Anticancer potential of *Magnolia champaca* and *Epipremnum aureum*

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Abstract

Cancer has remained the leading cause of death worldwide, significantly affecting life expectancy across all regions. Despite progress in multimodal treatment approaches, it continued to be the primary cause of mortality. The limitations of conventional therapies, including their adverse side effects and the emergence of resistance, highlighted the urgent need to explore alternative treatments. This research aimed to investigate the *in vivo* antineoplastic effects of methanolic extract of *Magnolia champaca* (MMC) and *Epipremnum aureum* (MEA) leaves in Dalton's lymphoma ascites (DLA) induced solid tumour model in Swiss albino mice and compared the effects of those with 5-fluorouracil. Acute oral toxicity studies of MMC and MEA were conducted as per OECD-423 guidelines (OECD-2001). No significant toxic effects and no death observed until the end of the study in both MMC and MEA treatment groups up to the dose of 2000 mg/kg body weight orally in mice which indicated that lethal dose of both the plant extracts were more than 2000mg/kg. Hence for the study, low dose was taken as 200 mg/kg and high dose taken as 400 mg/kg. Tumour was induced by Injecting viable DLA cells ($1x10^6$ cells/mouse) subcutaneously into the hind limb of six to eight-week-old Swiss albino mice. Tumour development was assessed by measuring the tumour. Thirty-six tumour positive animals were randomly divided into six groups comprising six animals each. Group I served as tumour control which received no treatment. Group II was administered with 5-FU at 20mg/kg. Groups III and IV received MMC at 200 mg/kg and 400 mg/kg respectively. MEA was administered to groups V and VI at 200 mg/kg and 400 mg/kg respectively. All the treatments were given orally for 10 days. Progression of the tumour was assessed by measuring the tumour volume. Animals were sacrificed and tumour masses were collected on day 11 to assess the effect of MMC and MEA on the expression of anti-apoptotic gene *Bcl-2* was studied using real time - quantitative polymerase chain reaction (RT-qPCR) with *GAPDH* as house-keeping gene.

Key words: Cancer; Swiss albino mice; Dalton's lymphoma ascites; GAPDH*; Bcl-2*

Introduction

Cancer remains a predominant global health burden, marked by dysregulated cellular proliferation and the capacity for invasion and metastasis to distant organ systems (Arnold et al., 2022). Among available therapeutic modalities, chemotherapy is extensively utilized as a first-line intervention (Faruk, 2021). Nevertheless, its clinical utility is frequently compromised by the emergence of multidrug resistance and substantial off-target toxicities due to its lack of selectivity, resulting in collateral damage to normal proliferative tissues (Israel et al., 2018). These limitations underscore the imperative for the development of novel therapeutic agents that possess enhanced efficacy with improved safety profiles. In this context, the investigation of bioactive compounds derived from natural products has emerged as a promising avenue for anticancer drug discovery (Ogbuewu et al., 2016).

Magnolia champaca (*M. champaca*) exhibits a broad spectrum of pharmacological effects, such as anti-inflammatory, wound-healing, antimicrobial, hypolipidemic, anti-ulcer, diuretic, anthelmintic, cardioprotective and antidiabetic activities (Raja and Koduru, 2014). *Epipremnum aureum (E. aureum)*, a member of the Araceae family, is known for its ability to purify indoor air and offers a variety of therapeutic benefits, including anti-inflammatory, antioxidant, antimicrobial and anti-termite properties (Meshram and Srivastava, 2016).

Apoptosis, a form of programmed cell death, is fundamental to the preservation of tissue homeostasis through the elimination of genetically compromised or superfluous cells. Perturbations in apoptotic pathways constitute a hallmark of oncogenesis, facilitating uncontrolled cellular proliferation. The B-cell lymphoma-2 (Bcl-2) protein family serves as a central regulator of apoptosis, encompassing both pro-apoptotic and anti-apoptotic members. Overexpression of anti-apoptotic Bcl-2 proteins inhibits apoptosis, thereby contributing to tumour cell survival and therapeutic resistance.

Materials and methods

Cell line

The cells used for the research was Dalton's Lymphoma Ascites (DLA) cells procured from Amala Cancer Research Centre, Amala Nagar, Thrissur. The DLA cells were maintained *in-vivo* in Swiss albino mice by intraperitoneal transplantation of 1×10^6 cells per mouse and were used for *in-vivo* study.

