Chemical Characterization and Evaluation of Biological Activities of Methanolic Extract of *Cycas revoluta* (Cycadaceae)

THESIS SUBMITTED

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Chemical Characterization and Evaluation of Biological Activities of Methanolic Extract of *Cycas revoluta* (Cycadaceae)"** *submitted by* ***Shri Samit Bera****, who got his name registered on* ***25.07.2011*** *for the award of Ph. D (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of* ***Prof.(Dr.) Amalesh Samanta*** *and that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.*

_____

Signature of the Supervisor
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Date: *(Samit Bera)*

Place: Jadavpur University, Kolkata

DEDICATED TO MY TEACHERS

AND MY FAMILY

CONTENTS

List of Abbreviations

5-FU	**:**	5-Fluoro-uracil
AP1	**:**	Activator Protein 1
APC	**:**	Adenomatous Polyposis Coli
b. w.	**:**	Body Weight
CAT	**:**	Catalase
Cdks	**:**	Cyclin-dependent kinases
CRC	**:**	Colorectal Cancer
DAB	**:**	Diaminobenzidine
DAPI	**:**	4, 6-Diamidino-2-phenylindole
DCFH-DA	**:**	2,7-Dichlorofluorescein diacetate
DED	**:**	Death Effector Domains
DMH	**:**	1,2-Dimethyl hydrazine
DW		Dry Weight
FACS	**:**	Fluorescence Assisted Cell Sorting
FADD	**:**	Fas-Associated Death Domain Protein
FAP	**:**	Familial Adenomatous Polyposis
GAE	**:**	Gallic Acid Equivalent
GC-MS	**:**	Gas Chromatography-Mass Spectroscopy
GSH	**:**	Reduced Gluta-thione
IBD	**:**	Inflammatory bowel disease
MAPK	**:**	Mitogen Activated Protein Kinases
MECR	**:**	Methanolic extract of *Cycas revoluta*.
MTT	**:**	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PET	**:**	Positron Emission Tomography
PI3K/Akt	**:**	Phosphoinositide 3-kinase / Akt (Protein Kinase B)
PI3K/PKC	**:**	Phosphoinositide 3-kinase / Protein kinase C
ROS	**:**	Reactive Oxygen Species
RUE	**:**	Rutin Equivalent
SOD	**:**	Super Oxide Dismutase
TNF	**:**	Tumor Necrosis Factor
TRADD	**:**	TNF Receptor-Associated Death Domain Protein
UHPLC	**:**	Ultra-high Performance Liquid Chromatography

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	E: DMH+5-FU Group (V) DMH group shows that tubular glands in mucosal and submucosal layer are not observed prominently. Tubular glands size is smaller in DMH Group than that of Control group. Degenerative changes observed at mucosal and submucosal layer in DMH Group. Tubular glands gradually appeared clearly as the doses of MECR increased. In DMH+5-FU group degenerative changes are least than those of DMH+MECR200 group and DMH+MECR400 group (magnification, 40x).	

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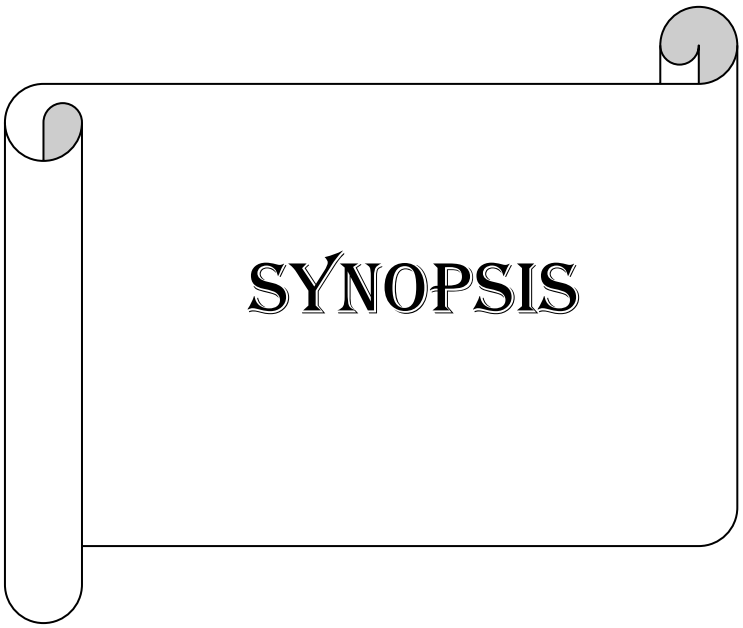
SYNOPSIS

SYNOPSIS

Objective: The objective of the study was to characterize chemically taking methanolic extract of *Cycas revoluta* followed by evaluation of the anti-colon cancer activity in-vitro and in-vivo model.

Chapter 1: This chapter deals with collection of *Cycas revoluta* male cone followed by extraction. Taking the methanolic extract of *Cycas revoluta* (MECR) total polyphenolics, total flvanoids are quantified. Then using GC-MS different types of metabolites (organic acids, amino acids, sugars, sugar alcohols, fatty acids, phenolic compounds and other compounds) were identified along with quantifications. It is already established that among the identified compounds, Myoinositol, Caffeic acid, Citric acid, Xylitol, Inositol-hexaphosphate, Caffeic acid , 4-Guanidinobutyric acid, Xylitol have cytotoxic activity against colon cancer cells. From the UHPLC study, the quantity of Parahydroxy Benzoic Acid, Catechin, Gallic Acid and Quercetin were determined using respective standards. All these compounds have anti-colon cancer activity. So the next work was motivated to determination of anti-colon cancer activity using MECR.

Chapter 2: This chapter deals with in-vitro colon cancer study of MECR taking colon cancer cell line (HCT-8) which was compared with normal colon cell line (CCD-18Co). Cytotoxicity was determined by MTT assay and Colony Formation Assay. Nuclear damage and degeneration was confirmed by DAPI Staining Assay and Tunel Assay. Cellular apoptotic study was accomplished by Flowcytometry using propidinium iodide (PI) stain. Flow Cytometry revealed that cell apoptosis had been occured at the sub-G_0 phase and the apoptosis generated due to more reactive oxygen species (ROS) production in cancer cell line(HCT-8) than that of normal colon cell (CCD-18Co).

Chapter 3: After getting satisfactory results of anti-cancer activity study in-vitro model, colorectal cancer preventive activity was done in wistar rat model using DMH as carcinogen. 5-Fluoro-uracil (5-FU) and MECR were used as standard drug and test drug respectively. After sixteen weeks treatment, the preventive results were taken on the basis of antioxidant status in colon tissue and compared with that of Normal Control group as well as DMH group. The preventive result also compared with respect to polyp formation and also on the basis of histopathological informations.

Conclusions:

MECR is rich in colorectal cancer protective phytochemicals which have antioxidant effects also. In summary, this thesis enlightens the result that MECR decreases proliferation and induces apoptosis by increasing ROS in human colon cancer cell line HCT-8 whereas no such activity was seen in normal colon cell line. As MECR also decreases polyp formation dose dependant manner and also boost up the antioxidant status in-vivo model, so it can be used as colon cancer protective agent. This work can be continued in the future to study and to make it clinically applicable for colon cancer protective activity in different experimental models.

CHAPTER 1

INTRODUCTION

1.1 Cancer

The development of cancer is a multistep process including different phenomena in normal cells leading to malignant development and progression (Aljarbou et al. 2018). The different factors include age, the presence of polyps, inflammatory bowel disease, lifestyle, genetic background and family medical history. Environmental factors such as obesity, physical inactivity, poor diet with low fibre containing, smoking and heavy alcohol consumption account for approximately 80% of all colorectal cancer cases (Svensson et al. 2016). Genetic susceptibility is associated with familial adenomatous polyposis (FAP) and Lynch Syndrome (hereditary non-polyposis colorectal cancer (HNPCC) which accounts for all colorectal cancer cases(Dashti et al. 2018). Individuals who have these diseases have an increased lifetime risk of CRC of up to 80% (Haggar & Boushey 2009).

1.2 TYPES OF TUMOUR

Tumors can be classified according to their migration status into benign or malignant. Benigns are primary tumors consist of abnormal cells that accumulate in a dormant state which originate in a specific tissue and do not migrate to different locations. However, malignant tumors migrate from their primary site, through the extracellular matrix (ECM), blood, lymph and the endothelium of the affected tissues to develop secondary tumor (Shenoy & Lu 2016).

1.3 TYPES OF CANCER

There are different types of cancer that have been classified based on their tissue origin. Carcinomas are types of cancer that originate from epithelial cells. Cancer which affects on lymphatic system is known as lymphoma. Bone and muscles are produced from mesoderm of which carcinoma is known as sarcomas , while adenocarcinomas are cancers that derived from glandular tissue such as breast (Alberts et al., 2002).

Cancer can be caused by several factors that can be classified into internal and external factors. Internal factors include genomic mutations that may occur during DNA replication. On the other hand, external factors include environmental causes such as ultraviolet rays, diet, smoking and obnoxious chemicals.

1.4 STATISTICS

Statistics from the International Agency for Research on Cancer (IARC), indicated that 14.1 million cases were diagnosed with cancer in 2012 worldwide. While total cancer deaths reached 8.2 million in the same year (International Agency for Research on Cancer, 2012). Terrifyingly the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths by 2030 according to American Cancer Society.

1.5 Colorectal Cancer

1.5.1 Functions of the Colon

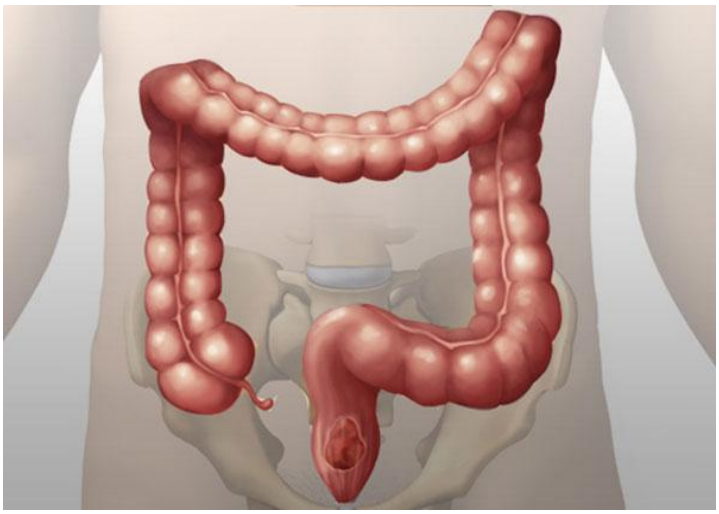

Figure 1: Position of colon in human abdomen

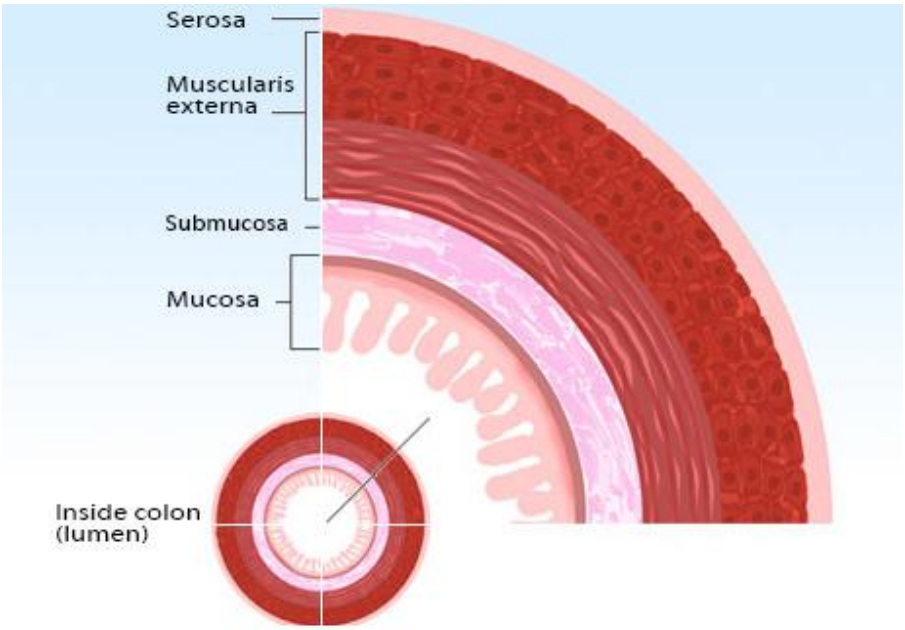

Figure 2: Different layers of colon

Colon is one part of the digestive system that functions to absorb water and store the digested materials until the body is ready to empty as excreta. Under normal condition, the colonic epithelium is constantly changed by crypt proliferation which migrate upward along the crypt-villi (Lipkin 1973). Around 25% of the colonic epithelium is rejuvenated every day. Moreover, the crypt epithelial cells of the entire colon and rectum are replaced every three to four days (Lipkin 1973). A diverse range of stimuli and disease processes are present to cause pathological epithelial cell shedding. These include TNF bacterial lipopolysaccharide (LPS), ischemia, ischemia-reperfusion injury, burn injury, trauma, etc. Normally enterocytes undergoing extrusion exhibit basolateral membrane detachment by the action of some proteolytic enzymes. Neighbouring cells extend cytoplasmic processes underneath the shedding cell as it leaves the monolayer to reform tight junctions and maintain epithelial contiguity. The replacement process is accelerated in pathological conditions(Williams et al. 2015).

1.5.2 Understanding Colorectal Cancer

Colorectal cancer is one of the most common cancer worldwide. According to the American Cancer Society, in 2012 colorectal cancer found to be the third most common cancer in men and the second in women. Approximately, 1.4 million cases of colorectal cancer occurred in 2012 worldwide. Around 693,900 deaths from colorectal cancer occurred in 2012 worldwide, which accounts for 8% of all cancer deaths.

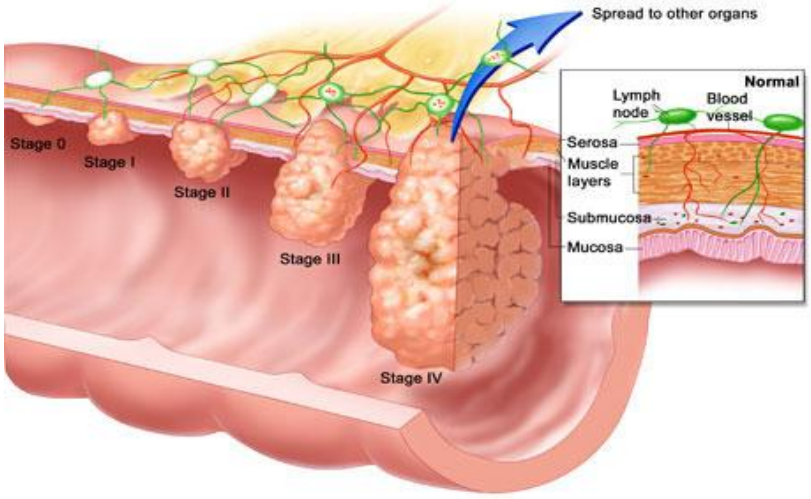

Figure 3: Different stages of colon cancer

Colorectal cancer induced from multiple genetic and epigenetic changes leading to the transformation of colon epithelial cells into invasive adenocarcinomas (Morelli et al. 2015). In addition, genetics has been attributed to colon cancer to either polyposis or non-polyposis syndromes. Polyposis syndrome is the familial adenomatous polyposis (FAP), which is associated with mutated gene or loss of FAP (also called the adenomatous polyposis coli (APC) gene. Non-polyposis colorectal cancer (HNPCC) syndrome is hereditary, which is associated with germ-line mutations in DNA mismatch repair genes (Lee et al. 2017).

1.5.3 Risk factors

1.5.3.1 Dietary factors and life-style

A “Western type” diet with high caloric diet like meat, animal-fat intake and with low fruit, vegetable, and fibre intake is a risk factor for CRC (Pan et al. 2018). Smoking increases the risk of adenomatous polyps and colorectal cancer as well (Dinicola et al. 2018). Obesity is associated with an elevated risk for CRC (Al-Sharif et al. 2018) . D-vitamin and calcium intake is inversely associated with CRC incidence (Bostick 2015). Heavy alcohol consumption is one of the known risk factors of CRC (Messina et al. 2013). Coffee consumption may play a protective role against CRC (Hu et al. 2018) . Nonsteroidal anti-inflammatory drugs(Kuo et al. 2018) and oestrogen replacement are also known to be preventive factors(Smirnoff et al. 1999) .

1.5.3.2 Hereditary syndromes

1.5.3.2.1 Lynch syndrome

Lynch syndrome is also known as hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant disease with risk for developing into colorectal cancer (Kóder et al. 2017). Mutations in this syndrome occur in DNA mismatch repair (MMR) genes: most are in MLH1 and MSH2 genes, but also in MLH3, MSH6, and PMS2 (Aarnio et al. 1999) (Cohen et al. 2017). In half of all HNPCC families, neither MMR mutations nor microsatellite instability is evident, in which case it is not called Lynch syndrome. About 2 to 5% of all colorectal cancers are HNPCC(Chen et al. 2018) .

1.5.3.2.2 FAP

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome caused by mutations in the adenomatous polyposis coli (APC) gene (Pang et al. 2018). Among colorectal cancers, it accounts for about 1%.

1.5.3.3 Inflammatory bowel disease (IBD)

Chronic inflammatory bowel disease (IBD) along with ulcerative colitis (UC) is a risk factor for CRC (Waldum et al. 1981). Unlike in other sporadic carcinomas, which typically develop via the adenoma-carcinoma sequence, a precancerous lesion in IBD can be flat, with a normal endoscopic appearance or can be a dysplasia-associated lesion or mass (DALM). Increased resistance to apoptosis and increased secretion of proinflammatory cytokines such as interleukin-6 lead to chronic activation of the mucosal immunosystem (Thompson et al. 2010). Proinflammatory cytokines can promote cancer development in UC (Lu et al. 2014), and risk factors for cancer are UC duration longer than 10 years (Yashiro 2014).

1.6 MITOTIC CELL CYCLE

Cells reproduce via duplicating their contents and dividing into two. This cycle of division along with duplication is known as a cell cycle. These processes are defined into two major phases which are S phase (DNA synthesis and chromosome duplication) and M phase (chromosome segregation and cell division). The M phase includes two major events; nuclear division (mitosis) and cell division (cytokinesis).

Most cell cycles contain extra phases that are called gap phases. These gap phases provide additional time for chromosomes duplication and segregation that are needed for cell growth generally. Additionally, these gaps serve as transition regulators in the cell cycle progression. The first gap phase, G1, found before S phase, while G2 occurs before M phase(Srivastava et al. 2014). The central components of the cell-cycle control system that include check points which regulate the transition from one phase to another are family of enzymes called cyclin-dependent kinases (Cdks). Cdks belong to serine/threonine protein kinases which are activated

at specific points during cell cycle. They get activated via binding to regulatory proteins called cyclins. Different cyclin-Cdks complexes are present at specific stage in the cell cycle and are important to regulate irreversible phase transition. Any defects in cell cycle regulation can result in developmental abnormalities and cancerous growth as well (Boxem 2006). The p16 (from INK family) and p21 (from Cip/Kip family) are proteins inhibitors that involved in regulating cyclin-Cdks activity (Wang et al. 2018).

1.7 APOPTOSIS

Apoptotic events are characterized by a series of distinct morphological changes that include blebbing, loss of cell membrane integrity, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation. Mitochondrial dysfunction, leading to cell death (apoptosis), produces reactive oxygen species (ROS) that influence numerous cell processes(Choi et al. 2012). Mitochondria are known to be a major source of intracellular ROS generation and are particularly vulnerable to oxidative stress (Chen & Zweier 2014). ROS may also act as intracellular messengers that are induced by a numerous range of stimuli and that trigger apoptosis. Although ROS have important roles in cell signaling, extended high levels of ROS can cause severe damage to DNA, RNA, and proteins, which eventually lead to cell death via either apoptotic or necrotic mechanisms (Wittgen & van Kempen 2007). Moreover during oxidative stress-induced cell death, ROS can target mitochondrial membrane potential. Apoptotic cell death activates caspases, the major executioners of these processes, initiated either through death receptor (extrinsic) or mitochondrial (intrinsic) pathways. Cells may be induced to undergo apoptosis by extracellular signals which is considered as an extrinsic pathway, while the other pathways induced by internal signals called intrinsic pathway.

1.7.1 The Extrinsic Pathway

External signals are part of the normal control which behave for the goodness of the organism as a whole, through surviving when they are needed and killing themselves when they are not. Several extracellular signals stimulate apoptosis, while other inhibit it (Grimm et al. 2011).

Some extracellular signals stimulate apoptosis such as death factors. Examples of death factors are Fas ligand or tumor necrosis factor (TNF) and both received through transmembrane death receptors such as Fas receptor or TNF receptor respectively. Fas ligand is bound to the plasma membrane of neighboring cells, while TNF is a soluble factor. Once ligand binds to the death receptor, the receptor undergoes conformational changes to transmit the signal into the cell. These conformational changes lead to enable intracellular adaptor proteins to bind through their death domains (Park et al. 2016). Examples of intracellular adaptor proteins are Fas-associated death domain protein (FADD) and TNF receptor-associated death domain protein (TRADD) (Scott et al. 2017). These adaptor proteins found to transduce the death signal from the receptor to caspases. The adapters recruit procaspase-8 molecules through death effector domains (DEDs). Once all the procaspases-8 molecules come close together, they become activated by self-cleavage. Caspase-8 is well-known to link between the receptor and the apoptotic proteases for that reason it’s called as an initiator caspase for the extrinsic cascade. Caspase activation is initiated by caspase-8 which in turn activate other caspases which called the executioner caspases (caspase 3, 6 and 7). These caspases lead to apoptosis(Weber et al. 2013).

