INTRODUCTION
Liver is a versatile organ of the body that regulates internal chemical environment [1]. Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide. HCCs are phenotypically (morphology and microscopy) and genetically heterogeneous tumours, possibly reflecting the heterogeneity of etiological factors implicated in HCC development, the complexity of hepatocyte functions and the late stage at which HCCs usually become clinically symptomatic and detectable[2, 3]. Hepatocarcinogenesis is a multi-factor, multi-step and complex process [4]. Malignant transformation of hepatocytes may occur, regardless of the etiological agent, through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response, and oxidative DNA damage [5-7]. Herbal compounds could affect all phases of HCC, including initiation, promotion and progression [8, 9]. Experimental studies show that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and prevent angiogenesis and metastatic spread, suggesting a potential role for antioxidants as adjuvants in cancer therapy.

Experimental studies support the role of reactive oxygen species in cancer, in part by showing that dietary antioxidants (eg, vitamin E, vitamin C, selenium, β-carotene, and other phytochemicals) as well as endogenous antioxidants (eg, glutathione) that neutralize or trap reactive oxygen species act as cancer preventive agents [10, 11]. Once cancer has occurred, however, treatment with radiation and chemotherapeutic agents that generate free radicals in cells largely relies on their oxidative damage to eradicate the cancerous cells [12]. Thus, the question arises whether antioxidants that protect normal cells from acute and long-term free radical damage may afford the same protection to tumor cells and hinder the overall outcome of cancer therapy [13].

It would be extremely beneficial in the field of cancer chemotherapeutics, if a natural anti-cancer agent can be discovered which also has notable antioxidant property. Our plant of interest, *Ruellia tuberosa* L. (Acanthaceae) is a medicinal plant which has strong anti-inflammatory property in-vivo [14]. In the present study we have investigated the anti-hepatocellular carcinoma property of methanolic extract of *Ruellia tuberosa* leaf on HepG2 (a perpetual cell line which was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma) along with that anti-carcinogenic property of methanolic extract of *Ruellia tuberosa* leaf & its various fractions on RAW 264.7 cell lysate have been studied.

MATERIALS & METHODS

Chemicals
The following chemicals were used: RPMI 1640 medium with L-glutamine, Fetal calf serum (FCS), Trypsin (Gibco, USA), HEPES, AnnexinV- FITC apoptosis detection kit, Ethidium bromide, Propidium iodide, Xanthine, Xanthine Oxidase purchased from Sigma (St. Louis, MO, USA), Sorafenib tosylate (Brand name: Nexavar, Bayer & Onyx Pharmaceuticals), Penicillin-Streptomycin (Biovest, Germany), Gentamycin (Nicholas, India), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]. RNase (SRL), Dimethyl sulfoxide (DMSO), Phenol, Chloroform (Qualigens Fine Chemicals), Trypan blue, Protease –K , Isoamyl alcohol, Agarose, Ethylene Diamine Tetra acetic Acid (EDTA) (Sisco R.L. Pvt Ltd), Sodium bi-carbonate, Super oxide dismutase (SOD) kit (Fluka), Vit C powder & Ethanol (Merck) and other chemicals and reagents were of analytical grade and purchased from local firms.

Cell Culture
Hepatocellular carcinoma cell line HepG2 and RAW264.7 cells (Macrophage cell line) were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were cultured and routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum [15], penicillin (100 units/ml), streptomycin (100μg/ml), Gentamycin (100μg/ml) and were incubated at 37 °C in a humidified atmosphere containing 5% CO2 inside a CO2 incubator. Both Hep-G2 & RAW 264.7 cells are adherent in nature. During sub culturing of the cells this adherent property can be diminished by adding 1x Trypsin solution in the cell.

ABSTRACT
Objective: The current study was planned to investigate the anti-carcinogenic & anti-oxidant property of *Ruellia tuberosa* L. (Acanthaceae) methanolic leaf extract (MERTL) on HepG2 cell line & RAW 264.7 murine macrophage cell lysate respectively.

Methods: The potent cytotoxicity activity of MERTL has been studied by cell growth inhibition study & MTT assay on metabolically active HepG2 cells. 1% Agarose gel electrophoresis, detection of apoptosis & cell cycle arrest by Flow cytometric analysis have been performed to determine the proportion & stages of cellular apoptosis on HepG2 cells. In anti-carcinoid study, Superoxide dismutase activity study of MERTL & its various fractions has been performed on RAW 264.7 cell lysate.