Plant materials

The leaves of *Magnolia champaca* (Chembakam) and *Epipremnum aureum* (Money plant) were collected locally from Wayanad, Kerala respectively during October and November (winter) 2023. *Magnolia champaca* is a tree. It is usually grown outdoors. *Epipremnum aureum* is a creeper. It is commonly grown indoors in pots, bottles, but also grow outdoors in warm, humid places with support.

The collected leaves were identified and authenticated by a botanist at St. Thomas college, Thrissur, Kerala. The herbarium of the leaves of *M. champaca* and *E. aureum* (Fig.1, Fig.2) were prepared and deposited as voucher specimens in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode.

Preparation of methanolic extract of *M. champaca* and *E. aureum* leaves

The extraction procedure was carried out as per the standard protocol of the hot continuous extraction method using the Soxhlet apparatus (Fig 3). The leaves of *M. champaca* and *E. aureum* were collected, cleaned off dust and debris and shade-dried by spreading on the paper. The dried leaves were coarsely pulverised using an electric pulveriser. Then, it was weighed and packed in a thimble and assembled in the Soxhlet apparatus with methanol (99 per cent, v/v) at 67°C. The methanolic extracts obtained were concentrated in the rotary vacuum evaporator (Fig 4) under reduced pressure and temperature (40°C) in pear shaped flask. The concentrated extracts were dried. The dried extracts were stored in a wide-necked container at -20°C in a deep freezer until further use.

Experimental animals

Eighty-seven Swiss albino mice, weighing about 20-25 g aged between six- eight weeks were procured from Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy under Kerala Veterinary and Animal Sciences University (KVASU). The animals were housed in polypropylene cages with stainless steel grill tops, bedded with paddy husk. The animals were acclimatised for a period of one week before the start of experiment. The animals were given pure water *ad libitum* and pellet feed procured from School of Animal Nutrition and Feed Technology, KVASU. The temperature was maintained between 22 ± 2˚C and relative humidity at 70 per cent with optimal air changes per hour and illumination cycle set to 12 h light and 12 h dark. The research was approved by Institutional Animal Ethics Committee (IAEC) of College of Veterinary and Animal Sciences, Pookode (reference number-IAEC/COVAS/PKD/22/8/2024).

Anti-neoplastic activity of extracts on solid tumour induced animal

Thirty-six tumour positive mice were chosen and randomly assigned to six groups of six animals each. Six animals were used in the normal control group (Group I). Based on the acute toxicity studies, the doses were fixed. The following were the treatment groups.

The first day of oral administration of the reference drug/test substance was designated as day one. Progression of tumour was evaluated by measuring the tumour volume on days one, five and eleven. On day 11,

animals were sacrificed and assessed relative gene expression studies of *Bcl-2* using real time PCR were also carried out.

