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.

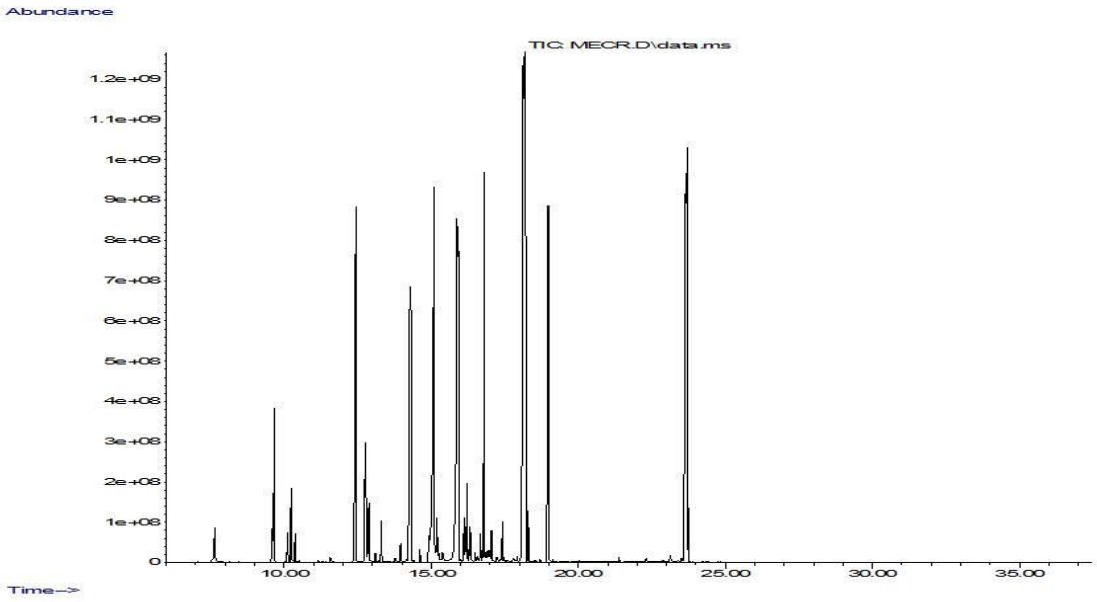

Figure S1: Peaks in GC-MS analysis of methanolic extract of *C. revoluta* (MECR)

