

Methanol Extract of *Caesalpinia bonducella* Induces Apoptosis via Up-Regulation of Bax and Activation of PARP in Ehrlich Ascites Tumor Cells

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Abstract

Nature has endowed plants with the power to cure multitude of diseases. In case of cancer treatment, the search for natural agents with potential antitumor effect has been intensified with time. Here we are reporting analysis of methanol extract of *Caesalpinia bonducella* (MECB) for its anti-proliferative and pro-apoptotic effect on Ehrlich ascites tumor (EAT) model *in-vivo*. MECB at 200 mg/kg concentration was responsible for decrease in the total percent of viable EAT cells (51.6%) and ascites volume (65%) in treated mice. MECB increased the rate of apoptosis with more number of cells exhibiting characteristic features such as membrane blebbing, apoptotic body formation and fragmented DNA which was evident by Giemsa and Acridine orange/Ethidium bromide (AO/EB) staining. Alongside, the mice survival time was increased upon MECB treatment. In addition, FACS data suggests that death of MECB treated EAT cells was due to apoptosis and not by necrosis. Furthermore the analysis for molecular mechanism revealed MECB decreased the level of anti-apoptotic Bcl-2 expression while increasing pro-apoptotic Bax level. The immunoblot analysis confirmed the activation PARP (Poly (ADP-ribose) polymerase), a substrate for executioner caspase-3, which brings about subsequent DNA fragmentation in apoptotic process. These results confirm the anti-proliferative and pro-apoptotic activity of MECB, which may further translated into therapeutic drug development to combat cancer.

Keywords: Methanol extract of *Caesalpinia bonducella* (MECB); Ehrlich ascites tumor (EAT); Bcl-2; Bax; PARP

Introduction

Cancer is a multifactorial disease having both genetic factors and environmental risk factors together contributing for the development of disease. Cancer has become second leading cause of death worldwide with higher incidence and mortality rates observed each year, especially in low and middle income countries. As a fact, one in seven deaths globally is attributed to cancer [1,2]. The recent global cancer estimates suggest that nearly 14 million people are diagnosed with cancer in a year and it is been predicted that the number of cancer cases are going to increase up to 19 million by 2025, 22 million by 2030 and 24 million by 2035 [3]. As a consequence, the global market for anti-cancer drugs was soared up to the value of \$91 billion in 2013 up from the previous \$71 billion in 2008 [4]. Chemotherapy is proved to be an effective and reliable treatment option in cancer therapy [5] which makes use of multiple drugs and hormonal agents alone or in combination [6]. Besides being toxic to cancer cells, the synthetic drugs used for chemotherapy also show lethal effect on normal cells due to their nonspecificity, which just act on dividing cells [7]. The action of these anti-cancer drugs manifests serious side-effects such as dry flaky skin, loss of hair, nausea and vomiting, changes in taste and appetite, blood clotting problems and may cause permanent damage to the kidneys, heart, lungs or reproductive system occasionally [8]. Naturally occurring phytochemicals such as vitamins, flavonoids, alkaloids, diterpenoids, and polyphenols from plants possess multitude of pharmacological effects which have been proved to be potent chemopreventive agents [9]. As a result, 74% of all anticancer drugs currently being used are of natural origin [10]. Yet the quest for medicinal plants with the effective anticancer property is intensified with time.

Caesalpinia bonducella of family Fabaceae (Caesalpiniaceae) is a prickly shrub or woody vine which is worldwide in its distribution [11]. It is widely used in ayurveda - The traditional Indian system of

medicine for treatment of variety of diseases such as inflammation, leprosy, malaria, hydrocele etc., [12]. The scientific studies have shown that different parts of the plant possess various pharmacological effects such as antitumor [13], anthelmintic, anti-amoebic and anti-estrogenic [14], analgesic, antipyretic [15], antiasthmatic [16], antibacterial, antidiarrhoeal, cytotoxic effect [17], Immunomodulatory activity [18].

The phytochemical profile of the *C. bonducella* confirms the presence of glycosides, alkaloids, cassane/voucapane diterpenoids such as caesalpin, β -caesalpin, α -caesalpin in seeds, bitter glycoside bonducin, sitosterol and heptacosane in seed kernel, cassane diterpene, caesaldekarsins -F, -G, and -C, caesalpinin, bonducellipins -A, -B, -C and -D in roots, homoisoflavonoids, 6-o-methylcaesalpinianone and caesalpinianone in bark, pinitol, brazillan and bonducin in leaves [12]. By considering all these facts, an effort was made to evaluate anti-proliferative and pro-apoptotic activity of MECB in Ehrlich ascites tumor (EAT) model and to understand the possible mechanism of cellular apoptosis.

