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RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

Biotransformation of Cardenolides from *Calotropis procera* and Their Cytotoxic Potential against Human Mammary Gland Carcinoma Cells

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10.18805/ag.D-5695

ABSTRACT

Background: *Poekilocerus pictus* (Fabricius 1771), a painted grasshopper, sequesters cardenolides from its food plant, the Apple of Sodom or Aak, *Calotropis procera* (Aiton) W.T. Aiton (Family-*Asclepiadaceae*). In our present investigation, we were able to isolate *Pseudomonas aeruginosa* KRK6 from the intestine of *Poekilocerus pictus* responsible for the biotransformation of cardenolides.

Methods: *Pseudomonas aeruginosa* KRK6 was grown in methanolic extracts of *Calotropis procera* and the modified cardenolides were detected by Liquid Chromatography-Mass Spectrometry (LCMS) and also used to induce apoptosis in cancer cells (MCF-7 cells and T-47 D).

Result: The modified cardenolides CPMEP6 was found to induce apoptosis in human breast adenocarcinoma cells (MCF cells-IC50 =6.31±0.4 µg/mL, T-47D cells-IC50 = 10.1±1.02 µg/mL). Phosphatidylserine exposure and DNA fragmentation suggested apoptosis in treated cancer cells. CPMEP6 induced apoptosis in cancer cells via the mitochondrial pathway by down-regulating BCL-2 protein expression and up-regulating BAX protein expression.

Key words: Apoptosis, Biotransformation, Cardenolide, *Calotropis procera*, *Poekilocerus pictus*.

INTRODUCTION

Cardenolides and cardiac glycosides found present in many plants in Asia and Africa (Lhinhatrakool and Sutthivaiyakit, 2006) including *Calotropis procera* (Aiton) W.T. Aiton, which are the proven anticancer agents (Juncker *et al*., 2009; Silva *et al*., 2010; Ibrahim *et al*., 2014). The painted grasshopper, *Poekilocerus pictus* contains the cardenolides in their body tissues and were sequestered from its food plant *Calotropis procera* (Aiton) (Common name-Apple of Sodom or Aak) belonging to the Family-*Asclepiadaceae* (Mathen and Hardikar, 2010). Moreover, a variety of microorganisms were found inhibited on the gut of insects, playing a major role on their internal physiology and nutrition. These intestinal microorganisms are found adapted to the gut contents of insects and helps in biotransformation of secondary metabolites obtained from the food plants regulating the internal physiology of insects (Gustafsson, 1968; Li *et al*., 2013). These intestinal microorganisms can modify the structure of secondary metabolites into more active pharmacophore units leading towards biosynthesis of clinically useful opiate drugs and new analogs in presence of specific enzymes (Long *et al*., 1995). There is a chance of apoptosis in cancer cells because of the presence of toxic cardenolides stored in the body tissues of *P. pictus* (Mijatovic *et al*., 2006, 2007; Prassas and Diamandis, 2008). *Pseudomonas aeruginosa* is one of the most abundant species found among intestinal isolates of *P. pictus*. Our present investigation aimed at isolation of intestinal flora of *Poekilocerus pictus* possibly responsible for biotransformation of cardenolides received from the host plant and its role in bringing apoptosis on cancer cells (MCF-7 and T-47D cells).

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How to cite this article: Kiran, R.K., Vinod, R.R. and Amol, R.K. (2023). Biotransformation of Cardenolides from *Calotropis Procera* and Their Cytotoxic Potential against Human Mammary Gland Carcinoma Cells. Agricultural Science Digest. doi: 10.18805/ag.D-5695.

Submitted: 30-09-2022 **Accepted:** 21-02-2023 **Online:** 02-05-2023

MATERIALS AND METHODS

Collection of plants and preparation of crude extract

The leaves and stems of *Calotropis procera* were collected from the Marathwada region of the Maharashtra State (India), sand powdered for their subsequent use for the preparation of the extract. About 50±10 mg methanolic extracts were prepared according to procedures described previously (Mijatovic *et al*., 2006; Mathen and Hardikar, 2010). Calotropis procera's shade-dried, ground-up aerial components were extracted in methanol at 60°C using a soxhlet equipment (Borosil, India). The extract was dried in desiccators, concentrated under vacuum (reduced pressure, Rotavac) and kept at 0°C for further use. This extract was identified as a Calotropis methanol extract (CME).