Results: Cytotoxicity study of MERTL has been confirmed by MTT assay & the IC50 value is calculated to be 54.95μg/ml on HepG2 cell line. Agarose gel electrophoresis study showed fragmented DNA in the form of ladder. Flow cytometric analysis showed appreciable number of cells in early apoptotic stage. The cells are getting arrested mostly in G0/G1, phase & also in G2/M phase of cell cycle. Anti-oxidant property of MERTL & its fractions has been confirmed by increased Super oxide dismutase activity.

Conclusion: *Ruellia tuberosa* L. (Acanthaceae) leaf extract possess potent apoptogenic activity on HepG2 cell line & the same showed notable anti-oxidant activity on RAW 264.7 cell lysate.

Keywords: *Ruellia tuberosa*, Hepatocellular carcinoma, Cytotoxicity, Apoptosis, Anti-oxidant, Superoxide dismutase.

ANTI-CARCINOGENIC ACTIVITY OF *RUHELLA TUBEROSA* L. (ACANTHACEAE) LEAF EXTRACT ON HEPATOMA CELL LINE & INCREASED SUPEROXIDE DISMUTASE ACTIVITY ON MACROPHAGE CELL LYSATE

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Received: 10 July 2013, Revised and Accepted: 04 Aug 2013
Collection of Test Sample (Ruellia tuberosa Plant)

Ruellia tuberosa (Acanthaceae) leaf has been collected from the nursery of Ramakrishna Mission, Narendrapur. The plant has been identified by Dr. K. Karthikeyan, Scientist C, Central National Herbarium, Botanical Survey of India, Howrah-711109.

Extraction, Fractionation & Preparation of Test sample

The Ruellia tuberosa leaf powder (1 kg) was defatted by using petroleum ether for 3 days and then soaked in about 5000 ml methanol for 7 days at room temperature with occasional shaking. The mixture was then filtered by filter paper and the solvent was evaporated by rotary vacuum evaporator & then lyophilized for 4 hrs to produce methanol free extract. The sticky melanocholic extract was obtained finally. It was kept in a container, sealed with parafilm & stored at 4°C in air tight container and was designated as methanolic (MERTL) extract of Ruellia tuberosa leaf for the experiments. 10gms of the Ruellia tuberosa leaf extract extracted with methanol termed as (MERTL) was suspended in 200 ml water & equivalent amount of pet ether, and then partitioned into water & ethyl acetate to get water fraction & EtAc fraction on the basis of polarity. Stock solution was prepared as 1mg/ml in PBS. PBS (10X) was taken as required for different experimentation. The fractions are termed as Ethyl acetate fraction of Ruellia tuberosa leaf (EF-RTL) & water fraction of Ruellia tuberosa leaf (WF-RTL). 1mg/ml stock solution of EF-RTL & WF-RTL was prepared as in phosphate buffer saline (PBS) from which desired doses were tested for the antioxidant study.

Anti-hepatocellular Carcinoma Study

Cell Viability and Cytotoxicity Study

HepG2 cells (1x10⁴) were seeded in 96-well sterile plates and were treated with different concentrations (5µg, 10µg, 25µg, 50µg, 75µg, 100µg, 150µg, 200µg) of MERTL for 24, 48 and 72 hrs and graphs are plotted against control cells & Sorafenib Tosylate (Standard) treated cells. For both cell viability and MTT study, after treatment for 24, 48, 72 hrs in respective time the media form upper layer has been removed & 1X Trypsin was added to cell viability study to minimize the adherence of the cells, then again 100µl RPMI 1640 media is added. The cell viability studies were done by trypan blue exclusion method using compound microscope. The cytotoxicity studies were performed by MTT assay. For MTT assay the absorbance of the colour solution can be quantified by measuring at a wavelength of 570 nm by microplate manager (Reader type: Model 680 XR Bio-Rad laboratories Inc) [17]. IC₅₀ value for HepG2 cell line is determined for 24 hrs. Another MTT study has been done taking the ½ IC₅₀ & 2 IC₅₀ doses for 24 hrs & the graph has been plotted.

Toxicity study on RAW 264.7 cell line by MTT assay

RAW264.7 cells (1x10⁴) were seeded in 96-well plates and incubated inside a CO₂ incubator for 24 hrs before treatment. The cells were treated with MERTL in doses of 5µg, 10µg, 25µg, 50µg, 75µg, 100µg, 150µg, 200µg for a period of 24 hrs at 37°C in a humidified atmosphere containing 5% CO₂ in air, untreated cells served as control. The cytotoxicity study was performed by MTT assay with slight modifications. 100 µl of the supernatant was removed, 20 µl of MTT (5mg/ml) was added to each well and 100 µl of fresh media was then added to each well because of the adherent capacity of RAW 264.7 cell line. The plate was then allowed to incubate for 3-4 hours at 37°C in 5% CO₂ incubator. 100 µl of DMSO was added to each well to dissolve the formazan crystal formed and the O.D values were recorded at 492 nm.

