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Paederia foetida induces anticancer activity by modulating chromatin modification enzymes and altering pro-inflammatory cytokine gene expression in human prostate cancer cells

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ABSTRACT

Aberrant epigenetic modifications are responsible for tumor development and cancer progression; however, readily reversible. Bioactive molecules from diets are promising to cure cancer by modulating epigenetic marks and changing immune response. These compounds specifically target the activity of DNMTs and HDACs to cure various human cancers. In view of this, we investigated the anticancer and epigenetic regulatory activities of an edible-plant *Paederia foetida*. The efficacy of methanolic extract of *P. foetida* leaves (MEPL) was tested for the modulation of epigenetic factors in gene silencing, i.e. DNMT and HDAC and expression pattern of certain tumor-suppressor genes. After treatment of prostate cancer cells (PC-3 and DU-145) with MEPL, lupeol and β-sitosterol; induction of apoptosis, decrease in cellular-viability and inhibition of cellular-migration were noticed. Simultaneously there was inhibition of DNMT1, HDACs and pro-inflammatory, IL-6, IL1-β, TNF-α and anti-inflammatory, IL-10 genes in cancer and THP1 cell lines. The DNMT1 protein content, enzyme activity and Bcl2 expression decreased significantly; however, expression of E-cadherin (CDH1) and pro-apoptotic gene Bax increased significantly after the treatment of cells with drugs. We conclude plant-derived compounds can be considered to target epigenetic machineries involved with malignant transformation and can open new avenues for cancer therapeutics provoking immune response.

1. Introduction

Prostate cancer is the most commonly diagnosed malignancy among men. It has been estimated that nearly 35 000 cases of prostate cancer are diagnosed every year and it accounts for approximately 25% of men death due to prostate cancer (Chen et al., 2012; Snyder et al., 2013). Several factors, such as dietary factors, life style related factors, environmental factors and androgens have been recognized as contributing risk factors associated with progression of prostate cancer. Over the past decade multiple evidences suggested that, about 5–10% of prostate cancer are hereditary and acquired by reversible epigenetic changes through the lifetime which can be preventable (Chiam et al., 2014; Labbe et al., 2015; Lee et al., 2015). Prostate cancer is a complex, multifactorial disease which arises through the acquisition of various genetic and epigenetic factors in its etiology. Proinflammatory states are associated with multiple tumor development and cancer progression, including prostate cancer. Proinflammatory cytokines represent important mediators of inflammation, which play a vital role in interaction between cancer and inflammation. It is evident that inflammatory cells play an important role in tumor development. In the

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process of neoplastic progression, inflammatory cells acts as tumor promoters by facilitating genomic instability and promoting angiogenesis; hence, inflammatory cells produces an attractive environment for tumor progression (Wang et al., 2018). Cytokines produced by the inflammatory cells influence the tumor bulk by regulating the growth, differentiation and migration of cells in tumor microenvironment. However, in the tumorigenic process, neoplastic cells distract the mechanism of inflammation such as selectin–ligand interactions and MMP production to produce a favorable condition for spread of neoplastic cells, the metastasis (Egeblad and Werb, 2002; Overall and Lopez-Otin, 2002). The pro-tumor actions of inflammatory cells play an important role in tumor development. Pro-tumor actions include releasing of growth and survival factors that promotes the process of angiogenesis and lymph angiogenesis, inducing DNA damage, remodeling of extra cellular matrix which in turn facilitates the mechanism of invasion and escaping host defense mechanisms (Coussens et al., 2002). Evidences are accumulating in favor that, under inflammatory microenvironment, exposure to reactive oxygen species or other pro-inflammatory cytokines that includes interleukin 6 (IL-6) affects the DNA methyltransferase 1 (DNMT1) protein function. This results in enhanced methylation level of tumor suppressor genes; hence plays an important role in carcinogenesis (Murata, 2018). Among different proinflammatory cytokines, IL-1β, IL-6 and TNF-α were major factors associated with carcinogenesis (Xu et al., 2015; Mukhopadhyay et al., 2015). THP1 designates a spontaneously immortalized monocyte-like cell line, derived from the peripheral blood of a childhood case of acute monocytic leukemia (M5 subtype). The use of cultured THP1 cells in vitro as a model for primary human monocytes ex vivo exemplifies the basic concept of translational research (Bouraoui et al., 2008; Torrealba et al., 2017).

Epigenetics is defined as the heritable alternations in cellular phenotype which results from heritable alternation in chromosome by mechanisms other than changes in the sequence of DNA (Okugawa et al., 2015). Among different epigenetic processes, methylation of DNA and modification of histones play a key role in the transcriptional activation or repression of oncogene or tumor suppressor genes respectively (Hegi et al., 2009). It has been reported that hypermethylation of tumor suppressor gene is associated with development of tumorigenesis (Counts and Goodman, 1995; Herman and Baylin, 2003). For example, in prostate cancer, hypermethylation of promoter region of GSTP1 and RARβ can be taken as specific marker for the diagnosis of early stage prostate cancer. Thus, drugs targeting these enzymes which are responsible for epigenetic gene silencing, such as, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) could be a promising area in cancer therapy by chemoprevention (Chen et al., 2011; Davis and Uthus, 2004). There are a number of DNA methylation inhibitors along with HDAC inhibitors such as, 5-Aza-2′-deoxycytidine and Trichostatin A respectively, which can inhibit cell proliferation by a well characterized mechanism(s) of action (Chen et al., 2013). However, the side effects of these drugs, such as toxic and nonspecific mechanism of action limited the use of these compounds as chemopreventive agents. Thus, there is great need of designing cost effective and fruitful non-toxic chemopreventive agent(s) as 'dietary-drugs' for prevention of cancer. Previously, it has been reported that several chemopreventive agents derived from daily consumption of fruits and vegetables act as DNMT inhibitors and can reactivate the expression of silenced tumor suppressor gene. It has been reported that EGCG from green tea polyphenol can inhibit DNMT and activate the expression of silenced tumor suppressor gene RARβ and P16 (CDKN2A) in esophageal cancer cells (Kim and Kim, 2013). In A431 skin cancer cells EGCG is also known to decrease the HDAC activity and increase the expression of active biomarkers resulting in inhibition of cellular proliferation and induction of apoptosis (Nandakumar et al., 2011). Curcumin, a diferuloylmethane derived from spice turmeric was found to induce global methylation of DNA in MV4-11 leukemia cells (Khor et al., 2011). Similarly, in LnCaP prostate cancer cells, sulforaphane (SFN) an organosulfur compound found in cruciferous vegetables can down-regulate the expression of DNMT1 and DNMT3A but not the expression of DNMT3B. It has also been seen that SFN possesses multi-target chemopreventive effect including the inhibition of HDAC activity (Myzak and Dashwood, 2006). Thus, now scientists are more interested in identification of novel 'botanical diets' which can effectively target the growth of tumor cells without any effect on normal human cells. With reference to the chemoprevention of prostate cancer, natural botanicals and dietary food components are gaining much more importance having the potentiality of acting as a chemopreventive agent.

