INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

Methanolic Extract of *Mallotus roxburghianus* Muell. Exhibit Anti-Cancer Activity against Dalton's Lymphoma Ascites (DLA) bearing Mice via Alterations of Apoptotic Genes E xpression and Redox-Homeostasis

Mary Zosangzuali[1], F Lalsangpuii[2], C Lalmuansangi[1], Lalchhandami Tochhawng[3], Amit Kumar Trivedi[1], Nachimuthu Senthil Kumar[4], Zothansiama[1]*

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

OPEN ACCESS

Received: 23-01-2023
Accepted: 09-06-2023
Published: 06-09-2023

Citation: Zosangzuali M, Lalsangpuii F, Lalmuansangi C, Tochhawng L, Trivedi AK, Kumar NS, Zothansiama (2023) Methanolic Extract of *Mallotus roxburghianus* Muell. Exhibit Anti-Cancer Activity against Dalton's Lymphoma Ascites (DLA) bearing Mice via Alterations of Apoptotic Genes E xpression and Redox-Homeostasis. Indian Journal of Science and Technology 16(SP1): 75-88. https://doi.org/10.17485/IJST/v16sp1.msc11

* **Corresponding author**.

zothans@gmail.com

Funding: DST for providing Inspire fellowship to Mary Zosangzuali (DST/INSPIRE Fellowship/ [IF170375]). UGC, Ministry of Tribal Affairs, Government of India, for providing fellowship to F. Lalsangpuii [201920-NFST-MIZ-02751] and C. Lalmuansangi [201718-NFST-MIZ-00902].

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Objectives: The current study examined the anti-cancer effects of methanolic extract of *Mallotus roxburghianus* (MRME) using Dalton's Lymphoma Ascites (DLA) bearing mice. **Methods:** According to OECD guidelines, the acute toxicity of MRME was assessed, and the LD_{50} of MRME was estimated using probit analysis. The effects of MRME on survival time, weight change, and antioxidant/oxidant status were determined in DLA mice by administering the animals with different doses of MRME, and doxorubicin was used as a standard reference drug. Cytotoxicity, activities of serum enzymes, and haematological parameters were also determined after treatment with MRME. Effects of MRME on DNA damage and the differential gene expression of apoptotic genes were also studied using Comet assay and qPCR techniques respectively. **Findings:** MRME significantly reduced tumour growth and extended the survival duration of tumour-bearing mice with increased in MST (26.0 ± 0.72), AST (23.0 ± 0.60), % IMLS (79.3 ± 0.58) and % IALS (50.0 ± 0.91) after treatment with 150 mg/kg. MRME also decreased antioxidant activities and enhanced lipid peroxidation. Decreased RBC levels and haemoglobin content in DLA mice were significantly reversed by MRME treatment. MRME also reduces AST, ALT, LDH, and CRE levels, which were otherwise increased in the DLA control animals. The apoptosis-based anti-cancer effects of *M. roxburghianus* were revealed by the induction of DNA damage, up-regulation of pro-apoptotic genes, and down-regulation of anti-apoptotic genes in DLA mice after *M. roxburghianus* treatment. MRME treatment increased the relative expressions of pro-apoptotic genes such as Apaf1, p53 and Bax by 3.19, 3.81 and

6.06 folds, respectively when compared to untreated control. **Novelty:** Our study demonstrates the anti-cancer activities of the methanolic extract of *M. roxburghianus* leaves thus potentiating the use of the plant for further development of anti-cancer agents.

Keywords: Mallotus roxburghianus; Dalton's Lymphoma Ascites; Anticancer; DNA damage; Apoptotic genes

1 Introduction

Mallotus roxburghianus (MR) is a shrub to a small tree belonging to the family Euphorbiaceae. It is locally known as Zawngtenawhlung and found widely in Mizoram, India, typically in the tropical evergreen forests and mixed bamboo forests. It is also found to be distributed within the Chittagong Hill tracts of Bangladesh and Myanmar. MR is traditionally used by the local people of Mizoram for the treatment of various ailments including fever, hypertension, inflammation, and diabetes [(1)]. Different species of *Mallotus* have been explored for their pharmaceutical applications by various researchers in the past few decades. *M. philippenensis* has been reported to possess antibacterial and antioxidant properties. Compounds isolated from *M. oppositifolius* showed potent antioxidant, antibacterial and antimalarial activities. The root extract of *M. apelta* and compounds isolated from *M. japonicus* showed significant antiviral activity [(2)]. *M. roxburghianus* has been reported to contain phenols, alkaloids, saponins, terpenes, tannins, triterpenoids, steroids, reducing sugars, coumarin, berginin, gallic acid, gentisic acid, gums, and a variety of other natural substances, particularly flavonoids like chrysin, hesperidin, naringenin, quercetin, and rutin. Furthermore, various extracts derived from *M. roxburghianus* have been reported to be associated with anti-inflammatory, antioxidant, anti-hemolytic, anti-pathogenic, antidiabetic, and recovery of testicular activity from the damaging influence of scrotal hyperthermia [(1)]. *M. roxburghianus* is found to be a valuable source of naturally occurring compounds with interesting pharmacological activities which created an interest in further investigations of the plant.

Based on statistics released by the World Health Organization, cancer is a leading cause of death globally in 2020, accounting for nearly 10 million deaths, or nearly one in six deaths. Cancer is a significant global health burden and about 70% of cancer fatalities take place in low- and middle-income countries, most likely as a result of factors such as rising pollution levels, lack of healthcare facilities, and expensive anti-cancer drugs. The development of anticancer drugs from natural sources, such as plants, is one strategy to overcome these challenges which may result in the creation of treatments that are cheaper for low- and middle-income nations [(3)].