DNA fragmentation by Agarose Gel Electrophoresis Study

HepG2 cells (1x10⁴) were treated with IC₅₀ dose of MERTL & Sorafenib Tosylate (20.0µg/ml) as standard for HepG2 were taken. After trypanineization cells were harvested and washed twice with PBS. The cells were resuspended in 500µl of lysis buffer (500Mm Tris-HCl, Pbs-8.0, 10Mm EDTA, 0.5%S DS) 10µg/ml of Proteinase K was added and kept for incubation at 37°C. After incubation was done by following the general phenol-chloroform extraction procedure [18] and kept at -20°C overnight. After centrifugation, DNA precipitates were washed with 70% ethanol dried and evaporated at room temperature and dissolved in TE buffer at 4°C overnight. To detect the DNA fragments the isolated DNA samples were electrophoreses overnight at 20V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.

Detection of Apoptosis by Flow Cytometry Analysis

In order to investigate the type of cell death induced by MERTL, flow cytometric analysis was done by performing dot plot assay [19-20]. The Hepatoma cells (1x 10⁵) were treated with individual IC₅₀ dose (18 hrs) of MERTL for 18 hrs. The cells were pelleted down, centrifuged at 2000 rpm for 8 min at 4°C and washed with Annexin -V-FITC binding buffer provided in apoptosis kit (Sigma). After centrifuging at 2000 rpm at 4°C, the cell pellets were dissolved in Annexin-V-FITC and Propidium iodide. After 15 min incubation in dark at room temperature flow cytometric analysis was done. All data were acquired with a Becton-Dickinson FACS LSRII Fortessa 4 laser cytometer. Flow-cytometry reading was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). Live statistics were used to align the X and Y mean values of the Annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with BD FACS Diva software program [21].

Study of Cell Cycle Arrest by Flow Cytometry Analysis

To assay the stage of cell cycle arrest in a flow cytometry [22], HepG2 (1x10⁵) cells were treated with MERTL (IC₅₀-dose) for 18 hrs. Cells were washed with PBS, fixed with cold methanol. They were then resuspended in cold PBS and kept at 4°C for 90 min. Cells were pelleted down, dissolved in cold PBS, treated with RNase for 30 min at 37°C and stained with Propidium iodide and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS (Becton Dickinson FACS Fortessa 4 laser cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using BD FACS Diva software (Becton Dickinson).

Antioxidant Study

Cell lysis and determination of Superoxide dismutase activity

RAW264.7 cells (1x10⁴) were seeded in 96-well plates and incubated for 24 hrs. The cells were washed twice with phosphate-buffered saline (PBS) and then treated separately with MERTL, EF-RTL, WF-RTL & Vitamin-C as standard (5, 10, 25, 50, 75, 100 µg/ml) for 24 hrs. The cells were then incubated with xanthine (100µM) and xanthene oxidase (25µg/ml) for 1 hr to generate oxidative stress by the production of reactive oxygen species (ROS). The RAW264.7 cells were harvested with chilled PBS, subjected to freeze-thaw process twice, sonicated thrice with 10 s bursts and then centrifuged at 10,000 g for 10 min at 4°C. The pellets (cellular debris) were rejected and the supernatants, i.e. the cell lysates were collected and assayed for Superoxide dismutase (SOD) activity [23]. The absorbance values were recorded at 450 nm and the SOD activities of different samples were thereby calculated according to the instructions provided with the kit (Sigma, U.S.A).

Statistical Analysis

Statistical analysis was done by Student’s t-test. P < 0.05 was considered as significant.

The percentage cell inhibition was calculated by the following formula:-

%Cell Inhibition = 100 × (O.D of control-O.D of treated)/O.D of control

O.D= Optical Density.

The percentage cell viability was calculated by the formula:- Viable cells (%) = (Total number of viable cells per ml/Total number of cells per ml) × 100.
RESULTS

Anti-hepatocellular Carcinoma Study

Cell Viability Study and Cytotoxicity Study

MERTL at concentrations of 5µg, 10µg, 25µg, 50µg, 75µg, 100µg, 150µg, 200µg significantly inhibited the growth of Hep-G2 cells compared with that of the control cells after 24, 48 and 72 hrs of treatment in a concentration-dependent manner (Fig. 1). In the MTT assay, there was significant concentration dependent reduction in the O.D values after treating the Hep-G2 cells with same concentrations of MERTL for 24, 48 and 72 hrs compared to the control cells (Fig. 2). These observations provided proof for cytotoxic nature of MERTL. The IC₅₀ calculated after MTT assay is 54.95µg/ml for HepG2 cells. Another graph of MTT assay taking ½ IC₅₀, IC₅₀ & 2 IC₅₀ doses for 24 hrs shows dose dependant decrease in O.D at 570 nm (Fig. 3).