Materials and Methods

Materials

Female Swiss albino mice (8-10 weeks old) were procured from

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Department of Biotechnology and Zoology, University of Mysore, Mysore, India. Ehrlich ascites tumor (EAT) cells were routinely maintained in our laboratory through *in-vivo* transplantation. Antibodies against Bcl2, Bax, PARP (Poly (ADP-ribose) polymerase) and β -actin were purchased from Cell Signaling Technology, USA. TRIzol reagent and Super-script III one-step RT-PCR system were obtained from Invitrogen, USA. Tetramethylrhodamine methyl ester (TMRM, $\geq 95\%$) was from Sigma-Aldrich. DMSO (Dimethyl sulphoxide) (cell culture grade) and all other chemicals, reagents were obtained from Hi-media, India.

Plant extract preparation

C. bonducella leaves were collected from Western Ghat region of Shimoga district in Karnataka. Dried leaf powder (25 g) was extracted in methanol at 50°C for 12 h (Soxhlet extraction). The solvent was subjected for evaporation at room temperature to get concentrated plant extract and the final yield was noted (4.1 g). Finally, the extract was dissolved in 1% DMSO for the treatment.

In-vivo EAT cell maintenance and treatment with MECB

Ehrlich ascites tumor (EAT), the murine mammary adenocarcinoma cells were grown in 8-10 weeks old female Swiss albino mice by i.p. injections (5×10^6 cells /mouse). The mice body weight was monitored regularly for the growth of tumor cells in the peritoneal cavity. After six days of inoculation, the mice were divided in to two groups as control and test. The test group mice received three doses of treatment (i.p.) with MECB (200 mg/kg) on every alternative day starting from day 6 till day 12, whereas control mice received no treatment. On day 13, mice were sacrificed by cervical dislocation and EAT cells along with ascites fluid were collected by making small cut at the peritoneal region, fluid was centrifuged at 2500 rpm for 5 min and the ascites volume was noted down. The cells were washed with physiological saline thrice and used for further analysis. The effect of MECB on cell viability was assessed by trypan blue dye exclusion method [10].

Mice survival analysis

The anti-tumor effect of MECB was also measured by means of survivability time. EAT bearing mice were divided in to two groups of control and treated. At least 10 mice were maintained in each group. The test mice received MECB (200 mg/kg) i.p. and control mice were kept untreated. Mice were monitored regularly with the records of their body weight as long as all mice were succumbed to death.

Apoptotic cell morphology assessment

Apoptotic cell morphology was studied by Giemsa stain and Acridine orange/ Ethidium bromide (AO/EB) double staining method. Briefly, EAT cells were collected by sacrificing both control and MECB treated mice after completion of treatment. Cells were washed several times with physiological saline and fixed in methanol:acetic acid (3:1, v/v). Thin smear of cells was made on glass slide and stained with Giemsa, slides were dipped in distilled water to remove excess dye and observed under light microscope. For acridine orange/ Ethidium bromide staining, the cells were treated with 100 μ g/mL AO/EB mixture and observed under fluorescence microscope [19].

Analysis of DNA ladder formation

DNA was isolated according to the procedure reported previously [20]. The cells were lysed with SDS (Sodium dodecyl sulfate, 0.5%) at 37°C and followed by treatment with potassium acetate (8M) for 1 h at 4°C. Cell suspension was centrifuged at 7000 rpm for

60 min, the supernatant obtained was subjected for washing with phenol:chloroform:isoamyl alcohol mixture (25:24:1) for thrice. At the final step of washing, the supernatant was treated with 20 μ g/mL RNase at 37°C and was followed with DNA precipitation in chilled ethanol. Finally, the DNA pellet was dissolved in $T_{10}E_1$ buffer for quantification and analysis. The DNA was subjected for agarose gel electrophoresis (1.2%) to assess the characteristic ladder pattern [21].

Detection of apoptosis by flow cytometry

Apoptosis induction by MECB in EAT cells was determined by flow cytometry as reported earlier [22]. Cells were assessed for the loss of mitochondrial membrane potential by staining with a fluorescent dye, tetramethyl-rhodamine methyl ester (TMRM). Cells which were positive for TMRM were considered to have intact mitochondria and the cells with lower fluorescence due to the loss of mitochondrial membrane integrity were served as a reliable marker for apoptosis. Control and MECB treated cells were harvested and washed with cold PBS (phosphate-buffered saline), then were stained with 100 nM TMRM (Molecular Probes-Invitrogen) for 15 minutes at 37°C. Finally the cells were washed with cold PBS and analyzed using BD FACS Calibur (BD Biosciences).