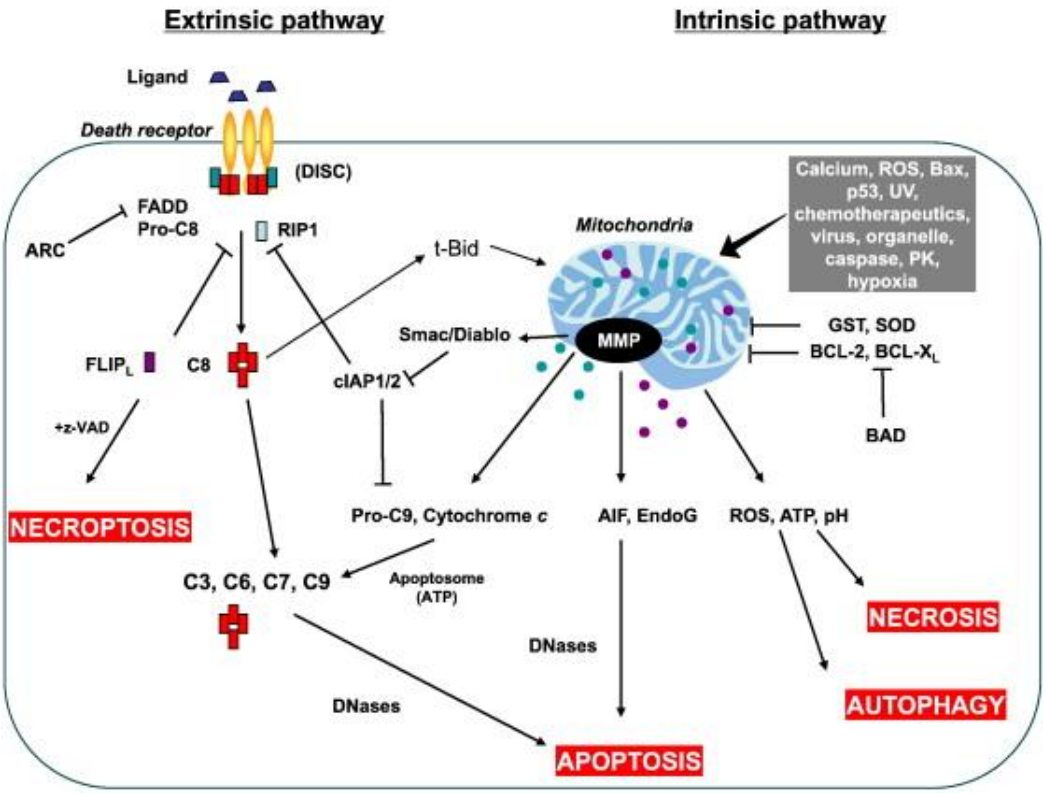

Figure 4: Apoptotic pathway

1.7.2 The Intrinsic Pathway

Cells can activate their apoptosis program internally, usually in response to injury or stresses (e.g., DNA damage or lack of oxygen or nutrients). The mitochondrion-mediated pathway begins with the disruption of the mitochondrial membrane potential (MMP) and release of apoptogenic proteins such as cytochrome C into the cytosol. Once in the cytosol, cytochrome C can activate caspase-9, which in turn cleaves and activates the executioner caspase-3. The caspase-cascade signalling system is regulated by a number of different molecules, such as proteins from the Bcl-2 and the inhibitor of apoptosis protein (IAP) families. The intrinsic pathway is regulated by the Bcl-2 family of proteins which has both anti-apoptotic (Bcl-2 and Bcl-xL) (Carpinelli et al. 2012) and pro-apoptotic (Bax, Bak, and Bid) members(Singh et al.

2015). These act on the mitochondrion to prevent or facilitate the release of apoptogenic factors (Al-Khayal et al. 2017) (Chakraborty et al. 2015).

1.8 Diagnosis of colorectal cancer

1.8.1 Symptoms and signs

Many colorectal cancer patients lack disease symptoms. Others suffer from anaemia along with altered bowel habits such as diarrhoea or constipation. Rectal tumours, mucous and bloody stools and urgency may occur. Fatigue, weight loss, abdominal distension or pain followed by liver enlargement or ascites in advanced cases, as are symptoms caused by metastatic disease (Cai et al. 2015).

1.8.2 Clinical examination

A large colonic tumour can be found by abdominal palpation. Over 50% of rectal cancer cases can be found by digital rectal examination.

1.8.3 Endoscopy

The most important diagnostic tool for CRC is colonoscopy. It allows evaluation of tumour size and location, and allows biopsies for histology of the tumour. In rectal cancer, the distance between tumour and anus is determined by rigid rectoscopy which enabling a choice of appropriate surgical technique.

1.8.4 Preoperative radiological staging

Preoperative thoracic and abdominal computerized tomography (CT) scan allows detection of metastatic disease and it may help in evaluating the location and size of the tumour, and even its invasion depth. These variables are important for preoperative staging and for determining

the proper surgical technique. In rectal cancer, CT is used for evaluating distant metastases, whereas magnetic resonance imaging (MRI) is the examination method of choice for local staging and evaluating tumour invasion depth also. Tumour distance from the anal canal, depth of invasion, and enlargement of lymph nodes is evaluated, all of which influence the choice of preoperative treatment and operative technique. Intraluminal endoscopic ultrasound (EUS) can serve for local staging of rectal cancer, but only for flat and distal tumours. In determination of T stage, accuracy has been reported at 90% (Massari 1998). EUS can find lymph nodes larger than 5 mm. EUS is, however, a very much operator-dependent examination. Positron emission tomography (PET/CT) visualizes metabolic changes in cancer cells, but its sensitivity is poor. It can be applied for detecting occult metastases, but it is unsuitable for preoperative local staging. Positron emission tomography together with MRI, a new alternative in evaluation of the local tumour staging, is under examination (Van Cutsem et al. 2016).

1.9 Treatments of Cancer

There are many types of cancer treatments, and these types are depending on the type of cancer and how advanced it is. These treatments include surgery, chemotherapy, radiation therapy, hormonal therapy or combined therapy. However, these kinds of therapies are known to have different side-effects. Hence, identification, isolation and development of new chemotherapeutic agents from plants “Phytochemicals” have gained significant recognition in the field of cancer therapy and become a major area of experimental cancer research (Singh et al. 2012). Recently, scientists all over the world are concentrating on the herbal medicines to fight against cancer. From different anticancer herbs, new novel herbal anticancer agents can be invented and might be designed to attack the cancerous cells without affecting normal cells of the body.

1.9.1 Phytochemicals

Plants have been shown to be an excellent source of new drugs, including anticancer agents. There are convincing evidences from experimental studies that highlighted the importance of naturally occurring compounds derived from plants in reducing the risk of several cancers and inhibit the development and spread of tumors. With the development of molecular and biochemical isolation and purification techniques, many anticancer agents derived from plants have been identified and developed.

During the last 20 years, more than 25% of drugs that are used were directly derived from plants, while the other 25% were chemically altered natural products (Cragg & Newman 2000). Different examples of anticancer drugs derived from plants are currently in use in clinical fields. These include terpene derivatives paclitaxel, microtubular inhibitor, derived from *Taxus brevifolia*.; vinca alkaloids vinblastine and vincristine, isolated from *Catharanroseus roseus,* is used for mitotic spindle inhibitor and the DNA topoisomerase I inhibitor camptothecin from *Camptotheca acuminata*. Besides, there are other types of promising bioactive phytochemicals which are currently in clinical trials or preclinical trials or undergoing further investigation. Examples include flavopiridol, roscovitine, silvestrol, combretastatin A-4 and betulinic acid. Different studies mentioned the importance of consuming fruits and vegetables, as a source of phytochemicals, in reducing the incidence of cancer. These phytochemicals include dietary polyphenols, flavonoids, phenolic acids indoles, vitamins (carotenoids) and phytoalexins, and sulfur rich compounds (Shankar et al. 2017) (Martínez et al. 2017) . The advantage of using such compounds for cancer treatment is their non-toxic nature. An ideal phytochemical for anticancer activity is one herbal drug having minimal or no toxicity along with anti-tumor properties with defined mechanism of action.

1.10 *Cycas revoluta*

1.10.1 Taxonomical classification of plant

Kingdom : Plantae

Division : Cycadophyta

Class : Cycadopsida

Order : Cycadales

Family : Cycadaceae

Genus : *Cycas*

Species : *Revoluta*

Binomial name: ***Cycas revoluta***

Figure 5: *Cycas revoluta* plant with male cone (Flower)

1.10.2 Plant description

Cycas, the only currently known genus of the Family Cycadaceae, are considered as fossil plants. The cycads are commonly called sago palm. These are widely distributed in the

Tropics, with species found in Asia, Africa, Southeast Asia, Pacific, and Australia. They also grow on volcanic, limestone, ultramafic, sandy, or even water-logged soils in grassland and forest habitats. *C. revoluta*, a widely cultivated species, is an introduced species from Japan and Taiwan. It is used as food by tribal people of North-East Asia, Africa. The demand of Cycas species is for domestic and international horticultural trade.

1.10.3 Economic Uses

As Food Plants: Cycadales are of definite importance as food plants and used variously as the source of food materials such as 'sago' and 'seed starch' vegetables, cakes, kaffir bread and poultry feed. The young leaves of *C. revoluta* is cooked and used as vegetables in several parts of the world including India, Malayasia, Philippines, Indonesia and Japan. The interior part of the stem of several cycads is ground boiled in water and used as feed of cattles, pigs and poultry.

Medicinal Uses

A decoction of the seeds of Dioon edule is used in Mexico for neuralgia. Young seeds of various species of Cycas are used in Sri Lanka, Fiji, India and some other countries as a remedy in bowel complaints and also as emetic and stomach purifiers. Cycas gum is used to cure malignant ulcers in some African Countries. The pounded stem of *Cycas pectinata* is used as a hair washes for diseased hair roots in Assam. Ulcerated wounds and swollen glands are treated in Cambodia from the mucilaginous terminal buds of *Cycas circinalis*. Because of the strongly narcotic nature of the pollen grains of *Cycas circinalis* and *C. rumohii*, the male cone scales of these species are sold in the market as an anodyne (a substance which relieves pain or diminishes distress). In Indonesia, Philippines and some other Asian countries, the

seeds of Cycas are made into a paste in coconut oil and used for wounds, swellings, sores, boils and various skin complaints.

As Source of Gum:

The gum, exuded from the injured petioles, stems or megasporophylls of *Cycas revoluta* and some other Cycads (*Dioon edule, Encephalartos lemarinelianus* and *Macrozamia spiralis*) is used variously as an adhesive, antidote for snake bites and insect bites, and on the malignant ulcers.

As Source of Fiber:

The surface fibers, obtained from the leaves of various species of Cycas (e.g. *C. circinalis, C. revoluta*) and *Macrozamia* are used for stuffing pillows, making mattresses and also for making cloth to some extent.As Source of Hats, Mats, Baskets and Paper Hats, mats, baskets, fences, brooms, cordage and twines are prepared from the leaves of various cycads, including *Cycas, Encephalartos and Macrozamia*. Paper is made from the fibers obtained from the stem of *Macrozamia spiralis*.

As Source of Oil:

An orange yellow oil is obtained from the seeds of *Cycas revoluta*. In some parts of the world, these oils are used as those of palm oil.

As Decorative Material:

The leaves of several cycads are cut, dried with special methods and used as decorative pieces in Japan, Ryukyu Islands and some other countries. It is developing as a good exporting

business in Ryukyu Islands and some other countries from where these leaves are exported to Germany, Switzerland and U.S.A.

1.10.3 Review on *Cycas revoluta*

Cycas revoluta, one of the most archaic living seed plants, contains edible starch in pith, and is used for making sago. Seeds of this plant contain cycasin which is a neurotoxin when consumed orally due to aglycone release from glycoside after acid hydrolysis in stomach (Hirayama et al. 1994). Though seeds contains toxin , the *C. revoluta* cone is used in the painful urination by the hilly people of North-East India (Rout et al. 2012). It is reported for the seeds to possess profound anticancer activity against colon cancer (Mandal et al. 2012a). From Chromatographic separation of the chloroform extract of *C. revoluta* leaflets provided 12 compounds, seven of which are reported for the first time from this species. The isolated compounds include 14 biflavonoids, three lignans, three flavan-3-ols, two flavone- C-glucosides, two Nor-isoprenoids, and one flavanone. Among the isolated compounds some showed moderate antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (Moawad et al. 2014). Bactericidal and proapoptotic peptide having DNA binding properties from *Cycas revoluta* seeds was identified and characterized. In this report, a small peptide (Ala-Trp-Lys-Leu-Phe-Asp-Asp-Gly-Val) with a molecular mass of 1,050 Da was isolated from Cycas revoluta seeds by using reversed-phase liquid chromatography. This peptide shows clear deleterious effects against human epidermoid cancer (Hep2) and colon carcinoma cells (HCT15). It caused inhibition of cancer cell proliferation and further breaking of nucleosome structures, inducing apoptosis by direct DNA binding. A remarkable antibacterial activity was also observed in this same peptide (Mandal et al. 2012b) . A chitinase from cycad, *Cycas revoluta*, (CrChi-A) is the first plant chitinase that has been found to possess transglycosylation activity (Taira et al. 2010). Besides mannose-specific lectins

(Nakamura et al. 2005), beta-D-glucosidase, azoxyglycoside (Yagi et al. 1985)etc had also been isolated from *C. revoluta*.. The ethyl acetate extract of leaflets showed weak cytotoxicity against HepG2 (IC_{50} = 207.6 μg/mL). Anti-leishmaniatic compound vitexin-2″-rhamnoside (IC_{50} = 13.8 μM, IC_{90} = 34.6 μM) has also been isolated from *C. revoluta.* Phytochemical investigation of the ethyl acetate extract of *Cycas revoluta* leaflets also provided five compounds including two new dihydroamentoflavone glucosides, (2S)-I-(2,3)-dihydro-I-7-O-β-D-glucopyranosylamentoflavone and (2S)-I-(2,3)-dihydro-I-7,II-7-di-O-β-D-glucopyranosylamentoflavone, in addition to the known compounds prunin, and protocatechuic acid. (Moawad et al. 2014). A recent study (Mandal et al. 2012a) has reported the anticancer activity of *C. revoluta* seeds on human colon carcinoma cells (HCT-15). However, till now, no reports are available on the colon cancer protective effect of *C. revoluta* cone (male flower) on the DMH induced colon cancer. Therefore, the present work was intended to evaluate efficacy of methanolic extract of *C. revoluta* (MECR) cone against colon cancer through *in-vitro* and *in-vivo* model.

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भारत सरकार
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वैज्ञानिक एफ का कार्यालय
केन्द्रीय राष्ट्रीय पादपाल
पत्रालय -बोटेनिक गार्डेन
हावड़ा -711 103

Ref. No. BSI/Pharma/SD/Tech./2016 Date: 31.10.2016

To

Mr. Samit Bera
Research Scholar
Department of Pharmaceutical Technology
Jadavpur University
Kolkata – 700032.

Sub: Identification of Plant sample – reg.

Sir,

With reference to your letter dated 16.08.2016, this is to inform you that the supplied plant sample has been studied pharmacognostically and confirmed as leaf and male cone of **Cycas revoluta** Thunb. (Cycadaceae).

In this connection, you are requested to make payment of **Rs. 500/-** (Rupees five hundred only) by **Cash** or by **Demand draft** drawn in favour of '**Accounts Officer, P.A.O. (BSI & ZSI), Kolkata**' payable at **Kolkata** towards identification charges.

Thanking you,

Yours faithfully,

A. B. D. Selvam 31/10/16

(A.B.D. SELVAM)
Scientist 'D'

CHAPTER 2

CHEMICAL CHARACTERIZATION OF MECR

2.1 Materials

Methanol, Sodium-bi-carbonate ($NaHCO_3$), Folin-Ciocalteu's reagent, Gallic acid, Rutin, N-Methyl-N-trimethyl silyl-trifluoro-acetamide (MSTFA), Methoxyamine hydrochloride, Catechin, Para-hydroxybenzoic acid, Quercetin, etc were purchased from Sigma Laboratories.

2.2 Plant Material Collection

Fresh male cone was collected from the village of Srirampur under East Midnapur district and authenticated (Voucher Ref. No.: BSI/Pharma/SD/Tech./2016) by botanist, Dr A B D Selvam of Botanical Survey of India (BSI), Shibpur, Howrah (West Bengal). The cones were cut into small pieces and dried in shade below 50°C. Then dried cone pieces were powdered in mixture grinder and stored in airtight container.

2.3 Extraction

The cones were cut into small pieces and were shade dried and then milled into a coarse powder. Then the air dried and powdered cones (175 gm) was first defatted with petroleum ether (60-80 °C) and then extracted with 2.5 litres of methanol (90%) using Soxhlet apparatus. After extraction, extract was filtered through Whatman No. 1 filter paper to remove the unsoluble particles. The solvent was then removed by rotary vacuum evaporator followed by repeated lyophilisation. After drying 11.7 gm (6.6% Yield) extract obtained.

2.4 Determination of total polyphenolic compounds

UV spectrophotometric method was used to determine the concentration of phenolics in the plant extracts (Stanković 2011). Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and

2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45 ^oC for 45 min. The absorbance was determined using spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of total phenolics in extracts was expressed as mg gallic acid equivalent (GAE) per g of dry weight of extract (mg GAE/g DW).

2.5 Determination of total flavonoid content

The content of flavonoids in plant extracts was determined using spectrophotometric method (Stanković 2011). The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, The content of flavonoids in extracts was expressed as mg of rutin equivalent (RUE) per g of dry weight of extract (mg RUE/g DW).

2.6 GC/MS analysis of MECR

2.6.1 Preparation of sample for identification of metabolites

10 mg of the dried sample of MECR was dissolved in MeOH:H_2O of HPLC grade in 1:1 ratio and then 50 µL of the dissolved sample was distributed into eppendorf tubes (3 x 50 µL) and evaporated to dryness. The residue was re-dissolved in 10 µL of methoxyamine hydrochloride (20 mg/mL in Pyridine) and subsequently shaken for 90 minutes at 30 ºC. Then 90 µL of N-Methyl-N-trimethyl silyl-trifluoro-acetamide (MSTFA) was added, and the mixture was shaken at 37 ºC for 30 minutes for trimethylsilylation of acidic protons to increase the volatility of metabolites and to enhance chromatographic separation of metabolites. Fatty Acid Methyl Esters (FAME) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{26} linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8 mg/mL (C_8 - C_{16}) and 0.4 mg/mL (C_{18} - C_{26}) was added(Kind et al. 2009).

2.6.2 Parameters for GC/MS analysis

GC/MS analysis was carried out following the method of Kind et. al. (Kind et al. 2009) after little modification (Das et al. 2016). HP-5 MS capillary column [Agilent J & W; GC Columns (USA), length 30 m plus Duragard 10 m, diameter 0.25 mm narrow bore, film 0.25 µm] was used. An injection was made in a sandwich mode with fast plunger speed without viscosity delay or dwell time. The analysis was performed under the following temperature programme: oven ramp 60 ºC (1 minute hold) to 325 ºC at 10 ºC /minute; 10 minute hold before cool down, 37.5 minutes runtime. The injection temperature was set at 250 ºC, the MS transfer line at 290 ºC, and the ion source at 230 ºC. Helium was used as the carrier gas at a constant flow rate of 0.723 mL/minute (carrier linear velocity 31.141 cm/sec).

1µL of samples were injected via the split mode (split ratio 1:5) onto the GC column. Prior to analysis, the method was calibrated with the FAME standards available with the Fiehn GC/MS Metabolomics library (2008) (Agilent Chem Station, Agilent Technologies Inc., Wilmington, USA). Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra and retention times (RT) with entries of mass spectra and retention time in Agilent Fiehn Metabolomics library using Agilent Retention Time Locking (RTL) method. Fiehn retention indices (RI) were also compared. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC/MS results and to identify chromatographic peaks.

2.7 Quantitative analysis by UHPLC

The identification and quantification of gallic acid, catechin, para-hydroxybenzoic acid and quercetin present in MECR were analyzed by ultra high-performance liquid chromatography (UHPLC) using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 autosampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Das et al. 2017). A standard stock solution (1 mg/mL) of gallic acid, catechin, para-hydroxybenzoic acid and quercetin were prepared in HPLC grade methanol and subsequently different concentrations (5, 10, 20, 30, 40 and 50 µg/mL**)** of standard solutions were prepared by diluting the standard stock solution for calibration curves in order to quantify the phenolic compounds present in MECR. The solutions for UHPLC analysis were filtered through a 0.45 µm membrane filter. The chromatographic separations were performed using a reversed phase C_{18} analytical column (250 mm × 4.6 mm internal diameter) with a particle size of 5 µm, Hypersil GOLD (Thermo Fisher Scientific, USA) and the column temperature was maintained at 25°C. The UHPLC analysis was performed using gradient elution as illustrated in Table 1 with a flow rate of 1.0 mL min^{-1} using 0.2 % (v/v) phosphoric

acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The injection volume was 5 μL and detector wavelength of 280 nm was used for the study. The quantification of phenolic compounds in MECR was calculated from the calibration curve. The chromatograms were processed using Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, USA).