Toxicity study on RAW 264.7 cell lines by MTT assay

Treatment with MERTL for 24 hrs did not show any cytotoxic activity on the RAW264.7 murine macrophage cells, rather in consecutive doses up to 100µg there is an increase in RAW 264.7 O.D. at 492 nm as observed by the MTT assay (Fig. 7).

Fig. 1: Histogram shows effect of MERTL on cell growth inhibition by measuring cell count, observed on HepG2 cell lines after 24, 48 and 72hrs of treatment. The cell count is compared to the untreated control cells and Sorafenib Tosylate treated cells. Reduction in no. of cells is observed in a time and concentration dependant manner. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values p<0.05.

Fig. 2: Histogram shows the effect of MERTL on cell cytotoxicity by MTT assay on HepG2 cell lines after 24, 48 &72 hrs. The O.D. at 570 nm is compared to the untreated cells and Sorafenib Tosylate treated cells. Reduction in the O.D. at 570 nm is observed in a time and concentration dependent manner. Data are mean ± S.E.M. * denotes significant decrease in O.D. at 570 nm from control values p<0.05.
Detection of apoptosis by DNA fragmentation and agarose gel electrophoresis

The gel pattern of the DNA samples isolated from untreated control Hep-G2 cells showed intact DNA bands whereas the gel pattern of the DNA samples isolated from MERTL (IC50 dose for cell line i.e. 54.95µg/ml, 24 hrs) treated Hep-G2 cells showed degraded DNA bands in the form of ladders (Fig. 4). So, the observations confirmed that the treatment with MERTL caused apoptosis in all the hepatoma cells.

Detection of Apoptosis by Flow Cytometric Analysis

In the flow cytometric analysis, double labelling technique, using Annexin V FITC and propidium iodide, was utilized. Lower left (LL) quadrant (Annexin V-/PI-) is regarded as the population of live cells, lower right quadrant (LR) (Annexin V+/PI-) is considered as the cell population at early apoptotic stage, upper right (UR) quadrant (Annexin V+/PI+) represents the cell population at late apoptotic stage and extreme upper right (UR) & upper left (UL) quadrant (Annexin V-/PI+) is considered as necrotic cell population. Flow cytometric data analysis revealed that after 18 h of treatment with IC50 dose of MERTL, 23.9% of HepG2 cells were in LR quadrant (early apoptotic stage) (Fig. 5).

Study of Cell Cycle Arrest by Flow Cytometric Analysis

Flow cytometric analysis showed that after 18 hrs treatment of Hep-G2 with MERTL at IC50 dose, G0/G1 peak was changed. DNA content increased by 1.24 fold in treated cells than that of control (67.5% against 55%) in G0/G1 phase, as well as in G2/M phase (15.3% against 11%) after MERTL treatment. These results indicated that drug treatment arrested the cell cycle of the cells mostly at G0/G1 phase and also in G2/M phase (Fig. 6).
Fig. 4: The gel pattern of DNA samples isolated from untreated control Hep-G2 cells (lane 1), HepG2 cells treated with 2.09μg/ml of standard anti-cancer drug, Sorafenib Tosylate (lane 2) HepG2 cells treated with IC₅₀ dose of MERTL (lane 3). Treatment with the standard anti-cancer drug and MERTL showed distinct DNA ladder formation indicating the process of apoptosis in human hepatoma cell line.

Fig. 5: Detection of apoptosis by Flow cytometric analysis in control (A) and treated (B) of HepG2 cells respectively after 18 hrs treatment at IC₅₀ dose with MERTL. Staining was done with Annexin V FITC and Propidium iodide. Dual parameter dot plot of FITC-fluorescence (x-axis) vs. PI-fluorescence (y-axis) shows logarithmic intensity.
Fig. 6: Flow cytometric analysis of cell cycle phase distribution in control (A) and treated (B) of HepG2 cells respectively after 18 hrs treatment at IC₅₀ dose of MERTL. Histograms represent various contents of DNA with actual number of cells (x-axis denotes fluorescence intensity of PE- Texas red and y-axis denotes count).