Natural compounds derived from medicinal plants appear to be the most promising among hundreds of chemicals that have been and are being tested for their anti-cancer activities as they are safer and exhibit positive long-term effects when compared to synthetic drugs [(4)]. Due to their wide range of pharmacological properties, phytochemical compounds derived from plants, such as alkaloids, flavonoids, triterpenoids, phenols, glucosinolates, quercetin, saponins, curcumin, myricetin, and tannins have drawn more attention in recent years [(5)]. These derived phytochemical compounds possess different biological activities including anticancer properties [(6)]. Furthermore, there is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumourigenesis and associated inflammatory processes, indicating the importance of plant products in cancer prevention and therapy. Various plant ingredients that possess anti-cancer activity have been translated from pharmacology investigations to clinical applications [(7)]. Approximately 60% of anticancer drugs such as allicin, beta-carotene, bleomycin, camptothecin, curcumin, dactinomycin, diosgenin, etoposide, genistein,

irinotecan, lycopene, paclitaxel, resveratrol, topotecan and vinca alkaloids (vincristine and vinblastine) used in clinical and preclinical trials are derived from plants[6].

Dalton's lymphoma is relatively inexpensive and easy to maintain; thus, served as an excellent model for studying the preclinical activity of an anti-tumour agent. Multiple studies have been conducted to elucidate the anti-cancer properties of several medicines as well as the subsequent mechanisms of action and their pathways involved using Dalton's lymphoma ascites (DLA) bearing mice[8]. With the aim of finding novel compounds without unfavorable side effects, the present study was carried out to investigate the anti-cancer efficacy of *M. roxburghianus* leaf extract in DLA-bearing Swiss albino mice.

2 Methodology

2.1 Chemicals

Bovine serum albumin (BSA), nitroblue tetrazolium (NBT), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), Folin-ciocalteu's reagent, n-butanol, thiobarbaturic acid (TBA), 5, 5' dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4 dinitrobenzene (CDNB) and phenazine methosulphate (PMS) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Doxorubicin (Getwell Oncology Pvt., Ltd. Haryana, India) was purchased from the local pharmacy. The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2 Collection of plant and preparation of extract

M. roxburghianus was collected from Lengpui, Mizoram. Identification and authentication were done by the department of Horticulture, Aromatic and Medicinal Plants (HAMP), Mizoram University, Aizawl. After drying at room temperature, the powdered leaves were then extracted in a Soxhlet apparatus at 40°C using 95% methanol for a minimum of 40 cycles. The liquid extract was filtered and concentrated under reduced pressure at 40°C for 5 h using a rotary evaporator (Buchi, Germany) and finally freeze-dried. The methanolic extract of *M. roxburghianus* (MRME) was then collected and stored at 4°C for further experiments.

2.3 Animals and tumour model

The animal care and handling were carried out in accordance with the guidelines issued by World Health Organization, Geneva, Switzerland, and were approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZUIAEC17-18-15) and CPCSEA (Committee for the Purpose of Control & Supervision of Experiments on Animals), New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA). Swiss albino mice (25-30g) were selected from an inbred colony maintained under controlled temperature ($25^{\circ}C \pm 2^{\circ}C$) and photoperiod of 12/12 h light/dark cycles (Frontier Euro Digital Timer, Taiwan) at the Animal Care Facility, Department of Zoology, Mizoram University, India. All animals were provided sawdust as bedding and had access to standard food pellets and water ad libitum. Dalton's Lymphoma Ascites (DLA) tumour has been maintained in 10-12 weeks old mice by serial intraperitoneal (i.p) transplantation of 1×10^6 viable tumour cells per animal (in 0.25 ml Phosphate-buffered saline (PBS), pH 7.4) under aseptic condition.

2.4 Preparation of Drug and mode of administration

Doxorubicin and MRME were dissolved in d. H_2O and 1% ethanol, respectively. Each animal from each group received an intraperitoneal (i.p) dose of treatments in accordance with body weight.

2.5 Acute toxicity study

The acute toxicity of MRME was determined as per OECD guidelines 420-425. Animals were divided into five groups of ten each, given intraperitoneal aliquot dosages of MRME (1.2, 1.4, 1.6, 1.8, and 2.0 g/kg b.wt), and monitored for 14 days after treatment for toxic symptoms and mortality. The LD_{50} value of the MRME was calculated using probit analysis. Prior to determining the probits, the mortality percentage for 0 and 100 are corrected as follows:

Corrected % formula for 0 and 100% mortality:

For 0% dead: 100 (0.25/n)

For 100% dead: 100 (n-0.25/n)

The dose corresponding to probit 5 i.e., 50%, was obtained by plotting the probit values against log doses. The standard error of the mean (SEM) of LD_{50} was calculated using the following formula:

$$Approx.\ SEM\ of\ LD50\ =\ \frac{(Log\ LD84 - Log\ LD16)}{\sqrt{2N}}$$

where N is the number of animals in each group.

2.6 Experimental design

For the evaluation of survival time and weight change, Swiss albino mice were randomly divided into six equal groups (n = 10). On day '0', all mice were transplanted (i.p) with 1×10^6 cells in 0.25 ml of PBS. After 24 h of tumour transplantation, each group received treatment for 9 consecutive days.

Group I (Control group): Mice were injected (i.p) with 0.5 ml of 1 % ethanol.

Group II-V (MRME group): Mice were injected (i.p) with MRME at the dose of 50, 100, 150, and 300 mg/kg b.wt, respectively.

Group VI (DOX group): Mice were injected (i.p) with doxorubicin (DOX) at the dose of 0.5 mg/kg b.wt as a standard drug.

A further experiment was carried out to assess antioxidant enzyme and lipid peroxidation, with the same grouping of DLA-bearing mice as previously described. The highest MRME dose (300 mg/kg b.wt) was excluded due to no improvement in the tumour response in vivo. After MRME (150 mg/kg b.wt) and doxorubicin administration, cytotoxicity, serum enzyme activity, and haematological parameters were also assessed in the DLA-bearing mice. The level of DNA damage as well as the expression of pro- and anti-apoptotic genes were also evaluated in the MRME (150 mg/kg b.w.t.) treated group in comparison to the control group.

