The Journal of Basic and Applied Zoology

RESEARCH

Open Access

Mussaenda macrophylla Wall. exhibit anticancer activity against Dalton's lymphoma ascites (DLA) bearing mice via alterations of redox-homeostasis and apoptotic genes expression

Marina Lalremruati[1], C. Lalmuansangi[1], Mary Zosangzuali[1], Lalchhandami Tochhawng[2], Amit Kumar Trivedi[1], Nachimuthu Senthil Kumar[3] and Zothan Siama[1*]

Abstract

Background: *Mussaenda macrophylla* is a shrub widely used in Mizo traditional practice for treatment of cancer, fever, cough, ulcer and dysentery. We have previously shown the antioxidant nature of the plant. In this study, we explore the anticancer activity of the aqueous extract of *M. macrophylla* (MMAE) using Dalton's lymphoma ascites (DLA) bearing mice as our model.

Results: MMAE significantly inhibited the tumor growth and increased the survival time of the tumor bearing DLA mice. MMAE significantly increased the glutathione (GSH) levels; and glutathione-s-transferase (GST) and superoxide dismutase (SOD) activities. Consistently, MMAE decreased lipid peroxidation levels in DLA mice. Reduced RBC and hemoglobin levels were significantly reversed by MMAE treatment. MMAE also lowers the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine (CRE) levels that were otherwise elevated in the DLA control animals. Induction of DNA damage, up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes in DLA bearing mice following MMAE treatment provide an insight into apoptosis based anticancer activities of *M. macrophylla*.

Conclusion: Our findings demonstrate the role of the aqueous extract of *M. macrophylla* as a potential anticancer agent possibly targeting the apoptotic pathway.

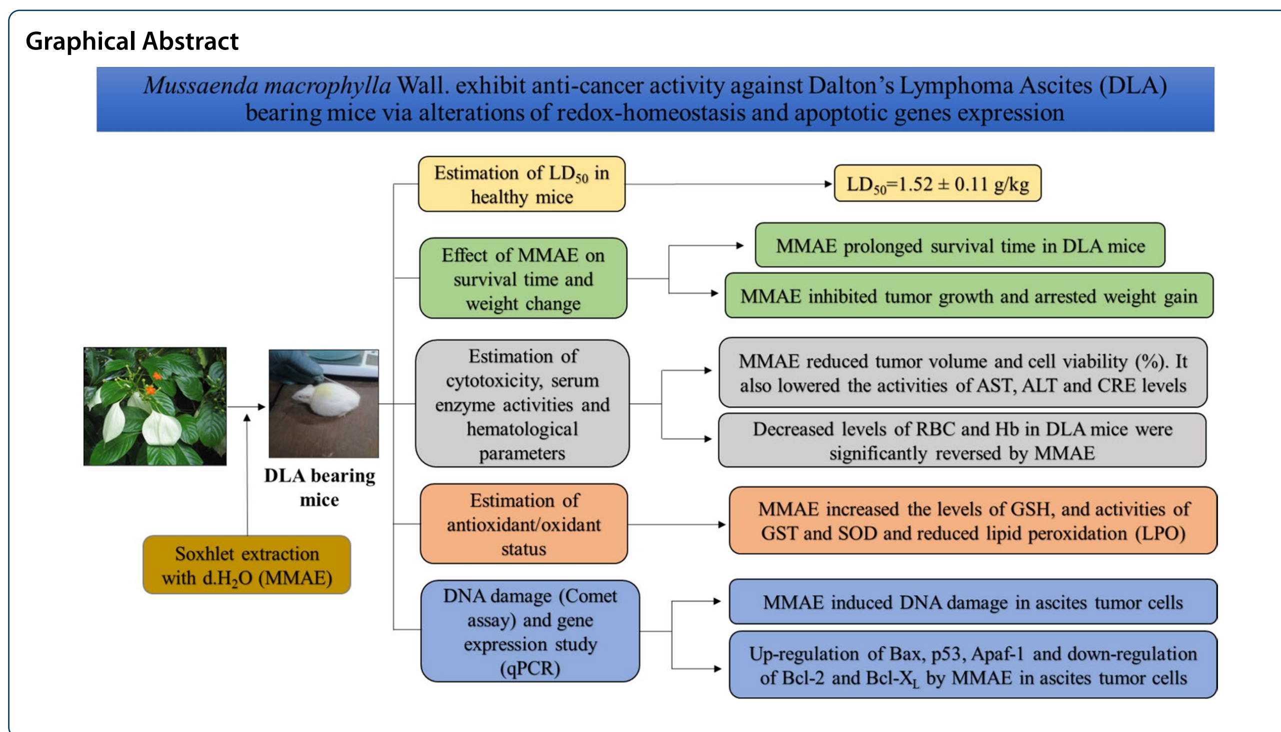
Keywords: *Mussaenda macrophylla*, Dalton's lymphoma ascites (DLA), Anticancer, DNA damage, Antioxidants, Apoptotic genes

*Correspondence: zothans@gmail.com
[1] Department of Zoology, Mizoram University, Aizawl 796 004, India
Full list of author information is available at the end of the article

Springer Open

© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

Graphical Abstract



Background

Cancer is a heterogeneous disease with multiple genotoxic and oncogenic aberrations characterized by uncontrolled proliferation, invasion and metastasis of cells (Hanahan & Weinberg, 2000). There are more than 36 major types of cancer with a striking fatality rate of 9.6 million people per year. It is a major health burden worldwide and it is expected to be the leading cause of death throughout the world in the 21st century (Bray et al., 2018; Rawla & Barsouk, 2019). Non-Hodgkin lymphoma is the 13th most frequently diagnosed cancer in the world and 11th leading cause of cancer mortality worldwide, accounting for 248,724 deaths (2.6% of the total) each year (Bray et al., 2018). Dalton's lymphoma, a murine non-Hodgkin's transplantable T-cell lymphoma, that originated at the National Cancer Institute (NCI), USA in 1947 in the thymus gland of a DBA/2 mouse. The line is then maintained by serial transplantation from mouse to mouse intraperitoneally (Chakrabarti et al., 1984). Dalton's lymphoma has served as a convenient model for studying various parameters of cancer progression, signalling mechanisms, and ultimately for screening of drugs for effective treatment (Das & Vinayak, 2014). In addition, Swiss albino mice serves as an excellent model for anticancer drug screening due to their easy accessibility and similarity with humans in terms of their genomic, anatomy and immunological system (Bernardi et al., 2002).

Of the hundreds of chemicals that have been and are being evaluated for their anticancer activities, natural compounds derived from medicinal plants offer a potential resource for development of new anticancer agent(s) due to their safety, efficacy and lesser side effects when compared with synthetic drugs (Thillaivanan & Samraj, 2014). Therapeutic drugs derived from different medicinal plants have been reported to play a crucial role as anticancer agents in various experimental models of cancer. In fact, about 60% of the currently available anticancer drugs are derived from plant sources (Kamal et al., 2014). Recent studies have demonstrated the anticancer properties of various plants such as *Emilia sonchifolia* (Shylesh & Padikkala, 2000), *Solanum pseudocapsicum* (Badami et al., 2003), *Astraeus hygrometricus* (Mallick et al., 2010), and *Sesbania grandiflora* (Laladhas et al., 2010) in Dalton's lymphoma ascites bearing mice.

Mussaenda macrophylla, locally known as Vakep, is a flowering shrub that belongs to the Rubiaceae family. It is endemic to southeast Asia and is known to occur in China, Myanmar and India (Manandhar, 2002). Traditionally, different health problems such as sour mouth, sour throat, oral infections, fever, cough, dysentery, diarrhea, indigestion, chronic ulcer, cancers and snake bites have been treated using various parts of this plant (Kim et al., 1999; Rosangkima & Jagetia, 2015). Our preliminary study revealed the presence of significant

number of phytochemicals including phenols, flavonoids, alkaloids, cardiac glycosides, saponins, steroids, tannins and terpenoids in *M. macrophylla* (Lalremruati et al., 2019). *M. macrophylla* have also been reported to show multi-pharmaceutical activities including anti-coagulant, anti-inflammatory and hepatoprotective activities (Dinda et al., 2008), anti-microbial (Chowdhury et al., 2013), antioxidant (Lalremruati et al., 2019), thrombolytic (Islam et al., 2013) and anti-diabetic activities (Bhandari et al., 2020). Given the present state of the scientific evidence on various pharmaceutical applications of *M. macrophylla*, high priority research is required to objectively assess the potential anticancer activity of *M. macrophylla*. Therefore, the present study is carried out to investigate the anticancer activity of *M. macrophylla* aqueous extract in Dalton's Lymphoma Ascites (DLA) bearing Swiss albino mice.