Paederia foetida (hereafter, P. foetida) commonly known as Ghandhali belongs to the family of Rubiaceae, commonly distributed all over the India. The plant has been used for the treatment of various diseases including treatment of diarrhea, dysentery, piles, and stomach ulcers and for various types of inflammation (Afroz et al., 2006; De et al., 1994; Khan et al., 2005). The plant is also known to possess antiviral, antidiarrheal, antitussive and antioxidant activity. It has been reported that the leaf of the plant possesses different chemical constituents comprising alkaloids, sitosterols, β-sitosterols, ascorbic acid, flavonoids, amino acids, iridoid glycosides and volatile oils. In this study, we report that the leaf extract of *P. foetida* exerts anticancer activity by modulation of mechanism and immunological potential in human prostate cancer, PC-3 and DU-145 and monocytic THP-1 cell lines.

2. Materials and methods

2.1. Preparation of methanolic extract of *P. foetida* leaves (MEPL)

50 g of dried powder leaves of *P. foetida* was soaked in 200 ml of methanol in a water bath for 24 h. The supernatant was collected and evaporated using a rotary evaporator. The concentrate was dried in lyophilizer and the dried powder was used for phytochemical screening.

2.2. Screening of phytochemicals

Preliminary phytochemical analyses of MEPL were done for identification of secondary metabolites (Detailed biochemical analysis is provided in supplementary data S1).

2.3. Thin layer chromatography

MEPL along with the expected reference compound (obtained from literature survey) was spotted on the TLC plate (TLC Silica gel 60 F 254, Merk, USA) and was developed in a solvent system containing ethyl acetate and hexane in 40:60 ratios (standardized after repeated experiment). Then the chromatograms were examined by putting the TLC plate in iodine chamber. The distance of the spot travelled from the origin on TLC plate was measured by calculating the Rf value of the compound i.e. (Distance travelled by the compound divided by the distance travelled by the solvent front from the origin).

2.4. Cell culture

Human prostate cancer cell lines PC-3, DU-145 and normal human keratinocytes, HaCaT were purchased from National Centre for Cell Science (NCCS), Pune, India. PC-3 cell lines were cultured in Ham F-12 whereas DU-145 and HaCaT were cultured in Dulbecco's-modified Eagle's Medium supplemented with 10% (v/v) Fetal bovine serum and penicillin (100 unit/ml) and streptomycin 0.1 mg/ml. Cells were grown at 37 °C in a 5% CO_2 cell culture incubator. Cells were harvested by trypsinization and number of living cells was calculated by trypan blue staining (0.2% v/v) using a hemocytometer. The human monocyte/macrophage cell line THP-1 was maintained and cultured in RPMI-1640 medium and were differentiated overnight into macrophages by adding 50 ng/ml of PMA.

2.5. Chemicals and antibodies

Stock solution of AZA (all purchased from Sigma Aldrich, St. Louis, MO, USA) were prepared by dissolving in Dimethylsulphoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA), stored at −20 °C and diluted in fresh medium immediately before use. Similarly, stock solutions of Lupeol and β-sitosterol were prepared by dissolving in 100% ethanol and dimethyl sulfoxide (DMSO, Himedia) in 1:1 ratio and used immediately or stored at −20 °C for further use. qRT- PCR primers of CDH1, Bcl2 and Bax were obtained from sigma Aldrich St. Louis, MO, USA. For Western blot analysis, antibodies of Rabbit polyclonal anti-DNMT1 (Santa Cruz) and E-cadherin (Abcam), Rabit polyclonal anti-Bcl2 (Santa Cruz) and mouse monoclonal anti-beta actin (Santa Cruz, Biotechnology, CA, USA) were used. DNMT and HDAC activity Assay kit were obtained from Epigenetek and Abcam respectively.

2.6. Cell viability assay

Cell viability was determined by MTT colorimetric assay. Briefly, 5×10^3 cells were seeded in each well of a 96-well plate. After 24 h of incubation growth medium was replaced with fresh medium containing different concentrations of MEPL (1–25 mg/ml), lupeol (5–60 μM) and β-sitosterol (10–120 μM) to determine the IC_{30} value. After 24 h of drug treatment cell proliferation was determined by adding 100 μl of MTT solution (0.8 mg/ml diluted in serum free media) allowing them to stand for 4 h in dark at 37 °C. Then MTT solution was removed and replaced by 100 μl of DMSO followed by incubation for 15 min in dark. Absorbance was taken at 595 nm by microplate reader spectrophotometer and mean of five experimental repeats were taken to obtain IC_{30} value for further experiments.

2.7. Chromatin condensation assay

PC-3 and DU-145 cells (10^5 cells/well) were seeded in 6 well cell culture plates and were treated with the MEPL, lupeol and β-sitosterol at their respective IC_{30} values for 24 h. After 24 h, cells were washed properly with PBS and stained with Hoechst 33342 (1 μg/ml, Invitrogen) followed by incubation for 10 min at 37 °C. Finally, images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71, excitation wavelength of 355–366 nm, emission wavelength of 465–480 nm) at 40× magnification. The number of condensed nuclei was counted against total number nuclei in the field and percentage of condensed nuclei were calculated and plotted graphically. Ten different fields, having 30–40 nuclei per field were counted performing three independent sets of experiments and gross average of the total number of cells in the field was taken.