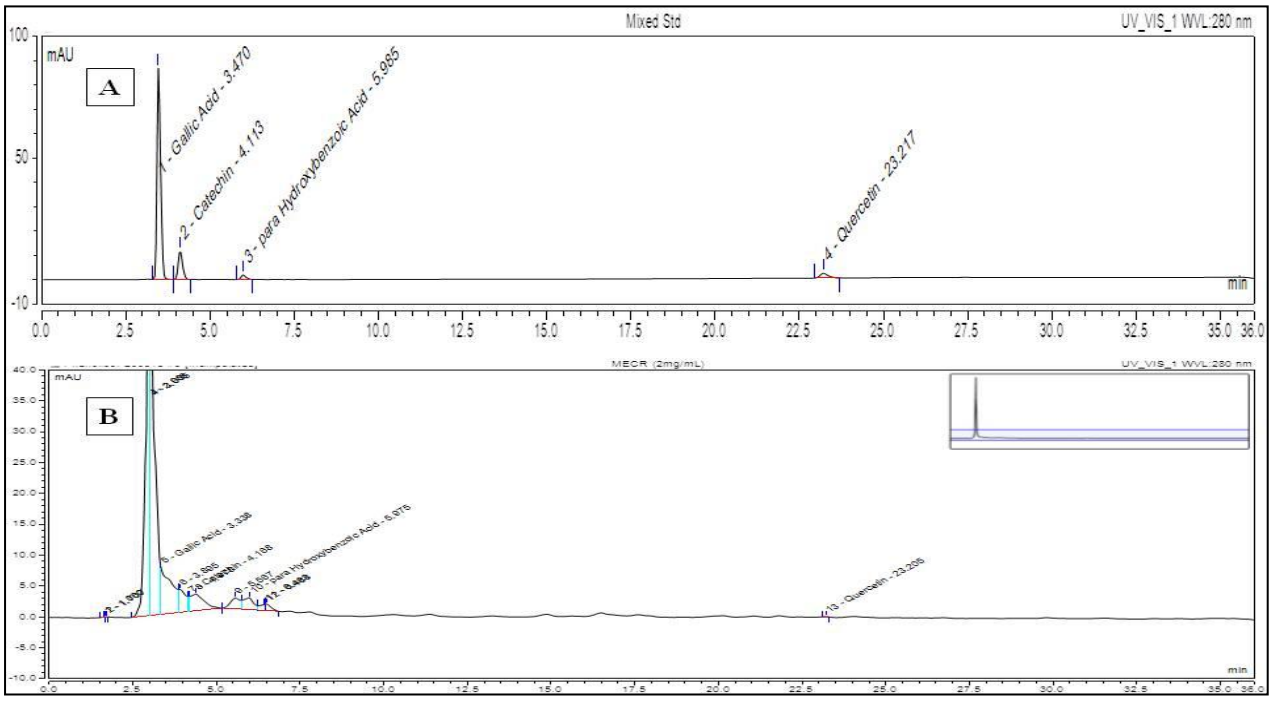

Figure S2: UHPLC chromatograms of a mixed standard of phenolic compounds**(A)** and the methanolic extract of *C. revoluta* (MECR) **(B)** as detected at 280 nm (1:Gallic acid, 2:Catechin, 3:Para-hydroxybenzoic acid, 4:Quercetin).

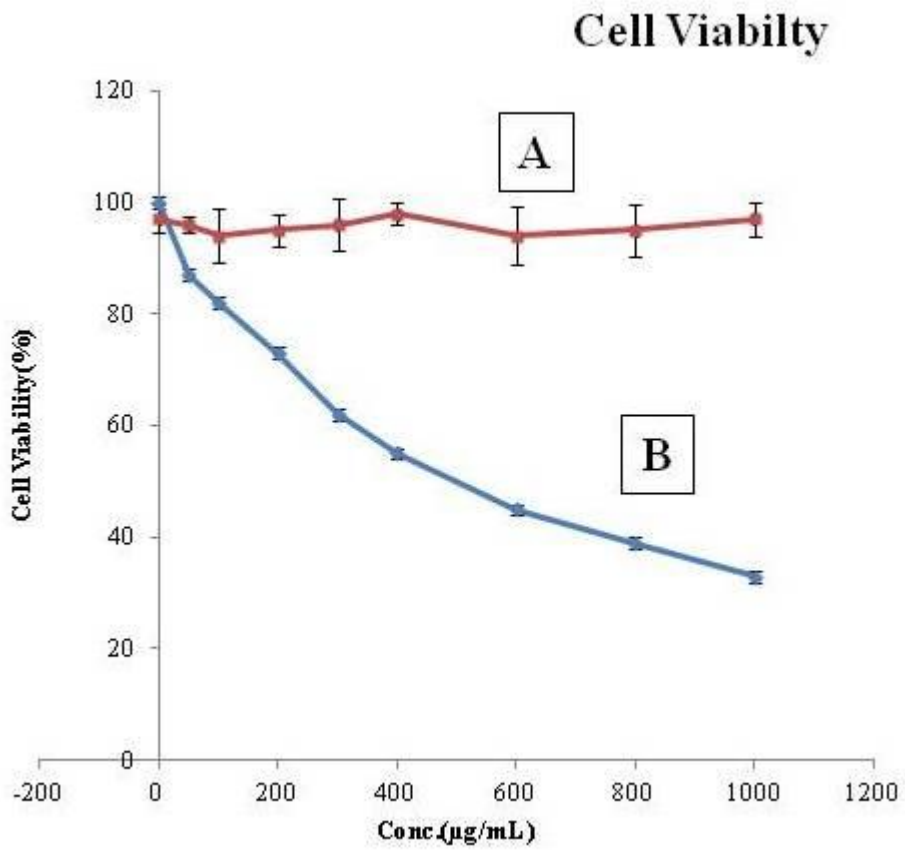

Figure S3: MTT Assay for Cell Viability Testing. **A:** Normal colon cell line (CCD-18Co). **B:** Colon cancer cell line (HCT-8). Values are expressed as mean ± SEM (n=3). Increased concentration decreased the viability in Colon cancer cell line (HCT-8) (IC_{50}=500±1.09 µg/mL).