Methods

Collection of plant and preparation of extracts

Mussaenda macrophylla leaves were collected from the community forest of Kolasib District, Mizoram, India. It was identified and authenticated by the Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl (voucher sample: MZU/HAMP/2018/026). The leaves were dried in shade at room temperature and powdered. The pulverized leaves were then first defatted using petroleum ether in a Soxhlet apparatus at 40 °C for 30 cycles and dried at 40 °C overnight to remove all the traces of petroleum ether. The powdered leaves were further extracted with chloroform, methanol and distilled water according to their increasing polarity using Soxhlet apparatus for a minimum of 40 cycles each. The liquid extracts were filtered and concentrated using a rotary evaporator (Buchi, Germany) under reduced pressure at 40 °C for about 5 h and finally freeze dried. The aqueous extract of *M. macrophylla* (MMAE), the most effective extract in the preliminary screening based on survival test, was subsequently used for further experiments.

Animals and tumor model

Swiss albino mice of both sexes (10–12 weeks old) weighing 25–30 g were selected from an inbred colony maintained under controlled conditions of temperature (23 ± 2 °C) and light (12 h of light and dark, respectively) at the Animal Care Facility, Department of Zoology, Mizoram University. The animals had free access to food and water. The animal care and handling were performed according to the guidelines of World Health Organization, Geneva, Switzerland. Dalton's lymphoma ascites (DLA) tumor has been maintained by serial intraperitoneal (i.p) transplantation of about 1×10^6 viable tumor cells in 10–12-weeks-old mice under aseptic condition.

Preparation of drug and mode of administration

The aqueous extract of *M. macrophylla* (MMAE) and doxorubicin (a standard drug) were dissolved in distilled water. Animal from each group received different dose of treatments according to body weight intraperitoneally (i.p).

Acute toxicity study

The acute toxicity study (Prieur et al., 1973) of MMAE was performed in Swiss albino mice as per the OECD guidelines 420–425. Animals of both sexes were randomly divided into four groups of ten animals each ($n = 10$), and treated with aliquot doses of MMAE intraperitoneally (1.2, 1.4, 1.6 and 1.8 g/kg b.wt) and monitored for mortality and toxic symptoms up to 14 days post-treatment. The LD_{50} value of the MMAE was calculated using probit analysis (Miller & Tainter, 1944). The probit values were then plotted against log-doses and the dose corresponding to probit 5, i.e., 50%, was calculated. The standard error of mean (SEM) of LD_{50} was calculated using the formula:

$$\text{Approx. SEM of } LD_{50} = (\text{Log LD84} - \text{Log LD16})/\sqrt{2N}$$

where N is number of animals in each group.

Experimental design

For the assessment of survival time and weight change, the animals were randomly distributed to five equal groups ($n = 6$). All mice were transplanted (i.p) with 1×10^6 cells in 0.25 mL of PBS on day '0'. Group I was treated as the control group, which received 0.25 mL of distilled water. Group II–IV were treated (i.p) with MMAE at the dose of 50, 100 and 150 mg/kg b.wt, respectively. Group V received doxorubicin (DOX) at the dose of 0.5 mg/kg b.wt as a standard drug. After 72 h of tumor transplantation, treatment was given to each group for 7 consecutive days.

Fresh experimental groups were formed as described above for the estimation of antioxidant status, lipid peroxidation, cytotoxicity, activities of serum enzymes, and hematological parameters. The expression of both pro-apoptotic and anti-apoptotic genes, and the level of DNA damage were also compared between the control group and MMAE (100 mg/kg b.wt)-treated group.

Estimation of survival time and weight change

The deaths, if any, of the tumor bearing mice were recorded daily and survival time was determined for all the experimental groups. The tumor response following

MMAE treatment was evaluated by calculating median survival time (MST) and average survival time (AST). The % increase in median life span (IMLS) and % increase in average life span (IALS) were also calculated using the standard formulae (Gupta et al., 2000).

T/C value, which is the MST of the treated group of animals (T) divided by that of control group (C), was also computed. The T/C ratio is given as a percentage and a compound is considered active if it shows T/C value $\geq$ 120% (National Cancer Institute Protocols). Animals from all the experimental groups were also monitored for alteration in body weight every 3 days up to 18 days post-tumor transplantation.

Processing of liver and tumor cell for biochemical assays

After 7 consecutive days of treatment with MMAE or DOX, each animal was euthanized through an overdose of ketamine followed by immediate excision of liver (Al-Batran et al., 2013) that were placed in pre-coded blinded petri -dish. 5% (w/v) tissue homogenate was prepared with ice cold buffer (5 mM EDTA, 0.15 M NaCl, pH 7.4) in a glass homogenizer followed by centrifugation for 30 min at 13,000 rpm at 4 °C. Then, the supernatants were immediately used for biochemical assays. The tumor cells were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. The tumor cells were pelleted, sonicated (PCi Analytics) and homogenized with ice cold buffer to produce 5% (w/v) homogenate. Cell homogenates were then centrifuged for 30 min at 10,000 rpm at 4 °C and the supernatants obtained were immediately used for the estimation of antioxidant status and lipid peroxidation.

Antioxidant assays

Protein contents were measured by the standard method (Lowry et al., 1951) using BSA as the standard. Glutathione (GSH) levels were measured by its reaction with DTNB in Ellman's reaction (Moron et al., 1979) to give a compound that absorbs light at 412 nm. GSH concentration was then calculated using the standard graph and represented in μmol/mg protein. Glutathione-s-transferase (GST) was measured using the standard method (Beutler, 1984). GST activity was calculated as follows: GST activity = (OD of test–OD of blank/9.6 × vol. of test sample) × 1000; where 9.6 is the molar extinction coefficient for GST. Superoxide dismutase (SOD) activity was determined by NBT reduction method (Fried, 1975). The enzyme activity was expressed in unit (1 unit = 50% inhibition of NBT reduction/mg protein).

Lipid peroxidation (LPO) assay

The level of lipid peroxidation (LPO) was assessed by the method of Beuege and Aust (1978). Malondialdehyde (MDA) is one of the toxic products formed from the oxidation of fatty acids such as phospholipids and has served as a convenient index for the assessment of the levels of lipid peroxidation reaction. MDA derived from LPO reacts with TBA to give a red fluorescent adduct absorbing at 535 nm. The concentration of MDA in the sample was then calculated using the extinction coefficient of $1.56 \times 10^6\ M^{-1}\ cm^{-1}$.

Effect of MMAE on cell toxicity and hematological parameters

The cytotoxic effect of MMAE was examined by studying tumor cell volume and the percentage of non-viable cell count using trypan blue dye exclusion test, in a hemocytometer. Red blood cell count (RBC), white blood cell count (WBC) and hemoglobin content were measured using standard protocols from the blood obtained by heart puncturing (D'Amour et al., 1965).

Measurement of serum ALT, AST and CRE

Activities of ALT (EC 2.6.1.2) and AST (EC 2.6.1.1), and the level of CRE were determined in serum using kits (Coral Clinical Systems, Uttarakhand, India).

Assessment of DNA damage using Comet assay

The alkaline single cell gel electrophoresis (Comet assay) was performed according to the standard method (Singh et al., 1988) with minor modifications. The tumor cells from both control and treatment groups were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. Briefly, 2×10^4 tumor cells were suspended in 75 μL of 0.5% low-melting point agarose prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose. Then, the slides were immersed in freshly prepared lysis buffer (10 mM Trizma base, 100 mM Na_2EDTA, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO, pH 10) for 2 h. After lysis, slides were kept on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH13) for 20 min that will allow unwinding of DNA. Electrophoresis was then carried out at 24 V and 300 mA for 30 min. Then, neutralization was done by washing with buffer (0.4 M Tris–HCl, pH 7.5) for 5 min. After neutralization, slides were washed with distilled water and then stained with ethidium bromide (EtBr) solution (2 μg/mL) for 5 min. Two slides were prepared for each animal and 100 randomly selected cells from each slide were examined using fluorescence microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615) with a magnification of 200×. Image capture and analysis were performed with Image J software.

qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression

The tumor cells from both control and treatment groups were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. The cells were pelleted and total RNA was extracted using Tri reagent (BR Biochem, Life Science Pvt. Ltd, R1022). Extracted RNA was quantified using Nanodrop Spectrophotometer (Nanodrop One C, Thermo Fisher Scientific) and RQ1 DNase kit (Promega, M198A, Madison, WI, USA) was used to remove the genomic contamination. cDNA was synthesized from 2 μg of total RNA using first-strand cDNA synthesis kit (Thermoscientific, K1621; Lithuania, Europe). Gene-specific primers (Table 1) were designed using Primer 3, Boston, MA, USA, and primers were obtained from Imperial Life Sciences Pvt. Ltd., Haryana, India. qPCR was performed using Quant-Studio 5 (ThermoFisher Scientific, Foster City, CA, USA). PCR reaction volume of 7 μL for each gene comprised of 1 μL each of cDNA, gene-specific forward and reverse primers, 3 μL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25742, Lithuania, Europe) and 1 μL of nuclease-free water (ThermoFisher Scientific, A19938, Bangalore, India). The cycling condition of qPCR was 1 cycle at 95 °C (20 s), 35 cycles at 95 °C (01 s), 60 °C (20 s) and 95 °C (01 s), additional melt curve plot step included 1 cycle of 60 °C (20 s) and 1 cycle of 95 °C (01 s) (Renthlei et al., 2018). Afterward, melting curves were generated to confirm a single uniform peak. GAPDH gene was used as a reference gene for determining the relative expression levels of specific target genes. Each sample was run in duplicate along with non-template and negative RT controls. The relative expression of genes was determined using ΔΔCt method (Livak & Schmittgen, 2001).