The dried *Calotropis procera* methanolic extract (CPME) 50 ug/ml was dissolved in water for further experimentation.

Bacterial isolates

Pseudomonas P6 was isolated from the intestine of *Poekilocerus pictus*(Kharat and Kharat, 2015). A P. pictus nymph was chosen and following a 24-hour hunger period, the insect was properly cleansed and moved to a sterile wax dish (dissection tray) filled with TC100 medium (HIMEDIA). The methods outlined by Lynn D.E. (Lynn, 1989, 1996, 2002) for *P. pictus* dissection were utilised with slight changes.

The isolated midgut was cut into multiple sections after being gently cleaned with saline (Himedia, India) and cut open with a microscissor in TC100 medium. The midgut was minced and then resuspended in 10 ml of saline.

The midgut suspension was inoculated in nutritional broth (Himedia, India) and cultured at 28°C for 72 hours to isolate the bacteria. On the nutritional agar (Himedia, India) plates, the 72-hour growth solution was disseminated after being diluted to 10-4. (Two plates for each pH 5, pH6, pH7, pH8, pH9 and pH10). The appearance of colonies was then monitored on these plates throughout a four-day incubation period at 28°C.

Biotransformation of cardiac glycosides

Pseudomonas *aeruginosa* strain P6 was inoculated and incubated for 24 h in Luria broth (Himedia, India) at pH 8.2 at 28°C. The broth culture was centrifuged at 6000x g and the pellet was washed twice with DPBS of pH 7.2 (Himedia, India) after 24 h of incubaton. CPME (1% w/v) was added to the suspension of *P. aeruginosa* strain KRK6 and incubated for 48 h at 28°C. A total of 100 µl of the culture broth was collected for 48 h at an interval of 12 h and the cardiac glycoside content was analysed using RP-HPLC.

Extraction the cardenolides

The method proposed by Kanojiya *et al*., (2012) was followed for extraction of cardenolide with some modification, wherecardenolide compounds were isolated from by adjusting the pH to 8.7 by 1M NaOH and extracted with 3 equal volumes of methanol. The organic phase was then dried with anhydrous Na_2SO_4 and the solvent was removed in *vacuo* by using rota evaporator. The extracted material 1 gm was re-dissolved in 10 ml of water.

Preparative biotransformation

The preparative scale biotransformation of cardenolides was carried out in three 1000 mL flasks, each containing 400 mL of M9-minimal medium broth. The *P. aeruginosa* strain KRK6 was incubated for 2 days before CPME (10% w/v) was added to each flask. The incubation conditions and the extraction process were the same as those described above and afforded a crude extract (3.46 g). CPMEP6. The extract (labelled as CPMEP6) was subjected to column chromatography on a silica gel column (60 g), with acetonitrile and water (6:4) as the mobile phase.

HPLC analysis

HPLC analysis of CPME and CPMEP6 was performed by an Agilent Prostar C-18 HPLC (equipped with Agilent autosampler and UV detector) using Agilent HPLC C18 column (250 × 4.6 mm, 5 µm). The mobile phase used was (A) acetonitrile (B) water. A linear gradient elution was used as a flow rate of 0.8 ml /min.The gradient was 0% to 30% B against 100% to 70% A from 0 to 14 min duration. The percentage of B was increased upto 55 till 25 min.

Integrations were performed with Agilent software and UV absorbance scans of the resultant peaks were measured between 190 and 220 nm.

