Anticancer potential of *Michelia champaca Linn.* bark against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice

Running title: In vivo Anti-cancer property of *M. champaca*bark against EAC cells

Ruksana Yesmin[1], Plabon Kumar Das[1], Hazrat Belal[1], Suraiya Aktar[1], Mst. Ayesha Siddika[1], SahariaYeasmin Asha[1], Farjana Habib[2], Md. Abdur Rakib[1], and Farhadul Islam[1]*

[1]Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6250, Bangladesh.
[2]Institute of Biological Science, University of Rajshahi, Rajshahi-6205, Bangladesh.

***Correspondence to: Farhadul Islam, PhD**
Associate Professor, Department of Biochemistry and Molecular Biology,
University of Rajshahi, Rajshahi-6205, Bangladesh
Email: farhad_bio83@ru.ac.bd
Telephone +880-721-711109
Fax +880-721-750064

Abstract

Background: Adverse side effects of currently available therapies against cancer, leads scientists to find effective compounds from natural sources.

Objective: In the present study, stem-bark of *Mycelia champaca* is subjected to evaluate its anti-proliferative effect against Ehrlich ascites carcinoma (EAC) cells. To date, anti-proliferative effects of *M. champaca* bark extract against EAC cell line has not been reported elsewhere. Therefore, we intended to investigate the anti-proliferative potential of M. champaca bark extract against EAC cells in vivo.

Methods: *In vivo* anticancer activity was evaluated against EAC cells bearing Swiss albino mice by monitoring parameters such as tumor cell proliferation, tumor weight measurement, and survival time etc. The mechanism of EAC killing was examined by observation of cell morphology and analysis the expression of certain cancer related genes. *In vitro* antioxidant potentiality was determined in terms of several common antioxidant assays. In addition, total phenolic and flavonoids contents were measured to insure the presence of phytochemicals.

Results: *M. champaca* bark extract showed strong antioxidant activities which were found to be strongly correlated ($P<0.001$) with phenolics and flavonoids contents. Furthermore, it was found that bark extract decreased tumor cell proliferation (77.46%; $P<0.01$), tumor weight (42.13%; $P<0.001$) and increased life span of tumor bearing mice (71.97%; $P<0.01$) at the dose of 250mg/kg (intraperitoneal; i.p.). *M. champaca* bark also altered the depleted hematological parameters such as red blood cell, white blood cell, hemoglobin (Hb%) towards normal in tumor bearing mice. In addition, upregulation ofp53, Bax and downregulation Bcl-2 followed by treatment indicated *M. champaca* bark could induce apoptosis of EAC cells.

Conclusion: These results indicated that MEMCB possesses significant cytotoxic activities against EAC cells and has a strong *in vitr*o antioxidant capacity. Therefore, bark of M.

champaca could be considered as a potential resource of anti-cancer agents, which might be used to formulate effective anticancer drugs.

Keywords: *Michelia champaca*, Chemotherapy, Natural products, Apoptosis, Antioxidant, EAC cells.

1. Introduction

Cancer is the second reason of death worldwide and imposes a serious problem on the public health [1-2]. Despite the availability of conventional chemotherapeutic approaches of cancer treatments, adverse side effects of these therapies lead researchers to search for new and effective drugs with less or no side effects [3]. Considering these limitations, scientists are in constant search of natural compounds with the capability of healing cancer. It was noted that plant derived natural products such as flavonoids, phenolics, alkaloids have received considerable attention due to their diverse pharmacological potentials, including cytotoxic and cancer chemo preventive properties [4-6]. In recent years, compounds from natural origin have been used as the prime source of medicine in cancer treatment [7]. Evidently, more than 60% of the recently used anticancer drugs are related to herbal origin [7]. Herbal products are worldwide accepted as a source of complementary and alternative medicine in various diseases especially in cancer, since they provide relatively safe and effective therapeutic options against cancer [8-10].

Micheliachampaca Linn, a plant belongs to the family of Magnoliaceae, have many traditional uses [11]. For example, it is used in the treatment of fever, colic, leprosy, eye disorders, inflammation, cough rheumatism, gonorrhea, cephalagia, gout and a number of other diseases, including inflammatory conditions [11]. It is an important source of biologically active compounds such as liridenine , parthenolide , guianolides , terpenoids , steroids , flavonoids , esters of benzoic acid, benzaldehyde, benzyl alcohol, quercetin, gallic acid *etc*., which have high antioxidant , antimicrobial, paraciticidal , antiulcer, anti-inflammatory, cytotoxic and anti-leprotic activities [12-14]. Leaves and flowers from *Michelia champaca* showed robust antihelmintic, anti-inflammatory, antidiabtic, antipyretic, antimicrobial, and antioxidant activities [15-17]. Stem bark aqueous extracts from the plant

78 exhibited a promising diuretic potential [16]. In addition, stem and root barks extracts
79 exhibited a broad spectrum of antibacterial activities [18].
80 To the best of our knowledge, limited data is available about antioxidant activity of *M.*
81 *champaca* bark in the literature. However, there is no report showing the anticancer effect of
82 *M. champaca* bark using animal model. Thus, herein, the present study focused on the
83 anticancer potential of methanol extract of *M. champaca* bark against EAC-cell bearing mice
84 along the possible underlying mechanisms of EAC killing properties. In addition, details
85 antioxidant potential of MEMCB was examined using in vitro assays.

2. Materials and Methods

2.1 Collection and authentication of *Michelia champaca*

Stem bark of *Michelia champaca* was collected from Rajshahi University Campus, Rajshahi, Bangladesh in October 2017 and was identified by an expert taxonomist from the Department of Botany, University of Rajshahi, Rajshahi, Bangladesh. Plant materials were then washed with fresh water and were shade dried for several days. The dried materials were grounded into coarse powder by grinding machine and the materials were stored at room temperature for future use.

2.2 Preparation of methanol extract of *Michelia champaca* bark (MEMCB)

About 200 g of dried powder of *M. champaca* bark was taken in amber colored extraction bottles. Methanol was used as solvent. The materials were soaked in 500ml methanol and kept for 7 days with occasional shaking and stirring. The extract was filtered through a filter paper and concentrated with a rotary evaporator under reduced pressure at 45°C. About 10 g of crude extract was obtained. The crude extract was then stored at 4°C for further analysis.

2.3 Chemicals and reagents

Folin-ciocalteu reagent (FCR), Sodium carbonate, Gallic acid, Catechin, Sodium hydroxide, Ascorbic acid, DPPH (2, 2-diphenyl-1-picryl-hydrazyl), BHT, Ascorbic acid, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA) were purchased from Sigma, MO, USA. Aluminum chloride and Sodium nitrite were purchased from Carl Roth, Karlsruhe, Germany. All chemicals used in this study were of reagent grade.

2.4 Test animals and care

Adult male Swiss albino mice about six to eight weeks old in average (25±5) g body weight were collected from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B) and used throughout the studies. Animals were kept in polypropylene cages containing sterile paddy husk as bedding material under hygienic

conditions. They were maintained under controlled conditions (12:12 hours light-dark), temperature (22±5) °C. The mice were fed with standard mice food-pellets (collected from ICDDR'B) and water was given in ad libitum.