Statistical analysis

All data were expressed as mean ± standard error of mean of pooled results obtained from three independent experiments. One-way ANOVA followed by Tukey's test was performed to test significant variations on survival time, change in body weight, antioxidants status, lipid peroxidation, tumor volume, cytotoxicity, hematological parameters, and activities of serum enzymes. Significance variation in relative gene expression and DNA damage were calculated using Student's *t* test between control and treatment groups. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 6.0 were used for statistical and graphical analyses. A *p* value of less than 0.05 was considered statistically significant.

Results

Acute toxicity test

In order to assess the acute toxicity of MMAE, four doses were chosen for the determination of LD_{50} starting from 0% mortality to 100% mortality. Thus, four doses were given intraperitoneally to 4 groups of 10 mice each. The dose corresponding to probit 5, i.e., 50%, was found to be 0.182 (log LD_{50}) with LD_{50} of 1.52 ± 0.117 g/kg b.wt.

Effects of MMAE on survival time and weight change

To understand the anticancer activity of MMAE on DLA bearing mice, the tumor bearing mice were treated with different doses of MMAE (Table 2). All untreated tumor bearing mice died within 19 days with MST and AST of 15.0 ± 0.57 days and 15.2 ± 0.46 days, respectively. Interestingly, treatment of DLA bearing mice with MMAE resulted in a dose-dependent increase in MST, AST, % IMLS and % IALS up to 100 mg/kg. However, reduction in life span of DLA mice was observed with the treatment of 150 mg/kg MMAE which may indicate that prolonged treatment with higher dose could have other effects. The results of the in vivo anticancer activity were also expressed as ratio of the median survival days of the treatment and control group (*T*/*C*) of DLA bearing mice. Treatment of DLA mice with MMAE at 50 and 100 mg/kg b.wt showed *T*/*C* values of 140.0% and 196.6%, respectively, indicating the effectiveness of MMAE as a potential anticancer agent. Treatment of DLA mice with MMAE at 150 mg/kg b.wt, however, reduced the *T*/*C* value, which is consistent with the MST, AST, % IMLS and % IALS

Table 1 Primer sequences and their product size used in qRT-PCR analyses of Dalton's lymphoma ascites (DLA) bearing mice treated with aqueous extract of *M. macrophylla* (100 mg/kg b.wt)

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)
P53	GTATTTCACCCTCAAGATCCGC	AGACTCCTCTGTAGCATGGG	100
Bax	CACCTGAGCTGACCTTGGAG	CAATCATCCTCTGCAGCTCCA	117
Apaf-1	ATGGAATTGGCAGACAGGGG	TTCCACACCTTCACCGTTCC	126
Bcl-2	GACTTCTCTCGTCGCTACCG	CTCTCCACACACATGACCCC	176
Bcl-X_L	AGGGGCTTAGCTGCTGAAAG	GTGGACAAGGATCTTGGGGG	81
GAPDH	AAAGGGTCATCATCTCCGCC	AGTGATGGCATGGACTGTGG	197

Table 2 Effect of MMAE and DOX treatment on DLA bearing mice on the tumor response assessment based on MST, AST, % IMLS, % IALS and *T/C* ratio

Dose (mg/kg b.wt)	MST	AST	% IMLS	% IALS	% *T/C* ratio
DLA Control	15.0 ± 1.57	15.2 ± 1.46	–	–	–
DLA + $MMAE_{50}$	21.0 ± 1.50**	21.3 ± 1.34**	40.0 ± 1.57[b]	40.7 ± 2.35[b]	140.0 ± 2.57*
DLA + $MMAE_{100}$	29.5 ± 1.28***	27.3 ± 1.91***	96.6 ± 1.88[c]	80.1 ± 1.96[c]	196.6 ± 3.33*
DLA + $MMAE_{150}$	24.0 ± 1.52***	22.5 ± 1.04**	60.0 ± 2.00[a]	46.4 ± 2.56[b]	160.0 ± 2.80*
DLA + $DOX_{0.5}$	22.5 ± 1.50**	24.2 ± 1.56***	63.3 ± 2.35[a]	59.4 ± 1.86[a]	163.3 ± 2.48*

DLA Control: DLA bearing mice without treatment; DLA + $MMAE_{50}$, DLA + $MMAE_{100}$, DLA + $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100 and 150 mg/kg, respectively. $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg). The results were expressed as percent (%) ± SEM, $n = 10$

**$p \leq 0.01$; ***$p \leq 0.001$ between the control and treatment groups

Different letters indicates significant variation between different treatment groups

* *T/C* ratio > 120

results (Table 2). DOX treatment also increased MST to 22.5 ± 0.50 days and AST to 24.2 ± 0.56 days, respectively. Consequently, DOX treatment caused an increase in % IMLS and % IALS. The DOX treatment also caused a significant increase in life span of DLA mice with *T*/*C* ratio of 163.3% (Table 2). Taking together, MMAE at the dose up to 100 mg/kg could increase the survival time of the animals showing the potential of the plant extract for future therapeutic use. Summary of the effects of MMAE and DOX on the survival of DLA bearing mice is given in Fig. 1A.

The weights of the animals in all the groups were recorded every 3rd day starting from the day of tumor transplant in order to determine the change in body weight. Due to the proliferation of the tumor cells, the untreated DLA bearing mice exhibited continuous weight gain until their survival. The treatment of DLA mice with 100 mg/kg MMAE after 72 h of tumor transplantation arrested the weight gain indicating inhibition of tumor cell proliferation and growth (Fig. 1B).

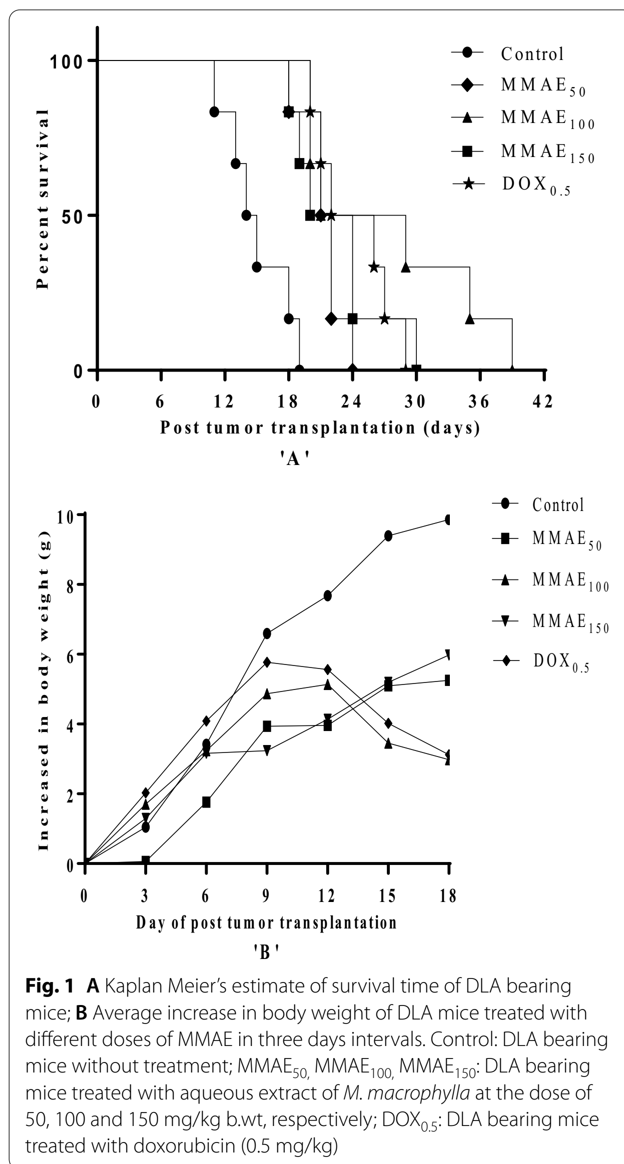


Fig. 1 **A** Kaplan Meier's estimate of survival time of DLA bearing mice; **B** Average increase in body weight of DLA mice treated with different doses of MMAE in three days intervals. Control: DLA bearing mice without treatment; $MMAE_{50}$, $MMAE_{100}$, $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100 and 150 mg/kg b.wt, respectively; $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg)

Effect of MMAE on antioxidants/oxidant status

The level and activities of antioxidants and oxidants in DLA bearing mice were measured in the tumor cells and liver after MMAE treatment. Treatment of DLA mice with MMAE results in increased antioxidant levels and activities both in tumor cells and liver when compared with the untreated control. MMAE treatment significantly increased glutathione content in a dose-dependent manner up to 100 mg/kg b.wt when compared to the control group (Fig. 2A). To determine the effect of MMAE on antioxidant enzymes, the activities of GST and SOD were assessed. In response to MMAE treatment, the antioxidant enzyme activities were significantly increased when compared with the control (Fig. 2B, C). To investigate whether MMAE treatment affects intracellular oxidant level, the level of lipid peroxidation (LPO) was assessed to indicate the level

of oxidative stress. Consistent to the increased antioxidant enzyme activities, oxidative stress was reduced significantly as evidenced from both the liver and tumor cells after treatment of DLA mice with MMAE. The decrease in LPO level was found to correspond to MMAE treatment in a dose-dependent manner up to 100 mg/kg b.wt (Fig. 2D).

