Purification, Chemical Characterization and Biochemical Evaluation of Betanin (A Betalain Pigment) from the fruits of *Opuntia ficus indica*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

SREEKANTH DEVALRAJU

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500046, INDIA

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Dedicated
to
Goddess Gayathri
&
Beloved Parents

University of Hyderabad

(A Central University established in 1974 by act of parliament)

HYDERABAD – 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Purification, Chemical Characterization and Biochemical Evaluation of Betanin (A Betalain Pigment) from the fruits of *Opuntia ficus indica*"** has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

Prof. P. Reddanna
(Research Supervisor)

Sreekanth Devalraju
(Research Scholar)

University of Hyderabad

(Central University established in 1974 by act of parliament)

HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that **Mr. Sreekanth Devalraju** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis entitled **"Purification, Chemical Characterization and Biochemical Evaluation of Betanin (A Betalain Pigment) from the fruits of *Opuntia ficus indica*"** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna _____
Supervisor

Head _____
Department of Animal Sciences

Dean _____
School of Life Sciences

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Sreekanth Devalraju ..✍

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ABBREVIATIONS

µM	**:**	**micro molar**
^{0}C	**:**	**degree centigrade/ degree celsius**
AA	**:**	**arachidonic acid**
ATP	**:**	**adenosine triphosphate**
BCIP	**:**	**5-bromo-4-chloro-3-indolyl phosphate**
COX	**:**	**cyclooxygenase**
cpm	**:**	**counts per minute**
DNA	**:**	**deoxy ribonucleic acid**
EDTA	**:**	**ethylene diamine tetra acetic acid**
FACS	**:**	**fluorescence activated cell sorter**
FBS	**:**	**fetal bovine serum**
g	**:**	**gram**
h	**:**	**hour(s)**
kDa	**:**	**kilodalton**
l	**:**	**litre**
mg	**:**	**milligram**
min	**:**	**minutes**
ml	**:**	**milliliter**
dl	**:**	**deciliter**
mM	**:**	**millimolar**
MTT	**:**	**3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide**
NBT	**:**	**nitroblue tetrazolium**
nm	**:**	**nanometers**
NSAIDs	**:**	**non-steroidal anti-inflammatory drugs**
N-terminal	**:**	**amino terminal**
OD	**:**	**optical density**
PAGE	**:**	**polyacrylamide gel electrophoresis**
PARP	**:**	**poly(ADP-ribose) polymerase**
PBS	**:**	**phosphate buffered saline**
pmole	**:**	**picomole**

rpm	**:**	**revolutions per minute**
SDS	**:**	**sodium dodecyl sulfate**
TEMED	**:**	**N,N,N',N'-tetramethylene diamine**
Tris	**:**	**tris-(Hydroxymethyl) aminoethane**
UV	**:**	**ultraviolet**
H_2O_2	**:**	**hydrogen peroxide**
$HO^{\cdot}$	**:**	**hydroxyl radical**
$HO_2^{\cdot}$	**:**	**hydroperoxyl, but is obsolete**
$NO^{\cdot}$	**:**	**nitric oxide, but is obsolete**
$O_2^{\cdot -}$	**:**	**superoxide anion radical**
$ONOO^-$	**:**	**peroxynitrite**
Asc; $AscH^-$; $Asc^{\cdot -}$	**:**	**ascorbate, general; ascorbate monoanion; ascorbate radical**
CAT	**:**	**Catalase**
DMPO	**:**	**5,5-dimethyl-pyrroline-1-oxide, a spin trap**
EPR	**:**	**electron paramagnetic resonance**
G	**:**	**gauss**
GPx	**:**	**glutathione peroxidase**
GR	**:**	**glutathione disulfide reductase ; often referred to as glutathione reductase (a misnomer)**
GSH	**:**	**glutathione, not reduced glutathione (a misnomer)**
GSSG	**:**	**glutathione disulfide; not oxidized glutathione (a misnomer)**
GST	**:**	**glutathione S-transferase**
H^+	**:**	**proton, hydron**
LDL	**:**	**low density lipoprotein**
MDA	**:**	**malondialdehyde**
$^1O_2$	**:**	**singlet oxygen**
$RO^{\cdot}$	**:**	**alkoxyl radical; not alkoxy**
$ROO^{\cdot}$	**:**	**alkyl dioxygen($^{\cdot}$), alkyldioxyl, alkylperoxyl radical; not peroxy**
ROS	**:**	**reactive oxygen species**
SOD	**:**	**superoxide dismutase**
CuZnSOD	**:**	**copper, zinc-superoxide dismutase (SOD-1)**

MnSOD	**:**	**manganese-superoxide dismutase (SOD-2)**
FeSOD	**:**	**iron-superoxide dismutase**
ECSOD	**:**	**extracellular superoxide dismutase (SOD-3)**
TBARS	**:**	**thiobarbituric acid reactive substances**

Introduction

1.1. Introduction to Ayurvedic system of medicine

Ayurvedic system of medicine is one of the most ancient traditional systems of medicine practiced widely in India, Srilanka and other countries. It has sound philosophical and experimental basis in treatment of diseases (Dahanukar et al, 2000). Atharvaveda (around 1200 BC), Charakasamhita and Sushrut samhita (Bhagavan Dash and Sharama, 2001) (1000-500 BC) are the main classics that provide detailed classification of over 700 herbs. Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on Ayurvedic medicinal plants in order to establish the scientific basis of their therapeutic potentials (Patwardhan, 2003).

1.2. Herbal medicine: research, development and markets

30% of the worldwide sales of drugs is based on natural products. Investigations on plants used in traditional and modern medicine in India and China serve as a source of inspiration and as models for the synthesis of new drugs from natural sources. They provide considerable market around the world for such compounds. For example, several million dollars worth of vincristine and vinblastine – the periwinkle derivatives used to treat childhood leukemia and Hodgkin's disease are marketed today. About 80 per cent of the population today relies on indigenous medicinal plants and the drugs. It is estimated that the global market of traditional medicine market is growing at the rate of 7-15 per cent annually. The US National Cancer Institute regularly year marks a large appropriation of their budget to screen about 50,000 natural substances for activity against cancer cell lines and the AIDS virus. India, China, Germany, and Japan, are the countries, which are exploring their traditional knowledge on herbal medicine as

well as the new plant sources for development of new drugs. It is estimated that India exports about Rs. 550 crores worth of herbal drugs. With rich and diverse botanical resources in our country, this is not an impressive export performance considering the worldwide herbal market which is of worth US $ 60 billions.

1.3. Natural products in drug discovery

Most of the potent drugs in use today are extracted from plants and several synthetic drugs are made from starting molecules extracted from plants. Up to 25% of all prescriptions in European and American countries include plant products or plant derivatives (Jaroszewski, 1984). In developing countries medicinal plants continue to be the main source of medication. In China alone 7295 plant species are utilized as medicinal agents. The World Health Organization estimated that for some 3.4 billion people in the developing world, plants represent the primary source of medicine. It is reported that at least 198 compounds derived from 90 plant species can be considered as important sources of drugs. Among the drugs that are currently in use in different countries, 77 % of them are being derived from plants used in traditional medicine (Fransworth et al., 1985, 1977). The importance of natural products is also evidenced by the fact that in 1991 nearly half of the best selling drugs were either natural products or their derivatives (O'Niell and lewis 1993).

Two approaches have been recommended for the exploitation of medicinal plants for drugs. One is to extract the active ingredients from the original traditional medicinal sources and identify their chemical nature, establish its detailed pharmacology and develop it into a drug for clinical trials and distribution. The other is to evaluate the herbal remedy as it is used, by traditional healer without purification. If the crude

product is found to be effective, further studies should focus on toxicity, dosage standardization, clinical trials and finally the inclusion of that preparation in modern Pharmacopoeia. Roughly four selection procedures can be identified for developing drugs from natural sources.

a. Selection based on traditional medical usage

If a given plant has been taken as medicine for generations, it is reasonable to assume that its constituents are not toxic at least in the form in which the preparation is used in traditional medicine.

b. Random selection

In this kind of selection the plants are randomly selected and the extracts are evaluated against one or more *in vitro* activity, and also for toxicity. This method is little tedious and expensive for commercial pharmaceutical organizations. Using this approach, Fansforth and his group (Fransworth et al., 1985, 1977) identified many compounds with anti-inflammatory activity from herbal origin.

c. Selection based on their chemical constituents

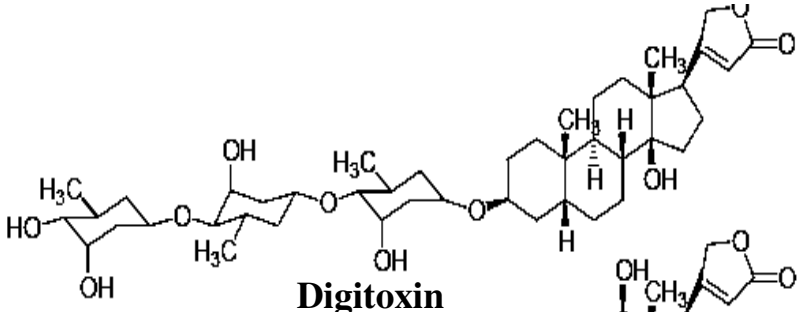
For this approach plants are screened for the presence of a particular type of chemical compound e.g. Alkaloid. Any plant showing the presence of alkaloids is then extracted and fractionated for active components with pharmacological properties. Some multinational pharmaceutical companies base their search for natural products on this approach.

d. Selection based on the combination criteria

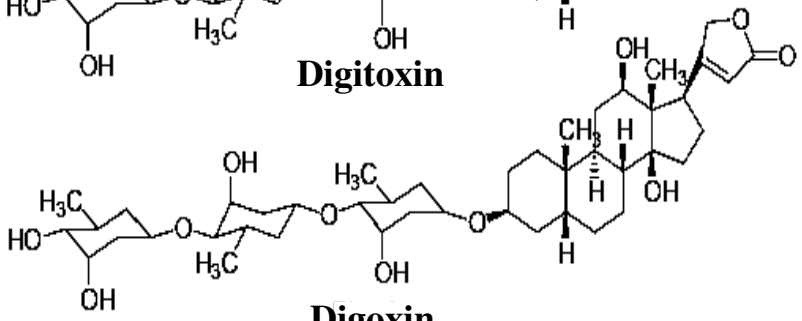
In this method a number of plants reputed to be effective in traditional or folkloric medicine are used to extract the known type of chemical constituent

(eg. Alkaloids) and subject the extracts to a variety of pharmacological screenings such as anti-inflammatory, antioxidant and antitumor etc.

It is possible nowadays to predict the most likely chemical constituents of different plant species from computer databases. Natural products alert or NAPRALERT is one such database (Fansworth, 1994). The compounds responsible for bioactivity of the herb are secondary metabolites. Herbs may contain saponins, resins, oleoresins, lactones and volatile oils. Complete phytochemical investigations of most of the medicinally important herbs have not been carried out so far. This would be beneficial in standardization and dose determination of herbal drugs, apart from identifying the active principles. Figure-1 illustrates some of the natural products that are isolated from the plants and presently utilized as potential drugs.



Digitoxin



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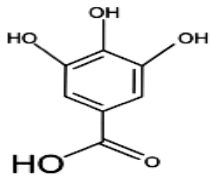
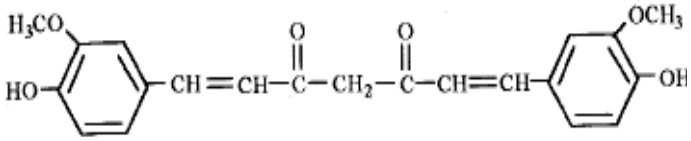
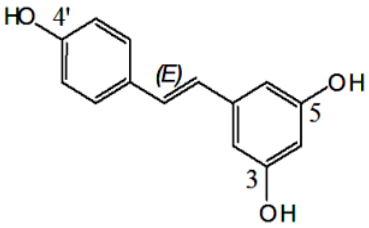


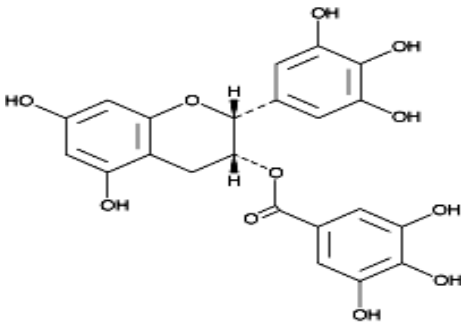
Gallic acid



Curcumin



Resveratrol



Epigallocatechin gallate

Fig. 1: Some important antioxidants from natural origin

1.4. Reactive oxygen species and oxidative stress

ROS are highly reactive oxidizing agents belonging to the class of free radicals. A free radical is any compound (not necessarily derived from oxygen) that contains one or more unpaired electrons generated in the aerobic act of life. The most common ROS that have potential implications in human disease include superoxide anion ($O_2^{\cdot -}$) hydrogen peroxide (H_2O_2), peroxyl (ROO-) radicals, and the very reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radical nitric oxide (NO.) and peroxynitrite anion (ONOO-) also appear to play a significant role in the pathogenesis of disease. The ultimate effects of NO depend upon its concentration and interactions with hydrogen peroxide. Peroxynitrite (oxo peroxonitrate) anion may be formed *in vivo* from superoxide and nitric oxide and actively reacts with glutathione, cysteine, deoxyribose, and other thiols/thioethers (Koppenol et al., 1992). This can form a strongly nitrating species in the presence of metal ions or complexes. In health, balance between production of ROS/RNS and antioxidant defenses lies slightly in favour of ROS/ RNS production. Oxidative stress occurs when there is an imbalance between free radical reactions and scavenging capacity of antioxidative defense mechanism of the organism (Sies, 1991). Oxidative stress is a severe disruption of balance in favour of ROS/RNS (Fig. 2). In principle, oxidative stress can result from increased production of ROS/RNS, excessive activation of phagocytic cells in chronic inflammatory diseases, diminished antioxidants e.g. mutations effecting antioxidant defense systems and depletions of dietary antioxidants and micronutrients.

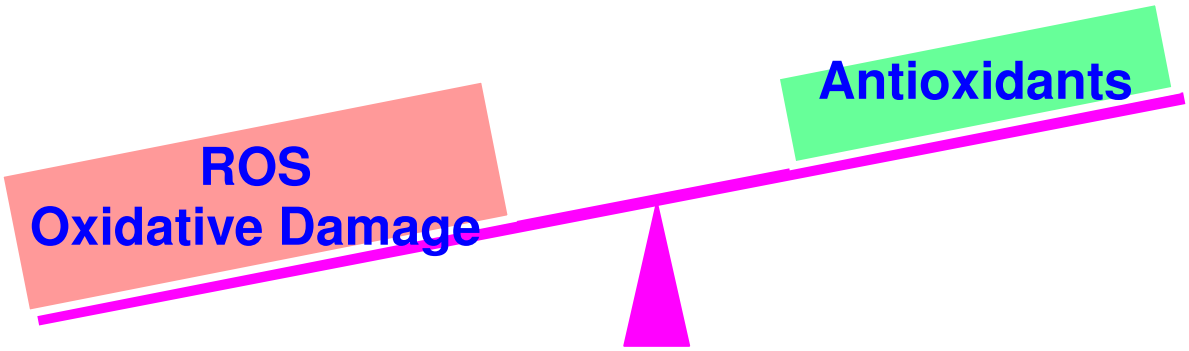


Fig. 2: Disruption of physiological balance in oxidative stress

1.4.1. Sources of ROS generation

In the aerobic act of life, both endogenous and exogenous sources are responsible for the generation of ROS.

1.4.1.1. Endogenous sources of free radicals

The endogenous systems that generate free radicals include: (i) cellular respiration in mitochondria involving the reduction of molecular oxygen (O_2) to water in the electron transport chain; (ii) functioning of the microsomal electron transport involving cytochrome P-450 during drug metabolism generating superoxide anion (O2**.** -). (iii) free metal iron, haemoproteins iron and copper salts promote generation of oxidizing radicals like OH. - from peroxides, by Fenton reaction;(iv) under certain conditions peroxisomes, organelles responsible for degradation of fatty acids and other molecules, produce H_2O_2 as a by-product and this ROS along with $O_2^{\cdot-}$ produce more potent $OH^{\cdot}$; (v) auto oxidation of natural compounds such as catacholamines, coenzyme Q10 and epinephrine generates $O_2^{\cdot-}$ (In blood vessels catacholamine autooxidation is an important source of ROS generation which is released under certain pathophysiological conditions) and (vi) oxidizing enzymes that produce ROS are diamine oxidase,

tryptophan dioxygenase, xanthine oxidase, lipoxygenase, cyclooxygenase, nitric oxide synthase (under low arginine conditions) guanylyl cyclase, glucose oxidase, myeloperoxidase, lacto peroxidase, chloro peroxidase etc. (Sies, 1996; Halliwell and Gutteridge, 1997).

1.4.1.2. Exogenous sources of free radicals

Other sources of free radicals include redox cycling of xenobiotics, exposure to physicochemical agents like ionizing radiations such as X-rays and γ-rays besides visible light or UV in the presence of oxygen and an endogenous compound or a drug that acts as photosensitizer. This can be in the form of cytochromes, porphyrins, riboflavin, tetracycline, phenothiazines, chemical toxicants or air pollutants. Most of the damage is induced by ionizing radiations in biological systems is indirect and mediated by products of radiolysis of water and their rearrangement reactions. These include hydrogen radical ($^{\cdot}H$), $^{\cdot}OH$, Hydrated electron (e_{aq}), H_2O_2, peroxyl radical ($ROO^{\cdot}$), $O_2^{\cdot -}$, Singlet oxygen ($^1O_2$) etc., (Von Sonntag, 1987; Devasagayam and Kesavan, 1996; Sarma et al., 1996).

1.4.2. Oxidative stress as a frequent complication in human disease

"Oxidative stress" is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as reactive oxygen species (Sikka et al., 1995). Reactive oxygen species (ROS) have been implicated in over a hundred of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, toxin exposure, physical injury, infection, and acquired immunodeficiency syndrome (Joyce, 1987) (Fig. 3).

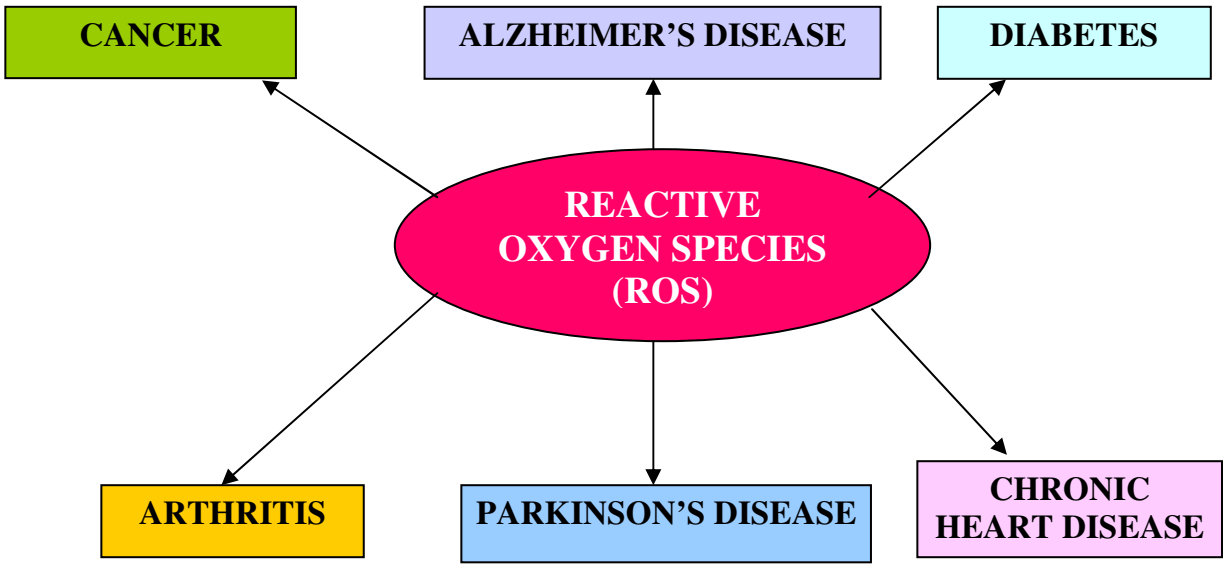


Fig. 3: Pathology of ROS

1.4.2.1. Oxidative stress in cancer

Free radicals are involved in the development of cancer (Cross et al., 1987). They attack many cellular targets including membranes, proteins and nucleic acids (Cerruti et al., 1994) and cause structural damage to the cellular DNA. These structural changes manifest as point mutations and chromosomal alterations in cancer- related genes (Cerruti et al., 1994). Consequently aged people predispose to the development of cancer. Cellular systems, however, are equipped with antioxidant defenses. Certain antioxidant supplements like Vitamin C and E can prevent much of the oxidative damage to DNA and thus reduce the ability of the oxidants to induce cancer (Ames et al., 1993).

1.4.2.2. Cardiovascular disease

Elevated levels of ROS coupled with low plasma levels of antioxidants were shown to be involved in the pathology of cardiovascular disease. Experimental evidences also confirm the oxidation of LDL by ROS. These oxidatively modified LDLs are taken up by macrophages to form foam cells, leading to the formation of plaques and hardening/ narrowing of the blood vessels, which impairs blood flow and deprives the heart of oxygen and nutrients (Cross et al., 1987; Ames et al., 1993). Reperfusion injury results when there is restoration of blood and oxygen supply to the heart after a period of ischemia. Antioxidants in the reperfusion medium, therefore, are known to prevent the tissue damage of this kind (Cross et al., 1987).

1.4.2.3. Oxidative stress and inflammatory diseases

Arthritis and inflammatory diseases of large intestine, such as Ulcerative colitis and Crohn's disease, are conditions in which oxidative damage is very much implicated (Cross et al., 1987; Ruan et al., 1997). One of the mechanisms responsible for the oxygen-mediated injury is tissue protein oxidation, otherwise referred to as protein carbonylation. It has been proposed that inflammatory bowel disease (IBD) may arise from the oxidation of proteins in the intestinal mucosa cells which there by disrupt the critical enzyme systems that are vital for the mucosal homeostasis or ion transport, both of which are impaired in IBD (Otamiri and Sjodhal, 1991).

1.4.2.4. Diabetes

Diabetes is a multi-systematic disease caused by a defect in glucose metabolism. Abnormally high blood sugar levels are a major clinical feature of the disease which is usually followed by accelerated onset and progression of atherosclerosis and other

diseases. In type-II diabetes, oxidative damage has been implicated in both the development of the disease as well as its many complications. Oxidants inhibit glucose metabolism in the glycolytic pathway and at the level of oxidative phosphorylation, there by causing a sugar overload in the blood. In hyperglycemia, increased blood sugar levels cause auto-oxidation of glucose and glycation of proteins, which have been implicated in the development of diabetic complications. Vitamin C & E and alpha lipoic acid have been found to be beneficial in these subjects (Paolisso and Giugliano, 1996).

1.4.2.5. Chemotherapy-Associated oxidative stress

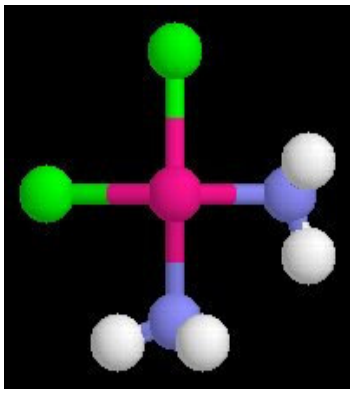
The drugs of many classes of antineoplastic agents are also known to generate high level of oxidative stress in biological systems (Conklin, 2000). These classes of drugs include the anthracyclines, most alkylating agents, platinum-coordination complexes and camptothecins. For these drugs, the hepatic microsomal monooxygenase system is a primary site where ROS are generated, although other enzymatic (e.g., xanthine oxidase) and nonenzymatic (Fenton and Haber- Weiss reactions) mechanisms also play a role. The electron transport system of cardiac mitochondria is another site where significant levels of ROS are generated by anthracyclines (Gille and Nohl, 1997). Although some classes of antineoplastic agents generate high levels of oxidative stress, others, including the taxanes, vinca alkaloids, antifolates, and nucleoside and nucleotide analogues, generate only low levels. Nevertheless, all drugs generate free radicals as they induce cytotoxicity in cancer cells.