RT-PCR for gene expression analysis

Total cellular RNA was extracted from the cells using Trizol reagent (Invitrogen), Gene expression analysis was performed using Invitrogen Super-script III one-step RT-PCR system. 1 μ g RNA was subjected for cDNA synthesis using primer sequences for Bcl-2 (Product No. B9179) forward sequence: 5'-CCT GTG GAT GAC TGA GTA CC-3'; Reverse sequence: 5'-GAG ACA GCC AGG AGA AAT CA-3', Bax (Product No. B8304) forward sequence: 5'-GTT TCA TCC AGG ATC GAG CAG-3'; Reverse sequence: 5'-CAT CTT CTT CCA GAT GGT, GA-3' and GAPDH (Product No. P7732) forward sequence: 5'-TGC MTC CTG CAC CAC CAA CT-3'; Reverse sequence: 5'-YGC CTG CTT CAC CAC CTT C-3' (M = A or Y = T or C). GAPDH was used as internal control.

Western blot analysis

Western blot analysis was performed following the methods reported elsewhere [22]. According to the method, total protein from the cells was isolated by using lysis buffer containing 0.02 M Tris, 2mM EDTA, 1% Triton X-100, 0.02mM phenylmethylsulfonyl fluoride, 0.1mM NaF, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin. Protein was quantified by Bradford protein assay and equal quantity of protein was resolved on 12% SDS-polyacrylamide gel electrophoresis. Protein was transferred on to a PVDF (Polyvinylidene difluoride) membrane and incubated with specific primary and secondary antibodies. The protein bands were detected by ECL plus kit (GE Healthcare).

Results and Discussion

MECB exerts anti-proliferative and cytotoxic effect on EAT cells *in-vivo*

To ascertain the anti-proliferative effect of MECB, EAT cells were grown in the peritoneal cavity of mice by injecting 5×10^6 cells/mouse (i.p.) and treated with test compound after 6 days of transplantation at the log phase of cell growth. Three doses of MECB (200 mg/kg body weight) was given every alternative day starting from 6th day, untreated mice were used as control. Mice body weight was monitored every day. As shown in Figure 1A there was considerable decrement in mice body weight upon MECB treatment when compared to untreated control

group where gradual increase in mice body weight was observed during the treatment period. The increasing body weight of tumor bearing mice is owing to accumulation of ascites fluid and proliferating EAT cells [23]. Hence the effect of MECB on ascites secretion was assessed (Figure 1B). The significant reduction in the ascites volume in treated mice (65%) in comparison with control mice was observed. The effect of MECB on EAT cell viability and their number was evaluated by trypan blue dye exclusion method. As a result of cytotoxic effect of MECB the great reduction by 51.6% in total number of viable cells was observed in treated mice when compared to control mice (Figure 1C).

Prolongation in mice survival time upon MECB treatment

Figure 2 illustrates that as a result of rapid proliferation of tumor cells in the peritoneal region, gradual increment in mice body weight was observed after of transplantation with EAT cells (i.p.). This was also evident from the observation of mice morphological appearance (Figure 2A). In comparison, the control mice gained significant amount of weight throughout the treatment period where as the EAT-bearing mice treated with MECB showed tremendous reduction in their body weight suggesting the anti-proliferative effect of plant. In addition, MECB considerably increased the survival time of EAT bearing mice up to 34 days but all control mice were died after 14 days of transplantation due to increase of ascites burden (Figure 2B).

MECB induce EAT cell death *in-vivo* by means of apoptosis

As a part of cancer treatment, it is imperative to induce apoptosis in tumor cells. Hence the pro-apoptotic effect of MECB was analysed by performing staining techniques and DNA fragmentation assay. Figure 3A and 3B depicts Giemsa staining and EB/AO staining respectively. MECB treated EAT cells displayed typical apoptotic morphologies such as cellular shrinkage, chromatin condensation and formation of apoptotic bodies in comparison with intact control cells. Further, the effect of MECB on induction of EAT cell apoptosis was confirmed by resolving DNA from both control and treated cells on agarose gel. MECB induced apoptosis was confirmed from the characteristic DNA ladder pattern which is one of the hallmark of apoptotic cells (Figure 3C).