Effect of MMAE on tumor volume and cell toxicity

Tumor volume and cell viability were assessed in order to determine the effect of MMAE on tumor load and cytotoxicity. Treatment of DLA mice with MMAE caused a significant reduction in the tumor volume in a dose-dependent manner when compared with the DLA control group (Table 3). The reduction in tumor volume with MMAE treatment was comparable to the reduction in tumor volume when DLA mice were treated with DOX. Similarly, percentage of non-viable cells has been shown to increase significantly in both MMAE- and DOX-treated DLA mice suggesting the cytotoxic effect of MMAE (Table 3).

Effect of MMAE on hematological and serum biochemical parameters

The DLA bearing mice was shown to have reduced RBC and hemoglobin levels as compared to the normal animals. Interestingly, the decrease in RBC and hemoglobin levels were reversed significantly by MMAE and DOX treatment when compared with the DLA control mice (Table 3). The level of reversal of RBC and hemoglobin contents by MMAE treatment is comparable to the reversal by treatment with the standard DOX treatment. Elevated levels of WBC were observed in the DLA bearing mice as compared to the normal control animals. However, with MMAE and DOX treatment, the WBC levels were significantly reduced to the level close to the normal control group (Table 3).

Serum biochemical parameters that include the enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine (CRE) level were found to be significantly increased in the DLA control mice as compared to the normal control group. Treatment with 100 and 150 mg/kg b.wt MMAE and DOX (0.5 mg/kg) were shown to significantly lower the activities of ALT, AST and CRE close to that of the normal control animals (Table 3).

Induction of DNA strand breaks by MMAE

The alkaline Comet assay was used to assess the level of DNA damage, both double stranded and single stranded, in DLA bearing mice after 7 consecutive days of treatment with MMAE (100 mg/kg). We found that MMAE induced DNA damage in ascites tumor cells which was indicated by significant increased tail length and olive moment in MMAE-treated group when compared to untreated control (Fig. 3).

Effect of MMAE on the expression of p53, Bax, Apaf 1, Bcl-2 and Bcl- X_L

The mRNA expression of both pro-apoptotic and anti-apoptotic genes were also investigated in DLA bearing mice using qPCR. We found that MMAE induced up-regulation of pro-apoptotic genes including p53, Bax and Apaf1 by 4.12-fold, 26.57-fold and 4.51-fold, respectively, and down-regulation of Bcl-2 and Bcl-X_L by 2.81-fold and 2.24-fold, respectively, when compared to untreated control (Fig. 4). The relative mRNA expression levels of pro-apoptotic genes (Bax, p53 and Apaf1) and anti-apoptotic genes (Bcl-2 and Bcl-X_L) in control and MMAE (100 mg/kg)-treated DLA bearing mice are given in Fig. 4.

Discussion

In order to increase the efficacy of cancer treatment, studies have recently been focused on drugs that have been used in traditional medicine (Singh et al., 2016). Study of the medicinal property of *M. macrophylla* has mostly been targeted on its antioxidant (Lalremruati et al., 2019), anti-microbial (Chowdhury et al., 2013), thrombolytic (Islam et al., 2013) and anti-diabetic (Bhandari et al., 2020) activities with no report on its anticancer activity. Therefore, in this study, we investigate the anticancer activities of the aqueous extract of *M. macrophylla* using DLA bearing mice as our model. Following the standard method of drug administration, DLA mice were treated with MMAE intraperitoneally. The dose used in this study was carefully selected after performing the acute toxicity that gave us the approximate LD_{50} which was found to be 1.52 ± 0.117 g/kg b.wt. In all the subsequent analyses, MMAE dose between 50 and 150 mg/kg b.wt was used which are all below the LD_{50}.

Several plants of the genus *Mussaenda* have been known for exhibiting anticancer activities. Mussaenin A, a compound isolated from *M. glabrata* has been shown to induce apoptosis in Hep G2 via up-regulation of pro-apoptotic genes (Bax, Bak and Bad) and down-regulation of anti-apoptotic genes (Bcl-2 and Cox-2) (Lipin & Darsan, 2017). Sanshiside D, an iridoid glycoside isolated

(See figure on next page.)

Fig. 2 Effects of aqueous extract of *M. macrophylla* on **A** glutathione level (GSH) (µmol/mg protein); **B** glutathione-s-transferase activity (GST) (unit/mg protein); **C** superoxide dismutase activity (SOD) (unit/mg protein); **D** lipid peroxidation (LPO) expressed in malondialdehyde (nmol/mg protein) in the fluid and liver of DLA bearing mice. Control: Dalton's lymphoma ascites bearing mice without treatment; $MMAE_{50}$, $MMAE_{100}$, $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100, 150 mg/kg b.wt, respectively. Means not sharing the same letter are significantly different at $p < 0.05$

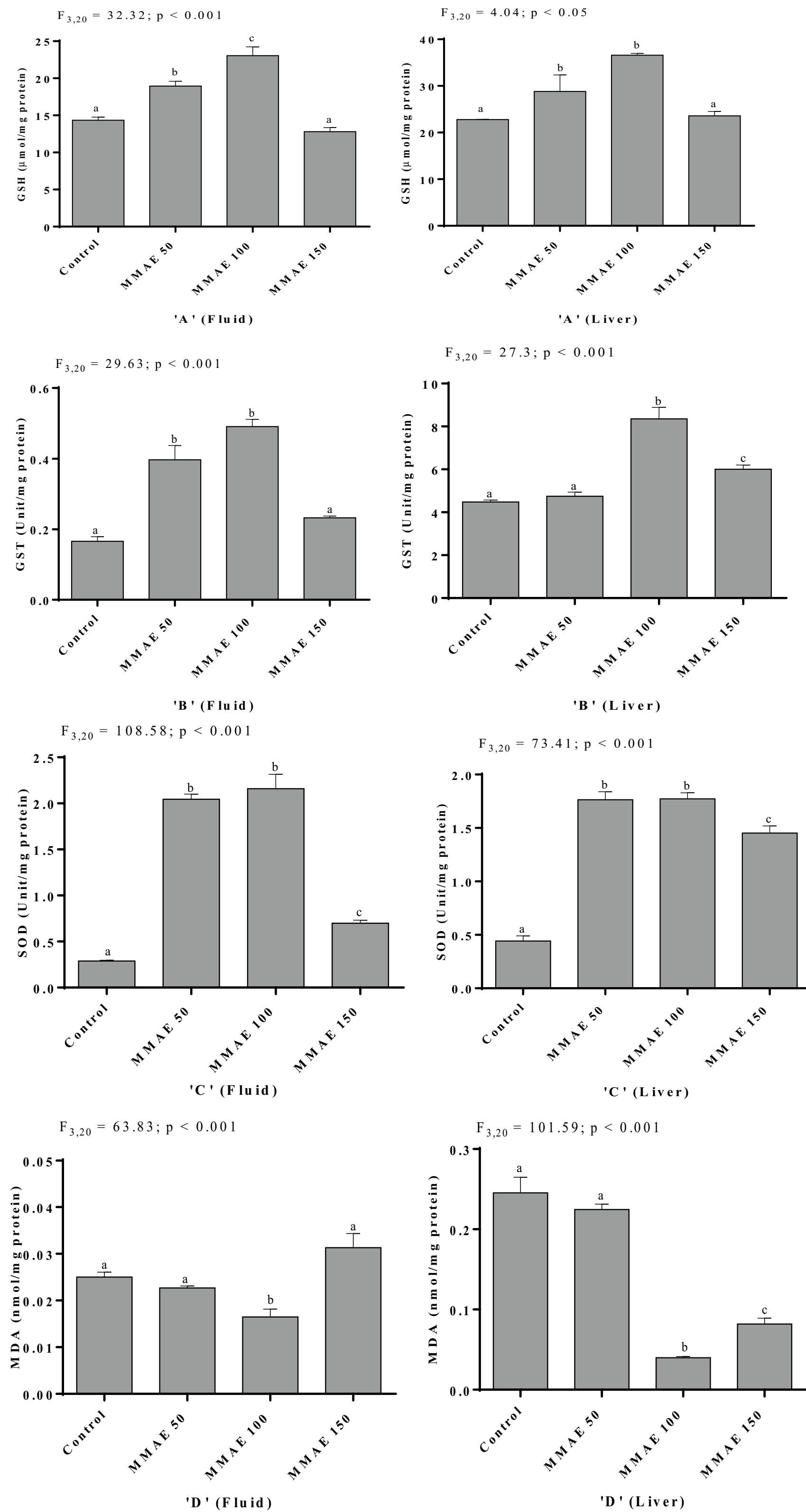


Fig. 2 (See legend on previous page.)