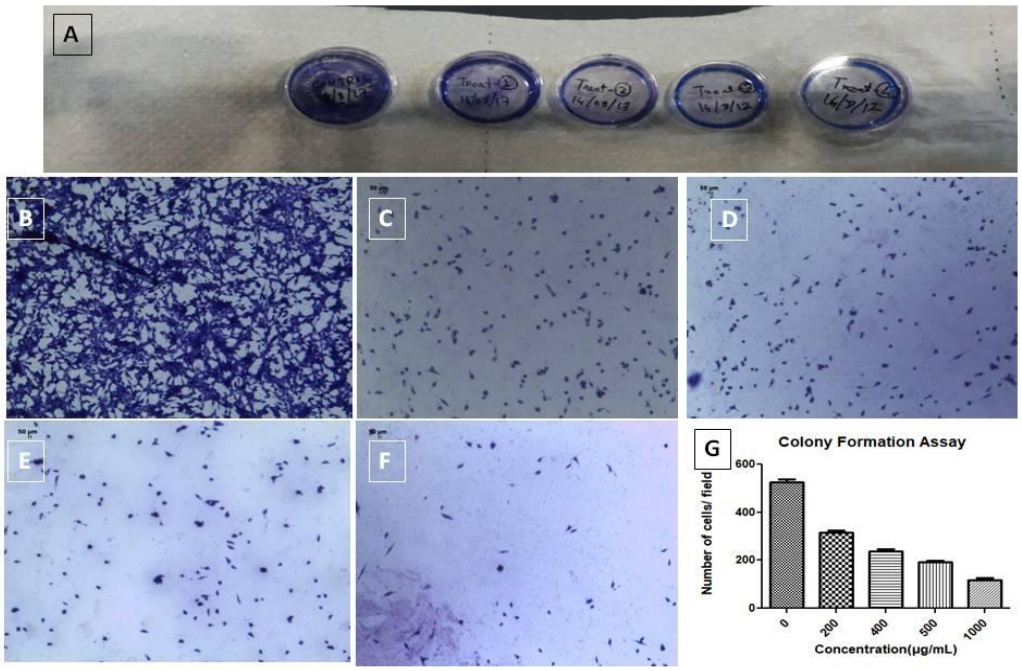

Figure S4: Colony formation assay. **A:** Petridish Plates (30 mm diameter). **B:** Control **C:** treatment with 200 µg/mL of MECR **D:** treatment with 400 µg/mL of MECR **E:** treatment with 500 µg/mL of MECR **F:** treatment with 1000 µg/mL of MECR **G:** Comparison of number of adherent colony forming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

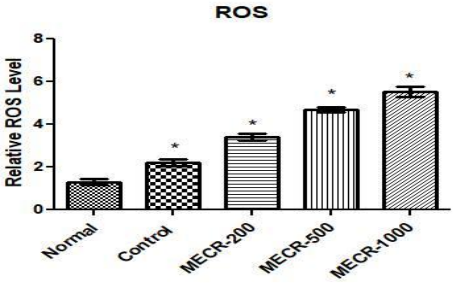

Figure S5: Relative Reactive Oxygen Species (ROS) level in Normal Colon Cell line (CCD-18Co) and Colon Cancer Cell line (HCT-8) along with treatment of MECR.Values were expressed as mean±SEM (n=3). *: Significantly ($p<0.05$) different from Normal Group (CCD-18Co).

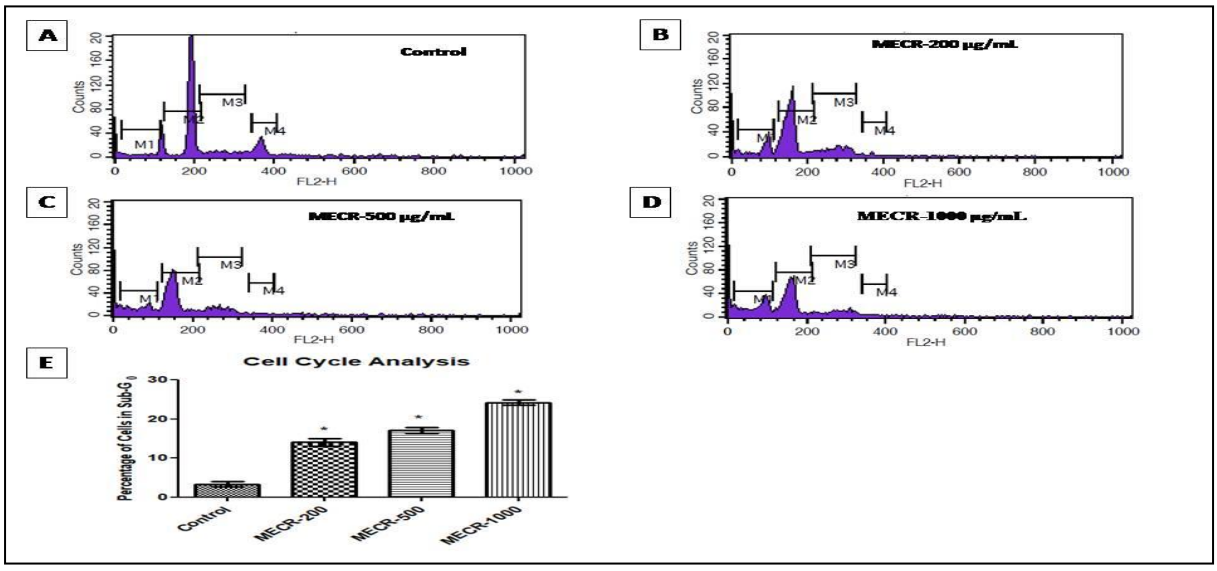

Figure S6: MECR induced apoptotic cell death in HCT-8 cells. **A, B, C, D:** Flowcytrometric analysis of HCT-8 cell cycle phase distribution after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. **E:** Histogram display percentage of sub-G_0 population at different concentration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