1.4.2.6. Cisplatin – induced nephrotoxicity is mediated by oxidative stress

Cisplatin (*cis* diammine dichloro platinum (II)) (Fig. 4) is an anticancer drug extensively used against a variety of cancers. Cisplatin chemotherapy is found to manifest nephrotoxicity that is characterized by decreased levels of antioxidant enzymes and increased lipid peroxidation. The usage of this potent anticancer agent is limited due to the adverse side effects that include cumulative nephrotoxicity (Safinstein et al., 1986), gastrointestinal toxicity and myelosuppression (Blakely et al., 1994; Waters et al., 1991). Accumulating evidences demonstrate that cisplatin exerts its nephrotoxicity and ototoxicity through oxidative stress generating reactive oxygen species (ROS) and interfering with the antioxidant defense mechanisms of these organs (Appenroth et al., 1993; Gabaizadeh et al., 1997; Ravi et al., 1995). Several antioxidants and oxygen radical scavengers have been reported to be effective in protection against these injuries (Rao and Rao, 1999). Antioxidants such as vitamim E, vitamin C (Appenroth et al., 1997), Selenium (Antunes et al., 2000), edravone (Sueishi et al., 2002), amifostine (Orditura et al., 1999), and Spirulina (Iyyapu et al., 2006), are reported to decrease the cisplatin-induced nephrotoxicity.

1.5. Antioxidants to circumvent oxidative stress

ROS are continuously generated in physiological conditions and are effectively eliminated by the intra cellular and extra cellular antioxidant defenses (Halliwell et al., 1992). Uncontrolled production of ROS often leads to damage of cellular macromolecules (DNA, lipids and proteins).



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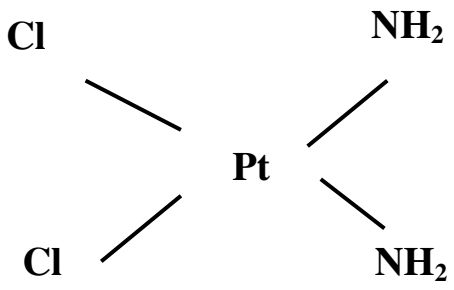


B

Fig. 4: Chemical structure of Cisplatin (cis-diammine dichloro platinum [II]; CDDP)

(A) Ball-and Stick model of cisplatin (B) Chemical structure of cisplatin revealing functional groups involved in toxicity of cisplatin

A number of cellular antioxidant defense mechanisms exist to neutralize the damaging effects of the free radicals. Enzymatic antioxidant systems Cu, Zn and Mn-superoxide dismutases (SOD), catalase, glutathione (GSH) peroxidase (GPx), and GSH reductase function by direct or sequential elimination of ROS, there by terminating their effects. On the other hand, non enzymatic antioxidants like scavenging molecules that are endogenously produced (GSH, ubiquinol, uric acid) or those derived from the diet (Vitamin C and E, carotenoids, alpha lipoic acid, Selenium etc) functioning as scavengers. These molecules donate electrons, and become free radicals that can either initiate chain reactions or conversely are regenerated.

1.6. Natural antioxidants and their therapeutic value

In view of deleterious side effects from synthetic anti-oxidant supplements on human health, the present day's focus is on anti-oxidants from natural sources (Dapkevicius et al., 1998; Frankel et al., 1995; Dimascio et al., 1989; Kerry et al., 1997). Fruits and vegetables form the primary source for these antioxidants with

nutraceutical values. Many natural anti-oxidants such as anthocyanidin, quercetin, rutin (Afanaslev et al., 1989; Hollman et al., 1997), kaempferol, catachin (Chisaka et al., 1988), catechol etc. have already been revealed to show therapeutic efficacy in various pathological conditions (Anderson et al., 1998; Bors et al., 1990; Cook and Samman 1996).

1.7. Plant pigments as another important class of natural antioxidants

While all color additives to foods are strictly regulated (Feord, 2003), naturally pigmented foods, and food products enhanced with pigments extracted from plants, are overwhelmingly preferred by consumers, especially in light of adverse publicity surrounding the consumption of some synthetic colorants. Natural pigments not only circumvent the putative health hazards posed by synthetic chemical colorants but also confer substantive and multifaceted health benefits (nutraceutical value) to the diet. The nutraceutical value of natural plant pigments such as carotenoids, anthocyanins, chlorophylls, and betalains is based on their recognized biologically active antioxidant properties. A wide range of bioassays and tests have been forwarded to establish the biological efficacy of natural pigments in human health intervention, including *in vitro* bioassays, *in vivo* (animal trials), epidemiological (population surveys) and more rarely, clinical (human intervention) trials. Pigments as health supplements or ingredients can be extracted from crop plants and byproducts, and several laboratories have explored bioreactor-based production of the same. For example, betalain pigments have been synthesized actively in hairy root cultures (Pavlov et al., 2002) as well as in suspension cultures (Akita et al., 2000) and large batch-type bioreactor cultures (Leathers et al., 1992), under sponsorship of pharmaceutical industries interested in expanding their

product portfolios to naturally occurring plant pigments with biomedicinal value. Four major challenges to health investigations involving natural pigments can be cited. These versatile compounds have unfortunately been extremely difficult to study due to their (1) often large, complex structures, (2) ephemeral nature (tendency to degrade during chemical separation), and (3) the fact that pigments can be highly metabolized after ingestion, making it very difficult to assign the contribution of particular constituents to human health intervention, or assess their absorption and bioavailability. In addition, (4) natural plant pigments often exert biological activity in concert with other co-occurring phytochemicals in a food or in the food matrix. Table-1 summarizes some of the important plant pigments with potential health benefits.

1.8. Antiproliferative effects of natural pigments is mediated by induction of Apoptosis

Many natural pigments are documented to induce apoptosis (programmed cell death) in a variety of cancer cells. Curcumin, a phenolic pigment from spice- turmeric, was found to induce apoptosis in transformed rodent and human cells in cell cultures (Jiang et al., 1996a; Wall and Wani, 1993; Samaha et al., 1997; Kuo et al., 1996; Jiang et al., 1996b). Curcumin was found to suppress two specific proteins normally part of an intracellular pathway that prevents apoptosis when stimulated. Curcumin partially inhibited NF-κB and strongly inhibited its upstream stimulator and another independent inhibitor of apoptosis, IKK (Doris et al., 2005). Proanthocyanidins, another class of polyphenolic plant pigments, when incubated with several cancer cell lines (breast, lung, gastric, and skin) induced apoptosis. The antiproliferative effects of these pigments appear to be mediated by inhibition of intracellular-signal transduction pathways (Jang et al., 1997).

1.9. Betalains - natural pigments that are not very well explored therapeutically

Betalains have not been as extensively studied in terms of health benefits as the other major plant-derived pigments; however, antiviral and antimicrobial properties are well documented (Strack et al., 2003). Commercially, betaxanthin pigments have been introduced as food supplements in order to fortify processed food products with a nutraceutical natural colorant containing essential amino acids (Lee and Min, 1990). The very potent radical scavenging activity of betalains has been demonstrated in a wide range of assays (Escribano et al., 1998; Zakharova and Petrova, 1998; Kanner et al., 2001; Kujala et al., 2001; Pavlov et al., 2002; Wettasinghe et al., 2002; Cai et al., 2003). Recently, betalain enrichment of human low-density lipoproteins effectively increased their resistance to oxidation (Tesoriere et al., 2003). A striking characteristic of betalain pigments is that they can be naturally absorbed by the body; at least 15% of consumers absorb large amounts resulting in temporary red urinary discharge, which indicates minimal transformation during metabolism (Wettasinghe et al., 2002). Beetroot extract, rich in betalain pigments, has demonstrated chemopreventive properties against both lung and skin cancers (Kapadia et al., 1996).

1.10. Betalains as a class of bioactive natural pigments

Betalain pigments are water-soluble vacuolar yellow (betaxanthins) and violet (betacyanins) pigments that replace anthocyanins in most of the plant families of the order caryophyllales. They are also found in some species of the fungal genera *Amanita* and *Hygrocybe.* Betalains are conjugates of the chromophore betalamic acid, which derives from 3-(3,4-Dihydroxy phenyl) alanine (DOPA) by an oxidative 4,5-estradiol ring-opening mechanism. The term betalain was introduced to describe these pigments

as derivatives from betalamic acid. Betalains were erroneously named 'nitrogenous anthocyanins' in the past and are today often referred to as 'chromo-alkaloids' due to the presence of a nitrogen atom in the chromophore.

1.11. Chemistry of Betalains

The main pigment of red beet, betanin (Schudel, 1919) has been used as a model for the determination of the structure and biosynthetic pathway of betalains. Research on betalain chemistry started in the late 1950s with the pioneering work of Dreiding's laboratory at the University of Zurich (Wyler et al., 1963). The common characteristic of all betalains is the presence of betalamic acid chromophore, a dihydropyridine moiety attached via a vinyl group to another nitrogenous group (Miller et al., 1968). Its lemon-yellow colour (λ max 424nm) results from the resonance system induced by the presence of three conjugated double bonds. Betaxanthins are formed by the condensation of an amino acid or an amine with the aldehyde group of betalamic acid, resulting in Schiff base. This structure is responsible for the strong yellow or yellow orange colours of betaxanthins and the maximum absorbance between 470 and 486nm. Betacyanins are also formed from a betalamic acid unit linked to a molecule of cyclo-DOPA (Wyler and Dreiding 1961). The later highly aromatic structure is responsible for the deep violet colour and a strong bathochrome shift of 60-70nm (λ max 534-554nm). The basic betacyanin and betaxanthin structures can be modified in numerous ways; conjugation reactions like glycosylation or acylation are common (Strack et al., 2003).

Betacyanins

Betacyanins are formed from two molecules of tyrosines as precursors. The simplest natural betacyanins are the non-glycosylated betanidin or isobetanidin chromophores obtained by the condensation of cyclo-DOPA with betalamic acid (Wyler et al., 1963). Both molecules differ only by the absolute configuration of their C_{15} chiral center (Wilcox et al., 1965). In beet hypocotyls the two compounds have a concentration ratio of about 4:1.

Other betacyanins are derived from these two isomers by *O*-glycosylation on one of the two free hydroxyl groups of cyclo-DOPA. Glycosylation at position 5 is called Betanin and is the major red beet pigment.

Majority of betacyanins are acylated by ferulic acid, or less frequently by cinnamic acid, on their glycoside part via an ester linkage. Malonylation is also present in betacyanin structure; it is known for stabilizing pigments in flowers, preventing anthocyanins from β-glycosidase attacks (Suziki et al., 2002). A new type of acylated betacyanin containing both an aliphatic and a hydroxy cinnomoyl aromatic acyl residue has been detected in *Phytolacca americana* (Schliemann et al., 1996). This kind of acylation is also observed in complex anthocyanins.

Betaxanthins

The yellow betaxanthins are immonium conjugates of betalamic acid with an amine or an amino acid. All protein amino acids and any of the 220 known non-protein amino acids found in plants can participate in the making of the betaxanthin molecules (Trezzini and Zryd, 1991b), therefore numerous betaxanthins can be found in plants, but only some have been fully characterized, most of them being present only in trace

amounts. The classification of betaxanthins distinguishes the aminoacid-derived compounds from the amine-derived conjugates. The first isolated structure was indicaxanthin from the fruits of cactus pear (*O. ficus indica*) (Piattelli et al., 1964). Numerous betaxanthins have been identified in the last 15 years. In *Portulaca grandiflora,* two pigments, one containing tyrosine (Portulacaxanthin II) and the other glycine (Portulacaxanthin III), were characterized (Trezzini and Zryd, 1991a). In Amaranthaceae, tryptophan-betaxanthin, first methylated betaxanthin, 3-methoxy tyramine-betaxanthin have been isolated from *Celosia argentea* (Schliemann et al., 2001).

Several betaxanthins have been identified in the fungus *Amanita muscaria*, including seven orange musca-aurins (λ max 480 nm) (Dopp and Musso, 1973a, 1973b; Trezzini and Zryd, 1991b). *Amanita muscaria* does not contain betacyanins but red and yellow betalamic acid-derived compounds, called respectively musca-purpurin (λ max 540 nm) and musca-flavin (λ max 420nm) (Terradas and wyler, 1991a; Mueller et al., 1997).

1.12. Biosynthesis of Betalains

Betalains are derived from tyrosine; this distinguishes them clearly from the phenylalanine-derived anthocyanins (Miller et al., 1968). The betalain biosynthetic pathway is rather simple. Only three to four enzymes are needed for the synthesis of the most simple betacyanin or betaxanthins (Fig. 5). They will catalyse (a) a ring opening reaction leading to the chromophore betalamic acid, (b) the formation of cyclo-DOPA, (c) a further glycosylation step in betacyanin synthesis, and finally (d) the transport of betaxanthins in the vacuoles. Betacyanin synthesis requires a minimum of two tyrosines

as precursors where as one is sufficient for betaxanthins. Feeding experiments with ^{14}C radiolabelled tyrosine showed that the entire C6-C3 skeleton of this amino acid is incorporated into betalmic acid and cyclo-DOPA molecules (Liebisch and Bohm 1981). Tyrosine hydroxylation by tyrosinase produces DOPA-a reaction that is very common in the plant kingdom.

Polyphenol-Tyrosinase reactions

Tyrosinases (Poly phenol oxidases, PPOs) are wide spread among plants and fungi. They are copper-containing enzymes that catalyse hydroxylation of phenols to *O*-diphenols (EC 4.14.18.1-monophenol: mono-Oxygenase) and their subsequent oxidation to *O*-quinones (EC 1.10.3.1-*O*-diphenol: oxygen Oxidoreductase). Another PPO called catechol oxidase catalyses only the oxidation of orthodiphenols.

The first step in betalain biosynthesis is the formation of DOPA from Tyrosine. DOPA is an important metabolic product of a large number of plant families and accumulates in large amounts in some leguminous species (e.g. *Vicia faba*) (Guggenheim, 1913). In betalain plants DOPA is then oxidized through a further diphenolase reaction to dopaquinone (rearranged to cyclo-DOPA) and modified by a ring-opening extra diol dioxygenase to *seco*-DOPA (rearranged to betalmic acid) (Fig. 5).

The ring-opening reaction

Betalamic acid is formed through an enzymatic cleavage of the DOPA aromatic ring at the postion 4,5; this produces an unstable *seco*-DOPA intermediate (Fischer and Dreiding, 1972; Terradas and Wyler, 1991b), which spontaneously closes on itself

(Schliemann et al., 1998). In *A. muscaria* an active 3, 4 DOPA extradiol-dioxygenase catalyses this extradiol cleavage both in the 4,5 and 2,3 positions (Girod and Zryd, 1991; Terradas and Wyler, 1991a). Depending on the pH, part of the substrate undergoes a 2,3 cleavage leading to the formation of the fungal pigment muscaflavin.

Glycosylation and other modifications

Most betacyanins are obtained by a final step involving the 5-or 6-*O*-glycosylation of betanidin or isobetanidin (Kobayashi et al., 2001). Two distinct enzymes have been isolated from *Dorotheanthus bellidiformis* cell suspensions (Heuer et al., 1996; Vogt et al., 1997). These enzymes catalyse the indiscriminate transfer of glucose from UDP-glucose to hydroxyl groups of betanidin, flavonols, anthocyanidins and flavones. Figure 5 shows the scheme of betalain biosynthesis.

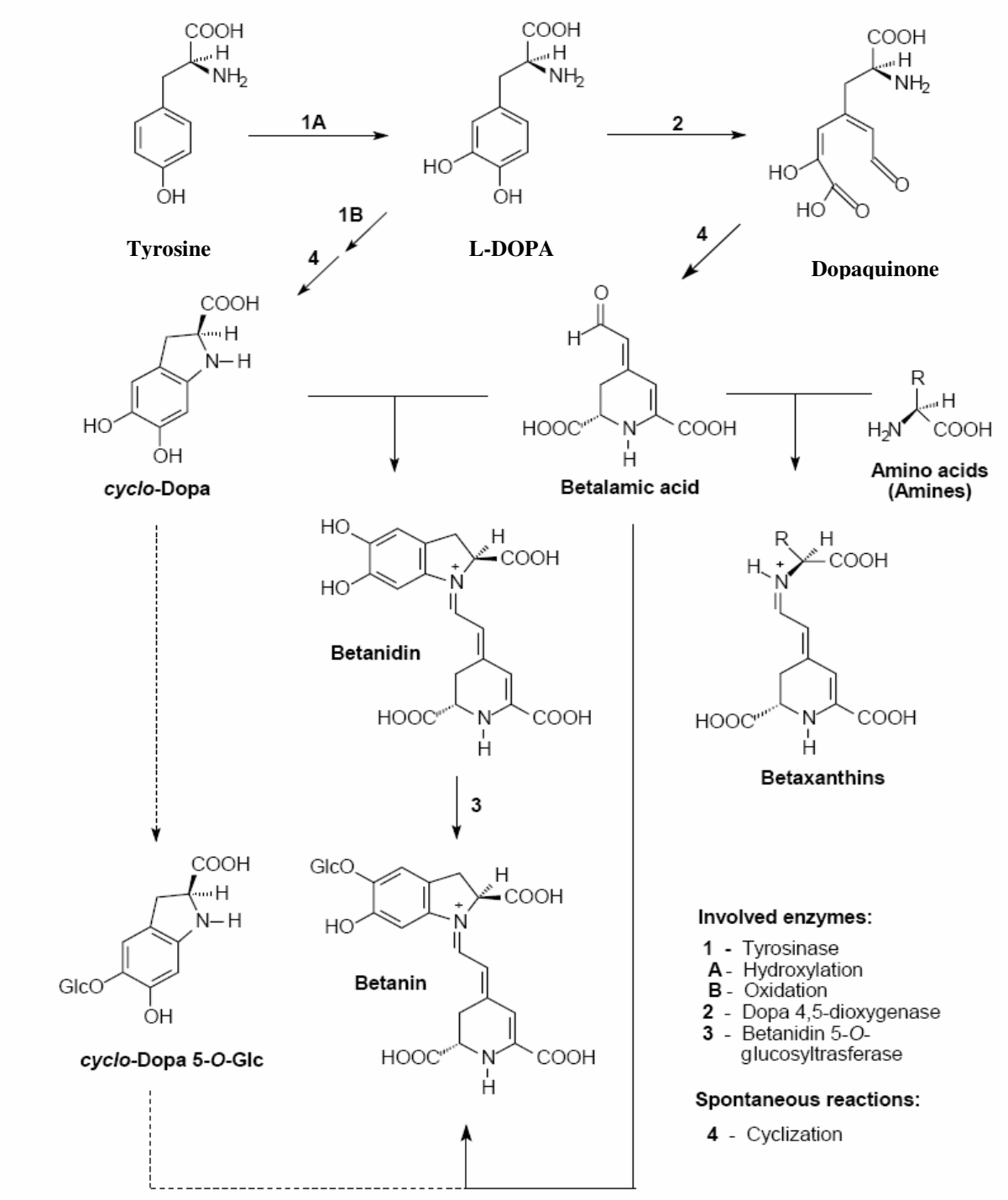


Fig. 5: Biosynthetic pathway of betalains

1.13. Cactus pear (*Opuntia sp.*) as an important source of Betalains

Cactus pear or prickly pear; a member of *Cactaceae* family is widely distributed and its commercial production is carried out in South America, California, Texas and Mediterranean countries (Tous, 1996). Recently the crop was introduced to India (Russel and Felker, 1987). Fig. 6 illustrates the *O. ficus indica* and its fruits with excessive betalain production. Cactus pear is very particular for the presence of betalains, a widely used natural food colorant in the food industry. These pigments are found predominantly only in 10 families of *Caryophyllaceae*. Betalains are generally extracted from red beet (*Beta vulgaris* (L) sub sp. *Vulgaris cv.rubra),* which contains up to 50 mg/100g of Betanin, a betacyanin. In contrast the fruits from *O. ficus indica* together from pulp and peel yields 113.9 mg/100g fresh fruit (Odoux and Dominguez, 1996). Compared to red beet, prickly pear offers considerable technological and sensory advantages as a source of betalains. The earth-like flavour of geosmin (Acree et al., 1976) and 3-sec-butyl-2-methoxypyrazine (Murray and Whitfield, 1975), high nitrite levels and microbial contamination (Henry, 1996) of red beets is not associated with cacti. Castellar et al., (2003) studied the stability of the red pigment extracted from three cactus-pear species (*O. ficus indica, O. stricta* and *O. undulata*) subjected to changes in the pH and temperature and found that the stability was pH dependent and showed a maximum at pH 5. Cactus-pear fruits could therefore, be an even better source of betalains than red beet. Table-2 shows the betalains found in different cactus-pear species. Several new forms of betalains have been detected so far which include Betanin, phyllocactin, plus C_{15} isomers and corresponding aglicons and neobetanin (Betacyanins) and five betaxanthins which include indicaxanthin, miraxanthin II, and vulgaxanthin I, II and IV (Stinzing et al., 2001; Stinzing et al., 2002).

Opuntia ficus indica

Fruits of *Opuntia ficus indica*

T.S of *Opuntia ficus indica* fruit

Fig. 6: *Opuntia ficus indica* showing the fruits

Table 1: Natural plant pigments and health intervention

Pigment	Disease condition or Health target	Selected research evidence supporting role of pigment in chronic diseases			
		In vitro	**In vivo**	**Epidemiological**	**Clinical**
Anthocyanins	Vision disorders	-----		Morazzoni & Bombardelli, 1996	Mastsumoto et al., 2001
	Neuroprotection	Youdim et al., 2000	Joseph et al., 1999 Youdim et al., 2000b	-------	-------
	Cardiovascular disease	Oak et al., 2003 Serraino et al., 2003	Nielsen et al., 2003	Havsteen, 2002 Duthie et al., 2000	Shanmuga nayagam et al., 2002
	Cancer	Kang et al., 2003 Naasani et al., 2003	Kang et al., 2003 Naasani et al., 2003	Horvathova et al., 2001 Duthie et al., 2000	-------
Carotenoids	Photooxidative damage	Sies and Sthal, 2003	-------	Khachik et al., 2002	Snodderly, 1995
	Immune system	Jyonouchi et al., 1996	------	--------	Hughes et al., 2003
	Cardiovascular disease	Fuhrman et al., 1997	Fuhrman et al., 1997	Delgado-Vargas et al., 2003 Platz et al.,2003	Platz et al., 2003
	Cancer	Kotake-Nara et al., 2001 Liu et al., 2003.	Wang et al., 1999 Jain et al., 1999 Teplizky et al., 2001	Goodman et al., 2003 Toniolo et al., 2001 Nyberg et al., 2003	Baron et al., 2003 Deming & Erdman, 1999 Kucuk et al., 2003
Chlorophylls	Cancer	Chung et al., 1999	Park &Surh, 1996 Rebeiz et al., 1996	------	------
Betalains	Cancer	Wattasinghe et al 2002	Kapadia et al.,1996	------	------

Table 2: Betalains in *Opuntia* species

Species	Betacyanins	Betaxanthins
O. bergeriana	betanidin betanin Phyllocactin	Indicaxanthin Miraxanthin Vulgaxanthin I Vulgaxanthin II
O. decumbens *O. dellenii* *O. guatemalensis*	betanin	---
O. engelmannii	betanin Phyllocactin	---
O. ficus indica	betanin isobetanin neobetanin	Indicaxanthin Miraxanthin Vulgaxanthin I Vulgaxanthin II Vulgaxanthin IV Adducts of betalamic acid with gammabutyric acid, phenylalanine, isoleucine, serine and valine
O. monacantha	betanidin betanin Phyllocactin	Indicaxanthin Vulgaxanthin I
O. paraguensis *O. riitteri*	betanin Phyllocactin	---
O. robusta *O. streptacantha*	betanin	---
O. stricta *O. tomentosa* *O. tomentella*	betanin neobetanin	---

O. vulgaris	betanin Phyllocactin	---
O. polycantha	betanidin betanin	Indicaxanthin Vulgaxanthin I

Source: Stinzing et.al. 2000

1.14. Scope and Objectives

Natural pigments are gaining much importance in view of their bioactivities and as food additives. Betalains are one such nutraceutical nitrogenous cationic natural pigments that are widely used as food additives. Unlike other pigments betalins are not very well studied with regard to their pharmacological properties and mechanism of action. Therefore the current study is designed to understand the nature of the water-soluble nitrogenous chromo alkaloids, betalins from fruits of *O. ficus indica*.

The specific Objectives of present study are

- To purify and chemically characterize and identify the betalain pigment from fruits of *Opuntia ficus indica.*
- To biochemically evaluate the
  - ❖ Antioxidant,
  - ❖ Nephroprotective and
  - ❖ Antiproliferative effects of purified betalain.