Influence of MECB on apoptotic rate of EAT cell

In the intrinsic pathway, DNA damage induces release of cytochrome c from the intermembrane space leading to failure in maintaining the mitochondrial membrane potential and ATP synthesis [24]. At the onset of apoptosis, activated caspase-3 cleaves PARP, which results in the formation of PARP cleavage products, a hallmark of apoptosis [25,26]. In the intrinsic or mitochondrial pathway of apoptosis, caspase activation is closely connected to the permeabilization of the outer mitochondrial membrane [27]. The changes in mitochondria

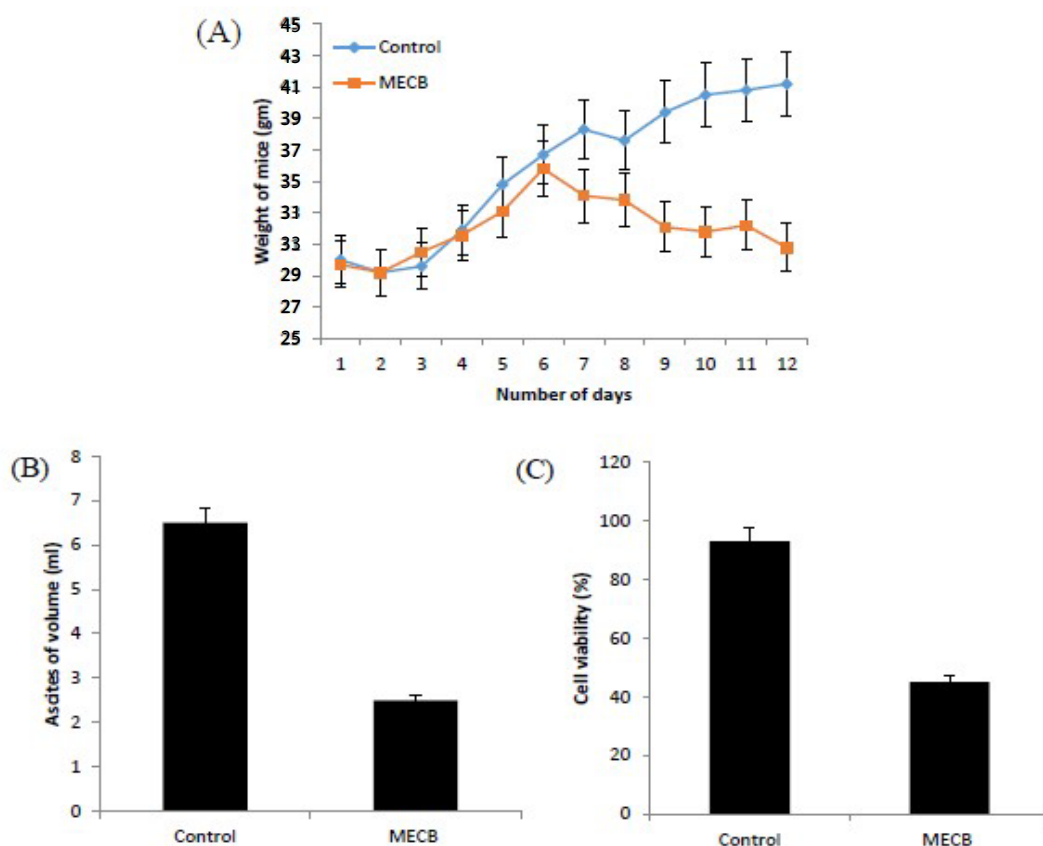


Figure 1: In-vivo anti-proliferative effect of MECB on EAT cell growth: Three doses of MECB (200 mg/kg body weight) was given every alternative day starting from 6th day of transplantation with 5×10^6 cells/mouse i.p., untreated mice were used as control. Minimum of five mice were maintained in both control and treated group. (A) Showing the effect of MECB on mice body weight. Significant decrease in mice body weight was observed upon treatment. (B) MECB reduces ascites secretion in treated mice (65%) in comparison with control mice. (C) Trypan blue dye exclusion method reveals that MECB was responsible for considerable decrement in total percent of viable cells.