2.8 Statistical analysis

All the data were evaluated with Graph Pad Prism version-5 software (GraphPad Software Inc, La Jolla, CA). Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Dennett's t-test to correct for multiple comparisons with acceptable statistical level significance ($p < 0.05$). Each experiment was presented as the mean ± SEM from triplicate experiments performed in a parallel manner.

2.9 RESULTS

2.9.1 Determination of total phenolic contents in MECR

By spectrophotometric method the total phenolic content was determined. The amount of total phenolic compounds in one gm of MECR was equivalent to 6.3±0.09mg of Gallic Acid (Table 1).

Table 1 Phytochemical content of methanolic extract of *Cycas revoluta* (MECR).

Sl No	Phytochemical constituents	Value
1	Total Phenolic Content (mg of GAE/g of DW extract)	6.3±0.09
2	Total Flavanoid Content (mg of Rutin/ g of DW extract)	4.6±0.06

GAE: Gallic Acid Equivalent. **DW:** Dry Weight. Values were expressed as mean±SEM (n=3)

2.9.2 Determination of total flavonoid content

The flavanoid was also determined by spectrophotometric method. The amount of total flavanoid content in one gm of MECR was equivalent to 4.6±0.06mg of Rutin (Table 1).

2.9.3 Determination of plant metabolites by GC-MS

Metabolites identification in MECR was accomplished using GC-MS analysis (Figure 6). A total forty-three (43) metabolites (organic acids, amino acids, sugars, sugar alcohols, fatty acids, phenolic compounds and other compounds) were identified as shown in Table 2. Among the identified compounds Myoinositol (IC_{50}: 1-10 µM) (Hu et al. 2000), Caffeic acid (IC_{50}: 8.01µM) (Rzepecka-Stojko et al. 2015), Citric acid (IC_{50}: 10 mM) (Lu et al. 2011), Xylitol (IC_{50}: 6.99) (Park et al. 2015) have cancer-protective activity. Inositol-hexaphosphate blocks the PI3K/Akt, AP-1, PI3K/PKC and MAPK signal transduction pathways blocking uncontrolled cell division and forcing malignant cells to either differentiate or enter apoptosis (Fu et al. 2016). Caffeic acid ester is an excellent inhibitor of beta-catenin/T-cell factor signaling in colon cancer cell apoptosis (Xiang et al. 2006). 4-Guanidinobutyric acid (IC_{50}: 43.7 µM) is reported to have cytotoxic activity against gastric cancer cells (Hwang & Jeong 2012). Xylitol provides a beneficial effect in improving health in anticancer treatment(Iwasa & Takahashi 1968) and shows apoptotic activity in the cancer cell (Wu et al. 2016).

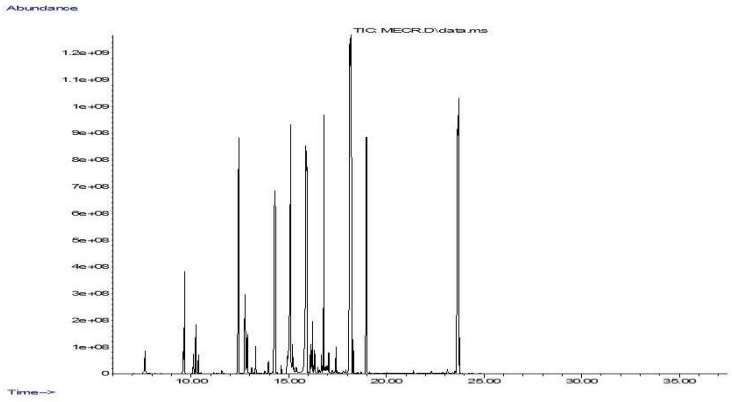


Figure 6: Peaks in GC-MS analysis of methanolic extract of *C. revoluta* (MECR)

Table 2: GC-MS identified metabolites in MECR

Metabolites Identified	Library RT	Observed RT	Peak area (%)
ORGANIC ACIDS			
Citramalic acid	12.63	12.18	0.015
Citric acid*	16.61	16.22	5.777
Dicrotalic acid	14.23	13.73	0.077
Fumaric acid	10.94	10.55	0.011
Gluconic acid 2	18.3	17.80	0.531
Glyceric acid	10.73	10.37	3.356
Glycolic acid	7.05	7.05	0.184
2-Isopropylmalic acid	13.84	13.44	0.037
Lactic acid	6.85	6.85	0.030
Maleic acid	10.32	10.04	0.080
D-Malic acid*	12.79	12.46	0.776
Oxalic acid*	7.88	7.88	0.197
D-Saccharic acid	18.61	16.69	0.609
Succinic acid*	10.51	10.14	0.039
AMINO ACIDS			
L-Allothreonine	16.36	16.18	2.733
Aspartic acid*	12	11.64	0.197
Aspartic acid 2	13.21	12.81	0.067
Beta-alanine	12.04	11.56	0.137

L-pyroglutamic acid	13.22	12.88	10.706
L-valine*	7.3	7.25	0.073
SUGARS and SUGAR DERIVATIVES			
Sucrose*	23.99	23.60	4.375
D-Trehalose*	24.75	24.37	0.106
Glycerol-1-phosphate	16.06	15.73	0.091
4- Methyl-beta-D-galactopyranoside	16.93	17.00	0.204
SUGAR ALCOHOLS			
D-threitol	12.95	12.74	9.989
Xylitol	15.38	15.19	6.875
D-mannitol	17.81	17.48	0.457
D-sorbitol	17.9	17.52	0.060
Allo-inositol	17.25	17.64	0.114
Myoinositol	19.35	18.99	28.303
FATTY ACIDS			
4-acetylbutyric acid	10.63	10.23	5.076
DL-3-aminoisobutyric acid	12.46	11.98	0.004
4-guanidinobutyric acid	13.35	12.88	10.131
Myristic acid*	16.89	16.32	4.914
Mucic acid	18.91	18.69	0.587
Stearic acid*	20.68	20.04	0.282
PHENOLS			
3-phenyllactic acid	13.98	13.54	0.006
Caffeic acid*	19.75	19.21	0.021

cis-4-hydroxycyclohexanecarboxylic acid	12.78	11.91	0.003
OTHERS			
Gluconic acid lactone 2*	17.43	17.59	0.542
Ribonic acid gamma lactone	15.05	14.61	2.192
Porphine	10.77	10.46	0.005
D-sphingosine	22.53	22.95	0.031

All the compounds were identified by comparing retention time (RT), retention index (RI) and mass spectra (MS) of Fiehn Library.

*** Compounds were further validated by comparing RT, RI and MS of standard compounds available in laboratory.**

2.9.4 Determination of phytochemicals by UHPLC

From the UHPLC study, the quantity of Parahydroxy Benzoic Acid (6380.7±5.13µg/g), Catechin (6604±3.60µg/g), Gallic Acid (2008.3±7.63µg/g) and Quercetin (44.7±4.04µg/g) were determined (Figure 7) (Table 3). Gallic Acid (IC_{50}: 740 µM) causes ROS dependent apoptosis and inhibited the growth of colon cancer cells (Subramanian et al. 2016). Catechin (IC_{50}: 20µg/mL) causes inhibition of cell growth, decreases in the levels of COX-2 and Bcl-xL proteins and apoptosis (Shimizu et al. 2005). Parahydroxy Benzoic acid has antiproliferative and proapoptotic activities through inhibition of histone deacetylases(Seidel et al. 2014). Quercetin (IC_{50}: 42.5µM) depends on COX-2 dependent ROS generation that induces apoptosis and inhibits cell survival (Raja et al. 2017).

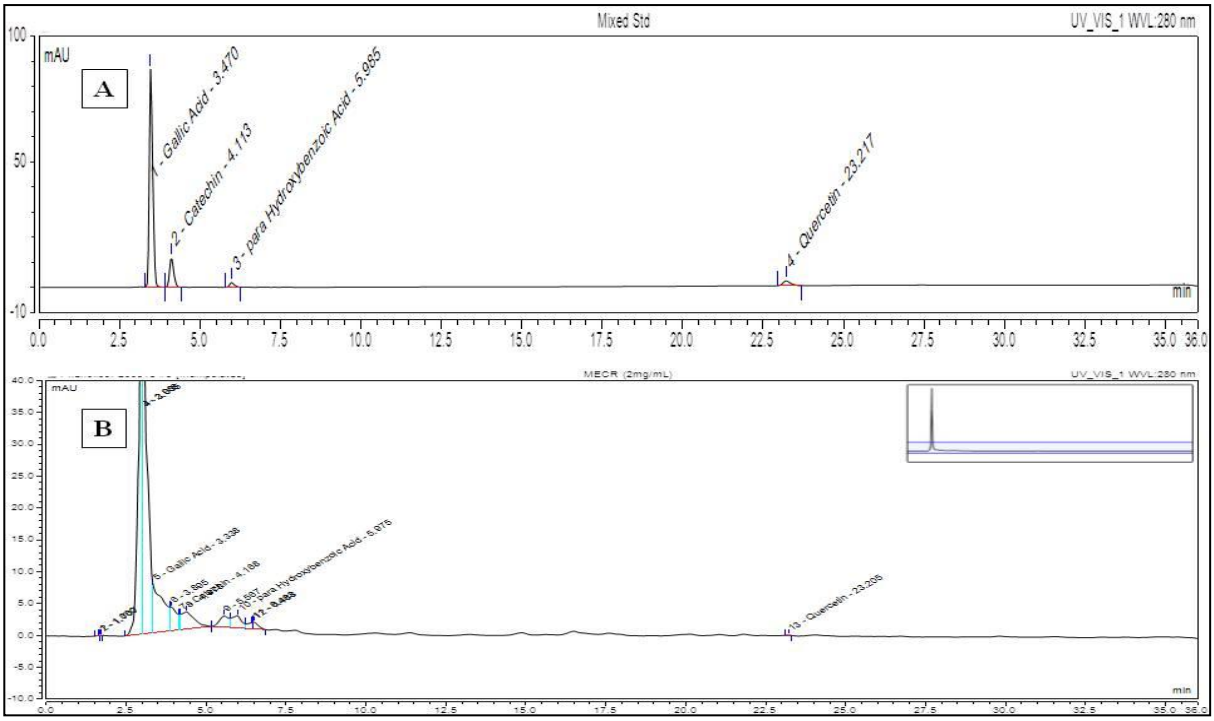


Figure 7: UHPLC chromatograms of a mixed standard of phenolic compounds (A) and the methanolic extract of *C. revoluta* (MECR) (B) as detected at 280 nm (1: Gallic acid, 2: Catechin, 3: Para-hydroxybenzoic acid, 4: Quercetin).

Table 3: UHPLC identified metabolites in MECR

SL No	Metabolites Identified	Quantity
1	Parahydroxy Benzoic Acid	6380.7±5.13µg/g
2	Catechin	6604±3.60µg/g
3	Gallic Acid	2008.3±7.63µg/g
4	Quercetin	44.7±4.04µg/g

Values were expressed as mean±SEM (n=3)

2.10 Discussion

Metabolites identification in MECR was accomplished using GC-MS analysis (Figure 6). A total forty-three (43) metabolites (organic acids, amino acids, sugars, sugar alcohols, fatty acids, phenolic compounds and other compounds) were identified as shown in Table 2. Among the identified compounds Myoinositol (IC_{50}: 1-10 µM) (Hu et al. 2000), Caffeic acid (IC_{50}: 8.01µM) (Rzepecka-Stojko et al. 2015), Citric acid (IC_{50}: 10 mM) (Lu et al. 2011), Xylitol (IC_{50}: 69mg/mL) (Park et al. 2015) have cancer-protective activity. Inositol-hexaphosphate blocks the PI3K/Akt, AP-1, PI3K/PKC and MAPK signal transduction pathways blocking uncontrolled cell division and forcing malignant cells to either differentiate or enter apoptosis (Fu et al. 2016). Caffeic acid ester is an excellent inhibitor of beta-catenin/T-cell factor signaling in colon cancer cell apoptosis (Xiang et al. 2006). 4-Guanidinobutyric acid (IC_{50}: 43.7 µM) is reported to have cytotoxic activity against gastric cancer cells (Hwang & Jeong 2012). Xylitol provides a beneficial effect in improving health in anticancer treatment(Iwasa & Takahashi 1968) and shows apoptotic activity in the cancer cell (Wu et al. 2016). From the UHPLC study, the quantity of Parahydroxy Benzoic Acid (6380.7±5.13 µg/g), Catechin (6604±3.60 µg/g), Gallic Acid (2008.3±7.63 µg/g) and Quercetin (44.7±4.04 µg/g) were determined (Figure 7). Gallic Acid (IC_{50}: 740 µM) causes ROS dependent apoptosis and inhibited the growth of colon cancer cells (Subramanian et al. 2016). Catechin (IC_{50}: 20 µg/mL) causes inhibition of cell growth, decreases in the levels of COX-2 and Bcl-xL proteins and apoptosis (Shimizu et al. 2005). Parahydroxy Benzoic acid has antiproliferative and proapoptotic activities through inhibition of histone deacetylases(Seidel et al. 2014). Quercetin (IC_{50}: 42.5 µM) depends on COX-2 dependent ROS generation that induces apoptosis and inhibits cell survival (Raja et al. 2017).

2.11 Concluding remarks

This chapter gives confirmation about the presence of different chemicals like organic acids, amino acids, sugars, sugar alcohols, fatty acids, phenolic compounds, flavanoids. Among the different compounds compounds Myoinositol (IC_{50}:1-10 µM), Caffeic acid (IC_{50}:8.01 µM), Citric acid (IC_{50}:10 mM), Xylitol (IC_{50}: 6.99) have colon cancer-protective activity. Besides these compounds it is reported that Parahydroxy Benzoic Acid (6380.7±5.13 µg/g), Catechin (6604±3.60 µg/g), Gallic Acid (2008.3±7.63 µg/g) and Quercetin (44.7±4.04 µg/g) of MECR have colorectal cancer protective activity. Hence, MECR might be anti-colon cancer agent and for this confirmation the work is extended to *in-vitro* and *in-vivo* model taking MECR as testing agent.

CHAPTER 3

IN-VITRO ANTI-COLON CANCER STUDY OF MECR

3.1 Materials

Dulbecco's Modified Eagle's medium (DMEM), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Foetal Bovine Serum (FBS), DCFH-DA (2',7-Dichlorofluorescein diacetate), Propidium iodide (PI), DAPI (4, 6-Diamidino-2-phenylindole), Horseradish peroxidise-conjugated anti-fluorescein antibody were purchased from Sigma Laboratories and TUNEL assay kit were purchased from Roche Molecular Biochemicals, Manheim, Germany.

3.2 Cell culture

Colon cancer cell line (HCT-8), normal colon cell line (CCD-18Co) were collected from were purchased from NCCS Pune, India. Cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in the air. When the cells were 60-70% confluent, the medium was aspirated, the cells were washed with phosphate-buffered saline (PBS), and fresh DMEM with or without antibiotic was added. Control plates were replenished with fresh medium and also incubated at similar conditions, as stated above (Singh et al. 2017).

3.3 Cell viability determination

Cell viability was quantified by the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed to evaluate the antiproliferative effect of the test material. HCT-8 cells was seeded into 96-well plates in the density of 1×10^4/well and incubated overnight. Then, 200 μL culture medium containing various concentrations (0, 50, 100, 200, 300, 400, 600, 800, 1000 μg/mL) of MECR extracts were added to each well. The test extracts were removed at the end of the incubation period. After 48 hrs of MECR

treatment, the MTT solution (5 mg/mL) was added to each well and incubated for 4 h. Cells were incubated with 5 mg/ml MTT solution (Sigma) for 2 h in a CO_2 incubator to allow the transformation of MTT dye to formazan salt (not dissolved in water). Finally, the MTT solution was removed and replaced by 150 µL DMSO each well to dissolve the formazan crystals and mixed for 10 min. The absorbance of the solution was determined by a Spectra Max M5 plate reader at 540 nm wavelength. The experiment was done with four replicates three times (Zhang et al. 2016).

3.4 Colony formation assay

HCT-8 cells were treated with different concentration (0, 200, 400, 500, 1000 µg/mL) of MECR for 48 hr and plated separately in a petridish plate (Plate diameter 30 mm) using 3 mL complete growth medium containing DMEM media with 10% foetal bovine serum (FBS) at a density of 1×10^3 cells/plate. After one week incubation, cells were stained with a crystal-violet solution [0.05% (w/v) crystal violet in 20% (v/v) methanol] for 10 min and then extra stain is washed by dipping the plate in distilled water (Du et al. 2017). The colonies were counted in a light microscope (DM 1000, Leica, Germany) using software Las EZ.

3.5 Reactive Oxygen Species (ROS) measurement

2',7'-dichlorofluorescein diacetate is a stable non-fluorescent, cell permeable compound, which on penetrating the cell is converted to $DCFH_2$ by intracellular esterases which is entrapped within the cell and is stable for a few hours. The de-esterified product on oxidation by ROS is converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF) and upon excitation green fluorescence which is proportional to the intracellular level of ROS. Thus, change in DCF fluorescence reflects mainly the intracellular accumulation of ROS.

HCT-8 cells were treated with different concentration (0, 200, 500, 1000 µg/mL) of MECR for 48 hr. and then washed in PBS 3 times. The ROS produced by HCT-8 cells was estimated using fluorescent dye DCFH-DA (2', 7-Dichlorofluorescein diacetate). In brief, after addition of 10 µl of DCFH-DA dye (final concentration of 20 µM) cells were incubated for 30 min in a CO_2 incubator (Heraeus, HERA cell). The fluorescence intensity was measured at an excitation and emission wavelength of 485 nm and 530 nm respectively using a spectrofluorimeter (Synergy HT, Biotek, USA). The data were expressed as percentage DCF fluorescence as compared to that of control. Simultaneously ROS level in normal colon cell (CCD-18Co) was also observed (Tiwari et al. 2010).

3.6 Detection of apoptosis by flow cytometry

The analysis of cell cycle phase distribution of HCT-8 was done according to the method of Holmes et al. with slight modification. HCT-8 cells were treated with different concentration (0, 200, 500, 1000 µg/mL) of MECR for 48 hr. followed by washing in PBS for 3 times. Then 1×10^6 cells were permeabilized with 70% ice-cold ethanol followed by staining with propidium iodide (PI, 20 µl of 1 mg/ml stock). Cell cycle phase distribution of nuclear DNA was determined on FACS (fluorescence-activated cell sorter) Calibur, fluorescence detector equipped with 488 nm argon-ion laser light source using Cell Quest software (Becton Dickinson, USA). A total of 10000 events were acquired for each sample analyzed. A histogram of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been displayed. The percentage of apoptotic cells was determined by measuring the fraction of nuclei that contained a sub-diploid DNA content(Manna et al. 2006).

3.7 DAPI staining assay

DAPI (4, 6-Diamidino-2-phenylindole) staining was used to assess nuclei morphology of cells. The logarithmic phase of HCT-8 cells was seeded into petridish plates (diameter 30 mm) in

the density of 1×10^3/plate and cultured overnight. After treatment with different concentrations (0, 200, 400, 800, 1000 μg/mL) of MECR extracts for 48 hr, the cells were stained with DAPI after fixing with 3.7% formaldehyde. The samples were then washed with PBS and detected by fluorescence microscope (Leica DM 4000B) (Zhang et al. 2016).

3.8 Tunel assay

Apoptosis analysis was done in the HCT-8 cells using TUNEL assay kit (Roche Molecular Biochemicals, Manheim, Germany) according to manufacturers' protocol. At first, HCT-8 cells were seeded separately in a petridish plate (30 mm) in the density 1×10^3/plate along with different concentrations (0, 200, 500, 1000 μg/ml) of MECR for 48 hrs. Then cells were trypsinized to cause detachment and cell smear was fixed in freshly prepared 4% paraformaldehyde in PBS at room temp followed by washing and endogenous peroxidase blocking with 3% H_2O_2 in methanol for 10 min at room temp. Then the cells were permeabilized with 0.5% Triton-X 100 and incubated with Tunel reaction mixture at 37 ºC for 60 min in a humidified chamber. After washing, cells were incubated with horseradish peroxidise-conjugated anti-fluorescein antibody at 37 ºC for 30 min in a humidified chamber. Stained cells were visualized after substrate diaminobenzidine (DAB) reaction by light microscope. For each sample, six randomly chosen fields were scored in a blinded manner. Mean percentage of apoptotic cells was determined(Basu et al. 2017).

3.9 Statistical analysis

All the data were evaluated with Graph Pad Prism version-5 software (GraphPad Software Inc, La Jolla, CA). Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Dennett's t-test to correct for multiple comparisons with acceptable statistical

level significance ($p < 0.05$). Each experiment was presented as the mean ± SEM from triplicate experiments performed in a parallel manner.