Fig.1 ***M. champaca***

Fig.2 ***E. aureum***

Fig.3 Soxhlet apparatus

Fig.4 Rotary vacuum evaporator

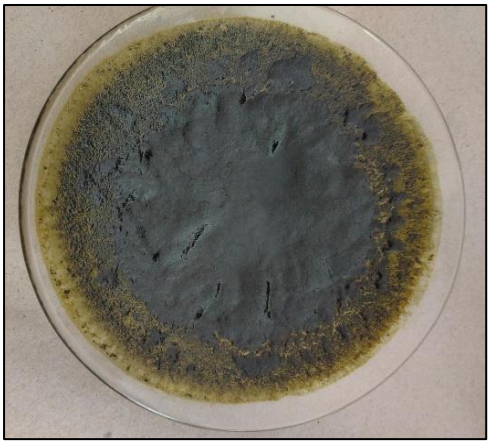

Fig.5 ***M. champaca*** **extract**

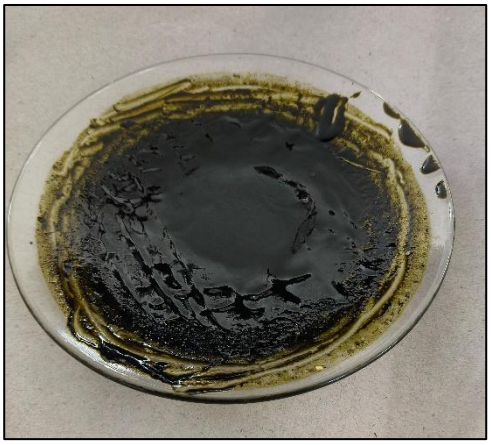

Fig.6 ***E. aureum*** **extract**

Table 1. Treatment groups

Group	Treatment
I	No treatment (Tumour control)
II	5-Fluorouracil@ 20 mg/kg orally for 10days
III	*M. champaca* @200mg/kg leaf extract orally for 10 days
IV	*M. champaca* @400mg/kg leaf extract orally for 10 days
V	*E. aureum* @200mg/kg leaf extract orally for 10 days
VI	*E. aureum* @400mg/kg leaf extract orally for 10 days

Acute oral toxicity studies

Acute oral toxicity was performed as per the Organization for Economic Co-operation and Development (OECD) guidelines 423 using acute toxic class method. The method provided information on the hazardous properties and allowed the substance to be ranked and classified according to the Globally Harmonized System (GHS) which caused acute toxicity. Six Swiss albino mice aged six to eight weeks were acclimatised for seven days. Before starting the dose, the animals were fasted overnight for food and water. The dose of 2000 mg/kg was administered orally as a single dose. The animals were observed for the toxicity signs. On the day of dosing, all the animals were observed for mortality and toxicity signs on the first 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after dosing and thereafter twice daily for mortality and once a day for clinical signs, for 14 days. There were no toxicity signs observed for the dose at 2000 mg/kg. Body weight of the animals was recorded on day zero (after overnight fasting), weekly thereafter and at termination of the study prior to sacrifice and subjecting the animals for complete gross pathological examination. Then, the compound was ranked and classified according to the (GHS) for the classification of chemicals which caused acute toxicity.

Collection of tumour mass

On day 11, after sacrificing the animals by CO_2 euthanasia, skin around the tumour mass was shaved, dissected and the tumour mass was collected in RNA later™ maintaining aseptic conditions for determining *Bcl-2* expression levels.

Relative gene expression study

The technique of real time - quantitative polymerase chain reaction (RT qPCR) was employed to study the relative expression of B-cell lymphoma-2 (*Bcl-2*) in tumour samples, keeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene.

Total RNA isolation

Total RNA was isolated from cell culture and tumour tissue samples using TRI Reagent®- RNA extraction method as per the manufacturer's protocol (M/s Sigma Aldrich, USA). Tumour samples stored in RNA later® was removed and thawed. Approximately, 100mg tissue was homogenised in one milliliter of ice-cold TRI reagent with power homogeniser and incubated for five minutes at room temperature. After incubation 0.2 mL of chloroform was added to each sample, the tubes were shaken vigorously for 15 sec and allowed to stand at room temperature for 10 min. The resultant mixture was centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase rich in RNA, was transferred to a fresh microcentrifuge tube and 0.5 mL of ice-cold isopropanol was added for precipitation of RNA. The tubes were incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was carefully decanted and RNA pellet was washed with one millilitre of 75 per cent ethanol. The samples were then centrifuged at 12,000 x g for five minutes at 4°C. The supernatant was discarded and pellet was air dried by inverting the tube on fresh paper towels for five minutes to remove traces of ethanol. The RNA pellet was dissolved in nuclease free water (40µL).

Assessment of purity and integrity of RNA isolated

After isolation, the RNA was quantified by spectrophotometric analysis with Nanodrop spectrophotometer, using the principle that one absorbance unit at 260 nm wavelength equals 40 µg RNA per µL.

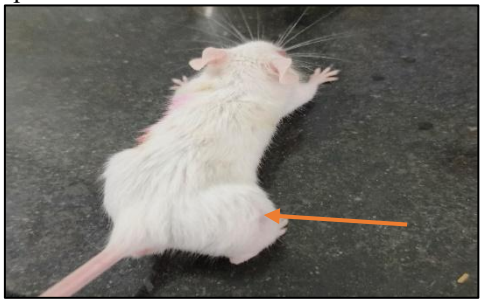

Fig.7 Tumour positive animal

The concentration of total RNA per microliter of the sample was obtained by placing one microliter of the sample into a nanodrop spectrophotometer after calibrating with nuclease free water (NFW) as blank. The purity of total RNA was assessed by using the ratio of absorbance of the samples taken at 260 nm, 280 nm and

230 nm in spectrophotometer. The samples showing A260/A280 and A260/A230 ratios between 1.8 - 2.0 or above were considered of ideal purity and taken for further study.