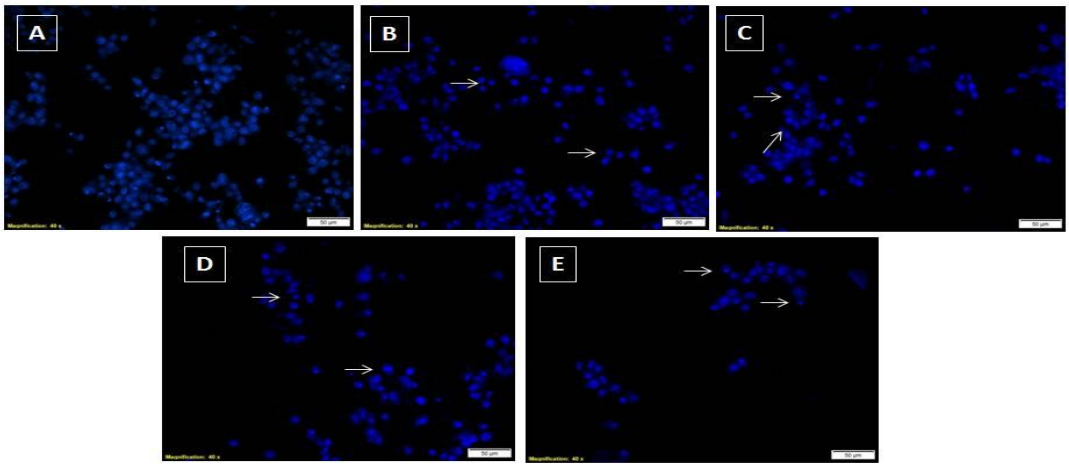

Figure S7: The morphological changes of nuclei were examined by fluorescence microscopy (magnification, 40x) using DAPI staining at various concentration of MECR treatment. **A:** 0 µg/mL (Control) **B:** 200 µg/mL **C:** 400 µg/mL **D:** 500 µg/mL **E:** 1000 µg/mL. The arrows indicate nuclear condensation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

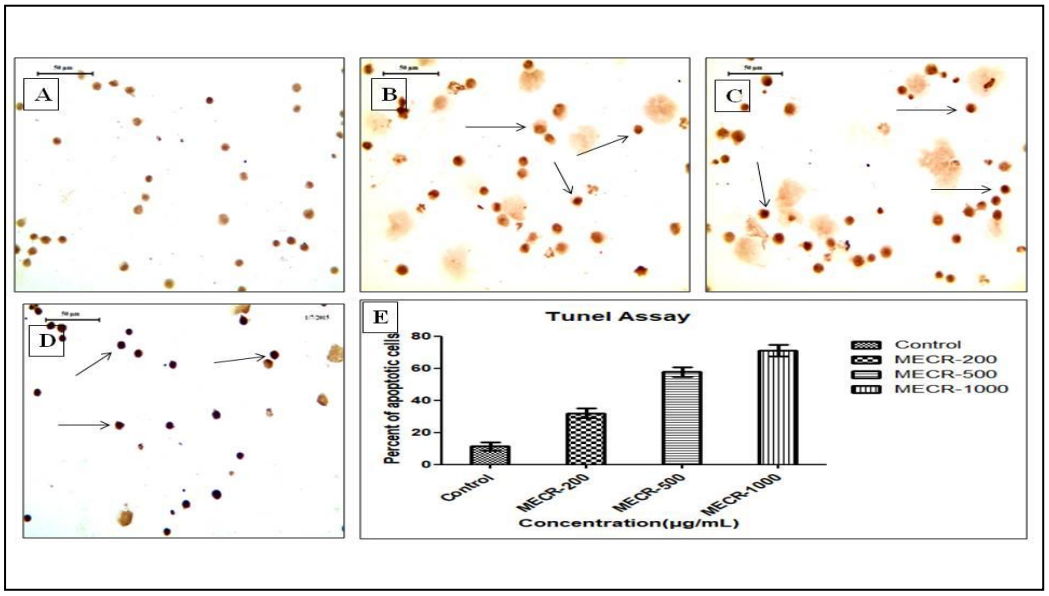

Figure S8: Induction of apoptosis (Tunel assay). **A, B, C, D:** Tunel Assay of HCT-8 cell after treatment with different concentration (0, 200, 500, 1000 µg/mL) of MECR. **E:** Histogram display the percentage of apoptotic cell

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).