2.7 Estimation of survival time and weight change

Daily records of tumour-bearing mice's deaths, if any, and survival times for all groups were maintained. The tumour response was calculated using the 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 formulae;

$$MST = \frac{First\ death\ +\ Last\ death\ in\ the\ group}{2}$$

$$AST = \frac{Sum\ of\ animals\ dead\ on\ different\ days}{Number\ of\ animals}$$

$$\%IMLS = \frac{MST\ of\ treated\ mice\ -\ MST\ of\ control}{MST\ of\ control} \times 100$$

$$\%IALS\ =\ \frac{AST\ of\ treated\ mice\ -\ AST\ of\ control}{AST\ of\ control} \times 100$$

T/C value, which is the MST of the treated group of animals (T) divided by that of the control group (C) was also computed. The T/C ratio is given as a percentage and a compound is considered active if it shows a T/C value $\geq$ 120% (National Cancer Institute Protocols). Alterations in the weight of the animals in all experimental groups were also evaluated periodically. Up to 18 days' post tumour transplantation, the animal's body weights were monitored every 3 days.

2.8 Processing of tumour cells for biochemical assays

A ketamine overdose was employed to euthanize the animals in each group at 6, 12, and 24 h after treatment. After aspiration and washing with NH_4Cl and 1X PBS twice, the tumour cells were pelleted, sonicated (PCi Analytics), and homogenized with ice-cold buffer (5 mM EDTA, 0.15 M NaCl, pH 7.4) yielding 5% (w/v) homogenate. The homogenates were centrifuged for 30 min at 4°C at 13,000 rpm, and the supernatants were then stored in aliquots at -80°C until used for biochemical assessments.

2.9 Biochemical assays

Protein contents were determined as described earlier [9] using BSA as standard.

2.9.1 ***Glutathione (GSH)***

Glutathione (GSH) levels were measured using the previously described method [9]. GSH was measured by its reaction with DTNB in Ellman's reaction to give a compound that absorbs light at 412 nm. Briefly, the cell homogenate (80 μl) was incubated with 0.02M sodium phosphate buffer (900 μl) and 10 mM DTNB (20 μl) for 2 min at room temperature. A mixture devoid of tissue lysates served as blank. The absorbance of the sample was taken against blank at 412 nm in a UV-visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). GSH concentration was calculated from the standard graph and expressed in μmol/mg protein.

2.9.2 ***Glutathione S-Transferase (GST)***

Glutathione-s-transferase (GST) was measured using previously described method [9]. Briefly, 20 mM CDNB (50 μl) was mixed with 0.1M phosphate buffer (850 μl, pH 6.5) and incubated at 37°C for 10 min. Then, 50 μl each of 20 mM GSH and cell homogenate was added to the mixture. A mixture devoid of tissue lysates served as blank. The absorbance was measured at 1 min intervals for 5 min at 340 nm. GST activity was measured as:

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.

2.9.3 ***Superoxide Dismutase (SOD)***

The activity of superoxide dismutase activity was measured by the NBT reduction method [9]. Briefly, cell homogenate (100 μl) and 186 μM PMS (100 μl) was mixed with 3 mM NBT (300 μl) and 780 μM NADH (200 μl). The mixture was incubated for 90 s at 30°C and the reaction was stopped by adding 1 ml of acetic acid and 4 ml of n-butanol. A mixture devoid of tissue lysates served as blank. The absorbance was measured at 560 nm and the enzyme activity was expressed in the unit (1 unit = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank-OD of sample/OD of blank) × 100

2.9.4 ***Catalase (CAT)***

CAT activity was estimated using the method described earlier [10] with minor modifications. Briefly, 3 % H_2O_2 (200 μl) was mixed with cell homogenate (50 μl) and 50 mM phosphate buffer (150 μl, pH7.0). The absorbance was taken at 240 nm, decomposition of H_2O_2 can be followed directly by the decrease in absorbance. The enzyme activity was expressed in units/mg protein. The catalytic activity of CAT at a time interval of 15 s was calculated by the following formula:

$K = 0.153 (\log Ao/A1)$

Where Ao is the absorbance at 0 s and A1 is the absorbance at 15s.

2.9.5 ***Lipid peroxidation (LPO)***

Lipid peroxidation (LPO) was measured using the method described earlier [9]. Briefly, 10% TCA, 0.8% TBA, and 0.02 N HCl were mixed with cell homogenate in a 1:2 ratio. After boiling for 10 min, the mixture was cooled immediately and centrifuged at 12000 rpm for 10 min. The absorbance of the supernatant was taken at 535 nm against blank. A mixture devoid of tissue lysates served as blank. The MDA concentration of the sample was calculated using the extinction coefficient of 1.56 x 10^6 $M^{-1}cm^{-1}$.

2.10 Effect of MRME on cell toxicity and haematological parameters

The cytotoxic effect of MRME was investigated by measuring the tumour cell volume and the percentage of viable and non-viable cell count using the trypan blue dye exclusion test, in a haemocytometer. Red blood cell count (RBC), white blood cell count (WBC), and haemoglobin content were measured from the blood collected by heart puncture as described earlier [9].

2.11 Measurement of serum ALT, AST, LDH, and CRE

Activities of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), LDH (EC 1.1.1.27), and CRE level were assessed in serum using commercially available kits (Coral Clinical Systems, Uttarakhand, India).

2.12 Assessment of DNA damage using Comet assay

The alkaline single-cell gel electrophoresis (Comet assay) was carried out as described earlier[9]. The tumour cells from both the control and treatment groups were washed with NH_4Cl and 1X PBS following aseptic aspiration. Briefly, 2 X 10^4 tumour cells were suspended in 0.5 % low-melting point agarose (75 μl) prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose. Slides were immersed for 2 h in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10). Slides were placed in a horizontal electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) after being lysed for 20 min to allow the unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA and the slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralisation, slides were washed with $d.H_2O$ and was stained for 5 min with ethidium bromide (EtBr) solution (2 g/ml). For each animal, two slides were prepared and 100 randomly selected cells from each slide were observed by a fluorescence microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615) with a 200x magnification. The images captured were analyzed with Image J software.