2. Materials and Methods

2.1. Materials

Cisplatin (catalogue # P-4394) was from Sigma-Aldrich (St.Louis MO, USA). Commercial kits to measure creatinine and urea were from Qualigens diagnostics - India. Spin trap, DMPO (catalogue # NS-811), for ESR analysis was acquired from Dojindo laboratories –Japan. Phosphate buffered saline (PBS), RPMI medium, Fetal bovine serum (FBS) were purchased from GIBCO Ltd (BRL Life Technologies Inc., Grand Island, NY). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Proteinase K, RNase A, Propidium iodide were from Sigma Chemical Co (St Louis, MO). The human chronic myeloid leukemia cell line, K562, was procured from National Center for Cell Science (NCCS), Pune, India. Nitrocellulose membranes were from Amersham Life Sciences; Monoclonal antibodies against cytochrome c and Bcl-2 were from Upstate Technologies (New York). Mouse monoclonal Bax antibodies were from Santa Cruz, CA, USA and polyclonal PARP antibodies were from R & D systems, USA.

2.2. Chemical characterization of betalains from fruits of *Opuntia ficus indica*

2.2.1. Isolation and purification of betalains from fruits of *Opuntia ficus indica* (prickly pear)

Pigment extraction from prickly pear fruits was performed according to Kanner et al., (2001), with minor modifications. Fruits were peeled and finely chopped. The fruit pulp (100g) was frozen in liquid nitrogen and homogenized in mortar. The resulting powder was suspended in methanol and allowed to stand at 4^{o}C for 2 hours with continuous stirring followed by filtration and centrifugation. The resulting residue was extracted twice with 80% aqueous methanol. The combined supernatants were taken to

dryness under reduced pressure. The residue was then dissolved in 60 ml of water. Three portions of 20 ml each were transferred to a sephadex LH20 (40 X 2.2 cm) column and eluted with water. Elution of betacyanin fractions were detected visually and collected for further purification on HPLC.

2.2.2. HPLC Purification of Betacyanins

The isolated pigments were submitted to preparative high performance liquid chromatography. A Shimadzu LC-6A system equipped with Rheodyne injector with a sample loop of 1 ml was used to separate the pigments. A Shim-pack PREP-ODS column with 20.0mm ID X 25 cm dimension was used and elution was carried out following a chromatographic program proposed by Strack et al. (1987). The program consisted of 55 min lenier gradient elution from solvent A (1% acetic acid in water) to solvent B (1% acetic acid in acetonitrile) with a flow of 5 ml/min. In each analysis, 250 µl of filtered extract was directly injected on to the chromatographic column. Monitoring was performed at 535 nm. The identities of the different chromatographic peaks were confirmed by their visible spectral characteristics in comparison to standards and retention times. The elution volumes relevant to betanin were collected and cryoessicated. For analysis, these cryoessicated samples were resuspended in phosphate buffer, and used. Quantification of betanin pigment was carried out using a UV-Vis spectrophotometer (Hitachi). Samples were diluted with 0.05M phosphate buffer (pH 6.5) as previously described (Stinzing et al., 2003) using the molar extinction coefficient of betanin ($\acute{\varepsilon}$ = 60000l/mol-cm; λ=535 nm; molecular weight=551 amu) (Wyler and Meur, 1979)

2.2.3. Co injection with standard

HPLC purified pigment fractions were collected, mixed with standard betanin (an authenticated standard isolated from red beet) in equal ratios and co injected into the HPLC in order to confirm the identity of the unknown pigment fraction.

2.2.4. NMR analysis

NMR spectra of purified fractions were recorded on a Bruker 400 MHZ NMR spectrometer. Proton chemical shifts were referenced to the residual solvent signal at δ=4.70 ppm (D_2 O). 1D NMR measurements were carried out using 100% D_2 O. Solvent and TFA suppression was achieved by implementing a pre saturation element in pulse sequences.

2.2.5. LC-MS analysis

LC-MS analysis was performed according to Stinzing et al (2004). Separation was performed using Shimadzu HPLC connected to UV –Vis detector in series with a Mass spectrometer fitted with ion spray source. Pigment separation was achieved on C18 column (250X4.6 mm, particle size 5 µm) fitted with a security guard C18 (4X3.0mm i.d.) operating at 25^{o}C and using 0.2% aqueous acetic acid (eluant A) and a mixture of acetonitrile and water (80/20, v/v, eluant B). The elution was carried out following a lenier gradient from 10% B to 27%B in 30 min and monitoring was performed at 535 nm for betacyanins. Positive ion mass spectra of eluting betacyanins was recorded setting the capillary temperature to 200^{o}C and keeping the ESI spray voltage at 4 KV. Spectra were monitored by injecting 50 µl of pre TFA acidified sample.

2.3. Antioxidant assays

2.3.1. 2,2-Diphenyl –1-picryl hydrazyl (DPPH) radical assay

The colorimetric method of DPPH assay is not applicable to colored substances, due to the interference of pigments. In order to prevent pigment interference, ESR based spin analysis technique of Hiroyuki et al., (2002) with little modification is used in the present study to evaluate the radical scavenging property of the betanin. The reaction mixture contained equal volumes of 0.5M DPPH in ethanol and different concentrations of betanin incubated for 15min in dark. Control sample contained equal volume of DPPH and ethanol. The percentage signal intensity was calculated relative to that of control.

Experimental conditions:

Analysis was performed with FR30 free radical monitor (JEOL, TOKYO, JAPAN) using Frequency-9420, Fixed magnetic field-335.3 mT, Microwave power-4mw, Amplitude-790.

2.3.2. Superoxide anion assay

The hypoxanthine and xanthine oxidase system is used for the generation of $O_2^{\cdot -}$ (Zang et al., 1995). The typical reaction mixture contained 0.125 units / ml XOD, 0.5 mM hypoxanthine and 0.67 M DMPO. The reaction was started with addition of xanthine oxidase from the stock solution prepared in phosphate buffered saline, pH 7.4. The assay mixture was transferred to a quartz capillary and fitted into the sample cavity of the ESR spectrometer. The instrumental conditions set were JEOL X-Band ESR spectrometer, modulation amplitude 0.1 mT (100 KHZ), scan range 10 mT, scan time 2 min, and microwave power 8mW.

2.3.3. The Nitric oxide scavenging assay

Sodium nitroprusside (10mM) in standard phosphate buffer solution was incubated with different concentrations of betanin dissolved in 0.025 M phosphate buffer pH 7.4 and the tubes were incubated at 25^0 C for 5 hours. Control experiment without test compound but with equivalent volume of buffer was conducted in an identical manner. After 5 hours of incubation, 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylene diamine dihydrochloride) was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with napthylene diamine was read at 546 nm (Sreejayan Rao, 1997).

2.3.4. The arachidonate peroxyl radical assay

To investigate the reaction of peroxyl solution with betanin, the arachidonic acid peroxyl radical intensity was monitored as a function of betanin concentration. The arachidonate peroxyl radical was generated mixing 0.67g/lit lipoxygenase in 0.2M borate buffer (pH 9.0) with 0.64mM arachidonic acid (Walee Chamulitrat et al., 1989). The reaction mixture was mixed for 100 ms and transferred into TM110 cavity of JEOL X-Band ESR spectrometer. The instrumental conditions set for recording of the spectra are modulation amplitude, 2.28G: power, 100 milliwatts: time constant,5S; scantime 167 s; scan range,100G.

2.3.5. Lipoxygenase assay

Lipoxygenase enzyme inhibitory activity was measured spectrophotometrically (Shimizu et al., 1984). Typical reaction mixture contained 2.9 ml of buffer (0.1M

phosphate buffer pH 6.3) and 100 µl of 5-LOX enzyme incubated with different concentrations of betanin. The sample was adjusted to zero absorbance by keeping the same reaction mixture as reference. Either 10 µl of 80 mM linoleic acid or 40 mM arachidonic acid was used to initiate the reaction. Reaction was followed for 2 min by monitoring the formation of conjugated dienes at 235 nm.

Enzyme activity was calculated by using following equation.

$$\text{LOX activity} = \frac{\text{Volume of the reaction mixture x absorbance difference per min}}{\text{ε x volume of the enzyme in ml}}$$

ε = Molar extinction coefficient of the hydroperoxides = 27, 500/ milli mole

2.4. Nephroprotective effects of betanin

2.4.1. Animals and treatment

Adult male Wistar rats (weight 200-250 g) were used in the present study. To investigate the cisplatin –induced nephrotoxicity, animals were divided into four groups of six animals each. The control group received normal saline orally. The second group received a single dose of cisplatin -16 mg/kg body weight i.p (Ravi et al., 1995). The third and fourth group received betanin -50 mg/kg and 100mg/kg body weight respectively, daily for five days and cisplatin -16 mg/kg body weight single dose. After five days, animals were humanely sacrificed using Phenobarbital anesthesia. Blood samples were obtained from animals and serum was used for measurement of urea and creatinine levels. The kidney specimens were dissected out. Surgically removed kidney specimens were subjected to fixation in 4% neutral formaldehyde for histopathological evaluation.

2.4.2. Renal function

Renal function was assessed by measurements of plasma urea nitrogen (PUN) and creatinine levels in the plasma. A 0.3 to 0.4 ml aliquot of blood was collected and the concentrations of PUN and creatinine in the blood plasma were measured using qualigens diagnostic kits according to manufacturers instructions.

2.4.3. Lipid peroxidation

The extent of lipid peroxidation was estimated in kidney homogenates by measuring the levels of malondialdehyde (MDA) formation using the modified thiobarbituric acid method (Ohkawa et al., 1979). Briefly, 10% (w/v) homogenates of the kidney tissues, were prepared in 1.15% KCl by using a Teflon homogenizer. 300 µl of this homogenate was added to 700 µl of water and the mixture was incubated for 30 minutes at 37^0 C. Two ml of thiobarbituric acid (0.375% TBA in 15% TCA) was added and the mixture was kept at 95^0 C for 30 minutes. To avoid evaporation of the samples, tubes were closed with marbles. After cooling at room temperature, the volume was checked and readjusted. The samples were centrifuged at 5000 rpm for 10 minutes and the absorbance of the supernatant was measured at 532nm. MDA values were calculated by using the standard 1,1,3,3, tetramethoxy propane.

2.4.4. Catalase activity staining

The method of catalase-activity staining was essentially that described by Clare et al. (1984). Equal amounts of proteins were applied to native electrophoresis, which was performed on 15% polyacrylamide gels. The gel was soaked in horseradish peroxidase (50 mg/ml) in 100 mM potassium phosphate buffer (pH 7.0) for 45 min. H_2O_2 was then added to a concentration of 5.0 mM and soaking was continued for 10 min. The gel was

then rapidly rinsed twice with distilled water and soaked in 0.5 mg/ml diaminobenzidine in potassium phosphate buffer until staining was complete.

2.4.5. Transmission electron microscopy (TEM)

For transmission electron microscopy, the kidney tissues were fixed in 3% glutaraldehyde in 0.15-mol/l sodium cacodylate buffer (Gove et al., 1997). Kidneys from saline, cisplatin and cisplatin + betanin treated rats were dissected out, immersed in same fixative and processed for embedding in plastic resin. Semi thin sections (0.1 µm) were stained with toludine blue. Selected areas were further sectioned and stained with uranyl acetate and lead citrate and examined under TEM (Hitachi).

2.5. Anti-proliferative effects of betanin

2.5.1. Cell culture and treatment

K562 cells were grown in suspension cultures employing RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ^{o}C. The cultured cells were passed twice each week, seeding at a density of about 2 x 10^5 cells/ml. For treatments, exponentially growing K562 cells were collected and re-suspended in fresh culture medium. A stock solution of 40 mM betanin was prepared in PBS from the cryoessicated purified samples and used in the experiments.

2.5.2. Cell proliferation assay

Cell proliferation was assessed using the 3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining method (Mossman, 1983). Briefly, 5 x 10^3 cells (K562) were incubated in 96-well plates in the presence or absence of betanin (10, 20,

40, 80 µM) for various time points in a final volume of 100 µl. At the end of the treatment, 20 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37^{o}C. The purple-blue MTT formazan precipitate was dissolved in 50 µl of lysis buffer (12 mM HCl, 5% isobutanol and 10% SDS). The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on micro titer plate reader (Microscan MS 5608A, ECIL Instruments).

2.5.3. Morphological differentiation

K562 cells were incubated with a fixed concentration of betanin (40 µM) and the appearance of morphological differentiation was assessed after 24 h. The cells were viewed on a phase contrast inverted microscope (400 X magnification) and photographed with Nikon F-601 AF Camera.

2.5.4. Scanning electron microscopy (SEM)

After treatment with betanin the cells were collected, washed with PBS and concentrated to 1 x 10^5 cells/ml. One drop of this suspension was placed on to a plastic coverslip that is previously coated with 1% poly-L-lysine. Cells were fixed with glutaraldehyde for 1 h and post fixed with 1% osmium tetroxide for 1 h. Cells were dehydrated by passing through graded alcohols and dried by the critical-point technique. After trimming, mounting, and coating with gold-platinum, the specimens were observed on SEM (JSM-5600, JEOL Co.)

2.5.5. Transmission electron microscopy (TEM)

Cell pellets were rinsed rapidly with PBS and fixed for 12 h at 4 ^{o}C in 2% vol / vol glutaraldehyde in 0.1% sodium cacodylate buffer, pH 7.4. Fixation was followed by 3-5 min rinses with 0.1% sodium cacodylate buffer, pH 7.4. Cells were post fixed with a

solution containing 1% osmium tetroxide (w/v) and 2% K_4Fe (CN) (w/v), and stained with 1% uranyl acetate, and pelleted in 2% agar. Pellets were dehydrated in graded ethanol solutions and embedded in spur resin. Ultra thin (60 nm) sections were cut on a Reichert Ultra cut microtome collected on Rhodanium 400-mesh grids, post-stained with uranyl acetate followed by lead citrate, and rinsed with water. The sections were examined in Philips CM-12 electron microscope at 80 KV.

2.5.6. Cell cycle analysis

To quantitate apoptosis, flow cytometric analysis was performed using propidium iodide as described previously (Reddy et al., 2003). Cells, which were less intensively stained than G1 cells (sub-G0/G1 cells) in flow cytometric histograms, were considered as apoptotic cells. Briefly, 3.5 x 10^6cells (K562) were plated in 6 well culture plates, cultured in 10% FBS with (40 µM) or with out betanin for 24 h. After treatment, cells were harvested and washed in PBS, and the viability was determined by tryphan blue exclusion method. For analyzing DNA content 10^6 cells were fixed in 70% ethanol, washed in PBS, incubated with 0.1 mg/ml RNase A and stained with propidium iodide (final concentration 50 µg/ml). The red fluorescence of individual cells was measured on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were analysed per sample.

2.5.7. Flow cytometric analysis of mitochondrial membrane potential

K562 cells were cultured and allowed to reach confluence for 24 h before treatment with betanin (40 µM). The cells were harvested and changes in the mitochondrial membrane potentials were measured by the uptake of cation Rhodamine 123 into mitochondria (Seuduto and Grotyohann, 1999). Untreated control cells were used to

determine the normal uptake of this cation, and the percentage of treated cells with a low membrane potentials were then calculated. Briefly, the cells were centrifuged at 800xg for 10 min and resuspended in 1 ml of Rhodamine 123 (10 µg/ml) for 30 min at room temperature, and washed once in PBS. The cells were resuspended in PBS. The samples (25000 events) were analyzed for fluorescence (FL-1 detector, filter 430/30 nm band pass) using a FACscan (Becton Dickson, San Jose, CA). The histograms of control and treated cells were compared.

2.5.8. Measurement of cytochrome c release using Western blot analysis

After cells were exposed to 40 µM betanin for various time points (0, 4, 8, 12, 24 h), cells were collected and washed once with PBS and subsequently with buffer A (0.25 M sucrose, 30 mM Tris–HCl, pH 7.9, 1 mM EDTA). Cells were then resuspended in buffer A containing 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin and homogenized with a glass dounce homogenizer. After centrifugation for 10 min at 21000xg, protein concentration of the supernatant was determined using the Bradford method (Bradford, 1976). The level of cytochrome c in the cytosolic protein extract (50 µg) was then analyzed by employing monoclonal antibody directed against human cytochrome c.

2.5.9. Western blot analysis

Cells were exposed to 40 µM betanin for various time points (0, 4, 8, 12, 24 h) and whole cell extracts were prepared based on the method of Pardhasaradhi et al. (2003). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin). After 30 min of shaking at 4 ^{o}C, the mixtures were

centrifuged (10000xg) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). An equal amount of total cell lysate was resolved on 8–12% SDS-PAGE gels and then transferred on to Nitrocellulose membranes. Membranes were stained with 0.5% ponceau in 1% acetic acid to confirm equal loading. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies (Bcl-2, Bax and PARP) in 10 ml of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 ^{o}C for 8–12 h and then incubated with respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

2.5.10. Intracellular localization of Betanin in K562 cells

The K562 cells treated with betanin (40 μM) for 24 h were washed in PBS and fixed in 4% formaldehyde, pH 7.4 for 20 min at 4 ^{o}C. After fixation, cells were washed twice in PBS and water. The cells were then viewed under confocal microscope with 506 nm and 529 nm of excitation and emission wavelengths.

2.6. Statistical analysis

Data reported as the mean ± SD of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). P-value of less than 0.05 was considered as significant.

3.0. Results

Betalains are the characteristic natural pigments present in fruits, flowers, tubers etc, of the Centrospermae members. They can be divided into two structural groups yellow betaxanthins and red purple betacyanins. Both the groups possess betalamic acid, in common whose chromophore is 1,7 –diazaheptamethinium system (Piatelli, 1976), while they differ mainly in the radicals bonded to the main structure. Their colour is due to the conjugation of a substituted aromatic nucleus to the diaza system that shifts the absorption maximum from 535 nm in betacyanins to 480 nm betaxanthins (Strack et al., 1993). In cactus pear (*Opuntia sp.*), these pigments are responsible for the purple, red, orange and yellow colours. Several kinds of betalains are known to exist and co-exist in the *Opuntia* species. The pigments are widely employed as food additives in view of their attractive colours and potent antioxidant properties. The aim of this study is to identify the kind of betacyanin pigment that is prominently present in the fruits of *Opuntia ficus indica* from Indian origin and to appreciate its bioactivities. Betanin, a betacyanin pigment was isolated and purified from fruits of *O. ficus indica* and its molecular structure was confirmed using various bio analytical techniques. The purified betanin was then subjected to evaluation of antioxidant, nephroprotective and antiproliferative effects as per the methods described in the methodology. Cisplatin induced nephrotoxicity in rats was used as model to evaluate the nephroprotective effects, while chronic myeloid leukemia cell line-K562 was employed to study antiproliferative effects of betanin.

3.1.1. HPLC purification of betalains

The betalains from *O. ficus-indica* fruit pulp were extracted twice with aqueous methanol and the extract was concentrated and then purified by gel filtration column chromatography on sephadex LH-20 column. The active fractions were pooled and then separated on HPLC as per the methodology described. The identities of betalains present in major proportion were confirmed by means of spectroscopic, co-chromatography and LC-MS analysis. The HPLC profile of the fruit extracts from *O. ficus-indica* on PREP-ODS column showed two peaks-peaks 1 (major) with a retention time of 27.2 min and peak 2 (minor) with a retention time of 28.2 min (Fig. 7). When these two peaks were monitored on UV-VIS spectrophotometer, they gave a characteristic betacyanin spectrum with absorbance maximum at 535 nm (Fig.8). Among these two peaks the major peak was collected and subjected to IR, ^{13}C NMR, Proton NMR and LC-MS analysis.

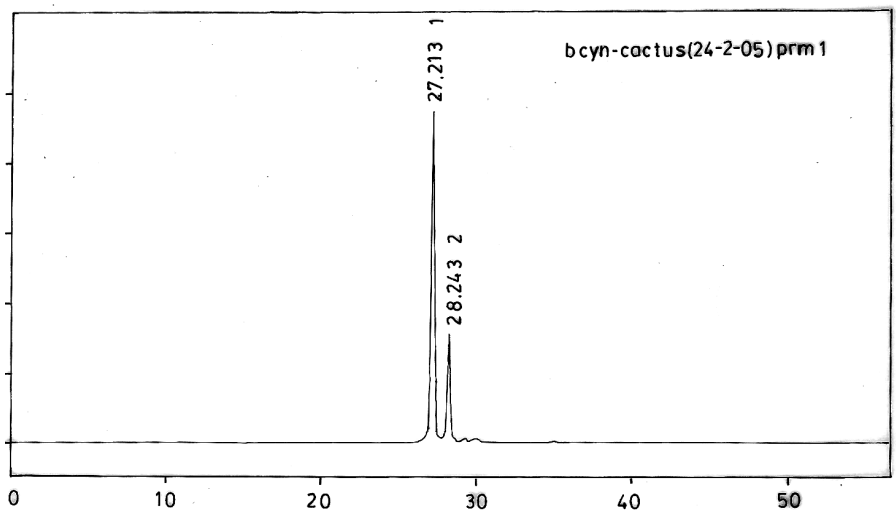


Fig. 7: HPLC profile of betalains from the fruit extracts of *Opuntia ficus indica*. 80% aqueous methanolic pigment extracts that were preliminarily purified using sephadex LH-20 gel filtration chromatography were passed through 0.2µ filters and injected on to the PREP-ODS column. Peak-1, $t_{R=}$27.21min, and Peak-2, $t_{R=}$28.24min.

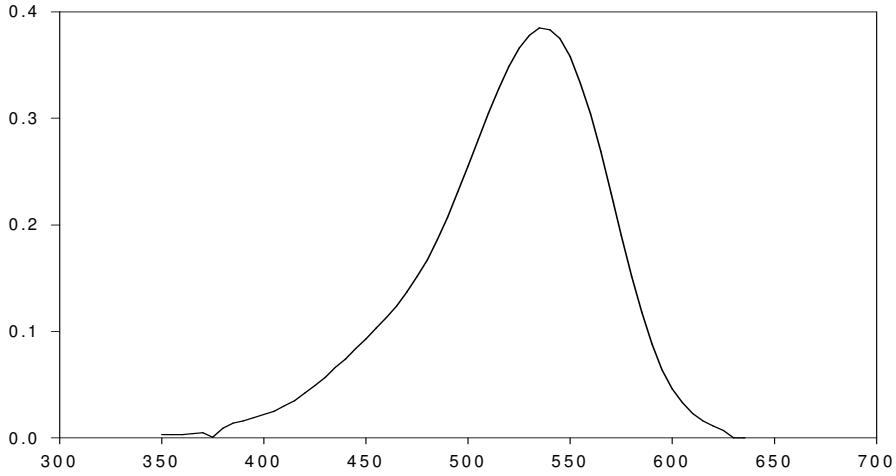


Fig. 8: Wavelength scan of peak-1 of HPLC purified fractions from *Opuntia ficus indica*.
Peak collected after HPLC separation was scanned over a wavelength range of 300nm-700nm. As a result characteristic betacyanin spectra with absorption maximum of 535 nm was observed.

3.1.2. IR, Proton NMR and ^{13}C NMR and analysis of Peak-1

The chemical structure of betacyanin present in fruits of *O.ficus indica* was established by Infra red (IR) spectroscopy, proton NMR and ^{13}C NMR analysis. The IR spectral bands at 3500, 1750 cm^{-1} indicated the presence of carboxylic acid groups that are present in the betanin (Fig.9). The proton NMR spectrum of the betacyanins showed a doublet peak at δ 5.0 ppm (d, J = 6.5 Hz,^{1} H) attributable to anomeric carbon and other signals at δ 8.2, 7.4, 6.6 and 4.4 ppm indicating the presence of aromatic component of betanin (Fig. 10). Further the structure of betacyanin was established by ^{13}C NMR analysis (Fig. 11).