ESI MS/MS method development and cardenolide analysis

CPMEP6 10 µl of the samples were analysed by Waters UPLC-TQD Mass spectrometer. HPLC mobile phases utilized were - Solution A: ACN:H_2O (5:95), Solution B: ACN, Solution C: Methanol, Solution D: 5 mM ammonium acetate (pH 6.5). The mobile phase flow rate was 0.45 ml/min and the gradient flow obtained with A:B:C:D at the ratio of 0.25:0:10:10 for 10 min on column Themo beta seal C18 100 × 2.1 cm of 3 um ID. The data so obtained were further analysed by Water's software and the extract ion current (EIC) of each cardenolide was quantified by their relativity of EIC to internal standard.

Induction of apoptosis in cancer cells

Cell culture

Human breast cancer cell lines T47D (ductal carcinoma) and MCF-7 (adenocarcinoma) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/ v^{-1}) foetal calf serum (FCS) and 2 mM glutamine. Cells were maintained at 37°C in a humidified atmosphere of air/CO_2 (19: 1) and were incubated with 1% FCS DMEM media during the experimentation.

Determination of cell viability and the MIC

CellTiter 96 ® AQueous One Solution Cell Proliferation Assay was used to evaluate the survival of CPMEP6-treated Breast cancer (MCF7 and T47D) cells (Promega, USA). Each well of a 96 well plate contained 1 × 10^3 cells and CPMEP6 at concentrations of 0.02, 4.0, 8.0, 16.4 and 64 g/mL was added to the DMEM medium (Life technologies Inc., USA). The cell viability was evaluated by MTT (5 mg/mL in PBS) after 24 hours in a CO_2 incubator at 37°C. A 96 well Multiscan Ascent was used to measure the absorbance at 490 nm (Thermo inc.USA). The percentage of live cells after treatment with CPMEP6 was compared to the percentage of live cells before treatment. The Paclitaxel were used as control drugs for the assay.

Detection of phsophatidylserine on membranes of apoptotic cells

The Annexin V-FITC Apoptosis Detection Kit (Life technologies Inc., USA.) was used for analysis of

phosphatidylserine exposure. Briefly, the cells were plated at ~ 1×10^5 cells in each well of 6 well plates in 500 µl DMEM medium with 10% v/v FBS and 0, 10, 20 µg/ml CPMEP6 was added to each well. The paclitaxel (20 µg/ml) were used as control drugs. Attune flow cytometer (Life technologies Inc., USA) was used to analyse the labelled cells and Attune cytometric software v2.1 was used to measure the results (Life technologies Inc. USA).

Expression of pro apoptotic and antiapoptotic proteins

Expression levels of RNA transcripts were quantitated in CPMEP6 treated and untreated cells. The MCF-7 and T-47D cells were plated at ~ 1×10^4 cells in each well of 12 well plates in 300 µl DMEM medium with FBS @ 10 % v/v and CPMEP6 @ 10 µg/ml was added to each well. The paclitaxel (20 µg/ml) was used as control drugs. Total RNA from cancer cells was isolated using iScript RT-qPCR sample preparation reagent (Biorad, USA). First strand cDNA was reverse transcribed from total RNA using the first strand cDNA synthesis kit (Biorad, USA). To analyse the expression of Bcl2, Bak1, Bax and GAPDH genes in the treated and untreated cells, cDNA was mixed with SYBR Green PCR master mix (Biorad, USA) and Real-time PCR was done according to kits manufacturer's instructions (Biorad, USA). Each run was subjected to a melting

Table 1: The primers used for the amplification of proapoptotic and antiapoptotic genes.

Name of the primer	Forward	Reverse
Bcl2	ATGTGTGTGGAGAGCGTCAA	ACAGTTCCACAAAGGCATCC
Bax	TTTTGCTTCAGGGTTTCATC	CAGTTGAAGTTGCCGTCAGA
Bak1	GCCTTTGCAGTTGGACTCTC	GGGTTGGGAGCAAGTGTCTA
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