Table 3 Effects of MMAE and DOX on tumor volume, cytotoxicity, hematological parameters and activities of serum enzymes in DLA bearing mice

Groups	Tumor volume (mL)	Non-viable cell (%)	Hematological Parameters			Serum enzymes		
			RBC (million/mm^3)	WBC (thousand/mm^3)	Hb (g%)	ALT (U/L)	AST (U/L)	CRE (mg/dL)
Normal control	–	–	5.4 ± 0.07^a	10.2 ± 1.23^a	20.2 ± 2.34^a	18.4 ± 1.78^a	90.4 ± 2.46^a	0.4 ± 0.03^a
DLA control	5.5 ± 0.28^a	1.1 ± 0.42^a	2.1 ± 0.42^b	29 ± 2.57^b	9.1 ± 1.37^b	29.4 ± 1.77^b	131.9 ± 3.66^b	0.9 ± 0.04^b
DLA + MMAE$_{50}$	3.4 ± 0.32^b	23.1 ± 0.52^b	2.8 ± 0.74^b	26.5 ± 1.15^b	10.6 ± 1.18^b	29 ± 2.68^b	126.9 ± 3.88^b	0.7 ± 0.05^c
DLA + MMAE$_{100}$	1.6 ± 0.39^c	35.4 ± 2.91^c	4.5 ± 0.89^c	14.4 ± 1.07^c	15.4 ± 1.60^c	20.8 ± 1.04^c	104.9 ± 2.65^c	0.5 ± 0.05^{c,d}
DLA + MMAE$_{150}$	1.7 ± 0.38^c	38.1 ± 3.90^c	4.2 ± 1.07^c	15.4 ± 1.08^c	14.1 ± 1.36^c	21.4 ± 2.29^c	109.4 ± 3.87^c	0.5 ± 0.04^d
DLA + DOX$_{0.5}$	1.5 ± 0.86^c	37.2 ± 2.13^c	4.4 ± 0.57^c	12.7 ± 1.78^a	16.9 ± 2.02^c	25.9 ± 1.09^{b,c}	113.9 ± 2.68^c	0.4 ± 0.04^{c,d}

Normal Control: Healthy mice without treatment; DLA Control: DLA bearing mice without treatment; DLA + MMAE$_{50}$, DLA + MMAE$_{100}$, DLA + MMAE$_{150}$: DLA bearing mice treated with 50, 100 and 150 mg/kg b.wt of aqueous extract of *M. macrophylla*; DLA + DOX$_{0.5}$: DLA bearing mice treated with 0.5 mg/kg b.wt of doxorubicin. Values are mean ± SEM ($n = 6$)

Different letters indicate significant variation at $p < 0.05$

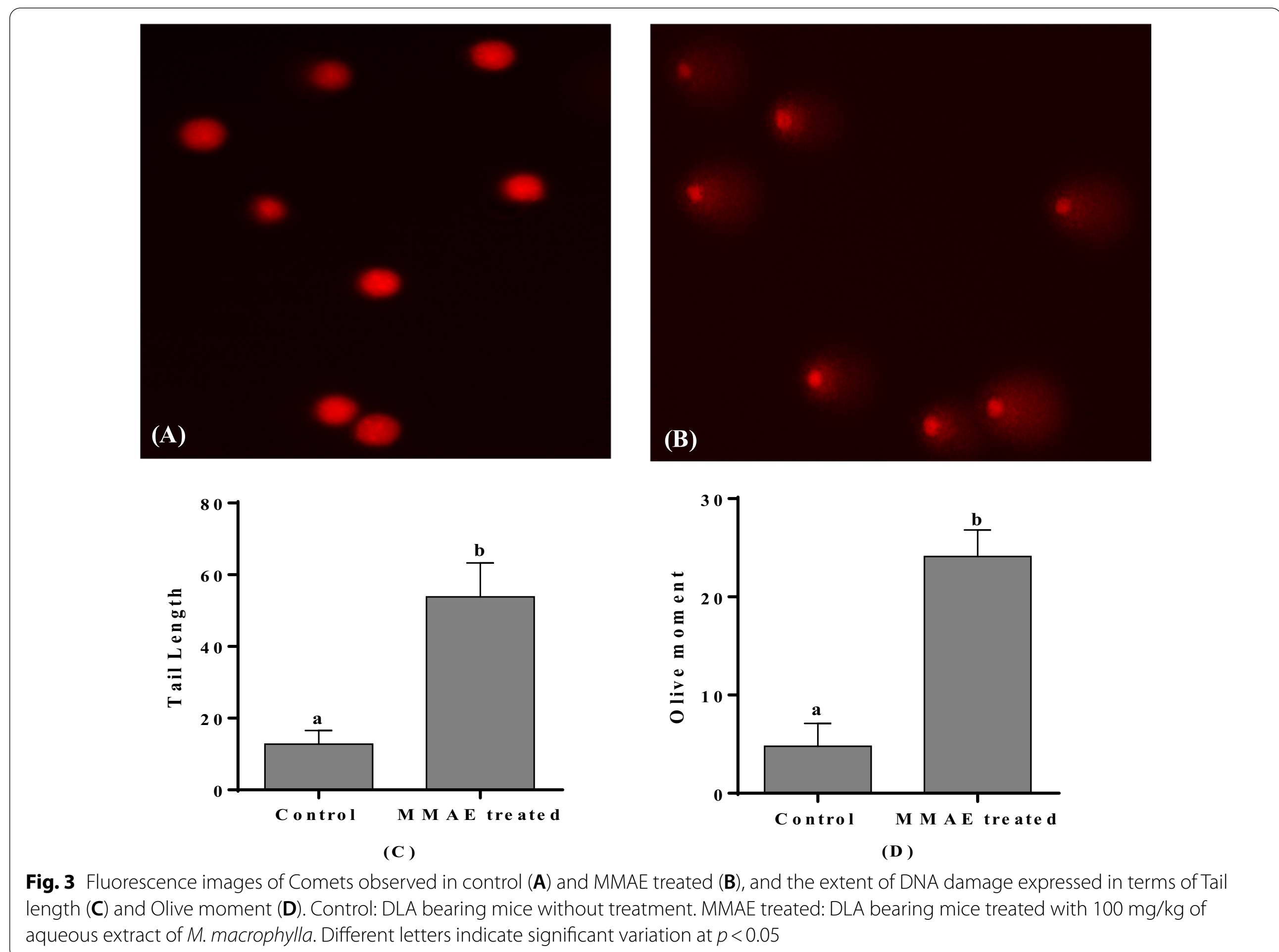


Fig. 3 Fluorescence images of Comets observed in control (**A**) and MMAE treated (**B**), and the extent of DNA damage expressed in terms of Tail length (**C**) and Olive moment (**D**). Control: DLA bearing mice without treatment. MMAE treated: DLA bearing mice treated with 100 mg/kg of aqueous extract of *M. macrophylla*. Different letters indicate significant variation at $p < 0.05$

from *M. dona aurora* displayed cytotoxicity and inhibition of cell growth in various cancer cells including Vero, HeLa and SMMC-7721 (Vidyalakshmi & Rajamanickam, 2009). The sepals of *M. phillipica* were shown to exhibit antitumor effects by triggering the antioxidant defense system in Caco-2 and MCF-7 bearing mice (Renilda &