2.5 Cell line and ethical permission

EAC cells were collected from Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as ascites tumor in Swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6cells/mouse. Permission to use mice model in this study was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentation on Animal, Human, Microbes and Living Natural Sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.6 Transplantation of ascetic tumor

Ascitic fluid was drawn out from EAC cell injected Swiss albino mice at the stage of log-phases of tumor cells. The freshly drawn fluids were diluted with normal saline (0.98% NaCl solution) and the tumor cells number was adjusted to approximately 2×10^6 cells/ml by counting with the help of a haemocytometer. The viability of tumor cells was observed by trypan blue dye (0.4%) exclusion assay. Cell sample showing above 90% viability were usedfor transplantation. Tumor suspension of 0.1 ml was injected intra-peritoneally (i. p.) to each mouse.

2.7 Antioxidant activity of MEMCB

2.7.1 Determination of total phenolics

Total phenolics content was determined by using Folin-Ciocalteu reagent following a slightly modified method of Wolfe [19].A 300µl of the extract/standard dissolved in methanol was mixed with 2 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 seconds and allowed to stand for

20 minutes at 25°C for color development. Absorbance was then measured at 760 nm UV-spectrophotometer (Hatch, Colorado, USA). Total phenolics content was expressed in terms of gallic acid equivalent, GAE (standard curve equation: $y = 0.0201x+0.0247$, $R^2 = 0.9923$), mg of GA/g of dry extract.

2.7.2 Determination of total flavonoids

Total flavonoids content was estimated using the method described by Ordonez with some modification [20]. To 0.5 ml of sample/standard, 1.5 ml of methanol, 100µl of 10% aluminum chloride, 100µl of 1M potassium acetate solution and 2.8 ml of distilled water was added. After 1 hour 30 minutes of incubation at room temperature, the absorbance was measured at 420 nm. Extract/standard was evaluated at a final concentration of 1 mg/ml. Total flavonoid contents were expressed in terms of catechin equivalent, CAE (standard curve equation: $y = 0.0043x+0.034$, $R^2 = 0.9919$), mg of CA/g of dry extract.

2.7.3 DPPH free radical scavenging assay

The free radical scavenging activity of MEMCB and standard solution (ascorbic acid) were investigated using DPPH radical scavenging method as reported in the literature [21]. In brief, 1ml of different extract solution in methanol and ascorbic acid were mixed with 3ml of DPPH solution (0.1mM) in methanol. The mixture was allowed to stand for 30 minutes to perform complete reaction. Finally, the absorbance of each extract was measured at 517 nm by using UV spectrophotometer. Free radical scavenging activity of each sample was calculated by using following formula:

DPPH Radical scavenging rate (%) = $[\{A_0 – A\}/ A_0] \times 100$

Where A_0(control)was the absorbance of DPPH blank solution, and A was the final absorbance of the tested sample after 30 minutes of incubation. The concentration, which caused a half-maximal reduced DPPH radical level (IC_{50}) was determined. Percentage (%) of

inhibition was plotted against concentration, and IC_{50} was calculated from the nonlinear regression curve using graph pad prism software version 7.0e.

2.7.4 Lipid peroxidation inhibition assay

Malondialdehyde content was estimated according to the methods described by Raniwith slight modification [22]. A 0.15% w/v Trichloroacetic acid, 0.375%w/v thiobarbituric acid and 0.25N Hydrochloric acid were mixed to form the stock Thio-barbituric acid (TBA)-Tricholoro acetic acid (TCA)-HCl reagent. This solution was mildly heated to assist the dissolution of TBA. Albino mice (20-25g) were used for the study. After decapitation, the brain was removed carefully. The tissue was immediately weighed and homogenized with cold 1.15%w/v KCl to make 10%v/v homogenate. The homogenate (0.5ml) was added to 1 ml of various concentrations of the extract and standard. Then the mixture was incubated for 30 minutes. The peroxidation was terminated by the addition of 2 ml of TBA-TCA-HCl reagent. The solution was heated for 15 minutes in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 minutes. The absorbance of the supernatant was measured at 535 nm. Qurecitin was used as reference standard. Inhibition percentage (%) of various concentrations was calculated by using the formula.

Lipid peroxidation inhibition activity (%) = $[\{A_0 – A\}/ A_0] \times 100$

Where A_0 is the absorbance of the control, and A is the absorbance of the extract/standard. Then percent (%) of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

2.7.5 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extract/standard was determined by the method described in literature with a slight modification [23]. A 0.5 ml of extract/standard at different concentrations was taken in different test tubes. 1 ml of Fe-EDTA solution (0.13% ferrous

ammonium sulphate and 0.26% EDTA), 0.5 ml of 0.018% EDTA solution, 1 ml of 0.85% DMSO solution and 0.5 ml of 22% AA were added into each of the test tubes. The test tubes were capped tightly and warm at 85°C for 15 minutes into the water bath. After incubation, the test tubes were uncapped and 0.5 ml ice cold TCA (17.5%) was added to each of test tubes immediately 0.3 ml of nash reagent (7.5 gm of ammonium acetate, 300 µl glacial acetic acid and 200µl acetyl acetone were mixed and made up to 100ml) was added into all of the test tubes and incubated at RT for 15 minutes. Absorbance was taken at 412 nm wave length by UV-spectrophotometer. Percentage (%) of hydroxyl radical scavenging activity was calculated by the following equation:

Hydroxyl radical scavenging activity (%) = $[\{A_0 – A\}/ A_0] \times 100$

Where A_0 is the absorbance of the control, and A is the absorbance of the extract/standard. Then inhibition percentage was plotted against concentration, and from the graph IC_{50} was calculated.

2.8 Brine shrimp lethality bioassay

Cytotoxicity of MEMCB was screened against *Artemia Salina* in a 1-day *in vivo* assay according to the standard protocol [24]. For the experiment 5 mg of the extract was dissolved in 1ml of distilled water to get a concentration of 5 µg/µl and by serial dilution technique, solutions of varying concentrations such as 10, 25, 50, 100 and 200 µg/ml were obtained. After 24hours of incubation, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50}value was determined using Probit analysis as described in statistical analysis section.

2.9 Determination of Anticancer properties of MEMCB

2.9.1 Determination of median lethal dose (LD_{50})

An acute toxicity study relating to the determination of LD_{50} was performed [25].MEMCB was dissolved in water and were injected intra-peritoneally to seven groups of mice (each

group containing four animals) at different doses such as 200, 400, 800, 1200, 1600, 2000 and 2500 mg/kg. $LD_{50}$was evaluated by recording mortality after 24hours.

2.9.2 Determination of tumor cell growth inhibition (*in vivo*)

Determination of *in vivo* tumor growth inhibition was carried out by the method as described in the previous study [26].To determine the cell growth inhibition of the tested plant extract, four groups of Swiss albino mice (6 in each group) weighing 25±5 gm were used. For therapeutic evaluation 1.36×10^6EAC cells in every mouse were inoculated on day "0". Treatments were started after 24 hours of tumor inoculation and continued for five days. Group one to two received the test compound at the doses of 150mg/kg and 250 mg/kg (i. p.). Group three received bleomycin at the dose of 0.3 mg/kg (i. p.) and group four was used as EAC control. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%NaCl). Viable cells were first identified using trypan blue and then counted by a hemocytometer. Total number of viable cells in every animal of the treated groups was compared with those of control (EAC treated only) group. The cell growth inhibition was calculated by using the formula:

% Cell growth inhibition = $(1- \frac{Tw}{Cw} \times 100)$

Where, Tw = Mean of number of tumor cells of the treated group of mice and Cw = Mean of number of tumor cells of the control group of mice.