3.10 RESULTS

3.10.1 Cytotoxicity

MTT assay was performed to determine the cytotoxicity effect using HCT-8 cancer cell line (Figure-8). Exposure of cells to different concentrations of the MECR (0-1000 µg/mL) affected significant cytotoxicity at concentrations up to 1000 µg/mL. A strong reduction in cell viability was observed in a dose-dependent manner. The toxic effect of MECR markedly decreased the cell viability to 31 ± 1.08 % at 1000 µg/mL. The IC_{50} value was found to be 500 ± 1.09 µg/mL against HCT-8 cell line .

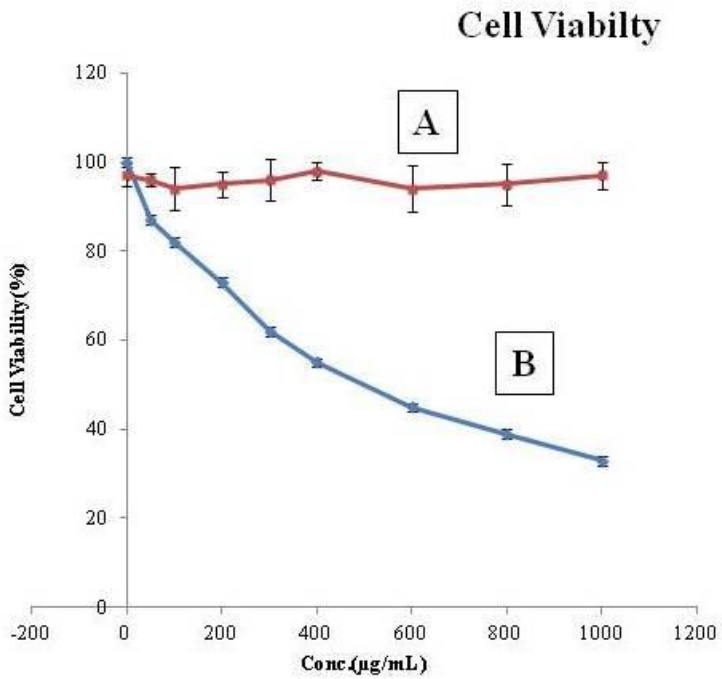


Figure 8: MTT Assay for Cell Viability Testing. A: Normal colon cell line (CCD-18Co). B: Colon cancer cell line (HCT-8). Values are expressed as mean ± SEM (n=3). Increased concentration decreased the viability in Colon cancer cell line (HCT-8) (IC_{50}=500±1.09 µg/mL).

3.10.2 Colony formation assay

This assay determines the number of adherent colony forming cells. The number of adherent colony forming cells decreased in a concentration (0-1000 µg/mL) dependant manner from 526 ± 12.02 to 116.7 ± 8.82 cells per field (Figure-9).

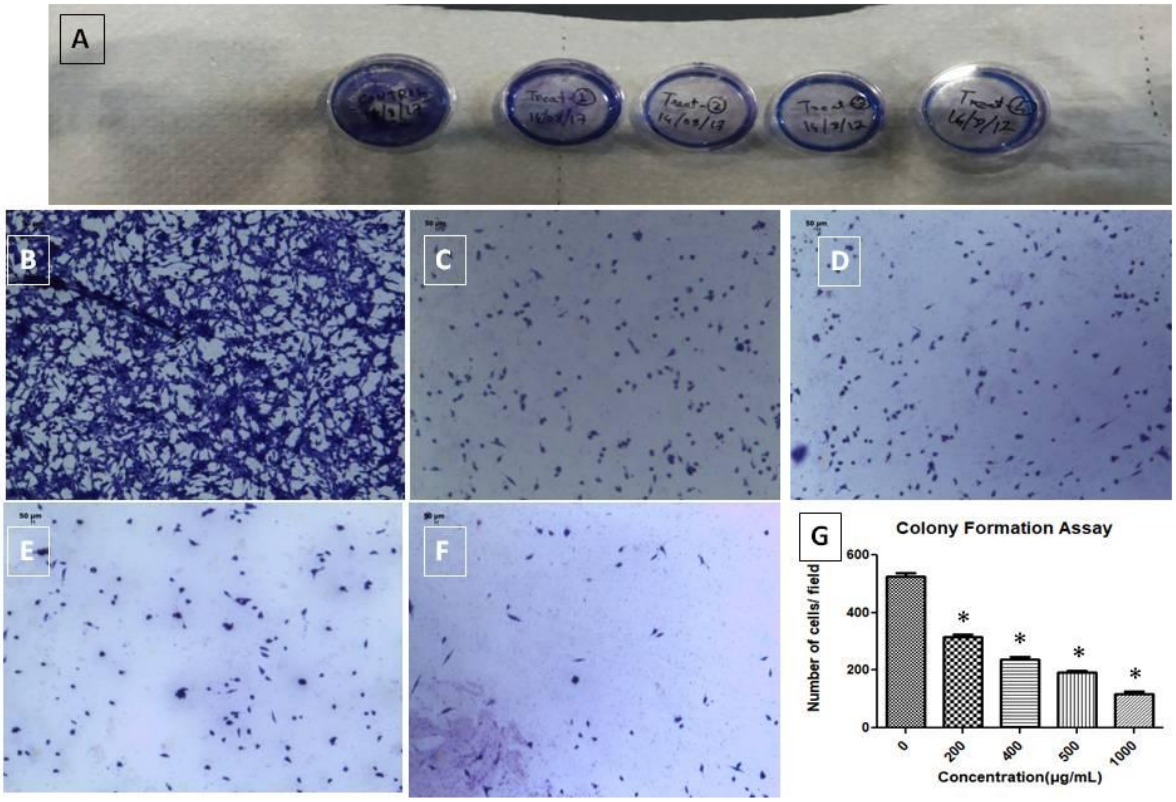


Figure 9: Colony formation assay. A: Petridish Plates (30 mm diameter). B: Control C: treatment with 200 µg/mL of MECR D: treatment with 400 µg/mL of MECR E: treatment with 500 µg/mL of MECR F: treatment with 1000 µg/mL of MECR G: Comparison of number of adherent colony forming cells as per different concentration of MECR. Values were expressed in mean ± S.E.M. (n=3). *:p<0.05 is considered significant.

3.10.3 Measurement of intracellular ROS levels:

Cell permeable fluorescent dye DCFH-DA enters the cell; the cytosolic esterases cleave the diacetate bond resulting in DCFH formation further this DCFH gets oxidized to fluorescent DCF by the ROS produced in the cell. The amount of fluorescence corresponds to the amount of intracellular ROS generation. The increase in oxidative stress is indicated by increase in Relative ROS levels (3.40±0.153, 4.70±0.115, 5.53±0.233) in the cells treated with MECR (200, 500, 1000 µg/mL) as compared to untreated control cells and simultaneously ROS level (1.30±0.153) of normal colon cells (CCD-18Co) was also observed (Figure-10).

ROS are constantly generated and eliminated in the biological system. ROS has different activity in normal cell and colon cancer cell. At low levels, ROS facilitates cancer cell survival since cell-cycle progression driven by growth factors and receptor tyrosine kinases (RTK) require ROS for activation. On the other hand, a high level, ROS can suppress tumour growth through the sustained activation of cell-cycle inhibitor and damaging macromolecules like DNA (Liang et al. 2016). At high level of ROS, lipid peroxidation also occurs at the cell membrane leading to cell death (Barrera 2012). In the experiment on HCT-8 cells, ROS is increased with the increase of concentration of MECR but remarkable level of it was not observed on normal colon cells (CCD-18Co). The ROS is increased in cancer cells due to polyphenolic compounds in it (Sharif et al. 2010). As ROS is increased with the concentration of MECR, so MECR is colon cancer preventive.

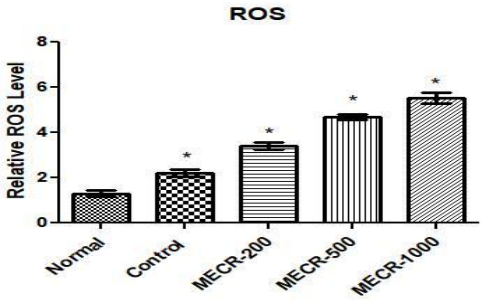


Figure 10: Relative Reactive Oxygen Species (ROS) level in Normal Colon Cell line (CCD- 18Co) and Colon Cancer Cell line (HCT-8) along with treatment of MECR.

Values were expressed as mean ± SEM (n=3).

***: (p<0.05) different from Normal Group (CCD-18Co).**

3.10.4 Effect of MECR on cell cycle progression and apoptosis

Effect of MECR on cell cycle progression of HCT-8 cells was analyzed by FACS using PI. It was evident from Figure-11 that treatment with MECR with increased concentration (0, 200, 500, 1000 µg/mL) resulted in increased percentage (3.40±0.656, 14.1±0.944, 17.2±0.761, 24.2±0.671) of apoptotic cells in the sub-G_0 phase (in figure-M_1) of the cell cycle.

In flowcytometry for cell cycle analysis, MECR was found to produce significant induction of apoptosis in HCT-8 cells by increasing sub-G_0 population. The occurance of apoptosis due to MECR was confirmed by DAPI staining assay and TUNEL assay as the DAPI stain and Tunel reagent bind with damaged DNA caused by ROS. MECR also significantly reduced the number of cells in the G_1/S phase. It seems that MECR did not block the cells in the G_1/S

phase, rather it shifted the cell population from G_1/S to sub-G_0 phase and as a result the number of apoptotic cells was increased in the sub-G_0 population.

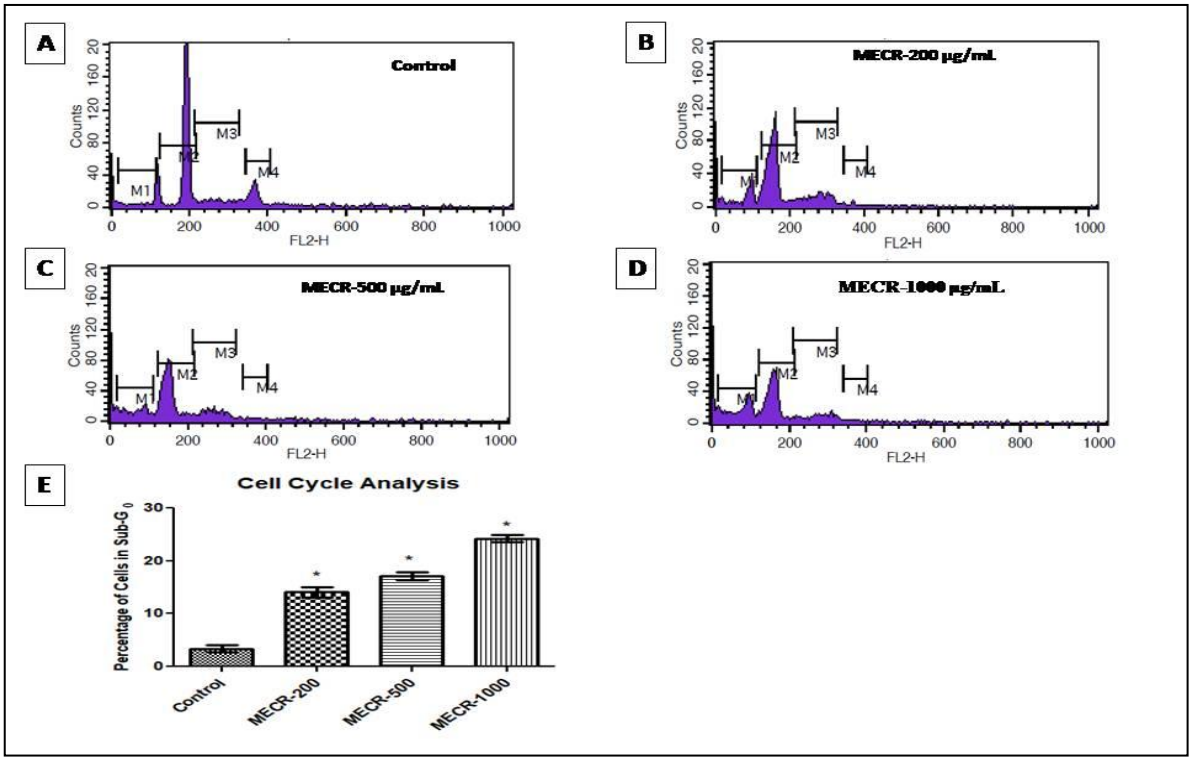


Figure 11: MECR induced apoptotic cell death in HCT-8 cells. A, B, C, D: Flowcytometric analysis of HCT-8 cell cycle phase distribution after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. E: Histogram display percentage of sub-G_0 population at different concentration of MECR treatment. Results are mean ± SEM(n=3). *: Significantly ($p<0.05$) different from Control.

3.10.5 DAPI staining assay

DAPI staining assay determines nuclear morphology. In this study nucleus condensed as the concentration of MECR were increased. Cell number also decreased in concentration

dependant manner. As the nucleus condensed the cytoplasm to nucleus ratio increased with the increase of concentration (Fig.-12).

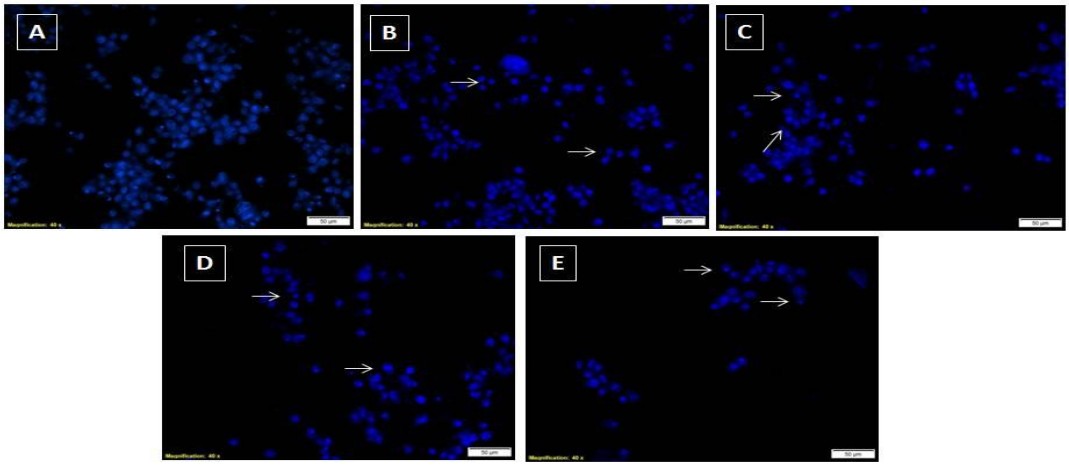


Figure 12: The morphological changes of nuclei were examined by fluorescence microscopy (magnification, 40x) using DAPI staining at various concentration of MECR treatment. A: 0 µg/mL (Control) B: 200 µg/mL C: 400 µg/mL D: 500 µg/mL E: 1000 µg/mL. The arrows indicate nuclear condensation and apoptotic bodies. Cell number also decreasing in a concentration dependant manner (magnification, 40x).

3.10.6 Tunel assay

This method detects the apoptotic DNA fragmentation, which is widely used to identify and quantify apoptotic cells, or to detect excessive DNA breakage in individual cells. The assay depends on the use of terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes attachment of deoxynucleotides, tagged with a fluorochrome or another marker, to 3'-hydroxyl termini of DNA double strand breaks. This method detects the apoptotic DNA fragmentation, which is widely used to identify and quantify apoptotic cells, or to detect

excessive DNA breakage in individual cells. In this study it was observed that percentage of apoptotic cells were increased in the treated (500 µg/mL) groups to 73.33 ± 3.53 % (Figure-13).

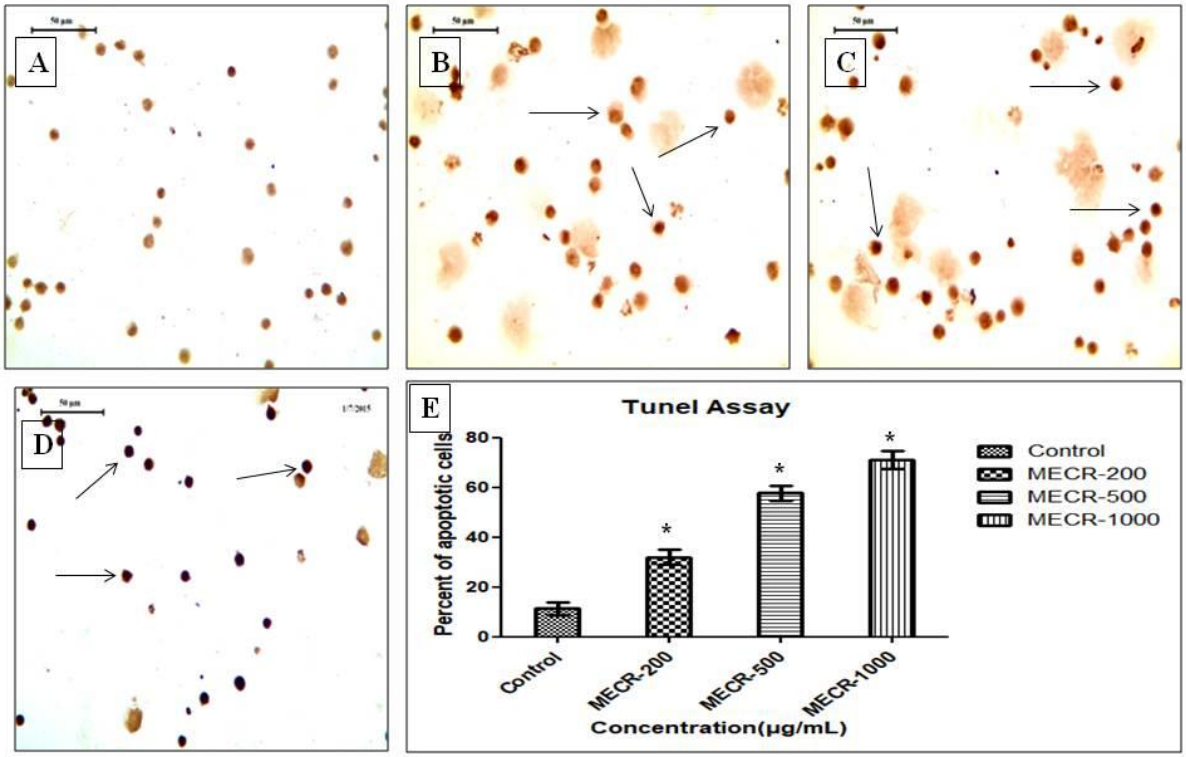


Figure 13: Induction of apoptosis (Tunel assay). A, B, C, D: Tunel Assay of HCT-8 cell after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. E: Histogram display the percentage of apoptotic cell population at different concentration of MECR treatment. Results are mean ± SEM (n=3). *: Significantly ($p<0.05$) different from Control. Arrows indicate in A, B, C and D, the cells with morphologically condensed nucleus were Tunnel positive, indicating the existence of fragmented DNA (magnification, 40x).

3.11 Discussion

The anti-colon cancer activities of MECR was confirmed by in-vitro assays like MTT assay for cell viability; Colony formation assay for cell proliferation inhibition; DAPI staining, Tunel assay and Cell Cycle Analysis for apoptotic study. MTT assay was performed to determine the cytotoxicity effect using HCT-8 cancer cell line (Figure 8). Exposure of cells to different concentrations of the MECR (0-1000 µg/mL) affected significant cytotoxicity at concentrations up to 1000 µg/mL. The toxic effect of MECR markedly decreased the cell viability to 31 ± 1.08% at 1000 µg/mL. The IC_{50} value was found to be 500 ± 1.09 µg/mL against HCT-8 cell line and this dosage was used as one of the dosages for subsequent analysis. Colony formation assay determines the number of adherent colony forming cells. The number of adherent colony-forming cells decreased in a concentration (0-1000 µg/mL) dependent manner from 526 ± 12.02 to 116.7 ± 8.82 cells per field (Figure 9). Cell-permeable fluorescent dye DCFH-DA gets oxidized to fluorescent DCF by the ROS produced in the cell. The amount of fluorescence compound is proportionally correlated to the amount of intracellular ROS generation. The increase in oxidative stress is indicated by increase in relative ROS levels (3.40±0.153, 4.70±0.115, 5.53±0.233) in the cells treated with MECR (200, 500, 1000 µg/mL) as compared to untreated control cells (HCT-8) and simultaneously ROS level (1.30±0.153) of normal colon cells (CCD-18Co) was also observed (Figure 10). The ROS is generated more in colon cancer cells due to the presence of phenolics in MECR (Kuete et al. 2016). Effect of MECR on cell cycle progression of HCT-8 cells was analyzed by FACS using PI. It was evident from Figure 11 that treatment with MECR with increased concentration (0, 200, 500, 1000 µg/mL) resulted in increased percentage (3.40±0.656, 14.1±0.944, 17.2±0.761, 24.2±0.671) of apoptotic cells in the sub-G_0 phase (in Figure M_1-parts) of the cell cycle. DAPI staining assay says about preliminary confirmation

about DNA damage. In this study nucleus condensed as the concentration of MECR were increased (Figure 12). Tunel assay detects the apoptotic DNA fragmentation, which is widely used to identify and quantify apoptotic cells or to detect excessive DNA breakage in individual cells. In this study, it was observed that percentage of apoptotic cells were increased in the treated (500 µg/mL) groups to 73.33 ± 3.53 % (Figure 13).