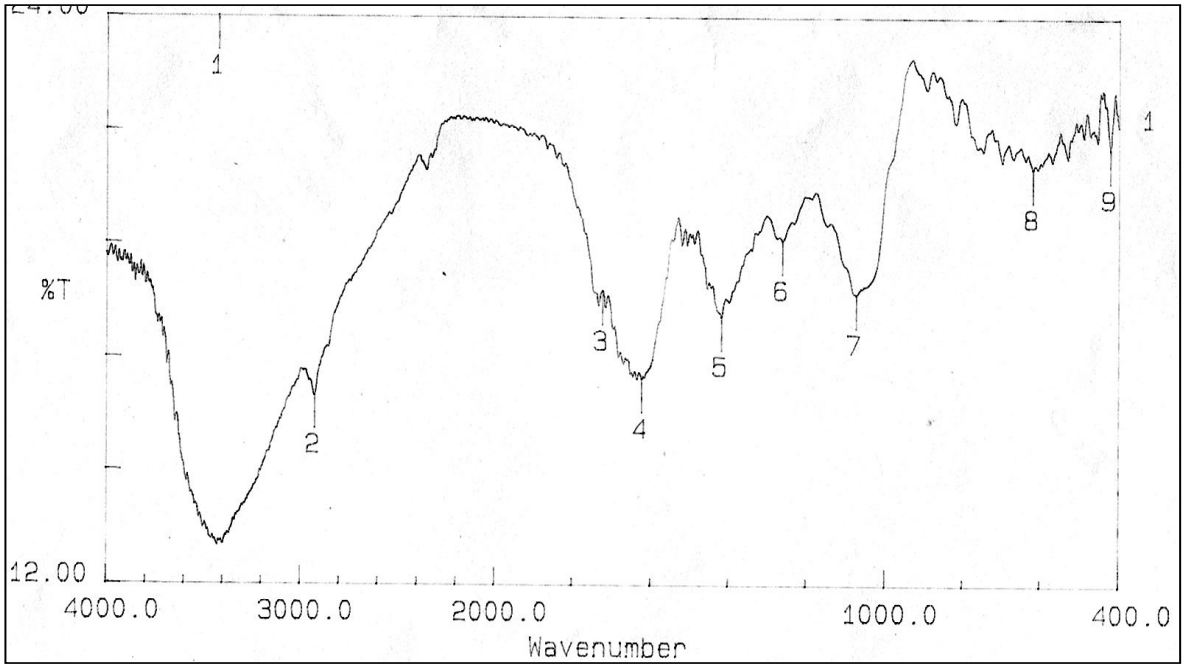


Fig. 9: IR spectral analysis of betacyanins purified from *Opuntia ficus indica*. Peak-1 fraction from HPLC was subjected to IR spectral analysis in KBr.

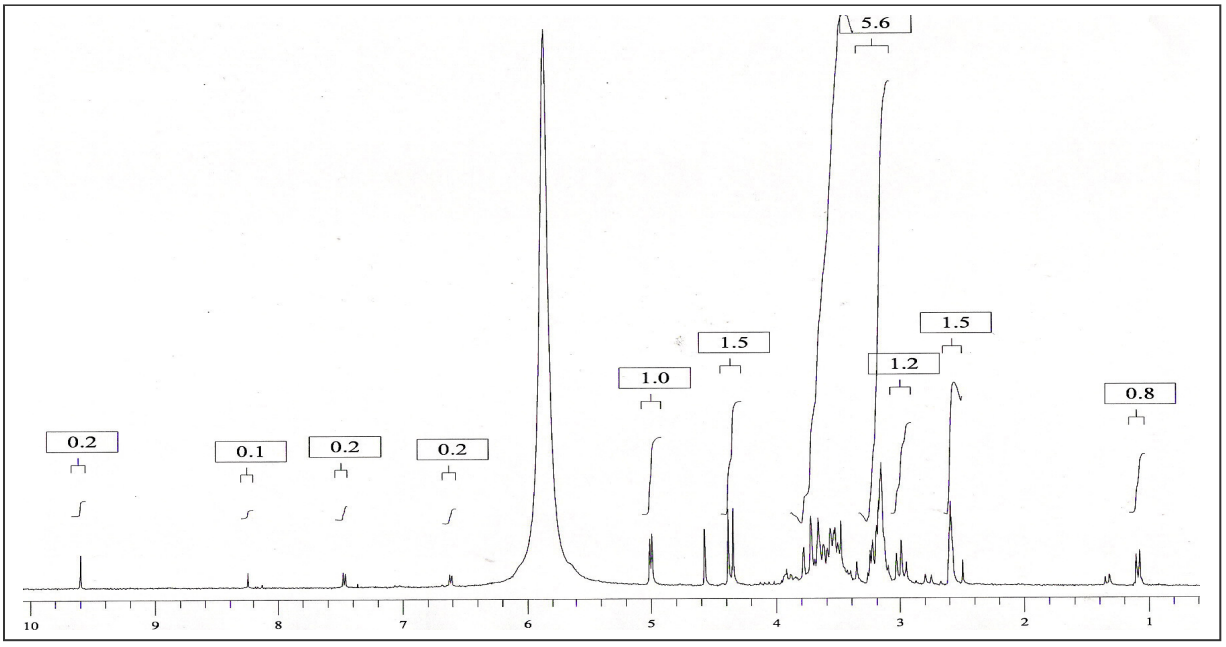


Fig. 10: Proton NMR analysis of betacyanin purified from *Opuntia ficus indica*. HPLC purified betacyanin pigment was subjected to proton NMR analysis. The data was acquired in TFA/D_2O solvent system (Scale = ppm)

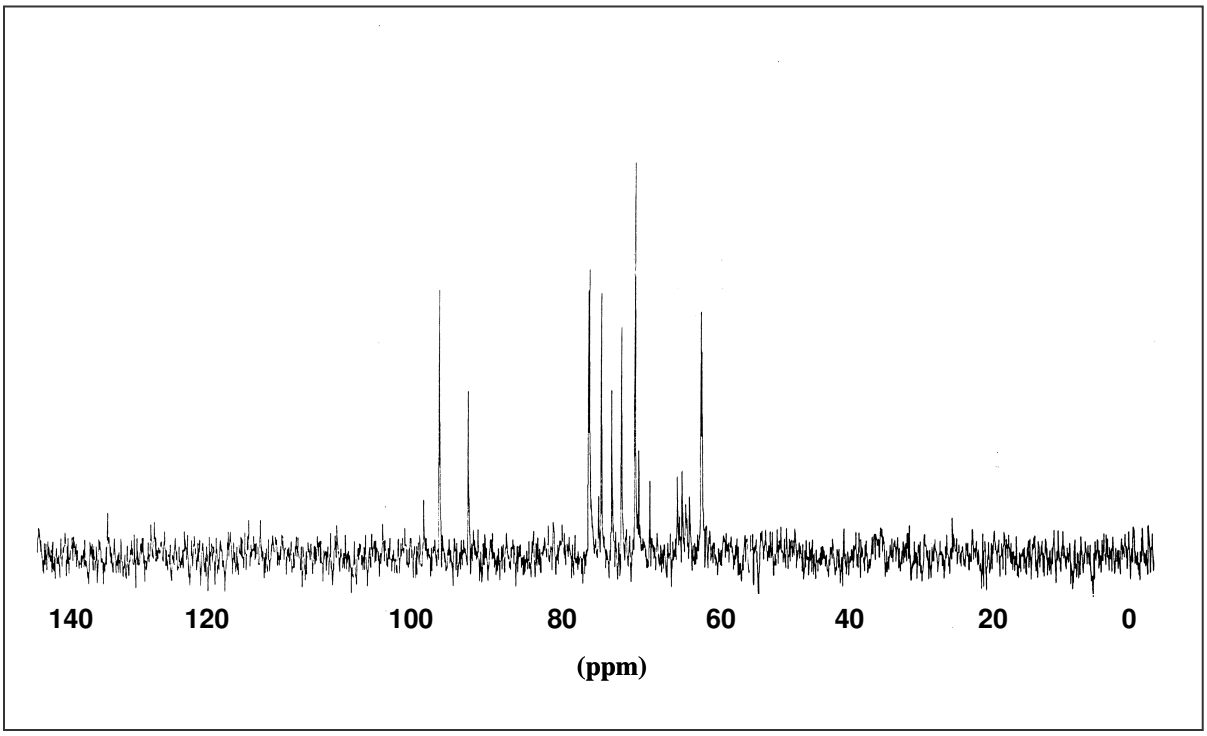


Fig.11: ^{13}C NMR analysis of Betacyanin purified from *Opuntia ficus indica*.
HPLC purified betacyanin pigment was subjected to proton ^{13}C analysis. The data was acquired in TFA/D_2O solvent system

3.1.3. Preparation of betacyanin standards

In order to confirm the identity of betacyanin pigment with that of betacyanin standards (Betanin and Isobetanin), HPLC analysis was carried out for the authenticated standards isolated from red beet, as per the method described by Kujala et al., 2001 with slight modification. In brief the red beetroots were collected, cut into small pieces and lyophilized. 500 mg of this lyophilized material was homogenized for 1 min in 10 ml of water. The homogenate was centrifuged for 10 min at 1500 x g .The clear supernatant thus obtained was further subjected to HPLC separation on Shim-pack PREP-ODS column with 20.0mm ID X 25 cm, using solvent A (1% acetic acid in water) and solvent B (1% acetic acid in acetonitrile) with a flow of 5 ml/min. Two peaks, peak-1 (major) for betanin and peak-2 (minor) for isobetanin were observed eluting at retention times of 27.2 and 28.2 min. respectively (Fig.12).

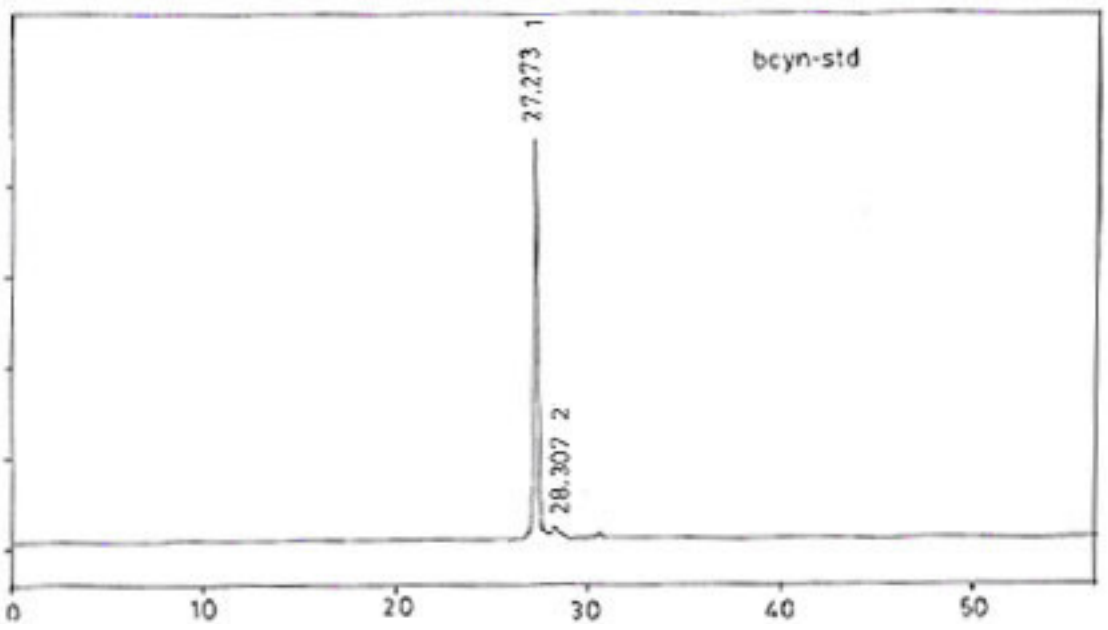


Fig. 12: HPLC analysis of betacyanin standards isolated from *Beta vulgaris* (red beet). 80% aqueous methanolic pigment extracts that were preliminarily purified using sephadex LH-20 gel filtration chromatography were passed through 0.2µ filters and injected on to the PREP-ODS column. Peak-1, Betanin with $t_{R=}$27.21min, and Peak-2, Isobetanin t $_{R=}$28.30min.

3.1.4. Co chromatography with betanin standard

In order to confirm the identity of the betacyanin pigment purified from fruits of *O. ficus indica,* the HPLC separated betanin standard from red beet was co injected along with the purified peak –1 of *O. ficus indica* on to the chromatographic column. As a result a solitary peak with a retention time of 27.03 min. corresponding to that of betanin was observed (Fig. 13). This further confirms the identity of betanin as a betacyanin pigment that is present in major proportion in the fruits of *O. ficus indica.*

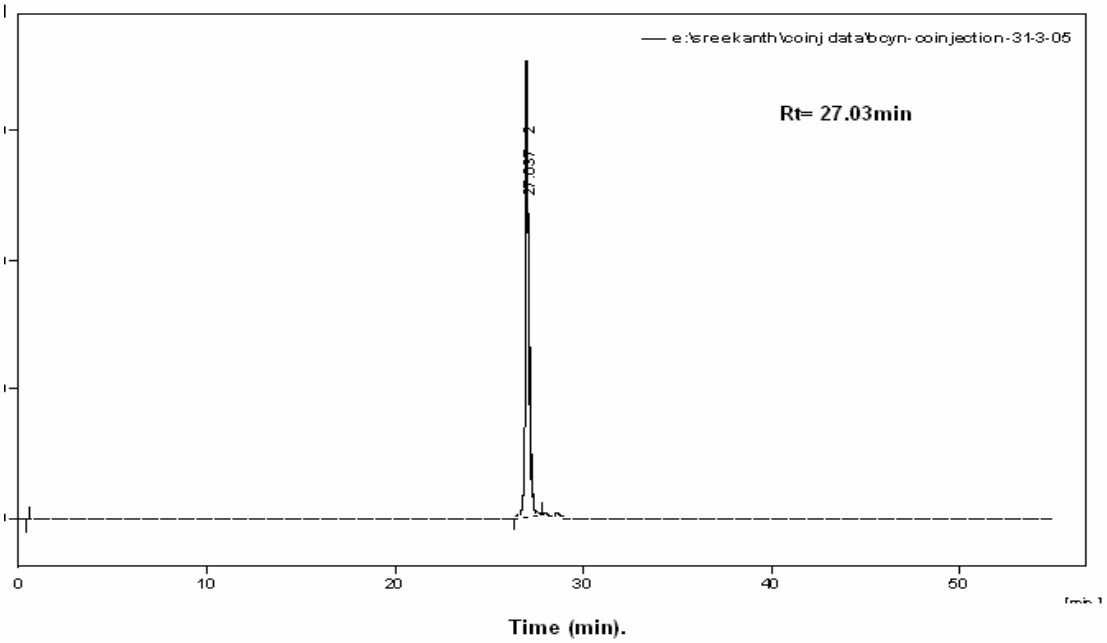


Fig.13: Co chromatography analysis with betanin standard
Betanin isolated from *Beta vulgaris* (red beet) by HPLC and peak-1 of HPLC separated betacyanin from *O. ficus indica* were co-injected on to the HPLC column. The peak represents the identity of betanin with $t_{R=}$27.03min

3.1.4. LC-MS analysis

The HPLC conditions used for the pigment analysis were adopted to LC-MS method and the molecular mass and the fragmentation pattern of the major betacyanin pigment present in fruits of *O. ficus indica* was determined. Taking the type of compound analyzed into account, a mass spectrometer with electrospray in positive ionization mode was selected. The mass spectrum of the peak-1 is shown in the Fig. 14 where a strong molecular ion $[M+H]^+$ of 551 amu was observed. These spectrums together with visible spectral and retention time data confirm its identity as betanin with a molecular mass of 551 amu. Figure 15 shows the final deduced structure of the betacyanin pigment betanin [Betanidin-5-O-β-glucoside].

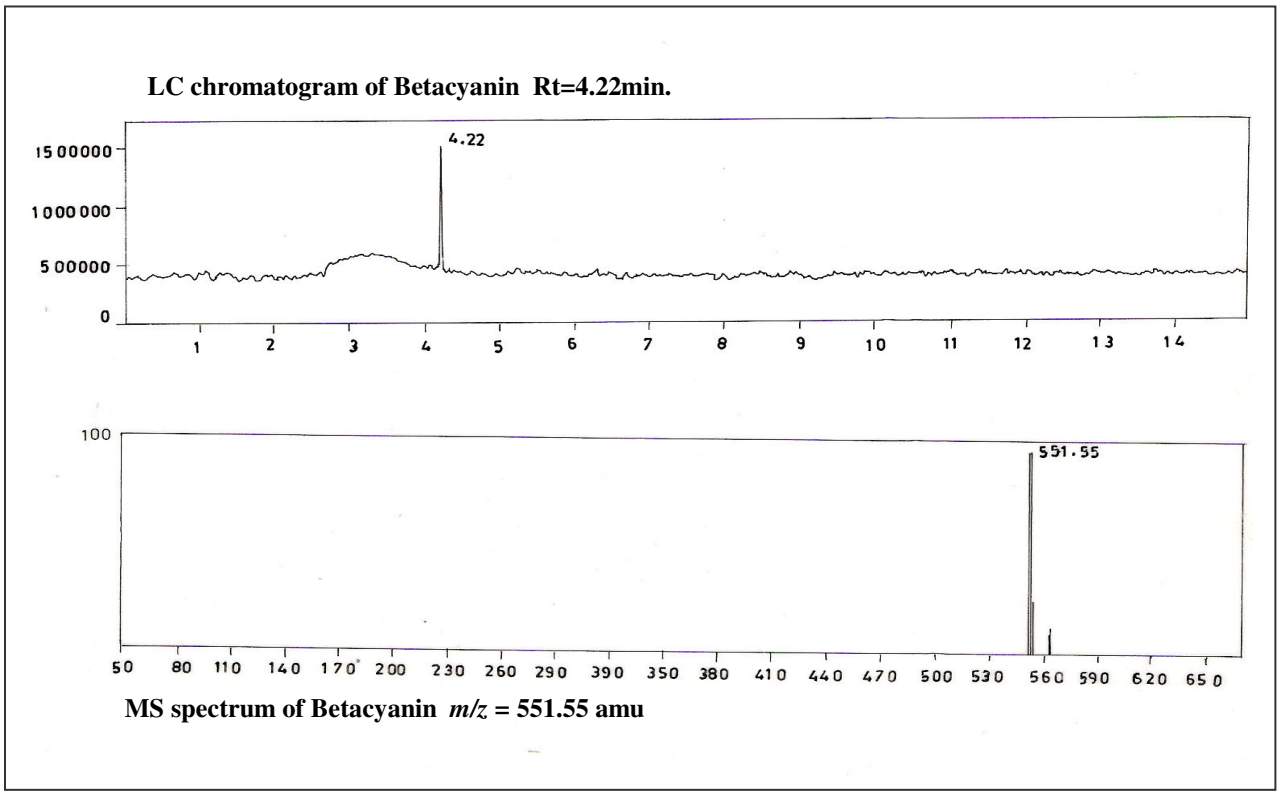
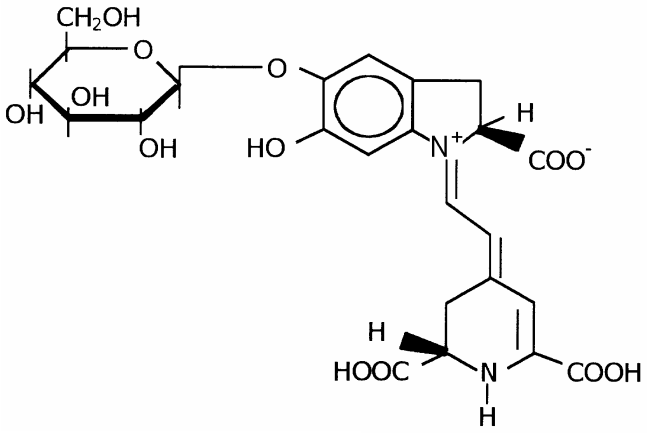


Fig. 14: Positive-ion electron spray, mass spectrum of Peak-1
HPLC separated Peak-1 fraction from *O. ficus indica* fruits was subjected to electron spray ionization LC-MS analysis. Strong molecular ion at [M+H$^+$] at 551 amu represents the presense of betanin



(C_{24} H_{26} $O_{13}N_2$): 551 amu

Fig. 15: Chemical structure of Betanin identified from fruits of *O. ficus indica.*
The betacyanin present in major proportion in the fruits of *O. ficus indica* is identified to be Betanin (C_{24} H_{26} $O_{13}N_2$) with 551 amu.

In this part of the study, The betalains from the *O.ficus indica* fruit pulp were extracted and purified by gel filtration chromatography and then on HPLC. Betanin (Betanidin 5-O-β–Glucoside), a preliminary betacyanin pigment present in major proportion was identified, whose chemical identity is established through IR, proton NMR, ^{13}C NMR analysis and co-chromatography studies with betacyanin standards. A strong molecular ion $[M+H]^+$ at 551 amu further confirmed the identity of betanin on the LC-MS studies. Betanin thus purified and chemically characterized is used to evaluate the antioxidant effects in quenching the free radicals that are generated *in vitro*.

In the first part of the study the molecular structure of betacyanin that is present in major proportion in the fruits of *O. ficus indica* was identified as betanin. Although, many studies on betacyanins revealed their antioxidant nature, none of the studies showed the free radical quenching effects of purified betacyanins in a systematic manner. Hence this part of the study was designed to focus on the specific free radical quenching effects of betanin, a preliminary betacyanin pigment on *in vitro* generated free radical systems such as DPPH, superoxide anion, nitric oxide and arachidonate peroxyl radicals. Apart from these, studies on the effect of betanin on inhibition of lipoxygenases, an enzymatic freeradical generator, was also evaluated.

3.2.1. 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical assay

Betanin showed potent scavenging effect of DPPH radical, as measured by ESR signals, in a concentration dependent manner with an IC_{50} of 25 µM (Fig. 16). At 85 µM, about 90% inhibition in the intensity of the ESR signal was obtained. Fig. 17 shows the characteristic spectral pattern of DPPH radical and its intensity when quenched by betanin, as measured by electron spin resonance spectroscopy.

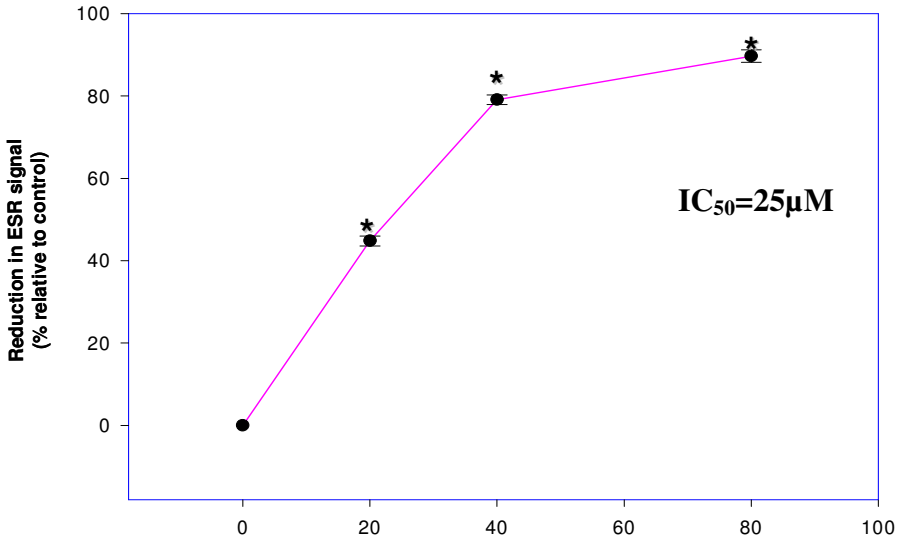


Fig. 16: Scavenging effect of betanin on DPPH radicals.
Data are mean ±SE from three independent experiments

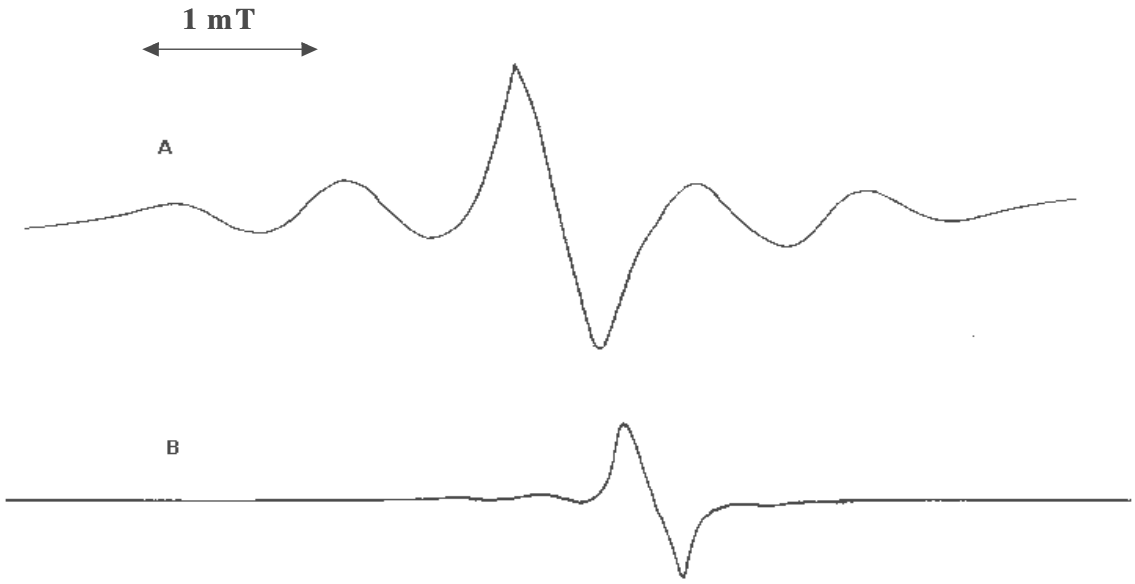


Fig. 17: ESR spectra of DPPH free radical in the absence and presence of betanin. (A) Control containing 0.5 M DPPH in ethanol; (B) DPPH 0.5 M solution incubated with 40 µM betanin. The spectra were recorded at frequency-9420, fixed magnetic field-335.3 mT, modulation width, 0.1mT, Microwave power-4mw, Amplitude-790.