2.9.3 Bioassay of EAC cells (transplantation ability of EAC cells)

The effect of MEMCB on transplant ability of EAC cells was carried out by the method as described in the literature [24]. Two groups of mice (n=4) were inoculated with 1.15×10^6 EAC cells. Group 1was treated with MEMCB at the dose of 250 mg/kg for 5 consecutive days and group 2 served as control (without treatment). On day 6, tumor cells from the mice were harvested in cold (0.98% NaCl) saline, pooled, centrifuged and re-inoculated into two

fresh groups of mice (n=4) as before. No further treatment was done on these mice. On day 5, they were sacrificed and viable tumor cells count/mice were estimated.

2.9.4 Determination of average tumor weight and survival time

The effect of MEMCB on transplant ability of EAC cells was carried out by the method as described in the literature [27]. Four groups of Swiss albino mice (6 in each group) were used for each fraction. For therapeutic evaluation 1.36×10^6EAC cells per mouse were inoculated into each group of mice on day '0'. Treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth were monitored by recording daily weight change and host survival was recorded and expressed as mean survival time in days and percent increase of life span was calculated by using the following formulae:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100$$

2.9.5 Monitoring of the hematological profile

Effects of MEMCB on hematological parameters such as WBC, RBC, Hb content, etc. were determined by the standard methods using cell dilution fluids and hemocytometer [28]. For this purpose, four groups (6 in each group) of mice were taken. Group 1; normal mice (without any treatment), group 2; EAC bearing control mice (only EAC treated), group 3and group 4; normal mice treated with MEMCB and EAC bearing mice treated with MEMCB at 250 mg/kg respectively. Blood was collected from mice of each group on day 10, 15and 25by tail puncture in anticoagulant containing tube.

2.9.6 Observation of morphological changes and nuclear damage of EAC cells

Cellular apoptosis induced by the MEMCB was studied by the method described in the literature [29]. Morphological observation of cells in absence and presence of extract

(250µg/ml) for 24 hours was studied using a fluorescence microscope (Olympus iX71, Korea). At first EAC cells were collected from culture disc (RPMI-1064 media) treated with the extract and saline (none treated control disc) and stained with 0.1 µg/ml of Hoechst 33342 at 37^{o}C for 20 minutes. Then the cells were washed with phosphate buffer saline (PBS) and re-suspended in PBS for observation of morphological changes under fluorescence microscopy.

2.9.7 Reverse transcriptase polymerase chain reaction

Total RNA from both treated and non-treated EAC cells was isolated using RNA extraction kit (Favorgen, Taiwan) following manufacturer's guidelines. The cDNA was prepared through reverse transcription PCR (polymerase chain reaction) using 2 µl total RNA, 1µl of each forward and reverse primer, 1µl dNTPs, 2µl $MgCl_2$, RNase inhibitor 0.50µl and 1µl of reverse transcriptase and 4 µl reaction buffer (Promega, Madison-Wiscosin, USA). Expression of apoptosis related gene such as p53, Bax and Bcl-2 was studied by PCR where GAPDH, a housekeeping gene, was used as control. The sequences of primer used in the experiment are shown in Table 1. Each 10 µl of PCR reaction mixture contained 5µl of master mix (Promega, Madison-Wiscosin, USA), 1µl each of forward and reverse primer, template 1 µl and 2µl of nuclease free water. Reaction conditions were initial PCR activation step of 3 minutes at 95°C, followed by 35 cycles of 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 1.00 minutes and a final extension of 72°C for10 minutes. PCR reactions were analyzed on 1 % agarose gel using Tiangen 1kb plus DNA ladder (Tiangen, Beijing, China) as DNA marker.

2.10 Statistical analysis

All analyses were carried out in triplicates and data were expressed as mean ± standard error of mean (SEM). Statistical analyses were performed by one way ANOVA. Duncan's test was used to determine the significance of differences between the groups. Differences at $p< 0.05$

were considered as statistically significant. Graph pad prism software version 7.0e was used to determination of IC_{50} value. Correlation coefficients were determined by using SPSS software. LC_{50} of compounds were determined using probit analysis application software.

3. Results

3.1 MEMCB exhibited strong antioxidant activity

Total phenolics and total flavonoids contents of the extract were expressed in gallic acid and catechin equivalents respectively and their values were determined in quantitative assay as following 176.63±4.60mg/g gallic acid and 100.54±5.70mg/g catechin equivalent. We investigated the antioxidant activity of MEMCB on the basis of DPPH, Lipid peroxidation and Hydroxyl radical scavenging assays. MEMCB exhibited dose dependent scavenging activity against DPPH free radical. The extract showed potent inhibition with IC_{50} value of43.15±2.721 µg/ml which was statistically less significant ($P<0.01$) to the IC_{50} value of AA (26.83±3.363µg/ml). However, MEMCB showed significantly comparable IC_{50}value with that of synthetic antioxidant BHT (38.76±4.547µg/ml) (Table 2). In lipid peroxidation scavenging assay MEMCB (300.8±5.718 µg/ml) showed less potent activity when compare to that of quercetin standard (251.7±4.130 µg/ml). Conversely, MEMCB exhibited significantly ($P<0.05$) higher antioxidant activity than AA (324.61±7.931 µg/ml) (Table 2). In hydroxyl radical scavenging activity assay the IC_{50} value were found 78.64±6.171, 50.23±5.758 and 41.75±2.432 µg/ml respectively by MEMCB, AA and BHT. Total phenolics and flavonoids content were strongly correlated ($P<0.001$) with their antioxidant activity (Figure 1).

3.2 MEMCB showed moderate cytotoxicity against brine shrimp

The brine shrimp lethality bioassay was done to assess the *in vitro* cytotoxic effect of the extract. Medium lethal concentration (LC_{50})of brine shrimp lethality was found to be 119.63 µg/ml and percent of mortality of nauplii were increased with the increase of concentration of MEMCB (Figure 2).

3.3 Anticancer activity of MEMCB

3.3.1 Lethal dose (LD_{50}) value

Lethal dose of MEMCB was found to be 2500 mg/kg body weight for intraperitoneal treatment in male Swiss albino mice. Toxicity was observed at this dose in experimental mice regarding to their body weight and general appearance.

3.3.2 MEMCB significantly inhibited EAC cells proliferations

Maximum cell growth inhibition was found with the treatment of MEMCB at the doses 250mg/kg and 150mg/kg, as evident from 77.46%and 52.11% reduction of tumor cells respectively whereas treatment with bleomycin at dose 0.3mg/kg showed 87.55 %cell growth inhibition (Table 3).

3.3.3MEMCB decreased the transplantation ability EAC cells

Transplantation ability of EAC cells treated with MEMCB was found to be decreased remarkably as 69.7%reduction in EAC cell growth was observed when 5days treated (at the dose 250mg/kg) EAC cells were re-inoculated into fresh mice and sacrificed on day 5.

3.3.4MEMCB dose dependently enhanced the survival time of EAC cell bearing mice

The effect of MEMCB on survival time at different doses has been summarized in Figure 3. It was observed that tumor induced mice treated with the MEMCB at doses 150mg/kg and 250mg/kg resulted in increased life span significantly, which were 41.01% and 71.97% respectively, when compared to that of control mice. Bleomycin increased the life span by 89.24 % as compared to control.

3.3.5 MEMCB decreased average tumor weight significantly

Effect of MEMCB and bleomycin on average tumor weight is shown in the Figure 4. Treatment of MEMCB on mice previously inoculated with EAC cells, resulted in the inhibition of tumor growth. In case of control (EAC bearing) group, the tumor weight was increased 75.34 % on day 20as compared to the normal mice. Mice treated with MEMCB at

doses 150 mg/kg and 250 mg/kg the tumor weight was increased by 42.13% and 33.08% respectively on day 20. Whereas, only 23.83% increased tumor weight was found after bleomycin treatment.