3.12 Concluding remarks

This chapter makes us ascertain about the anti-colon cancer activity of MECR on colon cancer cell line (HCT-8) but no cytotoxicity on normal colon cell line (CCD-18Co). MTT assay and colony formation assay determines cytotoxicity. By the MTT assay IC_{50} is determined. Subsequently the IC_{50} dose is used along with lower dose and higher dose of it for the determination of nuclear morphology by DAPI, DNA nicking by Tunel Assay, stage of apoptosis by Flowcytometry. DAPI and Tunel assay give confirmation about apoptosis in concentration dependant manner. In flowcytometry it gives information that cell apoptosis occurs by the MECR at the sub-G_0 phase in cell cycle. ROS is produced in significant amount in colon cancer cells to cause death, but no such effect was observed in normal colon cell line. So we can conclude MECR might be an ameliorating agent for the colorectal cancer prevention.

CHAPTER 4

IN-VIVO ANTI-COLON CANCER STUDY OF MECR

4.1 Materials & methods

4.1.1 Materials

DMH, methanol, 5-Fluoro Uracil (5-FU), phenazine methosulphate, NBT, Reduced nicotinamide adenine dinucleotide (NADH), glacial acetic acid, n-butanol, pure super oxide dismutase(SOD), H_2O_2, pure catalase (CAT) , phosphate buffer , ethylene diamine tetra acetic acid (EDTA), trichloroacetic acid (TCA), Tris-HCL, Dithio-bis (2-nitrobenzoic acid), pure reduced glutathione (GSH), sodium dodecyl sulphate were purchased from Sigma Laboratories, Germany.

4.1.2 Animals

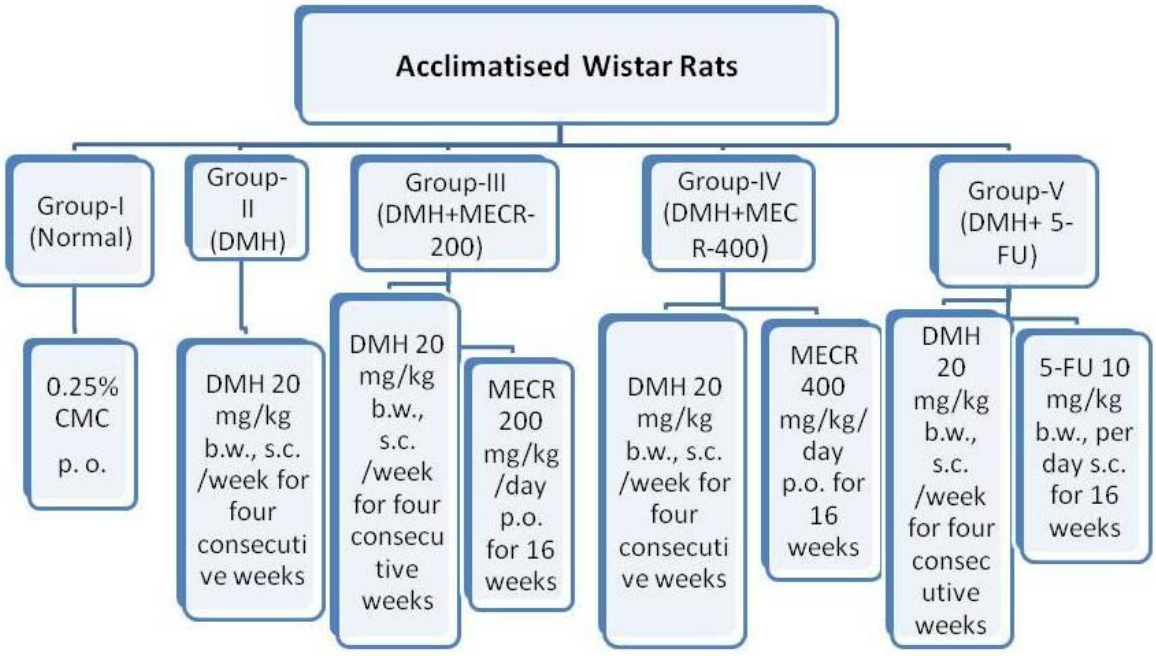
The experimental protocols involving animals were approved by the Institutional Animal Ethics Committee (NCPT/IAEC-15/2015) and the experiments on animals were performed in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. Experiments were performed on male Wistar rats (5 weeks old) procured from Indian Institute of Chemical Biology, Kolkata, India. The animals were fed standard balanced diet and drinking water and were maintained with 24 h day and night cycle. The animals were acclimatized for two weeks before starting of the experimentation.

4.1.3 Acute toxicity study

C. revoluta extract (MECR) in olive oil was administered orally to the animals in increasing doses upto 2000 mg/kg b.w. These animals were observed for 2 hr for behavioural, neurological and autonomic profiles and mortality and toxicity for 72 hr.

4.1.4 *In- vivo* experimental design for induction of colon cancer

Animals were divided into five groups (n=6). Group I serve as a vehicle control which received 0.25% carboxymethyl cellulose solution. Group II-V were treated with 1,2-Dimethyl hydrazine (DMH) was given at the dose of 20 mg/kg b.w., s.c. once a week for four consecutive weeks. Aqueous suspension of MECR at a dose of 200 mg/kg/day and 400 mg/kg were administered orally to the animals in group III to IV every day for 16 weeks. Group V received 5-FU as a standard drug at a dose of 10 mg/kg b.w., per day s.c. for 16 weeks(Sivaranjani et al. 2016).



Scheme 1: Flow chart of in-vivo colon cancer study using DMH

4.1.5 Macroscopic evaluation of the incidence of polyps

At the end of the experiment, rats were sacrificed and colons were taken out and flushed with phosphate buffer saline (PBS). The colons were cut to open longitudinally without disturbing the polyps and carefully counted through visual macroscopic examination. Then the colons were verified histopathologically.

4.1.6 *In vivo* antioxidant status determination

After 16 weeks of treatment, rats were kept one night fasting and then sacrificed to collect colons. After washing in ice-cold saline, colons were kept in deep freeze (-20°C) to conduct the different types of biochemical tests. Then the colon was taken out separately for each time just before the specific biochemical test to be conducted. Colon tissues were homogenated using homogenizer. For homogenization, 0.5 g of colon was taken in 5 ml (w/v) ice-cold saline followed by centrifugation at 2000 g for 10 min. The supernatant was used for evaluating the level of enzymatic antioxidant such as superoxide dismutase (SOD) and catalase (CAT) activity and non-enzymatic antioxidant such as reduced glutathione (GSH) (Arigesavan & Sudhandiran 2015).

4.1.6.1 Determination of SOD level

SOD activity was measured by the method of Marklund (Marklund & Marklund 1974) . The reaction mixture consisted of Tris–EDTA buffer (50 mM, pH 8.5), pyrogallol (24 mM) and 10 µl sample in a total volume of 0.2 ml. The enzyme activity was measured at 420 nm and was expressed as µmol/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation by 50%.

4.1.6.2 Determination of CAT level

Catalase activity was measured according to Aebi (Aebi 1984). It is based on the determination of the rate constant of decomposition of H_2O_2 by the enzyme. In brief, the assay mixture consisted of 1.9 ml phosphate buffer (50 mM, pH 7.4), 1 ml hydrogen peroxide (30 mM) and 0.1 ml of sample in a final volume of 3.0 ml. Absorbance were recorded at 240 nm over 3 min. The catalase activity was expressed as µmol/mg protein.

4.1.6.3 Determination of GSH level

GSH content was performed in the colon homogenates samples by the method of Ellman (Ellman 1959). Samples were subjected to 5% sulfosalicylic acid (v/v) and incubated for 1 h at 4 °C. The supernatant was allowed to react with 0.4% of Ellman's reagent (DTNB) in Tris buffer (0.2 M, pH 8) and optical density was assessed at 405 nm. The GSH content was calculated as nmol/mg protein.

4.1.7 Histological observation

Histological evaluation was performed in colon tissues. A portion of the specimen was fixed in 10% formalin and embedded in paraffin wax, sectioned at 4 µm thickness and were stained with haematoxylin & Eosin. Morphological changes of colon were evaluated by light microscopy method in control and experimental groups of animals (da Rocha et al. 2017).

4.1.8 Statistical analysis

All the data were evaluated with Graph Pad Prism version 5 software (GraphPad Software Inc, La Jolla, CA). Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Dunnett's t-test to correct for multiple comparisons with acceptable statistical level significance ($p < 0.05$). Each experiment was presented as the mean ± SEM.

4.2 Results

4.2.1 Acute toxicity study

In the acute toxicity assay, it was found that no mortality was observed up to doses 2000 mg/kg b.w., orally and were considered as safe and no lethality or any toxic reaction were found up to the end of the study period. By keeping $1/5^{th}$ (400 mg/kg) dose as highest, 200 mg/kg was selected as working doses for the present study.

4.2.2 Effect of DMH and MECR on polyps incidence

In the DMH-alone-treated rats (group II), 100% incidence of polyps was found (Table- 4). On supplementation with different doses of MECR (200 and 400 mg/kg b.w.) to DMH-induced rats (groups III–IV) the incidence of polyps was significantly reduced as compared to DMH-alone induced rats (group II). No specific changes were noticed control rats (groups I). 400 mg/kg b.w., MECR was found to be effective, the incidence of polyps being 33.33%, but it was slightly less effective than the synthetic standard drug 5-FU (10 mg/kg b. w.), the incidence of polyp being 13.33%.

Table-4: Effect of MECR on the incidence of colonic polyps in the different groups

Groups	No. of rats	No. of polyps bearing rats	Total no. of polyps	Average no. of polyps bearing rats [a]	Percentage incidence of polyps [b]	Percentage of polyps inhibition
Control (I)	6	0	Nil	Nil	-	-
DMH (II)	6	6	15	2.5	100	0
DMH+MECR200 (III)	6	5	9	1.8	60	40
DMH+MECR400 (IV)	6	3	5	1.67	33.33	66.67
DMH+5-FU (V)	6	2	2	1	13.33	86.67

DMH: 1,2-Dimethyl hydrazine (20 mg/kg b.w.), MECR200: Methanolic extract of *Cycas revoluta* (200 mg/kg b.w.), MECR400: Methanolic extract of *Cycas revoluta* (400 mg/kg b.w.), 5-FU: 5-Fluoro-uracil (10 mg/kg b.w.).[a]Total number of polyps/number of polyps-bearing rats in each group.[b](Total number polyps/total number of polyps in DMH group)×100.

4.2.3 Effect of MECR on different antioxidant enzyme activity

SOD, catalase and reduced gluta-thione (GSH) activity were decreased in DMH Group. All these parameters were restored significantly ($p<0.05$) towards the near normal value on supplementation with MECR (200 and 400 mg/kg b.w.) to DMH treated rats (Groups III and IV). In groupV, the synthetic drug 5-FU(10mg/kg b.w.) also increases the activities of SOD, CAT, GSH sigficantly ($p < 0.05$) more in DMH treated rats (Fig.-14).

SOD level was decreased to 0.55±0.007µmol/mg in case of only DMH treated group from the level 2.6±0.066µmol/mg in Normal Control group, but restored towards Normal Group level in dose dependant manner. When the treatments were accomplished with 200mg/kg b.w. and 400mg/kg b.w. then the levels of SOD were restored significantly ($p<0.05$) to 1±0.0034 µmol/mg and 1.5±0.095 µmol/mg respectively. These values were slightly less than the value (1.7±0.10 µmol/mg) of treated group with standard drug 5-FU (Fig. 14A).

In case of CAT determination, its level was decreased to 3.4±0.30µmol/mg in case of only DMH treated group from the level 19±0.99µmol/mg in Normal Control group, but restored towards Normal Group level in dose dependant manner treatment with MECR. When the treatments were accomplished with 200mg/kg b.w. and 400mg/kg b.w. of MECR then the levels of CAT were restored significantly ($p<0.05$) to 8.9±0.26 µmol/mg and 13±0.32 µmol/mg respectively. These values were slightly less than the value (17±0.36 µmol/mg) of treated group with standard drug 5-FU (Fig. 14B).

In case of non-enzymatic anti-oxidant GSH determination, the level of GSH was decreased to 2±0.37nmol/mg in case of only DMH treated group from the level 6.3±0.33nmol/mg in

Normal Control group, but restored towards Normal Group level in dose dependant manner treatment with MECR. When the treatments were accomplished with 200mg/kg b.w. and 400mg/kg b.w. of MECR then the levels of CAT were restored significantly($p<0.05$) to 2.3±0.33 nmol/mg and 3.2±0.31 nmol/mg respectively. These values were slightly less than the value (5.2±0.31 nmol/mg) of treated group with standard drug 5-FU (Fig. 14C).

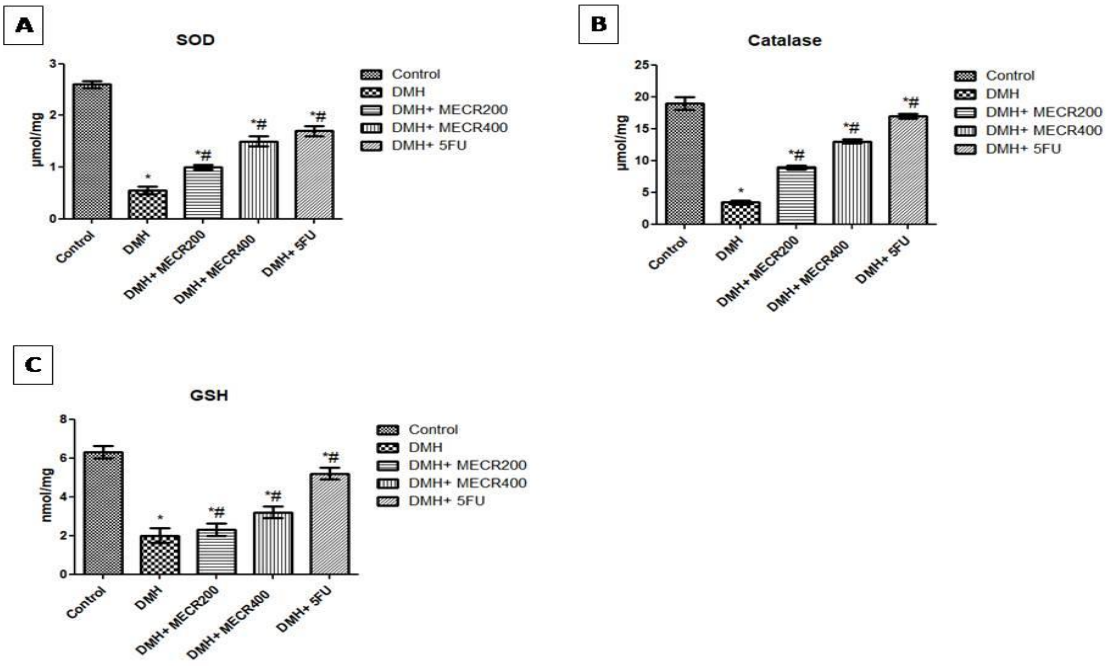


Figure 14: Evaluation of biochemical parameters in colon homogenate in Control (I), DMH (II), DMH+MECR200 (III), DMH+MECR400 (IV), DMH+5FU (V) groups.

A: Superoxide Dismutase (SOD).

B: Catalase (CAT).

C: Reduced Glutathione (GSH).

Values were expressed as mean±SEM (n=6). All groups are compared to Control Group. *: Significantly ($p<0.05$) different from Control group when compared with other groups by Dunnett's t-Test in one way ANOVA analysis. #: Treatment groups are compared with DMH Group ($p<0.05$) by Dunnett's t-Test in one way ANOVA analysis.

4.2.4 Histopathological evaluation

The colon of control group showed normal mucosa and sub mucosal layers and normal colonic architecture without apparent abnormality in (Figure 15A). DMH induced group showed clear degeneration as size of the cells smaller than that of normal (Figure 15B). In DMH+MECR200 group, mucosal and sub-mucosal layers were less ruptured compared to DMH induced group. In DMH+MECR400 group, the mucosal epithelial architecture along with sub-mucosal layer's integrity was better than that of DMH+MECR200 group. Tubular glands gradually appeared clearly as the doses of MECR increased. However, DMH+5FU treated group showed better results as compared to DMH+MECR400 group as tubular gland appeared clearly with arranged manner (Figure 15E).

Furthermore our histological observation revealed that clusters of abnormal degenerated glands and cells in the mucosal lining of the colon and rectum was observed in animals of DMH group along with severe mucosal and sub-mucosal damage. However, animals treated with orally administered MECR show replacement of abnormal cell in the DMH treated animals justifying the anticancer potential of MECR.

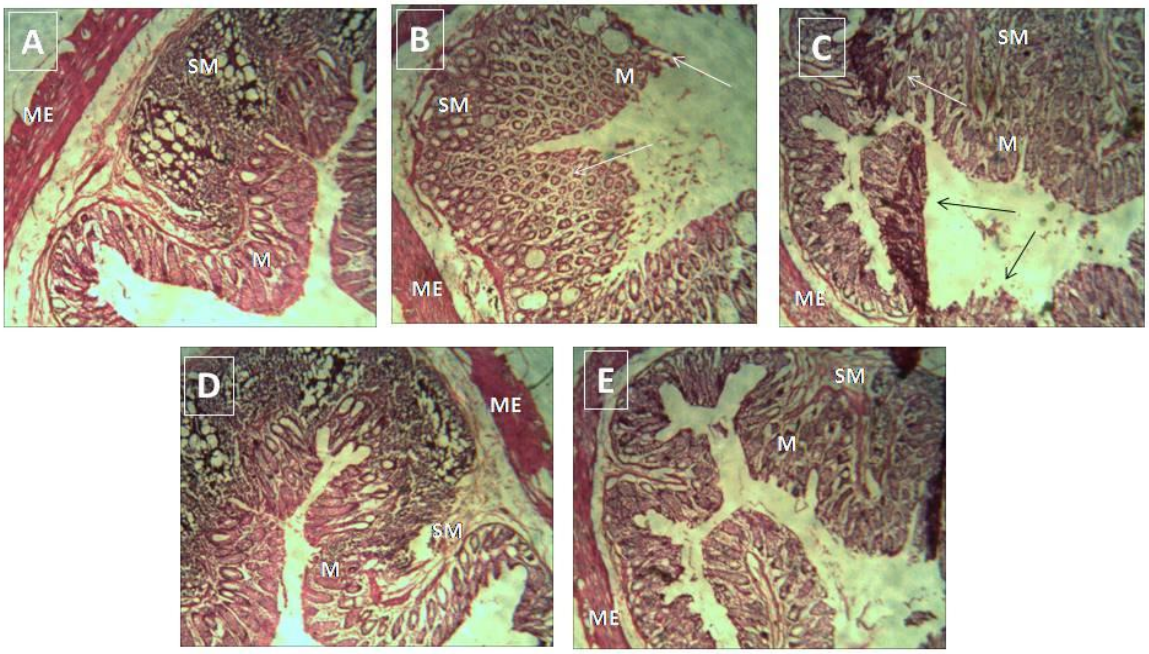


Figure 15: Histopathological evaluation of colon. M=Mucosal Layer, SM=Sub-Mucosal Layer, ME=Muscularis Externa. Arrows indicate the degenerative changes due to DMH.

A: Normal Control Group (I)

B: DMH Group (Toxic Control) (II)

C: DMH+MECR200 Group (III)

D: DMH+MECR400 Group (IV)

E: DMH+5-FU Group (V)

DMH group shows that tubular glands in mucosal and submucosal layer are not observed prominently. Tubular glands size is smaller in DMH Group than that of Control group. Degenerative changes observed at mucosal and submucosal layer in DMH Group. Tubular glands gradually appeared clearly as the doses of MECR increased. In DMH+5-FU group degenerative changes are least than those of DMH+MECR200 group and DMH+MECR400 group (magnification, 40 X).

4.3 Discussion

Colon cancer was induced with DMH in wistar rats through its metabolite azoxymethane which is potent genotoxic agents to trigger oxidative stress via DNA methylation of colonic epithelial cells(Hong et al. 1999). This adduct interferes with normal cell growth by altered normal gene transcription. Due to binding of DMH metabolite to DNA, gene transcription is modified to create oxidative stress through the production of Reactive Oxygen Species (ROS) inside the cells. Increase in ROS level is an important factor to develop colitis associated colon cancer (Liochev 2013).There are several endogenous antioxidant like SOD, CAT, and GSH increase in normal colon cell in response to small increase of ROS, but in DMH-induced rats due to modification gene transcription excessive ROS is produced crossing the level of endogenous antioxidant production. As a result, DNA damage occurs leading to colon cancer and less production of endogenous antioxidant. After the DNA damage, ROS is produced in fewer amounts for the survival of colon cancer cells. In the *in-vivo* model, the levels of endogenous antioxidant SOD, CAT, GSH were restored after administration of MECR in DMH-induced rats but not restored in only DMH-consumed rats. Endogenous antioxidant restoration by MECR occurs due to replacement of cancer cell in colon causing death in consequence of ROS increase by polyphenolic and flavanoid in MECR(Matsuo et al. 2005).On the other hand endogenous antioxidant increases in normal cell in response to ROS to reduce its level(Wattel et al. 2003). Hence colon cancer polyps are less observed in MECR treated group and polyp number decreases dose dependant manner.