Complementary DNA (cDNA) synthesis

The cDNA synthesis was carried out from total RNA using a verso cDNA synthesis kit as per the manufacturer's protocol. After thawing, mixed and briefly centrifuged all components of the kit and kept in ice. Ten microlitres of NFW were added to a 0.2 mL PCR tube. The template RNA (volume to get 500 ng) and one microlitre random hexamer to each tube, were added, mixed gently and the tubes were snap chilled on ice. To this, four microlitres of 5X cDNA synthesis buffer, two microlitres of dNTP mix and one microlitre each of RT enhancer and enzyme mix for each tube were added. The total reaction volume was made to 20 µL using sterile NFW, mixed gently and spun at 1000 rpm for about two minutes. Then the tubes were incubated at 42°C for 60 min. The reaction was terminated by heating the reaction mixture at 95°C for five minutes. The product was stored at -80°C until use.

Designing and selection of primers

Gene specific primers (table 2) were designed using online "Primer 3" primer design software (Primer3, http://bioinfo.ut.ee/primer3/) and specificity was checked using Primer3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer blast/).

Synthesis and dilution of the primers

The primers were obtained from Integrated DNA Technologies, India in lyophilized form and were centrifuged prior to opening to prevent the loss of contents. To prepare stock solution of the primers, they were reconstituted in sterile NFW to a concentration of 100 µM. The solutions were incubated at room temperature for one h and then stored at -20^{o}C. Working solutions of each primer was prepared by mixing five microlitres of stock solution to 45 µL of NFW to obtain a working solution concentration of 10 µM.

Standardisation of pcr conditions using gradient pcr

Optimum annealing temperature for the primers to amplify *Bcl-2* and *GAPDH* gene was determined by gradient PCR (Biorad, M/s Thermal cycler, USA). The conditions were optimised by setting different temperatures for annealing process. The temperature that gave the best result for amplification was taken for further studies. Reaction mix for gradient PCR reaction. The PCR reaction mix used are given in table 3. The tubes were spun briefly and placed in a thermal cycler. The details of gradient PCR conditions for amplification are given in table 4. The amplified products were stored at -20°C till further processing.

Checking for amplification of target DNA

The amplified products were checked in 1.5 percent agarose gel (1X TBE) electrophoresis. Five microlitres of the product were mixed with one microliter of 6X gel loading dye and loaded into the wells carefully. As a molecular size standard, one microlitre of 100bp DNA marker was also loaded into one of the wells. Electrophoresis was then carried out at 50V until bromophenol blue dye migrated more than a four-centimetre length of gel in 1X TBE buffer. The gel was visualised under a UV transilluminator and documented in a gel documentation system (BioRad, Gel Doc 2000TM).

Real time – quantitative PCR (RT-QPCR)

The expressions of *Bcl-2*and *GAPDH* were studied using SYBR green chemistry (Maxima SYBR green qPCR master mix) (M/s Thermo scientific, USA). The reaction was carried out in triplicates (Table 5). The expression of target gene *Bcl-2* was compared with a reference gene *GAPDH* to calculate ΔCt and the expression of same gene in treatment sample versus control samples to calculate ΔΔCt. The method used is referred to as comparative Ct method.

