Evaluation of chemical composition and anticancer properties of *Citrullus colocynthis*

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By

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DECLARATION

I declare that the dissertation entitled **"Evaluation of chemical composition and anticancer properties of *Citrullus colocynthis* (L.)."** has been prepared by me under the guidance of Dr. Pankaj Bhardwaj, Assistant Professor, Centre for Biosciences, Centre for Biosciences and Dr. Sandeep Singh, Centre for Genetic Diseases and Molecular Medicine, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

It is to be certify that **Jatinder Kaur** has prepared her dissertation entitled **"Evaluation of chemical composition and anticancer properties of *Citrullus colocynthis* (L.)",** for the award of M.Sc degree of the Central University of Punjab. She has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

"Evaluation of chemical composition and anticancer properties of *Citrullus colocynthis* (L.)"

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Key words : Colocynthis, anticancer, apoptosis, antioxidant, Extracts, secondary metabolites

Colocynth (*Citrullus colocynthis* (L.)) is medicinal plant of family Cucurbitaceae which is the native of Turkey. This plant has been used as anti-inflammatory agent, for the treatment of jaundice, skin infections, and urinary diseases from ancient times. It is anti-diabetic, anti-microbial, anti-parasitic, antifungal and anti-oxidant in nature and thought to possess anti-cancer properties also. There is no proper study available regarding its anticancer nature. The aim of the study was to estimate the chemical composition of *C*. *colocynthis* fruit and the effect of different extracts prepared from *C*. *colocynthis* pulp and seeds on various cancer cell lines. These extracts were found to contain number of secondary metabolites which may further have useful and healing properties. When cancer cells were treated with different prepared extracts, the cell viability of various cancer cells was reduced effectively. Effect of p53 gene was also analysed by using intact p53 as well as p53 mutant cell lines. Antioxidant activity of *C. colocynthis* was also determined by performing H_2DCFDA. Assessment of various antioxidant enzymes such as Catalase, SOD, and Glutathione reductase was also done and found to be present in this fruit. We also analysed the protective effect of *C. colocynthis* by first treating the cells with pesticides and then with colocynthis extracts. Results indicate that *C. colocynthis* neutralized the harmful effect of pesticides. The results obtained are encouraging and confirm the value of the use of *C. colocynthis* as an anticancer agent for fighting against various cancers. It may be helpful to find the exact component responsible for the protective action of *C. colocynthis*.

Signature of Student Signature of Supervisor Signature of co-supervisor

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviations
1.	Deoxyribonucleic acid	DNA
2.	3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide	MTT
3.	2′, 7′-dichlorodihydrofluorescein diacetate	H_2DCFDA
4.	Centre for Jatropha Promotion	CJP
5.	B-cell lymphoma 2	Bcl-2
6.	N-nitroso-N-methylurethane	NNMU
7.	Pancreatic cancer cells	PANC-1 cells
8.	Signal transducer and activator of transcription 3	STAT3
9.	Total cholesterol	TC
10.	Triglycerides	TG
11.	Free fatty acids	FFA
12.	Very low-density lipoprotein	VLDL
13.	High-density lipoprotein	HDL
14.	Dulbecco’s Modified Eagle’s Media	DMEM
15.	Phosphate buffer solution	PBS
16.	Dimethyl Sulfoxide	DMSO

17.	Ethylene diamine tetra acetic acid	EDTA
18.	Bovine Serum Albumin	BSA
19.	Dinitrosalicyclic acid	DNSA
20.	Fetal Bovine Serum	FBS
21.	Wild type	wt
22.	Ultraviolet	UV
23.	Infrared	IR
24.	Reactive Oxygen Species	ROS
25.	Thioredoxin reductase	TrxR
26.	Thioredoxin	Trx
27.	Nicotinamide adenine dinucleotide phosphate	NADPH
28.	Selenocysteine	Sec
29.	Nuclear factor Kappa Beta	NF-κB

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Chapter 1

Introduction

1.1 Origin of the Problem

Cancer is one of the major problem among various deadly and epidemic diseases therefore its consequences can be seen worldwide. More than twelve million new cases are diagnosed yearly and the rate is increasing (Hegde *et al.,* 2009). The large number of patients die after developing cancer, despite the availability of various treatments; therefore, there is an increase demand for developing new approaches for cancer therapy. There are a number of treatments available for such deadly diseases, but the problem widens due to number of side effects. Therefore, there is a need for development of treatments or drugs, which must have very minimal side effects. Ayurveda is such a blessing to human kind where drugs and various treatments are made possible by using various plants and herbs with minimal or no side effects. Although, there are number of treatments currently available such as Chemotherapy, Surgery and Radiotherapy for cancer, but these are not fully effective and safe. Therefore, Development of a successful therapeutic approach remains one of the most challenging issues. Most commonly used drugs in cancer treatment are synthetic such as methotrexate, fluorouracil, mercaptopurine, abiraterone acetate, chlorambucil, cabazitaxel, Ixabepilone etc. which ultimately decreases blood cell count, causes nausea, vomiting, mouth ulcers, skin rashes, photosensitivity, dizziness, headache, drowsiness, kidney damage (with a high-dose therapy) liver damage, hair loss, mutations and DNA damage. Medicinal plants are widely used in traditional cultures all over the world and they are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals. Drugs derived from plants such as quinine, morphine, codeine, colchicine, atropine and reserpine are of precious values. Plant medicines have subtle effects on various different biochemical pathways in the human body, which directly or indirectly helps to restore the balance and equilibrium of the body (Fig-1.1)

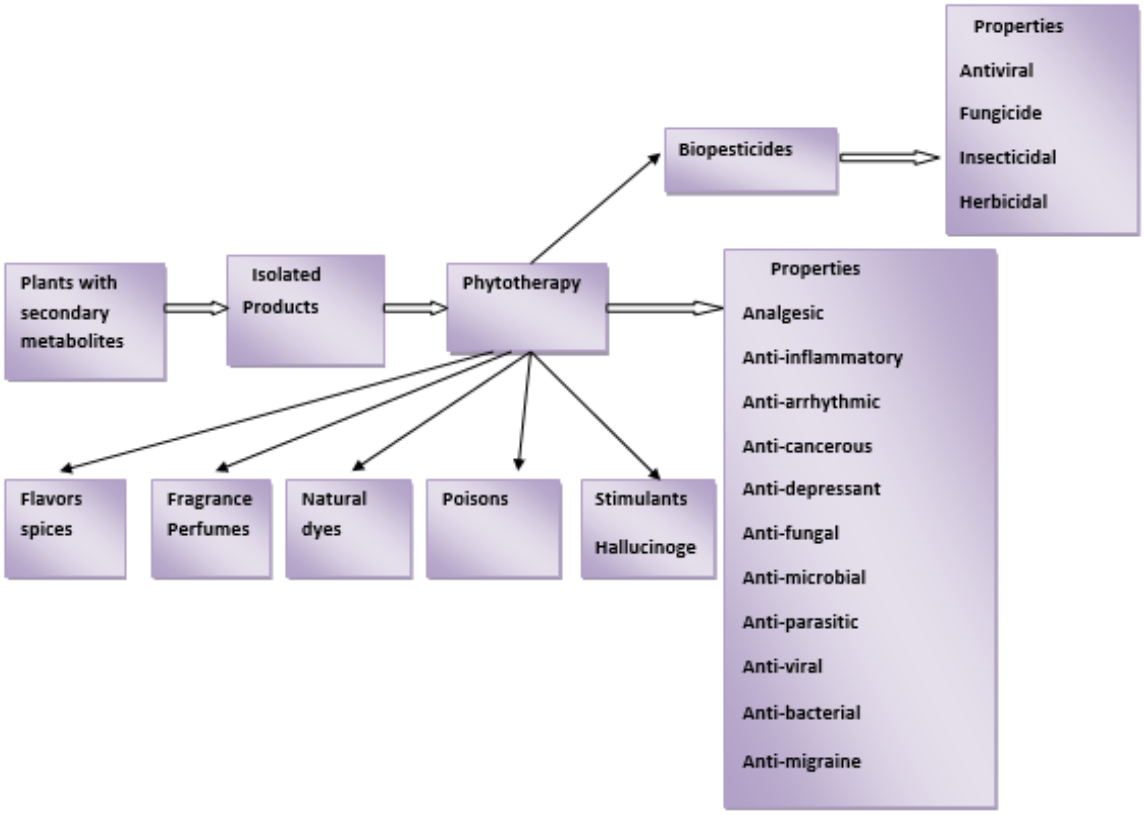


Fig-1.1 Diversity of natural products and their biological properties

The present study proposes to study one such plant's fruit *Citrullus colocynthis* (L.) which is widely used for various homemade remedies and known worldwide for its antioxidant properties.

The study proposes to test the anticancer nature (if any) of the plant's extracts.

1.2 Background

The ancient Indians had a great passion for the nature and were much closer to the plant and ecosystem, quite contrary to the modern world. The plant kingdom was not meant only to satisfy hunger, but also to serve relief and remedy for diseases. Several plant products were included in day-to-day life such as in food and in other dietary habits for health care. There are number of reports suggests that many plants used as rejuvenation medications are potent antioxidants. Neem appears to have beneficial properties in curing number of diseases (Mulla *et al.,* 1999). Turmeric and Curcumin have shown effectiveness in preventing cancer *in vitro* (Chattopadhyay *et al.,* 2004).

The synthetic chemicals has wide applications in day to day life of human, but the use of such deadly chemicals has now been widen and unlimited which has resulted in various side effects, residual persistence, developing resistance in microbes and pests, increase in atmospheric pollution and even affects the biodiversity of plant and animals, also affects the quality of air, water and soil. It is now being globally realized that inspite dependence on synthetic chemicals, plant derivatives are natural and safer for use. The Indian trees, herbs, shrubs etc. were popular from ancient period and are well known for their action and uses. *C. colocynthis,* commonly known as the colocynth, bitter apple, bitter cucumber, desert gourd, egusi, or vine of Sodom is nature's gifts (Fig-1.2). This is a desert plant native to the Mediterranean Basin and Asia, especially Turkey. This plant has been well recognized in traditional medicine for the treatment of diabetes mellitus as a purgative (L Harvey A, 2010), treatment of rheumatism, snakebite (Bhardwaj *et al.*, 2011) and have been thought to harbor anti-tumor properties (Newman *et al.,* 2000). Due to their special importance in safety of communities, this plant receives attention to research centers (Okigbo *et al*., 2009; Karthikeyan *et al*., 2009; Lozoya *et al.*, 1989). This plant have valuable properties due to the presence of various complex chemical substances, which are named as secondary metabolites (Karthikeyan *et al*., 2009; Lozoya *et al.,* 1989). They are categorized as alkaloids, glycosides, flavonoids, saponins, tannins, carbohydrates and contain essential oils (Sharafzadeh *et al*., 2012). Plant based natural constituents can be derived from any part of this plant like bark, leaves, flowers, roots, fruits, seeds, etc. (Gordon *et al*., 2001).

a.  c. 

b.  d. 

Fig-1.2 a. *C. colocynthis* (fresh) **b.** *C. colocynthis* (Dry form) and its black seeds **c.** *C. colocynthis* (Seeds and Interior view) **d.** Flower of *C. colocynthis*

It is an annual or a perennial plant (in wild) in Indian arid zone and has a great survival rate under extreme xeric conditions. It can tolerate annual precipitation of 250 to 1500mm and an annual temperature of 14.8 to 27.8°C. Its pH ranges between 5.0 and 7.8. It has characteristics features like yellow flowers and smooth fruit, which on ripening contains a white spongy pulp enclosing numerous compressed white or brownish seeds. Colocynth is an important ayurvedic herb of Cucurbitacae family (Bode and M. 2004). Colocynth contains the chemical cucurbitacin, which is extremely irritating to the mucous membranes of stomach and intestines (Gry *et al.,* 2006).

1.3 Chemical composition

The seeds of *C. colocynthis* have been subjected to a range of pharmacological, phytochemical and nutritional investigations in recent years. It has been shown to contain 17% of a fixed oil with high proportion of unsaturated fatty acids, mainly linoleic acid (60-70%), oleic acid (11.7-15%) and a very low n-3 poly-unsaturated Fatty Acid level (0.5%) (Gurudeeban *et al.,* 2010). It is also rich in antioxidants e.g. tocopherol, polyphenol and plant sterol. Colocynthis seeds contain 8.25% protein

content and rich in lysine, leucine and sulfo amino acids (Javed *et al*., 1994). Egusi (colocynthis) kernels contain 52% oil, 28.5% protein, 2.7% fiber and 8.2% carbohydrates (Al-Khalifa*,* 1996). The leaves and flowers contain mainly two important flavonoids, named quercetin and kaempferol (Harsh *et al.,* 1988). Fruit of colocynthis contains iso-vitexin, iso-orientin and iso-orientin 3'-methylether, while the aerial parts contain three C-p-hydroxy benzyl derivatives viz., 8-C-p-ydroxybenzylisovitexin, 6-C-p-hydroxybenzylvitexin and 8-C-p-hydroxybenzylisovitexin 4'-O-glucoside (Maatoq *et al.*, 1997).