4.4 Concluding remarks

This chapter clearly demonstrate that the supplementation of MECR modulates the DMH induced effects. The severity of DMH induced effects in MECR treated groups are reduced in

dose dependant manner. In histopathological evaluation, the treated group with MECR showed less mucosal and sub-mucosal damage compared to DMH induced group. Tubular glands gradually appeared clearly as the doses of MECR increased. In the *in-vivo* model, the levels of endogenous antioxidant SOD, CAT, GSH were restored after administration of MECR in DMH-induced rats but not restored in only DMH-consumed rats. CAT, SOD stands in forefront of defence against oxidative stress and plays a vital role in maintaining homeostasis.GSH acts as second line of defence against generated free radicals and inhibit alteration of membrane fluidity, cellular redox imbalance. Hence we conclude MECR is a potential agent for the the prevention of colorectal cancer (CRC).

The present study opens many new areas of research work. This work can be continued in the future to study and to make it clinically applicable for colon cancer protective activity in different experimental models and also to isolate, identify and standardize the active principle(s) that are responsible for colorectal cancer preventive activity.

SUMMARY

In summary, this thesis enlightens the result that MECR decreases proliferation and induces apoptosis by increasing ROS in human colon cancer cell line HCT-8 whereas no such activity was seen in normal colon cell line (CCD-18Co). Gallic Acid, Catechin, Quercetin of MECR are mainly responsible for ROS generation in cancer cell. Besides those metabolites, Myoinositol, Caffeic acid, Citric acid, Xylitol, 4-Guanidinobutyric acid of MECR have anti-colon cancer activity. As MECR also decreases polyp formation dose dependant manner and also boost up the antioxidant status like SOD, Catalase, GSH level in-vivo model, so it can be used be used as colon cancer protective agent. Therefore, MECR ameliorates to combat colon related health issues naturally and dilutes the overall oxidative stress, acting in colorectal cancer (CRC) management. Hence, this work can be continued in the future to study and to make it clinically applicable for colon cancer protective activity in different experimental models.

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Research Article

PREVENTIVE EFFECT OF *CYCAS REVOLUTA* IN 1,2-DIMETHYLHYDRAZINE-INDUCED COLON CANCER IN WISTAR RAT MODEL

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ABSTRACT

Objective: The aim of this study was to evaluate the colon cancer protective activity of *Cycas revoluta* (Cycadaceae).

Methods: Methanolic extracts of *C. revoluta* (MECR) were assessed for total polyphenols and total flavonoids content. For the *in vivo* study, animals were divided into five groups (n=6). Group I serves as control which received 0.25% carboxymethyl cellulose solution. Groups II-V were treated with 1,2-dimethylhydrazine (DMH) which was given at the dose of 20 mg/kg b.w., s.c. once a week for 4 consecutive weeks. Aqueous suspension of MECR at a dose of 200 mg/kg/day and 400 mg/kg was administered orally to the animals in Groups III-IV every day for 16 weeks. Group V received 5-fluorouracil (5-FU) as a standard drug at a dose of 10 mg/kg b.w., per day s.c. for 16 weeks. After that, animals are sacrificed and colons are taken separately to evaluate biochemical parameters and morphological and histopathological changes.

Results: MECR contains total polyphenols (6.3±0.09 mg of gallic acid equivalent /g) and total flavonoids (4.6±0.06 mg of rutin equivalent/g). The *in vivo* study revealed that superoxide dismutase (SOD), catalase, and reduced glutathione (GSH) activity were decreased in DMH Group. All these parameters were restored significantly ($p<0.05$) toward the near normal value on supplementation with MECR (200 and 400 mg/kg b.w.) to DMH-treated rats (Groups III and IV). In Group V, the synthetic standard drug 5-FU (10 mg/kg b.w.) also increases the activities of SOD, CAT, and GSH significantly ($p<0.05$) more in DMH-treated rats.

Conclusions: It can be concluded that MECR protects rat from DMH-induced colon cancer.

Keywords: *Cycas revoluta*, Methanolic extracts of *C. revoluta*, 1, 2-Dimethylhydrazine, Colon cancer, 5-Fluorouracil.

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INTRODUCTION

Colon cancer is the most fatal issues throughout the world and a leading cause of cancer-related mortality. Oxidative stress is one of the main causes of colon cancer [1]. The gastrointestinal tract is more susceptible to toxic chemical exposure which may execute the chronic inflammation, and later, it becomes tumour in the gastrointestinal tract [2]. Over the past few years, the first-line clinical treatments for colon cancer patients are cytoreductive surgery and combined chemotherapy [3]. Drug resistance results in a poor overall survival rate [4]. Therefore, the development of effective and less toxic drugs is urgent for colon cancer patients.

The colon-specific carcinogen 1,2-dimethylhydrazine (DMH) induces the formation of methyl adducts with DNA bases and causes point mutations [5]. This adduct interferes with normal cell growth by altered normal gene transcription. After the Phases I and II metabolism, carcinogenic metabolites are produced from DMH. The activation of carcinogen is done by Phase I enzymes. The pro-carcinogen DMH undergoes hydroxylation in Phase I reactions catalyzed by cellular microsomal monooxygenases to produce strong electrophiles. The produced strong electrophiles are capable of interacting with cellular nucleophiles such as DNA to form adducts causing mutagenesis and neoplastic transformation. By Phase II biotransformation, these electrophilic intermediates are detoxified by enzymes such as glutathione (GSH) S-transferase [6]. Due to binding of DMH metabolite to DNA, gene transcription is modified to create oxidative stress through the production of reactive oxygen species (ROS) inside the cells. The ROS has pathological status by inducing oxidative stress-mediated inflammatory response in several tissues [7]. Increase in ROS level is an important factor to develop colitis-associated colon cancer [8]. There are several endogenous antioxidant such as superoxide dismutase (SOD), CAT, and GSH increase in normal colon cell in response to small increase of ROS, but in DMH-induced rats due to modification gene transcription, excessive ROS is produced crossing the level of endogenous antioxidant production. As a result, DNA damage occurs leading to colon cancer and less production of endogenous antioxidant. After the DNA damage, ROS is produced in fewer amounts for the survival of colon cancer cells.

Cycas revoluta, one of the most primitive living seed plants, contains edible starch in pith, and is used for making sago. Seeds of this plant contain cycasin which is a neurotoxin when consumed orally due to aglycone release from glycoside after acid hydrolysis in the stomach [9]. Although seeds contains toxin, the *C. revoluta* cone is used in the painful urination by the hilly people of Northeast India [10]. It is reported for the seeds to possess profound anticancer activity against colon cancer [11]. The plant contains several important chemicals including dihydroamentoflavone glucosides [12], peptides [11], chitinase [13], mannose-specific lectins [14], and beta-D-glucosidase [15]. *C. revoluta* has been reported to have many pharmacological effects such as antioxidant [16], anti-inflammatory, anticancer, antileishmaniasis [12], and antimicrobial [11]. A recent study [11] has reported the anticancer activity of *C. revoluta* seeds on human colon carcinoma cells (HCT-15). However, till now, no reports are available on the colon cancer protective effect of *C. revoluta* cone (male flower) on the DMH-induced colon cancer. Therefore, the present work was intended to evaluate the efficacy of methanolic extract of *C. revoluta* (MECR) cone against DMH-induced colon cancer in Wistar rat model.

MATERIALS AND METHODS

Materials

DMH, methanol, 5-fluorouracil (5-FU), phenazine methosulphate, NBT, reduced nicotinamide adenine dinucleotide, glacial acetic acid,

n-butanol, pure SOD, H_2O_2, pure catalase (CAT), phosphate buffer, ethylenediaminetetraacetic acid, trichloroacetic acid, Tris-HCL, Dithiobis (2-nitrobenzoic acid), pure reduced GSH, anddd sodium dodecyl sulfate were purchased from Sigma Laboratories, Germany.

Animals

The experimental protocols involving animals were approved by the Institutional Animal Ethics Committee (NCPT/IAEC-15/2015), and the experiments on animals were performed in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals guidelines. Experiments were performed on male Wistar rats (5 weeks old) procured from Indian Institute of Chemical Biology, Kolkata, India. The animals were fed standard balanced diet and drinking water and were maintained with 24 h day and night cycle. The animals were acclimatized for 2 weeks before starting of the experimentation.

Plant material

Fresh male cone was collected from the village of Srirampur under East Midnapur district and authenticated (Voucher Ref. No.: BSI/Pharma/SD/Tech./2016) by botanist, Dr. A B D Selvam of Botanical Survey of India (BSI), Shibpur, Howrah (West Bengal). The cones were cut into small pieces and dried in the shade below 50°C. Then, dried cone pieces were powdered in mixture grinder and stored in airtight container.

Extraction of plant extract

The cones were cut into small pieces and were shade dried and then milled into a coarse powder. Then, the air dried and powdered cones (175 g) were first defatted with petroleum ether (60–80°C) and then extracted with 2.5 L of methanol (90%) using Soxhlet apparatus. The solvent was then removed with repeated lyophilization. After drying, 11.7 g (6.6% yield) extract was obtained.

Determination of total polyphenolic compounds

UV spectrophotometric method was used to determine the concentration of phenolics in the plant extracts [17]. The content of total phenolics in extracts was expressed as mg gallic acid equivalent (GAE) per g of dry weight of extract (mg GAE/g DW).

Determination of total flavonoid content

The content of flavonoids in plant extracts was determined using spectrophotometric method [17]. The content of flavonoids in extracts was expressed as mg of rutin equivalent (RUE) per g of dry weight of extract (mg RUE/g DW).

Acute toxicity study

MECR in olive oil was administered orally to the animals in increasing doses up to 2000 mg/kg b.w. These animals were observed for 2 h for behavioral, neurological, and autonomic profiles and mortality and toxicity for 72 h.

***In vivo* experimental design for induction of colon cancer**

Animals were divided into five groups (n=6). Group I serves as a vehicle control which received 0.25% carboxymethyl cellulose solution. Groups II-V were treated with DMH was given at the dose of 20 mg/kg b.w., s.c. once a week for 4 consecutive weeks. Aqueous suspension of MECR at a dose of 200 mg/kg/day and 400 mg/kg was administered orally to the animals in Groups III to IV every day for 16 weeks. Group V received 5-FU as a standard drug at a dose of 10 mg/kg b.w., per day s.c. for 16 weeks [18].

Macroscopic evaluation of the incidence of polyps

At the end of the experiment, rats were sacrificed and colons were taken out and flushed with phosphate-buffered saline. The colons were cut to open longitudinally without disturbing the polyps and carefully counted through visual macroscopic examination. Then, the colons were verified histopathologically.

In vivo antioxidant status determination

After 16 weeks of treatment, rats were kept one night fasting and then sacrificed to collect colons . After washing in ice-cold saline, colons were kept in deep freeze (−20°C) to conduct the different types of biochemical tests. Then, the colon was taken out separately for each time just before the specific biochemical test to be conducted. Colon tissues were homogenated using homogenizer. For homogenization, 0.5 g of colon was taken in 5 ml (w/v) ice-cold saline followed by centrifugation at 2000 g for 10 min. The supernatant was used for evaluating the level of enzymatic antioxidant such as SOD and catalase (CAT) activity and non-enzymatic antioxidant such as reduced GSH [19].

Histological observation

Histological evaluation was performed in colon tissues. A portion of the specimen was fixed in 10% formalin and embedded in paraffin wax, sectioned at 4 μm thickness, and was stained with hematoxylin and eosin. Morphological changes of colon were evaluated by light microscopy method in control and experimental groups of animals [20].

Statistical analysis

All the data were evaluated with Graph Pad Prism version 5 software (GraphPad Software Inc, La Jolla, CA). Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Dunnett's t-test to correct for multiple comparisons with acceptable statistical level significance ($p<0.05$). Each experiment was presented as the mean ± SEM.

RESULTS

Total polyphenolic and total flavonoid content

MECR contains total polyphenols (6.3±0.09 mg of GAE/g) and total flavonoids (4.6±0.06 mg of RUE/g) (Table 1).

Acute toxicity study

In the acute toxicity assay, it was found that no mortality was observed up to doses 2000 mg/kg b.w. orally and was considered as safe, and no lethality or any toxic reaction was found up to the end of the study period. By keeping $1/5^{th}$ (400 mg/kg) dose as highest, 200 mg/kg was selected as working doses for the present study.

Effect of DMH and MECR on polyps incidence

In the DMH-alone-treated rats (Group II), 100% incidence of polyps was found (Table 2). On supplementation with different doses of MECR (200 and 400 mg/kg b.w.) to DMH-induced rats (Groups III-IV), the incidence of polyps was significantly reduced as compared to DMH-alone induced rats (Group II). No specific changes were noticed control rats (Groups I). 400 mg/kg b.w., MECR was found to be effective, the incidence of polyps being 33.33%, but it was slightly less effective than the synthetic standard drug 5-FU (10 mg/kg b.w.), the incidence of polyp being 13.33%.

Effect of MECR on different antioxidant enzyme activity

SOD, catalase, and reduced GSH activity were decreased in DMH Group. All these parameters were restored significantly ($p<0.05$) toward the near normal value on supplementation with MECR (200 and 400 mg/kg b.w.) to DMH-treated rats (Groups III and IV). In Group V, the synthetic drug 5-FU (10 mg/kg b.w.) also increases the activities of SOD, CAT, and GSH significantly ($p<0.05$) more in DMH-treated rats (Fig. 1).

Table 1: Phytochemical content of MECR

Phytochemical constituents	Value
Total phenolic content (mg of GAE/g of DW extract)	6.3±0.09
Total flavonoid content (mg of rutin/g of DW extract)	4.6±0.06

Values were expressed as mean±SEM (n=3). MECR: Methanolic extract of *Cycas revoluta, C. revoluta: Cycas revoluta*

Table 2: Effect of MECR on the incidence of colonic polyps in the different groups

Groups	Number of rats	Number of polyps bearing rats	Total number of polyps	Average number of polyps bearing rats[a]	Percentage incidence of polyps[b]	Percentage of polyps inhibition
Control (I)	6	0	Nil	Nil	-	-
DMH (II)	6	6	15	2.5	100	0
DMH+MECR200 (III)	6	5	9	1.8	60	40
DMH+MECR400 (IV)	6	3	5	1.67	33.33	66.67
DMH+5-FU (V)	6	2	2	1	13.33	86.67

DMH: 1,2-dimethylhydrazine (20 mg/kg b.w.), MECR200: Methanolic extract of *Cycas revoluta* (200 mg/kg b.w.), MECR400: Methanolic extract of *Cycas revoluta* (400 mg/kg b.w.), 5-FU: 5-Fluoro-uracil (10 mg/kg b.w.). [a]Total number of polyps/number of polyps-bearing rats in each group. [b](Total number polyps/total number of polyps in DMH group) ×100

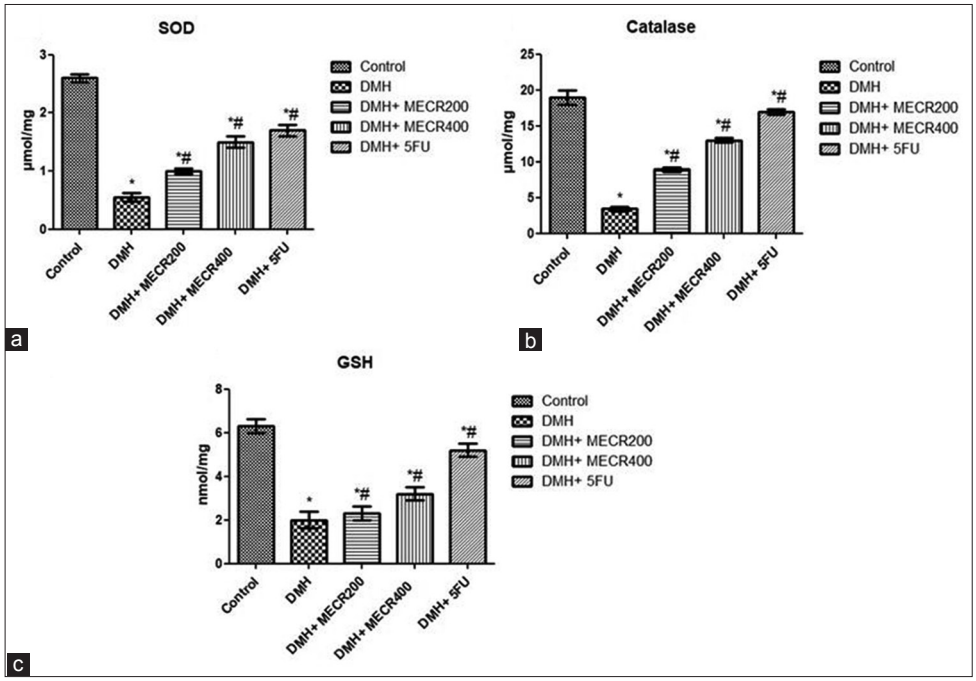

Fig. 1: Evaluation of biochemical parameters in colon homogenate in control (I), DMH (II), DMH+MECR200 (III), DMH+MECR400 (IV), DMH+5FU (V) groups. (a) Superoxide dismutase, (b) catalase, (c) reduced GSH. Values were expressed as mean±SEM (n=6). All groups are compared to control group. *Significantly (p<0.05) different from control group when compared with other groups by Dunnett's t-test in one-way ANOVA analysis. #Treatment groups are compared with DMH Group (p<0.05) by Dunnett's t-test in one-way ANOVA analysis

Histopathological evaluation

The colon of control group showed normal mucosa and submucosal layers and normal colonic architecture without apparent abnormality in (Fig. 2a). DMH-induced group showed clear degeneration as the size of the cells smaller than that of normal (Fig. 2b). In DMH+MECR200 group, mucosal and submucosal layers were less ruptured compared to DMH-induced group (Fig. 2c). In DMH+MECR400 group, the mucosal epithelial architecture along with sub-mucosal layer's integrity was better than that of DMH+MECR200 group (Fig. 2d). Tubular glands gradually appeared clearly as the doses of MECR increased. However, DMH+5FU-treated group showed better results as compared to DMH+MECR400 group as tubular gland appeared clearly with arranged manner (Fig. 2e).

DMH group shows that tubular glands in mucosal and submucosal layer are not observed prominently. Tubular glands size is smaller in DMH Group than that of control group. Degenerative changes was observed at mucosal and submucosal layer in DMH Group. Tubular glands gradually appeared clearly as the doses of MECR increased. In DMH+5-FU group, degenerative changes are least than those of DMH+MECR 200 group and DMH+MECR 400 group (magnification, ×40).

DISCUSSION

Colon cancer was induced with DMH in Wistar rats through its metabolite azoxymethane which is potent genotoxic agents to trigger oxidative stress through DNA methylation of colonic epithelial cells [21]. This adduct interferes with normal cell growth by altered normal gene transcription. Due to binding of DMH metabolite to DNA, gene transcription is modified to create oxidative stress through the production of ROS inside the cells. Increase in ROS level is an important factor to develop colitis-associated colon cancer [8]. There are several endogenous antioxidants such as SOD, CAT, and GSH increase in normal colon cell in response to small increase of ROS, but in DMH-induced rats due to modification gene transcription, excessive ROS is produced crossing the level of endogenous antioxidant production. As a result, DNA damage occurs leading to colon cancer and less production of endogenous antioxidant. After the DNA damage, ROS is produced in fewer amounts for the survival of colon cancer cells. In the *in vivo* model, the levels of endogenous antioxidants SOD, CAT, and GSH were restored after administration of MECR in DMH-induced rats but not restored in only DMH-consumed rats. Endogenous antioxidant restoration by MECR occurs due to replacement of cancer cell in colon causing death in consequence of ROS increase by polyphenolic and flavonoid in MECR [22]. On the other hand, endogenous antioxidant increases in normal cell in response to ROS to reduce its level [23]. Polyphenolics and flavonoids of MECR are antioxidant [24] which perform anticancer [25] activity ultimately in rat colon. Hence, colon cancer polyps are less observed in MECR-treated group and polyp number decreases dose-dependent manner.

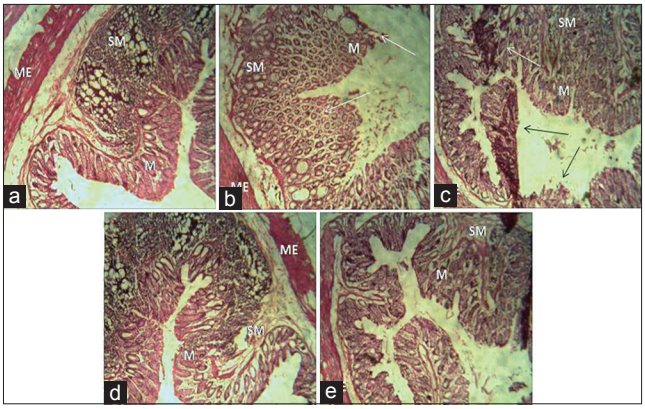

Fig. 2: Histopathological evaluation of colon. M - mucosal layer, SM - submucosal layer, ME - muscularis externa. Arrows indicate the degenerative changes due to DMH. (a) Normal control Group (I), (b) DMH Group (toxic control) (II), (c) DMH+MECR200 Group (III), (d) DMH+MECR400 Group (IV), (e) DMH+5-FU Group (V)

Furthermore, our histological observation revealed that clusters of abnormal degenerated glands and cells in the mucosal lining of the colon and rectum were observed in animals of DMH group along with severe mucosal and submucosal damage. However, animals treated with orally administered MECR show replacement of abnormal cell in the DMH-treated animals, justifying the anticancer potential of MECR.