Table 2. Description of primers used

Gene	Primer sequence (5"- 3")	size
GAPDH	F: TCTCCACTTTGCCACTGCAA	65
	R: GAACGGATTTGGCCGTATTG	
Bcl-2	F: GTCCCGCCTCTTCACCTTTCAG	148
	R: GATTCTGGTGTTTCCCCGTTGG	

Table 3. Reaction mix for gradient PCR reaction

Components	Volume (µL)
Template (cDNA)	1.00
PCR Master mix	10.00
Forward primer (10 pM/µl)	1.00
Reverse primer (10 pM/µl)	1.00
Nuclease free water	7.00
Total	20.00

Table 4. Gradient PCR conditions for amplification

Sl. No.	Steps	Temperature		Time
1.	Initial denaturation	95ºC		5 min
2.	Denaturation	95ºC		45 sec
3.	Annealing	*Bcl-2*	63˚C to 70.2°C	30 sec
		GAPDH	54.65˚C to 64.65˚C	
4.	Extension	72ºC		1 min
5.	Step 2 to 4 repetition → 34 cycles			
6.	Final extension	72ºC		10 min

Separate PCR reactions were set up for genes. Each sample was amplified in triplicate (technical replicates). In addition, one non-template control (NTC) for each gene and negative control (with only NFW) were also included in the reaction. A suitable plate setting was done before the start of the experiment. master mix with template DNA was loaded into the designated well of PCR strips (8 tubes/strip). The strips were centrifuged at 250 g for 30 sec and were placed in a StepOne Plus® Realtime Thermal cycler. The thermal cycler was pre-programmed for temperature and cycling conditions specified in table 6.

Fluorescence signals were measured in each cycle. For each sample, the curve was generated after the completion of amplification and was analysed in positive and negative controls to detect the specificity of PCR reaction.

Melt curve analysis

A melt curve analysis was performed after the reaction for checking the specificity of amplification. The programme for melt curve analysis consisted of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec followed by 95°C for 15 sec. Data acquisition was performed during the final denaturation step.

Relative quantification

Relative quantification describes the change in expression of the target sample relative to some reference group such as an untreated control. *Bcl-2* quantification was done by Ct (Cycle Threshold) comparative method and was expressed as 'n' fold up-regulation of the transcribed gene to the calibrator. For relative quantification by comparative method, the values were expressed relative to the control sample called the calibrator. *GAPDH* was used as the internal control. The Ct of the target gene and Ct of the control gene were determined for each sample and calibrated (ΔΔCt method).

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (test sample)} - \Delta Ct \text{ (control sample)}$$

$$\text{Relative quantification, } RQ = 2^{-\Delta\Delta Ct}$$

Statistical analysis

All results were expressed as Mean ± SE with 'n' equal to the number of replicates. All the statistical analysis was conducted using statistical package for social sciences (SPSS) software version 24. The intergroup comparison was assessed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison for pairwise analysis. Analysis of variance (ANOVA – one way) followed by Tukey's multiple comparison tests was used to compare the significant differences among various treatments on relative gene expression.

Table 5 Optimised concentrations of RT-qPCR Master Mix

Components	Volume (µL)
Template (cDNA)	1.0
Maxima SYBR Green qPCR Master Mix (2X)	6.25
Forward Primer (10 pM/µL)	0.5
Reverse primer (10 pM/µL)	0.5
NFW	4.25
Total	12.5

Table 6. RT-qPCR conditions for *Bcl-2* and *GAPDH* gene

Steps		Temperature		Time
Initial denaturation		95°C		3 min
30 cycles of	Denaturation	*Bcl- 2*	63°C	30 sec
	Annealing	*GAPDH*	61°C	
Extension		72°C		35 sec

Results

Relative gene expression study

The relative expression of *Bcl-2* in tumour tissue samples collected from control and treatment groups were analysed by real time - quantitative polymerase chain reaction (RT-qPCR).

Total RNA isolation

The samples were found to have total RNA concentration of 200-500 ng/µL with A_{260}/A_{280} and A_{260}/A_{230} ratios around 1.8 – 2.0. This indicated acceptable purity and was used for c-DNA synthesis.

Standardisation of PCR conditions using gradient PCR

The optimum annealing temperature for the genes *Bcl-2* and *GAPDH* (housekeeping gene) were standardised by employing gradient PCR. Annealing temperature that gave the best result for amplification by gradient PCR was selected for real time PCR.

