, 20.46, 36.6 and 43.9% reduction of blood glucose, serum cholesterol, triglycerides and LDL (low-density lipoprotein) respectively whereas HDL (high-density lipoprotein) cholesterol was found to be improved 12.7% when compared with STZ treated rats. GSH, SOD and CAT activity of liver homogenate was improved 33.46, 49.36 and 52.48% respectively while the TBARS decreased 36.18% with same treatment. Decreased levels of TBARS and increase of GSH, SOD and CAT activity indicated a reduction in free radical formation in liver of diabetic rats. The present study demonstrated that CE fruit extract possess good antidiabetic potential and could improve lipid profile and oxidative stress efficiently during diabetic condition.

Key Words: hypolipidemic, hypoglycemic, glibenclamide

INTRODUCTION

The term diabetes mellitus is the most prevalent metabolic disorder characterized with increased blood sugar level and improper primary metabolism [1]. It is the most common disease in the world affecting 25% of population and troubles 150 million people and is set to rise to 300 million by 2025 [2]. Diabetes also gives rise to various secondary problems such as retinopathy, peripheral vascular insufficiencies and neuropathy. These secondary problems take place due to the oxidative stress and DNA damage caused by the generation of free radicals in the cells [3]. Diabetes is still not completely curable by the present antidiabetic agents. Insulin therapy is the only satisfactory approach in diabetes mellitus, even though it has several drawbacks like insulin resistance, anorexia, brain atrophy and fatty liver in chronic treatment [4]. Diabetes mellitus is associated with increased oxidative stress. Free radicals are continuously produced in the body as the result of normal metabolic processes and interaction with environmental stimuli. The level of lipid peroxidation in the cell is controlled by various cellular defense mechanisms consisting of enzymatic and non-enzymatic scavenging systems during oxidative stress mediated propagation of reactive oxygen and nitrogen species (RONS), which is always related with diabetes [5,6]. But disturbances of innate antioxidant defense mechanism in prolonged diabetic condition showed alteration in antioxidant enzyme levels such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and

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Research Article

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITIES OF TWO SELECTED 'BIHI' FRUITS USED AS VEGETABLES IN DARJEELING HIMALAYA

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ABSTRACT

The aim of this study was to determine the antioxidant activity as well as total phenol (TPC) and total flavonoid content (TFC) in two fruits, *Solanum anguivi* and *Solanum incanum* grown in Darjeeling Himalaya and used as vegetables in Nepali recipes. The antioxidant activities were examined by five different methods namely DPPH free radical scavenging activity, reducing power, iron chelation, anti-lipid peroxidation, and nitric oxide scavenging activity. The results showed that considerable amount of TPC and TFC was present in these fruit extracts as well as these vegetables contain a vast array of different phytochemicals in their dry form. *Solanum incannum* showed higher antioxidant activity than *Solanum anguivi.* Significant correlations were obtained between free-radical scavenging capacity and TFC, indicating that flavonol group of metabolites were the chief performers of antioxidant activity. Principle Component Analysis (PCA) indicated that polar metabolites along with hydrophilic radical scavengers contributed to the major variability in the antioxidant activity of the plants. Overall, the present results provided basic data for choosing these fruits with high antioxidant capacity for consumption or for the development of antioxidant based medicines as value-added products.

Keywords: *Solanum anguivi, Solanum incanum,* Antioxidants, Vegetables, DPPH, Oxidative stress.