2.8. Cell migration analysis by wound healing assay

PC-3 and DU-145 cells were allowed to grow up to 90% confluency and scratch was made on the uniform monolayer of cells using a sterile 200 μl pipette tip. Cell debris was removed by washing with PBS. The scratched monolayer of cells was then treated with MEPL, lupeol and β-sitosterol at proper concentration. Images were taken after 24 h of drug treatment to measure width of the wound.

2.9. Clonogenic assay

Cells were seeded, at a density of 500 cells/well in a 6-well plate and allowed to grow up to 8 h for attachment. Then cells were treated with MEPL, lupeol and β-sitosterol at their respective IC_{30} values and allowed to grow up to two weeks until distinct colonies were formed in control (untreated cells). Cells were then fixed with a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 2 h and washed in distilled water followed by air dried and evaluated for colony formation.

2.10. Immunofluorescence microscopy

Prostate cancer cells (PC-3 and DU-145) were seeded in glass coverslips in a 6-well plate and treated with MEPL, lupeol and β-sitosterol for 24 h at their respective IC_{30} values. After proper washing cells were fixed with ice cold methanol and were permeablized with 0.25% Triton X. Then blocking was done with 3% BSA in PBST for 30 min. Cells were then incubated with CDH1 primary antibody for overnight at 4 °C. After PBS wash, cells were incubated with FITC conjugated secondary antibody (Santa Cruz Biotech) for 2 h followed by PBS wash. Then, cells were treated with DAPI (1 mg/ml) for 10 min. Immunofluorescent proteins were visualized, detected and photographed using epifluorescence microscope (Olympus IX71) after proper washing.

2.11. RNA extraction and analysis of mRNA expression by qRT-PCR

PC-3 and DU-145 cell lines were treated with MEPL, lupeol, β-sitosterol at their respective IC_{30} values and total RNA was extracted using TRI Reagent (Sigma Aldrich St. Louis, MO, USA) according to the manufacturer's instruction. Then total RNA was reverse-transcribed with oligo-dT using Revert Aid first strand cDNA synthesis kit (Thermo-Scientific). qRT-PCR was performed with a realplex4 Eppendorf system using SYBR Green Jump startTM Taq Ready mix to study the expression of following genes (CDH1, Bcl2, Bax, IL-6, IL-10, IL1-β and TNF-α) using specific primer pairs. Primer sequences for qRT-PCR analyses of genes are given in Table-1. PCR was started with 5 min at 95 °C for denaturation, followed by 40 cycles. A final extension step at 72 °C for 7 min was included after the final cycle to complete polymerization. The mRNA level was normalized to GAPDH as an internal control (Pradhan et al., 2019).

2.12. Protein extraction and western blot analysis

About 80–85% confluent cells treated with different drugs were harvested and cellular proteins were extracted using RIPA buffer containing cocktail of protease inhibitor (Sigma). Protein was centrifuged for 20 min at 14000 g at 4 °C. The protein concentration was measured by Bradford method. Equal amount of protein was electrophoresed in SDS-polyacrylamide gel at a constant voltage of 120 V in the running buffer and then transferred to a PVDF membrane (Millipore). Protein containing membranes were then blocked with 3% Bovine Serum Albumin (BSA) in phosphate buffer saline containing 0.1% Tween-20 (PBST) for 2 h at RT. Then the membranes were incubated overnight at 4 °C with primary antibodies. After subjected to proper washing membrane was incubated with secondary antibody coupled to Horseradish peroxidase (HRP-conjugated). Then Immunoreactive proteins were developed and detected in X-Ray film using ECL (Thermo Scientific).

Table 1
List of primer sequence used for qRT- PCR.

Gene	Primer sequence
E-cadherin	F 5′-CGAGAGCTACACGTTCACGG-3′
	R 5′-GGGTGTCGAGGGAAAAATAGG-3′
BCL2	F 5′-CCTGTGGATGACTGAGTACC-3′
	R 5′-GAGACAGCCAGGAGAAATCA-3′
Bax	F 5′-TTCATCCAGGATCGAGCAG-3′
	R 5′-CGCTCAGCTTCTTGGTGG-3′
GAPDH	F 5′-GGAGCGAGATCTCCCCAAAAT-3′
	R 5′-GGCTGTTGTCATACTTCTCATGG-3′
IL-6	F 5′-CCTGAACCTTCCAAAGATGGC-3′
	R 5′-TTCACCAGGCAAGTCTCCTCA-3′
IL1-β	F 5′-ATGATGGCTTATTACAGTGGCAA-3′
	R 5′-GTCGGAGATTCGTAGCTGGA-3′
TNF-α	F 5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′
	R 5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′
IL-10	F 5′-GTGATGCCCCAAGCTGAGA-3′
	R 5′-CAGGGGCTTGCTCTTGTTTT-3′

2.13. DNMT activity assay

PC-3 and DU-145 cells treated with different concentrations of MEPL, lupeol, β-sitosterol and AZA (for 24 and 72 h respectively) were harvested after proper washing and nuclear extract were prepared. AZA was taken as a positive control as it is a well-known DNMT inhibitor. Then DNMT activity was measured using DNMT activity Assay kit (Epigentek) as per manufacturer's instructions. Finally, absorbance was recorded using microplate reader spectrophotometer (PerkinElmer, Walthman, MA-USA) at 450 nm.

2.14. HDAC activity assay

HDAC activity was quantified using colorimetric HDAC Activity Assay Kit. PC-3 and DU-145 cell lines were treated with MEPL, Lupeol and β-sitosterol at different concentration for 24 h. Nuclear extracts were prepared after proper washing. TSA was taken as positive control as it is a well-known HDAC inhibitor. Then HDAC activity was carried out using HDAC activity assay kit as per manufacturer's instructions. Absorbance was recorded using microplate reader (PerkinElmer, Walthman, MA-USA) at 450 nm.

2.15. Statistical analysis

Statistical analyses of the data were performed by student's *t*-test using SPSS software. All data are presented as mean ± SD. Variations with $p < 0.05$ were considered as significant for all experiments.