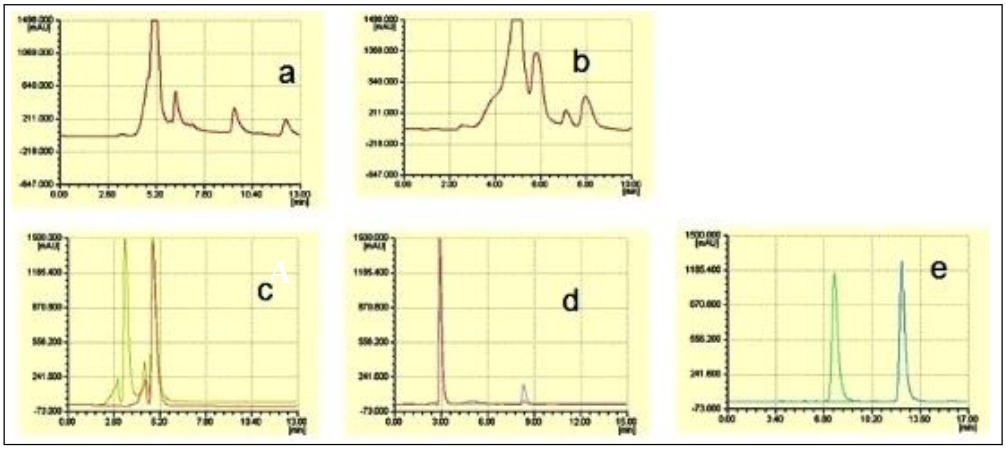

Fig 1: (a) HPLC spectra of calotropis methanolic extract for bacterial growth (b) the modification in cardenolides, (c), (d) and (e) cardenolides separated from growth medium of *Pseudomonas* P6. Mobile phase Acetonitrile: water a) b) c) 60:40, d) 80:20; e) 40:60.

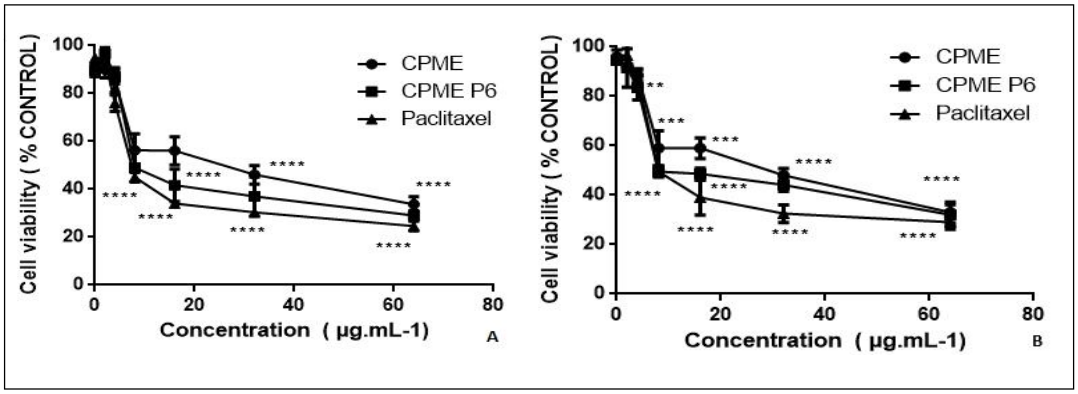

Fig 2: The cytotoxic effect of CPMEP6 on human mammary gland carcinoma cells –(A) MCF-7 and (B) T47-D. Cell viability was estimated by the MTS assay. Cells were treated with concentrations of CPMEP6 0, 5,10,20,40 µg/ml for 24 h. * P<0.05, ** P<0.01, *** P<0.001 or **** P<0.0001 vs. drug-untreated group. Data represent the mean ± S.E.M. of three different experiments with triplicate sets in each assay.

curve to confirm the amplification and all assays revealed only one peak. Table 1 lists the primers used.

Tunel assay

After 24 h of incubation in presence of CPMEP6 @ 10 µg/ml, the cells were washed twice with the DPBS. The cells were fixed in 70% (v/v) ice-cold ethanol at 4°C for 24 h. Propidium iodide (Life technologies Inc., USA) was used to dye the fixed cells, which were then passed through a 50 m filter. Apo-BRDU tunel assay was performed after staining and determined using the Attune flow cytometer (Life technologies Inc. USA).