INTRODUCTION

Free radicals which are atomic or molecular chemical species with unpaired electrons are highly unstable and can react with other molecules by giving out or accepting single electron [1]. Oxidation processes are one of the most important routes for producing free radicals in food, drugs and even living systems. These unstable molecules are capable of causing cellular damage, which leads to cell death and tissue injury [2]. Free radicals are linked with the majority of human diseases like ageing, atherosclerosis, cancer, diabetes, liver cirrhosis, cardiovascular disorders, etc.[3, 4]. The most common reactive oxygen species (ROS) are superoxide anion (O_2·-), hydrogen peroxide (H_2O_2), peroxyl radical (ROO·) highly reactive hydroxyl radical (OH·), nitric oxide (NO) and peroxynitrite anion (ONOO·). Antioxidants cease direct ROS attacks and radical mediated oxidative reactions and are important in the prevention of many diseases and health problems[5]. The antioxidants are not only required by our body to combat ROS but also equally important as food additives either in synthetic or naturally occurring forms[6]. Epidemiological and *in vitro* studies on medicinal plants, fruits and vegetables strongly supported the idea that plant constituents with antioxidants are capable of exerting protective effects against oxidative stress in biological systems[7, 8, 9] as well as can be used to prevent complex diseases like Alzeimer's disease and cancer[10]. Many naturally occurring antioxidants from plant sources have been identified as free radical scavengers or active oxygen-scavengers[11, 12]. Recently, natural antioxidants have attracted considerable interest among nutritionists, food manufacturers and consumers, due to their presumed safety and prospective therapeutic values[13].

Solanum anguivi Herb. Lam. ex Dum and *Solanum incanum* L. are two plants locally known as 'Bihi' and 'Bara Bihi' respectively by the people of Darjeeling hills. These plants are shrub like in habit under the family of Solanaceae and are widely cultivated in Darjeeling hills. Fruits of these plants are popular for vegetables of this area. The roots of *S. anguivi* plant are used for curing diarrhoea and skin ailments[14]. The fruits of *S. incanum* are extensively used for the treatment of cutaneous mycotic infections and other pathological conditions[15] and these fruit extracts have high antifungal and DPPH scavenging activity[16]. Due to presence of glycosidal alkaloids the members of Solanaceae family are recognized with antibiotic activity[15] and most of the *Solanum* plants may scavenge reactive oxygen species[17,18,16]. But the detailed information about phytochemical profile of bioactive fractions obtained from the fruits of these two *Solanum* species are still lacking and addressing the functional aspects of health-promoting components like scavenging and detoxification of specific oxygen-based radicals require better understanding and in-depth investigation of antioxidants present in them for channeling their use as functional foods and as ingredients in pharmaceutical and nutracutical industry.

In this study, we investigated the DPPH· radical scavenging, reducing power, metal chelating, nitric oxide scavenging, superoxide scavenging and lipid-peroxidation assay as well as the phytochemical compositions and polyphenol content of the fruits of *S. anguivi* and *S. incanum*. These multiple methods are recommended to determine antioxidant capability of food materials that reflect their potential defensive functions against various oxidative stress induced diseases.

MATERIALS AND METHODS

Plant materials

The fruits of *S. incanum* L. and *Solanum anguivi* Herb. Lam. ex Dum (Figure 1) were collected form from Sorang Basti, Darjeeling, West Bengal, India. Taxonomic position was authenticated by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The materials were deposited in the 'NBU Herbarium' and recorded against the accession no 9542 and 9574 dated 04-03-09.

Animal material

Goat liver, used for anti-lipid peroxidation assay, were collected from slaughter house immediately after slay and the experiment was conducted within one hour after collection.

Chemicals

Methanol (M), 2.2-diphenyl-1-picryl hydrazyl (DPPH), nitroblue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), phenazine methosulphate (PMS), sulfanilamide, glacial acetic acid, napthylethylenediamine dihydrochloride, ferrozine, ferrous chloride, trichloroacetic acid (TCA), thiobarbituric acid (TBA), $FeSO_4.7H_2O$, KOH, KH_2PO_4, ethylene-diamine tetra acetic acid (EDTA), ascorbic acid, vitamin-E, 2-deoxyribose, potassium ferricyanide, ferric chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), sodium nitroprusside, gallic acid, Folin-Ciocaltue reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), acetone, petroleum ether, Sodium sulphate, and Sodium hydroxide (NaOH) were either purchased from Sigma Chemicals (USA), or of Merck analytical grade.