3. Results

3.1. TLC analyses of phytochemicals from MEPL

Phytochemicals from MEPL were analysed by qualitative chemical tests, which reveals the presence of alkaloids, flavonoids, terpenoids, steroids and cardiac glycosides (Table 2, see also supplementary protocols S1). The TLC analysis confirms the presence of lupeol and β-sitosterol (by comparing the Rf value of spots developed from MEPL with the Rf value of commercially available lupeol and β-sitosterol taken as reference compounds). The Rf value of standard lupeol and standard β-sitosterol was 0.84 and 0.65 respectively which corresponds to the Rf value of crude extract (Table 3 and Fig. 1). This confirms the presence of lupeol and β-sitosterol.

3.2. MEPL, lupeol and β-sitosterol exhibit cytotoxic activity to prostate cancer cells

The cytotoxic activity of MEPL, lupeol and β-sitosterol was examined by colorimetric MTT assay in both prostate cancer cell lines, PC-3 and DU-145 as well as in normal keratinocyte cells (HaCaT). A remarkable decrease in cellular viability was observed with increase in the concentration of drugs in prostate cancer cell lines. But in case of normal HaCaT cells no significant lethal effect was observed as compared to other two drugs and almost all cells remain viable even at a higher concentration of all drugs. The IC_{30} values of MEPL (Fig. 2A), lupeol (Fig. 2B) and β-sitosterol (Fig. 2C) for PC-3 cells were found to be 20 mg/ml, 50 μM and 80 μM respectively and for DU-145 values were 15 mg/ml, 40 μM and 70 μM respectively. Similarly, in THP1 cell line, cell viability assay was performed with varying concentration of MEPL, lupeol and β-sitosterol for 24 h. We observed reduction in cell viability in a dose-dependent manner. IC_{30} values of MEPL, lupeol and β-sitosterol in THP1 cell line were found to be 25 mg/ml, 40 μM and 120 μM respectively. We used IC_{30} of drugs because of having more cells to obtain more DNA, RNA and enzymes for subsequent analyses. Hence, these concentrations were chosen for further experiments in THP1 cell line (as represented in Fig. 10).

Table 2
List of phytochemicals present in the methanolic extract of *Paederia foetida* leaf by phytochemical screening.

Sl. No	Types of phytochemicals	Remarks
1	Alkaloid	+
2	Flavonoid	+
3	Terpenoid	+
4	Steroid	+
5	Cardiac glycoside	+
6	Tannin	–
7	Ellagic Acid	–

Table 3
Rf value of compound present in the MEPL with the Rf value of reference compounds.

Rf value	Plant extract	Lupeol	β- sitosterol
A	0.15	–	
B	0.28	–	
C	0.48	–	
D	0.52	–	
E	0.65	–	0.65
F	0.84	0.84	–
G	0.91	–	–
H	0.95	–	–

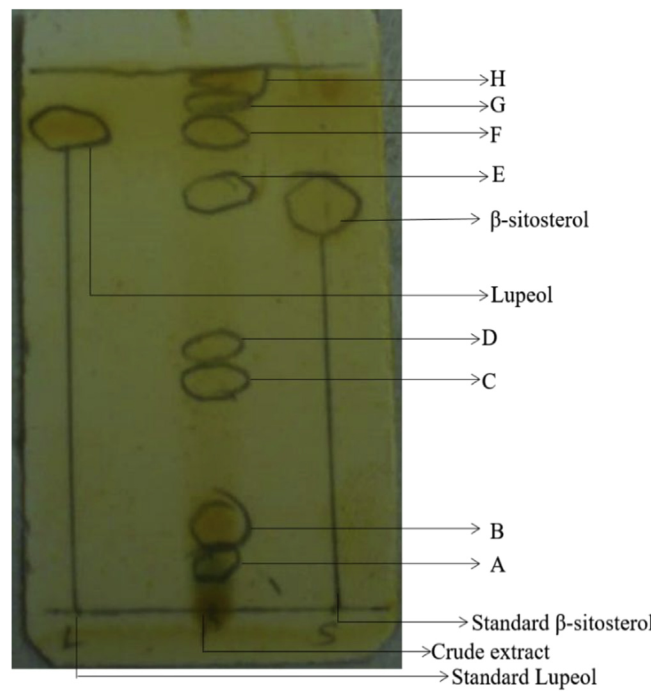

Fig. 1. TLC analyses of methanolic extract of *Paederia foetida* leaves.
For identification of the active molecules several TLC were executed, a representative TLC image is shown in Fig. 1.

3.3. MEPL, lupeol and β-sitosterol induces apoptotic cell death in prostate cancer

To understand the possible mechanism of cell death in PC-3 and DU-145 cells, apoptotic activity of these drugs was analysed by chromatin condensation assay. The number of condensed nuclei in MEPL, lupeol and β-sitosterol treated cells was much more than the control cells in both PC-3 and DU-145 cell lines (Fig. 3A and B). The percentage of condensed nuclei in MEPL, lupeol and beta-sitosterol treated PC-3 cells were 69.18%, 78.23% and 71.65%, respectively whereas in DU-