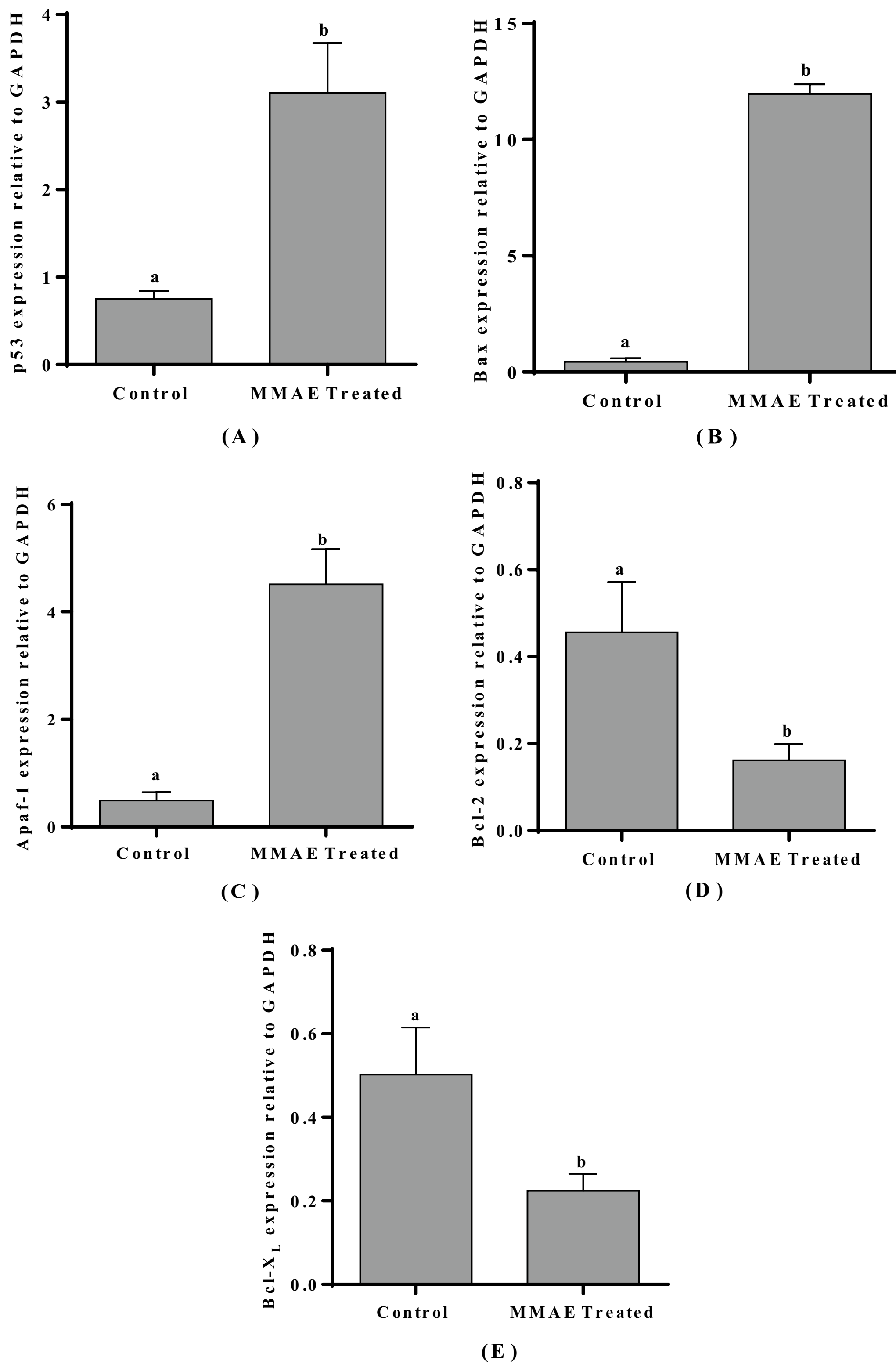


Fig. 4 Effects of aqueous extract of *M. macrophylla* on mRNA expression levels of **A** p53; **B** Bax; **C** Apaf1; **D** Bcl-2; **E** Bcl- X_L in Dalton's Lymphoma Ascites (DLA) bearing mice. Control: DLA bearing mice without treatment. MMAE treated: DLA bearing mice treated with 100 mg/kg of aqueous extract of *M. macrophylla*. Different letters indicate significant variation at $p < 0.05$

Fleming, 2016). *M. roxburghii* (Chowdury et al., 2015) and *M. luteola* (Shylaja & Sathiavelu, 2017) have also been shown to possessed anticancer activities. Interestingly, similar to the other members of the *Mussaenda* genus, *M. macrophylla* shows anticancer potential as evidenced from our current study. Our results indicate that MMAE increased the life span of DLA bearing mice as shown by the increase in MST, AST, % IMLS and % IALS (Table 2). Increase in life span of animals is an important criterion for the efficacy of an anticancer agent (Gupta et al., 2004). The body weight of DLA bearing mice generally increases due to the increased cell proliferation of the cancer cells. The potential of MMAE as an anticancer agent was also shown by its ability to suppress the weight gain in DLA bearing mice possibly by inhibiting proliferation of cancer cells in vivo. Cytotoxicity of plant extract is another important feature for consideration as anticancer agent. MMAE treatment was found to exhibit cytotoxicity against ascitic tumors as the treatment reduced tumor volume and increased the percentage of non-viable cells (Table 3). Therefore, our findings suggest that the chemoprotective effects of MMAE could be linked to its role in inhibiting cancer cell proliferation, reduction in tumor load and cytotoxicity which led to increase in life span of the cancer bearing animals.

Majority of cancer cells are marked with alteration in hematological parameters (Dongre et al., 2008; Thavamani et al., 2014) as is observed in the case of DLA bearing mice. The hematological changes include reduced levels of RBCs and hemoglobin content accompanied by increased levels of WBC count. Treatment of DLA bearing mice with MMAE showed a pronounced effect in restoring the levels of RBC, hemoglobin and WBC close to that of the normal control levels (Table 3). Inflammation of liver is another feature generally observed in ascitic tumors which could be assessed by determining the levels and activities of key liver enzymes such as AST, ALT, and CRE. In the DLA bearing mice, all these enzymes were highly elevated which was however significantly lowered with MMAE treatment (Table 3). Taking together, MMAE could influence and restore the altered hematological and biochemical parameters in ascitic tumors. The aspect that MMAE could reinstate the altered biochemical and hematological profiles of the DLA bearing mice suggests a promising aspect of *M. macrophylla*as an anticancer agent.

The balance between oxidants and antioxidants in the cell of many cancer types remains key to disease progression or improvement. This is accounted to the known role of accumulation of intracellular ROS and its association with cancer progression. Many cancer cells are shown to have higher levels of ROS as compared to the normal cells (Tafani et al., 2016). Therefore, to counterbalance the increased ROS levels in the cancer cell, the cells antioxidant mechanisms have to be efficient. Unfortunately, in many cancers, the activities of the antioxidant system are overwhelmed by the high ROS levels. Therefore, many cancer treatments require external agent to assist the cellular antioxidants. Since high ROS ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$) levels have close association with tumor initiation, angiogenesis, cell invasion, metastasis and chemoresistance in different cancer models (Galadari et al., 2017) use of antioxidants or agents that enhance antioxidant system may provide an opportunity to reduce intracellular ROS-mediated tumorigenesis and cancer progression. In fact, natural products including plant extract have demonstrated antioxidant efficacy such as sesamol, curcumin, ascorbic acid and vitamin E for cancer treatment both in vitro and in vivo (Galadari et al., 2017). Furthermore, a large variety of antioxidants, either alone or in combination with conventional anticancer agents, have been carried out clinically for their use toward anticancer therapeutics in different types of cancer including Acute lymphoblastic leukemia (Al-Tonbary et al., 2009), breast cancer (Zhang et al., 2012) and ovarian cancer (Ma et al., 2014). Consistently, in the present study, augmentation of GSH level and activities of GST and SOD, and decreased lipid peroxidation as evidenced by the significant decrease in MDA levels after MMAE treatment clearly demonstrate its antioxidant nature which may be responsible for its anticancer activity in DLA bearing mice. Over-expression of antioxidant enzymes such as SOD1, SOD2, SOD3,GPx3 and Prx6 has been reported to induce cell death, decrease survival time and suppression of metastasis in various cancer cells (Galadari et al., 2017). Plants such as *Hypericum hookerianum* (Dongre et al., 2008), *Aegle marmelos* (Chockalingam et al., 2012), *Cyathula prostrate* (Mayakrishnan et al., 2014) and *Cocculus hirsutus* (Thavamani et al., 2014) have been reported to possess anticancer activities in DLA bearing mice via elevation of antioxidant defense system and reduction in lipid peroxidation. Injection of SOD has also been reported to significantly inhibit the peroxidation, metastatic tumor growth and extended the survival period of mice inoculated with B16-BL6 cells (Hyoudou et al., 2008).

DNA damage and induction of apoptosis in response to anticancer agents are another important factor in anticancer therapy. In our study, we observe that MMAE treatment induces significant DNA damage in the ascites tumor cells (Fig. 3). Several plant-derived anticancer drugs have been reported to show similar effects in various cancer types including Dalton's lymphoma (Fattahi et al., 2013; Madunić et al., 2018). The Bcl-2 (B-cell lymphoma/leukemia-2) family of both pro- and anti-apoptotic proteins, through their interactions, plays

central roles in regulation of diverse cell death mechanisms including apoptosis (Reed, 2008). Alterations in the expression of these genes contribute to the pathogenesis and progression of cancers, thus providing targets for anticancer drug discovery. Altered expression of anti-apoptotic genes such as Bcl-X_L and Bcl-2, and pro-apoptotic genes such as Bid, Bax and Apaf1 have been documented in several human cancers (Sung et al., 2016). Pharmacological inhibition of anti-apoptotic gene expression in cancer has emerged as major strategies for inducing apoptosis and ultimately causing tumor regression (Fesik, 2005). In order to assess the effect of MMAE in inducing apoptosis, the expression levels of apoptotic genes including p53, Bax, and Apaf-1 and anti-apoptotic genes such as Bcl-2 and Bcl-X_L were determined. Our result shows up-regulation of pro-apoptotic gene expression while the anti-apoptotic gene expressions were down-regulated (Fig. 4). It is thus plausible that MMAE triggers apoptotic response in DLA mice and offers protective effects in the animals.

Conclusion

Our finding through in vivo cancer model depicts the ability of *M. macrophylla* to reduce tumor load, prolong the life span of the cancer bearing animals, and activation of apoptotic pathway through DNA damage. It would be interesting to further explore the mechanisms and pathways through which *M. macrophylla* exerts anticancer effects. Furthermore, it would be important to conduct a bioassay guided fractionation study in order to isolate and characterize the active ingredient that possesses the anticancer activity.