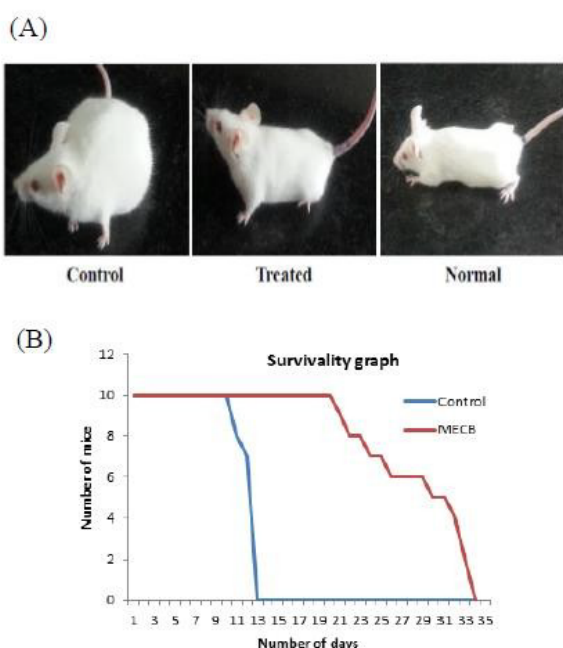


Figure 2: Effect of MECB on mice survival time: Mice were injected intraperitoneally with 5×10^6 cells, treatment with MECB was started from 6th day till 12th day in an alternative manner, mice without treatment were considered as control. (A) Picture shows the effect of MECB on mice morphological appearance. (B) After treatment both control and treated mice were kept under observation for the effect of MECB on mice survival time. As depicted in graph the plant increased the survival time of treated mice up to 34 days in comparison with control group which succumb to death after 14 days of transplantation.

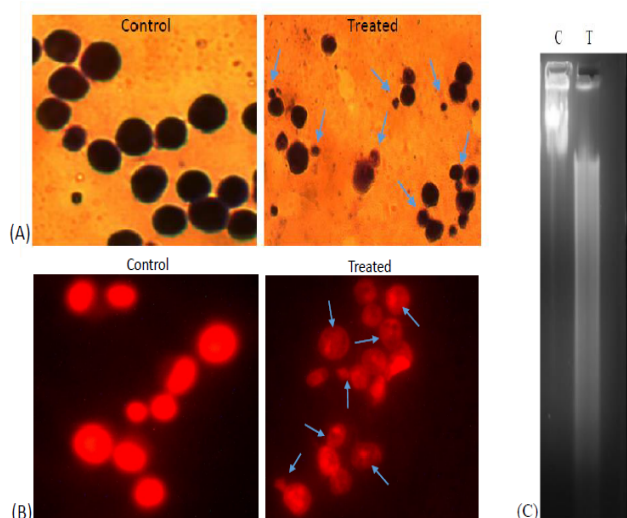


Figure 3: MECB induces apoptosis of EAT cells. Pro-apoptotic effect of MECB was analysed by staining techniques and DNA fragmentation assay after treatment. (A) and (B) Illustrates Giemsa and EB/AO staining respectively. Cells are exhibiting typical apoptotic morphologies such as cellular shrinkage, chromatin condensation, and apoptotic bodies upon treatment when compared to control cell morphology. (C) DNA from the cells of both control and treated mice was isolated and separated on 1.2% agarose gel. As shown in the Figure MECB induce fragmentation of DNA which is one of the hallmark of apoptotic cells where C: Control; T: MECB treated.

membrane potential were investigated using TMRM staining. The rate of apoptosis induction by MECB was assessed by FACS analysis. EAT Cells from control and treated mice were stained with 100 nM TMRM for 15 min and pro-apoptotic effect was determined by measuring mitochondrial membrane potential. As illustrated in Figure 4, the rate of EAT cell apoptosis was remarkably higher in MECB (200 mg/kg) treated mice, over 43.7% of total cell population were observed. We found that MECB induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) in EAT cells, which indicated that the mitochondrial apoptotic death-signal pathway plays a critical role in MECB induced apoptosis in EAT cells.