Statistical analysis

Data on each parameter were replicated thrice and subjected to analysis of variance (ANOVA) following one way completely randomized block design. The p-values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Poekilocerus pictus feeding on *Calotropis* sp. sequesters the toxic cardenolides on its body parts during the process of digestion and absorption of food particles. Subsequently, the midgut flora of *P. pictus* get exposed to the cardenolides inherited from the food content. Previous reports revealed that the glucosinolates were degraded by gut microbiota and the hydrolytic products underwent absorption in the colon (Sikorska-Zimny and Beneduce, 2021) . This is the first report of cardenolide biotransformation by the midgut bacterial isolates of *P. pictus* .

Growth and identification of bacterial isolates

Pseudomonas aeruginosa strain KRK6 was grown in CPME containing Kings A medium, was used for the further study and the GenBank accessionnumber of the isolated *P. aeruginosa* strain KRK6 was HM366592.1.

Cardenolides from CPME

The preliminary HPLC analysis confirmed the presence of cardenolides in the medium (Fig 1 A, B and C). The major cardenolides observed in CPME were digoxin and strophanthin (Fig 1 D and E) and the bio-transformed cardenolides were presented in Fig 1 B, C and D).

ESI-MS/MS analysis

The biotransformed cardenolides by *Pseudomonas aeruginosa* strain KRK6 identified from CPME were digoxin and K-strophanthin. The main product of microbial transformation of Digoxin was reported to be D1 and the the principal bioconversion products of K-strophanthin was S1.

The main biotransformation products obtained from CPME by *Pseudomonas* sp. were D1 (18.0%), D2 (10.6%), S1 (5.8%), H1 (3.9%) and H2 (3.2%) (Fig S1 to 4). The experimental work suggested that microbial transformation of cardenolides with *Pseudomonas* sp. caused an oxidation reaction that resulted in more cytotoxic products. Similarly, the biotransformation of compounds like veraguensin, galbelgin and galgravin was reported for more bioactive derivatives. In addition, Kasahara *et al*., (1997) reported biotransformation by fungal isolates were achieved by the substitution of functional groups .

Growth inhibitory effect of CPMEP6 extract on MCF-7 and T-47D breast cancer cells

To examine the anti-proliferative action of CPMEP6 on breast cancer cells, the cells were treated with CPMEP6 for 24 h and growth inhibition was assessed by MTS assay. It was evident from our present investigation that exposure of the two breast cancer cell lines to CPMEP6, reduces the cellular viability in concentration and time-dependent manners. CPMEP6 at concentrations of 0, 1, 2, 4, 8, 10 µg/mL caused a dose- and time-dependent decrease of cell viability with IC_{50} value of 6.3±0.6 (MCF-7) and 10 ±1 µg/mL (T-47D) cells. As shown in Fig 2, CPMEP6 (10 µg/mL) caused a 50±4% and 51.3±2.2 % population of apoptotic in MCF-7 and T-47D cells, respectively, as compared to the controls. Based on the determined IC_{50} for each cell line, it appears that T-47D and MCF-7 cells exhibit a greater sensitivity to CPMEP6 compared to the CPME or Paclitaxel. In addition, cytotoxicity results indicated a higher sensitivity of MCF-7 and T-47D cells to CPMEP6 than CPME.

CPMEP6 induces apoptosis in Mammary gland carcinoma cells (MCF-7 and T-47D cells)

To analyse the mechanism of CPMEP6 induced cell death, induction of apoptosis was measured by annexin V/ propidium iodide (PI) staining, which detects the externalization of phosphatidylserine (PS), a characteristic feature of cells entering apoptosis. CPMEP6 (10 µg/ml) induces apoptosis in growing MCF-7 and T-47D cells. The live cell population of T-47D cells (85.3%) (Fig 3A) decreased to 77.2% and 63.5% in presence of the 10 ìg/ml CPMEP6 (Fig 3B) and paclitaxel (Fig 3C), respectively. About 21.4% apoptotic cells population was detected in 20 µg/ml of CPMEP6 treated T-47D cells (Fig 3B). The apoptotic cell percentage in MCF-7 was found decreased from 94.6% (Fig 3D) up to 45.2% in presence of the 10 ìg/ml of CPMEP6 (Fig 3E). However, 44.45% of apoptotic cells (MCF-7) were found in presence of the 10 µg /ml of the Paclitaxel (Fig 3F).