3.3.6 MEMCB restored the depleted hematological parameters towards normal

The hematological parameters like RBC, WBC, Hb*etc*., of both treated and non-treated mice were examined. For normal mice receiving MEMCB at 250mg/kg/day, all the examined parameters were found to be slightly changed from normal values during the treatment period. After 25days of the treatment they were restored to almost normal values. In case of parallel treatment of EAC-bearing mice, these parameters were found to be significantly deteriorated as compared to those of the normal mice due to tumorigenesis. But these deteriorated parameters reversed towards normal values when treated with MEMCB at dose 250 mg/kg. All the experimental data are presented in (Table 4).

3.3.7MEMCB induced morphological changes of EAC cells

Morphological changes of EAC cells were examined by Hoechst 33342 staining after culturing the cells with and without MEMCB for 24 hours. EAC nuclei were round, regular and homogeneously stained with Hoechst 33342 in control group as shown in Figure 5(A). Whereas MEMCB treated EAC cells showed manifest fragmented DNA in nuclei as shown in Figure 5(B). Apoptotic feature including membrane blebbing and nuclear condensation were also observed clearly by fluorescence microscopy. These results indicate that MEMCB could induce apoptosis of EAC cells.

3.3.8 MEMCB induced the expression of apoptotic genes

We hypothesized that cells growth inhibitory effect of MEMCB is mediated by the apoptotic induced cell death. Therefore, we intended to evaluate the expression level of apoptosis related genes. It was found that between the two doses 250mg/kg is more effective. Therefore, it was chosen to evaluate the gene expression levels of EAC cells. As it is well established

that various genes play crucial roles in apoptosis, we examined whether or not MEMCB affected the expression of pro-apoptotic genes like p53, and Bax and the expression of anti-apoptotic gene Bcl-2. GAPDH was used as control and was amplified to test the successful conversion of mRNA into cDNA. Both the control and treated cells showed the identical bands at similar level at 475bp position in the gel that is shown in Figure 6 (A). Another gene namely p53, a tumor suppressor gene, and its over expression in treatment indicates inhibiting the growth of cancer cells. In Figure 6(B), the expression of p53 gene was higher compared to control at the 458bp position on the gel. Consistently, the expression of pro-apoptotic gene Bax was increased at the 479 bp level in MEMCB treated EAC cells compared to control EAC cells. On the other hand, the Bcl-2 is an anti-apoptotic gene and its altered expression plays a critical role in regulating the cell's apoptosis. Interestingly, the expression of Bcl-2 gene was lower compared to control at the 304bp position on the gel which is shown in Figure 6 (C). The results of gene amplification study reported that the treated mice showed upregulation of p53 and pro-apoptotic gene Bax mRNA level and down regulation of Bcl-2 mRNA level (when compared with their respective control) which indicated that the experimental extract work to cause mitochondrial mediated apoptosis of EAC cells [7].

4. Discussion

Many plants are used to treat tumors in traditional system of medicine, but most of the plants have not been scientifically evaluated. The antitumor effect of plant extracts is widely studied due to their low toxicity and less side effects [6]. Accordingly, plant materials or their derivate contribute significantly in developing new therapeutics against various pathophysiological conditions, including cancer.

The present study for the first time examined the potency and efficacy of the *M. champaca* bark extract as a potential resource for anticancer agent by measuring reduction of average tumor weight, tumor cell growth inhibition and increase of life span of the EAC-cell bearing mice as well as measuring hematological parameters after treatment. For tumor bearing mice, body weight found to be increased gradually with time. However, treatment with MEMCB reduced the growth rate significantly ($P<0.001$). MEMCB also inhibited the tumor cell proliferation effectively, as much as 77.46% ($P<0.001$) inhibition was achieved at dose 250 mg/kg which was quite comparable to that of bleomycin (a clinically used anti-cancer drug). These results could indicate direct cytotoxic effect of MEMCB. MEMCB also increased the life span of tumor bearing mice very effectively. An agent is used as a chemo-preventive anticancer agent, which can be judged by assigning its ability to enhance the life span of tumor bearing mice [31]. In this respect, as MEMCB was found to be increased the life span of tumor bearing mice, therefore it could be considered as a source of anticancer agents.

Myelosuppression is one of the major drawbacks found in cancer chemotherapy followed by the progression of anemia due to the reduction of RBC and hemoglobin contents in host [32]. The treatment with MEMCB also reversed all the depleted hematological parameters back towards almost normal level, which indicated that it could have protective effect on haemopoetic system. Thus, the potential anti-cancer agents derived from this plant could generate more tolerable drugs with more protective benefits to the patients with cancer.

To confirm the anti-proliferative effect we opted to study molecular mechanism of the MEMCB induced cell growth inhibition. Not surprisingly, MEMCB found to be induced apoptotic features in EAC cells, which were visualized under fluorescence microscope. Apoptosis is a sequential event that selectively eliminates unnecessary cells or unhealthy cells without affecting surrounding normal cell [33]. Inhibition of apoptosis is the critical early event in tumor development, which allows the cell to proliferate abnormally and leading to the development of cancer [33]. We hypothesized that apoptosis pathway which modulates cells death was responsible for the dose dependent depletion of EAC cells upon MEMCB treatment.

Molecular studies indicate that apoptosis is an ideal process to detect cell death into the body which is controlled by some regulatory proteins like Bcl-2, Bax, p53 *etc*. The downregulation of Bcl-2 and upregulation of p53 mRNA represent mitochondria mediated cell apoptosis.[7] A previous study reported that the various phytochemicals like favonoids, alkaloids, glycosides, etc. are responsible for inducing upregulation of p53 gene that can cause the DNA damage in cancer cell [34]. In this investigation, the expression level of p53 and Bcl-2 mRNA of MEMCB treated mice were evaluated along with control. The upregulation of p53 and Bax mRNA and downregulation of Bcl-2 mRNA were a reliable indication of mitochondrial apoptosis of EAC cells.

The cytotoxicity and anticancer activity of MEMCB is probably due to presence of flavonoids and phenolics [35-37]. Our study demonstrated significant antioxidant activity of MEMCB which was found to be strongly correlated ($P<0.01$) with flavonoids and phenolic contents. Therefore, we strongly believe that the presence of flavonoids, phenolics and also the cytotoxic nature of the extract could be attributed to the anticancer effects of *M. Champaca*. . The current investigation provides a solid evidence that bark extract of this plant can render significant health benefit as far as the cancer is concerned.

5. Conclusion

In the light of above observations it can be concluded that antioxidant and anticancer abilities of the methanolic extract of *Michelia champaca* bark renders its suitability to be considered as a source for the development of anticancer drug. In order to ascertain this as novel potential anticancer drugs, it is necessary to carry out further experiments against other cancer cell lines with higher test animals and with advanced techniques. These findings will definitely give positive support to carry out further researches in a way to formulate novel anticancer drugs.

Availability of Data and Materials

The data presented in the present study was generated from primary research and no data were used from database sources. Therefore, all the data containing this report is original thus no data deposition is applicable.

Conflict of interest

The authors declare no conflict of interest

References

1. Tsai, C.J; Riaz, N.; Gomez, S.L. Big Data in Cancer Research: Real-World Resources for Precision Oncology to Improve Cancer Care Delivery. *Semin Radiat Oncol*.2019, 29, 306-310.

2. Bosacki C, Vallard A, Gras M, Daguenet E, Morisson S, Méry B, Jmour O, Guy JB, Magné N. Complementary and alternative medicines in cancer patients. *Bull Cancer*.2019, 106, 479-491.