Research Article

ANTIOXIDANT ACTIVITIES OF DIFFERENT PARTS OF TREE TOMATO FRUIT

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ABSTRACT

Cyphomandra betacea (Cav.) Sendtn. (Solanaceae) is commonly known as 'Tree Tomato'. It is a common shrub, which widely grows in the Darjeeling Himalaya. The fruits are good sources of provitamin A, vitamin C, B_6, E and iron. This study was conducted to determine the antioxidant activities of methanol extracts from five different parts (placenta, endocarp, epicarp, seed and mesocarp) of this fruit. Antioxidant activities were evaluated by DPPH (2,2-diphenyl-1-picryllhydrazyl) radical, nitric oxide (NO), hydroxyl radical (OH), superoxide scavenging activity, metal chelating, reducing power (RP) and anti-lipid peroxidation. Placenta and endocarp showed significantly higher superoxide scavenging and metal chelating activity whereas inhibition of lipid peroxidation was much better in mesocarp. Therefore, different parts of *C. betacea* fruit mainly placenta, endocarp and epicarp are potential functional food ingredient and their incorporation into human diets might provide protection and help to reduce oxidative damage in different vital organs.

Keywords: *Cyphomandra betacea*, Antioxidant, Free-Radical, Phytochemicals.

INTRODUCTION

Cyphomandra betacea (Cav.) Sendtn. is domesticated plant in the hills of Darjeeling and commonly known as 'Tree Tomato'. The fruits are eaten fresh, cooked in stews and sauces, prepared as chutney, pickles as well as directly consumed with salads. *C. betacea* mature fruit juice is traditionally used in Ecuador for the treatment of tonsillitis, high cholesterol and stomach pain.[1] This fruit, naturally acidic, is recommended for its nutritional qualities, as a good source of provitamin A, vitamin E, C, B_6 and iron.[2-5] The fruit has significant amount of bioactive phytochemicals like carotenoids, anthocyanins and phenolic compounds.[6] Fruits and vegetables may protect against numerous chronic diseases including carcinoma, cerebro- and cardiovascular stroke, ocular and neurological disorders[7,8] due the presence of such type of antioxidant constituents, like vitamin C, R-tocopherol, carotenoids, glutathione, flavonoids, and phenolic acids.[9] The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from chronic diseases has also raised interest among scientists and food manufacturers as consumers move towards functional foods with specific health effects.[10] A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. Oxidative process is one of the most important routes for producing free radicals in foods, drugs and even in living systems.[11] The most effective path to eliminate and diminish the action of free radicals which cause the oxidative stress is antioxidative defense mechanisms. It has been established that oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, *diabetes mellitus*, cancer, immunosuppression, neurodegenerative diseases and others.[12] Several widely consumed vegetables are rich in various phenolic compounds and vitamins which are the main source of natural antioxidant. Among vegetables, tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), chilli pepper (*Capsicum annuum* L.), and potato (*Solanum tuberosum* L.), which belong to the solanaceae family, are important for their richness with healthy components, due to which they are also widely consumed.[13] But *C. betacea* is a plant which belongs to this family and its use is mainly restricted only in the hilly region as vegetables.

The aim of the present study was to investigate the different parts of the fruits of *C. betacea* from Darjeeling hills as a potential functional food and antioxidant source, as an alternative to synthetic compounds. In this work we have determined the radical scavenging efficacy of different parts of this fruit as well as the phytonutrients like total carotene, lycopene, anthocyanin, total phenolics and flavonol.

MATERIALS AND METHODS

Plant materials

Ripe and fresh *C. betacea* fruits were collected from Sorang Basti, Darjeeling, West Bengal, India. Different parts of this fruits like placenta, endocarp, epicarp, seed and mesocarp were surgically separated. The plant material was authenticated from Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material was deposited in the 'NBU Herbarium' and recorded against the accession no 9579 dated 04-03-09.