1.4 Uses of *C. colocynthis*

C. colocynthis is being used for different purposes. It is useful in different types of medicine preparations and as an energy source, e.g. oilseed and biofuel. Dried pulp of unripe fruit is used as a medicine for its drastic laxative and hydragogue (causing a discharge of water), cathartic action on the intestinal tract (Mohammed *et al.,* 2004). After drying the fruit, its pulp and seeds are grinded separately. This powder is used as a drastic purgative.

Plant figures into remedies for carcinoma, endothelioma, leukemia, tumors of the liver, spleen and various eye problems. It contains three antitumor ingredients i.e. cucurbitacin B, cucurbitacin E and the D-glucoside of beta-sitosterol (Madari *et al.,* 2004). The pulp or leaves are a folk remedy for cancerous tumors. A decoction of the whole plant, made in juice of fennel, is said to help indurations of the liver (Uma *et al.,* 2014). Roots may be used as purgative against ascites (accumulation of fluid in the peritoneal cavity, causing abdominal swelling), for jaundice, urinary diseases, rheumatism and for snake-poison (Mood *et al.,* 2008). It has antimicrobial activity, antidiabetic activity and anesthetic activity (a substance that induces insensitivity to pain (Heydari *et al.,* 2014). It can be used in the beginning of pregnancy, to cause an abortion (Cole *et al.,* 1968). Methanolic extracts of *C. colocynthis* was found to have antiulcer and antioxidant activity (Gill *et al.,* 2011; Reddy *et al.,* 2012). Extract of *C. colocynthis* leaves was evaluated for antiasthmatic activity, through antiallergic effects. Petroleum ether extract and ethanol extract of the plant showed significant antiallergic activity in both milk-induced leukocytosis (antistress) and eosinophilia (antiallergic) in mice. Thus, it is founded that the leaves of *C. colocynthis* possess antiallergic activity and properties in the treatment of Asthma (Talole *et al.,* 2010). Recent study shows that consumption of *C. colocynthis* with radioactive radiations

has effects on growth of tumors such as larynx cancer (Al-Zahrani *et al.,* 2006; Delazar *et al.,* 2006; Abed *et al*., 2000; Usman *et al*., 2003). The dried pulp of *C. colocynthis* has been used for constipation, edema, bacterial infections, cancer and diabetes. The aqueous leaves extract of *C. colocynthis* showed antimicrobial and anti-inflammatory effects (Marzouk *et al.,* 2013). The dried fruits are grinded added with black salt, ajawain and fed to animals for control of stomachache, diarrhea and as deworming agent. Its roots contain some resins and are used in ayurvedic medicines for hypertension, constipation, jaundice, urinary disorders and arthritis. Seeds of this fruit contain 15–20% oil and this oil has useful properties such as use in manufacturing soaps. The fruit is used to repel moths from wool. In India, the vine is planted as a sand binder. Seed, often removed from the poisonous pulp and eaten in Central Sahara regions, contains a fixed oil. *C. colocynthis* seeds are rich in oil and protein and can be utilized on an industrial scale; such oil composition resembles safflower oil. Considering *C. colocynthis* potential as an oil seed feedstock for biodiesel CJP (Centre for Jatropha Promotion) has honor to establish this untapped resource as alternative source for Bio-Diesel industry of future (Fig-1.3) (Giwa *et al.,* 2010).

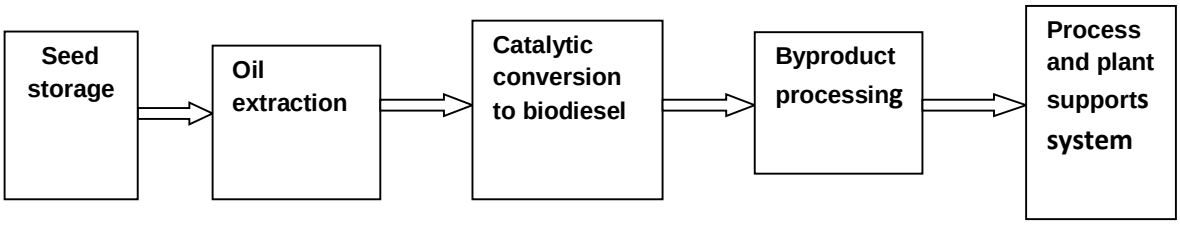


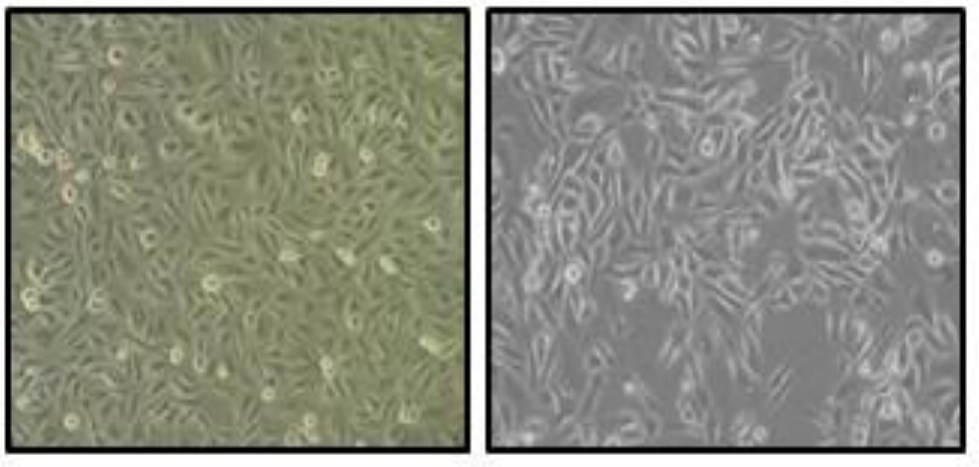
Fig-1.3 Mechanism of processing of *C. colocynthis* products

Cancer "remedy" contains three antitumor ingredients named cucurbitacin B (active against PS-134 and KB tumor systems), cucurbitacin E (active against LL and KB systems) and the D-glucoside of beta sitosterol. The pulp or leaves are a folk remedy for cancerous tumors. Results from preliminary studies clearly indicate that *C. colocynthis* might be beneficial in attenuating the elevated biochemical parameters during DEN/PB induced hepatic damage and suggested that ability of the extracts to provide defense against oxidative stress by quenching free radicals which indicates it has anti-oxidant properties (Rajangam *et al.,* 2013;). One of the study revealed that *C. colocynthis* ability to control and manipulate the accumulation of nanoparticles for an extended period inside a cell could lead to improvements in

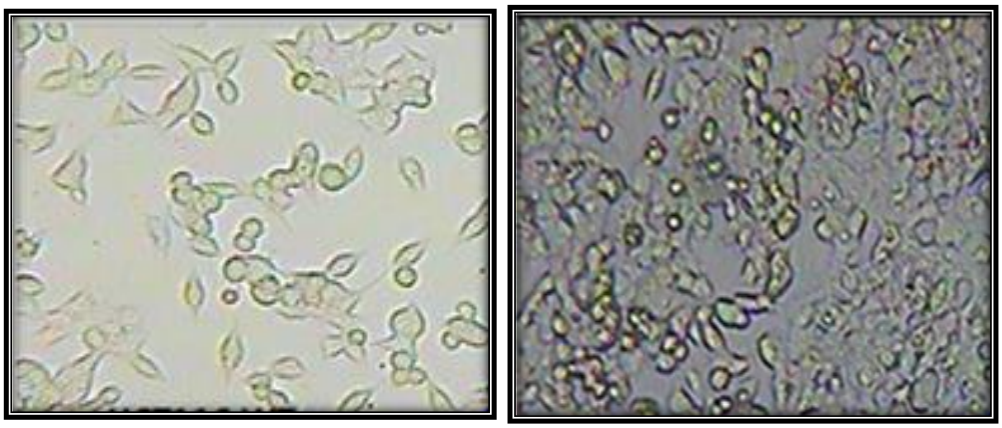
diagnostic sensitivity and therapeutic efficiency. This can eliminate the use of expensive drugs for cancer treatment (Vidyanathan *et al.,* 2009). The plant has also been reported to possess a wide range of traditional medicinal uses in common cold, cough, bronchitis, jaundice, joint pain, cancer, toothache, wound, mastitis, and in gastrointestinal disorders such as indigestion, constipation, dysentery, gastroenteritis and colic pain (Hussain *et al.,* 2014).

Table1.1 Cell lines selected for study

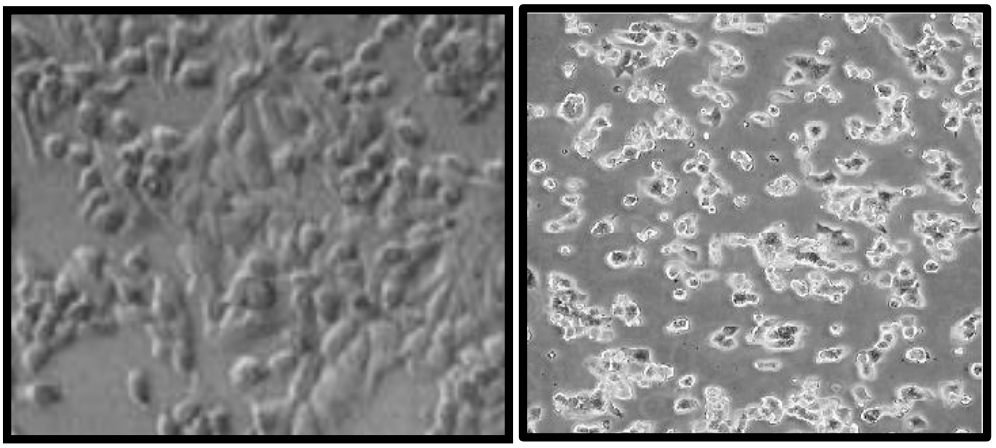
Sr. No.	**Cell line**	**Origin/ Type**	**Characteristics**
1.	HCT116 (wt & p53 mutant)	Colon	Epithelial
2.	MCF7	Breast	Epithelial
3.	HepG2	Liver	Epithelial
4.	PC3	Prostate	Epithelial
5.	H460	Lung	Epithelial



a. MCF7 **b.** PC3



c. HCT116 (wt) **d.** HCT116 (p53 mutant)



e. HepG2 **f.** H460

Fig-1.4 a. MCF7, **b.** PC3, **c.** HCT116 (wt), **d.** HCT116 (p53 mutant), **e.** HepG2, **f.** H460 at 20X

1.4 Hypothesis

Extracts of *C. colocynthis* plant are known to possess antioxidant properties and are thought to have potent anticancer action but no proper study is available regarding anticancer nature and mechanism of action. Extracts will be tested against various cancer cell lines (Colon, breast, lung, liver and prostate) and its antioxidant and anticancer property will be determined.

1.5 Objectives of the study

1.5.1 Objectives

To achieve the aim of research goals, the following objectives are formulated:

a. Evaluation of Phytochemicals present in various extracts of *C. colocynthis.*

b. To elucidate the antioxidant activity of C*. colocynthis.*

c. To check the anticancer activity of *C. colocynthis* on various cancer cell lines.

1.5.2 Conceptual Framework

Reason behind the current research is to investigate the beneficial effects of the fruit *C. colocynthis* on different types of cancers such as lung cancer, breast cancer, colon cancer etc. as well as to study its antioxidant properties. The fruit of *C. colocynthis* is common and it does not require much attention for its growth. This fruit is known from ancient times for its valuable medicinal properties and being natural is one of the reasons, which enhance its useful properties. Aim behind this study involves the use of different extracts of this plant in order to suppress the generation of reactive oxygen species and to study its effect on various cancer cell lines. Being natural, it may have very less or negligible side effects as compare to toxic chemicals, which has abundant of side effects. Other useful property of this fruit is that it is inexpensive and affordable by poor as well as common people.

Chapter 2

Review of literature

Cucurbitaceae is a largest family containing 120 genera and approximately 825 species which are typically distributed in the tropical countries, poorly represented in temperate regions (Takhtadzhian *et al.,* 1997). *C. colocynthis* is used in folk medicine in rural areas as a purgative, anti-rheumatic and a remedy for skin infections. It also has antipyretic, analgesic, antimicrobial and anti-inflammatory properties (Lee *et al.*, 2010). This plant contains cucurbitacins A, B, C and D, a-elaterin (Tannin-Spitz *et al.,* 2007).