3.2.2. Superoxide Anion assay

The ability of betanin to scavenge the superoxide anion free radical, generated by hypoxanthine-xanthine oxidase system, was monitored using DMPO spin trapping technique. The ESR signal intensity of DMPO-OOH adduct generated from hypoxanthine-xanthine oxidase system was inhibited by betanin very effectively with an IC_{50} value of 4µM (Fig. 18). Figure 19 shows the ESR spectrum of DMPO-OOH adduct formed from the reaction of hypoxanthin-xanthine oxidase, and its quenching pattern when incubated with betanin.

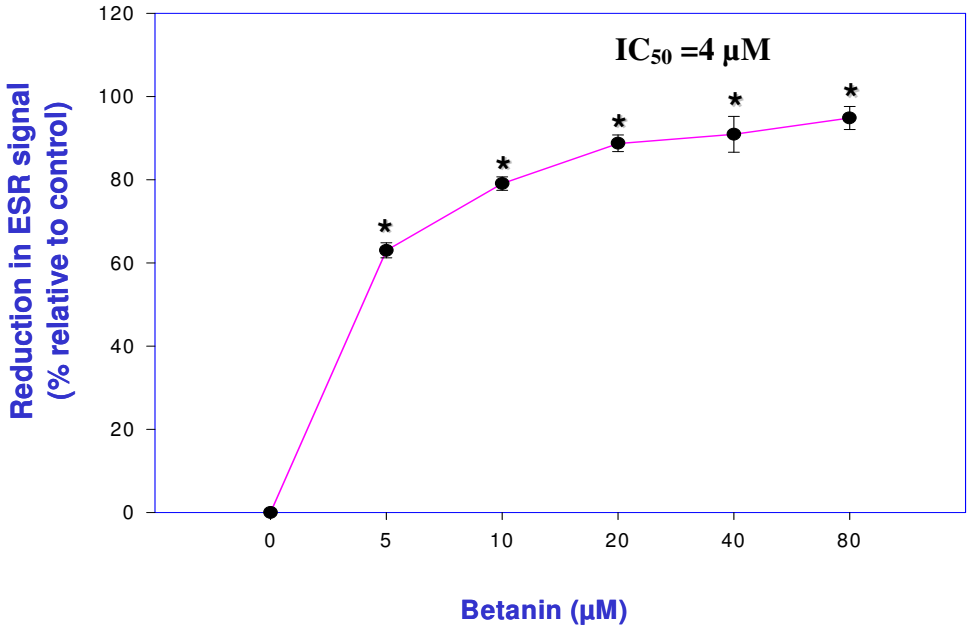


Fig. 18: Effect of Betanin on relative signal intensity of DMPO-OOH adduct formed from Superoxide anion and DMPO.
Data are expressed as mean ±SE. (n=3) (*) p<0.05 compared with control

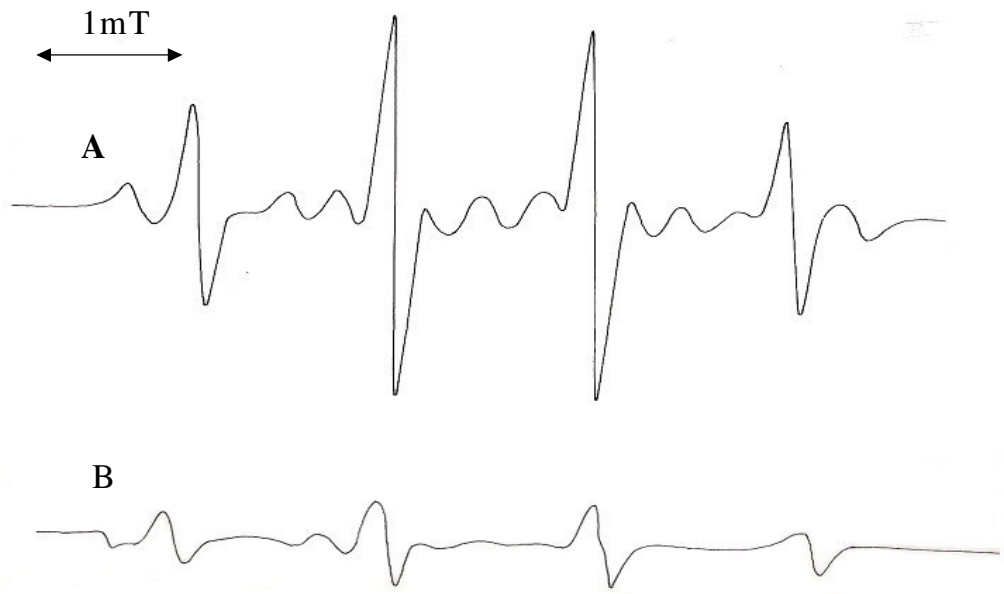


Fig. 19: ESR signals of DMPO-OOH adduct formed from Superoxide anion and DMPO.
(A) In the absence of betanin (B) In presence of betanin. The instrumental conditions set were JEOL X-Band ESR spectrometer, modulation amplitude 0.1 mT (100 KHZ), Scan range 10 mT, Scan time 2 min, Microwave Power 8mW.

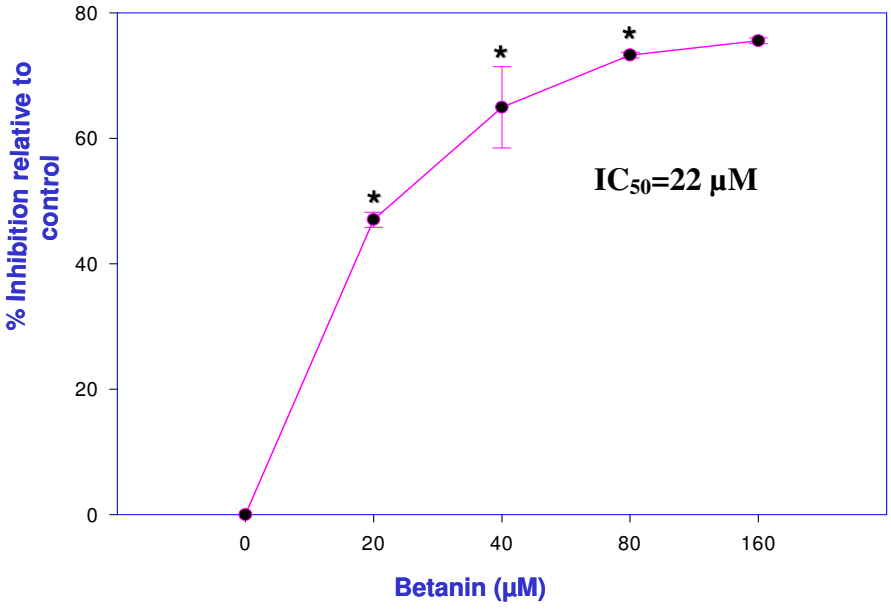


Fig. 20: Effect of Betanin on scavenging of nitric oxide radical generated from sodium nitroprusside.
Data are expressed as % inhibition of mean ±SE. (n=3) (*) P<0.05 compared with control

3.2.1. Nitric oxide scavenging assay (spectrophotometry)

Sodium nitroprusside serves as a chief source of nitric oxide radicals. The absorbance of the chromophore formed during diazotization of the nitrite with sulphinilamide and subsequent coupling with naphthalene diamine is used as the marker for nitric oxide scavenging activity. Betanin showed significant nitric oxide radical scavenging activity (up to 75%) at a concentration of 160 µM with an IC_{50} value of 22 µM. The % inhibition relative to control by various Betanin concentrations is plotted in Fig.20.

3.2.2. Effect of Betanin on arachidonate peroxyl radical

To investigate the reaction of peroxyl radical with antioxidant, the arachidonic acid peroxyl radical intensity was monitored as a function of betanin. Typical assay mixture contained 0.67g/lit lipoxygenase in 0.2M borate buffer pH (pH 9.0) with 0.64mM arachidonic acid which was mixed for 100ms and analyzed.

In the presence of Betanin, the decrease in the intensity of peroxyl radical signal correlates well with an increase in intensity of a secondary radical species with hyperfine split pattern that is characteristic of phenoxyl radicals, (Fig. 21).

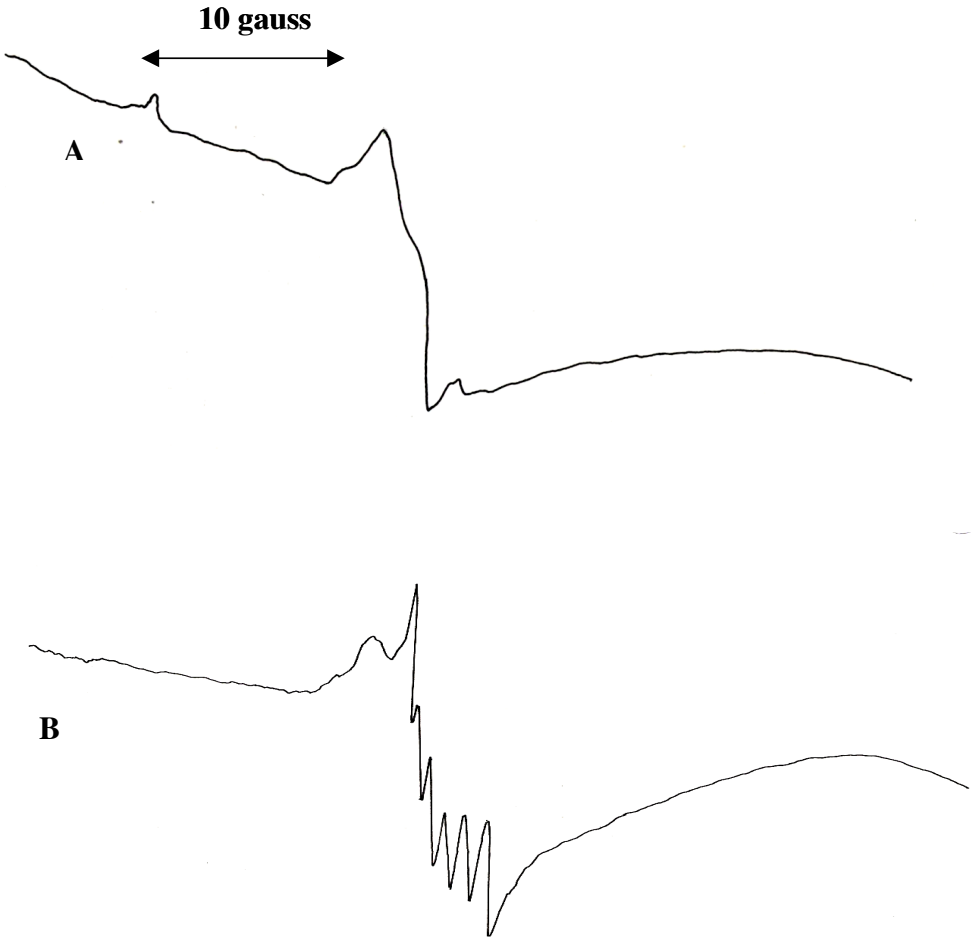


Fig. 21: Direct ESR spectra of the arachidonate peroxyl radical formed by mixing arachidonic acid with lipoxygenase in air-saturated 0.2M borate buffer (pH 9.0).
(A) 0.64mM arachidonic acid (containing 0.25% ethanol) mixed with 0.67-g/lit lipoxygenases, (B) Same as A in the presence of 40µM betanin. The spectra were recorded at gain, 6.3 X 10^6: modulation amplitude, 2.28G: power, 100 milliwatts; time constant, 5S; scan time 167 s and scan range, 100G.

3.2.3. Inhibition of 5-Lipoxygenase activity

The activity of 5- lipoxygenase (5-LOX) plays a vital role in initiation and progression of lipid peroxidation. As betanin showed potent antioxidant properties, its effect on 5-Lipoxygenase, the enzyme that initiates lipid peroxidation, was analyzed.

Typical reaction mixture contained 2.9 ml of buffer (0.1M phosphate buffer pH 6.3) and 100µl of enzyme 5-LOX incubated with different concentrations of betanin.10µl of 80mM arachidonic acid was used to initiate the reaction. Betanin inhibited the 5- lipoxygenase activity with an IC_{50} value of 7.5 µM (Fig. 22).

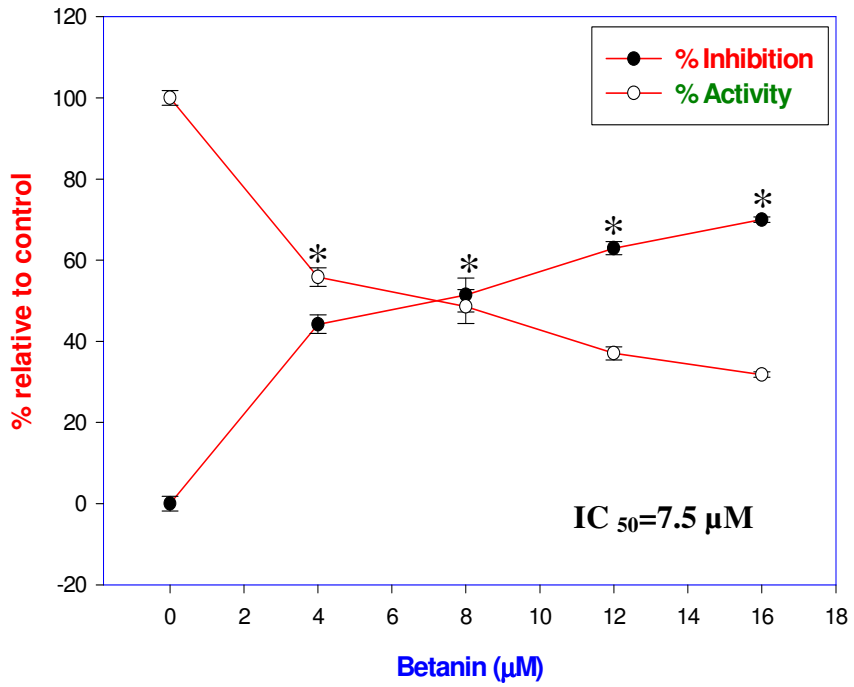


Fig. 22: Effect of betanin on 5-Lipoxygenase activity.
The reaction mixture contained 2.9 ml of buffer (0.1M phosphate buffer pH 6.3) and 100µl of enzyme 5-LOX incubated with different concentrations of betanin. The reaction is initiated with 10µl of 80 mM linoleic acid and monitored at 235 nm. Data are expressed as mean ±SE. (n=3) of % activity and % inhibition. (*) P<0.05 compared with control

To sum up, this part of the study evaluates the quenching effects of betanin to wards specific free radicals that are generated *in vitro*. Another important finding of the study is the inhibition of 5-lipoxygenase by betanin and the mechanism of inhibition. In view of the potent free radical scavenging effects of betanin, it is quite likely that betanin could play a protective role in many free radical mediated disorders.

The fore going studies demonstrate the potent antioxidant properties of betanin, isolated from fruits of *O. ficus indica*, under *in vitro* conditions. Hence, further studies were undertaken to appreciate the protective effects of betanin against free radical mediated disorders, under *in vivo* conditions. To evaluate this, Cisplatin- induced nephrotoxicity (mediated by oxidative stress) in rats was selected as a model of study.

3.3.1 Cisplatin induced nephrotoxicity

Cisplatin (cis-diamminedichloroplatinum [II]; CDDP) is a widely used chemotherapeutic agent for various cancers. As a side effect the drug induces nephrotoxicity and ototoxicity that is mediated by enhanced oxidative stress. In this study adult male rats were treated with a single dose of cisplatin (16 mg/kg body weight). The curative group received betanin 50 and 100 mg/Kg body weight for five days along with a single dose of cisplatin (16 mg/kg body weight). After 5 days the animals were sacrificed for evaluation of ultra structural changes in the kidneys and biochemical parameters such as renal function, lipid peroxidation and antioxidant enzyme activity levels.

3.3.2 Effect of betanin on renal function of rats treated with cisplatin

In order to test the effects of betanin on cisplatin-induced nephrotoxicity, adult male rats were treated with a single intraperitoneal dose of cisplatin (16 mg/kg body weight) and after 5 days the animals were sacrificed for evaluation of renal function. Rats treated with cisplatin showed typical signs of renal dysfunction as evident from the elevated levels of plasma creatinine and urea levels compared to that of cisplatin alone treated controls ($P < 0.05$) (Fig. 23 & 24). Treatment of animals with a single dose of cisplatin (16 mg/kg body weight) and betanin (50 and 100 mg/Kg body weight/ day for 5 days)

reduced the elevated levels of plasma creatinine and urea levels ($P < 0.05$).Both 50 and 100mg/ kg bw betanin treated groups showed similar decrease in plasma urea and creatinine levels, showing no dose-dependent effects.

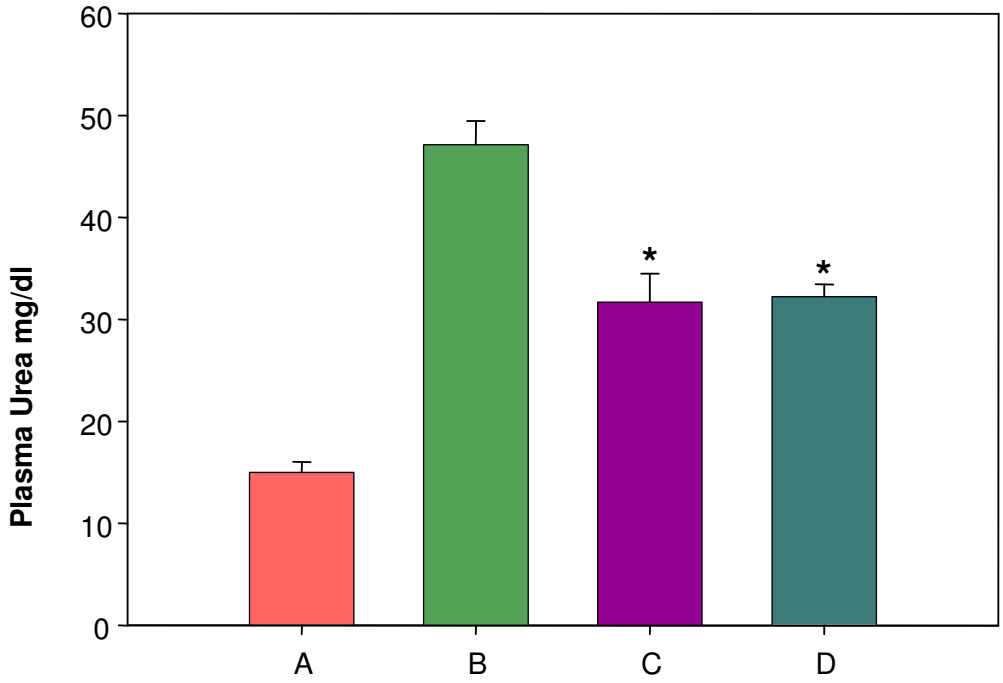


Fig. 23: Effect of Betanin on plasma urea levels in the rats treated with cisplatin. (A) Saline treated (B) Cisplatin (a single i.p dose of 16 mg/kg BW) treated (C) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (50 mg/Kg BW/day for 5 days) treated (D) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (100 mg/Kg BW/day for 5 days) treated. Data are expressed as mean ±SE. (n=6) (*) P<0.05 compared with cisplatin treated.

3.3.3. Effect of betanin on lipid peroxidation in rats treated with cisplatin

Lipid peroxidation as an index of oxidative stress was measured in terms of malondialdehyde (MDA) levels in the kidney. Elevated MDA levels were observed in the kidney tissues of the cisplatin treated group, in comparison to that of saline treated control. (Compare B to A in Fig. 25). Administration of betanin along with cisplatin decreased the MDA levels significantly compared to that of cisplatin treated, in a dose dependent manner (Fig. 25).

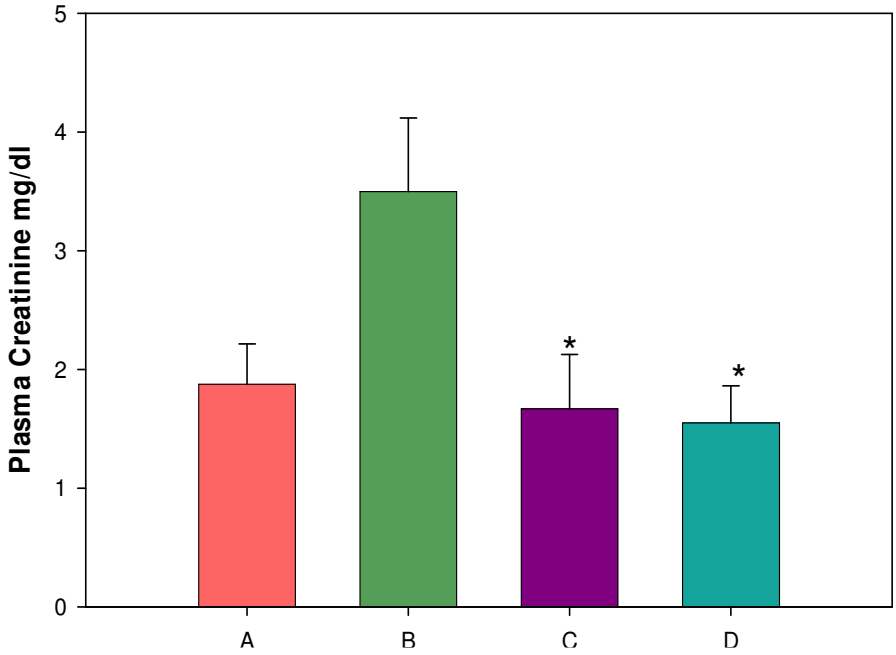


Fig. 24: Effect of betanin on plasma creatinine levels in the rats treated with cisplatin
(A)Saline treated (B) Cisplatin (a single i.p dose of 16 mg/kg BW) treated (C) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (50 mg/Kg BW/day for 5 days) treated (D) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (100 mg/Kg BW/day for 5 days) treated. Data are expressed as mean ±SE. (n=6) (*) P<0.05 compared with cisplatin treated.

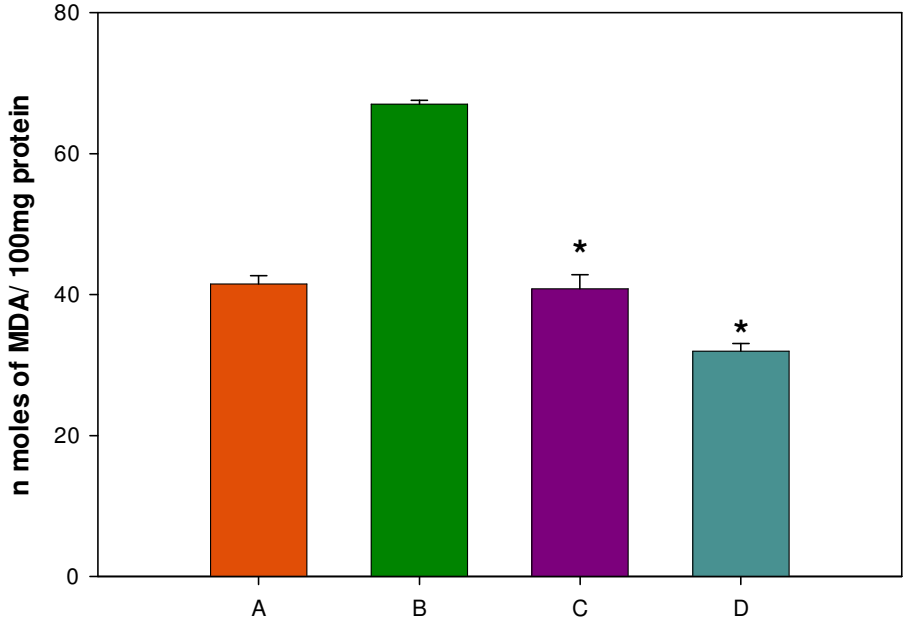


Fig. 25: Effect of betanin on MDA levels in the rats treated with cispaltin
(A) Saline treated (B) Cisplatin (a single i.p dose of 16 mg/kg BW) treated (C) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (50 mg/Kg BW/day for 5 days) treated (D) Cisplatin (a single i.p dose of 16 mg/kg BW) + Betanin (100 mg/Kg BW/day for 5 days) treated. Data are expressed as mean ±SE. (n=6) (*) P<0.05 compared with cisplatin treated.