Real-Time quantitative PCR (RT-qPCR)

The relative gene expression of *Bcl-2,* in tumour tissue of animals exposed to various treatments was compared to control, keeping *GAPDH* as the reference gene. The results are presented in table 7 and figure 8. The melt curves of the genes in real time PCR of tumour samples are depicted in figure 7. The expression of *Bcl-2* in control group was normalised to unity. In comparison with control, the expression of *Bcl-2* was significantly ($p<0.05$) downregulated in all treatment groups. The fold change in expression of *Bcl-2* in group-II, group-III, group-IV, group- V and group- VI were 0.762 ± 0.56, 0.218 ± 0.05, 0.272 ± 0.04, 0.373± 0.08 and 0. 487± 0.16 respectively. The group treated with lower dose *M. champaca* showed a significant ($p<0.05$) decrease in relative expression when compared to other groups. Among the two doses, lower doses of both plants showed significant ($p<0.05$) downregulation than higher doses.

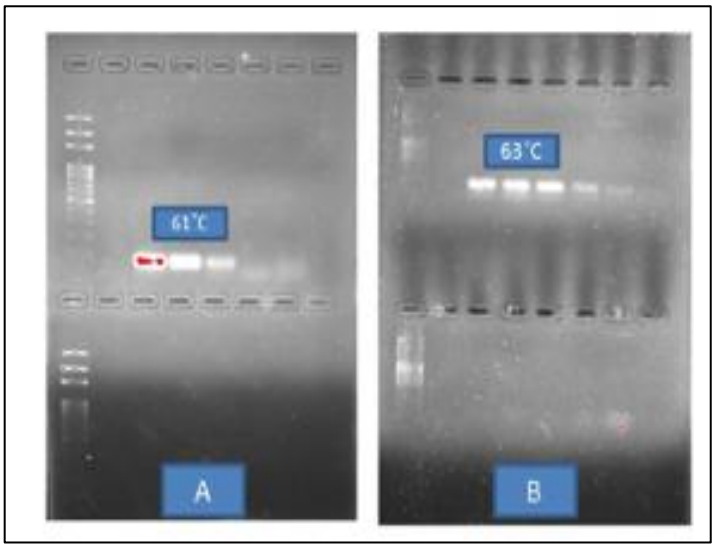

Fig 6. (A). *GAPDH* showing maximum amplification at annealing temperature 61°C;
(B). *Bcl-2* showing maximum amplification at annealing temperature 63°C.

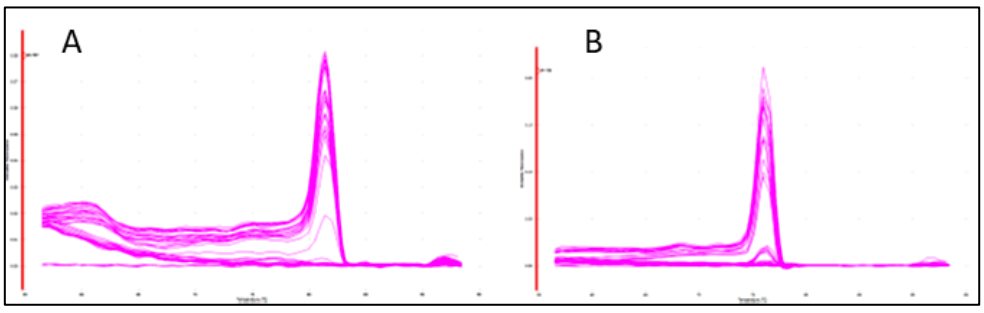

Fig 7. Melt curve for *GAPDH* (A) and *Bcl-2* (B) gene

Table 7. Relative gene expression of *Bcl-2*

Groups	Fold change
I	1[a]
II	0.762 ± 0.56[b]
III	0.218 ± 0.05[f]
IV	0.272 ± 0.04[e]
V	0.373 ± 0.08[d]
VI	0. 487± 0.16[c]

Values expressed as mean ± SEM, n=3, Means bearing similar superscript (a-f) within each column do not differ significantly at ($p<0.05$)