2.1 Identification

C. colocynthis species are easy to identify due to its specialized features of plant, fruit and leaves.

2.1.1 Different names in India

C. colocynthis is found in different parts of India and are known by various different names as described in Table 2.1

Table 2.1 Various names of *C. colocynthis* in different regions

Sr. No.	Botanical Name	***Citrullus colocynthis***
1.	Sanskrit	Indravaruni, visala, mahendra varuni
2.	Hindi	Badi-Indrayan, tumba
3.	English	Colocynth, bitter apple
4.	Bengali	Makhal
5.	Gujarati	Indrayan
6.	Marathi	Kadu – indravani
7.	Malyalam	Paikummatti
8.	Tamil	Paedikari Attutummatti
9.	Punjabi	Kodtuma

2.1.2 Roots and stems

C. colocynthis plant has large perennial roots that send out long and slender, angular, tough, rough vine like stems.

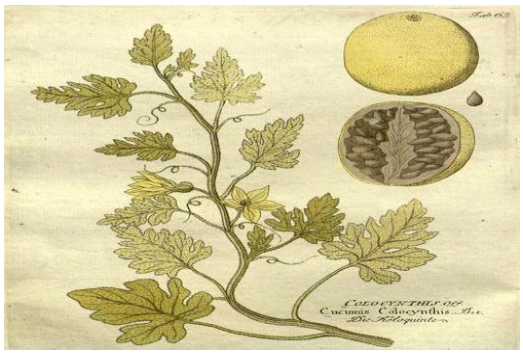
a.  b. 

Fig-2.1 Stem of *C. colocynthis* plant

The stems normally spread on the ground and have tendency to climb over herbs and shrubs by their axillary branching tendrils. The stems are herbaceous and beset with rough hairs; the leaves stand alternatively on long petioles (Fig-2.1).

2.1.3 Leaves

Leaves are about 5-10 cm in length and have around 3 to 7 lobes. Sometimes the middle lobe has an ovate structure. The leaves are triangular with many clefts and have a rough, hairy texture with open sinuses. The upper surface of the leaves is green in color and the lower surface is comparatively pale. The segment is obtuse, petioles are 1.3-2.5 cm and densely hairy (Borhade *et al.,* 2013) (Fig-2.2).

Fig-2.2 Leaves of *C. colocynthis* plant

2.1.4 Flowers

The flowers of *C. colocynthis* are yellow in colour and are borne by yellow-greenish stem. Each has a five-lobed corolla and a five-parted calyx.

Fig-2.3 Flower of *C. colocynthis* plant

They are monoecious (having both the male and female reproductive organs in the same individual); therefore, the male and the female reproductive parts (stamen in male, pistils and ovary in female) are borne in different flowers on the same plant. The male flowers calyx is shorter than the corolla. They have five stamens, four of which are coupled and one is single with monadelphous (united by their filaments to form one group) anther. The female flowers have three staminoids and a 3-carpels ovary (Fig-2.3). The two sexes are distinguishable by observing the globular and hairy inferior ovary of the female flowers.

2.1.5 Seeds

The seeds are 5mm long and 3mm wide in size, smooth, compressed and ovoid shaped (Fig-2.4). They are located on the parietal placenta. The seeds are light yellowish orange to dark brown in color. They are edible but similarly bitter, nutty-flavored and rich in fat and protein. These are eaten as whole or used as an oilseed. The oil content of the seeds is 17-19% (w/w), consisting of 67-73% linoleic acid, 10-16% oleic acid, 5-8% stearic acid, and 9-12% palmitic acid (Akobundu *et al,.* 1982). It is estimated that the oil yield is approximately 400L/hectare (Henning and R, 2000).

Fig-2.4 Seeds of *C. colocynthis* plant

2.1.6 Fruits

The fruit is globular and smooth. It is filled with a soft, white pulp in which numerous seed are embedded. The dried pulp of *C. colocynthis* has been used for constipation, edema, bacterial infections and diabetes (Talole *et al.,* 2013). Recently, the antioxidant effects and the effect of the aqueous extract of the pulp on kidney and liver functions were reported (Ramanathan *et al.,* 2011).

2.2 Area and production

C. colocynthis found throughout India and Ceylon, both wild and cultivated. It is also indigenous in the Arabia, West Asia, tropical Africa, Mediterranean region. It occupies the vast area extending from the west coast of Northern Africa (Senegambia, Morocco and the Cape Verde islands), eastward through the Sahara, Egypt, Arabia, Persia, and Baluchistan and through India, as far as the Coromandel Coast and Ceylon, touching northward the Mediterranean and Caspian seas. Colocynth is a desert plant, giving evidence of the dominion of life even in such arid regions. The fruit is used in Morocco for protecting woolen clothing from moths (Kress *et al.,* 1995). A brief account of the growth of colocynth in Palestine has more recently appeared in the United States consular reports (1895) which reported the following points:

a. The fruit grows abundantly between the mountains of Palestine and the eastern shore of the Mediterranean, from the city of Gaza northward to Mount Carmel.

b. The plant thrives without any attention whatever on the part of the farmer, since the climate and soil are all sufficient for its perfect growth (the natural requirements includes merely a sandy soil, warm climate and little moisture.)

c. The fruit, which is also known as the Turkish colocynth, is collected by the native peasants in July and August, before it is quite ripe, sold to Jaffa dealers, who peel it and dry the pulp in the sun, it is then molded into irregular small balls, packed in boxes and exported, mostly via England.

2.3 Cultivation

C. colocynthis is a perennial plant that can propagate both by generative and vegetative means. In the Indian arid zone, the growth takes place between January and October, but the most favorable period for the vegetative growth is during

summer, which coincides with the rainy season. *C. colocynthis* can be sown mixed with crops like bajra, moth, mung, etc. preferably on sandy soils. Soils with high moisture contents are not suitable. Mainly 4-5 kg seed is required per hectare for direct seeding (Lloyd *et al.,* 1898). Field is prepared by deep ploughing during June and leaving the field open under the sun. After receiving two subsequent rains, the field is ploughed twice. Fresh and healthy seeds are taken and soaked for 10-12 hours in warm water, wrapped in gunny bags and buried in soil at a depth of 1.5-2 feet. The place is moistened regularly for 4-5 days. To enhance production, an organic fertilizer can be applied. Colocynth is also commonly cultivated together with cassava (Intercropping) in Nigeria (Olaoye *et al.,* 2012). Thinning is done to keep plant to plant at a distance of 2m. The fruits ripe and turns to yellow color at the month of November. The seeds are separated from the pulp and then are used for various purposes. The studies related to *C. colocynthis* are highlighted in Table 2.2.

Table 2.2 Various studies highlighted the protective effects of *C. colocynthis* in various experiments

Sr. No.	Plant material	Results	References
1.	*C. colocynthis* distilled water extracts	Antigenotoxic effect and Genoprotective effect against cyclophosphamide induced oxidative DNA damage in mice	Shokrzadeh *et al.,* 2013
2.	*C. colocynthis* benzene, ethylacetate, petroleum ether and methanol extracts	Larvicidal, ovicidal and repellent activities against the mosquito *Culex quinquefasciatus*	Mullai *et al.,* 2007
3.	*C. colocynthis* crude methanolic fruit extract (CME) and its fractions	Inhibited the *L. major* parasities and showed antileishmanial activity	Baloch *et al.,* 2013

4.	*C. colocynthis* petroleum ether, acetone, hexane, ethyl acetate and methanol extracts	Inhibited the Larvicidal activity of *C. colocynthis* in *Culex quinquefasciatus*	Rahuman *et al.,* 2008
5.	*C. colocynthis* Triterpenoids cucurbitacin	Inhibited tumor growth and growth of CT-26 cells (N-nitroso-N-methylurethane (NNMU), undifferentiated colon carcinoma cell line) in syngenic BAL B/c mice *in vivo*	Kim *et al.,* 2014
6.	*C. colocynthis* Triterpenoids cucurbitacin I	Inhibited MCF-7 cell migration in a dose-dependent manner, with an IC5050.03mM *in vivo*	Lopez-Haber *et al.,* 2013
7.	*C. colocynthis* Triterpenoids cucurbitacin E	Significantly enhanced the growth inhibitory effect of cisplatin on breast cancer cells	Lan *et al.,* 2013
8.	*C. colocynthis* aqueous and diluted acetone extracts	Antibacterial and anticandidal activity against Gram-negative and Gram-positive bacteria and various Candida spp*. in vitro*	Marzouk *et al.,* 2009
9.	*C. colocynthis* Triterpenoids cucurbitacin B	Laryngeal squamous carcinoma cells and decrease of Bcl-2 and induction of apoptosis	Zhang *et al.,* 2009
10.	*C. colocynthis* Triterpenoids cucurbitacin E	Inhibited the growth of PANC-1 cells (Pancreatic cancer cells) by inducing apoptosis. Inhibited the STAT3 phosphorylation and upregulated expression of p53	Sun *et al.,* 2010

11.	*C. colocynthis* silver nano particles	Silver nanoparticles reduce viability of the HEp-2 cells in a dose-dependent manner *in vitro*	Satyavani *et al.,* 2011
12.	*C. colocynthis* methanolic extracts	No death in any of the groups of BALB mice after receiving methanolic extract of *C. colocynthis* fruits	Rajangam *et al.,* 2013
13.	*C. colocynthis* methanolic extracts	Significantly decreased the elevated levels of cholesterol (TC), triglycerides (TG), free fatty acids (FFA), very low-density lipoprotein (VLDL) and significantly increased the level of high-density lipoprotein (HDL) at dose (200,400 mg/kg)	Rajangam *et al.,* 2013

Chapter 3

Material and Methods

3.1 Materials

3.1.1 Chemicals

Dulbecco's Modified Eagle's Media (DMEM), Fetal Bovine Serum (FBS), Antibiotic (Penicillin/streptomycin, ciprofloxacin), trypsin MTT 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Phosphate buffer solution (PBS), DMSO (Dimethyl Sulfoxide), Agarose, NaOH, NaCl, Na_2EDTA, Sodium Dodecyl Sulphate (SDS), EDTA, H2DCFDA dye, Triton X-100,

3.1.2 Instruments

Table 3.1 Various instruments were being used in entire research work listed below:

Sr. No.	**Name Of Instruments**	**Manufacturing Company**
1.	Analytical balance TE214, S	Sartorius
2.	Autoclave (vertical) NSW-227	Calton
3.	CO_2 incubator	New Brunswick, UK
4.	ELISA Reader 642	Systronics
5.	Flourescent Microscope aided with computer	Olympus Magnus
6.	Hot Plate	Tarsons
8.	Light Binocular Microscope	Olympus Magnus
9.	Mini Centrifuge MC-02	Spinwin Daikan Scientific Co. Ltd.
10.	pH Meter	Mettler Toledo
11.	Rectangular water bath	Julabo
12.	Refrigerated Centrifuge 5430R	Eppendorf, Germany

13.	UV-VIS double beam 2202 Spectrophotometer	Systronics

Methods

3.2 Experimental design and Protocols used in whole experimentation

Brief Plan of work is given below:

Experiment 1: Phytochemical analysis of extracts of *C. colocynthis*.

Experiment 2: Biochemical analysis of various compounds present in fruit of *C. colocynthis.*

Experiment 3: To estimate the antioxidant activity of *C. colocynthis*

3a: Estimation of free radical stress response of *C. colocynthis.*

3b: Assessment of antioxidant enzymes (Catalase, SOD, Glutathione reductase) activity of *C. colocynthis.*

Experiment 4: To check the anticancer activity of *C. colocynthis* by performing MTT assay on HCT116 (colon cancer), MCF7 (human breast adenocarcinoma), HepG2 (human liver cancer), PC3 (human prostate cancer cell lines), H460 (lung cancer).

Experiment 5: To elucidate the protective effect of *C. colocynthis* in pesticides pretreated cells.

3.3 Sample Collection and extracts preparation

The samples were collected from nearby places of Bathinda at different time points. The samples were allowed to dry in the presence of sunlight.

a. The seeds were separated from the fruit of *C. colocynthis,* which was dried and then both fruit pulp and seeds were ground separately.

b. Very fine powder of pulp and seeds were obtained and filtered using sieve of particular required size.

c. Powder was prepared and weighed.

d. Different extracts were prepared using different solvents. These extracts were then used for various tests.

3.4.1 Extracts (Sultan *et al.,* 2010)

C. colocynthis pulp water and seeds extract, ethanol pulp and seeds extract, hydro methanol pulp and seeds extract were prepared as follow:

Table 3.2 Extracts of *C. colocynthis*

Sr. No.	Pulp	Sample Code	Sr. No.	Seeds	Sample Code
1.	Water	WP	4.	Water	WS
2.	Ethanol	EP	5.	Ethanol	ES
3.	Hydro-methanol	HP	6.	Hydro-methanol	HS

3.4.1.1 Water extract

Powder of pulp and seeds (each 500mg) were weighed and dissolved in 10ml of distilled water separately, and vortexing was done and left at rotor for 24 hours for preparation of water extract.

3.4.1.2 Ethanol extract

Powder of pulp and seeds (each 500mg) were weighed and dissolved in 5ml 70% ethanol separately, and vortexing was done and left at rotor for 24 hours.

3.4.1.3 Hydro methanol extract

Powder of pulp and seeds (each 500mg) were weighed separately and hydromethanol solvent is prepared by adding required quantity of water and methanol (H_2O/CH_3OH, 30/70 (v/v)) and vortexing was done and left at rotor for 24 hours for proper dissolving.