3.3.4. Effect of betanin on activity levels of catalase in kidney of rats treated with cisplatin

Under stress conditions the cellular antioxidant defenses get disturbed. In the present study cisplatin administration markedly decreased the activity levels of catalase (compare lane-2 to lane-1 in Fig. 26), an endogenous antioxidant enzyme (Fig. 26), suggesting impaired antioxidant defenses. Betanin treatment (50 and 100 mg/Kg body weight) along with cisplatin restored the catalase activity levels to a maximum extent to that of saline treated control.

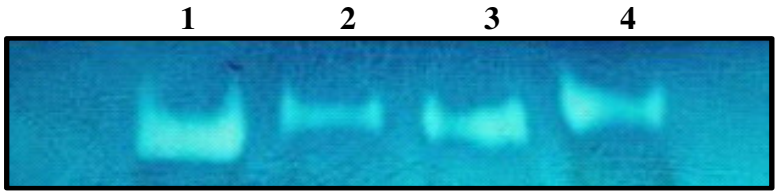


Fig. 26: (A) Effect of Betanin on catalase activity levels in the rats treated with cispaltin (Activity staining)
(1) Saline treated; (2) Cisplatin (a single i.p dose of 16 mg/kg BW) treated; (3) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (50 mg/Kg BW/day for 5 days) treated; (4) Cisplatin (a single i.p dose of 16 mg/kg BW) +Betanin (100 mg/Kg BW/day for 5 days) treated.

3.3.5. Effect of Betanin on ultra structural changes in kidney of rats treated with cisplatin

The impaired renal function induced by cisplatin was further confirmed by the changes observed in the morphological features as indicated by the transmission electron microscopic pictures (Fig. 27). Cisplatin treatment resulted in the loss of brush border membrane, alterations in mitochondrial shape, and changes in thickness of the basement membrane. Administration of betanin along with cisplatin, on the other hand, showed only minimal changes in the ultra structure of kidneys in a dose dependent manner.

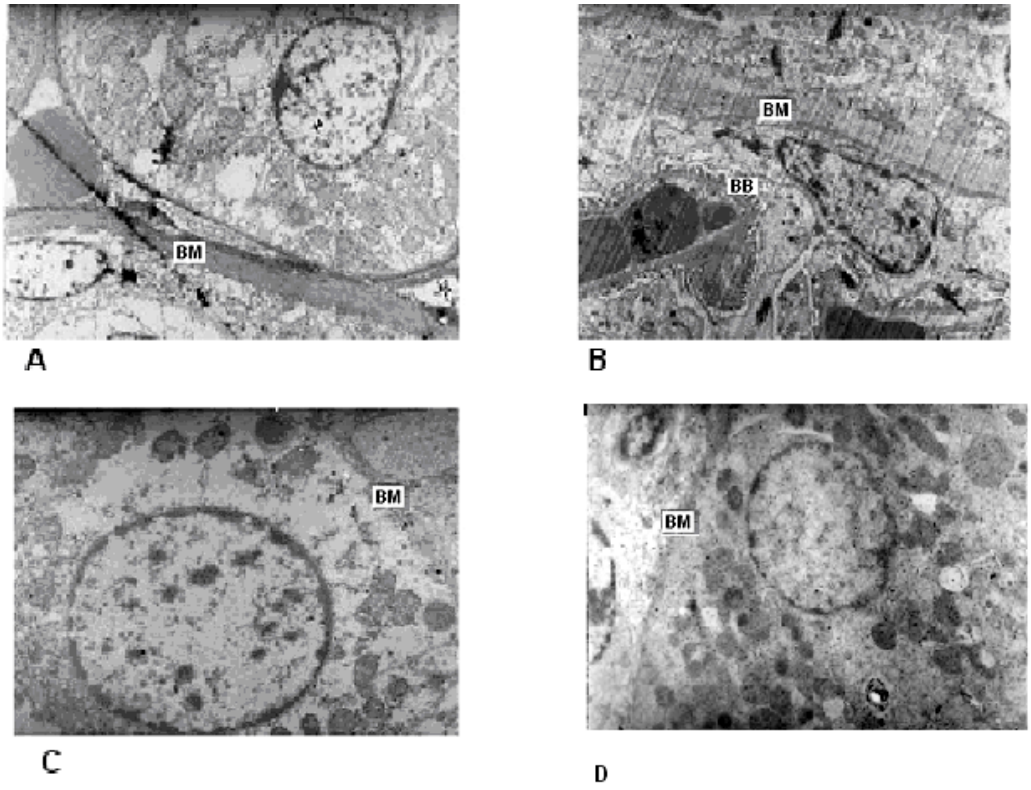


Fig. 27: Effect of Betanin on Ultra structural changes in the kidneys treated with cisplatin

(A) Saline treated (5700 X) (B) Cisplatin (a single i.p dose of 16 mg/kg BW) treated (5700 X) (C) Cisplatin (a single i.p dose of 16 mg/kg BW) + Betanin (50 mg/Kg BW/day for 5 days) treated (9000 X) (D) Cisplatin (a single i.p dose of 16 mg/kg BW) + Betanin (100 mg/Kg BW/day for 5 days) treated (6700 X). BM=Basement membrane; BB=Brush border membrane.

Majority of the plant pigments identified so far showed anti-proliferative effects when tested on a wide range of cancer cell lines *in vitro*. The examples of such pigments include anthocyanins, chlorophylls, carotenoids etc. In this connection studies were initiated to know whether betanin, being an antioxidant pigment could induce any antiproliferative effects in cancer cells. To achieve this human chronic myeloid leukemia cell line K562 was employed.

3.4.1. Effect of Betanin on the growth of K562 cell line

The effect of betanin on growth of K562 cells was evaluated by MTT assay. The cells were treated with different concentrations of betanin (0, 20, 40, 80 µM) for 12, 24 and 48 h. A dose dependent decrease in K562 cell growth until 48 h after betanin treatment was observed, with 50% decrease in cell proliferation at 40 µM betanin for 24 h (Fig. 28). Further studies were carried out to analyze the mode of cell death on cells exposed to 40 µM betanin for 24 h.

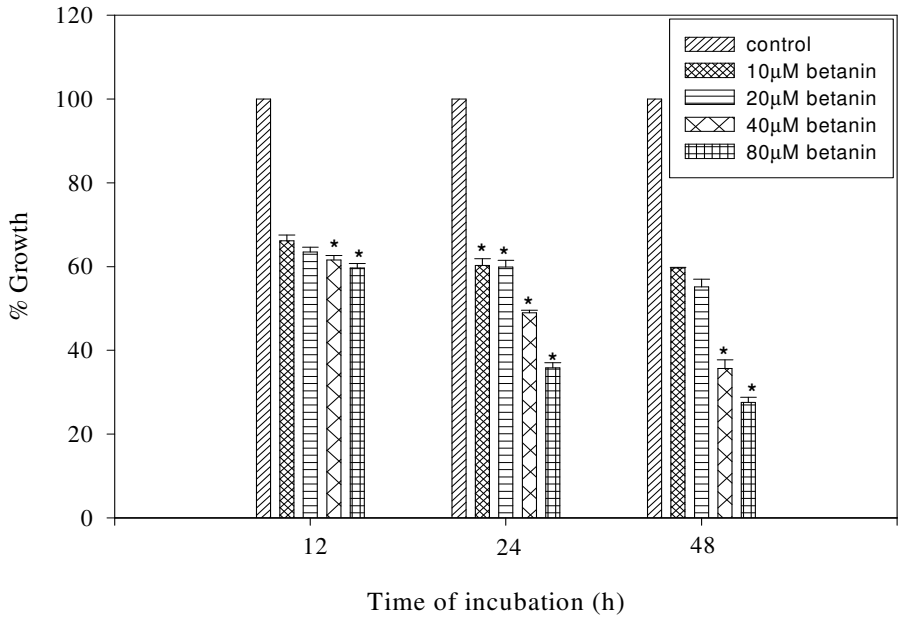


Fig. 28: Effect of betanin on proliferation of K562 cells.
The cells were cultured in 10% FBS medium and treated with betanin (0, 10, 20, 40, 80 µM) for 12, 24 and 48 h. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. Values were expressed as mean ± SD of three independent experiments (* P < 0.05).

3.4.2. Morphological and ultrastructural changes induced by betanin in the K562 cells

K562 cells treated with (40 µM) or without betanin for 24 h were viewed under phase contrast microscope. Betanin treated cells showed web like activated membrane structures associated with fragmented nuclei (Fig. 29). Scanning and transmission electron microscopic studies were carried out to observe the morphological and ultrastructural changes induced by betanin in the K562 cells. Morphological features pertaining to apoptotic cell death such as membrane blebbing was clearly observed (Fig. 30). The transmission electron microscopic studies revealed the condensation of chromatin and loss of intra cellular details (Fig. 31).

A **B**

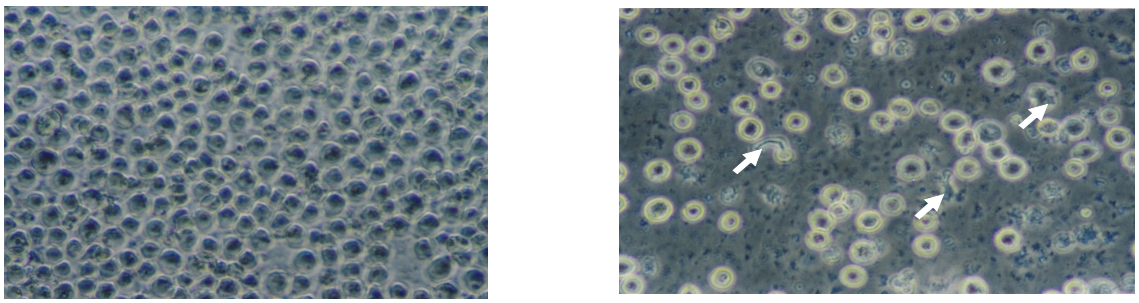


Fig. 29 Light microscopic pictures (40 X magnification) showing the morphology of K562 cells treated with betanin.
(A) Control K562 cells (B) K562 cells treated with 40 µM betanin for 24 h. Arrows indicate the apoptotic bodies or nuclear condensation.

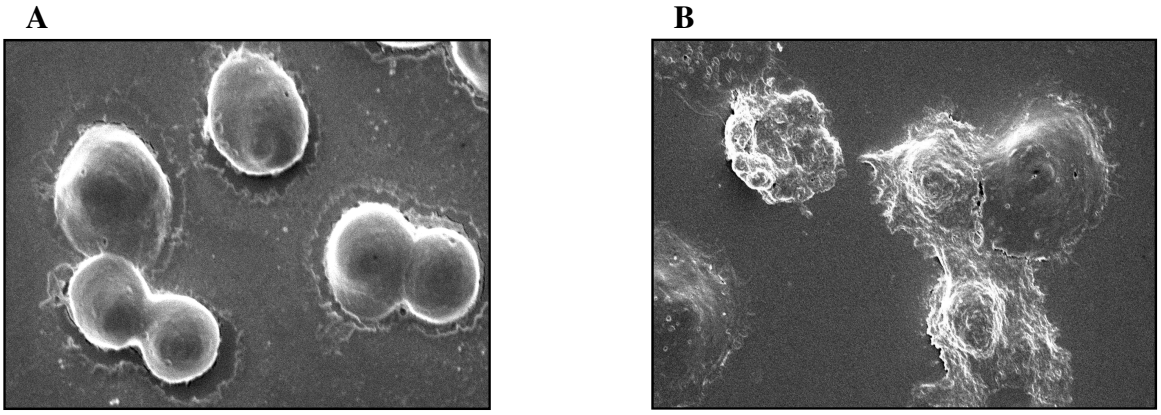


Fig. 30: Scanning electron microscopic studies showing ultra structural morphology in K562 cells treated with 40 µM betanin.
(A) Control K562 cells (B) K562 cells treated with 40 µM betanin for 24 h. Membrane blebbing is seen in the betanin treated cells (2700 X).

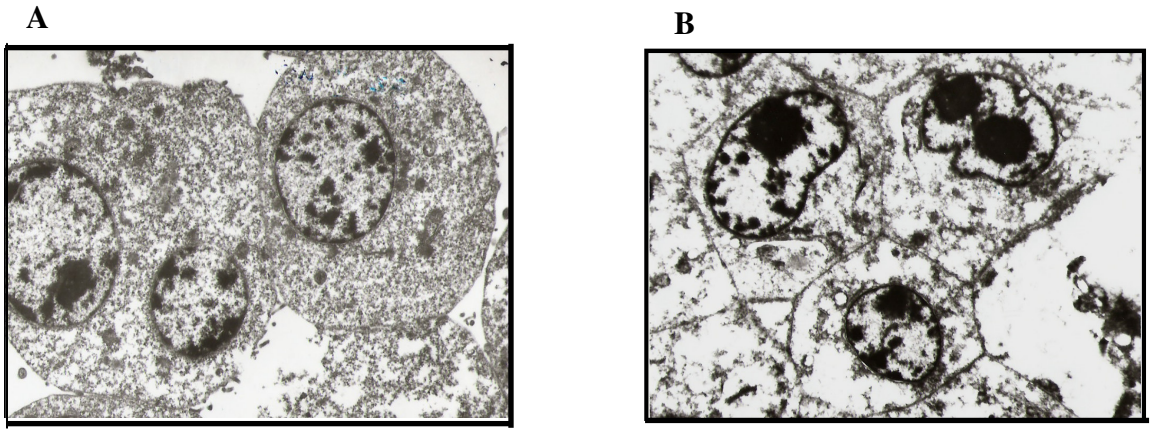


Fig. 31: Transmission electron micrographs of K562 cells treated with 40 µM betanin.
(A) Control K562 cells (B) K562 cells treated with 40µM betanin for 24 h. Nuclear condensation and loss of intracellular details are clearly observed in the betanin treated cells (3000 X).

3.4.3. Effect of betanin on cell cycle profile of K562 cells (Flow cytometric analysis)

The induction of apoptosis in cells treated with betanin was further confirmed by flow cytometric analysis of DNA content. K562 cells treated with (40 µM) or without betanin for 24 h were stained with propidium iodide and subjected to FACS analysis. Fig. 32 illustrates the DNA content histograms of these cells. These studies showed 28.4% of betanin treated cells at sub G0/G1 phase, when compared to 3% in untreated cells.

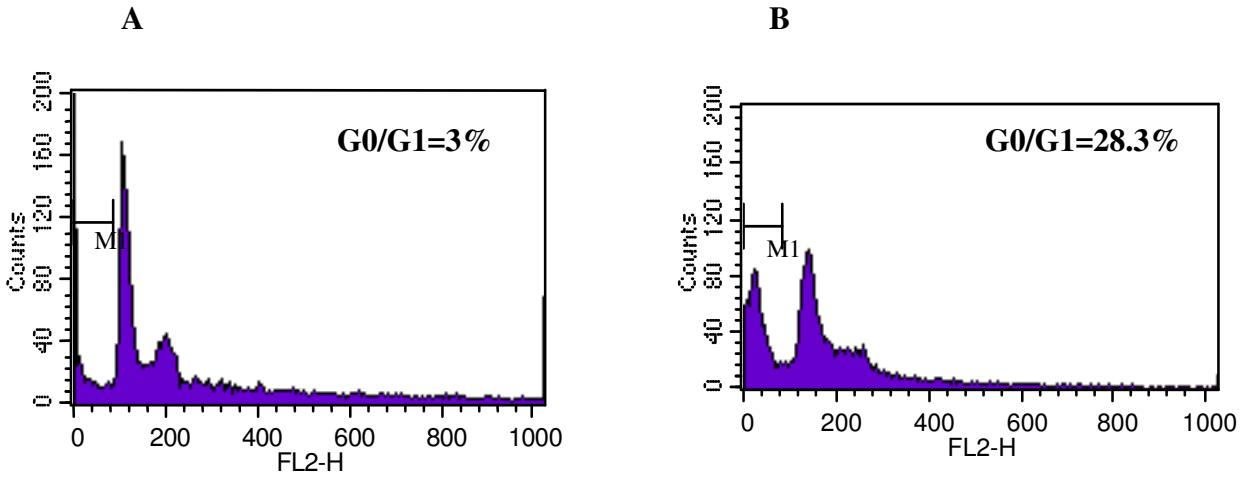


Fig. 32: Flow cytometric analysis of the control and betanin treated K562 cells. Cells treated with or with out betanin for 24 h were fixed and stained with propidium iodide and the DNA contents were quantified by Flow cytometer. The number of hypo diploid (sub-G0/G1 phase) cells is expressed as a percentage of the total number of cells. (A) Control K562 cells (3%), (B) K562 cells treated with 40 µM Betanin (28.4%).

3.4.4. Mitochondrial membrane potentials (Flow cytometric analysis)

The decrease in mitochondrial membrane potential ($\Delta\Psi m$) is associated with mitochondrial dysfunction (Seuduto and Grotyohann, 1999). In the present study changes in the membrane potential of K562 cells exposed to 40 µM Betanin was investigated. The cells were harvested and changes in the mitochondrial membrane potentials were measured by the uptake of lipophilic cation- Rhodamine 123 into mitochondria.

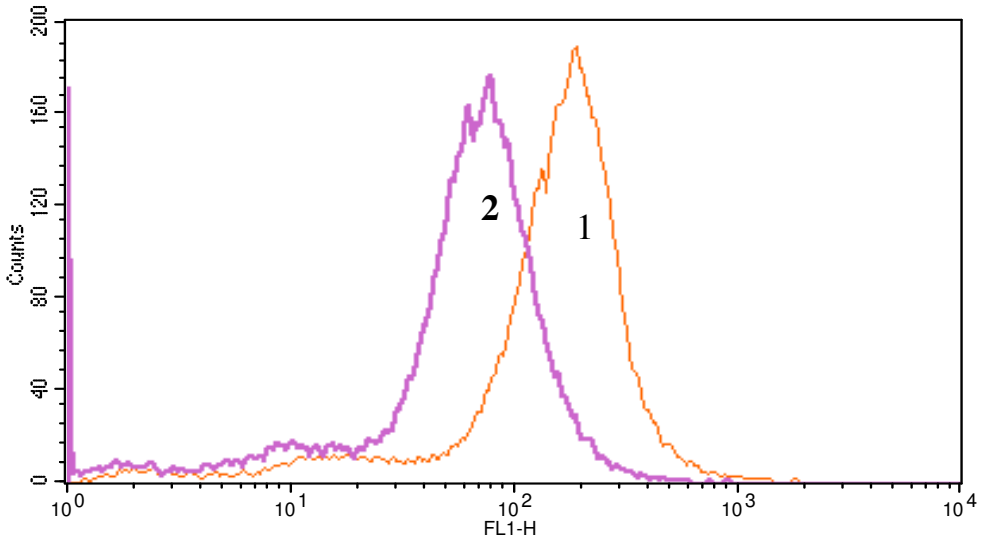


Fig. 33: Flow cytometric analysis showing the disruption of mitochondrial membrane potentials:
Induction of mitochondrial dysfunction and relative mitochondrial membrane potential (ΔΨm) was measured by fluorescent emission from rhodamine 123 uptake by mitochondria. K562 cells were treated with 40 µM betanin for 24 h and stained with rhodamine 123 and subjected to FACS analysis. Histogram-1 represents fluorescence intensity from control K562 cells. Histogram-2 represents fluorescence intensity from K562 cells treated with betanin 40 µM.

Untreated control cells were used to determine the normal uptake of rhodamine and the percentage of treated cells with low membrane potentials was calculated. Fig. 33 shows the decrease of 70 % of fluorescence intensity in the cells treated with 40 µM betanin, compared to that of control, indicating the leakage in the mitochondrial membrane potentials.

3.4.5. Betanin effects on cytochrome c release, Bcl-2 and Bax proteins in K562 cells.

The process of apoptosis is associated with the disruption of mitochondrial membrane potentials, which results from the opening of permeability transition pores in the mitochondrial membrane leading to the release of cytochrome c. To determine whether there is any release of cytochrome c from the mitochondria into the cytosol, cytosolic fractions from the cells treated with 40 µM betanin for indicated times, were subjected to Western blot analysis. A time dependent elevation in the cytosolic levels of cytochrome c with maximum increase at 12 and 24 h after betanin treatment was observed (Fig. 34A). Furthermore, the expression levels of Bcl-2 and Bax proteins are associated with mitochondrial membrane integrity and play a crucial role in the regulation of apoptosis. Time dependent decrease of Bcl-2 protein levels was observed after betanin treatment (Fig. 34 A), but no appreciable change in the levels of Bax protein was observed at all the time periods (Fig. 34 A). These results suggest a disturbed Bcl-2/Bax ratio in the cells treated with betanin.

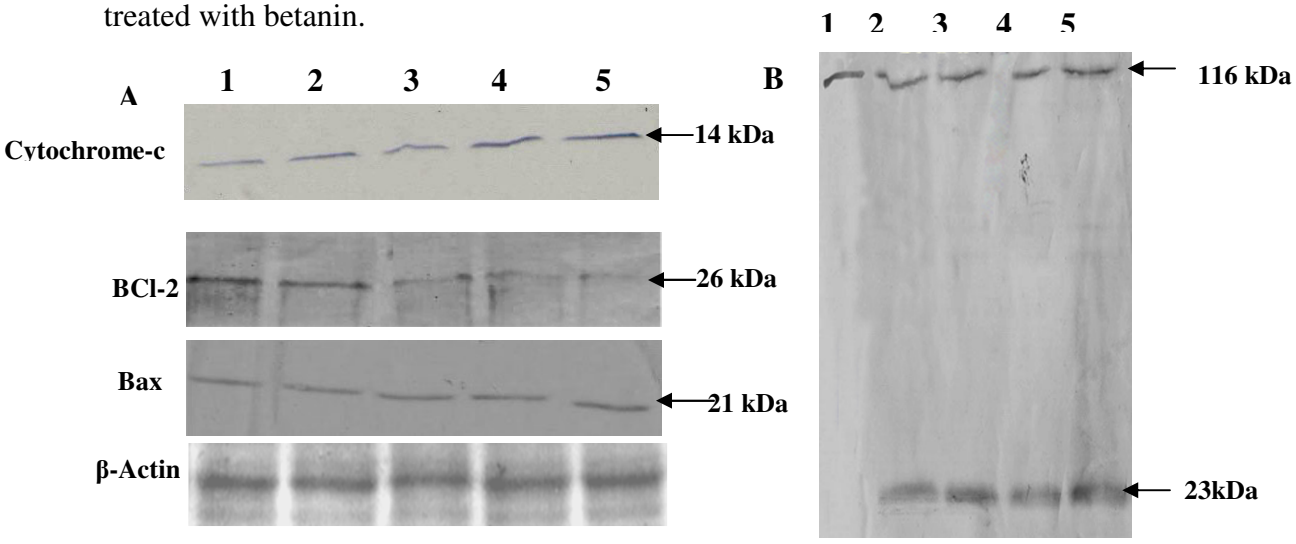


Fig. 34: Western blot analysis showing the effect of betanin on (A) cytochrome c release, Bcl-2 protein, Bax protein, Beta actin and (B) PARP (Poly (ADP-ribose) polymerase), cleavage. Equal amounts of protein (50 µg) from the K562 cells treated with 40 µM betanin for the indicated times (0, 4, 8, 12, 24 h) were analyzed by 15% SDS–PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with monoclonal cytochrome c, Bcl-2 and Bax antibodies and polyclonal PARP antibodies respectively. Lane 1: 0 h; lane 2: 4h; lane 3: 8h; lane 4: 12 h; lane 5: 24 h.

3.4.6. PARP cleavage in response to betanin treatment

PARP, poly (ADP ribose) polymerase, is a nuclear enzyme implicated in many cellular processes including apoptosis and DNA repair. During apoptosis PARP (116 kDa) is cleaved to yield two fragments of 85 and 23 kDa. To determine whether PARP is cleaved in betanin induced cell death, K562 cells were treated with 40 μM betanin for indicated times (0, 4, 8, 12, 24 h) and PARP cleavage was monitored by employing PARP antibodies that recognize the 23 kDa fragment of cleaved PARP and uncleaved 116 kDa full length PARP. The data presented in Fig. 34 B show the gradual increase in the appearance of 23 kDa signature fragment of PARP cleavage at all the indicated times (lanes 2-5). However, in control cells (lane-1) no 23 kDa fragment of PARP was observed, except the uncleaved 116kDa protein.

3.4.7 Intracellular localization of Betanin in K562 cells

In order to find out whether betanin enters the K562 cells, confocal microscopic studies were undertaken on cells exposed to betanin (40 μM) for 24 h and washed to remove non-specific binding of betanin. These studies showed a strong fluorescence signal in the cytoplasm of the cells treated with the betanin (Fig. 35 B), with no such signal in the untreated cells (Fig. 35 A).