CONCLUSION

From this study, it can be concluded that MECR might be a colon cancer protective agent. The present study opens many new areas of research work. This work can be continued in the future to study and to make it clinically applicable for colon cancer protective activity in different experimental models and also to isolate, identify, characterize, and standardize the active principle(s) that are responsible for this activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Metabolite profiling and *in-vitro* colon cancer protective activity of *Cycas revoluta* cone extract

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SHORT COMMUNICATION

Metabolite profiling and *in-vitro* colon cancer protective activity of *Cycas revoluta* cone extract

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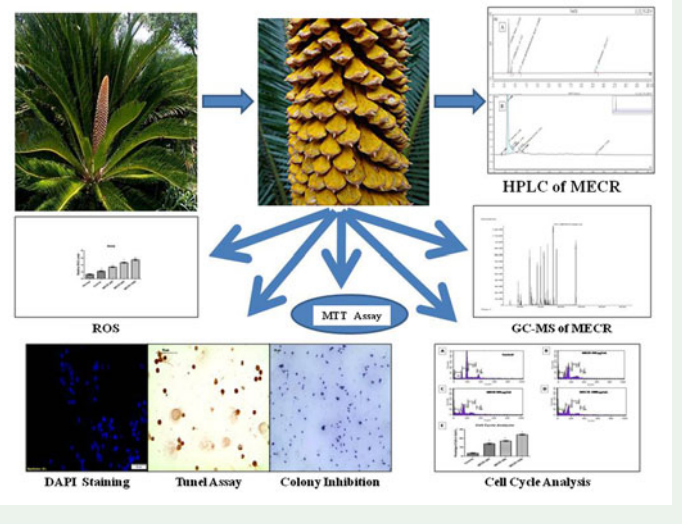
ABSTRACT

The methanolic extract of *Cycas revoluta* cone (MECR) was analyzed by GC-MS and UHPLC for metabolite profiling and was evaluated for anti-colon cancer property by using in vitro assays like Cell Viability Assay, Colony Formation Assay, ROS Determination, Flowcytometry, DAPI staining assay, Tunel assay. GC-MS and HPLC analysis confirmed the presence of different phytochemicals in the extract of *Cycas revoluta* cone. *In-vitro* studies showed MECR extract showed significant anti-colon cancer activity by reducing proliferation and inducing apoptosis in colon cancer cell (HCT-8) line, but no such activity was seen in normal colon cell (CCD-18Co) line. The investigation confirms that MECR may be a promising candidate in colon cancer protection.



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KEYWORDS
Cycas revoluta; colon cancer; reactive oxygen species (ROS); GC-MS

List of Abbreviations: MECR: Methanolic extract of *Cycas revoluta*; ROS: Reactive Oxygen Species; DAPI: 4, 6-Diamidino-2-phenylindole; UHPLC: Ultra-high Performance Liquid Chromatography; PI3K/Akt: Phosphoinositide 3-kinase/Akt (Protein Kinase B); AP1:

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Activator Protein 1; PI3K/PKC: Phosphoinositide 3-kinase/Protein kinase C; MAPK: Mitogen Activated Protein Kinases; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA: 2,7-Dichlorofluorescein diacetate; FACS: Fluorescence Assisted Cell Sorting; GC-MS: Gas Chromatography-Mass Spectroscopy

1. Introduction

Chemotherapy of cancer is yet to be improved with respect to its side effects. Oxidative stress is one of the main causes of colon cancer (Martín et al. 2016). Over the past few years, the first-line clinical treatments for colon cancer patients are cytoreductive surgery and combined chemotherapy (Glehen et al. 2004). Drug resistance results in a poor overall survival rate (Mirakhorli et al. 2013). Therefore, development of effective and less toxic drugs is urgent for colon cancer patients.

Cycas revoluta is commonly used as traditional herbal medicine for various diseases like urinary disorders (Rout et al. 2012), neuralgia, malignant ulcer, etc and also as food by North-East Asian people. From an extensive literature review, it was observed that the plant contains several important chemicals including peptides (Mandal et al. 2012), chitinase (Taira et al. 2010), beta-D-glucosidase (Yagi et al. 1985) etc. *C. revoluta* has been reported to have many pharmacological effects such as antioxidant (Lolodi & Eriyamremu 2013), anti-leishmaniasis (Moawad et al. 2014), antimicrobial (Mandal et al. 2012) etc. A recent study (Mandal et al. 2012) has reported the anticancer activity of *C. revoluta* seeds on human colon carcinoma cells (HCT-15). However, till now, no reports are available on the colon cancer protective effect of *C. revoluta* cone (male flower). Therefore, the present work was intended to identify the different bioactive constituents in methanolic extract of *C. revoluta* (MECR) along with the determination of colon cancer protective activity using *in-vitro* model.

2. Results and discussion

Metabolites identification in MECR was accomplished using GC-MS analysis (Figure S1). A total forty-three (43) metabolites (organic acids, amino acids, sugars, sugar alcohols, fatty acids, phenolic compounds and other compounds) were identified as shown in Table S1. Among the identified compounds Myoinositol (IC_{50}:1–10 μM) (Hu et al. 2000), Caffeic acid (IC_{50}:8.01 μM) (Rzepecka-Stojko et al. 2015), Citric acid (IC_{50}:10 mM) (Lu et al. 2011), Xylitol (IC_{50}:6.99) (Park et al. 2015)have cancer-protective activity. Inositol-hexaphosphate blocks the PI3K/Akt, AP-1, PI3K/PKC and MAPK signal transduction pathways blocking uncontrolled cell division and forcing malignant cells to either differentiate or enter apoptosis (Fu et al. 2016). Caffeic acid ester is an excellent inhibitor of beta-catenin/T-cell factor signaling in colon cancer cell apoptosis (Xiang et al. 2006). 4-Guanidinobutyric acid (IC_{50}:43.7 μM) is reported to have cytotoxic activity against gastric cancer cells (Hwang & Jeong 2012). Xylitol provides a beneficial effect in improving health in anticancer treatment(Iwasa & Takahashi 1968) and shows apoptotic activity in the cancer cell (Wu et al. 2016). From the UHPLC study, the quantity of Parahydroxy Benzoic Acid (6380.7 ± 5.13 μg/g), Catechin (6604 ± 3.60 μg/g), Gallic

Acid (2008.3 ± 7.63 μg/g) and Quercetin (44.7 ± 4.04 μg/g) were determined (Figure S2). Gallic Acid (IC_{50}:740 μM) causes ROS dependent apoptosis and inhibited the growth of colon cancer cells (Subramanian et al. 2016). Catechin (IC_{50}:20 μg/mL) causes inhibition of cell growth, decreases in the levels of COX-2 and Bcl-xL proteins and apoptosis (Shimizu et al. 2005). Parahydroxy Benzoic acid has antiproliferative and proapoptotic activities through inhibition of histone deacetylases(Seidel et al. 2014). Quercetin (IC_{50}:42.5 μM) depends on COX-2 dependent ROS generation that induces apoptosis and inhibits cell survival (Raja et al. 2017).

The anti-colon cancer activities of MECR was confirmed by in-vitro assays like MTT assay for cell viability; Colony formation assay for cell proliferation inhibition; DAPI staining, Tunel assay and Cell Cycle Analysis for apoptotic study. MTT assay was performed to determine the cytotoxicity effect using HCT-8 cancer cell line (Figure S3). Exposure of cells to different concentrations of the MECR (0–1000 μg/mL) affected significant cytotoxicity at concentrations up to 1000 μg/mL. The toxic effect of MECR markedly decreased the cell viability to 31 ± 1.08% at 1000 μg/mL. The IC_{50} value was found to be 500 ± 1.09 μg/mL against HCT-8 cell line and this dosage was used as one of the dosages for subsequent analysis. Colony formation assay determines the number of adherent colony forming cells. The number of adherent colony-forming cells decreased in a concentration (0–1000 μg/mL) dependent manner from 526 ± 12.02 to 116.7 ± 8.82 cells per field (Figure S4). Cell-permeable fluorescent dye DCFH-DA gets oxidized to fluorescent DCF by the ROS produced in the cell. The amount of fluorescence compound is proportionally correlated to the amount of intracellular ROS generation. The increase in oxidative stress is indicated by increase in relative ROS levels (3.40 ± 0.153, 4.70 ± 0.115, 5.53 ± 0.233) in the cells treated with MECR (200, 500, 1000 μg/mL) as compared to untreated control cells (HCT-8) and simultaneously ROS level (1.30 ± 0.153) of normal colon cells (CCD-18Co) was also observed (Figure S5). The ROS is generated more in colon cancer cells due to the presence of phenolics in MECR (Kuete et al. 2016). Effect of MECR on cell cycle progression of HCT-8 cells was analyzed by FACS using PI. It was evident from Figure S6 that treatment with MECR with increased concentration (0, 200, 500, 1000 μg/mL) resulted in increased percentage (3.40 ± 0.656, 14.1 ± 0.944, 17.2 ± 0.761, 24.2 ± 0.671) of apoptotic cells in the sub-G_0 phase (in Figure M_1-parts) of the cell cycle. DAPI staining assay says about preliminary confirmation about DNA damage. In this study nucleus condensed as the concentration of MECR were increased (Figure S7). Tunel assay detects the apoptotic DNA fragmentation, which is widely used to identify and quantify apoptotic cells or to detect excessive DNA breakage in individual cells. In this study, it was observed that percentage of apoptotic cells were increased in the treated (500 μg/mL) groups to 73.33 ± 3.53% (Figure S8).

3. Conclusion

From the result, it can be concluded that MECR decreases proliferation and induces apoptosis by increasing ROS in human colon cancer cell line HCT-8 whereas no such activity was seen in normal colon cell line. Hence MECR might be considered as a colon cancer protective agent.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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SUPPLEMENTARY MATERIAL

Metabolite Profiling and *in-vitro* Colon Cancer Protective Activity of *Cycas revoluta* Cone Extract

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ABSTRACT

The methanolic extract of *Cycas revoluta* cone (MECR) was analyzed by GC-MS and UHPLC for metabolite profiling and was evaluated for anti-colon cancer property by using in vitro assays like Cell Viability Assay, Colony Formation Assay, ROS Determination, Flowcytometry, DAPI staining assay, Tunel assay. GC-MS and HPLC analysis confirmed the presence of different phytochemicals in the extract of *Cycas revoluta* cone. *In-vitro* studies showed MECR extract showed significant anti-colon cancer activity by reducing proliferation and inducing apoptosis in colon cancer cell (HCT-8) line, but no such activity was seen in normal colon cell (CCD-18Co) line. The investigation confirms that MECR may be a promising candidate in colon cancer protection.

Key Words: *Cycas revoluta*; Colon cancer; Reactive oxygen species (ROS); GC-MS.

Experimental

Materials

Methanol; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Propidium iodide (PI); 2,7-Dichlorofluorescein diacetate (DCFH-DA); 4, 6-Diamidino-2-phenylindole (DAPI) Stain were purchased from Sigma Laboratories, Germany. TUNEL assay kit purchased (Roche Molecular Biochemicals, Manheim, Germany).

Plant Material

Fresh male cone was collected from the village of Srirampur under East Midnapur district and authenticated (Voucher Ref. No.: BSI/Pharma/SD/Tech./2016) by botanist, Dr A B D Selvam of Botanical Survey of India (BSI), Shibpur, Howrah (West Bengal). The cones were cut into small pieces and dried in shade below 50 °C. Then dried cone pieces were powdered in mixture grinder and stored in airtight container.

Extraction

The cones were cut into small pieces and were shade dried and then milled into a coarse powder. Then the air dried and powdered cones (175 gm) was first defatted with petroleum ether (60-80°C) and then extracted with 2.5 litres of methanol (90%) using Soxhlet apparatus. The solvent was then removed by rotary vacuum evaporator followed by repeated lyophilisation. After drying 11.7 gm (6.6% Yield) extract obtained.

GC/MS analysis of MECR

Preparation of sample for identification of metabolites

10 mg of the dried sample of MECR was dissolved in MeOH:H_2O of HPLC grade in 1:1 ratio and then 50 µL of the dissolved sample was distributed into eppendorf tubes (3 x 50 µL) and evaporated to dryness. The residue was re-dissolved in 10 µL of methoxyamine hydrochloride (20 mg/mL in Pyridine) and subsequently shaken for 90 minutes at 30 ºC. Then 90 µL of N-Methyl-N-trimethyl silyl-trifluoro-acetamide (MSTFA) was added, and the mixture was shaken at 37 ºC for 30 minutes for trimethylsilylation of acidic protons to increase the volatility of metabolites and to enhance chromatographic separation of metabolites. Fatty Acid Methyl Esters (FAME) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{26} linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8 mg/mL (C_8 - C_{16}) and 0.4 mg/mL (C_{18} - C_{26}) was added(Kind et al. 2009).

Parameters for GC/MS analysis

GC/MS analysis was carried out following the method of Kind et. al.(Kind et al. 2009) after little modification(Das et al. 2016). HP-5 MS capillary column [Agilent J & W; GC Columns (USA), length 30 m plus Duragard 10 m, diameter 0.25 mm narrow bore, film 0.25 µm] was used. An injection was made in a sandwich mode with fast plunger speed without viscosity delay or dwell time. The analysis was performed under the following temperature programme: oven ramp 60 ºC (1 minute hold) to 325 ºC at 10 ºC /minute; 10 minute hold before cool down, 37.5 minutes runtime. The injection temperature was set at 250 ºC, the MS transfer line at 290 ºC, and the ion source at 230 ºC. Helium was used as the carrier gas at a constant flow rate of 0.723 mL/minute (carrier linear velocity 31.141 cm/sec).

1µL of samples were injected via the split mode (split ratio 1:5) onto the GC column. Prior to analysis, the method was calibrated with the FAME standards available with the Fiehn GC/MS Metabolomics library (2008) (Agilent Chem Station, Agilent Technologies Inc., Wilmington, USA). Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra and retention times (RT) with entries of mass spectra and retention time in Agilent Fiehn Metabolomics library using Agilent Retention Time Locking (RTL) method. Fiehn retention indices (RI) were also compared. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC/MS results and to identify chromatographic peaks.

Quantitative analysis by UHPLC

The identification and quantification of gallic acid, catechin, para-hydroxybenzoic acid and quercetin present in MECR were analyzed by ultra high-performance liquid chromatography (UHPLC) using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 autosampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Das et al. 2017). A standard stock solution (1 mg/mL) of gallic acid, catechin, para-hydroxybenzoic acid and quercetin were prepared in HPLC grade methanol and subsequently different concentrations (5, 10, 20, 30, 40 and 50 µg/mL**)** of standard solutions were prepared by diluting the standard stock solution for calibration curves in order to quantify the phenolic compounds present in MECR. The solutions for UHPLC analysis were filtered through a 0.45 µm membrane filter. The chromatographic separations were performed using a reversed phase C_{18} analytical column (250 mm × 4.6 mm internal diameter) with a particle size of 5 µm, Hypersil GOLD (Thermo

Fisher Scientific, USA) and the column temperature was maintained at 25°C. The UHPLC analysis was performed using gradient elution as illustrated in Table 1 with a flow rate of 1.0 mL min^{-1} using 0.2 % (v/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The injection volume was 5 µL and detector wavelength of 280 nm was used for the study. The quantification of phenolic compounds in MECR was calculated from the calibration curve. The chromatograms were processed using Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, USA).

Cell culture

Colon cancer cell line (HCT-8), normal colon cell line (CCD-18Co) were collected from were purchased from NCCS Pune, India. Cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum, 100 µg/mL of penicillin, and 100 µg/mL of streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in the air. When the cells were 60-70% confluent, the medium was aspirated, the cells were washed with phosphate-buffered saline (PBS), and fresh DMEM with or without antibiotic was added. Control plates were replenished with fresh medium and also incubated at similar conditions, as stated above(Singh et al. 2017).

Cell viability determination

Cell viability was quantified by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed to evaluate the antiproliferative effect of the test material. HCT-8 cells was seeded into 96-well plates in the density of 1×10^4/well and incubated overnight. Then, 200 µL culture medium containing various concentrations (0, 50, 100, 200, 300, 400, 600, 800, 1000 µg/mL) of MECR extracts were added to each well. After 48 hrs of MECR treatment, the MTT solution (5 mg/mL) was added to each well and incubated for 4 h. Finally, the MTT solution was removed and replaced by 150 µL DMSO each well to dissolve the formazan crystals and mixed for 10 min. The absorbance of the solution was determined by a Spectra Max M5 plate reader at 540 nm wavelength. The experiment was done with four replicates three times (Zhang et al. 2016).

Colony formation assay

HCT-8 cells were treated with different concentration (0, 200, 400, 500, 1000 µg/mL) of MECR for 48 hr and plated separately in a petridish plate (Plate diameter 30 mm) using 3 mL complete growth medium containing DMEM media with 10% foetal bovine serum (FBS) at a density of 1×10^3 cells/plate. After one week incubation, cells were stained with a crystal-violet solution [0.05% (w/v) crystal violet in 20% (v/v) methanol] for 10 min and then extra

stain is washed by dipping the plate in distilled water (Du et al. 2017). The colonies were counted in a light microscope (DM 1000, Leica, Germany) using software Las EZ.

Reactive Oxygen Species (ROS) measurement

HCT-8 cells were treated with different concentration (0, 200, 500, 1000 µg/mL) of MECR for 48 hr. and then washed in PBS 3 times. The ROS produced by HCT-8 cells was estimated using fluorescent dye DCFH-DA (2',7-Dichlorofluorescein diacetate). In brief, after addition of 10 µl of DCFH-DA dye (final concentration of 20 µM) cells were incubated for 30 min in a CO_2 incubator (Heraeus, HERA cell). The fluorescence intensity was measured at an excitation and emission wavelength of 485 nm and 530 nm respectively using a spectrofluorimeter (Synergy HT, Biotek, USA). The data were expressed as percentage DCF fluorescence as compared to that of control. Simultaneously ROS level in normal colon cell (CCD-18Co) was also observed (Tiwari et al. 2010).

Detection of apoptosis by flow cytometry

The analysis of cell cycle phase distribution of HCT-8 was done according to the method of Holmes et al. with slight modification. HCT-8 cells were treated with different concentration (0, 200, 500, 1000 µg/mL) of MECR for 48 hr. followed by washing in PBS for 3 times. Then 1×10^6 cells were permeabilized with 70% ice-cold ethanol followed by staining with propidium iodide (PI, 20 µl of 1 mg/ml stock). Cell cycle phase distribution of nuclear DNA was determined on FACS Calibur fluorescence-activated cell sorter (FACS), fluorescence detector equipped with 488 nm argon-ion laser light source using Cell Quest software (Becton Dickinson, USA). A total of 10000 events were acquired for each sample analyzed. A histogram of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been displayed. The percentage of apoptotic cells was determined by measuring the fraction of nuclei that contained a sub-diploid DNA content(Manna et al. 2006).

DAPI staining assay

DAPI (4, 6-Diamidino-2-phenylindole) staining was used to assess nuclei morphology of cells. The logarithmic phase of HCT-8 cells was seeded into petridish plates (diameter 30 mm) in the density of 1×10^3/plate and cultured overnight. After treatment with different concentrations (0, 200, 400, 800, 1000 µg/mL) of MECR extracts for 48 hr, the cells were stained with DAPI after fixing with 3.7% formaldehyde. The samples were then washed with PBS and detected by fluorescence microscope (Leica DM 4000B) (Zhang et al. 2016).

Tunel assay

Apoptosis analysis was done in the HCT-8 cells using TUNEL assay kit (Roche Molecular Biochemicals, Manheim, Germany) according to manufacturers' protocol. At first,

HCT-8 cells were seeded separately in a petridish plate (30 mm) in the density 1×10^3/plate along with different concentrations (0, 200, 500, 1000 μg/ml) of MECR for 48 hrs. Then cells were trypsinized to cause detachment and cell smear was fixed in freshly prepared 4% paraformaldehyde in PBS at room temp followed by washing and endogenous peroxidase blocking with 3% H_2O_2 in methanol for 10 min at room temp. Then the cells were permeabilized with 0.5% Triton-X 100 and incubated with Tunel reaction mixture at 37 ºC for 60 min in a humidified chamber. After washing, cells were incubated with horseradish peroxidise-conjugated anti-fluorescein antibody at 37ºC for 30 min in a humidified chamber. Stained cells were visualized after substrate (DAB) reaction by light microscope. For each sample, six randomly chosen fields were scored in a blinded manner. Mean percentage of apoptotic cells was determined(Basu et al. 2017).

Statistical analysis

All the data were evaluated with Graph Pad Prism version-5 software (GraphPad Software Inc, La Jolla, CA). Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Dennett's t-test to correct for multiple comparisons with acceptable statistical level significance ($p < 0.05$). Each experiment was presented as the mean ± SEM from triplicate experiments performed in a parallel manner.