These prepared extracts were then filtered by using syringe filters and stored the filtered extracts at room temperature. Filtered extracts were then used for various assays.

3.4.2 Phytochemical Assays of *C. colocynthis*

3.4.2.1 Flavonoids Assay (Singh *et al.,* 2012)

Small quantities of the extracts were heated with 10ml of ethyl acetate in boiling water for 3min. The mixture was filtered differently and the filtrates were Tprepared filtrates were shaken with 1ml of dilute ammonia solution. The layers were allowed to separate by letting it stand for some time and color was observed.

3.4.2.2 Terpenoids Assay (Edeoga *et al.,* 2005)

Different prepared extracts of *C. colocynthis* were used to find out whether terpenoids are present or not by mixing the extracts with 2ml of chloroform and concentrate H_2SO_4 (3ml) is carefully added to form a layer and color was observed.

3.4.2.3 Steroids Assay (Singh *et al.,* 2012)

Acetic anhydride (2ml) and 2ml of H_2SO_4 was added to extracts and observed for color changes.

3.4.2.4 Tannins Assay (Bachaya *et al.,* 2009)

Small quantity of the extracts was boiled with 5ml of 45% ethanol solution for 5min. Each of the mixture was cooled and filtered. Filtrate was diluted with distilled water and added two drops of ferric chloride and observed if any precipitation takes place which is the indicator for the presence of Tannins.

3.4.2.5 Saponins Assay (Singh *et al.,* 2012)

Small quantity of extracts was diluted with 4ml of distilled water. The mixture was shaken continuously and then observed on standing for stable brake.

3.4.2.6 Diterpenes Assay (Bachaya *et al.,* 2009)

Small amount of extracts of *C. colocynthis* fruit was dissolved in 5ml of distilled water. Few drops of copper acetate solution were added and observed for color changes. Green color is indicator of the presence of diterpenes.

3.4.3 Biochemical analysis of various compounds present in fruit of *C. colocynthis*

3.4.3.1 Estimation of Proteins (Bradford Assay) (Kruger *et al.,* 2002)

To 100µl different extracts, 100µl of 1M NaOH was added followed by 3ml Bradford reagent to it. Mixed well, and read at 595nm and compared with standard curve for determination of concentration of proteins in the sample (BSA is taken as standard).

3.4.3.2 Estimation of Phenolic Compounds (Ghimeray *et al.*, 2010)

To 200µl of different extracts, 1ml of Sodium carbonate and 1ml of water was added. Mixed well and add 0.1ml Folin Ciocalteau reagent (2N). Kept it for 1hr, read the OD at 765nm and compared with standard curve for determination of phenols in the sample (Gallic acid 10mg/10ml was used as standard).

3.4.3.3 Estimation of Reducing sugars (Odunfa *et al*., 1985)

Reducing sugars estimation was done by Dinitrosalicyclic acid (DNSA) method. 1ml of DNSA was mixed with 200µl of 80% hot ethanol extract and boiled for 12min. It was cooled at room temperature after adding 2ml of distilled water. Read at 560nm spectrophotometrically compared with standard curve of reducing sugars. Final quantity of reducing sugars was expressed in g/100g of dry weight.

3.4.3.4 Estimation of Starch (Cready *et al.*, 1950)

Ethanol extract (about 200µl) was mixed with 3ml distilled water and 4ml 52% perchloric acid. Kept in ice bath for 20min. Now 200µl of this extract was mixed with 2ml distilled water and 3ml of anthrone reagent. Again kept in ice box for 5min. Boiled the sample mixture at 100°C for 8min. Cooled to room temperature. Read at 630nm and compared with the standard curve of glucose for determining the concentration of starch in the sample.

3.4.1 Culturing of the Cell lines

Cell lines were passaged in flasks. This is done by adding Trypsin which causes detachment of cells from the surface of flasks. Trypsin needs to be actively inactivated to stop the reaction. It was inactivated by addition of DMEM media containing FBS. Cells with media were taken and centrifuged at 1200RPM, 4°C for 4min. Then, supernatant was discarded and pellets were resuspended in 4ml fresh DMEM media. Cells were cultured in fresh flasks.

3.4.2 Maintenance and sub-culturing of cell lines

Once confluent, old media was completely removed from the cells. Then, 1ml trypsin for T-25 flask and 2ml trypsin for T-75 flaks was added, fresh media was added and cells were centrifuged at 1200g 4°C for 5min. Supernatant was discarded and pellets were resuspended in complete media that contains DMEM Media, 10% FBS, 1X penicillin/streptomycin and ciprofloxin. Cells were transferred to T-25 and T-75 flasks. 4ml media was added in T-25 flasks and 10ml for T-75 flasks. Flasks were

incubated in incubators at 37°C, containing 5% CO_2 and 95% humidity. These cells were cultured by adding fresh media for every three days.

3.4.3 Cryopreservation and thawing of cell lines

Cell lines were preserved in cell freezing media with 10% DMSO in cryovials. Thawing of these vials is done at 37°C, resuspended in 4ml DMEM media in 15ml centrifuge tube, and centrifuged at 1200g for 10min. Supernatant was discarded and pellets were resuspended in 2ml media, seeded in 35mm culture dish and incubated at 37°C.

3.4.4 Estimation of antioxidant activity of *C. colocynthis* by using H2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) dye (Baviskar *et al.,* 2013)

Reactive oxygen species (ROS) are byproduct of a number of cellular processes such as oxygen metabolism, stress conditions etc. They can also result by exposure of cells to UV light, IR etc. In turn, these ROS such as the hydroxyl radical (-OH), hydrogen peroxide (H_2O_2), superoxide radical (O^{2-}) which causes large proportion cellular damage. Antioxidant proteins and systems within cells generally neutralize these ROS species, but overproduction of ROS can lead to cell death. ROS are highly reactive and can damage DNA, protein and lipids. These ROS species are genotoxic, i.e. they induce DNA modifications and subsequently mutations. Therefore, the cellular generation of ROS constitutes a serious threat to the integrity of the cellular genome, despite of the existence of efficient defense mechanisms (antioxidants, specific DNA repair), and it is supposed to be causally involved in the generation of cancer and other various age-correlated diseases (Fig-3.1). H2DCFDA is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells to detect the generation of reactive oxygen intermediates in cells. Upon cleavage of the acetate groups by intracellular estrases, the nonfluorescent H2DCFDA is converted to the 2', 7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound, which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495nm and 529nm respectively.

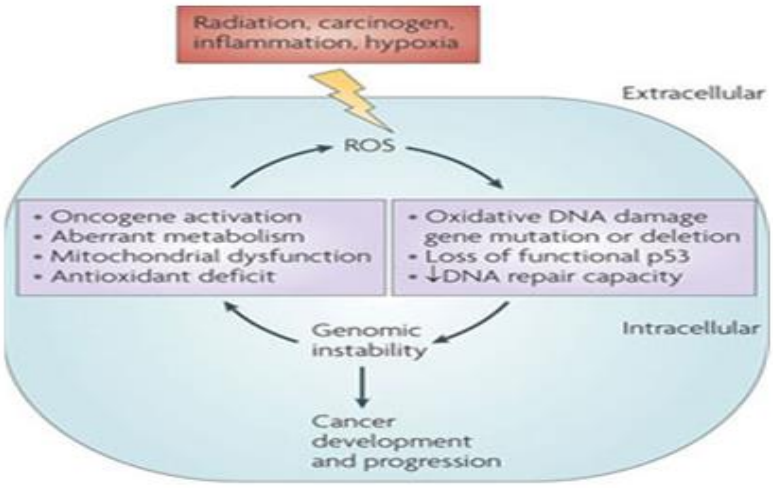


Fig-3.1 Continuous generation of ROS species causes DNA modifications by specific mechanisms. These DNA modifications contribute to increase the spontaneous mutation frequency of the cells during replication, which can activate tumor genes and thereby can result in cancer.

Materials

H2DCFDA, Phosphate buffer solution

Procedure

Two 96 well plates were seeded with 100µl of 6.0 x10^4 cells/ml of cell lines HCT116 (wt) and HCT116 (p53 mutant) in laminar airflow and incubated at 37°C in incubator for 24 hrs. Then, next day, cells were treated with different extracts of *C. colocynthis.* Treated cells were again incubated for 24hrs in incubator. Then after 24 hours, 50µl of H2DCFDA containing PBS was added in each well and kept for 20min in dark. After 20min, media was discarded and 50µl fresh PBS was added in each well. Absorbance was recorded at 470, 530, 590 and 650 nm.

3.4.5 Assesment of antioxidant enzymes (Catalase, SOD, Glutathione reductase) in *C. colocynthis*

Preparation of cell lysates

Five 96 well plates were seeded with HCT116 cell lines in Laminar air flow and incubated at 37ºC in incubator for 24hrs. Next day, the cells were treated with different extracts of *C. colocynthis* as per the experimental design. Treated cells were again incubated for 24hrs in incubator. Cells were harvested and then Triton was added. Cells were then freezed at -4ºC. After freezing, cells were thawed and

again freeze the cells and again thawed the cells. The cells were centrifuged at 14000RPM for 5min and supernatant was stored at -80ºC for further use.

1. Superoxide Dismutase (SOD) assay (Marklund *et al.,* 1974).

This is an important antioxidant defense enzyme in nearly all cells exposed to oxygen. Superoxide dismutase is an enzyme that helps in breakdown of potentially harmful oxygen molecules in cells, thus can prevents tissues from getting damage. It plays a critical role in reducing the oxidative stress implicated in various life-threatening diseases. Studies have shown that SOD can play a critical role in reducing internal inflammation and pain associated with conditions such as arthritis.

$$2o^{2-} + 2H^{+} \Longrightarrow O_2 + H_2O_2$$

Materials

6mM EDTA, 6mM Pyragallol, 0.1M Tris Hcl buffer

Procedure

In 100µl sample, 1.5ml Tris HCL buffer, 0.5ml 6mM EDTA and 1ml of 6mM Pyragallol was added and then mixed properly and absorbance was taken at 420nm on UV-Spectrophotometer.

Calculations

$$\% \text{ inhibition} = \frac{\text{Control - Treatment}}{\text{Control}} \times 100 = x$$

50% inhibition = X/50 = Y U/ml

2. Catalase (Chance *et al.,* 1955)

Catalase is an enzyme, present in peroxisome, which catalyzes the decomposition of hydrogen peroxide to water and oxygen. It helps in protecting the cell from oxidative damage by various reactive oxygen species. One catalase molecule can convert many molecules of hydrogen peroxide to water and oxygen each second.

$$2H_2O_2 \Longrightarrow 2H_2O + O_2$$

Materials

50mM Sodium Phosphate buffer (pH-7.5), Hydrogen peroxide, Cell lysate, extracts

H_2O_2 solution 39mM

0.2ml of H_2O_2 was diluted to 50mM Sodium Phosphate buffer (pH-7.5)

Extraction

Enzyme was extracted with 50mM Sodium Phosphate buffer (pH-7.5) containing 1% polyvinylpyrolidine (PVP).

Procedure

In 1.8ml, 50mM Sodium Phosphate buffer, 0.2ml of sample (enzyme extract) was added. Then, Sodium Phosphate buffer containing H_2O_2 was added. Decrease in absorbance was measured at 240nm by taking readings at intervals of 30sec for 3min.

Extinction coefficient

0.0394m/M/cm

Calculations

Catalase activity was calculated by using following formula:

Volume activity (Unit/ml) = [(A/min (Blank) – A/min (Sample)].d .1)/ V X 0.0436

Where, A/min = Change in Absorbance per min

d = dilution of original sample for Catalase Reaction

V = sample volume in Catalase Reaction, (x ml = 0.00x ml)

0.0436 = e^{mM} for hydrogen peroxide

1 = reaction volume in ml

3. Glutathione Reductase assay (Sedlak *et al.,* 1968)

Glutathione reductase is an enzyme that catalyzes the reduction of oxidized glutathione to reduced glutathione using NADPH as hydrogen donor, so it acts as indirectly acting antioxidant. Glutathione reductase plays an important role in preventing the oxidative damage by maintaining the glutathione level in the cells. With the help of glutathione peroxidase enzyme glutathione is able to minimize the hydrogen peroxide level (Fig-3.2).

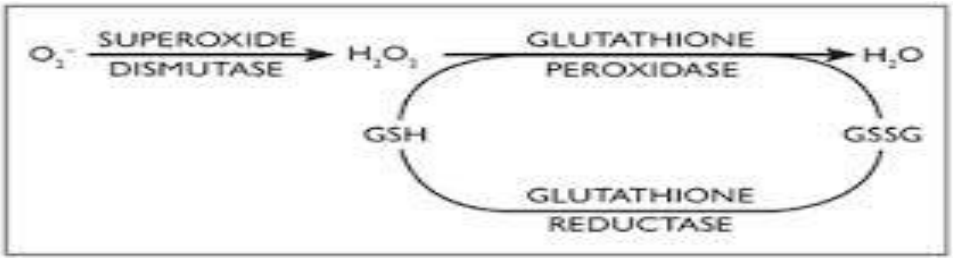


Fig-3.2 Conversion of oxidized form of Glutathione to reduced form

Materials required

0.1M Potassium phosphate buffer of pH 7.5, 0.2mM EDTA, 1.5mM $Mgcl_2$, 0.5mM NADPH, 2mM Glutathione oxidized, extracts.