3. Suja, K.P.;Jayalekshmy, A.;Arumughan, C. Free radical scavenging behavior of antioxidant compounds of sesame (Sesamumindicum L.) in DPPH center dot system. J Agric Food Chem, 2004, 52, 912-915.

4. Rajkumar, V.;Guha, G.; Kumar, A. Antioxidant and anti-neoplastic activities of Picrorhizakurroa extracts. *Food Chem. Toxicol*,2011, 49, 363-369.

5. Mann, C.D.; Neal, C.P.;Garcea, G.; Manson, M.M.; Dennison, A.R.; Berry, D.P. Phytochemicals as potential chemopreventive and chemotherapeutic agents in hepatocarcinogenesis.*EUR J CANCER PREV*. 2009, 1, 13-25.

6. Doughari, J.H.; Human, I.S.;Bennade, S.;Ndakidemi, P.A. Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. *J. Med. Plants Res,* 2009, 3, 839-848.

7. Ernst, E.;Cassileth, B.R. The prevalence of complementary/alternative medicine in cancer: a systematic review. *Cancer*, 1998, 83, 777–782.

8. Al-Mamun, M.A.;Husna, J.;Khatun, M. Assessment of antioxidant, anticancer and antimicrobial activity of two vegetable species of Amaranthus in Bangladesh.*BMC Complement Altern Med*, 2016, 16, 157.

9. Safarzadeh, E.;Shotorbani, S.S.;Baradaran, B. Herbal medicine as inducers of apoptosis in cancer treatment. *Adv Pharm Bull*, 2014, 4, 421–427.

10. Rajesh, K.;Chitra, P.;Paarakh, M.; and Chidambaranathan, N. Anticancer activity of aerial parts of Aervalanata Linn Juss ex Schult against Dalton's Ascitic Lymphoma.*Eur. J. Integr. Med*,2011, 3, 245–250.

11. Nayak, S.;Behera, S.K.; Mishra, M.K. Ethno-medico-botanical survey of Kalahandi district Orissa. *Indian J Trad Knowledge*, 2004, 3, 72-79.

12. Ahmed, H.; Mishra, A.; Gupta, R.;Saraf, S.A. Determination of quercetin in *Micheliachampaca* L. (CHAMPA) leaves and stems bark by HPTLC.*Int J Pharm Bio Sci*. 2011, 2, 388-397.

13. Gupta, S.;Mehla, K.; Chauhan, D.; Nair, A. Anti-inflammatory activity of leaves of *Micheliachampaca* investigated on acute inflammation induced rats.*LAT AM J PHARM*.2011, 30, 819-822.

14. Mullaicharam, A.R.; Kumar, M.S. Effect of *Micheliachampaca* Linn onpylorous ligated rats. *J. Appl. Pharm*,2011, 1, 60-64.

15. Gupta, R.;Saraf, S.A. Antidiabetic activity of flower buds of *Micheliachampaca* Linn. *Indian J.Pharmacol*, 2008, 40, 256-26.

16. Dama, G.;Bidkar, J.;Deore, S.;Jori, M.; Joshi, P. Helmintholytic Activity of the Methanolic and Aqueous Extracts of Leaves of *Micheliachampaca*.*Res J Pharmacol&Pharmacody*,2011, 3, 25-26.

17. Vimala, N.;Nagarajan, S.;Alam, M.Antiinflammatory and antipyretic activity of *Micheliachampaca*Linn.,(white variety), IxorabrachiataRoxb. And Rhynchosiacana (Willd.)D.C. flower extract.*Indian J Exp Biol*. 1997, 35, 1310-4.

18. Khan, M.R.;Kihara, M.;Omoloso, A.D. Antimicrobial activity of *Micheliachampaca*. *Fitoterapia*, 2002, 22, 744-48.

19. Wolfe, K.; Wu, X.; Liu, R.H. Antioxidant activity of apple peels. *J Agric Food Chem*, 2003, 51, 609–614.

20. Ordonez, A.A.L.; Gomez, J.D.;Vattuone, M.A.; Isla, M.I. Antioxidant activities of Sechiumedule (Jacq.) Swart extracts. *Food Chem*, 2006, 97, 452–458.

21. Cheel, J.;Theoduloz, C.; Rodriguez, J.;Schmeda-Hirschmann, G. Free radical scavengers and antioxidants from Lemongrass (Cymbopogoncitratus (DC.) Stapf.).*J AgricFood Chem*. 2005, 53, 2511-2517.

22. Rani, P.;Unni, K.M.;Karthikeyan, J. Evaluation of antioxidant properties of berries.*Indian J ClinBiochem*, 2004, 19(2), 103-10.

23. Klein, S.M.; Cohen, G.;Cederbaum. Production of formaldehyde during metabolism of dimethyl sulphoxide by hydroxyl radical generating system. *Biochemistry*, 1981, 20, 6002–6012.

24. Khanam, J.A.; Islam, M.F.;Jesmin, M.; Ali, M.M. Antineoplastic activity of acetone semicarbazone (ASC) against Ehirlich ascites carcinoma (EAC) bearing mice. *J NATL SCI FOUND SRI*, 2010, 38, 225-231.

25. Litchfield, J.T.; Wilcoxon, F.A. Simplified method of evaluating dose effect experiments.*J. Pharmacol. Exp. Ther*, 1949, 96, 99-133.

26. Islam, F.; Ghosh, S.;Khanam, J.A. Antiproliferative and hepatoprotective activity of metabolites from Corynebacteriumxerosis against Ehrlich Ascites Carcinoma cells. *Asian Pac J Trop Biomed*, 2014, 284-92.

27. Rasida, P.; Islam, F.;Khanum, J.;Yeasmin, T. Preventive effect of Ethanol Extract of *Alpiniacalcarata*Rosc on Ehrlich's ascitic carcinoma cell induced malignant ascites in mice.*Asian Pac J Trop Biomed*. 2005, 5, 121-125.

28. Kabir, S.R.; Islam, M.F.;Alom, M.J.;Zubair, M.A.;Absar, N. Purification, characterizations of a snake guard seeds lectin with antitumor activity against Ehrlich ascites carcinoma cells *in vivo* in mice. *Protein Pept Let*, 2012, 19, 360-368.

29. Rhma, S.N.;Wahab, N.W.; and Malek, S.N.A. *In Vitro* morphological assessment of apoptosis induced by antiproliferativeconstituents from the rhizomes of *Curcuma zedoaria*.*Evid Based ComplementAlternatMed*,2012, 1-14.

30. Alam, A.K.; Hossain, A.S.; Khan, M.A.;Kabir, S.R.; Reza, M.A.; Rahman, M.M.; Islam, M.S.; Rahman, M.A.; Rashid, M.;Sadik, M.G. The antioxidative fraction of white mulberry induces apoptosis through regulation of p53 and NFκB in EAC cells. *PLoS One*, 2016, 11, e0167536.

31. Clarkson, D.;Burichenal, J.H. Preliminary screening of antineoplastic drugs. *ProgClin Cancer*, 1965, 1, 625-629.

32. Aravind, S.R.;Joseph, M.M.; Varghese, S.;Balaram, P.;Sreelekha, T.T. Antitumor and immunopotentiating activity of polysaccharide PST001 Isolated from the seed kernel of *Tamarindusindica*: an *in vivo* study in mice. *Sci. World J*, 2012, 361-382.

33. Brown, J.M.;Attardi, L.D. The role of apoptosis in cancer development and treatment response.*Nat. Rev.* Cancer, 2005, 5, 231–7.