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Table S1: GC-MS identified metabolites in MECR

Metabolites Identified	Library RT	Observed RT	Peak area (%)
ORGANIC ACIDS			
Citramalic acid	12.63	12.18	0.015
Citric acid*	16.61	16.22	5.777
Dicrotalic acid	14.23	13.73	0.077
Fumaric acid	10.94	10.55	0.011
Gluconic acid 2	18.3	17.80	0.531
Glyceric acid	10.73	10.37	3.356
Glycolic acid	7.05	7.05	0.184
2-Isopropylmalic acid	13.84	13.44	0.037
Lactic acid	6.85	6.85	0.030
Maleic acid	10.32	10.04	0.080
D-Malic acid*	12.79	12.46	0.776
Oxalic acid*	7.88	7.88	0.197
D-Saccharic acid	18.61	16.69	0.609
Succinic acid*	10.51	10.14	0.039
AMINO ACIDS			

L-Allothreonine	16.36	16.18	2.733
Aspartic acid*	12	11.64	0.197
Aspartic acid 2	13.21	12.81	0.067
Beta-alanine	12.04	11.56	0.137
L-pyroglutamic acid	13.22	12.88	10.706
L-valine*	7.3	7.25	0.073
SUGARS and SUGAR DERIVATIVES			
Sucrose*	23.99	23.60	4.375
D-Trehalose*	24.75	24.37	0.106
Glycerol-1-phosphate	16.06	15.73	0.091
4- Methyl-beta-D-galactopyranoside	16.93	17.00	0.204
SUGAR ALCOHOLS			
D-threitol	12.95	12.74	9.989
Xylitol	15.38	15.19	6.875
D-mannitol	17.81	17.48	0.457
D-sorbitol	17.9	17.52	0.060
Allo-inositol	17.25	17.64	0.114
Myoinositol	19.35	18.99	28.303
FATTY ACIDS			
4-acetylbutyric acid	10.63	10.23	5.076
DL-3-aminoisobutyric acid	12.46	11.98	0.004
4-guanidinobutyric acid	13.35	12.88	10.131
Myristic acid*	16.89	16.32	4.914
Mucic acid	18.91	18.69	0.587
Stearic acid*	20.68	20.04	0.282
PHENOLS			
3-phenyllactic acid	13.98	13.54	0.006
Caffeic acid*	19.75	19.21	0.021
cis-4-hydroxycyclohexanecarboxylic acid	12.78	11.91	0.003
OTHERS			
Gluconic acid lactone 2*	17.43	17.59	0.542
Ribonic acid gamma lactone	15.05	14.61	2.192
Porphine	10.77	10.46	0.005
D-sphingosine	22.53	22.95	0.031

All the compounds were identified by comparing retention time (RT), retention index (RI) and mass spectra (MS) of Fiehn Library.

* Compounds were further validated by comparing RT, RI and MS of standard compounds available in laboratory.

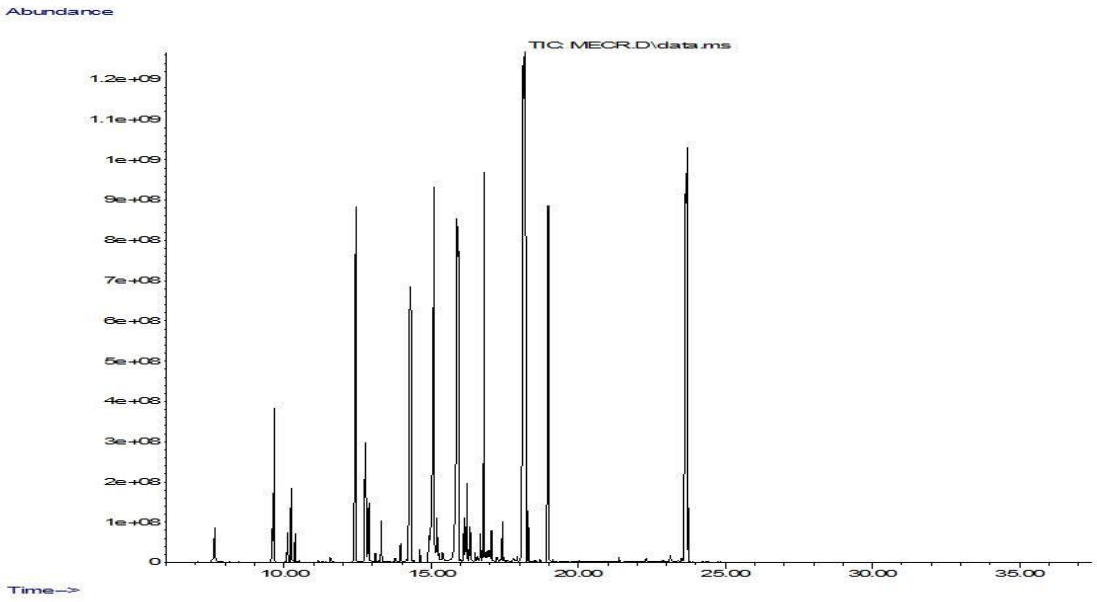

Figure S1: Peaks in GC-MS analysis of methanolic extract of *C. revoluta* (MECR)

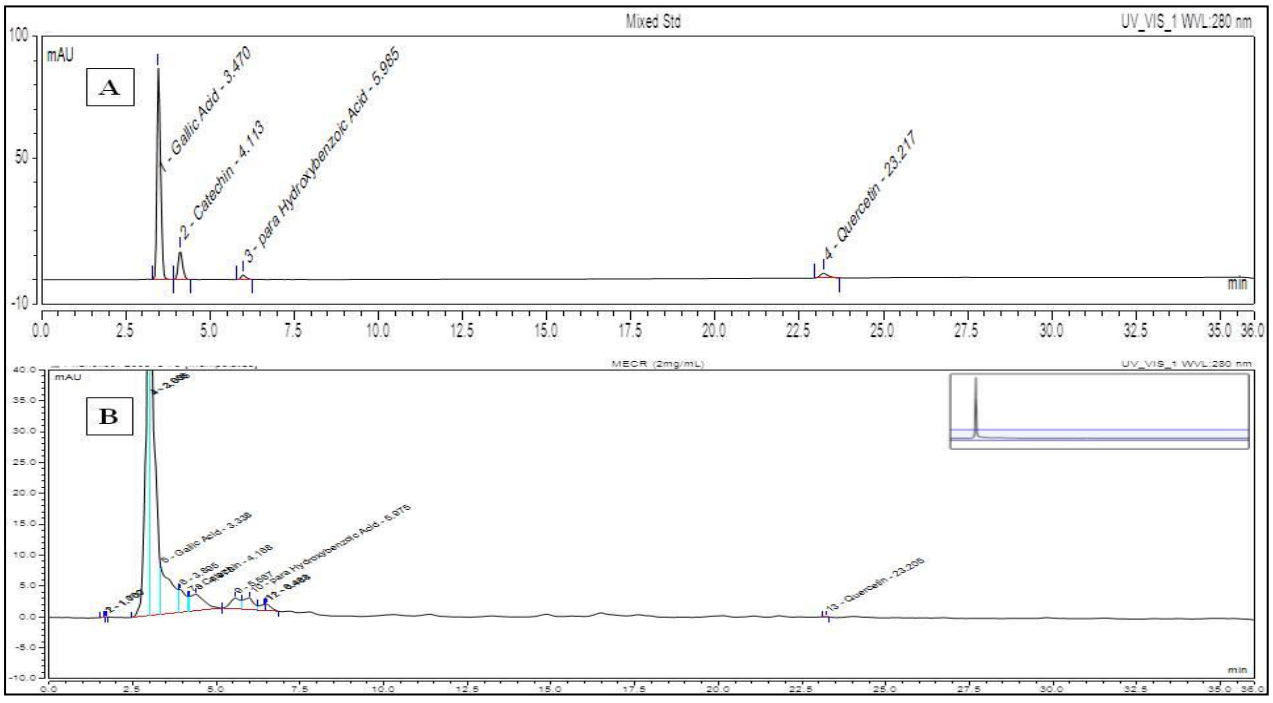

Figure S2: UHPLC chromatograms of a mixed standard of phenolic compounds**(A)** and the methanolic extract of *C. revoluta* (MECR) **(B)** as detected at 280 nm (1:Gallic acid, 2:Catechin, 3:Para-hydroxybenzoic acid, 4:Quercetin).

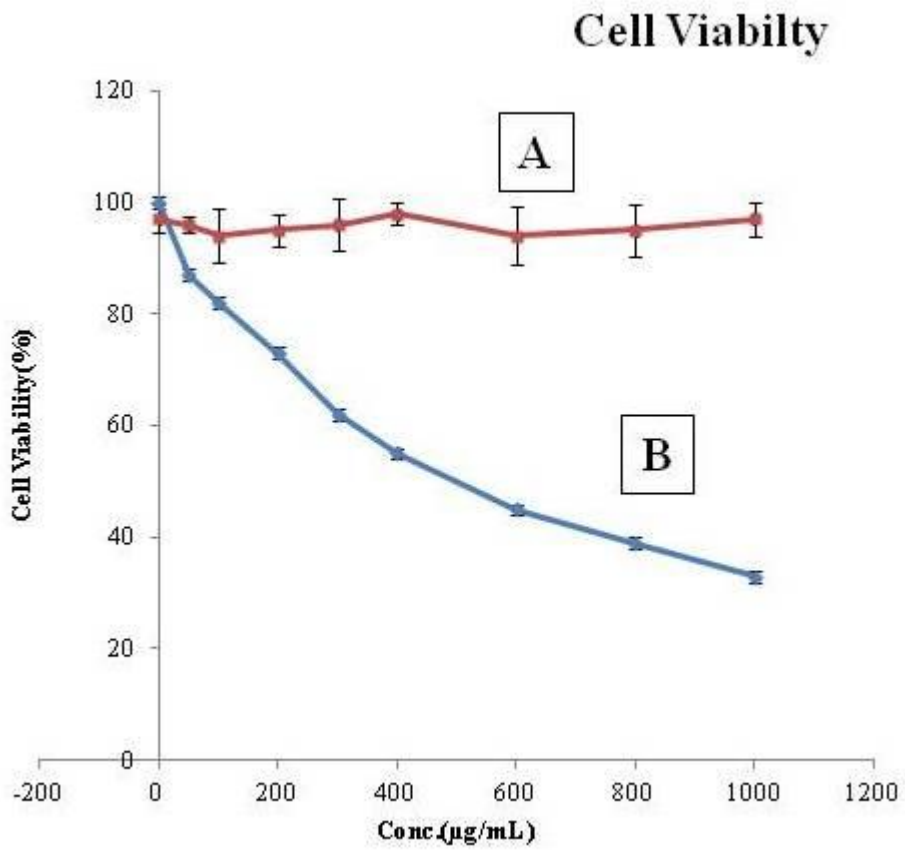

Figure S3: MTT Assay for Cell Viability Testing. **A:** Normal colon cell line (CCD-18Co). **B:** Colon cancer cell line (HCT-8). Values are expressed as mean ± SEM (n=3). Increased concentration decreased the viability in Colon cancer cell line (HCT-8) (IC_{50}=500±1.09 µg/mL).

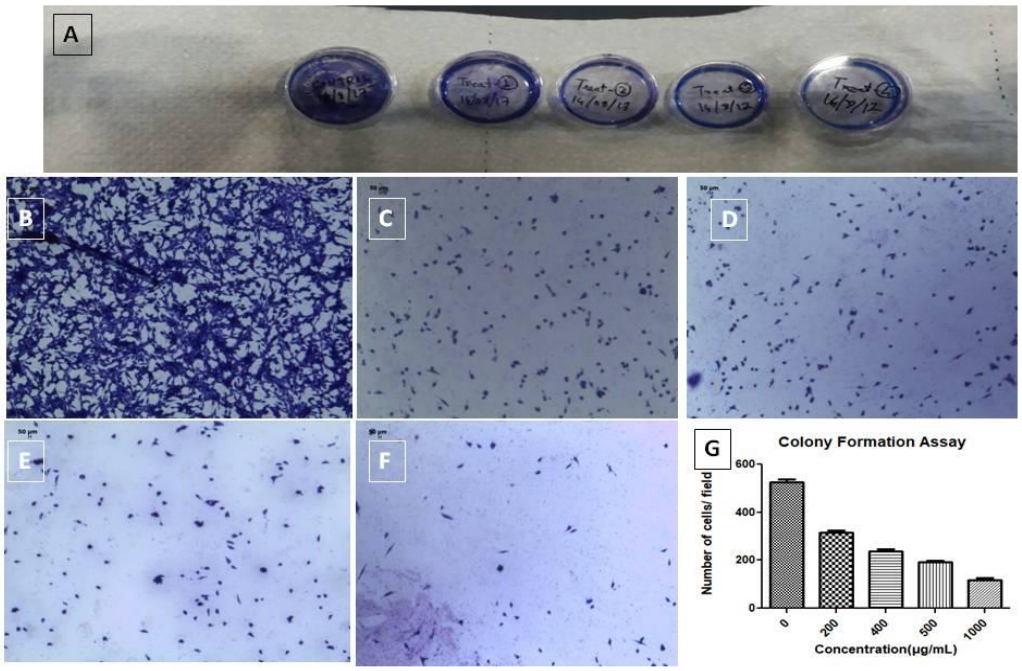

Figure S4: Colony formation assay. **A:** Petridish Plates (30 mm diameter). **B:** Control **C:** treatment with 200 µg/mL of MECR **D:** treatment with 400 µg/mL of MECR **E:** treatment with 500 µg/mL of MECR **F:** treatment with 1000 µg/mL of MECR **G:** Comparison of number of adherent colony forming cells as per different concentration of MECR. Values were expressed in mean ± S.E.M. (n=3). *:$p<0.05$ is considered significant.

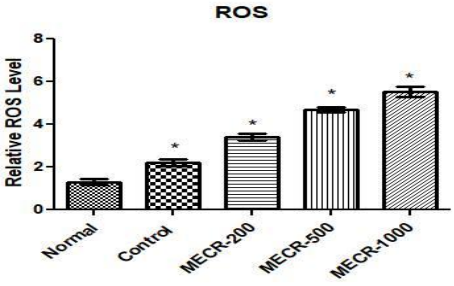

Figure S5: Relative Reactive Oxygen Species (ROS) level in Normal Colon Cell line (CCD-18Co) and Colon Cancer Cell line (HCT-8) along with treatment of MECR.Values were expressed as mean±SEM (n=3). *: Significantly ($p<0.05$) different from Normal Group (CCD-18Co).

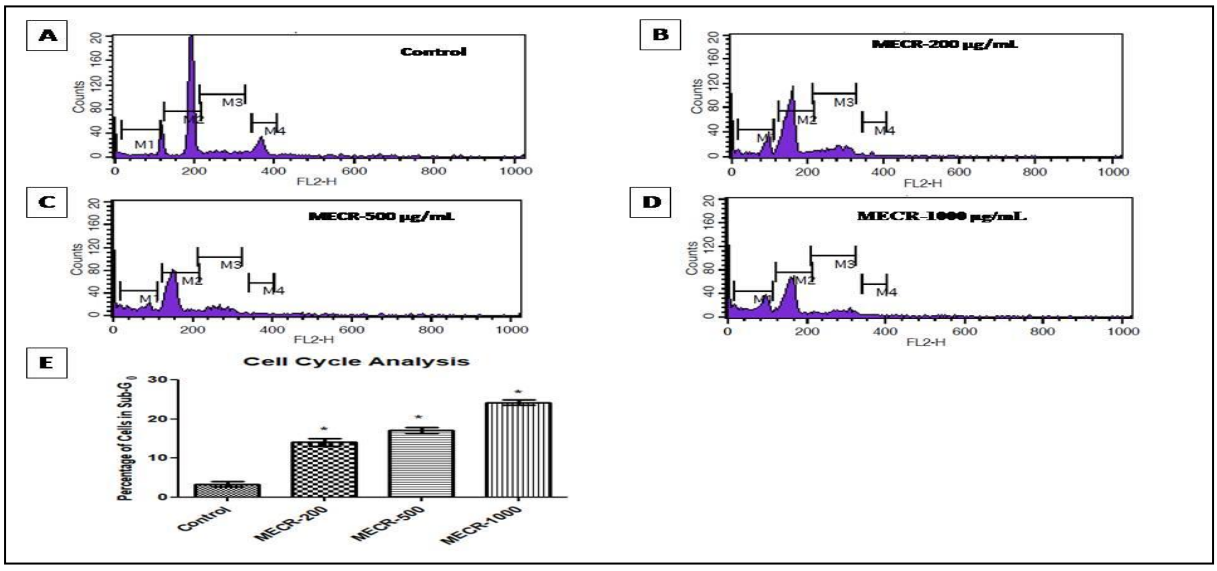

Figure S6: MECR induced apoptotic cell death in HCT-8 cells. **A, B, C, D:** Flowcytrometric analysis of HCT-8 cell cycle phase distribution after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. **E:** Histogram display percentage of sub-G_0 population at different concentration of MECR treatment. Results are mean ±SEM(n=3). ∗: Significantly ($p<0.05$) different from Control.

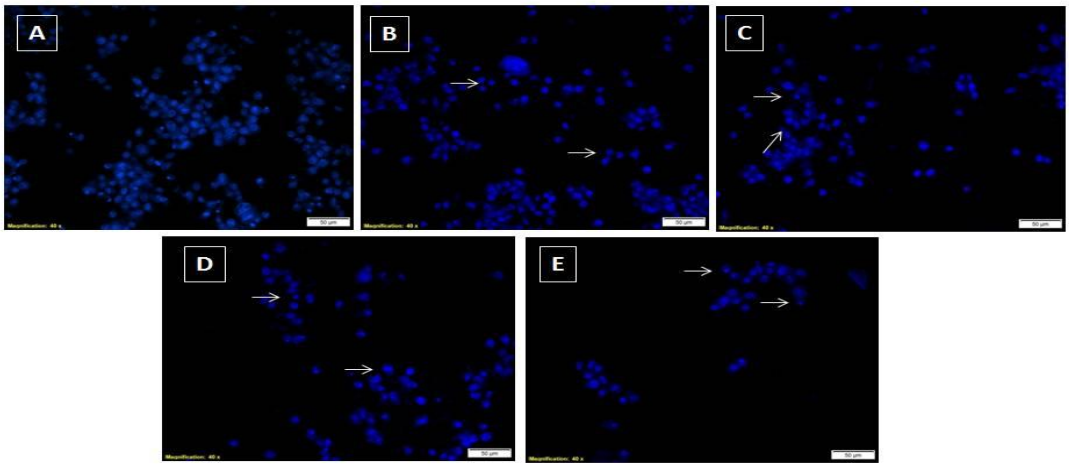

Figure S7: The morphological changes of nuclei were examined by fluorescence microscopy (magnification, 40x) using DAPI staining at various concentration of MECR treatment. **A:** 0 µg/mL (Control) **B:** 200 µg/mL **C:** 400 µg/mL **D:** 500 µg/mL **E:** 1000 µg/mL. The arrows indicate nuclear condensation and apoptotic bodies. Cell number also decreasing in a concentration dependant manner (magnification, 40x).

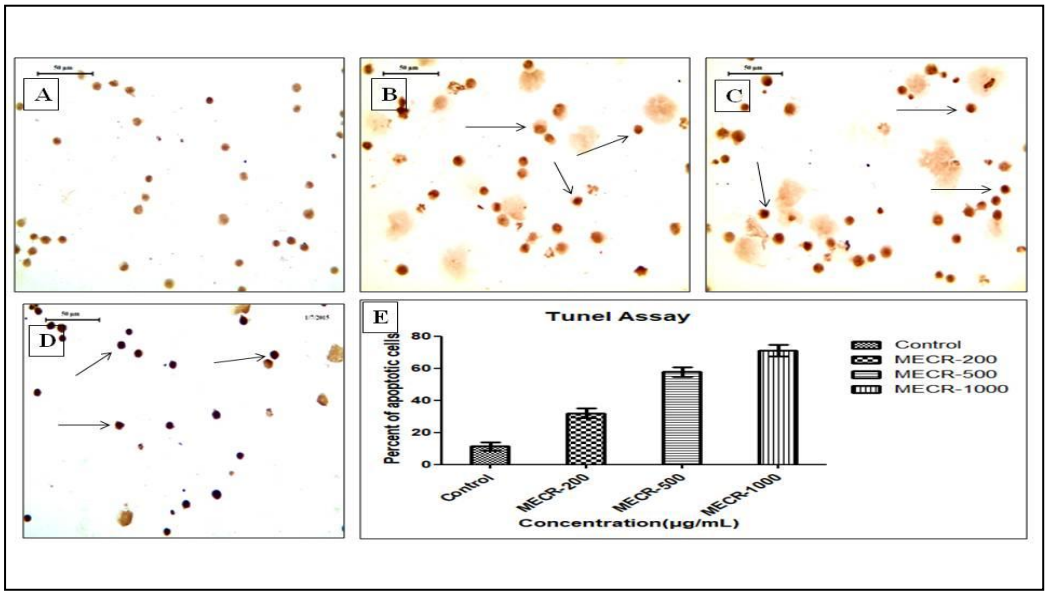

Figure S8: Induction of apoptosis (Tunel assay). **A, B, C, D:** Tunel Assay of HCT-8 cell after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. **E:** Histogram display the percentage of apoptotic cell

population at different concentration of MECR treatment. Results are mean ±SEM (n=3). ∗:Significantly ($p<0.05$) different from Control. Arrows indicate in **A, B, C** and **D**, the cells with morphologically condensed nucleus were Tunnel positive, indicating the existence of fragmented DNA (magnification, 40x).