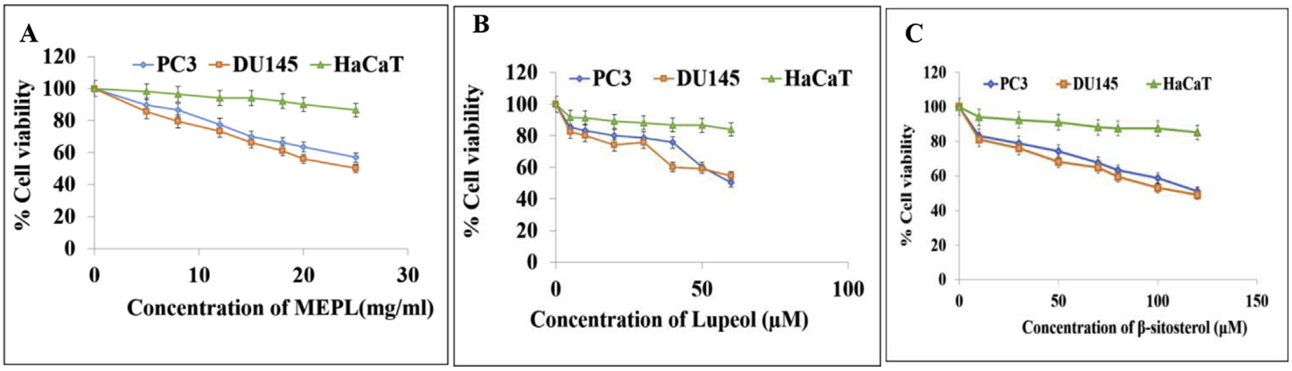

Fig. 2. Cytotoxicity activity of MEPL, Lupeol and β-sitosterol in PC-3 and DU-145 prostate cancer cells, and normal skin keratinocytes, HaCaT cells. Representative plots on the effect of (A) MEPL, (B) lupeol, and (C) β-sitosterol in the viability and growth of PC-3 and DU-145 prostate cancer cells, and normal skin keratinocytes, HaCaT cells. Both types of the cell lines were treated with drugs shown at the concetration for 24 h. The IC_{30} value of MEPL, lupeol and β-sitosterol for PC-3 cells were found to be 20 mg/ml, 50 µM and 80 µM respectively and for DU-145 it was 15 mg/ml, 40 µM and 70 µM respectively. Data are represented as mean ± SD of three different experiments.

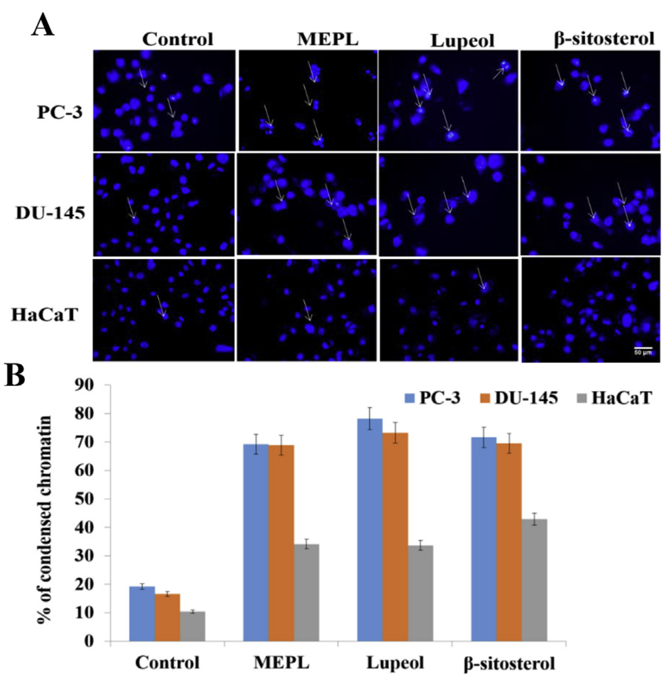

Fig. 3. Analysis of chromatin condensation by Hoechst staining. Formation of condensed chromatin (represented by Arrow) in MEPL, lupeol and β-sitosterol treated and untreated (A) PC-3, DU-145 and HaCaT cell lines. More number of condensed chromatins were formed in drug treated cells as compared to untreated one. (B) Graphical representation of % of condensed chromatin in PC-3, DU-145 and HaCaT cell lines. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$. Scale Bar = 50 µm.

145 cells it is 68.92%, 73.18% and 69.45%.

3.4. MEPL, lupeol and β-sitosterol inhibits migration and proliferation of prostate cancer cells

Increased invasion and cell motility is an important factor contributing to cancer progression and metastasis. Taking this fact into consideration, anti-migratory activity of MEPL, lupeol and β-sitosterol against prostate cancer cell lines was evaluated by scratch wound healing assay. Results indicate that drugs significantly inhibits the migration of prostate cancer cells as compared to untreated (control) one. The number of invaded cells in PC-3 cell line was 189, 32, 72, 26 (Fig. 4A) whereas in DU-145 it was 71, 16, 19, 20 (Fig. 4B) after treatment with control, MEPL, lupeol and β-sitosterol respectively.

Clonogenic assay also reveals that MEPL, lupeol and β-sitosterol inhibit proliferation of PC-3 and DU-145 cells as compared to untreated one which was confirmed by the formation of less number of colonies in drug treated cells in comparison to control (Fig. 4C and D).

3.5. MEPL, lupeol and β-sitosterol induce apoptosis by modulation of Bcl2 and Bax in prostate cancer cells

In order to investigate the possible molecular mechanism of apoptosis induced by MEPL, lupeol and β-sitosterol in PC-3 and DU-145 prostate cancer cells, expression of mitochondria-associated protein i.e. Bcl2 and Bax was analysed. qRT-PCR and western blotting data suggested the decreased expression of Bcl-2 in both PC-3 (Figs. 5B and 6C) and DU-145 (Figs. 5D and 6D) cell lines. Similarly, induced expression of Bax in transcript level was observed in drug treated PC-3 (Fig. 5A) and DU-145(Fig. 5C) cells as compared to control one.

3.6. MEPL, lupeol and β-sitosterol inhibits migration of prostate cancer cells by inducing CDH1 expression

To determine the inhibitory effect of drugs on metastatic properties of prostate cancer, expression of CDH1 was evaluated, since E-cadherin acts as a potent regulator of cell migration. Treatment with MEPL, lupeol and β-sitosterol significantly upregulated the expression of CDH1 both in PC-3 (Fig. 6A and C) and DU-145 (Fig. 6B and D) cell lines in terms of mRNA and protein level respectively as compared to untreated one. Immunofluorescence data also confirmed the induced expression of CDH1 in drug treated cells as compared to untreated (control) one (Fig. 6E and F).

3.7. MEPL, lupeol and β-sitosterol inhibits the enzymatic activity as well as m-RNA and protein level expression of DNMTs in prostate cancer cells

DNMT plays an important role in DNA methylation. Therefore, DNMT activity assay was determined after treatment with MEPL, lupeol and β-sitosterol at different concentrations. Results obtained showed decreased DNMT activity in MEPL, lupeol and β-sitosterol treated PC-3 (Fig. 7A, B and 7C) and DU-145 (Fig. 7D, E and 7F) prostate cancer cells in a dose dependent manner. Similar decrease in DNMT activity was also observed in AZA treated cells (Fig. 7G). This reduction in DNMT activity is also consistent with reduced protein level expression of DNMTs. Western blotting data also revealed the decrease in protein level expression of DNMT1 in drug treated PC-3 (Fig. 8A) and DU-145 (Fig. 8B) cells as compared to untreated one.