34. Quideau, S.;Defeux, D.;Douat-Casassus, C.;Pouysegu, L. Plant polyphenols: chemical properties, biological activities, and synthesis.*ChemInt Ed*,2011, 50, 586–621.

35. Zheng, W.; Wang, S.Y.Antioxidant activity and phenolic compounds in selected herbs. *J Agric Food Chem*, 2001, 49, 5165–5170.

36. Fotsis, T.; Pepper, M.S.;Aktas, E.;Breit, S.;Rasku, S.;Adlercreutz, H.Flavonoid,dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res*,1995, 57, 2916–2921.

37. Wagner, H.; Geyer, B.;Yoshinobu, K.;Govind, S.R. Coumestans as the main active principles of liver drugs *Ecliptaalba* and *Wedelicacalendulaceae*. *PlantaMedica*. 1986, 5, 370–372.

38. Islam, M.; Rahi, M.; Jahangir, C. A.; Rahman, M. H.; Jerin, I.; Amin, R.; & Reza, M. A. In Vivo Anticancer Activity of Basellaalba Leaf and Seed Extracts against Ehrlich's Ascites Carcinoma (EAC) Cell Line. *Evid Based Complement Alternat Med*, 2018, 2018.

549 **Figure legends**

550 **Figure 1**: Relationship of (A) total phenolic contents with % radical scavenging activity and
551 (B) total flavonoid contents with % radical scavenging activity. Data expressed as mean ± SD
552 (n=3, **p< 0.01).

553 **Figure 2:** Effects of MEMCB on the mortality of brine shrimp naupli.

554 **Figure 3:** Effects of MEMCB on survival time of tumor bearing mice. Results are shown as
555 mean ± SEM, where significant values are *P<0.01 and **P<0.001 when compared with
556 control (EAC bearing only). Where C, S, MEMCB1 and MEMCB2 indicate EAC bearing
557 control, Bleomycin treated and methanol extract of *Micheliachampaca* bark treated mice at
558 doses of 150 and 250 mg/kg/day body weight respectively.

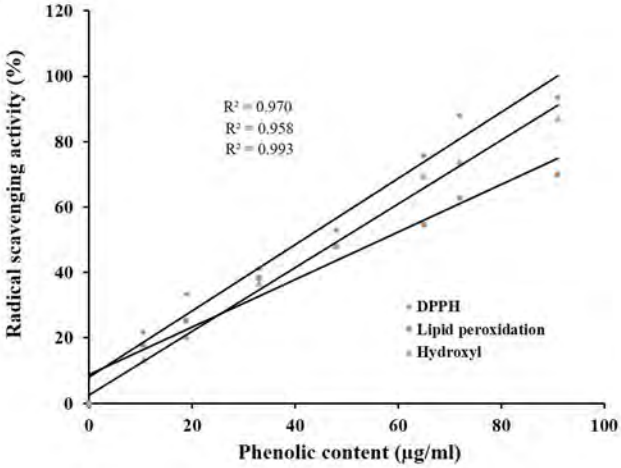
559 **Figure 4:** Effects of MEMCB on tumor weight of EAC bearing mice. Results are shown as
560 mean ± SEM, where significant values are *P<0.01and **P<0.001when compared with
561 control (EAC bearing only).

562 **Figure 5:** (A) Fluorescence microscopic view of control group round shaped, regular nuclei
563 and (B) MEMCB treated EAC cells with apoptotic features including condensed chromatin,
564 apoptotic bodies, membrane blebbing and nuclear fragmentation.

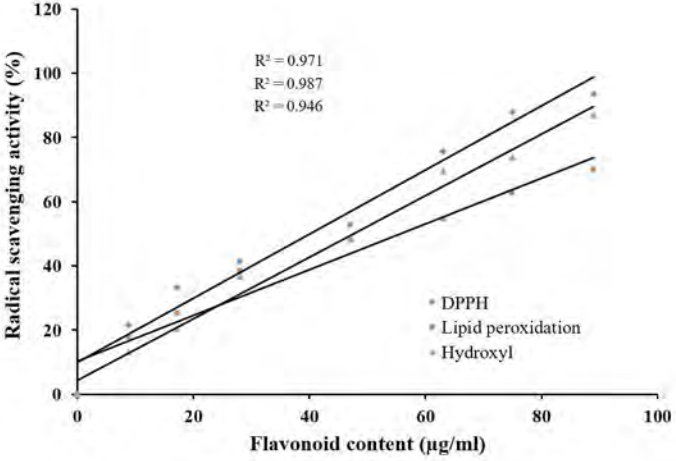
565 **Figure 6:** Amplification of apoptosis-related genes p53, Bax, and Bcl-2 and control gene
566 GAPDH. Total RNA extracted from treated and untreated EAC cells were reverse-
567 transcribed, and PCR reaction was carried out using primers specific p53, Bax, and Bcl-2 and
568 GAPDH. PCR reaction products separated on agarose gel stained with ethidium bromide. M
569 DNA ladder, T RNA from EAC cells of MEMCB treated mice, C RNA from EAC cells of
570 MEMCB untreated mice