Role of mitochondrial membrane proteins like BCL2 and BAX in apoptosis induction by CPMEP6 in MCF-7 and T-47D cells was determined by Realtime qPCR. Several cytoplasmic proteins, particularly members of the BCL2 family, are critical to apoptosis regulation. In which, the pro-apoptotic subgroup, including BAX, Bad and Bak, promotes cell death; while the anti-apoptotic subgroup, including BCL2 and BCL-XL, inhibits apoptosis. Our data shows that CPME down regulated the expressions of these pro-survival proteins in a dose dependent manner (Fig 4).

CPMEP6 changes the ratio of the mitochondrial proteins in Human breast cancer cells. In untreated T-47D cells, the BCL2 expression was found upregulated and the BAX was found down regulated. A down regulated BCL2 expression was observed in presence of CPMEP6 than untreated T-47D

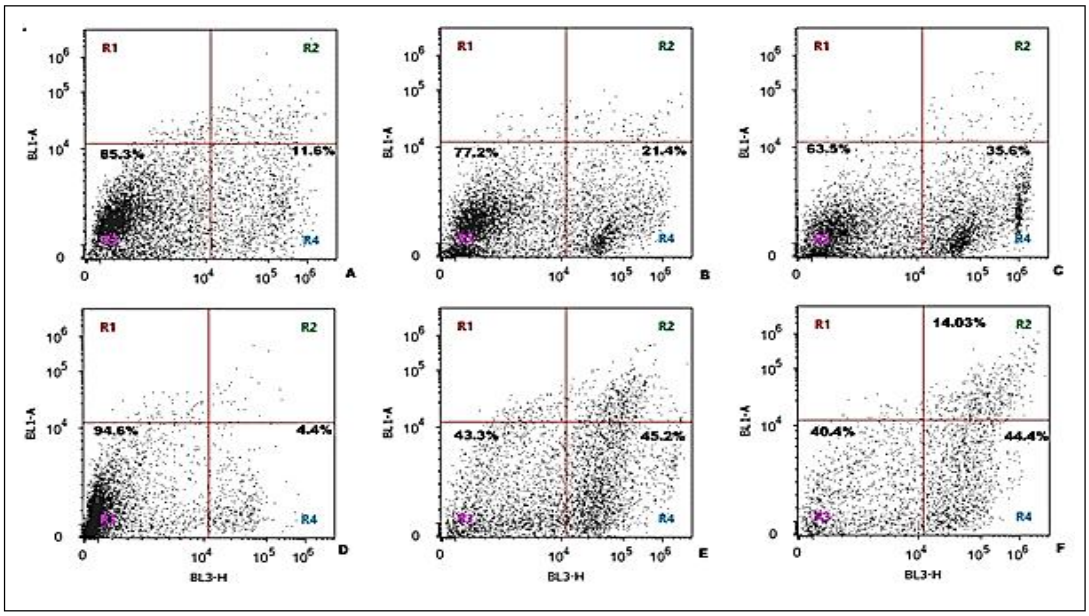

Fig 3: Apoptosis induced by CPMEP6 in Human breast cancer cells. Apoptosis in MCF- and T-47D cells was detected by flow cytometic assay of annexinV and PI. Where, (A) T47-D cells without drug, (B) T47-Dcells with CPMEP6 (C) T47-D cells with paclitaxel, (D) MCF-7 cells without drug, (E) MCF-7cells with CPMEP6, (F) MCF-7cells with paclitaxel. Numbers indicate the percentage of cells in each quadrant. Quadrant analysis of fluorescence intensity of gated cells in FL-1 (Annexin V-FITC) and FL-3 (PI) channels was from 10,000 events. The lower left quadrant (R8) shows viable cells; the lower right quadrant (R9) shows viable cells in early stages of apoptosis; the upper right quadrant (R7) shows cells in the later stage of apoptosis; and the upper left (R6) quadrant shows dead cells.