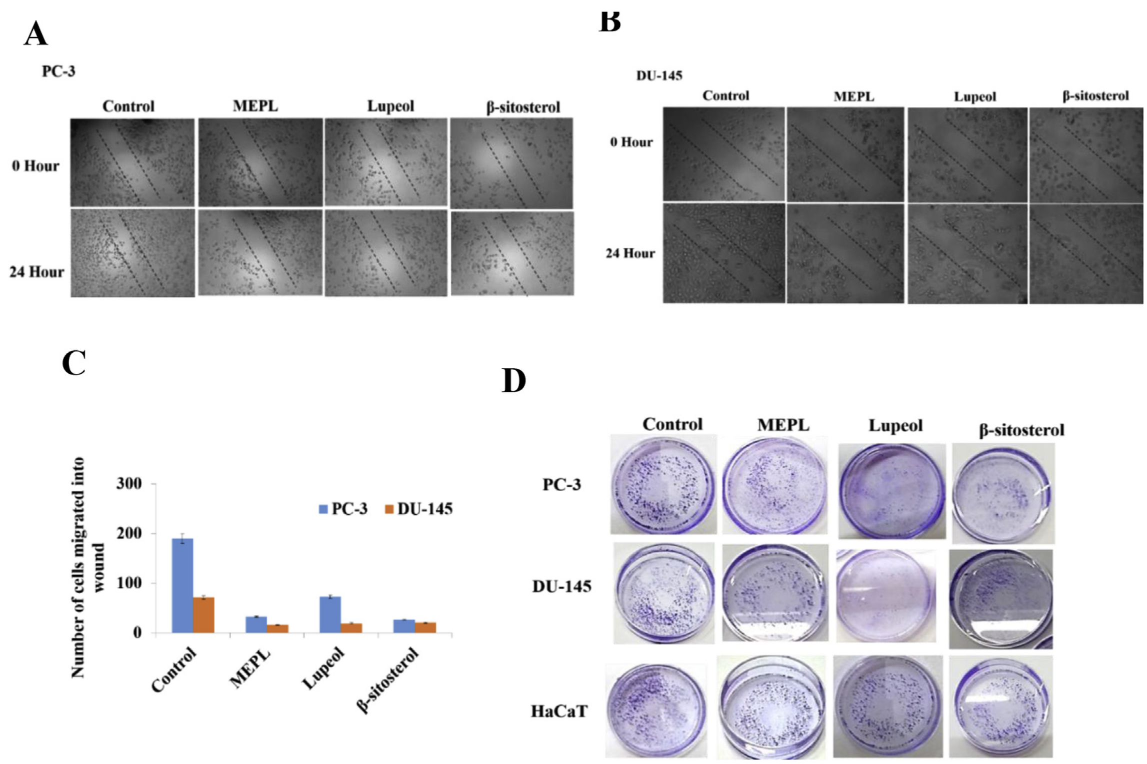

Fig. 4. Scratch assay in prostate cancer cell lines after treatment with MEPL, Lupeol and β-sitosterol.
Prostate cancer cells were treated with MEPL, lupeol and β-sitosterol at their respective IC_{30} value. Representative images of cellular migration in (A) PC-3, (B) in DU-145 cell line. The uniform monolayer was scratched and width of the wound areas was photographed at the indicated time points. (C) Graphical representation of number of migrated cells in wound region of both untreated and treated PC-3 and DU-145 prostate cancer cell lines. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$. (D). Clonogenic Assay showing colony formation in MEPL, lupeol and β-sitosterol treatment.

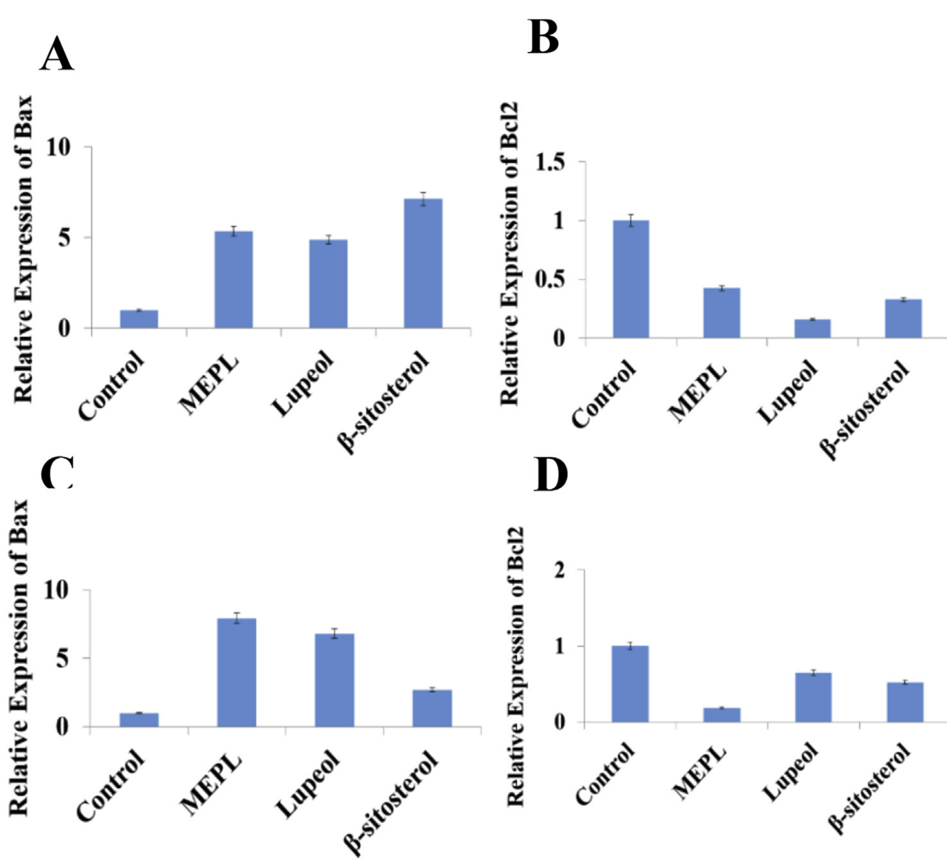

Fig. 5. qRT-PCR analysis of Bcl2 and Bax in PC-3 and DU-145 prostate cancer.
(A and B) Relative expression of Bax and Bcl2 in PC-3; (C and D) in DU-145 prostate cancer cell lines respectively after MEPL, lupeol and β-sitosterol drug treatment. The expression of Bax was upregulated whereas Bcl2 exhibited decrease in expression at transcript level after drug treatment in both the cell lines as compared to control (untreated) one. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$.