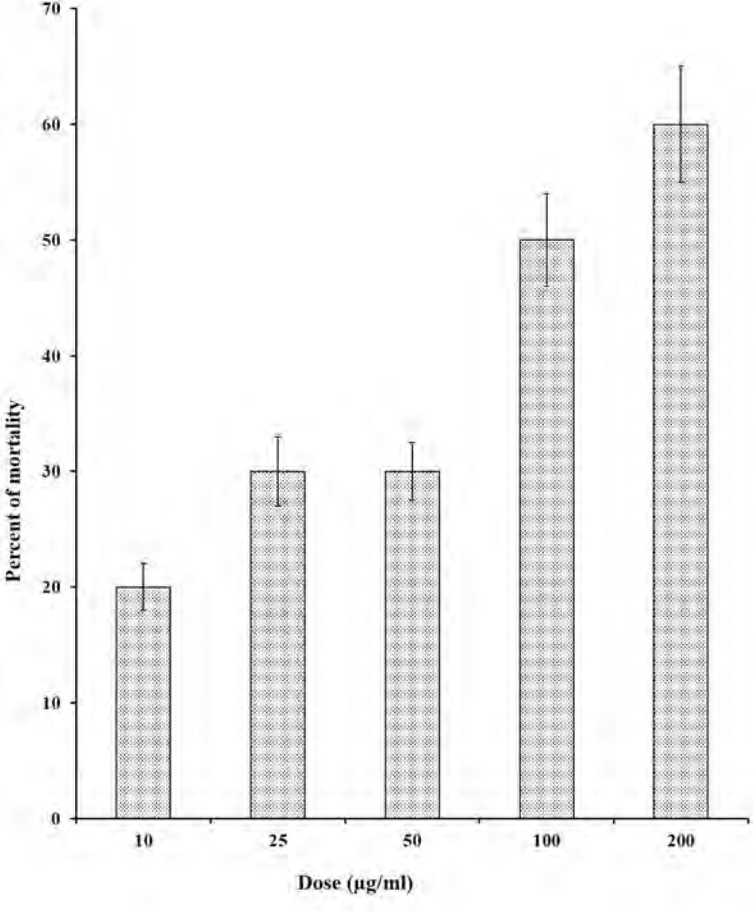
571

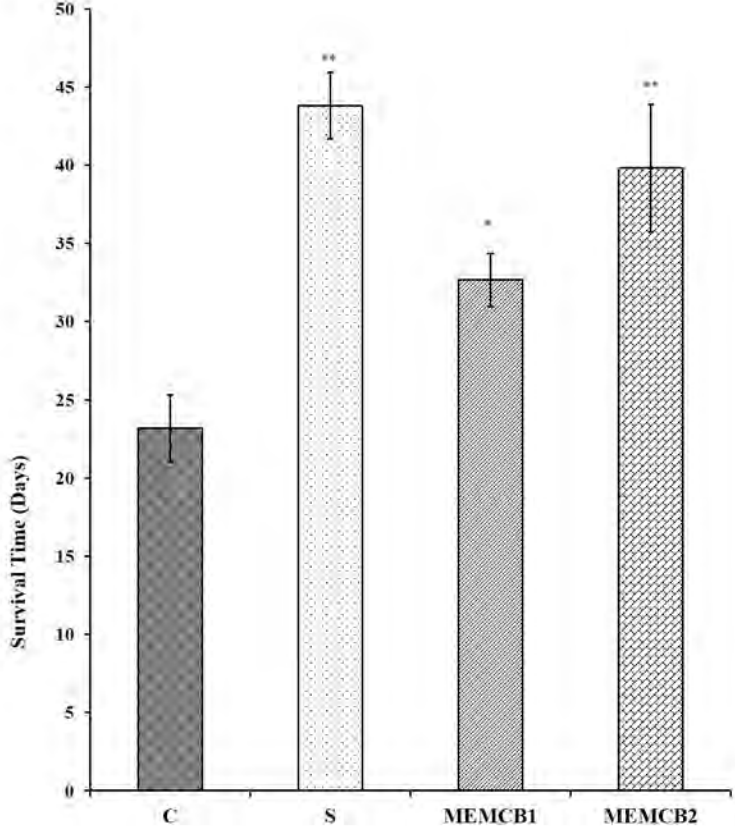
A)

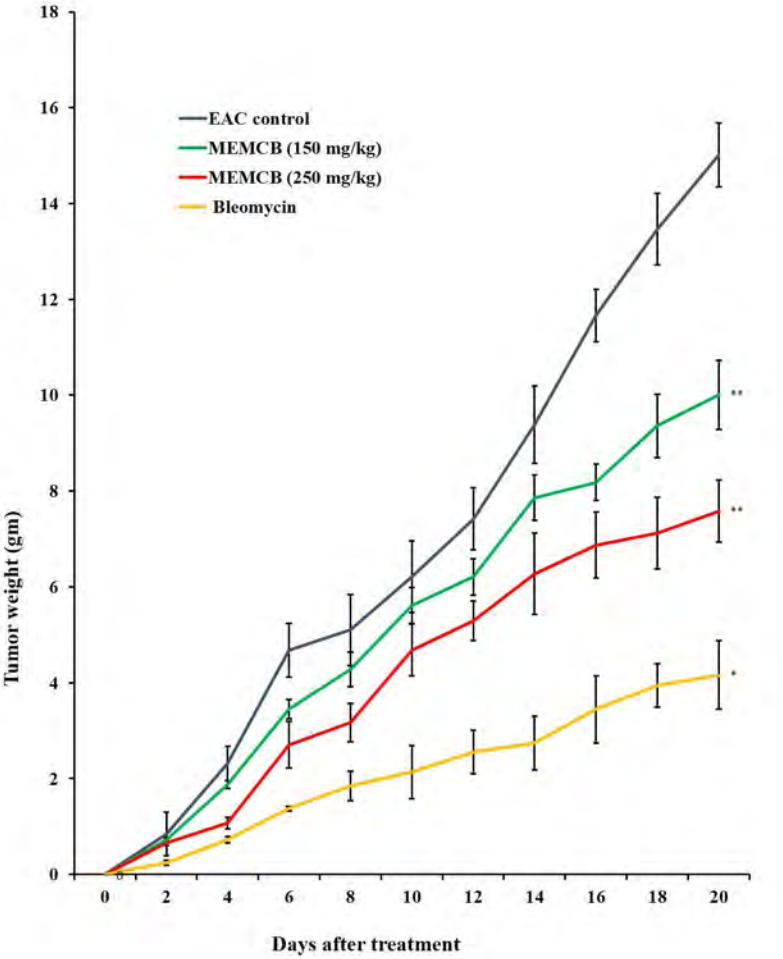

Radical scavenging activity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Phenolic content (µg/ml)
R² = 0.970
R² = 0.958
R² = 0.993
DPPH
Lipid peroxidation
Hydroxyl

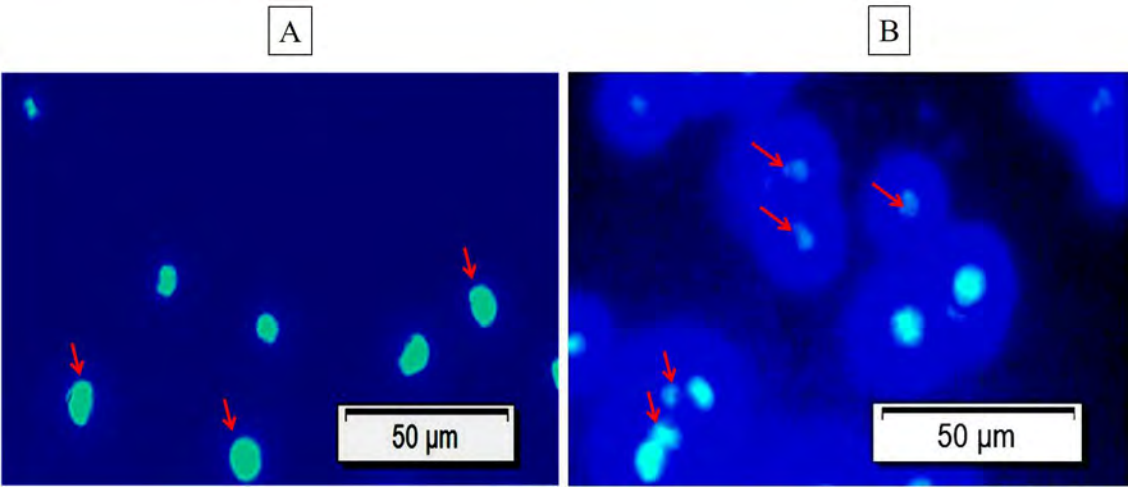
B)


Radical scavenging activity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Flavonoid content (µg/ml)
R² = 0.971
R² = 0.987
R² = 0.946
DPPH
Lipid peroxidation
Hydroxyl


70
60
50
40
30
20
10
0
Percent of mortality
10
25
50
100
200
Dose (µg/ml)


Survival Time (Days)
50
45
40
35
30
25
20
15
10
5
0
**
*
**
C
S
MEMCB1
MEMCB2


EAC control
MEMCB (150 mg/kg)
MEMCB (250 mg/kg)
Bleomycin
Tumor weight (gm)
Days after treatment
0
2
4
6
8
10
12
14
16
18
20
**
**
*