Antioxidant Study

Determination of SOD activity

The RAW 264.7 cells after treatment with METRL, EF-RTL, WF-RTL & Vit C as standard (5, 10, 25, 50, 75, 100 μg/ml in PBS) for 24 hrs shows increased level of SOD activity in the cell lysate compared with that of the control untreated RAW 264.7 cells. Comparative study of % SOD Activity of METRL, EF-RTL, WF-RTL & Vitamin C (standard) with respect to control cells plotted (Fig. 8).

Fig. 8: Line diagram shows comparative % increase in Superoxide Dismutase activity on RAW 264.7 cell lysate by using various concentrations of MERTL, EF-RTL, WF-RTL & Vit. C (standard). Data are mean ± S.E.M.
DISCUSSION
Natural products provide an appreciable percentage of new active lead molecules, clinical candidates and drugs despite competition from different methods of drug discovery. The number of natural product derived drugs present in the total drug launches from 1981 to 2002 was recently analysed [24, 25] and it was concluded that natural products are still a significant source of new drugs, especially in the anti-cancer and anti-hypertensive therapeutic areas [24]. Chemoprevention is a novel approach emphasizing on the prevention or delay of carcinogenesis by means of pharmacological, biological, and nutritional intervention and recently, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control [26]. Practical experience has shown that once a plant extract is found to possess anti-inflammatory activity, it is better to test whether it possesses any anti-cancer activity because it is now pretty well established that inflammation and cancer go hand in hand. Since the anti-inflammatory activity of *Ruellia tuberosa* L. extract has already been reported [14]. It is likely that the methanolic extract of *Ruellia tuberosa* L. (Acanthaceae) leaf (MERTL) may also have anti-hepatocellular carcinoma activity. The present investigation confirmed the anti-proliferative, cytotoxic and apoptogenic ability of methanolic extract of *Ruellia tuberosa* L. (Acanthaceae) leaf (MERTL) on HepG2 (Human liver carcinoma cell line).

The anti-proliferative and the cytotoxic activities of MERTL have been supported by the observations in cell viability study and in MTT assay respectively. MERTL inhibited the growth and the metabolic activities of HepG2 cell line in a concentration & time dependent manner. 10μg dose of MERTL on HepG2 cell line are determined. MERTL (same concentrations as used in HepG2 study) also shows positive effects on RAW 264.7 murine macrophage cell line (no significant toxicity). This finding reveals that MERTL preferentially acts on human liver carcinoma cells.

Further evidence in support of the apoptogenic activity of MERTL was obtained from the gel patterns of Agarose gel electrophoresis. MERTL treated cells (HepG2) showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator.

Dual staining with Annexin V FITC and propidium iodide in dot plot assay made it possible to identify live, early apoptotic and late apoptotic cells [27, 28]. Experiments showed increased number of cells in the early and late apoptotic stage (mostly early) after treatment with MERTL implying the fact that apoptosis has been triggered by the treatment with MERTL in HepG2 cells. Cell cycle analysis revealed that treatment with MERTL arrested the HepG2 cell population in both the G0/G1 & G2/M phase of cell cycle.

Oxidants are capable of stimulating cell division, which is a critical factor in mutagenesis. When a cell with a damaged DNA strand divides, cell metabolism and duplication becomes deranged. Thus, a mutation can arise which in turn is an important factor in carcinogenesis. It is believed that antioxidants exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increases in cell division. Both cigarette smoking and chronic inflammation are two of the major causes of cancer and have strong free radical components in their mechanisms of action. Some research has indicated that people who smoke tend to have lower antioxidant levels than non-smokers and are at an increased risk for both cancer and cardiovascular disease. Well over 100 studies have reported that reduction in cancer risk is associated with a diet high in vitamin C[29].

In anti-oxidant study, oxidative stress has been generated by adding xanthene & xanthene oxidase to RAW 26:4.7 macrophage cell line. By inducing this oxidative stress the anti-oxidant property of MERTL, EF-RTL, and WF-RTL have been studied by analyzing the Super Oxide Dismutase activity (SOD kit used). Vit C is used as standard antioxidant. The result of the study goes like this in % SOD activity Vit-C> EF-RTL> MERTL> WF-RTL. Though Vit-C (standard) has the highest % of SOD activity, but still MERTL & its two fractions also notably increased the % SOD activity from control cell lysate, especially EF-RTL.

CONCLUSION
The present investigations confirmed that *Ruellia tuberosa* L. (Acanthaceae) leaf extract possess potent apoptogenic activity against hepatoma cell line & the same along with its various fractions showed notable anti-oxidant activity on RAW 26:4.7 cell lysate.

ACKNOWLEDGEMENT
The authors of this paper are very much thankful to CSIR, Indian Institute of Chemical Biology, Kolkata for providing the funding to perform the research work.

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