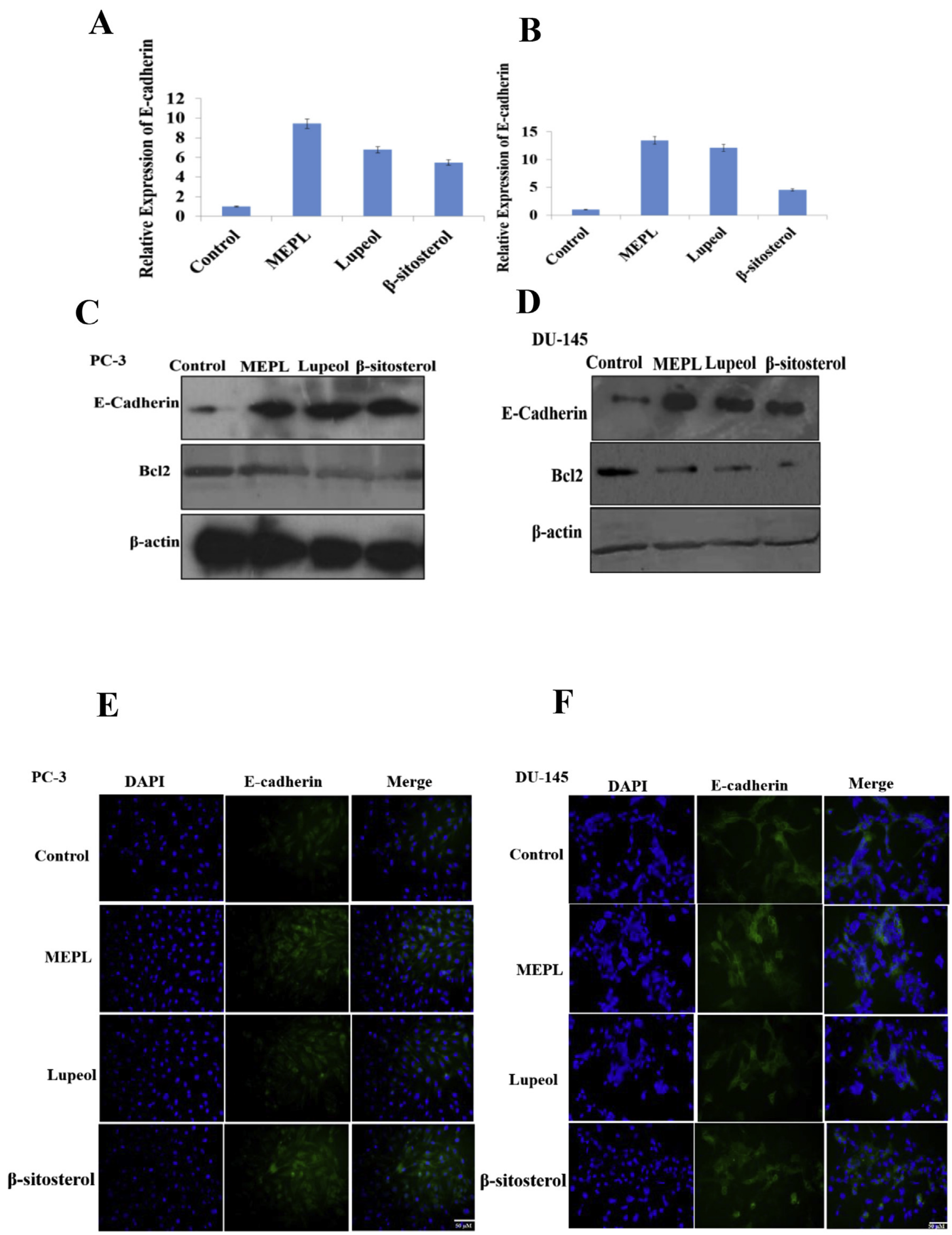

Fig. 6. Effect of MEPL, lupeol and β-sitosterol on CDH1 and Bcl2 expression in PC-3 and DU-145 prostate cancer cell lines.
Relative expression of CDH1 at the mRNA level was measured by qRT-PCR in (A) PC-3 and in (B) DU-145 cell line respectively. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$. Protein expression of CDH1 and Bcl2 by Western blot analysis (C) in PC-3 and (D) In DU-145 prostate cancer cell line respectively after MEPL, lupeol and β-sitosterol drug treatment. β-actin was taken as internal control. Immunofluorescence analysis of CDH1 in (E) PC-3 (F) in DU-145 prostate cancer cell line. CDH1 expression was enhanced after drug treatment in both the cell lines. Scale Bar = 50 μm.

3.8. MEPL, lupeol and β-sitosterol inhibits expression and activity of HDACs in prostate cancer cells

DNA methylation and histone deacetylation co-operatively plays significant role in regulating gene silencing/expression during tumor development and cancer progression. Histone deacetylation allows DNA to tightly wrap around the histone core and hence prevents binding of transcription factor on promoter site to inhibit transcription. Thus to examine whether MEPL, lupeol and β-sitosterol has any role in HDAC inhibition, we analysed prostate cancer cells for in vitro HDAC activity. Results obtained showed, MEPL, lupeol and β-sitosterol inhibit the HDAC activity in PC-3 (Fig. 9A, B and 9C) and DU-145 (Fig. 9D, E