2.13 QRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression

Tumour cells from both the control and treatment groups were washed with NH_4Cl and 1X PBS after aspiration in an aseptic condition. Total RNA was extracted from the pelleted cells using the 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. 2 μg of total RNA was used to synthesize cDNA using a first-strand cDNA synthesis kit. (Thermoscientific, K1621; Lithuania, Europe). Gene-specific primers [enlisted in Table 1] were designed using Primer 3, Boston, MA, USA. 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^{TM} SYBR^{TM} 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). Afterwards, melting curves were generated to confirm a single uniform peak. The GAPDH gene is used as a reference gene to calculate 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 the ΔΔCt method.

Table 1. Primer sequences used in qRT-PCR analyses of Dalton's lymphoma ascites (DLA) bearing mice treated with methanolic extract of *M. roxburghianus* (150 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

2.14 Statistical analysis

All data were expressed as mean ± standard error of the mean. Significance variation in survival time, change in body weight, antioxidants status, lipid peroxidation, relative gene expression, and DNA damage were calculated using Student's t-test between control and treatment groups. One-way ANOVA followed by Tukey's test was performed to test significant variations in tumour volume, cytotoxicity, haematological parameters, and activities of serum enzymes. 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.

3 Results and Discussion

3.1 Acute toxicity test

To investigate the acute toxicity, the approximate LD_{50} of MRME was calculated as a pilot study by the so-called 'staircase method'. Five doses ranging from 0% to 100% mortality were selected to determine the LD_{50}. Five doses were administered intraperitoneally to 5 groups of 10 mice each [Table 2]. Plotting the probit values against log doses revealed that the dose equivalent to probit 5, or 50% death, was 0.223 (log LD_{50}), with an LD_{50} of 1.67 ± 0.15 g/kg b.wt. All subsequent experiments in this study were performed with MRME at doses between 50 and 300 mg/kg body weight, which are all below the LD_{50}.

Table 2. Results of the lethal dose of methanolic extract of *M. roxburghianus*(MRME) for the determination of LD $_{50}$ in healthy Swiss albino mice

Groups	Dose (g/kg)	Log dose	% dead	Corrected %	Probits
1	1.2	0.08	0%	2.50%	3.03
2	1.4	0.14	20%	20.00%	4.16
3	1.6	0.2	40%	40.00%	4.75
4	1.8	0.26	80%	80.00%	5.84
5	2	0.3	100%	97.50%	6.96

3.2 Effects of MRME on survival time and weight change

To investigate the anti-cancer activity of MRME in DLA-bearing mice, different doses were given to the animals [Table 3]. All untreated DLA-bearing mice died in less than 18 days with MST and AST of 14.5 ± 0.84 days and 15.8 ± 1.23 days, respectively. A dose-dependent elevation in AST, MST, % IALS, and % IMLS up to 150 mg/kg was observed in MRME-treated DLA-bearing mice. However, a reduction in DLA-mice life span was observed with the treatment of 300 mg/kg MRME, revealing the possibility of adverse effects from prolonged use of a higher dose of MRME. Results of the *in vivo* anti-cancer activity in DLA-bearing mice were also expressed as the ratio of the median survival days for the treatment and control groups (T/C value). The T/C values of 134.48 %, 141.37 %, and 197.31 % were observed in DLA mice treated with MRME at the doses of 50,100, and 150 mg/kg b.wt. respectively, showing the viability of MRME as a potential cancer treatment. However, treatment of DLA-bearing mice with 300 mg/kg of MRME reduced the T/C value to 127.5 % which is consistent with the results of the AST, MST, % IALS and % IMLS. DOX treatment also elevates MST to 22.5 ± 0.71 days and AST to 22.4 ± 1.21 days, respectively. Consequently, DOX treatment increased % IMLS and % IALS to 55.17% and 41.77%, respectively [Table 3]. The potential of the plant extract for possible therapeutic applications is shown by the ability of MRME to increase animal survival time at doses up to 150 mg/kg. Figure 1 summarizes the effects of MRME and DOX on the survival of DLA-bearing mice. Our results indicate that life span was increased significantly in DLA-bearing mice as shown by an increase in AST, MST, % IALS and % IMLS.

Table 3. Effect of MRME and DOX treatment on DLA bearing mice on the tumour response assessment based on MST, AST, % IMLS, % IALS and T/C ratio. DLA Control: DLA bearing mice without treatment; DLA +$DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg); DLA + $MRME_{50,}$ DLA + $MRME_{100,}$ DLA + $MRME_{150,}$ DLA + $MRME_{300}$: DLA bearing mice treated with methanolic extract of *M. roxburghianus* at the dose of 50, 100, 150 and 300 mg/kg, respectively. The results were expressed as percent (%) ± SEM, n=10.

Dose (mg/kg b. wt)	MST	AST	% IMLS	% IALS	% T/C ratio
DLA Control	14.5 ± 0.84	15.8 ± 1.23	-	-	-
DLA +$DOX_{0.5}$	22.5 ± 0.71***	22.4 ± 1.21***	55.2 ± 0.31[a]	41.7 ± 1.24 [a]	155.1 ± 1.00★
DLA +$MRME_{50}$	19.5 ± 0.63**	18.5 ± 0.72**	34.4 ± 1.02[b]	17.8 ± 0.96 [b]	134.4 ± 1.98★
DLA +$MRME_{100}$	20.5 ± 0.32**	21.3 ± 0.50***	41.3 ± 0.54[c]	34.8 ± 0.90 [c]	141.3 ± 1.59★
DLA +$MRME_{150}$	26.0 ± 0.72***	23.0 ± 0.60***	79.3 ± 0.58[d]	50.0 ± 0.91 [d]	197.3 ± 2.81★
DLA +$MRME_{300}$	18.5 ± 0.92*	18.1 ± 0.69**	27.5 ± 1.45[e]	14.5 ± 0.84 [b]	127.5 ± 1.52★

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 between the control and treatment groups.
'★' indicates a T/C ratio > 120
Different letters indicate significant variation between different treatment groups.