Procedure

To analyze the enzyme activity, 0.2ml Sample was taken, in this added 0.2M Potassium Phosphate buffer, 0.2mM EDTA, 1.5mM $Mgcl_2$, 0.5mM NADPH and 2mM Glutathione oxidized, then mixed properly and absorbance was taken at 340nm on UV-Spectrophotometer.

Calculation

Glutathione reductaseactivity was calculated by using following formula:

$$\text{Units/ml} = \triangle A/\text{min}/6.22X10^{-3}.d$$

Where $\triangle$ A= Change in Absorbance per min

d = dilution of original sample

$6.22x10^{-3}= e^{mM}$ for NADPH

3.4.6 MTT 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide Assay (Baviskar *et al.,* 2013)

MTT is a colorimetric assay; it is used to assess cell viability as a function of redox potential. Actively respiring cells convert the water-soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to an insoluble purple formazan by mitochondrial succinate dehydrogenase after entering in cell.The formazan was then solubilized with an organic solvent like DMSO and its concentration was determined by using spectrophotometer.

Chemicals required

MTT Reagent, Phosphate buffer solution, DMSO (Dimethyl Sulfoxide).

Procedure

Two 96 well plates was seeded with 100µL of 10^5 cells per ml, one plate seeded with HCT116 wt (wild type) cell line and another plate with HCT116 (p53 mutant). These plates were incubated in incubator for 24 hours. After 24 hours, cells were treated with 6 different concentrations of *C. colocynthis* extracts, out of which there was 3 different concentrations of *C. colocynthis* pulp extracts and 3 different concentrations of seeds extracts. Treated cells were left for incubation for about 48 hours. After that media was completely removed from the wells and cells were washed with 1x phosphate buffer solution (PBS). Then 100µl of (0.5mg/ml) MTT

was added in each well. Then Plates were incubated for approximately 2-4 hours at 37°C. MTT solution was then discarded from wells and DMSO solution was added in order to dissolve the intracellular precipitates in DMSO solution. After 20 min, Purple color was observed then the absorbance of the samples was measured at 570nm**.** Similarly, 96 well plates were seeded with MCF7 cell lines (human breast cancer cells), HepG2 cell lines (human liver cancer cells), PC3 (human prostate cancer cell lines), H460 cell lines (human lung cancer cells) by repeating the same procedure.

Chapter 4

Results

Results of the entire research work have been divided into following sections:

a. Qualitative estimation of the presence and absence of Biochemical compounds in *C. colocynthis (*Phytochemical assay).

b. To determine the chemical composition of various compounds present in *C. colocynthis* (Biochemical assay).

c. Determination of antioxidant or free radical scavenging activity in *C. colocynthis*

d. Assessment of various antioxidant enzymes present in *C. colocynthis* (Catalase, SOD, Glutathione reductase activity).

e. Determination of anticancer activity in *C. colocynthis.*

f. Determination of protective effect of Citrullus colocynthis extracts on pesticides treated cells.

Section a

Results of phytochemical assays

4.1 Flavonoids Assay

Flavonoids are a large family of polyphenolic compounds synthesized by plants. These are important as they have antioxidant and free radical scavenging effects as well as they have potential estrogenic and anticancer activities (Springob *et.,al* 2002).

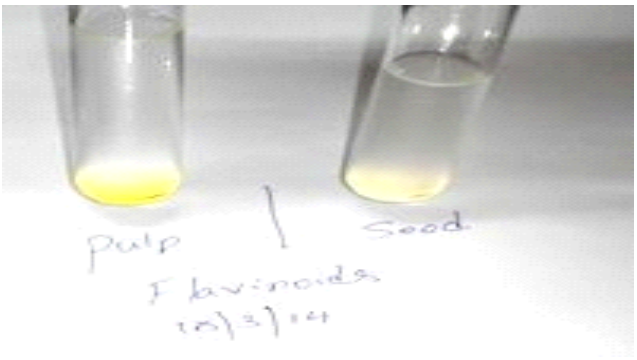


Fig-4.1 Light yellow color indicates present of flavonoids

We observed light yellow color, which indicates the presence of Flavonoids in *C. colocynthis* (Fig-4.1).

5.2 Terpenoids Assay

Terpenoids are plant's secondary metabolites along with alkaloids and flavonoids. These are also called as Isoprenoids as they are derived from five carbon isoprene units.

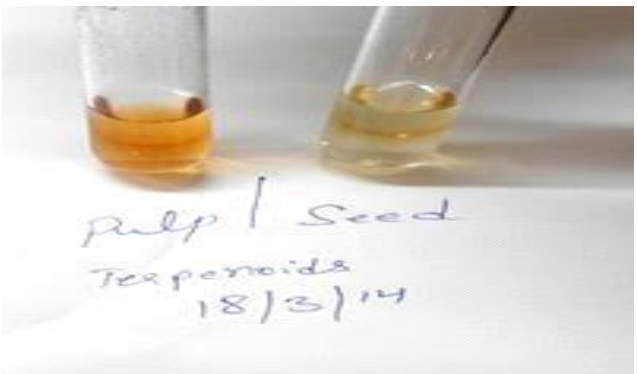


Fig-4.2 Reddish brown layer formation indicates presence of terpenoids

We observed reddish brown layer formation, which indicates the presence of Terpenoids in *C. colocynthis* (Fig-4.2).

4.3 Tannins Assay

Tannins are naturally occurring plant polyphenols. They bind and precipitate proteins and have large influence on the nutritive value of many foods eaten by humans.

Fig-4.3 Red precipitates indicates presence of tannins

We observed light green color of the solution as well as red precipitates indicated the presences of tannins (Fig-4.3).

4.4 Saponins Assay

There was no froth formation in both pulp and seeds indicating the absence of saponins.

4.5 Diterpenes Assay

These are naturally occurring organic compounds that comprise of two monoterpene molecules found in some essential oils. They have antibacterial, antiviral, antifungal and antioxidant properties. Dark green colour indicated the presence of Diterpenes.

4.6 Steroids Assay

These are large class of organic compounds with a characteristic molecular structure containing four rings of carbon atoms (three six-membered and one five). They include many hormones, alkaloids, and vitamins. Dark reddish brown color indicated the presence of steroids.

Table 4.1 Results of various phytochemical tests

Sr. No.	Phytochemical Assay	Pulp		Seed	
		Observation	**Presence**	**Observation**	**Presence**
1	Flavonoids Assay	Light yellow color	**++**	very light yellow color	**+**
2	Terpenoids Assay	Reddish brown color	**++**	Very light reddish brown color	**+**
3	Steroids Assay	Dark Reddish brown	**++**	Light Reddish brown color	**+**
4	Tannins Assay	Light green color and dark red ppt	**++**	Light green color and dark red ppt	**+**
5	Diterpenes Assay	Dark Green color	**++**	Light green color	**+**
6	Saponins Assay	No froth formation	**-**	No froth formation	**-**

(+refers to presence of compound in average amount, ++ refers to presence of compound in good amount, - refers to absence of compound)

Phytochemical results indicates that there is presence of flavonoids, Terpenoids, Steroids, tannins, diterpenes in both *C. colocynthis* pulp as well as seeds but saponins were found to be completely absent in both *C. colocynthis* pulp as well as seeds.

Section b

Results of Biochemical assays

A. Estimation of Protein content in *C. colocynthis*

Estimation of proteins was done for different extracts of *C. colocynthis* (samples) i.e. HS, HP, ES, EP, WS, and WP. Among these samples, WP showed highest protein content 0.5µg/µl and ES showed lowest amount of proteins i.e. about 0.1µg/µl. HS has about 0.33µg/µl concentration of proteins (Table 4.2).

Table 4.2 Protein concentration in samples of *C. colocynthis*

Sr. No.	Sample	Protein concentration (µg/µl)
1	Hydromethanol seeds (HS)	0.33
2	Hydromethanol pulp (HP)	0.3
3	Ethanol seeds (ES)	0.1
4	Ethanol pulp (EP)	0.075
5	Distilled water seeds (WS)	0.375
6	Distilled water pulp (WP)	0.5

On the other hand, HP showed about 0.3µg/µl of the proteins. Similarly, EP showed about 0.075µg/µl concentration of proteins present in *C. colocynthis.* Consequently, WS showed about 0.35 µg/µl concentration of proteins. Based on these results, we can say that water extract of the fruit *C. colocynthis* is rich in proteins.

B. Estimation of Phenolic Compounds in *C. colocynthis*

Estimation of phenolic compounds was done for different extracts of *C. colocynthis* (samples) i.e. HS, HP, ES, EP, WS, and WP. Among these samples, WP showed highest phenolic compounds content about 0.5375µg/µl and ES showed lowest amount of phenolic compounds i.e. about 0.25µg/µl. HS showed about 0.212µg/µl concentration of phenolic compounds (Table 4.3).

Table 4.3 Estimation of Phenolic Compounds in samples of *C. colocynthis*

Sr. no.	Sample	Phenolic compounds concentration (µg/µl)
1	Hydromethanol seeds (HS)	0.2125
2	Hydromethanol pulp (HP)	0.5
3	Ethanol seeds (ES)	0.125
4	Ethanol pulp (EP)	0.3625
5	Distilled water seeds (WS)	0.475
6	Distilled water pulp (WP)	0.5375

On the other hand, HP showed about 0.5µg/µl of the phenolic compounds. Similarly, EP showed about 0.3625µg/µl concentration of phenolic compounds present in *C. colocynthis.* Consequently, WS showed about 0.53µg/µl concentration of phenolic compounds. Based on these results, we can say that water extract of the fruit *C. colocynthis*is rich in phenolic compounds.

C. Estimation of Starch in *C. colocynthis*

Estimation of starch was done for ethanol extracts of *C. colocynthis* (samples) i.e. EP, ES. Here only the samples containing ethanol were used for starch estimation because ethanol is necessary for the extraction of starch present in sample. Among these samples, EP showed highest starch content 0.5µg/µl and EP showed about 0.25µg/µl. Based on these results, we can say that pulp of the fruit *C. colocynthis* contains high amount of starch than its seeds (Table 4.4).

Table 4.4 Estimation of Starch in samples of *C. colocynthis*

Sr. No.	Sample	Starch Concentration (µg/µl)
1	Ethanol seeds (ES)	0.25
2	Ethanol pulp (EP)	0.5

D. Estimation of Total sugars in *C. colocynthis*

Estimation of total sugars was done for ethanol extracts of *C. colocynthis* (samples) i.e. EP, ES (Table 4.5).

Table 4.5 Estimation of Total sugars in samples of *C. colocynthis*

Sr. No.	Sample	Total sugars concentration (µg/µl)
1	Ethanol seeds (ES)	4.3
2	Ethanol pulp (EP)	5.5

Among these samples, EP showed highest total sugars content i.e. 5.5µg/µl and ES showed about 4.3µg/µl. Here also, only the samples containing ethanol were used for sugar estimation because ethanol is necessary for the extraction of total sugars present in sample. Based on these results, we can say that pulp of the fruit *C. colocynthis* contains high amount of total sugars than its seeds.

E. Estimation of Reducing sugars in *C. colocynthis*

Estimation of reducing sugars was carried out for two samples of *C. colocynthis* i.e. EP and ES (Table 4.6).

Table 4.6 Estimation of reducing sugars in samples of *C .colocynthis*

Sr. No.	Sample	Reducing sugars concentration (µg/µl)
1	Ethanol seeds	0.02
2	Ethanol pulp	3.0

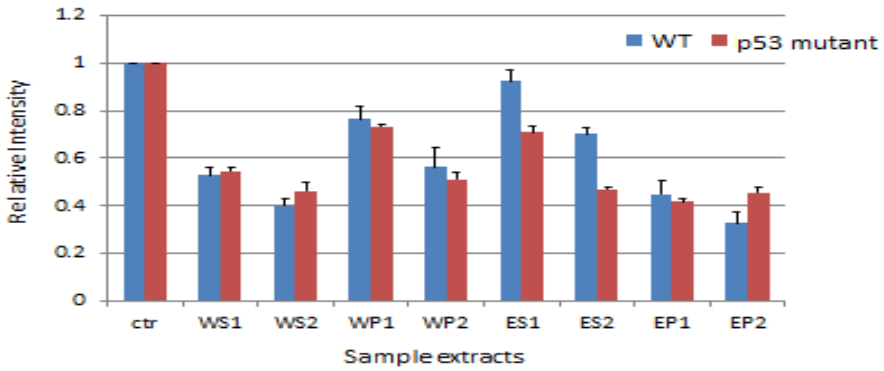
Among these Samples, high amount of reducing sugars was observed in the ethanol pulp extract of the *C. colocynthis.* On the other hand, ES showed very less amount of reducing sugars i.e. only about 0.02µg/µl as compare to pulp .Overall, it seems pulp is rich in various phytochemical compounds as compared to seeds.