Abbreviations

MMAE: *Mussaenda macrophylla* aqueous extract; DLA: Dalton's lymphoma ascites; DOX: Doxorubicin; BSA: Bovine serum albumin; GSH: Glutathione reduced; NADH: Nicotinamide adenosine dinucleotide; NBT: Nitroblue tetrazolium; TBA: Thiobarbaturicacid; PMS: Phenazine methosulphate; CDNB: 1-Chloro-2,4 dinitrobenzene; DTNB: 5, 5′ Dithio 2-nitrobenzoic acid; PBS: Phosphate-buffered saline; TCA: Trichloroacetic acid; HCl: Hydrochloric acid; DMSO: Dimethyl sulfoxide; OECD: The Organisation for Economic Co-operation and Development; LD_{50}: Median lethal dose; MST: Median survival time; AST: Average survival time; % IMLS: Increase in median life span; % IALS: Increase in average life span; GST: Glutathione-s-transferase; SOD: Superoxide dismutase; LPO: Lipid peroxidation; MDA: Malondialdehyde; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CRE: Creatinine; qRT-PCR: Quantitative real time polymerase chain reaction; ANOVA: Analysis of variance; SPSS: Statistical Package for the Social Sciences; ROS: Reactive oxygen species.

Acknowledgements

We thank the Department of Science and Technology (DST), Government of India, for providing Inspire fellowship to Mary Zosangzuali (DST/INSPIRE Fellowship/[IF170375]). We also thank the University Grant Commission, Ministry of Tribal Affairs, Government of India, for providing fellowship to C. Lalmuansangi (201718-NFST-MIZ-00902) and Marina Lalremruati (UGC-MZU Fellowship). The authors thank DBT, New Delhi for the support through Advanced State Biotech Hub at Mizoram University.

Authors' contributions

ML, CL, MZ, AKT and ZS conducted the experiment and performed data analysis. Experimental design, statistical analysis and manuscript preparation were done by ZS and ML. Manuscript was critically reviewed by LT and NSK. All authors reviewed the data and approved the final manuscript.

Funding

This work was financially supported by the Directorate of Science and Technology, Government of Mizoram, India (Vide Grant Number B. 13012/1/217/DST) for purchasing of chemicals and other reagents.

Availability of data and materials

Supplementary information or data can be obtained from the author on request.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZU-IAEC/2018/09) and CPCSEA (Committee for the Purpose of Control & Supervision of Experiments on Animals), New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to disclose.

Author details

[1]Department of Zoology, Mizoram University, Aizawl 796 004, India. [2]Mizoram Science, Technology and Innovation Council, Aizawl, Mizoram 796001, India. [3]Department of Biotechnology, Mizoram University, Aizawl 796 004, India.

Received: 20 May 2021 Accepted: 8 February 2022
Published online: 19 February 2022

References

Al-Batran, R., Al-Bayaty, F., Jamil Al-Obaidi, M. M., Abdualkader, A. M., Hadi, H. A., Ali, H. M., & Abdulla, M. A. (2013). In vivo antioxidant and antiulcer activity of *Parkia speciosa* Ethanolic leaf extract against ethanol-induced gastric ulcer in rats. *PLoS ONE, 8*(5), e64751. https://doi.org/10.1371/journal.pone.0064751

Al-Tonbary, Y., Al-Haggar, M., El-Ashry, R., El-Dakroory, S., Azzam, H., & Fouda, A. (2009). Vitamin E and N-acetylcysteine as antioxidant adjuvant therapy in children with acute lymphoblastic leukemia. *Advances in Hematology*. https://doi.org/10.1155/2009/689639

Badami, S., Manohara, S. A., Kumar, E. P., Vijayan, P., & Suresh, B. (2003). Antitumor activity of total alkaloid fraction of *Solanum pseudocapsicum* leaves. *Phytotherapy Research, 17*(9), 1001–1004. https://doi.org/10.1002/ptr.1229

Bernardi, R., Grisendi, S., & Pandolfi, P. P. (2002). Modelling haematopoietic malignancies in the mouse and therapeutical implications. *Oncogene, 21*, 3445–3458. https://doi.org/10.1038/sj.onc.1205313

Beuege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 30*, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Beutler, E. (1984). *Red cell metabolism: A manual of biochemical methods* (3rd ed., p. 188). Grune and Stratton Inc. https://doi.org/10.1042/bst0131259

Bhandari, R., Shrestha, D., Pandey, J., Gyawali, C., Lamsal, M., Sharma, S., Rokaya, R. K., Aryal, P., & Khadka, R. B. (2020). Study of in vitro anti-oxidant and anti-diabetic activity by *Mussaenda macrophylla* root extracts. *International Journal of Current Pharmaceutical Research, 12*(4), 70–74. https://doi.org/10.22159/ijcpr.2020v12i4.39085

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394–424. https://doi.org/10.3322/caac.21492

Chakrabarti, S., Chakrabarti, A., & Pal, A. K. (1984). Chromosome analysis of Dalton's lymphoma adapted to the Swiss mouse: Clonal evaluation and

Cheterochromatin distribution. *Cancer Genetics and Cytogenetics, 11*(4), 417–423. https://doi.org/10.1016/0165-4608(84)90022-0

Chockalingam, V., Kadali, S., & Gnanasambantham, P. (2012). Antiproliferative and antioxidant activity of Aegle marmelos (Linn.) leaves in Dalton's Lymphoma Ascites transplanted mice. *Indian Journal of Pharmacology, 44*(2), 225–229. https://doi.org/10.3892/mmr.2014.2015

Chowdhury, S. R., Akter, S., Sharmin, T., Islam, F., & Quadery, T. M. (2013). Antimicrobial activity of five medicinal plants of Bangladesh. *Journal of Pharmacognosy and Phytochemistry, 2*(1), 164–170.

Chowdury, I. A., Alam, M. N., Chowdhury, S., Biozid, S., Faruk, Md., Mazumdar, M. U., & Chowdhury, A. I. (2015). Evaluation of ex-vivo anti-arthritic, anti-inflammatory, anti-cancerous and thrombolytic activities of *Mussaenda roxburghii* Leaf. *European Journal of Medicinal Plants, 10*(4), 1–7. https://doi.org/10.9734/EJMP/2015/20483

D'Amour, F. F., Blood, F. R., & Belden, D. A. (1965). *The manual for laboratory work in mammalian physiology* (3rd ed.). The University of Chicago Press.

Das, L., & Vinayak, M. (2014). Long term effect of curcumin in regulation of glycolytic pathway and angiogenesis via modulation of stress activated genes in prevention of cancer. *PLoS ONE, 9*(6), e99583. https://doi.org/10.1371/journal.pone.0099583

Dongre, S. H., Badami, S., & Godavarthi, A. (2008). Antitumor activity of *Hypericum hookerianum* against DLA induced tumor in mice and its possible mechanism of action. *Phytotherapy Research, 22*, 23–29. https://doi.org/10.1002/ptr.2248

Fattahi, S., Ardekani, A. M., Zabihi, E., Abedian, Z., Mostafazadeh, A., Pourbagher, R., & Akhavan-Niaki, H. (2013). Antioxidant and apoptotic effects of an aqueous extract of *Urtica dioica* on the MCF-7 human breast cancer cell line. *Asian Pacific Journal of Cancer Prevention, 14*(9), 5317–5323. https://doi.org/10.7314/apjcp.2013.14.9.5317

Fesik, S. W. (2005). Promoting apoptosis as a strategy for cancer drug discovery. *Nature Reviews Cancer, 5*, 876–885. https://doi.org/10.1038/nrc1736

Fried, R. (1975). Enzymatic and non-enzymatic assay of superoxide dismutase. *Biochimie, 7*, 657–660. https://doi.org/10.1016/S0300-9084(75)80147-7

Galadari, S., Rahman, A., Pallichankandy, S., & Thayyullathil, F. (2017). Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radical Biology and Medicine, 104*, 144–164. https://doi.org/10.1016/j.freeradiomed.2017.01.004

Gupta, M., Mazumder, U. K., Rath, N., & Mukhopadhyay, D. K. (2000). Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. *Journal of Ethnopharmacology, 72*(1–2), 151–156. https://doi.org/10.1016/s0378-8741(00)00227-0

Gupta, S., Zhang, D., Yi, J., & Shao, J. (2004). Anticancer activities of *Oldenlandia diffusa*. *Journal of Herbal Pharmacotherapy, 4*(1), 21–23. https://doi.org/10.1080/j157v04n01_03

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57–70. https://doi.org/10.1016/s0092-8674(00)81683-9

Hyoudou, K., Nishikawa, M., Kobayashi, Y., Ikemura, M., Yamashita, F., & Hashida, M. (2008). SOD derivatives prevent metastatic tumor growth aggravated by tumor removal. *Clinical and Experimental Metastasis, 25*(5), 531–536. https://doi.org/10.1007/s10585-008-9165-3