Effect of MECB on Bcl-2, Bax and PARP expression in EAT cells

Bcl-2 and Bax are the important signalling factors of intrinsic apoptotic (mitochondria mediated) process [28] that regulate cytochrome *c* release, activation of caspases and DNA fragmentation [29]. Bcl-2, the member of Bcl-2 family is an oncoprotein that resist cell death where as Bax, another constituent of the Bcl-2 family acts as pro-apoptotic factor by inducing apoptosis. Studies have shown that the expression of pro-apoptotic proteins such as Bax or Bak is very necessary to induce cellular apoptosis [30]. The ratio of bcl-2 (anti-apoptotic) / bax (pro-apoptotic) majorly determines the sensitivity of a cell to death signals [31]. Alteration in the Bcl2 family protein expression has been associated with many cancer types whereas Bcl2 over expression in tumor cells is regarded as a common attribute [32]. Previous reports have shown the effects of medicinal plants in down regulating Bcl-2 protein expression in EAT cells [19,33]. To determine the effect of MECB on Bcl-2 and Bax, total cellular RNA was extracted and subjected for reverse transcription-PCR using specific primers. As it is shown in Figure 5A, MECB reduced anti-apoptotic Bcl-2 expression to great extent in EAT cells while upregulating the expression of pro-apoptotic protein Bax. Thus MECB is shown to have modulatory effect on apoptosis signalling proteins.

Poly (ADP-ribose) polymerase (PARP) is a DNA repair enzyme and is cleaved by activated effector caspases-3, activated PARP brings about subsequent DNA degradation in apoptosis process [34]. To determine the involvement of MECB in caspase induced DNA fragmentation immunoblot analysis was performed. Figure 5B reveals that MECB induce the cleavage of PARP and confirms the likely activation of executioner caspase-3.

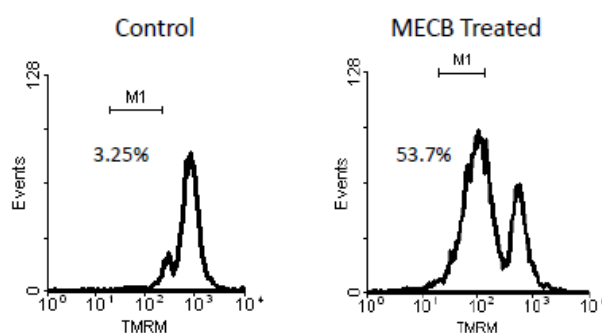
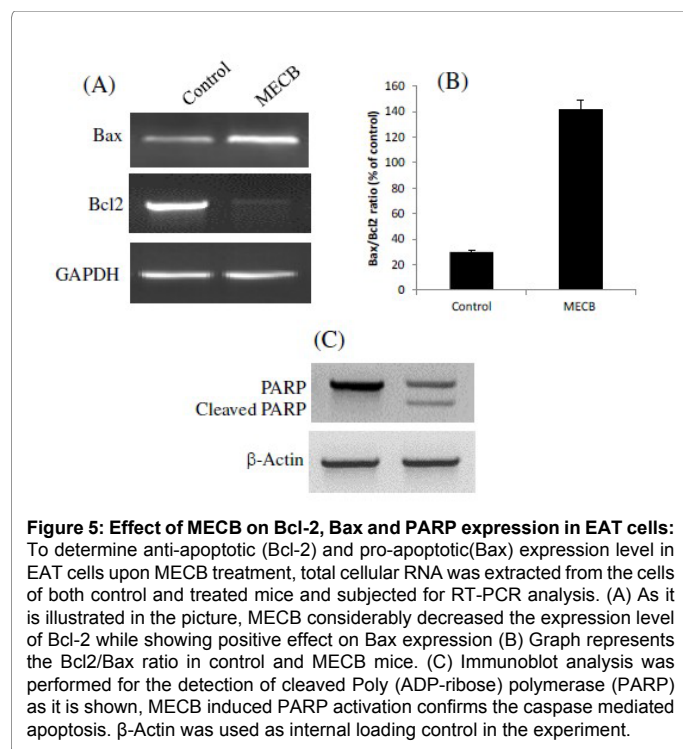


Figure 4: In-vivo effect of MECB on induction of apoptosis rate: The rate of apoptosis induction by MECB was assessed by FACS analysis. Cells from control and treated mice were stained with 100 nM TMRM. As it is illustrated in Figure, the rate of apoptosis was remarkably higher in MECB treated mice (200 mg/kg) in comparison with control mice alongside. Apoptosis was detected by measuring mitochondrial membrane potential.



Conclusion

The present study emphasizes *in-vivo* cytotoxic effect of methanol extract of *C. bonducella* leaves on EAT cell model. The experimental results shows that induction of apoptosis by mitochondrial pathway which is mediated by alteration mitochondrial membrane potential and Bax up regulation. The upregulated Bax leads to the caspase-3 activation, which in turn cleaves of PARP. Further the identification bio active components which brings cell apoptosis is essential for a promising source for developing novel therapeutics to combat cancer.

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