Section c

Antioxidant activity or free radical scavenging activity in *C. colocynthis*.

C. colocynthis is known for its antioxidant properties. Antioxidant property of *C. colocynthis* was measured in HCT116 cell lines (wt and p53 mutant). HCT116 cell lines (colon cancer) were treated in 96 well plates with different concentration of different extracts of *C. colocynthis* (10µl-20µl) for 24 hours. The cell lines used were of two different categories, one was wild type i.e. having p53 gene and other having mutant p53 gene.H2DCFDA dye was used to measure the ROS production of cells and protective effect of extracts of *C. colocynthis* was checked.

a.



b.

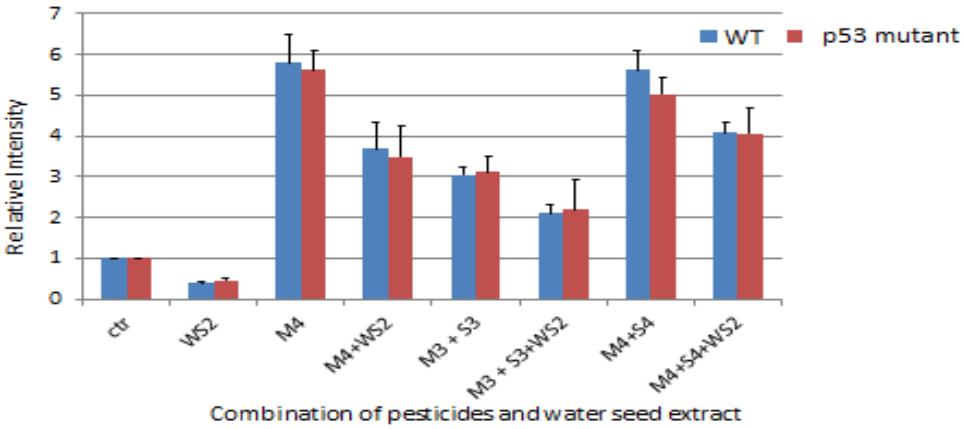


Fig-4.8a. Percent scavenging activity of HCT116 cells (colon cancer) in response to different concentrations of extracts individually. **b.** Percent scavenging activity of HCT116 cells (colon cancer) in response to different concentrations their treatment by pesticides (Monocrotophos and Malathion) and observe the protective effect of

water seed extract of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3).

When treatment to HCT116 (wt) cell lines was given with 10µl water seed extract of *C. colocynthis.* It was observed that there was about 60.2 to 62.8% reduction in ROS within the cells. When concentration of water seeds extract was increased to 20µl, effect was more increased and reduction in ROS level is 68.5 to 69.7%. In contrast to this, when treatment was given with water pulp extract of 10µl, there was about 41 to 45.8% reduction in ROS. When same sample was taken in higher concentration i.e. 20µl, the antioxidant effect was more observed. There was approximately 63.5 to 65.5% reduction in ROS. Similarly, when 10µl ethanol seed extract was added, there was approximately 9.1 to 14.6% reduction in level of ROS. When same extract in higher concentration i.e. 20µl was used, more effect was observed, there was about 15.2 to 19.8% reduction in level of ROS. When 10µl ethanol pulp extract was added, there was about 67.5 to 73.2% decrease in ROS level. When the same extract was used in higher concentration, there was about 69.6 to 79.8% decrease in ROS level (Fig- 4.8a).

We also did an experiment by treating the cells with pesticides Monocrotophos and Malathion. Pesticides are known to cause oxidative stress in cells (Bassil*et al.,* 2007). Cells were treated with 250µM Monocrotophos (M4) which results in higher percentage of ROS generation. Further, this was used as control and after that combination of 250µM Monocrotophos (M4) and 20µl *C. colocynthis* water seed extract was added to HCT116 (wt) cell lines and we observed that ROS effect generated by Monocrotophos was reduced by *C. colocynthis* water seed extract upto 23.1 to 25.7%. Then we used combination of both the pesticides (M3+S3) (100µM Monocrotophos + 50µM Malathion) and this was used as a control in order to evaluate the antioxidant activity of *C. colocynthis* water seed extract and concentration of water seed extract was used about 20µl, there was about 25.3 to 29.8% reduction in the level of ROS as compared to control. When we treated cells with high concentration of pesticides as compare to previous ones (M4+S4) (250µM Monocrotophos+100µM Malathion), which was again used as control for comparing the antioxidant activity of *C. colocynthis* water seed extract, there was about 20 to 26.55% reduction in the amount of ROS level (Fig- 4.8b).

Section d

Assesment of antioxidant enzymes in *C. colocynthis*

1. Superoxide Dismutase (SOD) assay

Superoxide dismutase enzyme catalyzes the dismutation of superoxide (O2−) into oxygen and hydrogen peroxide which is further processed into water and oxygen by next enzyme named Catalase. Thus, they are important antioxidant defense in nearly all cells. To analyze enzyme activity present in *C. colocynthis,* different extracts were taken and after making the reaction mixture, activity was observed by taking the absorbance at 420nm wavelength on spectrophotometer.

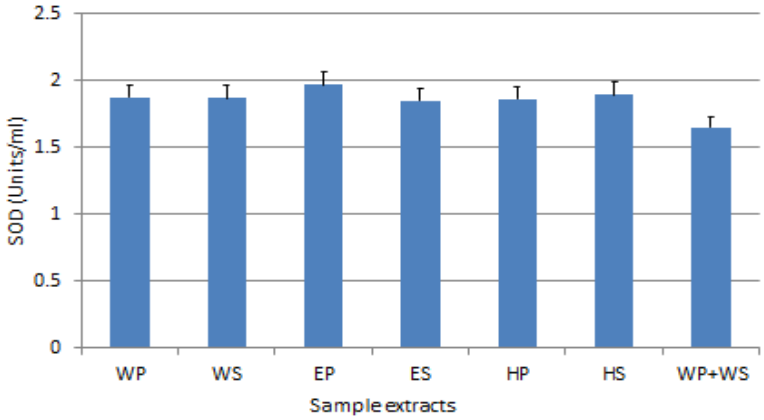


Fig-4.4 SOD enzyme activity in *C. colocynthis*

Results indicate that *C. colocynthis* increases SOD enzyme activity several folds. Highest SOD activity was present in EP and HS extracts. On the other hand, WP, WS, ES, HP extracts showed almost similar amount of SOD activity (Fig 4.4).

2. Catalase assay

Catalase is an antioxidant enzyme which converts the hydrogen peroxide into oxygen and water. Thus, reducing the number of ROS species. Enzyme activity was observed at 240nm.

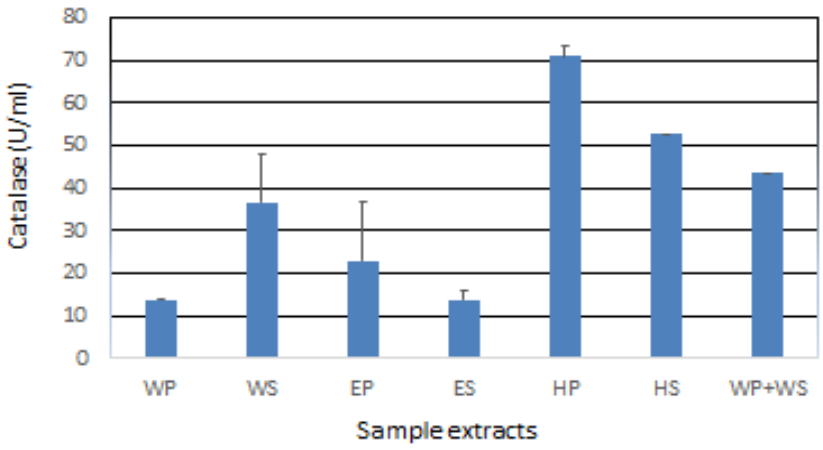


Fig- 4.5 Catalase activity in different extracts of *C. colocynthis*

All the extracts showed the Catalase activity (Fig-4.5). Highest Catalase activity is found to be present in hydromethanol pulp (HP) extract i.e. near about 70.5 to 71.6%. Subsequently; Catalase is found to about 51.5 to 52.8% in hydromethanol seeds (HS) extract. On the other hand, water seeds extract showed about 35.7 to 37.8% of Catalase activity whereas Water pulp extract showed about 11.5 to 13.4% Catalase activity. Ethanol seeds extract showed about 11.4 to 12.7% Catalase activity whereas Ethanol Pulp extract showed about 21.5 to 22.5% of Catalase activity (Fig-4.4). Interestingly, one combination of mixture of water Pulp and Seeds extract showed about 41.5 to 43.6% of Catalase activity

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3. Glutathione reductase assay

Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH). This enzyme is essential for the GSH redox cycle which helps in maintaining the adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. Firstly the enzyme concentration was checked in various prepared extracts of *C. colocynthis* extracts.

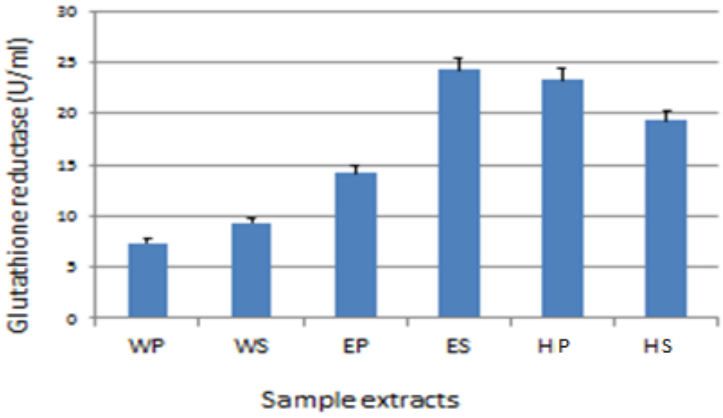

Fig- 4.6 Glutathione reductase activity in different extracts of *C. colocynthis*

Result indicates that highest amount of Glutathione reductase was found to be present in ethanol seed extract (Fig-4.6). Hydromethanol pulp and seed extract also showed presence of Glutathione reductase enzyme. Same experiment was also performed in cell lysate by treating with extracts of *C. colocynthis.*

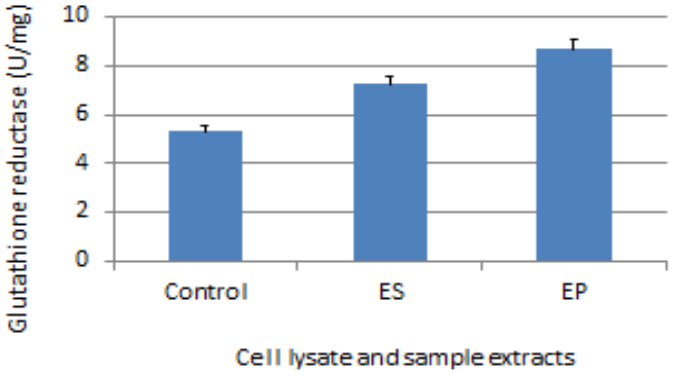

Fig- 4.7 Glutathione reductase activity in prepared cell lysate (ethanol extracts of *C. colocynthis*)

When the prepare cell lysate was treated with ethanol pulp and seed extract in order to assessment of Glutathione reductase enzyme (Fig-4.7). It was found that there was about 22.3 to 23.5% increase in the enzyme concentration in the ethanol seed whereas in case of ethanol pulp, there was about 50.5 to 51.6% increase. So, we can say that after the treatment with extracts of *C. colocynthis,* there was increase in enzyme concentration.

Section e

Determination of anticancer activity in *C. colocynthis* in cancer cell lines

C. colocynthis is known for its anticancer properties. Anticancer activity of *C. colocynthis* was evaluated by measuring the cell viability of cancer cells. Cancer Cell viability (HCT116 cell lines) was measured by treating them with various prepared different extracts of *C. colocynthis* at different concentrations (MTT assay).

1. MCF7 cell lines

Cell viability of cancer cells (MCF7 cell lines) was measured by treating them with various prepared different extracts of *C. colocynthis* at different concentrations. MCF7 cell lines (breast cancer) were treated in 96 well plates with different concentration of different extracts of *C. colocynthis* (range 5µl-10µl) for 24 hours.