Animal material

Goat liver, which was used for anti-lipid peroxidation assay, was collected from slaughter house immediately

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In vitro antioxidant activity of two edible Timbur fruits of Darjeeling Himalaya

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Abstract
Free radicals are implicated for many chronic, painful and near-fatal diseases including Diabetes mellitus, arthritis, cancer, apoptosis, neurodegenerative disorders etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance and current research is now directed towards finding naturally occurring antioxidants of plant origin. Edible Timburs namely *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are traditionally used as spices in different Nepali recipes. In the present study, the fruits of these plants were extracted with aqueous methanol (1:4) to examine the *in vitro* antioxidant property, phenol content and phytochemical constituents. The scavenging activities on DPPH free radicals, superoxide anions and per-oxidized lipid molecules were determined as well as the flavonoid and phenolic constituents of the extracts. The extracts exhibited significant scavenging activity towards DPPH free radicals and high anti-lipid peroxidation values due to the presence of relatively high total phenol contents. Also, these spices contain a vast array of different phytochemicals in their dry form. These results suggest that both *Zanthoxylum acanthopodium* and *Litsea cubeba* fruits are endowed with antioxidant phytochemicals and could serve as basal ingredients for nutraceutical formulations.

Keywords: Antioxidant, DPPH, *Zanthoxylum acanthopodium*, *Litsea cubeba*

An extensive diversity of medicinal plants and edible fruits is observed in Darjeeling hills. Two plants of these hills viz. *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are locally known as 'Boke Timbur' and 'Sil Timbur' respectively. These plants are wild, tree like in habit under the families of Rutaceae and Lauraceae and are widely spread in the forest of Darjeeling hills. Fruits of these plants ('Timburs') have been used traditionally for healing diarrhea, vomiting, and gastric ulcer and as warm killer. These fruits are also used as spices in different Nepali recipes. Several studies have been conducted to determine the antioxidant properties of many plants, especially those used in traditional medicine (Jang *et al.* 2007, Surveswaran *et al.* 2007). Currently, there is a great interest in the field of antioxidant substances mainly due to the findings concerned with the effects of free radicals in the organism. Free radicals have significant role in creation of several metabolic, mutagenic and age-related disorders like diabetes, cirrhosis, cancer and cardiovascular diseases (Hertong and Feskns 1993). Reactive oxygen species (ROS), which include free radicals such as superoxide anion (SO^{2-}), hydroxyl radicals (OH) and non-free-radical species like H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen (Gulcin *et al.* 2002, Halliwell and Gutteridge 1999, Yildirim *et al.* 2000). It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation risks and therefore, help to prevent cancer or heart diseases. Recently, there is a growing attention on the discovery of natural antioxidants because epidemiological and clinical evidences suggest that consumption of vegetables and fruits reduce the risk of developing chronic diseases like cancer and in this respect phytochemicals are generally safer than synthetic chemicals (Dastmalchi *et al.*, 2007). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Plants contain a wide variety of free radical scavenging molecules like phenols, flavonoids, vitamins, terpenoids etc. that are rich in antioxidant activity (Cai *et al.*, 2003). However, the use of natural antioxidants are limited due to lack of knowledge about their molecular composition and dynamics, amount of active ingredients in the source material and the availability of relevant toxicity data (Shahidi *et al.* 1994). Natural antioxidants tend to be safer and they also posses antiviral, anti-inflammatory, anti-tumour and hepatoprotective properties (Lim and Murtijaya, 2007). Information related to antioxidant activity and phenolic compounds on traditional Darjeeling medicinal and underexplored edible plants is scarce. Literature survey revealed no relevant phyto-pharmacological records on *Zanthoxylum acanthopodium* and *Litsea cubeba*, and the fruits of these plants have not yet been screened for their antioxidant activity. This present study, therefore investigated the phytochemical compositions and polyphenol content, the *in vitro* antioxidant, lipid peroxidation and superoxide scavenging potential of this plant.

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