Due to the increased cell proliferation of the cancer cells, the body weight of DLA-bearing mice increases, and they exhibited constant weight gain till their mortality [Figure 2]. In the present study, both the control and treatment groups of DLA-bearing mice exhibited no spontaneous tumour regression. However, MRME-treated groups (100, 150, and 300 mg/kg b.wt.) showed a significantly reduced average rate of weight gain at three-day intervals up to the 18th day post-transplantation of tumour when

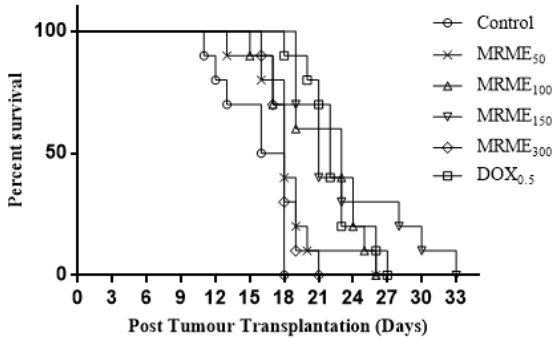

Fig 1. Kaplan Meier's estimate of survival time of DLA bearing mice. Control: DLA bearing mice without treatment; $MRME_{50}$, $MRME_{100}$, $MRME_{150}$, $MRME_{300}$: DLA bearing mice treated with methanolic extract of *M. roxburghianus*at the dose of 50, 100, 150 and 300 mg/kg b. wt, respectively; $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg).

compared to the control group [Table 4]. This suggests that MRME treatment has the potential to lower tumour load possibly the cancer cell proliferation *in vivo*.

Table 4. Average increase in weight of DLA mice treated with different doses of MRME and DOX at 3 days' intervals up to the18th day of post tumour inoculation. DLA Control: DLA bearing mice without treatment; DLA + $MRME_{50}$, DLA + $MRME_{100}$, DLA + $MRME_{150}$, DLA + $MRME_{300}$: DLA bearing mice treated with methanolic extract of *M. roxburghianus*at the dose of 50, 100, 150 and 300 mg/kg, respectively; DLA + $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg).

Dose (mg/kg b. wt)	Weight gain (g) (Mean ±SEM)
DLA Control	2.84 ± 0.46
DLA + $MRME_{50}$	1.73 ± 0.28
DLA + $MRME_{100}$	1.50 ± 0.32*
DLA + $MRME_{150}$	1.45 ± 0.26*
DLA + $MRME_{300}$	1.47 ± 0.22*
DLA + $DOX_{0.5}$	1.57 ± 0.39*

* significant variation ($p<0.05$) between control and treatment groups.

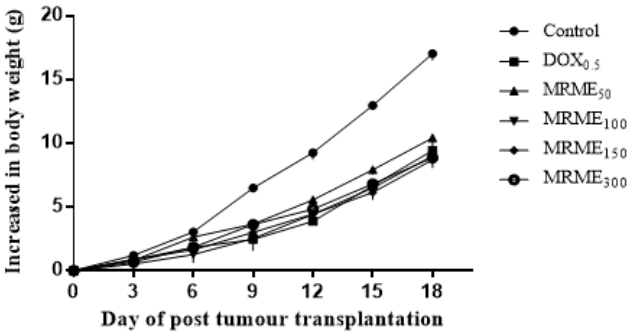

Fig 2. Average increase in body weight of DLA mice treated with different doses of MRME (50, 100, 150 and 300 mg/kg b. wt) and DOX (0.5 mg/kg b. wt) in three days' intervals (The initial weight of all the animals was taken as '0'g).

3.3 Effect of MRME on Antioxidants/Oxidant status

The antioxidant levels and activities in DLA mice were assessed after treatment with MRME to determine the effect of MRME on antioxidants and oxidant levels in tumour cells. When compared to the control, all experimental groups receiving MRME and DOX treatment had lower antioxidant levels and activity. Glutathione content was substantially reduced in DLA mice treated with MRME and DOX compared to the control group, and this reduction was time- and dose-dependent [Table 5]. The GST, SOD, and CAT activities were measured to assess the effect of MRME on antioxidant enzymes. All antioxidant enzyme activities were considerably lower following MRME treatment compared to the control. The DOX treatment also showed reduced antioxidant enzyme activities, which is consistent with the effect of MRME. It's worth emphasizing that the MRME treatment had a higher inhibitory effect on SOD and CAT activities than the standard DOX [Table 5].

Table 5. Alteration in the antioxidant enzymes and lipid peroxidation in the DLA bearing mice treated with various doses of MRME (50, 100, 150 and 300 mg/kg b. wt) and DOX (0.5 mg/kg b. wt). Values are expressed as mean ± SEM (n = 6)

Treatment	PTT (hr)	GSH	GST	SOD	CAT	LPO
Control	6	3.01 ± 0.02	0.50 ± 0.02	0.93 ± 0.03	0.016 ± 0.02	0.015 ± 0.06
	12	3.10 ± 0.05	0.56 ± 0.05	0.74 ± 0.01	0.014 ± 0.01	0.021 ± 0.01
	24	2.86 ± 0.03	0.67 ± 0.03	0.81 ± 0.07	0.015 ± 0.02	0.023 ± 0.02
MRME (50 mg/kg)	6	1.89 ± 0.02***	0.23 ± 0.05*	0.34 ± 0.01**	0.008 ± 0.01***	0.069 ± 0.03**
	12	1.76 ± 0.01***	0.12 ± 0.05**	0.27 ± 0.05**	0.007 ± 0.05**	0.089 ± 0.05***
	24	1.31 ± 0.02***	0.11 ± 0.02**	0.21 ± 0.02**	0.006 ± 0.02***	0.094 ± 0.07***
MRME (100 mg/kg)	6	1.52 ± 0.02***	0.12 ± 0.01**	0.31 ± 0.05**	0.007 ± 0.03**	0.074 ± 0.03**
	12	1.42 ± 0.02***	0.10 ± 0.07**	0.20 ± 0.02**	0.006 ± 0.02***	0.091 ± 0.02***
	24	1.23 ± 0.03***	0.09 ± 0.02**	0.19 ± 0.07**	0.005 ± 0.01***	0.103 ± 0.05***
MRME (150 mg/kg)	6	1.42 ± 0.04***	0.11 ± 0.04**	0.24 ± 0.06**	0.006 ± 0.03***	0.082 ± 0.06**
	12	1.32 ± 0.03***	0.09 ± 0.08**	0.20 ± 0.01**	0.005 ± 0.01***	0.102 ± 0.05**
	24	1.23 ± 0.02***	0.06 ± 0.02***	0.18 ± 0.02**	0.003 ± 0.02***	0.127 ± 0.03***
DOX (0.5 mg/kg)	6	2.04 ± 0.04***	0.23 ± 0.01**	0.42 ± 0.03**	0.008 ± 0.03***	0.051 ± 0.03**
	12	1.90 ± 0.06***	0.15 ± 0.03**	0.32 ± 0.01**	0.008 ± 0.03**	0.055 ± 0.01**
	24	1.73 ± 0.04***	0.13 ± 0.07**	0.27 ± 0.02**	0.006 ± 0.02***	0.058 ± 0.02**