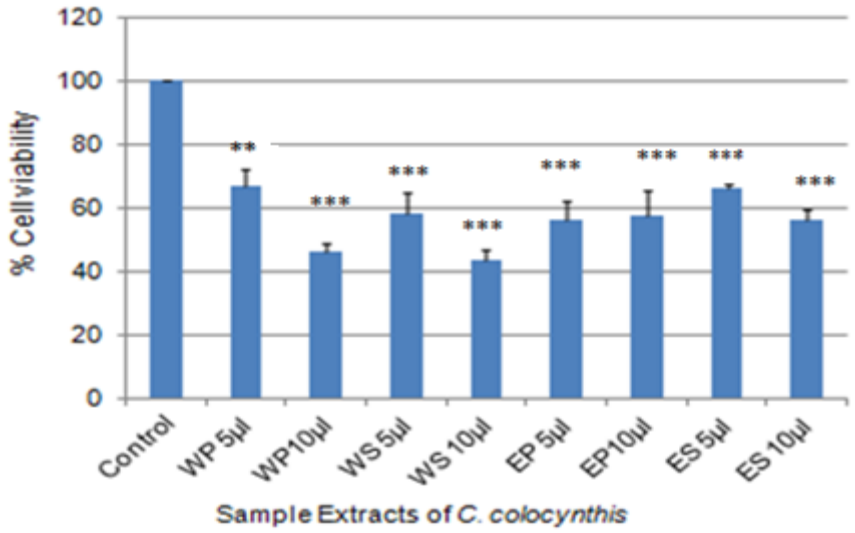

Fig-4.9 Percent viability of MCF7 cell lines (colon cancer) after treating them with various different extracts of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3).

Result indicates that all extracts of *C. colocynthis* successfully reduced the breast cancer cells. On treating with 5µl water pulp extract, MCF7 showed about 35.2 to 36.6% reduction. When the MCF7 cell lines were treated with 10µl of the same sample, cell viability was reduced to about 64.7 to 65.4%. Consequently, treatment with 5µl water seed extract reduced the cell viability about 40.6 to 43.7%. When the same sample was used in higher concentration i.e.10µl, cell viability was reduced to about 54.4 to 5.5%. On the other hand, when the MCF7 cells were treated with

5µl ethanol pulp extract, cell viability of breast cancer cells was reduced to about 37.5 to 39.4%.When the ethanol pulp was used in higher concentration i.e. 10µl, cell viability was reduced to about 34.4 to 35.6%. Similarly, when 5µl ethanol seed extract were treated with MCF7 cell lines, cell viability was reduced to about 33.4 to 34.5%. Interestingly, treatment was also given by using 10µl water seed extract. This extract reduced the cell viability about 41.5 to 42.7%. Here, highest anticancer activity against breast cancer cell was found to be present in water seed extract of the fruit *C. colocynthis* (Fig-4.9).

2. PC3 cell lines

Cell viability of prostate cancer was also measured in order to study the protective effect of the fruit *C. colocynthis.* Here also, prepared different extracts were used and cells were treated with extracts at different concentrations in the same manner as mentioned above.

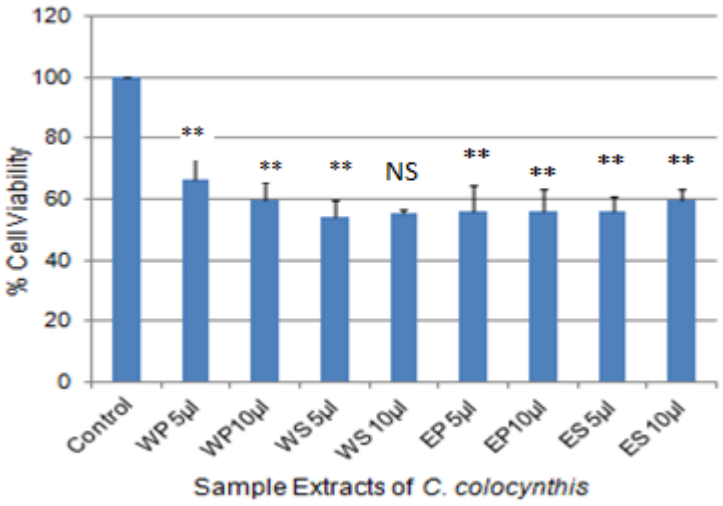

Fig-5.0 Percent viability of PC3 cells (prostate cancer) after treating them with various different extracts of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3).

Here all the extracts showed remarkable results by reducing the viability of prostate cancer cells. When the PC3 cells were treated with 5µl Water pulp extract, it was observed that there was about 28.5 to 35% decrease in cell viability. Moreover, when the same extract was used in higher concentration i.e. 10µl, it was found that there was about 36.7 to 40% decrease in cell viability. On the other hand, when water seed extract was used at concentration 5µl, it was observed that there was

about 40.3 to 44.4% reduction in cell viability. However, effect remained almost same, when the concentration of the same extract was increased to 10µl. When ethanol pulp extract was added at concentration 5µl, there was about 37.1 to 43.4% reduction in cell viability. The effect was similar, when the same extract was used in higher concentration i.e. 10µl. Extract of ethanol seeds at 5µl concentration showed greater effect by reducing the cell viability to about 39.5 to 43% as compare to when it was used in higher concentration i.e. 10µl (Fig-5.0).

3. HepG2 cell lines

Protective effect of the fruit *C. colocynthis* was also observed by using HepG2 cell lines (human liver cancer). Here also, HepG2 cells were treated with extracts at different concentrations in the same manner as mentioned above.

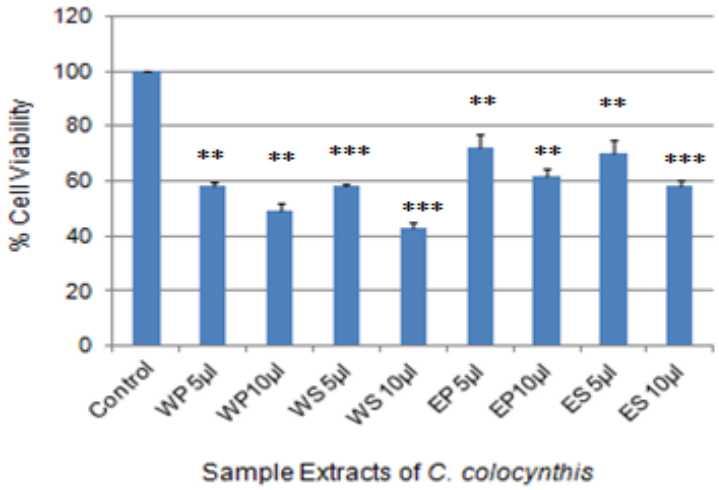

Fig-5.1 Percent viability of HepG2 cell lines (liver cancer) after treating them with various different extracts of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3).

Result indicates that all the extracts showed effective results by reducing the cell viability of liver cancer cells. It was observed that when cells were treated with water pulp extract at concentration 5µl, there was about 38.4 to 40.4% reduction in cell viability. Moreover, when cells were treated with higher concentration of water pulp extract i.e.10µl, there was about 51.5 to 54.8% decrease in cell viability. Furthermore, when water seed extract was used at 5µl concentration, cell viability was found to be reduced to about 40.4 to 42.7%. Similarly, when the same extract was used in 10µl concentration, there was about 56.8 to 59.4% decrease in cell viability. When HepG2 cells were treated with 5µl ethanol pulp, there was about 21.7

to 23.4% decrease in cell viability. Interestingly, when the same extract was used in10µl concentration, there was about 37.5 to 39.3% reduction in cell viability of cancer cells. Extract of ethanol seed at 5µl concentration was found to reduce the cancer cell viability up to 30.4 to 33.5%. Similarly, when the same extract was used in higher concentration, there was about 40.1 to 43.5% decrease in cell viability of cancer cells (Fig-5.1).

4. H460 cell lines

We also analyzed the potential and protective role of *C. colocynthis* by treating the H460 cells with different prepared extracts of *C. colocynthis.*

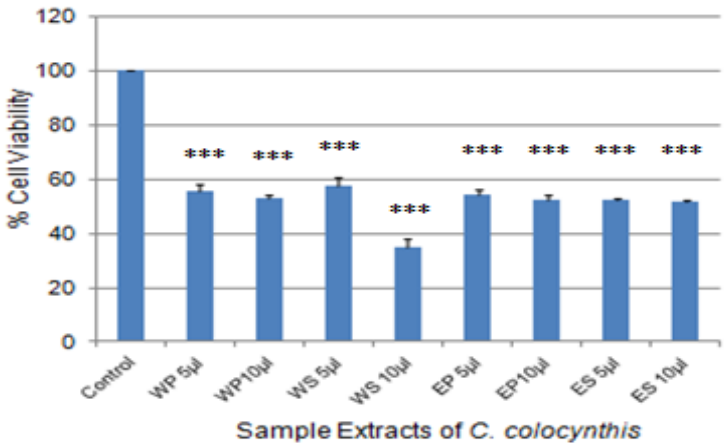

Fig-5.2 Percent viability of H460 cell lines (lung cancer) after treating them with various different extracts of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3).

When cells were treated with 5µl water pulp extract, cell viability was decreased up to 42.6 to 45.4% (Fig-5.2). Interestingly, when the cells were treated with high concentration of water pulp extract i.e. 10µl, there was about 45.4 to 47.8% reduction in cell viability of lung cancer cells. When cells were treated with 5µl water seed extract, there was approximately 40.2 to 42.6% decrease in cancer cell viability. Similarly, when the same extract was used in higher concentration i.e. 10µl, there was about 61.4 to 64.6% decrease in cancer cell viability. Consequently, when cells were treated with 5µl ethanol pulp, there was about 41.4 to 43.5% reduction in cell viability of lung cancer cells. When higher concentration of the same extract was used, it was found that cancer cell viability was reduced approximately about 43.5 to 45.6%. On the other hand, ethanol seed extract was used which shows about

44.5 to 45.3% decrease in cell viability at 5µl concentration. When the same extract was used in higher concentration cancer cell viability was found to be reduced to about 30.5 to 31.7%

5. HCT116 (wt and p53 mutant)

HCT116 cell lines (colon cancer) were treated in 96 well plates with different concentration of different extracts of *C. colocynthis* (range 5µl-10µl) for 24 hours. The cell lines are of two different categories, one was wild type i.e. having p53 gene. p53 is also known as tumor suppressor protein, is a gene that encodes for a protein that helps in regulating the cell cycle. It plays an important role in causing apoptosis of various disfunctioning or old cells in normal form, but if the p53 gene gets mutated it can cause cancer by allowing abnormal cells to proliferate. In normal cells, the p53 amount is low. When there is DNA damage or cell faces any stress conditions, its amount increases. It functions in causing growth arrest, DNA repair and apoptosis. On the other side, there were cells that contains mutant p53 gene.

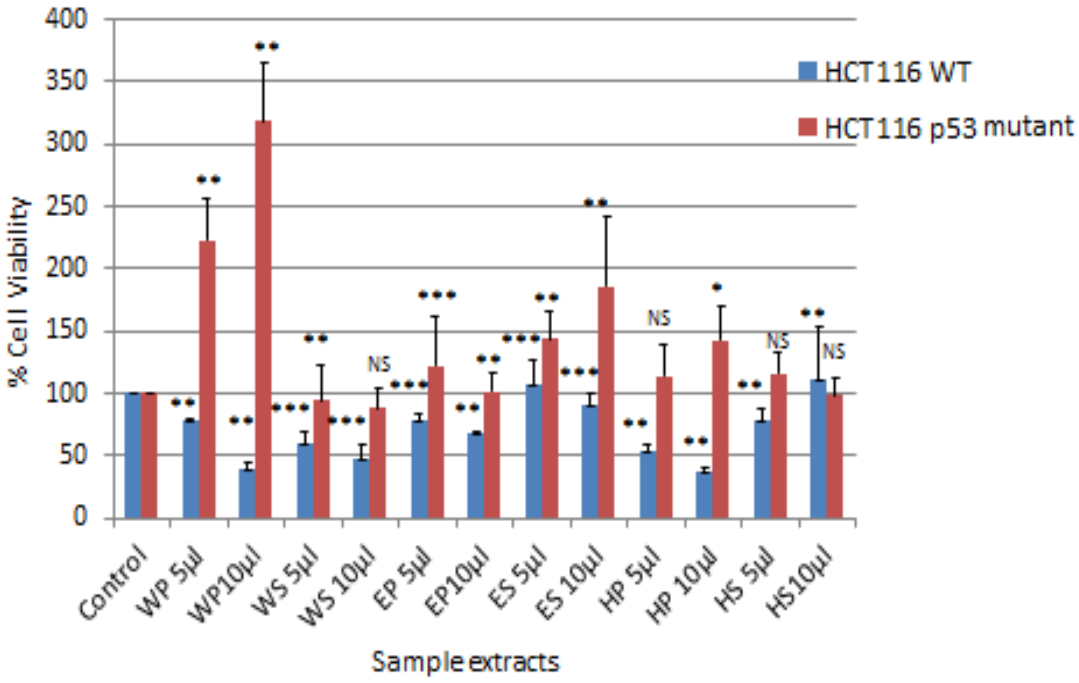

Fig-5.3 Percent viability of HCT116 cells (colon cancer) after treating them with various different extracts of the fruit *C. colocynthis* at different concentrations. Data is expressed as mean values ± S.E. (n=3). Student t test was applied in order to get significant values.