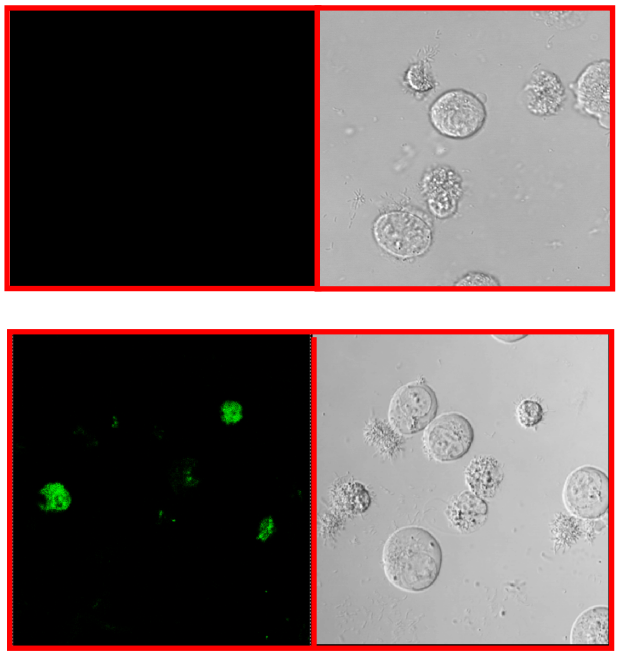


Fig. 35: Confocal image showing the localization of betanin in K562 cells (A) Control cells (B) Betanin (40 µM) treated cells for 24 h showing intracellular localization of betanin.

In summary, betanin showed potent antiproliferative effects in human chronic myeloid leukemia cell line-K562. It is also evident from the experimental results that betanin executes apoptosis through intrinsic pathway of cell death, by altering the mitochondrial membrane integrity, cytochrome-c leakage and PARP cleavage. These antiproliferative effects of betanin further adds value to its nutarceutical value in addition to its use as a food colorant.

4.0. Discussion

The process of oxidation is essential to many living organisms to derive energy that fuels various biological processes. However, oxygen-centered free radicals and other reactive oxygen species that are generated continuously *in vivo* are highly reactive causing oxidative damage to the cells and tissues. Although all the organisms are well equipped with antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage, specifically under stress conditions. This oxidative damage was shown to be responsible for ageing and age related disorders such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). However, antioxidant supplements, or foods containing antioxidants may be used to reduce the oxidative stress and there by prevent the related disorders.

In view of serious side effects posed by the antioxidant supplements from synthetic origin, the focus is now on antioxidants from natural origin. Dietary intake of a number of phytochemicals was shown to have health benefits by limiting the oxidative damage exerted by reactive oxygen species. Natural pigments are important in showing various beneficial biological activities. These include C-Phycocyanin (Subhashini et al., 2004; Pardhasaradhi et al., 2005), Anthocyanidins (Kamei et al., 1995; Bomser et al., 1996; Nagase et al., 1998; Hou et al., 2001; Meiers et al., 2001) and Curcumin (Samaha et al., 1997; Kuo et al., 1996; Jiang et al., 1996 b). Apart from their antioxidant potentials, these natural pigments exert antiproliferative effects. The mechanisms involved in these effects include: (i) induction of alterations in the cell differentiation pattern, which plays a vital role in the invasiveness and metastatic progression of the tumors, (ii) blockade of

pre neoplastic cell expansion or induction of apoptosis, and (iii) intervention of metabolic activation of carcinogens by scavenging ROS.

Betalains are another class of important natural pigments which are found in the plants of the order caryophyllales (Clement and Mabry 1996) and in some genera of the besidiomycetes. Betalains comprise red-purple betacyanins and the yellow betaxanthins. Betalains find their importance as food colorants as they provide attractive bright hues for food products (Stinzing, 1999). Recently pharmacokinetic studies on these pigments in human subjects were conducted (Netzel et al., 2005). There has been growing interest in the use of these natural pigments for food coloring, since synthetic dyes are becoming more and more critically assessed by the consumer, both from the ends of quality and health. Apart from serving as food additives, the natural pigments also play a vital nutraceutical role in circumventing the health hazards posed by synthetic and chemical colorants. Betalains are one such nutraceutical nitrogenous cationic natural pigments that are widely used as food additives.

Opuntia ficus indica is a perennial plant that grows all over the world and belongs to the family of cactaceae. A wide range of studies demonstrated the usage of this herbal extracts in management of many disorders. Ethanol extracts of *O. ficus indica* showed potent anti-inflammatory properties (Park et al., 2001a). Pre-treatment with lyophilized cladode extracts in the rats revealed a protective action against ethanol-induced ulcer formation (Galati et al., 2001). In another study the methanolic extracts of stems reported to possess wound-healing properties (Park et al., 2001b). The fruits of *O. ficus indica* are very good source of water-soluble nitrogenous chromo alkaloids called betalains. Unlike other pigments betalains are not very well studied with regard to their pharmacological

properties and mechanism of action. Hence, in the present study we evaluated the antioxidant, nephroprotective and anti-proliferative effects of betanin, a principle betacyanin pigment isolated from the fruits of *O. ficus indica.*

4.1 Isolation and identification of betacyanins present in the fruits of *Opuntia ficus indica*

A number of different forms of betacyanins are known to exist and coexist in the *Opuntia* species. However the concentration and kind of the betacyanin present in the species is dependent on factors like environment and geographical origin. In the present study the betacyanins isolated from the fruits of *O. ficus indica* were identified to be betanin and Isobetanin based on the retention times exhibited by the compounds in the HPLC analysis and co-chromatography with standards. The absorbance maxima at 535nm confirmed the presence of betacyanins. Betanin, a primary betacyanin pigment appears to be present in a major proportion whose chemical identity is established through IR, proton NMR and ^{13}C NMR studies. A strong molecular ion $[M+H]^+$ at 551 amu further confirmed the identity of betanin in the LC-MS studies. Presence of betanin in higher quantities in the fruits of *O. ficus indica* is in agreement with the earlier reports (Stinzing et al 2001, 2002).

4.2. Antioxidant effects of Betanin

Betalain pigments are reported to possess potential antioxidant properties (Kanner et al., 2001). In agreement with these studies, the present study specifically demonstrated the scavenging activity of betanin upon various types of free radicals. Betanin inhibited DPPH radical with an IC_{50} value of 25 µM while superoxide and nitric oxide radicals were inhibited with IC_{50} values of 4 µM and 22 µM respectively. Betanin decreased the

signal intensity of arachidonate peroxyl radical generated from 5-lipoxygenase reaction and resulted in a secondary radical species with characteristic hyperfine splitting. This observation further draws support from the inhibition of 5-Lipoxygenase activity *in vitro* with an IC_{50} value of 7.5 µM. Lipoxygenase proteins have a single polypeptide chain with a molecular mass of 75-80 kDa in animals and 94-104 kDa in plants. The proteins have an N-terminal β-barrel domain and a larger catalytic domain containing a single atom of non-heme iron. The metal is liganded to conserved histidines and to the carboxyl group of a conserved isoleucine at the C-terminus of the protein. The enzymes are usually in the ferrous (inactive) form when isolated. Oxidation to the active ferric enzyme is required for catalysis. This strong lipoxygenase inhibition by betanin seems to be achieved through the reduction of iron to the ferro inactive form. It is already proposed that the capacity to reduce the iron ion determines the antioxidant activity of some phenolic compounds to inhibit soybean lipoxygenase type-I (Kemal et al 1987; Vanderzee et al., 1989) or 5- lipoxygenase (Laughton et al., 1991).

4.2 Nephroprotective effects of betanin

Cisplatin is a widely used chemotherapeutic agent for various cancers. But its usage is limited due to its associated complications such as nephrotoxicity and ototoxicity (Kuhlmann et al., 1997). A number of studies have demonstrated the role of peroxidative damage and impaired antioxidant systems in the cisplatin induced renal failure. Reactive oxygen species (ROS) including the super oxide anion ($O_2^{\cdot -}$), and hydroxyl ($HO^{\cdot}$) radical and their metabolites (H_2O_2, HOCl) have been implicated in the pathogenesis of cisplatin induced renal failure (Inselmann et al., 1995; Yilmaz et al., 2004; Mansour et al., 2002; Baek et al., 2003). It has been found that the renal content of total nitrate/nitrite

is increased in cisplatin treated rats (Yildirim et al., 2003; Ozen et al., 2004), suggesting that nitric oxide production is enhanced in these animals. Administration of amino guanidine (Mansour et al., 2002) or N [ω]-nitro-L-arginine methyl ester (L-NAME) (Srivastava et al., 1996), which are potential inhibitors of nitric oxide synthase, was shown to decrease renal damage induced by cisplatin, suggesting that nitric oxide is playing a crucial role in these experimental models. Similarly supplementation with antioxidants such as melatonin (Sener et al., 2000), Selenium (Antunes et al., 2000) Vitamin E, and Vitamin C (Appenroth et al., 1997), dithiocarbamate (Somani et al., 1995) and Mn SOD (Davis et al., 2001) also prevented the cisplatin-induced nephrotoxicity, which further confirms the role of oxy radicals in cisplatin-induced nephrotoxicity. Present study is to evaluate the antioxidant potentials of betanin, a primary betacyanin pigment isolated from fruits of *O. ficus indica*, and evaluate its usefulness in ameliorating the cisplatin induced renal damage.

Administration of betanin along with cisplatin in rats prevented the cisplatin-induced increase in plasma urea nitrogen (PUN) and creatinine, the markers of kidney damage, This property of betanin appears to be mediated by the improvement in antioxidant defenses as evidenced by the increase in the activity of catalase, with concomitant decrease in the lipid peroxidation in the renal tissue upon treatment with betanin. The cytoprotective effects of betanin can also be explained by its ability to inhibit 5-lipoxygenase, an enzyme that is implicated in the initiation and progression of lipid peroxidation (Kanner et al., 2001). Restoration in the levels of catalase suggests that the betanin treatment is capable of protecting the redox state of the kidneys during cisplatin intoxication. Superoxide and nitric oxide radicals have a potential role in the

pathogenesis of cisplatin-induced nephrotoxicity (Yolanda et al., 2004). The *in vitro* results obtained in the present investigation showed dose dependent scavenging of superoxide anion and nitric oxide radicals by betanin, which further supports the preventive role of betanin in cisplatin-induced nephrotoxicity. This effective scavenging of superoxide anion and nitric oxide radicals by the treatment of betanin may be responsible for preventing the formation of peroxynitrite radicals, which play a pivotal role in renal toxicity. Transmission electron microscopic studies on renal tissues of rats treated with cisplatin indicate the loss of brush border membrane, alterations in mitochondrial shape, and changes in thickness of the basement membrane. Betanin treatment along with cisplatin prevented cisplatin-induced ultra structural changes in the renal tissue in a dose dependent manner.

In summary the above studies demonstrated that betanin, an antioxidant pigment purified from fruits of *O. ficus indica,* prevents cisplatin-induced nephrotoxicity in rats by its free radical quenching antioxidant properties. Thus betanin has a promising role in the treatment of acute renal injury induced by cisplatin. Further studies are required to study the clinical applications and underlying molecular mechanisms by which betanin confers cyto protection from nephrotoxicants.

4.3 Anti-proliferative effects of betanin

Cancer chemoprevention is defined as inhibition of tumor initiation, promotion and progression by employing pharmacologic or natural agents that prevent the metabolic activation of procarcinogens (Hong and Sporn, 1997). A number of natural phytochemicals and antioxidants such as C-phycocyanin (Subhashini et al., 2004; Pardhasaradhi et al., 2005), Gallic acid (Inone et al., 1994), Capsaicin (Wolvetang et al.,

1996), Gingerol (Samaha et al., 1997), -(-) epigallocatechin gallate (Suganuma et al., 1996, 1999), Resveratrol (Clament et al., 1998) etc., were shown to have anticancer properties. In the present study we have analyzed the effects of betanin, a principle betalin pigment isolated from *O. ficus indica,* on human chronic myeloid leukemia cell line, K562. Further in-depth studies were taken up to understand the molecular mechanisms involved in betanin-induced cell death. We found that betanin induced dose dependent and time dependent inhibition in the growth of K562 cells, with IC_{50} of 40 µM. Apoptosis is a process of cell death that is critically regulated based on the expression of cell's intrinsic suicide machinery (Vaux and Korsmeyer, 1999), which further leads to the characteristic pattern of morphological, biochemical, and molecular changes. The present study showed typical apoptotic characteristics such as membrane blebbing and chromatin condensation in the cells treated with betanin. The flow cytometric analysis of betanin treated cells showed an increase in the hypo diploid apoptotic DNA content with a decrease in the number of cells at the S and G2 phases of the cell cycle. Accumulating scientific evidences indicate the pivotal role of mitochondria in the execution of apoptosis of the cells exposed to various stimuli (Desagher and Martinou, 2000; Green and Reed, 1998). The present studies showed decreased mitochondrial membrane potential with simultaneous appearance of cytochrome c in the cytosolic fractions of cells exposed to betanin. These studies suggest that betanin, after gaining entry into the cell, somehow induces the mitochondrial disruption leading to decreased membrane potentials and leakage of mitochondrial cytochrome c into the cytosol. Confocal microscopic studies support such a possibility of entry of betanin into the cells. Further decrease in expression of Bcl-2 protein coupled with a decrease in the

Bcl-2/Bax ratios strongly support mitochondrial membrane alterations in the cells treated with betanin. Caspase-3, an executioner caspase in apoptotic cascades is implicated in the cleavage of a number of proteins, including Poly (ADP-ribose) polymerase (PARP), which is a hallmark of apoptosis. In the present study a time dependent increase in the PARP cleavage in cells treated with betanin (40 μM) was observed, suggesting the activation of caspase-3 activity. The foregoing studies, thus, clearly demonstrate that betanin induces apoptosis in K562 cells by intrinsic death pathway. Further studies, however, are required to understand the mechanisms involved in betanin-induced alterations in mitochondrial membrane integrity in K562 cells.

This part of the study demonstrates that betanin, a natural betacyanin pigment isolated from the fruits of *O. ficus indica*, enters K562 cells and alters mitochondrial membrane integrity, leading to cytochrome c leakage, activation of caspases and nuclear disintegration. These biochemical alterations are reflected in morphological and ultra structural changes, typical of cells undergoing apoptosis. These findings on anticancer effects of betanin, further adds value to the nutritional characteristics of the fruits of *O. ficus indica.*

Conclusions

1. Oxidative stress is an imbalance between cellular antioxidants and prooxidants that tilts either towards excess generation of reactive oxygen species (ROS) and/or decreased antioxidants. Oxidative stress is associated with the onset and progression of majority of the human disease conditions that range from coronary heart diseases to cancers.
2. Although the cell is equipped with cellular antioxidant defense mechanisms to prevent and counteract free radical generation, these defense mechanisms are insufficient to control under stress conditions eventually leading to disturbed cellular homeostasis.
3. Antioxidant supplementation, therefore, plays a critical role in the prevention and/or delay the progression of the degenerative diseases. In view of serious side effects of various synthetic antioxidants, there is growing demand for the natural antioxidants.
4. Betalains are pigmented chromo alkaloids with potent antioxidant properties. Red-purple betacyanins and yellow betaxanthins are the two important classes of betalains found in the members of caryophyllales.
5. In order to identify and understand the bioactivities of pigmented antioxidants, we have selected, *O. ficus indica*, which is a rich source of betalain pigments. Preparative HPLC was carried out to purify the betalain pigments present in the fruits of *O. ficus indica.*

6. The structural identification of betalain pigments was carried out by employing analytical techniques such as IR, Proton NMR, ^{13}C NMR, LC-MS and HPLC.
   a) The HPLC profile of betalains from *O. ficus indica* showed two peaks with retention times 27.2 min and 28.2 min. that exactly correlated with that of betanin and isobetanin standard retention times.
   b) The major peak (peak-1) with retention time of 27.2 min from HPLC analysis showed maximum absorbance at 535 nm, characteristic of betacyanin pigments.
   c) IR spectrum showed the presence of bands at 1700 and 3500 cm^{-1} corresponding to carboxylic acid groups of betanin.
   d) ^{13}C NMR spectra showed the allocation of carbon atoms in the betacyanins, while the Proton NMR spectrum showed peaks at δ 5.0 ppm (d, J = 6.5 Hz, ^{1} H) attributable to anomeric carbon and other signals at δ 8.2, 7.4, 6.6 and 4.4 ppm indicating the presence of aromatic component of betanin.
   e) HPLC co-chromatography studies of peak-1 and standard betanin resulted in a single peak, suggesting their co-elution.
   f) LC-MS analysis of betacyanins showed a strong molecular ion $[M+H]^{+}$ at 551 amu, which further confirmed the identity of peak-1 as betanin.
   g) Based on the above studies, the major pigment present in the fruits of *O. ficus indica* was identified as betanin, with a molecular mass of 551 amu.

7. The antioxidant effects of betanin, against various radical species were evaluated. Betanin showed potent scavenging effect on DPPH, superoxide anion and nitric oxide radicals with an IC_{50} values of 25 µM, 4 µM and 22 µM respectively. Betanin also inhibited the signal intensity of arachidonate peroxyl radical generated from

5-lipoxygenase reaction and resulted in the formation of a secondary radical species with a hyperfine splitting pattern that is comparable with that of phenoxyl radicals. Betanin also inhibited 5-lipoxygenase with an IC_{50} value of 7.5 µM.

8. In the light of potent antioxidant properties of betanin recorded *in vitro*, further studies were undertaken to evaluate its efficacy in preventing the tissue oxidative damage under *in vivo* conditions. As cisplatin is known to induce nephrotoxicity mediated by oxidative stress, an animal model of cisplatin-induced nephrotoxicity was employed for evaluating the protective effects of betanin.
    a) Administration of single dose of cisplatin (16-mg/Kg bodyweight) in Wistar rats resulted in increase in the nephrotoxicity that is mediated by oxidative stress. Administration of betanin (50 and 100 mg/kg body weight) along with cisplatin in rats prevented the increase in plasma urea nitrogen (PUN) and creatinine, the markers of kidney damage.
    b) Betanin treatment restored the levels of catalase, an antioxidant enzyme whose levels were down regulated in the group of animals treated with cisplatin alone.
    c) Increased lipid peroxidation was observed in the kidney tissues of animals treated with cisplatin alone indicating enhanced oxidative stress. Betanin treatment along with cisplatin, on the other hand, prevented the increase in lipid peroxidation.
    a) Transmission electron microscopic studies on kidney of rats treated with cisplatin showed marked ultra structural changes such as loss of border membrane, alterations in the mitochondrial shape and changes in the

thickness of basement membrane. Betanin treatment along with cisplatin prevented cisplatin-induced ultra structural changes in a dose dependent manner.

9. It is very well established from earlier studies that pigmented antioxidants such as anthocyanins, chlorophylls, carotenoids etc., were successful in inducing antiproliferative effects. In this connection studies were initiated to know whether betanin, being an antioxidant pigment can induce antiproliferative effects in cell lines such as chronic myeloid leukemia K562.
    a) Betanin induced dose and time dependent inhibition in the growth of K562 cells, with IC_{50} of 40 µM.
    b) Treatment of K562 cells with betanin (40 µM) resulted in marked morphological changes such as web like activated membrane structures associated with fragmented nuclei when compared to untreated control cells.
    c) Scanning electron microscopic studies showed membrane blebbing in the cells treated with betanin compared to untreated control cells
    d) Transmission electron microscopic studies on betanin treated cells revealed the condensation of chromatin and loss of intracellular details compared with untreated controls.
    e) betanin treatment also showed an increase in the hypo diploid apoptotic DNA content with a decrease in the number of cells at the S and G2 phases of the cell cycle compared to untreated control cells as evidenced by flow cytometric analysis.

f) Decrease in mitochondrial membrane potentials in the cells treated with betanin, as evidenced by the leakage of cytochrome-c, into the cytosol. No such leakage of cytochrome-c was observed in untreated cells.

g) Betanin showed changes in the ratios of Bcl2/Bax in the Western blot analysis and also induced the activation of caspase-3 which is evident from the cleavage of PARP in the betanin treated cells compared with the untreated control cells.

h) Confocal microscopic studies on Betanin treated cells confirmed the entry of the betanin into the intracellular space, suggesting that betanin enters K-562 cells and executes anti-proliferative effects.

10. The above studies thus reveal that betanin, isolated from fruits of *O. ficus indica*, is a potent antioxidant and exhibits nephroprotective properties in rats. Like other plant pigments betanin also showed anti-proliferative effects in the Chronic myeloid leukemia cell line K562.

5.0 References

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Betanin a betacyanin pigment purified from fruits of *Opuntia ficus-indica* induces apoptosis in human chronic myeloid leukemia Cell line-K562

Devalraju Sreekanth, M.K. Arunasree, Karnati R. Roy, T. Chandramohan Reddy, Gorla V. Reddy, Pallu Reddanna*

School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Abstract

Betalains are water-soluble nitrogenous vacuolar pigments present in flowers and fruits of many caryophyllales with potent antioxidant properties. In the present study the antiproliferative effects of betanin, a principle betacyanin pigment, isolated from the fruits of *Opuntia ficus-indica*, was evaluated on human chronic myeloid leukemia cell line (K562). The results show dose and time dependent decrease in the proliferation of K562 cells treated with betanin with an IC_{50} of 40 μM. Further studies involving scanning and transmission electron microscopy revealed the apoptotic characteristics such as chromatin condensation, cell shrinkage and membrane blebbing. Agarose electrophoresis of genomic DNA of cells treated with betanin showed fragmentation pattern typical for apoptotic cells. Flow cytometric analysis of cells treated with 40 μM betanin showed 28.4% of cells in sub G0/G1 phase. Betanin treatment to the cells also induced the release of cytochrome *c* into the cytosol, poly (ADP) ribose polymerase (PARP) cleavage, down regulation Bcl-2, and reduction in the membrane potentials. Confocal microscopic studies on the cells treated with betanin suggest the entry of betanin into the cells. These studies thus demonstrate that betanin induces apoptosis in K562 cells through the intrinsic pathway and is mediated by the release of cytochrome *c* from mitochondria into the cytosol, and PARP cleavage. The antiproliferative effects of betanin add further value to the nutritional characteristics of the fruits of *O. ficus-indica*.

Keywords: *Opuntia ficus-indica*; Betanin; Apoptosis; Chronic myeloid leukemia

Introduction

Natural products with high medicinal values are gaining much importance in light of serious side effects posed by the medicinal derivatives from chemical origin. Natural pigments are another class of natural products that have attracted considerable attention as antioxidants that induce beneficial effects on human health and disease prevention. Carotenoids (Sies and Stahl, 2003) and anthocyanins (Matsumoto et al., 2001) are such natural pigments that have proven to possess health benefits. Recently we have reported that C-Phycocyanin, a natural pigmented antioxidant, isolated from *Spirulina platensis* induces apoptosis in mouse macrophage cell line RAW 264.7 stimulated with LPS (Reddy et al., 2000) and human chronic myeloid leukemia cell line-K 562 (Subhashini et al., 2004). Betalins are another class of natural pigments with reddish purple (betacyanins) or yellow (betaxanthins) nitrogenous vacuolar components that are widely used as food colorants. In contrast to other natural pigments such as carotenoids and anthocyanins, the physiological effects of betalains are not well studied. In a recent study

*Corresponding author. Tel./fax: +91 40 23010745.
E-mail address: prsl@uohyd.ernet.in (P. Reddanna).

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the antiviral and antimicrobial effects of betalain pigments have been reported (Strack et al., 2003). Furthermore, the antioxidant properties of betalins has been demonstrated in a wide range of assays (Zakharova and Petrova, 1998; Kanner et al., 2001; Gentile et al., 2004) and it was reported that enrichment of human low-density lipoproteins by betalains, effectively increased resistance to oxidation (Tesoriere et al., 2003). In addition, a role for betalain pigments in the chemoprevention against lung and skin cancers has been documented (Kapadia et al., 1996). It is recently documented that natural food colors such as betanin can inhibit the cell proliferation of a variety of human tumor cells (Muntha Reddy et al., 2005), however; the exact mechanism of action for the betalain pigments has not been addressed in any of the foregoing studies. In the present study we report anti-proliferative effects of betanin, a principle betacyanin pigment isolated from the fruits of *Opuntia ficus-indica*, on human chronic myeloid leukemia cell line-K562 and elucidate the molecular mechanisms involved.