PTT=Post Treatment Time; MRME = Methanolic extract of *M. roxburghianus*; GSH = Glutathione (μmol/mg protein); GST =Glutathione-s-transferase (Unit/mg protein); SOD = Superoxide dismutase (Unit/mg protein); CAT = Catalase (Unit/ mg protein); LPO = Lipid peroxidation (nmol of MDA/mg protein). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ between the control and treatment groups

The level of lipid peroxidation (LPO), a biomarker of oxidative stress, was evaluated to determine whether MRME treatment causes a rise in intracellular oxidant levels. The levels of oxidative stress were significantly higher following MRME treatment in DLA mice, which is consistent with the decreased antioxidant enzyme activities. The increase in LPO was shown to be time- and dose-dependent in response to MRME treatment. When compared to DOX, it was determined that MRME was more effective at elevating LPO [Table 5].

Reactive oxygen species (ROS) production and elimination must be balanced as excessive ROS production has been linked to the pathophysiology of several diseases, including cancer. The remarkable array of antioxidant systems, both enzymatic (like SOD, CAT, GST, and GPx) and non-enzymatic (such as GSH, ascorbic acid, and lipoic acid) maintains this redox balance in a cell [(9)]. Cancer cells have a higher level of ROS than normal cells due to their increased metabolism and mitochondrial malfunction [(11)]. Increased ROS levels have been linked to biochemical and molecular alterations crucial for tumour initiation, development, and progression as well as resistance to treatment. Indeed, such a correlation has been observed in several malignancies, including melanoma, leukemia, hepatoma, glioma, and cancers of the breast, lung, bladder, colon, pancreas, and prostate [(12)]. Consequently, agents that either increase ROS production to severely toxic levels or suppress the antioxidant defense mechanism may offer a chance to target cancer cells. A wide range of anti-cancer medications, even some under clinical testing, efficiently kill cancer cells and make them more susceptible to chemotherapeutic treatments by increasing ROS generation and/or decreasing the antioxidant defense mechanism [(13)]. MRME treatment decreased glutathione levels and antioxidant enzyme activity in DLA mice, including GST, SOD, and CAT [Table 5]. The levels of lipid peroxidation consistently increased after MRME treatment, which may have been caused by reduced antioxidant activities that allowed cellular ROS to build up. The cytotoxic effects of MRME on the DLA tumour in the current study may, in turn, be caused by the elevation in ROS levels. Similar circumstances exist in other anti-cancer drugs, including doxorubicin, whose primary mechanism of toxicity is the generation of ROS [(14)].

It has also been observed that decreased GSH levels and the activity of GSH-related enzymes like GST increase tumour cell death[15]. The current study also demonstrated a notable MRME-induced lipid-damaging effect in DLA-bearing mice. Lipid peroxidation is a significant event related to cell death that has been shown to cause substantial impairment in membrane function via increased membrane permeability, membrane protein oxidation, and DNA damage, ultimately leading to cell death[16].

3.4 Effect of MRME on tumour volume and cell toxicity

To ascertain the impact of MRME on tumour load and cytotoxicity, the tumour volume and cell viability were evaluated. When compared to the DLA control group, treatment with MRME (150 mg/kg b. wt) resulted in a substantial reduction in tumour volume [Table 6]. The reduction in tumour volume observed after MRME treatment is relatively similar to the reduction in tumour volume reported after DOX treatment of DLA mice. Similar to this, the percentage of cell viability considerably declines in both MRME and DOX-treated DLA mice, indicating the cytotoxic nature of MRME. Cytotoxicity is another crucial feature to be considered while selecting a plant extract as an anti-cancer drug. Ascitic tumours were found to be responsive to MRME treatment, which decreased tumour volume and raised the proportion of non-viable cells. Our findings thus imply that the chemoprotective benefits of MRME may be related to its involvement in reducing tumour load, cytotoxicity, and tumour growth, all of which contribute to the prolongation of life in cancer-bearing animals. A relevant criterion for assessing the potency of an anti-cancer drug is the prolongation extending of the animal's lifespan[17].

Table 6. Effects of MRME and DOX on tumour volume and cytotoxicity in DLA bearing mice. Values are mean ± SEM (n = 6)

Groups	Tumour Volume (ml)	Viable cell (%)	Non-viable cell (%)
DLA Control	3.70 ± 0.08^{a}	81.60 ± 2.94^{a}	18.40 ± 2.94^{a}
DLA + $MRME_{150}$	1.24 ± 0.05^{b}	30.33 ± 1.15^{b}	69.66 ± 1.15^{b}
DLA + $DOX_{0.5}$	1.13 ± 0.12^{b}	28.33 ± 0.94^{b}	71.66 ± 0.94^{b}

Results were compared with DLA control group. DLA + $MRME_{150}$: DLA bearing mice treated with150 mg/kg of methanolic extract of *M. roxburghianus*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg of doxorubicin. Different letters indicate significant variation.