It was observed that there was decrease in cell viability of HCT116 (wt) cell lines with an increase in extracts concentrations of *C. colocynthis.* The results suggest that cell viability reduced from 25.1 to 28% when HCT116 (wt) cell lines were treated with *C. colocynthis* water pulp extract of concentration 5µl. Furthermore, cell viability was much reduced when HCT116 (wt) treated with *C. colocynthis* water pulp extract of concentration 10µl (Fig-5.3). There was approximately 50.5 to 60.1% decrease in cell viability, which signifies the remarkable results. Similarly, the HCT116 (wt) cell lines showed a significant decrease in cell viability when treated with 5µl *C. colocynthis* water seed extracts. There was approximately 38.5 to 40.2% decrease in cell viability when treated with 5µl of *C. colocynthis* water seed extract of concentration 5µl. There was approximately 51.8 to 53.5% decrease in cell viability when the 10µl concentration of *C. colocynthis* water seed extract was added. Similarly, *C. colocynthis* ethanol pulp extract of concentration 5µl showed 23.5 to 26.5% decrease in cell viability and when its concentration was doubled i.e. 10µl, then cell viability decreases up to 33.3 to 36.45%.Furthermore, when treatment was given with *C. colocynthis* ethanol seed extract of concentration 10µl, there was approximately 16.1 to 18.9% decrease in cell viability. Consequently, 5µl hydro-methanol pulp extract treated cells showed significant decrease in cell viability up to 49.8 to 50.3%. When the same extract treatment was given in higher concentration i.e. 10 µl, cell viability shows effective results. There was approximately 63.8 to 68.4% decrease in cell viability. When cells were treated with 5µl of hydro-methanol seed extract of *C. colocynthis,* there was near about 24.1 to 25.5% decrease in cell viability.

Interesting fact is that only the HCT116 (wt) cell lines showed the decrease in cell viability of cancer cells. On the other hand HCT116 (p53 mutant) does not show any cell death. Infact, there was two to three fold increase in cell.

Section f

Determination of protective effect of *C. colocynthis* extracts on pesticides treated cells.

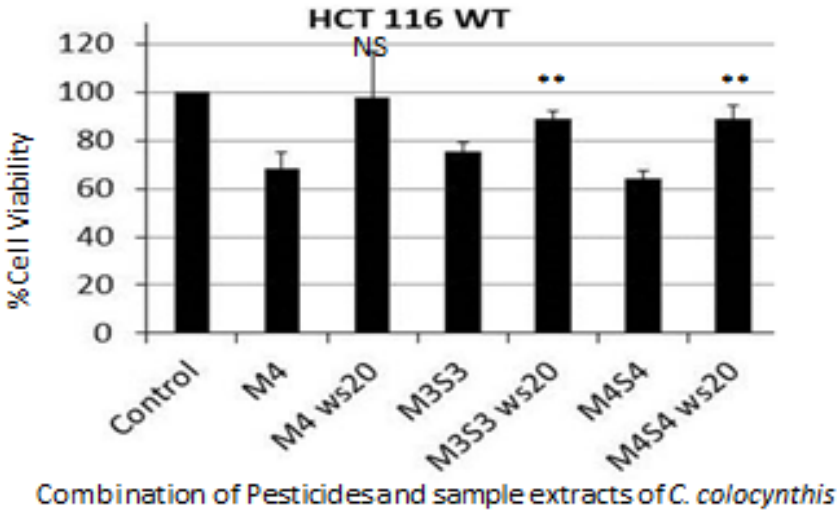

Fig-5.4 Effect of pesticides and protective effect of water seed extract was analyzed in HCT116 wt cell lines. Data is expressed as mean values ± S.E. (n=3). Student t test was applied in order to get significant values.

When cells were treated with pesticides Monocrotophos and Malathion at different concentrations, say M4 (250µM Monocrotophos), there was about 27.4 to 29.5% decrease in cell viability of HCT116 (wt) cells (Fig-5.4). Thus, it induced the cell death. In order to see the protective effect of *C. colocynthis*, cells were treated with 20µl water seed extract and it was observed that there was about 23.5 to 24.6% increase in cell viability. Similarly, cells were treated were combination of M3S3 (100µM Monocrotophos and 50µM Malathion), it was resulted in 16.7 to 19.5% decrease in cell viability. Protective effect of water seed extract was then observed, there was about 8.4 to 9.5% increase in cell viability as compare to M3S3 control. Furthermore, cells were treated with another concentration of combination of pesticides M4S4 (250µM Monocrotophos, 100µM Malathion). There was about 35.4 to 38.5% decrease in cell viability and when cells were treated with water seed extract; there was about 26.4 to 27.5% increase in cell viability.

Effect of pesticides treatment and protective effect of *C. colocynthis* was also observed in p53 mutant cell lines.

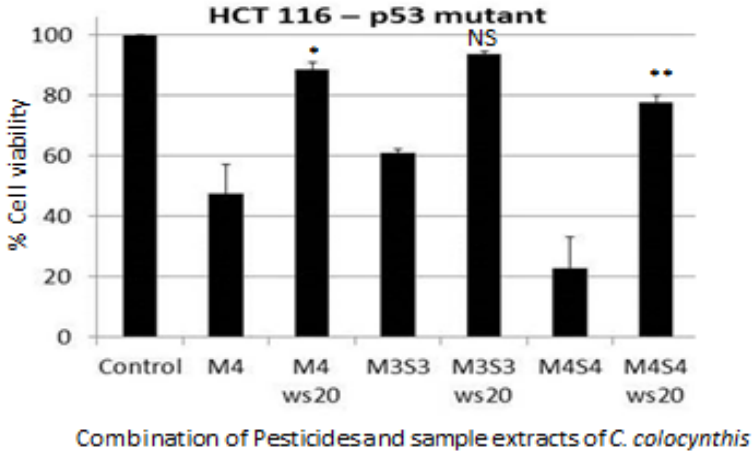

Fig-5.5 Effect of pesticides and protective effect of water seed extract was analyzed in HCT116 p53 mutant cell lines. Data is expressed as mean values ± S.E. (n=3). Student t test was applied in order to get significant values.

When cells were treated with say, M4 (250µM, Monocrotophos) there was 52.5 to 53.6% decrease in cell viability of p53 mutant cells. When 20µl water seed extract was added, the cell viability was increased upto 47.4 to 48.8% as compare to M4. Similarly, When cells were treated with M3S3 i.e. (100µM Monocrotophos, 50µM Malathion, cell viability of p53 mutant cells was decreased about 38.5 to 39.6%, there was 25.5 -27.7% increase in cell viability when 20µl water seed extract was added (Fig-5.5). Another combination of pesticides M4S4 (250µM Monocrotophos, 100 µM Malathion) was also used; it shows that there was about 74.6 to 78.5% decrease in cell viability. There was about 54.6 to 57.4% increase in cell viability, when the cells were treated with 20µl concentration of water seed extract.

Chapter 5

Discussions

C. colocynthis is a medicinal plant which is known for its precious properties. This plant is native of turkey and has been the basis of medical treatments throughout the human history. The phytochemical screening and quantitative estimation of the chemical constituents of the *C .colocynthis* plant showed that the fruit pulp is rich in flavonoids, terpenoids, diterpenes, tannins, steroids whereas seeds of this fruit contains only trace amount of these compounds. Flavonoids, tannins, terpenoids, diterpenes are known to exhibit antioxidant as well as potent anticancer activities (Kathiresan *et al.,* 2006; Heber *et al.,* 2004; Kaur *et al.,* 2002). Tannins are common in fruits, tea, chocolate, legume forages, trees etc. They are composed of a very diverse group of oligomers and polymers. Tannins showed higher cytotoxic activity against human oral squamous cell carcinoma and salivary gland tumor cell lines than against normal human gingival fibroblasts (Sakagami *et al*., 2000). On the other hand, Terpenoids are known for their extensive role in various traditional medicines as well as in synthetic drugs targeting various diseases. Important terpenoids include the curcuminoids which is found in turmeric and mustard seeds, citral, menthol, camphor, salvinorin A. Study revealed that terpenoids, had shown immunomodulatory and antitumor activities. These were able to induce apoptosis in various cancer cells by activating various proapoptotic signaling cascades. Many of the terpenoids found to inhibit metastatic progression and tumor-induced angiogenesis. The molecular mechanisms that involved in these activities include inhibition of various oncogenic and anti-apoptotic signaling pathways and suppression or nuclear translocation of various transcription factors including nuclear factor Kappa Beta (NF-κB) (Kuttan *et al.,* 2011). Flavonoids are phenolic compounds synthesized by plants. These play an important role as antioxidants and cure cancer by protecting the cells from damage caused by free radicals (Miller and A. L., 1996). One of the study revealed that the flavonoids inhibited the mammalian TrxR, which is supposed to be cancer chemoprevention agents because of their antioxidant activities. They are also used in the treatment of rheumatism, asthma and hay fever and various others health problems. Alkaloids, flavonoids are found in colocynth (Haby *et al.,* 1960) and have purgative action (Dafni *et al.,* 1984; Burkill *et al.,* 1985). Results of estimation of compounds indicate that water pulp extract

was rich in the concentration of proteins, polyphenols whereas total sugars, reducing sugars and starch concentration were high in Ethanol pulp extracts.

C. colocynthis known for its various potential roles in treatment and cure of various health problems but there are few reports of antioxidant and anticancer activity in the *in vitro* models. Thus, studies on antioxidant and anticancer activity of *C. colocynthis* are important to evaluate their response in cancer cells. In the present investigation, attempts were made to study the effect of *C. colocynthis* extracts on various cancer cell lines. Pesticides are known to cause free radicals (Devasagayam *et al.,* 2004). Thus, the protective effect of *C. colocynthis* was also estimated by inducing ROS by treating the cells with different concentrations of Monocrotophos and Malathion. Results indicate that all the extracts significantly able to quench the impact of ROS induced by pesticides. Results of present study suggest that *C. colocynthis* has the property to protect the cell from oxidative stress which was induced by pesticide treatment. Here, Water pulp and water seed extracts showed the most significant results and confirmed the antioxidant property of *C. colocynthis.* Assessment of various antioxidant enzymes was done in order to find out concentration of t the antioxidant enzymes such as Catalase, SOD and Glutathione reductase. Results of various enzymatic assays suggest that *C. colocynthis* is rich in these antioxidant enzymes. Maximum Catalase activity was present in HP and WP extracts. On the other hand, quantity of SOD enzyme was observed to be present in almost similar in all the Extracts. Glutathione reductase was found to be present in high quantity in Ethanol seed extract as well as Hydromethanol pulp and seed extracts. Antioxidant enzymes assay was also performed by treating the cell lysate with ethanol extracts. Here, the antioxidant enzymes Glutathione reductase was found to be present in high amount in ethanol pulp extract. Similarly attempts were made to study the anticancer potential of *C. colocynthis* on various cancer cell lines. The results suggest that there was significant reduction in viability of PC3, MCF7, HepG2, H460 cancer cell lines on treating with *C. colocynthis* extracts. Significant reduction in cell viability was also observed in HCT116 (wt) but there was no significant reduction in cell viability of HCT116 (p53 mutant). Reports suggest that more than 50% cancer p53 gene become mutated and thus alteration or inactivation of p53 can lead to cancer (Greenblatt *et al.,* 1994). These mutations seem to be the most common genetic

change in human cancers. It can be depicted from the results that p53 is important protein involved in cell death pathway. Thus, results suggest that *C. colocynthis* can be better therapeutic agent and holds a potential for a treatment of cancer with minimal side effects. It is abundantly available plant that can be included in diet after its proper processing, may help to fight against cancer.

Chapter 6

Conclusions and Future Perspectives

Chemical compounds present in *C. colocynthis* include Flavonoids, Terpenoids, Tannins, Steroids, Diterpenes. Among various extracts used maximum amount of these compounds were present in water extracts of pulp and seed. These chemical compounds may be responsible for its protective role and potential activity such as anticancer activity and antioxidant activity on various cancer cells.

When, we treated HCT116 WT as well as p53 mutant cell lines with extracts of *C. colocynthis,* only the cells having intact p53 showed decrease in viability. It can be concluded that p53 may be responsible for cell death of various cancer cell lines. *C. colocynthis* also contains antioxidant properties which may help in scavenging the ROS generation in our body. Here also, water pulp and seed extracts were observed to be more effective in causing reduction in cell viability of cancer cells. This indicates that drug can be formulated from the *C. colocynthis* which will be cost efficient and more useful than other expensive synthetic drugs.

The results obtained are encouraging and confirm the value of the use of *C. colocynthis* as an anticancer agent for fighting against various cancers. There is requisite to further investigate the effect of *C. colocynthis* other extracts prepared from different parts of plant such as roots, leaves, etc on various cancer cell lines. The study we performed *in vitro* can further be performed *in vivo* to better analyse the effects of this fruit. It may be helpful to find the exact component responsible for the protective action of *C. colocynthis.* There is also need of understanding the mechanism and mode of action of *C. colocynthis* i.e. how it causes cell death of cancer cells as well as various signalling pathways involved can be studied more deeply.

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