A
B
50 µm
50 µm

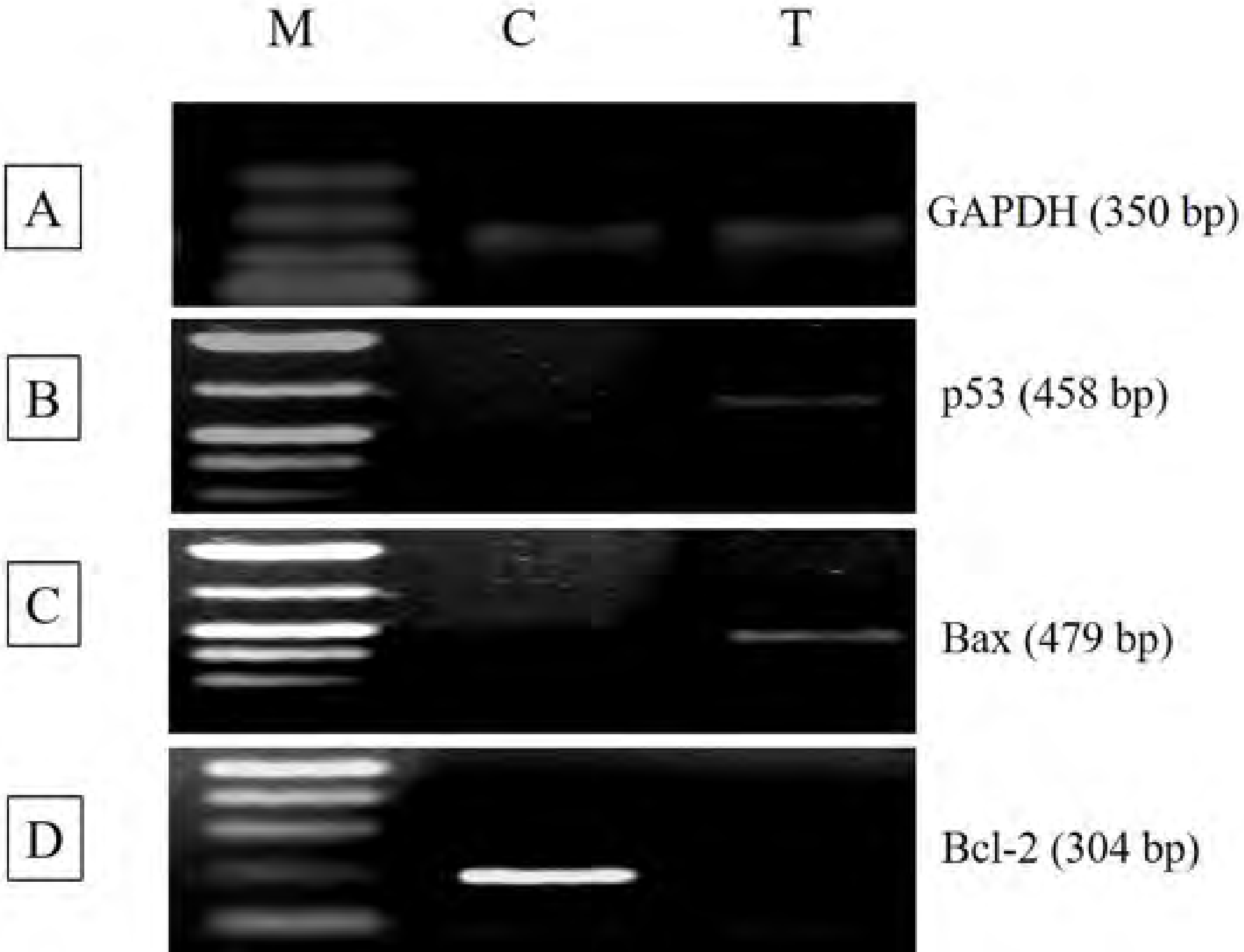

M
C
T
A
GAPDH (350 bp)
B
p53 (458 bp)
C
Bax (479 bp)
D
Bcl-2 (304 bp)

Table 1: The sequence of primers used for PCR amplification.

Gene name	**Primer sequence**	References
GAPDH	Forward: 5′-GTGGAAGGACTCATGACCACAG-3′	[38]
	Reverse: 5′-CTGGTGCTCAGTGTAGCCCAG-3′	
p53	Forward: 5′-CACAAAAACAGGTTAAACCCAG-3′	[30]
	Reverse: 5′-AGCACATAGGAGGCAGAGAC-3′	
Bcl-2	Forward: 5′-GTGGAGGAGCTCTTCAGGGA-3′	[30]
	Reverse: 5′-AGGCACCCAGGGTGATGCAA-3′	
Bax	Forward: 5′-GGCCCACCAGCTCTGAGCAGA-3′	[30]
	Reverse: 5′-GCCACGTGGGCGTCCCAAAGT-3′	

Table 2: Antioxidant activity of MEMCB

Extract/Standards	IC_{50}(µg/ml) value		
	DPPH	Lipid Peroxidation Scavenging Assay	Hydroxyl radical scavenging activity
MEMCB	43.15±2.721[a]	300.8±5.718[b**]	78.64±6.171[a**]
AA	26.83±3.363	324.61±7.931	50.23±5.758
BHT	38.76±4.547	-	41.75±2.432
QUERCETIN	-	251.7±4.130	

Results are shown as mean ± SEM, where significant values are [a]P<0.01 and [b]P<0.05 when MEMCB compared with standard AA and **P<0.001 when MEMCB compared with BHT and Quercetin. Where MEMCB; methanol extract of *Micheliachampaca* bark, AA; Ascorbic acid, BHT; Butylated hydroxytoluene.

Table 3: Effect of MEMCB on EAC cell growth inhibition (*in vivo*).

Name of Exp.	Nature of the drug	Dose in mg/kg/day (i.p)	No of EAC cells in mouse on day 6 after tumour cell inoculation	% of cell growth inhibition
Control (EAC cell bearing mice)	-	-	$(4.26 \pm 0.28) \times 10^{6a}$	-----
Bleomycin	Antibiotic	0.3	$(0.53 \pm .025) \times 10^{6b}$	87.55%
MEMCB	Plant extract	150	$(2.04 \pm 0.07) \times 10^{6ab}$	52.11%
		250	$(0.96 \pm 0.06) \times 10^{6cb}$	77.46%

Number of mice in each case (n=6); the results were shown as mean ± SEM. Where significant values are, $^{a}p<0.001$ comparison to Bleomycin, $^{b}p<0.05$ comparison to Bleomycin and $^{c}p<0.001$ comparison to Control.

Table 3: Effects of MEMCB on hematological parameters in normal and experimental mice

Name of Exp.	Days	RBC Cells/ml	WBC Cells/ml	Hb (gm/dl)
Normal mice	0	$(6.19\pm0.14)\times10^{9}$	$(9.26\pm0.26)\times10^{6}$	14.75±0.19
Normal mice + MEMCB	10	$(5.23\pm0.23)\times10^{9a}$	$(8.54\pm0.38)\times10^{6b}$	11.56 ± 0.86^{a}
	15	$(5.72\pm0.53)\times10^{9b}$	$(7.08\pm0.34)\times10^{6a}$	12.64 ± 0.66^{a}
	25	$(5.85\pm0.34)\times10^{9}$	$(6.89\pm0.97)\times10^{6a}$	13.39 ± 0.39^{b}
EAC control	10	$(3.92\pm0.15)\times10^{9}$	$(13.33\pm1.52)\times10^{6}$	12.00±1.31
	15	$(2.98\pm0.26)\times10^{9}$	$(15.20\pm1.22)\times10^{6}$	9.75±1.20
	25	$(2.30\pm0.33)\times10^{9**}$	$(18.73\pm2.30)\times10^{6**}$	$7.70\pm0.40^{*}$
EAC+ MEMCB	10	$(4.01\pm0.33)\times10^{9}$	$(11.92\pm0.52)\times10^{6}$	8.16±0.60
	15	$(4.56\pm0.28)\times10^{9}$	$(10.13\pm0.21)\times10^{6}$	9.51±0.24
	25	$(4.90\pm0.16)\times10^{9}$	$(9.45\pm1.61)\times10^{6}$	10.15±0.25