Materials and methods

Phosphate buffered saline (PBS), RPMI medium, fetal bovine serum (FBS) were purchased from GIBCO Ltd. (BRL Life Technologies Inc., Grand Island, NY). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], proteinase K, RNase A, propidium iodide were from Sigma Chemical Co. (St Louis, MO). Nitrocellulose membranes were from Amersham Life Sciences; monoclonal antibodies against cytochrome *c* and Bcl-2 were from Upstate Technologies. monoclonal Bax antibodies were from Santa Cruz, CA, USA and polyclonal PARP antibodies were from R&D Systems, USA.

Extraction, purification and characterization of betanin from fruits of *O. ficus-indica*

O. ficus-indica (prickly pear) fruit pulp (100 g) was homogenized, extracted into 100 ml of aqueous methanol and concentrated in vacuo at 25 °C to 3–4 ml.

Prior to preparative HPLC, the concentrated fractions were purified by gel filtration on sephadex-LH 20 column (40 × 2.2 cm). The betacyanin fractions thus obtained were lyophilized. The purified betacyanin fractions were subjected to preparative HPLC on Shimadzu ODS-HPLC column (20 mm × 25 cm) and elution was carried out following a gradient program (Strack et al., 1987). The structural identity of betanin was further confirmed by NMR and LC–MS studies (data not shown).

Cell culture and treatment

K562 cells were grown in suspension cultures employing RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cultured cells were passed twice each week, seeding at a density of about 2×10^5 cells/ml. For treatments, exponentially growing K562 cells were collected and re-suspended in fresh culture medium. A stock solution of 40 mM betanin was prepared in PBS from the cryodessicated purified samples and used in the experiments.

Cell proliferation assay

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining method (Mossman, 1983). Briefly, 5×10^3 cells (K562) were incubated in 96-well plates in the presence or absence of betanin (10, 20, 40, 80 μM) for various time points in a final volume of 100 μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 50 μl of lysis buffer (12 mM HCl, 5% isobutanol and 10% SDS). The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on micro titer plate reader (Microscan MS 5608A, ECIL Instruments).

Scanning electron microscopy

After treatment with betanin (40 μM, 24 h) the cells were collected, washed with PBS and concentrated to 1×10^5 cells/ml. One drop of this suspension was placed on to a plastic coverslip that is previously coated with 1% poly-L-Lysine. Cells were fixed with glutaraldehyde for 1 h and post fixed with 1% osmium tetroxide for 1 h. Cells were dehydrated by passing through graded alcohols and dried by the critical-point technique. After trimming, mounting, and coating with gold–platinum, the specimens were observed on SEM (JSM-5600, JEOL Co.)

Transmission electron microscopy

Cell pellets were rinsed rapidly with PBS and fixed for 12 h at 4 °C with 2% vol/vol glutaraldehyde in 0.1% sodium cacodylate buffer, pH 7.4. Fixation was followed by 3–5 min rinses with 0.1% sodium cacodylate buffer, pH 7.4. Cells were post fixed with a solution containing 1% osmium tetroxide (w/v) and 2% $K_4Fe(CN)$ (w/v), and stained with 1% uranyl acetate,

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and pelleted in 2% agar. Pellets were dehydrated in graded ethanol solutions and embedded in spur resin. Ultra thin (60 nm) sections were cut on a Reichert Ultra cut microtome collected on Rhodanium 400-mesh grids, post-stained with uranyl acetate followed by lead citrate, and rinsed with water. The sections were examined in Philips CM-12 electron microscope at 80 kV.

Cell cycle analysis

To quantitate apoptosis, flow cytometric analysis was performed using propidium iodide as described previously (Reddy et al., 2003).

Detection of DNA fragmentation

Cells were treated with betanin (0, 20, 40, 80 μM) for 24 h and the fragmented DNA was isolated using the SDS/Proteinase K/RNase A extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA (Reddy et al., 2003). The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180–200 bp multiples on the agarose gel.

Flow cytometric analysis of mitochondrial membrane potential

K562 cells were cultured with betanin (40 μM for 24 h). The cells were harvested and changes in the mitochondrial membrane potentials were measured by flow cytometric analysis (Seuduto and Grotyohann, 1999).

Western blot analysis

Cells were exposed to 40 μM betanin for various time points (0, 4, 8, 12, 24 h) and whole cell extracts were prepared based on the method of Pardhasaradhi et al. (2003). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin). After 30 min of shaking at 4 °C, the mixtures were centrifuged ($10{,}000 \times g$) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford (1976) method. An equal amount of total cell lysate (50 μg) was resolved on 8–12% SDS-PAGE gels and then transferred on to nitrocellulose membranes. Membranes were stained with 0.5% ponceau in 1% acetic acid to confirm equal loading. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies (Cyt-c, Bcl-2, Bax and PARP) in 10 ml of antibody-dilution buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 °C for 8–12 h and then incubated with respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

Intracellular localization of betanin in K562 cells

The K562 cells treated with betanin (40 μM) for 24 h were washed in PBS and fixed in 4% formaldehyde, pH 7.4 for 20 min at 4 °C. After fixation, cells were washed twice in PBS and water. The cells were then viewed under confocal microscope with 506 and 529 nm of excitation and emission wavelengths.

Statistical analysis

Data reported as the mean ± SD of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). *P*-value of less than 0.05 was considered as significant.

Results

Isolation and identification of betanin

The betalains from *O. ficus-indica* fruit pulp were extracted and purified by gel filtration chromatography and then on HPLC to yield a prominent betacyanin pigment (data not shown). The prominent betacyanin pigment was identified as betanin–betanidin 5-*O*-beta glucoside (Fig. 1) based on mass spectral and NMR studies. The absorption spectrum of purified betanin showed maximum absorbance at 535 nm (data not shown). This purified betanin pigment with a molecular weight of 551 was employed in the present study.

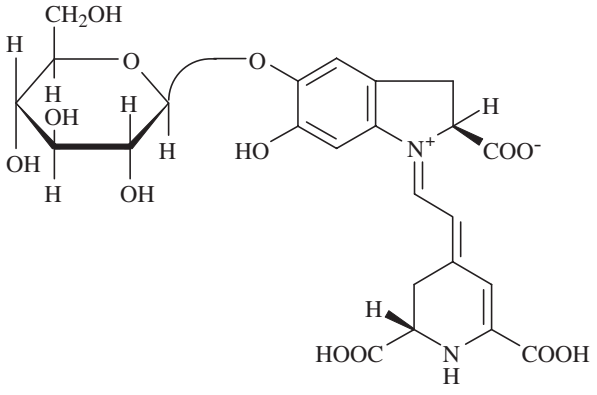

Fig. 1. Structure of betanin isolated from the fruits of *O. ficus-indica*.

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Betanin inhibited the K562 cell growth

The effect of betanin on growth of K562 cells was evaluated by MTT assay. We observed a dose dependent decrease in K562 cell growth until 48 h after betanin treatment (Fig. 2), with 50% decrease in cell proliferation at 40 μM betanin for 24 h. Further studies were carried out to analyze the mode of cell death on cells exposed to 40 μM betanin for 24 h.

Betanin induced ultra structural changes in the K562 cells

Scanning and transmission electron microscopic studies were carried out to observe the morphological and ultra structural changes induced by betanin in the K562 cells. Morphological features pertaining to apoptotic cell death such as membrane blebbing was clearly observed (data not shown). The transmission electron microscopic studies revealed the condensation of chromatin and loss of intra cellular details (Fig. 3).

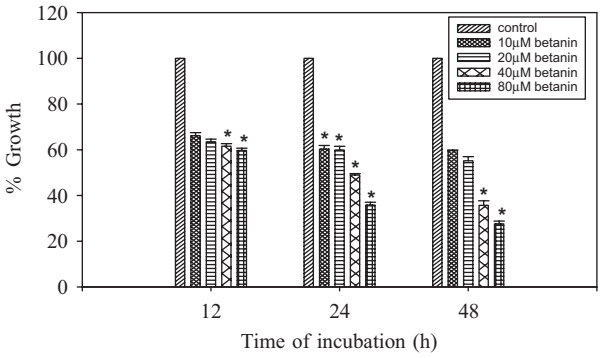

Fig. 2. Effect of betanin on proliferation of K562 cells. The cells were cultured in 10% FBS medium and treated with betanin (0, 10, 20, 40, 80 μM) for 12, 24 and 48 h. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. Values were expressed as mean ± SD of three independent experiments (*$P < 0.05$).

Betanin induced DNA fragmentation of K562 cells

DNA extracted from K562 cells treated with betanin, at indicated concentrations (0, 20 40, 80 μM) for 24 h revealed a progressive increase in the 180–200 bp ladder fragments. Such a pattern corresponds to inter nucleosomal cleavage, which is characteristic of apoptosis. Control cells did not exhibit any such DNA fragmentation (data not shown).

Effect of betanin on cell cycle profile of K562 cells

The induction of apoptosis in cells treated with betanin was further confirmed by flow cytometric analysis of DNA content. Fig. 4 illustrates the DNA content histograms of K562 cells treated with (40 μM) or without betanin for 24 h. These studies showed 28.4% of betanin treated cells at sub G0/G1 phase, when compared to 3% in untreated cells (Fig. 4).

Betanin caused reduction in cell mitochondrial membrane potentials (flow cytometric analysis)

The decrease in mitochondrial membrane potential ($\Delta\Psi m$) is associated with mitochondrial dysfunction (Seuduto and Grotyohann, 1999). In the present study we investigated the changes in the membrane potential of

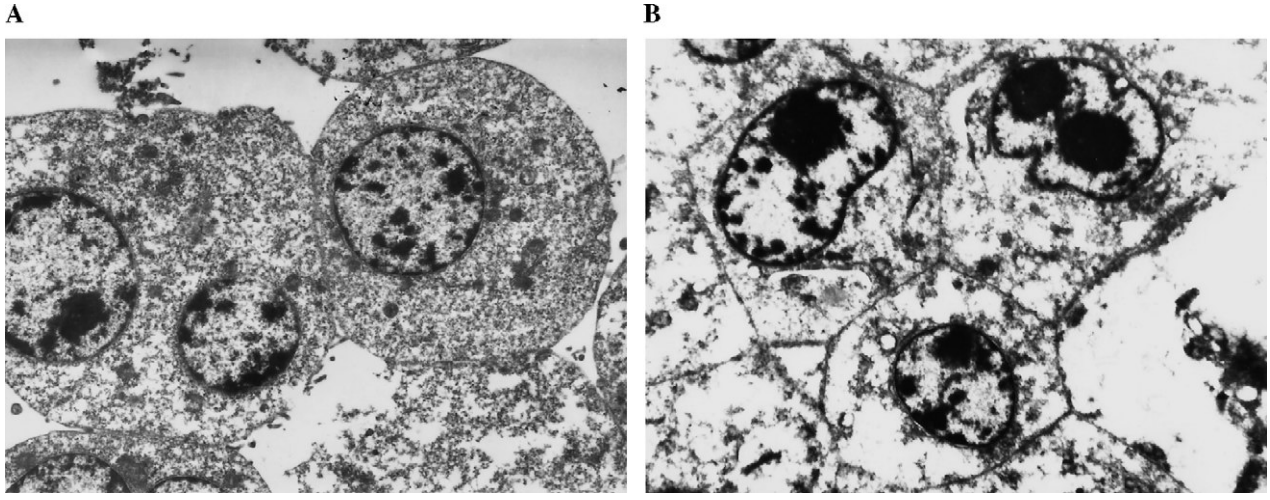

Fig. 3. Transmission electron micrographs of K562 cells treated with 40 μM betanin. (A) Control K562 cells, (B) K562 cells treated with 40 μM betanin for 24 h. Nuclear condensation and loss of intracellular details are clearly observed in the betanin treated cells (3000 ×).

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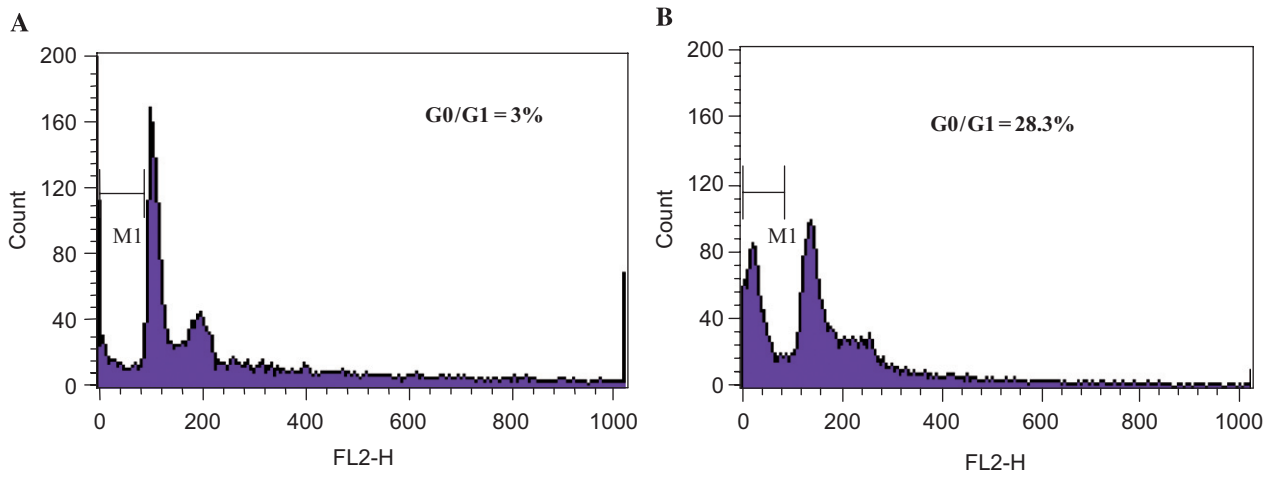

Fig. 4. Flow cytometric analysis of the control and betanin treated K562 cells. Cells treated with (40 μM) or without betanin for 24 h were fixed and stained with propidium iodide and the DNA content was quantified by Flow cytometer. The number of hypo diploid (sub-G0/G1 phase) cells is expressed as a percentage of the total number of cells. (A) Control K562 cells (3%), (B) K562 cells treated with 40 μM betanin (28.3%).

K562 cells exposed to 40 μM betanin. The cells were harvested and changes in the mitochondrial membrane potentials were measured by the uptake of lipophilic cation Rhodamine 123 into mitochondria (Kroemer and Reed, 2000). Untreated control cells were used to determine the normal uptake of this cation, and the percentage of treated cells with low membrane potentials was calculated. Shift in percentage of fluorescence intensity from 96.1% in control to 89.1% in the cells treated with 40 μM betanin was observed (data not shown).

Betanin effects on cytochrome *c* release, Bcl-2 and Bax proteins in K562 cells

The process of apoptosis is associated with the disruption of mitochondrial membrane potential which results from the opening of permeability transition pores in the mitochondrial membrane leading to the release of cytochrome *c*. To determine whether there is any release of cytochrome *c* from the mitochondria into the cytosol, cytosolic fractions from the cells treated with 40 μM betanin for indicated times, were subjected to Western blot analysis. We observed a time dependent elevation in the cytosolic levels of cytochrome *c* with maximum increase at 12 and 24 h after betanin treatment (Fig. 5A). Furthermore, the expression levels of Bcl-2 and Bax proteins are associated with mitochondrial membrane integrity and play a crucial role in the regulation of apoptosis. Time-dependent decrease of Bcl-2 protein levels was observed after betanin treatment (Fig. 5B), but no appreciable change in the levels of Bax protein was observed at all the time periods (Fig. 5C). These results suggest a disturbed Bcl-2/Bax ratio in the cells treated with betanin.

PARP cleavage in response to betanin treatment

PARP, poly (ADP ribose) polymerase, is a nuclear enzyme implicated in many cellular processes including apoptosis and DNA repair. During apoptosis PARP (116 kDa) is cleaved to yield two fragments of 85 and 23 kDa. To determine whether PARP is cleaved in betanin induced cell death, we treated K562 cells with 40 μM betanin for indicated times (0, 4, 8, 12, 24 h) and PARP cleavage was monitored by employing PARP antibodies that recognize the 23 kDa fragment of cleaved PARP and uncleaved 116 kDa full length PARP. The data presented in Fig. 5D show the gradual increase in the appearance of 23 kDa signature fragment of PARP cleavage at all the indicated times (lanes 2–5). However, in control cells (lane 1) no 23 kDa fragment of PARP was observed, except the uncleaved 116 kDa protein.

Intracellular localization of betanin in K562 cells

In order to find out whether betanin enters the K562 cells, confocal microscopic studies were undertaken on cells exposed to betanin (40 μM) for 24 h. These studies showed a strong fluorescence signal in the cytoplasm of the cells treated with the betanin, with no such signal in the untreated cells (Fig. 6).

Discussion

Cancer chemoprevention is defined as inhibition of tumor initiation, promotion and progression by employing pharmacologic or natural agents that prevent the metabolic activation of procarcinogens (Hong and

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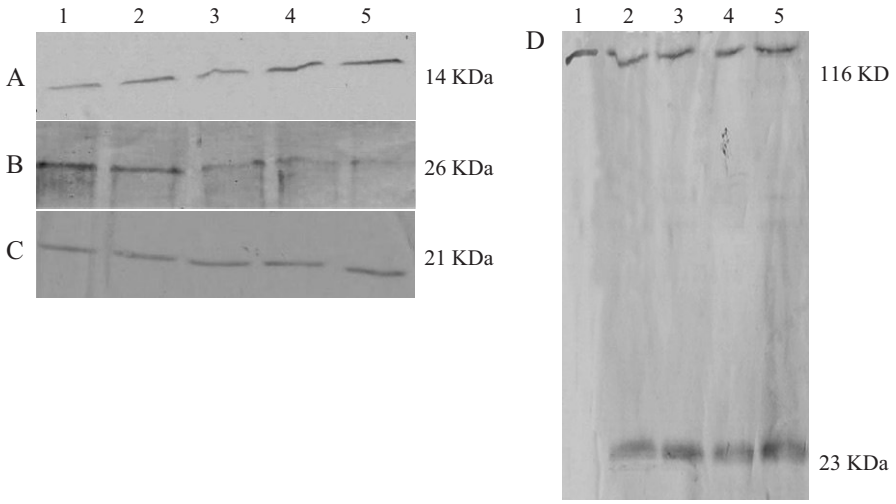

Fig. 5. Western blot analysis showing the effect of betanin on (A) cytochrome c release, (B) Bcl-2 protein expression, (C) Bax protein expression levels, (D) PARP cleavage. Equal amounts of protein (50 μg) from the K562 cells treated with 40 μM betanin for the indicated times (0, 4, 8, 12, 24 h) were analyzed by 15% SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with monoclonal cytochrome c, Bcl-2, Bax antibodies and polyclonal PARP antibodies, respectively. Lane 1: 0 h; lane 2: 4 h; lane 3: 8 h; lane 4: 12 h; lane 5: 24 h.

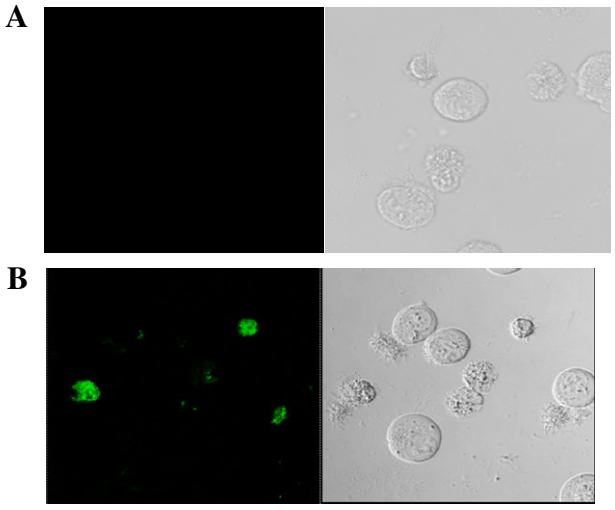

Fig. 6. Confocal image showing the localization of betanin in K562 cells. (A) Control cells, (B) betanin (40 μM) treated cells for 24 h showing intracellular localization of betanin.

Sporn, 1997). A number of natural phytochemicals (Katdare et al., 1998) and natural antioxidants such as C-Phycocyanin (Subhashini et al., 2004; Pardhasaradhi et al., 2005), Anthocyanidins (Hou et al., 2001; Meiers et al., 2001; Nagase et al., 1998; Bomser et al., 1996; Kamei et al., 1995), Curcumin (Kuo et al., 1996; Jiang et al., 1996), fungal cytochalasins (Nagasawa et al., 2000) etc., were shown to have anticancer properties. The mechanisms responsible for executing the anti-proliferative effects include: (i) induction of alterations in the cell differentiation pattern, which plays a vital role in the invasiveness and metastatic progression of the tumors, (ii) blockade of pre neoplastic cell expansion or induction of apoptosis, and (iii) intervention of metabolic activation of carcinogens by scavenging ROS. In the present study we have analyzed the effects of betanin, a principle betacyanin pigment isolated from *O. ficus-indica,* on human chronic myeloid leukemia cell line, K562.

O. ficus-indica is a perennial plant that grows all over the world and belongs to the family of Cactaceae. A wide range of studies demonstrated the usage of this herbal extracts in management of many disorders. Ethanol extracts of *O. ficus-indica* showed potent anti-inflammatory properties (Park et al., 2001). Pre-treatment with lyophilized cladode extracts in the rats revealed a protective action against ethanol-induced ulcer formation (Galati et al., 2001). In another study the methanolic extracts of stems reported to possess wound-healing properties (Park and Chun, 2001). The fruits of *O. ficus-indica* are very good source of water-soluble nitrogenous chromo alkaloids called betalains. Betalains find their importance as food colorants as they provide attractive bright hues for food products (Stinzing, 1999). In a recent study the pharmacokinetic effects of these pigments in human subjects was demonstrated (Netzel et al., 2005). There has been growing interest in the use of these natural pigments for food coloring, since synthetic dyes are becoming more and more critically assessed by the consumer, both from the ends of quality and health. Apart from serving as food additives, the natural pigments also play a vital nutraceutical role in circumventing the health hazards posed by synthetic and chemical colorants. Betalains are one such nutraceutical nitrogenous cationic natural

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pigments that are widely used as food additives. Unlike other pigments betalains are not very well studied with regard to their pharmacological properties and mechanism of action.

In the present study we evaluated the anti proliferative effects of betanin, a principle betacyanin pigment isolated from the fruits of *O. ficus-indica,* on chronic myeloid leukemia cell line, K562. Further in-depth studies were taken up to understand the molecular mechanisms involved in betanin-induced effects. We found that betanin induced dose dependent and time dependent inhibition in the growth of K562 cells, with IC_{50} of 40 μM. Apoptosis is a process of cell death that is critically regulated based on the expression of cell's intrinsic suicide machinery (Vaux and Korsmeyer, 1999), which further leads to the characteristic pattern of morphological, biochemical, and molecular changes. The present study showed typical apoptotic characteristics such as membrane blebbing, laddering of DNA and chromatin condensation in the cells treated with betanin. The flow cytometric analysis of betanin treated cells showed an increase in the hypo diploid apoptotic DNA content with a decrease in the number of cells at the S and G2 phases of the cell cycle. Accumulating scientific evidences suggest the pivotal role of mitochondria in the execution of apoptosis of the cells exposed to various stimuli (Desagher and Martinou, 2000; Green and Reed, 1998). Our present studies showed decreased mitochondrial membrane potential with simultaneous appearance of cytochrome *c* in the cytosolic fractions of cells exposed to betanin. We hypothesize that betanin, after gaining entry into the cell, induced the mitochondrial disruption leading to decreased membrane potentials and leakage of mitochondrial cytochrome *c* into the cytosol. Confocal microscopic studies support such a possibility of entry of betanin into the cells. Further decrease in expression of Bcl-2 protein and the alterations in the Bcl-2/Bax ratios strongly support mitochondrial membrane alterations in the cells treated with betanin. Caspase-3, an executioner caspase in apoptotic cascades is implicated in the cleavage of a number of proteins including Poly (ADP-ribose) polymerase (PARP), which is a hallmark of apoptosis. In the present study a time dependent increase in the PARP cleavage in cells treated with betanin (40 μM) was observed, suggesting the activation of caspase-3 activity. The foregoing studies clearly demonstrate that betanin induces apoptosis in K562 cells by intrinsic death pathway. Further studies, however, are required to understand the mechanisms involved in betanin-induced alterations in mitochondrial membrane integrity in K562 cells.

In conclusion, the present study demonstrates that betanin, a natural betacyanin pigment isolated from the fruits of *O. ficus-indica*, enters K562 cells and alters mitochondrial membrane integrity, leading to cytochrome *c* leakage, activation of caspases and nuclear disintegration. These biochemical alterations are reflected in ultra structural changes, typical of cells undergoing apoptosis. These findings on anticancer effects of betanin, further add value to the nutritional characteristics of the fruits of *Opuntia ficus-indi*ca.

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