Islam, F., Chowdhury, S. R., Sharmin, T., Uddin, M. G., Kaisar, M. A., & Rashid, M. A. (2013). In vitro membrane stabilizing and thrombolytic activities of *Ophirrhiza mungos*, *Mussaenda macrophylla*, *Gmelina philippensis* and *Synedrella nodiflora* growing in Bangladesh. *Journal of Pharmacy and Nutrition Sciences, 3*(1), 71–75. https://doi.org/10.6000/1927-5951.2013.03.01.8

Kamal, A., Ashraf, M., Vardhan, M. V., Faazil, S., & Nayak, V. L. (2014). Synthesis and anticancer potential of benzothiazole linked phenylpyridopyrimidinones and their diones as mitochondrial apoptotic inducers. *Bioorganic and Medicinal Chemistry Letters, 24*, 147–151. https://doi.org/10.1016/j.bmcl.2013.11.057

Kim, N. C., Desjardins, A. E., Wu, C. D., & Kinghorn, A. D. (1999). Activity of triterpenoid glycosides from the root barks of *Mussaenda macrophylla* against oral pathogens. *Journal of Natural Products, 62*(10), 1379–1384. https://doi.org/10.1021/np9901579

Laladhas, K. P., Cheriyan, V. T., Puliappadamba, V. T., Bava, S. V., Unnithan, R. G., Vijayammal, P. L., & Anto, R. J. (2010). A novel protein fraction from *Sesbania grandiflora* shows potential anticancer and chemopreventive efficacy, in vitro and in vivo. *Journal of Cellular and Molecular Medicine, 14*(3), 636–646. https://doi.org/10.1111/j.1582-4934.2008.00648.x

Lalremruati, M., Lalmuansangi, C., & Zothansiama. (2019). Free radical scavenging activity and antioxidative potential of various solvent extracts of *Mussaenda macrophylla* Wall: An in vitro and ex vivo study. *Journal of Applied Pharmaceutical Science, 9*(12), 94–102. https://doi.org/10.7324/JAPS.2019.91213

Lipin, D. S., & Darsan, B. M. (2017). Mussaenin A isolated from *Mussaenda glabrata* induces apoptosis in the liver cancer cells via mitochondrial pathway. *International Journal of Pharmacognosy and Phytochemical Research, 9*(9), 1266–1273. https://doi.org/10.25258/phyto.v9i09.10315

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ddC(T)) method. *Methods, 25*, 402–408. https://doi.org/10.1006/meth.2001.1262

Lowry, O. H., Rosebrough, N. J., & Randall, R. J. (1951). Protein measurement with the Folin-phenol reagent. *Biochemistry, 193*, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Ma, Y., Chapman, J., Levine, M., Polireddy, K., Drisko, J., & Chen, Q. (2014). High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy. *Science Translational Medicine, 6*(222), 222ra18. https://doi.org/10.1126/scitranslmed.3007154

Madunić, J., Madunić, I. V., Gajski, G., Popić, J., & Garaj-Vrhovac, V. (2018). Apigenin: A dietary flavonoid with diverse anticancer properties. *Cancer Letters, 28*(413), 11–22. https://doi.org/10.1016/j.canlet.2017.10.041

Mallick, S. K., Maiti, S., Bhutia, S. K., & Maiti, T. K. (2010). Antitumor properties of a heteroglucan isolated from *Astraeus hygrometricus* on Dalton's lymphoma bearing mouse. *Food and Chemical Toxicology, 48*(8–9), 2115–2121. https://doi.org/10.1016/j.fct.2010.05.013

Manandhar, N. P. (2002). Plants and people of Nepal. *Journal of Ethnobiology, 23*, 313–314.

Mayakrishnan, V., Kannappan, P., Shanmugasundaram, K., & Abdullah, N. (2014). Anticancer activity of *Cyathula prostrata* (Linn) Blume against Dalton's lymphoma in mice model. *Pakistan Journal of Pharmaceutical Sciences, 27*(6), 1911–1917.

Miller, L. C., & Tainter, M. L. (1944). Estimation of LD_{50} and its error by means of log-probit graph paper. *Proceedings of the Society for Experimental Biology and Medicine, 57*, 261.

Moron, M. S., Depierre, J. W., & Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione-s-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta, 582*, 67–78. https://doi.org/10.1016/0304-4165(79)90289-7

Prieur, D. J., Young, D. M., Davis, R. D., Cooney, D. A., Homan, E. R., Dixon, R. L., & Guarino, A. M. (1973). Procedures for preclinical toxicologic evaluation of cancer chemotherapeutic agents: Protocols of the laboratory of toxicology. *Cancer Chemotherapy Reports, 3*(4), 1–39.

Rawla, P., & Barsouk, A. (2019). Epidemiology of gastric cancer: Global trends, risk factors and prevention. *Przeglad Gastroenterologiczny, 14*(1), 26–38. https://doi.org/10.5114/pg.2018.80001

Reed, J. C. (2008). Bcl-2-family proteins and hematologic malignancies: History and future prospects. *Blood, 111*, 3322–3330. https://doi.org/10.1182/blood-2007-09-078162

Renilda, A. J., & Fleming, A. T. (2016). Cytotoxicity of *Mussaenda philippica* against *Artemia salina* and cancer cell lines. *International Journal of Pharma and Bio Sciences, 7*(2), 63–67.

Renthlei, Z., Gurumayum, T., Borah, B. K., & Trivedi, A. K. (2018). Daily expression of clock genes in central and peripheral tissues of tree sparrow (*Passer montanus*). *Chronobiology International, 36*(1), 110–121. https://doi.org/10.1080/07420528.2018.1523185

Rosangkima, G., & Jagetia, G. C. (2015). In vitro anticancer screening of medicinal plants of Mizoram state, India, against Dalton's lymphoma, MCF-7 and Hela cells. *International Journal of Recent Scientific Research, 6*(8), 5648–5653.

Shang, A., Lu, W. Y., Yang, M., Zhou, C., Zhang, H., Cai, Z. X., Wang, W. W., Wang, W. X., & Wu, G. Q. (2018). miR-9 induces cell arrest and apoptosis of oral squamous cell carcinoma via CDK 4/6 pathway. *Artificial Cells, Nanomedicine and Biotechnology, 46*(8), 1754–1762. https://doi.org/10.1080/21691401.2017.1391825

Shylaja, G., & Sathiavelu, A. (2017). Cytotoxicity of endophytic fungus *Chaetomium cupreum* from the plant *Mussaenda luteola* against breast cancer cell line MCF-7. *Bangladesh Journal of Pharmacology, 12*, 373–375. https://doi.org/10.3329/bjp.v12i4.33596

Shylesh, B. S., & Padikkala, J. (2000). In vitro cytotoxic and antitumor property of *Emilia sonchifolia* (L.) DC in mice. *Journal of Ethnopharmacology, 73*(3), 495–500. https://doi.org/10.1016/s0378-8741(00)00317-2

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*, 184–191. https://doi.org/10.1016/0014-4827(88)90265-0

Singh, S., Sharma, B., Kanwar, S. S., & Kumar, A. (2016). Lead phytochemicals for anticancer drug development. *Frontiers in Plant Science, 7*, 1667. https://doi.org/10.3389/fpls.2016.01667

Sung, B., Chung, H. Y., & Kim, N. D. (2016). Role of apigenin in cancer prevention via the induction of apoptosis and autophagy. *Journal of Cancer Prevention, 21*, 216–226. https://doi.org/10.15430/JCP.2016.21.4.216

Tafani, M., Sansone, L., Limana, F., Arcangeli, T., De Santis, E., Polese, M., Fini, M., & Russo, M. A. (2016). The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression. *Oxidative Medicine and Cellular Longevity, 3907147*, 1–18. https://doi.org/10.1155/2016/3907147

Thavamani, B. S., Mathew, M., & Palaniswamy, D. S. (2014). Anticancer activity of *Cocculus hirsutus* against Dalton's lymphoma ascites (DLA) cells in mice. *Pharmaceutical Biology, 52*(7), 867–872. https://doi.org/10.3109/13880209.2013.871642

Thillaivanan, S., & Samraj, K. (2014). Challenges, constraints and opportunities in herbal medicines: A review. *International Journal of Herbal Medicine, 2*(1), 21–24.

Vidyalakshmi, K. S., & Rajamanickam, G. V. (2009). An iridoid with anticancer activity from the sepals of *Mussaenda 'dona aurora'*. *Indian Journal of Chemistry, 48*, 1019–1022.

Zhang, G., Wang, Y., Zhang, Y., Wan, X., Li, J., Liu, K., Wang, F., Liu, Q., Yang, C., Yu, P., Huang, Y., Wang, S., Jiang, P., Qu, Z., Luan, J., Duan, H., Zhang, L., Hou, A., Jin, S., … Wu, E. (2012). Anti-cancer activities of tea epigallocatechin-3-gallate in breast cancer patients under radiotherapy. *Current Molecular Medicine, 12*(2), 163–176. https://doi.org/10.2174/156652412798889063

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen journal and benefit from:

► Convenient online submission
► Rigorous peer review
► Open access: articles freely available online
► High visibility within the field
► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com