3.5 Effect of MRME on haematological and serum biochemical parameters

The DLA-bearing mice had decreased RBC and haemoglobin levels in comparison to normal mice. Interestingly, MRME (150 mg/kg b. wt) and DOX treatment significantly increased RBC and haemoglobin levels when compared to the DLA control mice [Table 7]. RBC and haemoglobin reversal levels following MRME treatment are comparable to those following normal DOX treatment. When compared to the normal control mice, the DLA-bearing mice had elevated WBC counts. However, the WBC counts have significantly lowered with MRME and DOX treatment to a level comparable to that of the normal control group [Table 7]. Our study is in agreement with Aynalem *et al*[18], where cancer treatment exhibit changes in haematological parameters. Similar alterations occur in ascites tumour, with decreasing levels of RBCs and haemoglobin content accompanied by elevated WBC count. The treatment of DLA-bearing mice with MRME had a significant effect in restoring the levels of RBC, haemoglobin, and WBC close to normal control levels [Table 7].

Table 7. Effects of MRME and DOX on haematological parameters of DLA bearing mice. Values are mean ± SEM (n = 6)

Groups	RBC	WBC	Hb (g %)
	(million/ mm^3)	(thousand/mm^3)	
Normal Control	3.36 ± 0.09^{b}	9.20 ± 0.23^{b}	15.20 ± 0.34^{b}
DLA Control	1.70 ± 0.11^{a}	34.43 ± 0.57^{a}	8.70 ± 0.26^{a}
DLA + $MRME_{150}$	2.77 ± 0.04^{c}	14.75 ± 0.97^{c}	12.60 ± 0.11^{c}
DLA + $DOX_{0.5}$	2.70 ± 0.05^{c}	10.43 ± 0.86^{b}	12.93 ± 0.37^{c}

Results were compared with DLA control group. Normal Control: Healthy mice without treatment; DLA + $MRME_{150}$: DLA bearing mice treated with150 mg/kg of methanolic extract of *M. roxburghianus*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg of doxorubicin. Different letters indicate significant variation.

Serum biochemical parameters such as enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatinine (CRE) level were observed to be significantly elevated in the DLA control mice when compared to the normal control group. The activities of ALT, AST, LDH, and CRE levels are significantly reduced

with treatment with MRME (150 mg/kg b. wt) and DOX to levels comparable to the normal control mice [Table 8]. Another characteristic of ascitic tumours that are frequently observed is liver inflammation, which may be evaluated by determining the levels of ALT, AST, LDH, and CRE. All of these enzymes were significantly increased in DLA-bearing mice but were significantly reduced by MRME treatment to levels that were comparable to those of the normal control groups [Table 8]. Taking together, biochemical and haematological parameters that were altered in ascitic tumours could be influenced and restored by MRME. The aspect that MRME could restore the altered biochemical and haematological profiles of DLA-bearing mice suggests that *M. roxburghianus* has a promising future as an anti-cancer agent.

Table 8. Effects of MRME and DOX on activities of serum enzymes of DLA bearing mice. Values are mean ± SEM (n = 6)

Groups	ALT (U/L)	AST (U/L)	LDH (U/L)	CRE (mg/dL)
Normal Control	46.43 ± 3.40^{b}	87.40 ± 3.45^{b}	1240.61 ± 14.65^{b}	0.46 ± 0.03^{b}
DLA Control	96.89 ± 4.34^{a}	149.62 ± 6.54^{a}	3148.14 ± 9.21^{a}	1.02 ± 0.02^{a}
DLA + $MRME_{150}$	50.80 ± 3.11^{b}	111.35 ± 7.23^{c}	2216.17 ± 11.45^{c}	0.65 ± 0.02^{c}
DLA + $DOX_{0.5}$	53.67 ± 3.47^{b}	116.91 ± 3.65^{c}	2292.72 ± 8.74^{c}	0.52 ± 0.03^{b}

Results were compared with DLA control group. Normal Control: Healthy mice without treatment; DLA + $MRME_{150}$: DLA bearing mice treated with150 mg/kg of methanolic extract of *M. roxburghianus*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg of doxorubicin. Different letters indicate significant variation.

3.6 Induction of DNA strand breaks by MRME

The alkaline Comet assay was used to assess DNA damage in DLA-bearing mice after 9 days of MRME (150 mg/kg) treatment. Our findings demonstrated that MRME caused DNA damage in ascites tumour cells, as indicated by the increased tail length and olive moment in the MRME-treated group compared to the untreated control [Figure 3]. Anti-cancer treatment includes factors like DNA damage and the initiation of apoptosis in response to anti-cancer drugs. According to our study, MRME treatment causes significant DNA damage in the ascites tumour [Figure 4]. Various anti-cancer drugs based on plants have been reported to have similar effects on a wide range of cancer forms, including Dalton's lymphoma[9].

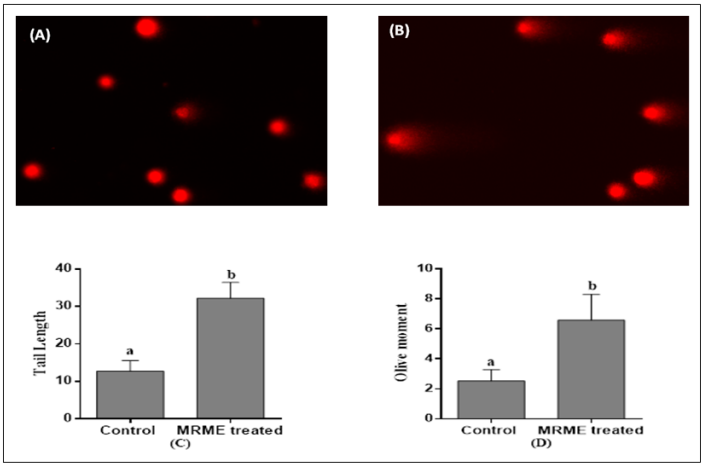

Fig 3. Florescence images of Comets observed in control (A) and MRME treated (B), and the extent of DNA damage expressed in terms of Tail length (C) and Olive moment (D). Control: DLA bearing mice without treatment. MRME treated: DLA bearing mice treated with 150 mg/kg of methanolic extract of *M. roxburghianus* . Different letters indicate significant variation