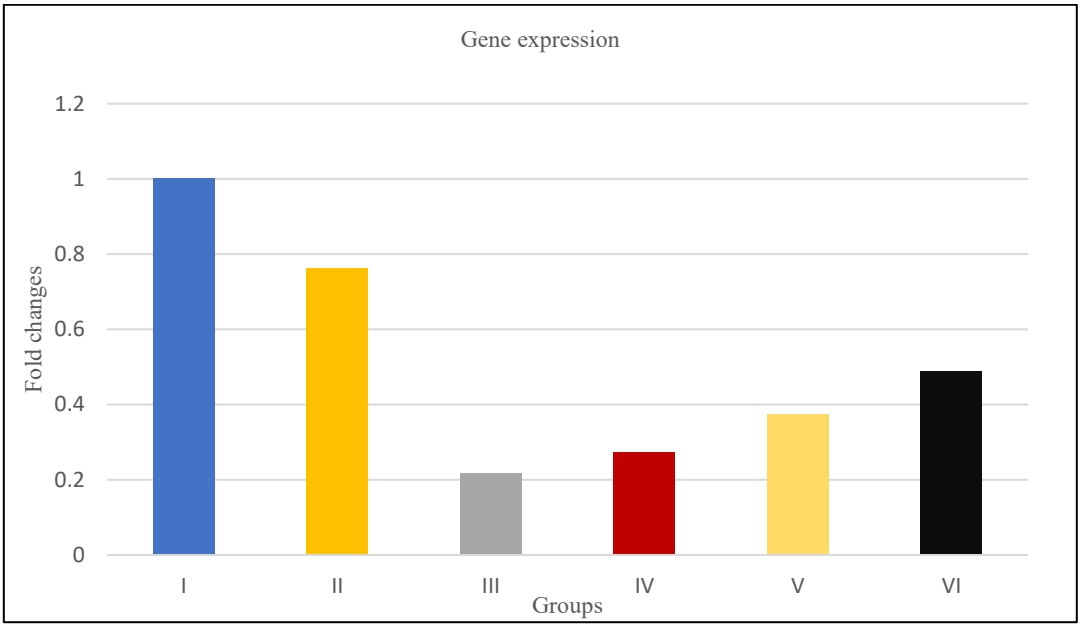

Fig 8. Relative gene expression of *Bcl-2*

Discussion

Apoptosis, or programmed cell death, plays a crucial role in regulating cell growth and tissue development. The disruption of apoptosis can lead to the onset, growth and advancement of tumours. Therefore, targeting the mechanisms of apoptosis offers a promising approach to creating new cancer treatments that can effectively hinder tumour development (Fiandalo and Kyprianou, 2012).

The Bcl-2 family of proteins is comprised of pro-apoptotic (eg. Bak, Bax) and anti-apoptotic (eg. Bcl-2) proteins that play a pivotal role in the regulation of apoptosis, especially *via* the intrinsic pathway at the mitochondria level. This study evaluated the relative expression of *Bcl-2* in tumour tissue in response to various treatments, compared to the control, using *GAPDH* as the reference gene. In comparison to the control, *Bcl-2* expression was significantly downregulated in all treatment groups. Notably, the group treated with the lower dose of *M. champaca* exhibited a significant reduction in relative *Bcl-2* expression compared to the other groups. This study highlights the remarkable pro-apoptotic activity of the plant extracts through the downregulation of the anti-apoptotic *Bcl-2*. Lalremruati *et al*. (2022) observed similar downregulation of *Bcl-2* gene expression in the treatment groups compared to the untreated group.

The results of the present study suggest that lower doses of both extracts exerted a more pronounced effect compared to higher doses. This finding aligns with previous research showing that certain plant extracts demonstrate significant anti-proliferative effects at lower IC_{50} values. Tesfaye *et al.* (2021), reported that, methanol extracts of *Euphorbia. schimperiana* exhibited IC_{50} values ranging from 1.85 ± 0.44 to 3.28 ± 1.2 µg/mL against A427, SiSo and RT-4 cell lines and *Kalanchoe crenata* possessed potent cytotoxic activity, at IC_{50} value of 2.33 µg/mL against mesothelioma, further highlighting the effectiveness of lower concentrations in eliciting anti-cancer effects. However, further studies on the efficacy of low doses are needed to fully explore therapeutic potential of MMC and MEA for development as cancer treatment agents.

Conclusion

Acute oral toxicity study identified *M. champaca* and *E. aureum* as safe antineoplastic agents. Results of the current study revealed both *M. champaca* and *E. aureum* leaf extracts exhibited antineoplastic potential in DLA cell induced solid tumour in mice. Relative gene expression study revealed a significant downregulation of the antiapoptotic gene, *Bcl-2*. MMC was found to be comparatively more potent than MEA.

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