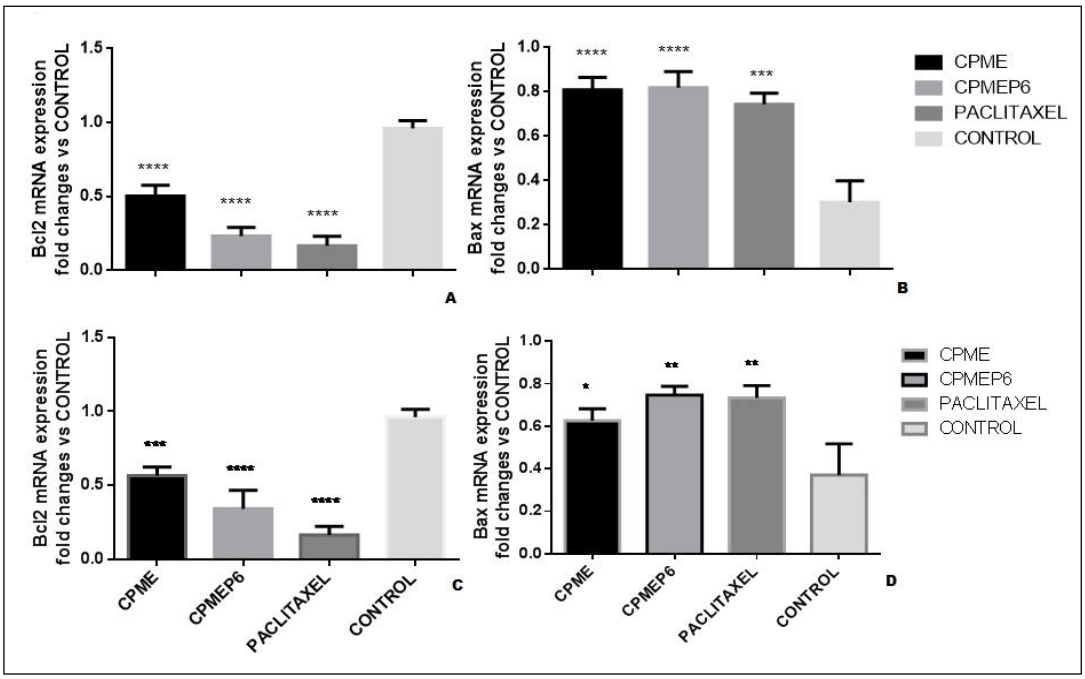

Fig 4: Upregulation of pro apoptotic proteins in CPME P6 treated human breast cancer cells where (a) T47-D (b) MCF-7. The primers used for the amplification are shown in Table 1. The bars represent the mean number of triplicates wells from three independent experiments; the statistical significance of differences between untreated and treated groups at different times was determined. Values are expressed as mean ±SD, * P<0.05, ** P<0.01, *** P<0.001 or **** P<0.0001.