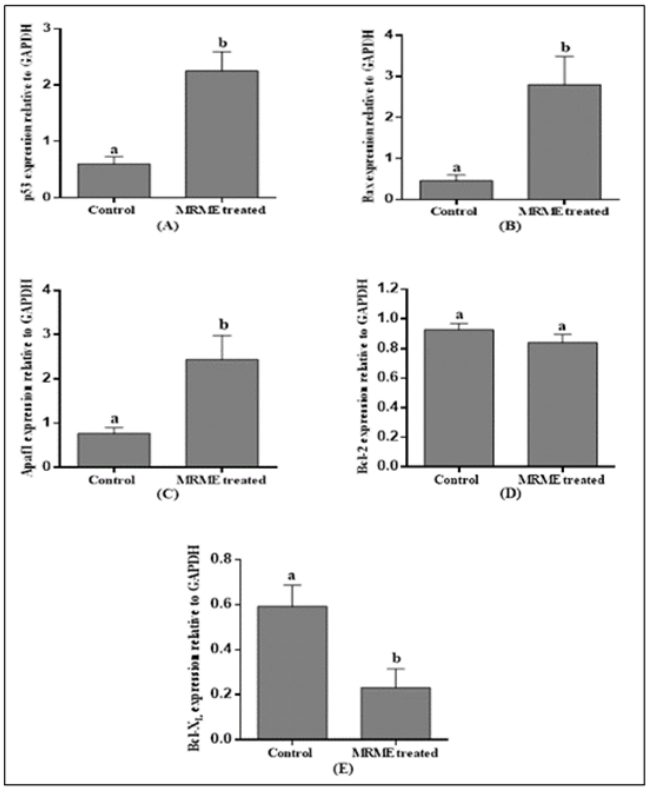

Fig 4. Effects of methanolic extract of *M. roxburghianus* on mRNA expression levels of (A) p53; (B) Bax; (C) Apaf1; (D) Bcl-2; (E)Bcl-X_L in Dalton's Lymphoma Ascites (DLA) bearing mice. Control: DLA bearing mice without treatment. MRME treated: DLA bearing mice treated with 150mg/kg of methanolic extract of *M. roxburghianus*. Different letters indicate significant variation

3.7 Effect of MRME on the expression of p53, Bax, Afap1, Bcl-2, and Bcl-XL

Using qPCR technique, the mRNA expression levels of both pro- and anti-apoptotic genes were analyzed in DLA-bearing mice. When compared to the untreated control, it was observed that MRME treatment increased the expression of pro-apoptotic genes such as Afap1, p53, and Bax by 3.19 folds, 3.81 folds, and 6.06 folds, respectively, and decreased the expression of Bcl-X_L by 2.56 folds. MRME, did not, however, affect Bcl-2 expression at the mRNA level. The relative mRNA expression of pro-apoptotic genes (Apaf1, Bax and p53) and anti-apoptotic genes (Bcl-X_L and Bcl-2) in control and MRME (150 mg/kg) treated DLA-bearing mice is given in Figure 4.

The interactions of the Bcl-2 (B-cell lymphoma/leukaemia-2) family of pro- and anti-apoptotic proteins play significant roles in the regulation of numerous cell death mechanisms, including apoptosis [19]. The altered expressions of these genes contribute to the pathogenesis and development of cancers, making them potential targets for the discovery of anti-cancer drugs. Several human cancers have been related to altered expression of anti-apoptotic genes including Bcl-X_L and Bcl-2 and pro-apoptotic genes like Apaf1, Bax, and Bid [20,21]. In cancer, inhibition of anti-apoptotic gene expression has emerged as a major strategy for the induction of apoptosis and, ultimately, tumour regression [22]. To determine the effect of MRME on inducing apoptosis, the expression levels of apoptotic genes such as Apaf1, p53, and Bax, as well as anti-apoptotic genes such as Bcl-X_L and Bcl-2 were determined. Our findings demonstrate the up-regulation of pro-apoptotic gene expression and the downregulation of anti-apoptotic gene expression [Figure 4]. Therefore, it is possible that MRME induces an apoptotic response in DLA mice and provides protective effects on the animals.

4 Conclusion

The methanolic extract of *M. roxburghianus* from our study demonstrates a novel finding indicating its potential as an anti-cancer agent. *M. roxburghianus* has been shown to have medicinal benefits in studies using animal models, particularly in the treatment of diabetes, however, research on *M. roxburghianus* for the treatment of cancer is still in its early stages. Our results

from an *in vivo* cancer model show that *M. roxburghianus* has the ability to decrease tumour loads, lengthen the lifespan of cancer-bearing animals, and induce the apoptotic pathway through DNA damage, potentially via an increase in ROS levels in the cell. MRME treatment increased the relative expressions of pro-apoptotic genes such as Apaf1, p53 and Bax by 3.19, 3.81 and 6.06 folds, respectively, and decreased the expression of Bcl-X_L by 2.56 folds when compared to untreated control. Further research into the mechanisms and pathways through which *M. roxburghianus* exerts its anti-cancer properties would be noteworthy. Consequently, it would be crucial to perform a bioassay-guided fractionation study in order to identify and characterize the active component with anti-cancer activity.

5 Declaration

Presented in 4th Mizoram Science Congress (MSC 2022) during 20th & 21st October 2022, organized by Mizoram Science, Technology and Innovation Council (MISTIC), Directorate of Science and Technology (DST) Mizoram, Govt. of Mizoram in collaboration with science NGOs in Mizoram such as Mizo Academy of Sciences (MAS), Mizoram Science Society (MSS), Science Teachers' Association, Mizoram (STAM), Geological Society of Mizoram (GSM), Mizoram Mathematics Society (MMS), Biodiversity and Nature Conservation Network (BIOCONE) and Mizoram Information & Technology Society (MITS). The Organizers claim the peer review responsibility

6 Acknowledgment

The authors thank the Department of Science and Technology (DST), Government of India, for providing Inspire fellowship to Mary Zosangzuali (DST/INSPIRE Fellowship/[IF170375]). The authors also thank the University Grant Commission, Ministry of Tribal Affairs, Government of India, for providing fellowship to F. Lalsangpuii [201920-NFST-MIZ-02751] and C. Lalmuansangi [201718-NFST-MIZ-00902].

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