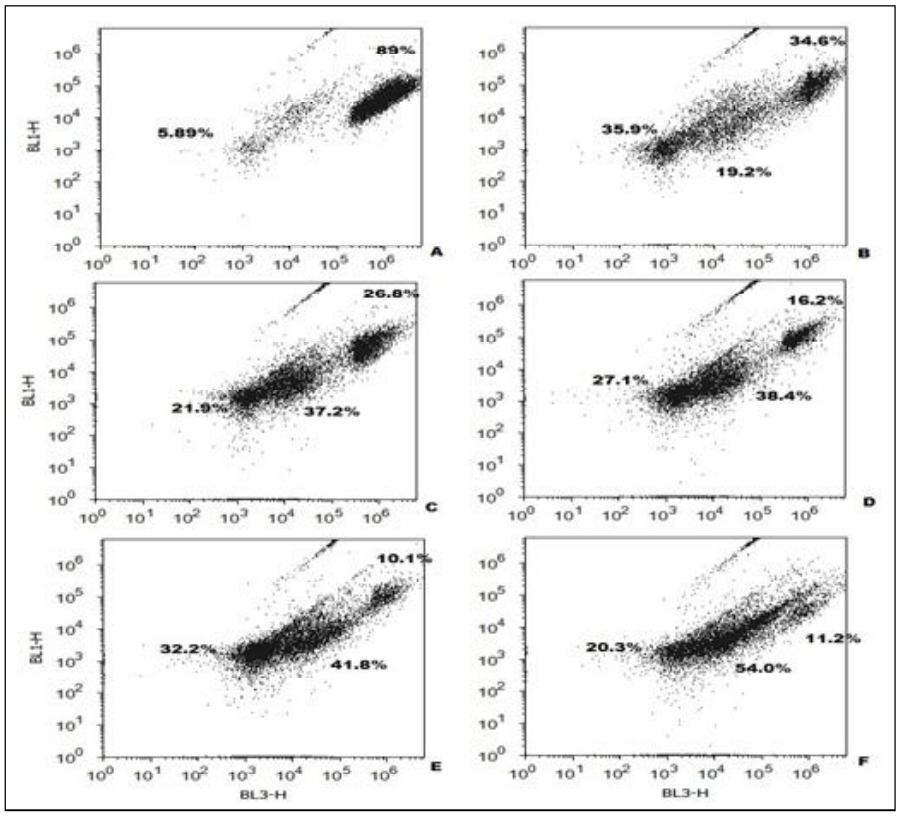

Fig 5: APOBRdU Tunel assay in MCF-7 and T47-D cells. Where (A) Negative control (B) Positive control (C) T47-D cells treated with 10 µg/ml CPMEP6. (D) T47-D cells treated with 10 ìg/ml Paclitaxel. (E) MCF-7 cells treated with 10 µg/ml CPMEP6. (F) MCF-7 cells treated with 10 µg/ml Paclitaxel.

cells (Fig 4A). Paclitaxel can induce the up regulation of the BAX in T-47D cells (Fig 4B). Upregulated proapoptotic BAX expression indicates the apoptosis in CPMEP6 treated T-47D and MCF-7 cells (Fig 4B and 4D). The low relative fold expression of the BCL2 was found in CPMEP6 treated cells than the CPME treated cells (Fig 4A and C).

DNA fragmentation (TUNEL assay) in apoptotic MCF-7 and T-47D cells

Apoptosis induced by CPMEP6 was analysed for the DNA fragmentation in T-47D and MCF-7 cells. The ApoBRdU tunel assay kit was used for the detection of fragments. TUNEL positive cells (21.9%) were found in CPMEP6 treated T-47D cells more than the negative (5.9%) control used in the experiments (Fig 5C). In MCF-7 cells treated with CPMEP6, 32.2% cells were found TUNEL positive and 41.85 cells were found in early apoptotic phase (Fig 5E).

CPMEP6 triggers the DNA degradation in apoptotic Human breast cancer (T-47D and MCF-7) cells (Fig 5 A,B,D,F). Furthermore, we found that TUNEL positive-DNA fragmentation and phosphatidylserine externalization were significantly increased in CPMEP6-treated cells in a dose-dependent manner compared to controls (Fig 5 C and E).

CONCLUSION

From our present investigation, it could be concluded that the biotransformed CPMEP6 induces the DNA degradation leading to apoptosis in Human breast cancer (T-47D and MCF-7) cells. Moreover, the TUNEL positive-DNA fragmentation and phosphatidylserine externalization were significantly increased in CPMEP6-treated cells in a dose-dependent manner as compared to the control**.** The CPMEP6 induced apoptosis in cancer cells via the mitochondrial pathway by down-regulating BCL-2 protein expression and up-regulating BAX protein expression.

ACKNOWLEDGEMENT

Authors are thankful to SERB, GOVT. OF INDIA for financial support under the project no. SR/FT/LS-11/2011.

Conflict of interest: None.

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