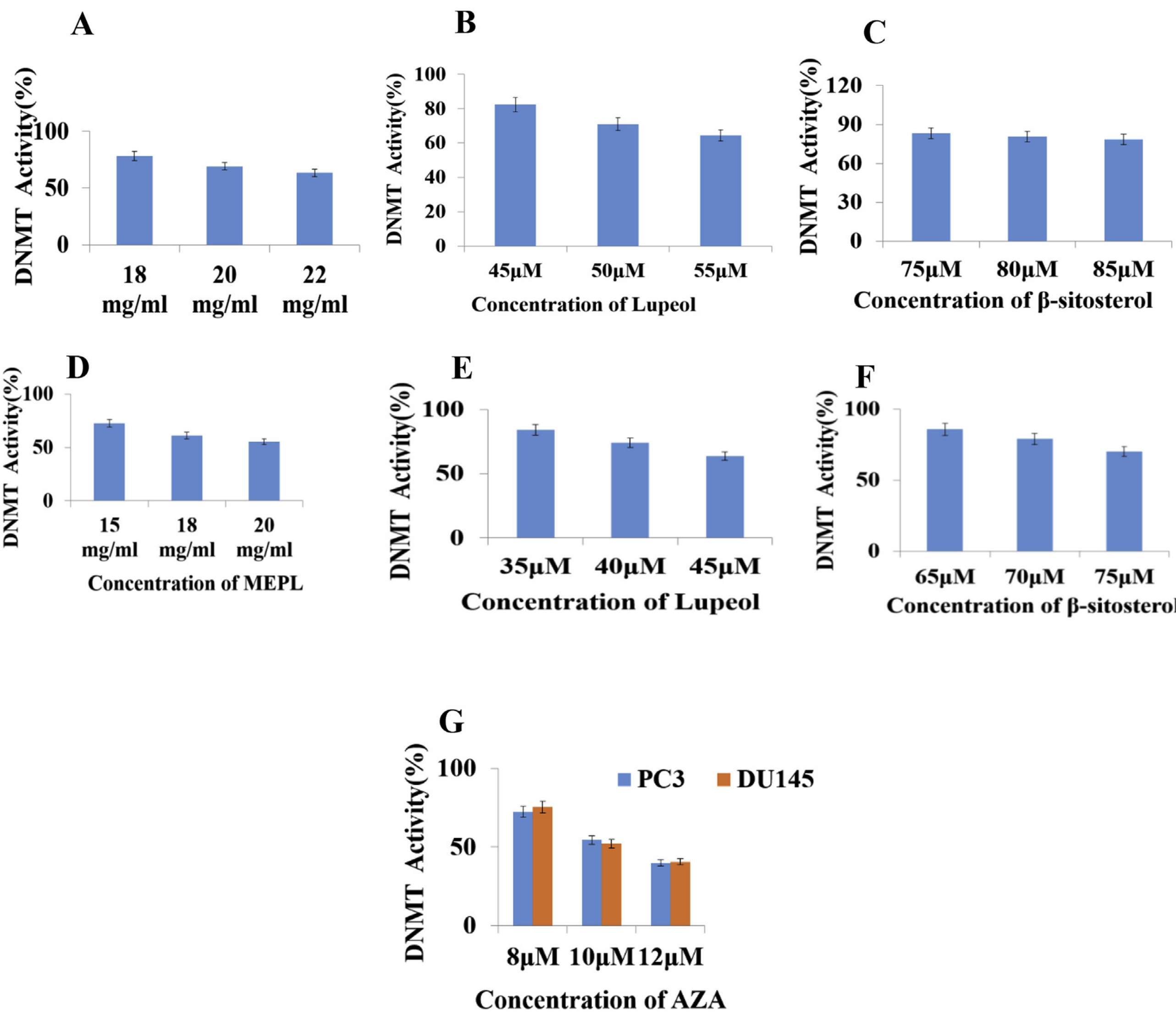

Fig. 7. MEPL, lupeol and β-sitosterol inhibits the DNMT activity in prostate cancer cells.
MEPL, lupeol and β-sitosterol inhibit DNMT activity in PC-3 and DU-145 cell lines in a dose dependent manner. DNMT activity assay performed in (A) MEPL, (B) lupeol and (C) β-sitosterol treated PC-3 cell lines; DNMT activity Assay performed in (D) MEPL, (E) lupeol and (F) β-sitosterol treated DU-145 prostate cancer cell lines, (G) DNMT activity Assay of 5-AZA-2′-deoxycyidine in PC-3 and DU-145 prostate cancer cell lines. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$.

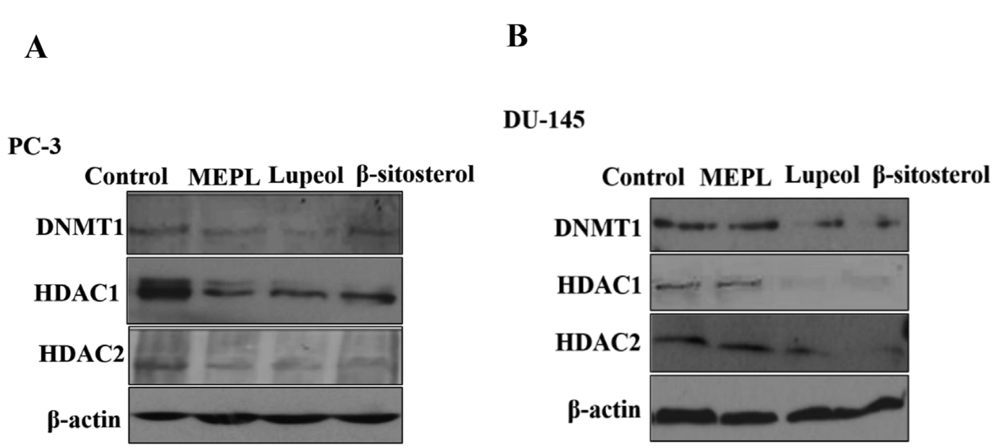

Fig. 8. Effect of MEPL, lupeol and β-sitosterol on DNMT1, HDAC1 and HDAC2 expression in PC-3 and DU-145 prostate cancer cell line.
(A) Protein expression of DNMT1, HDAC1 and HDAC2 by Western blot analysis after treatment with MEPL (20 mg/ml), lupeol (50 µM) and β-sitosterol (80 µM) in PC-3 cell line. (B) Protein expression of DNMT1, HDAC1 and HDAC2 by Western blot analysis after treatment with MEPL (15 mg/ml), lupeol (40 µM) and β-sitosterol (70 µM) in DU-145 prostate cancer cell line.

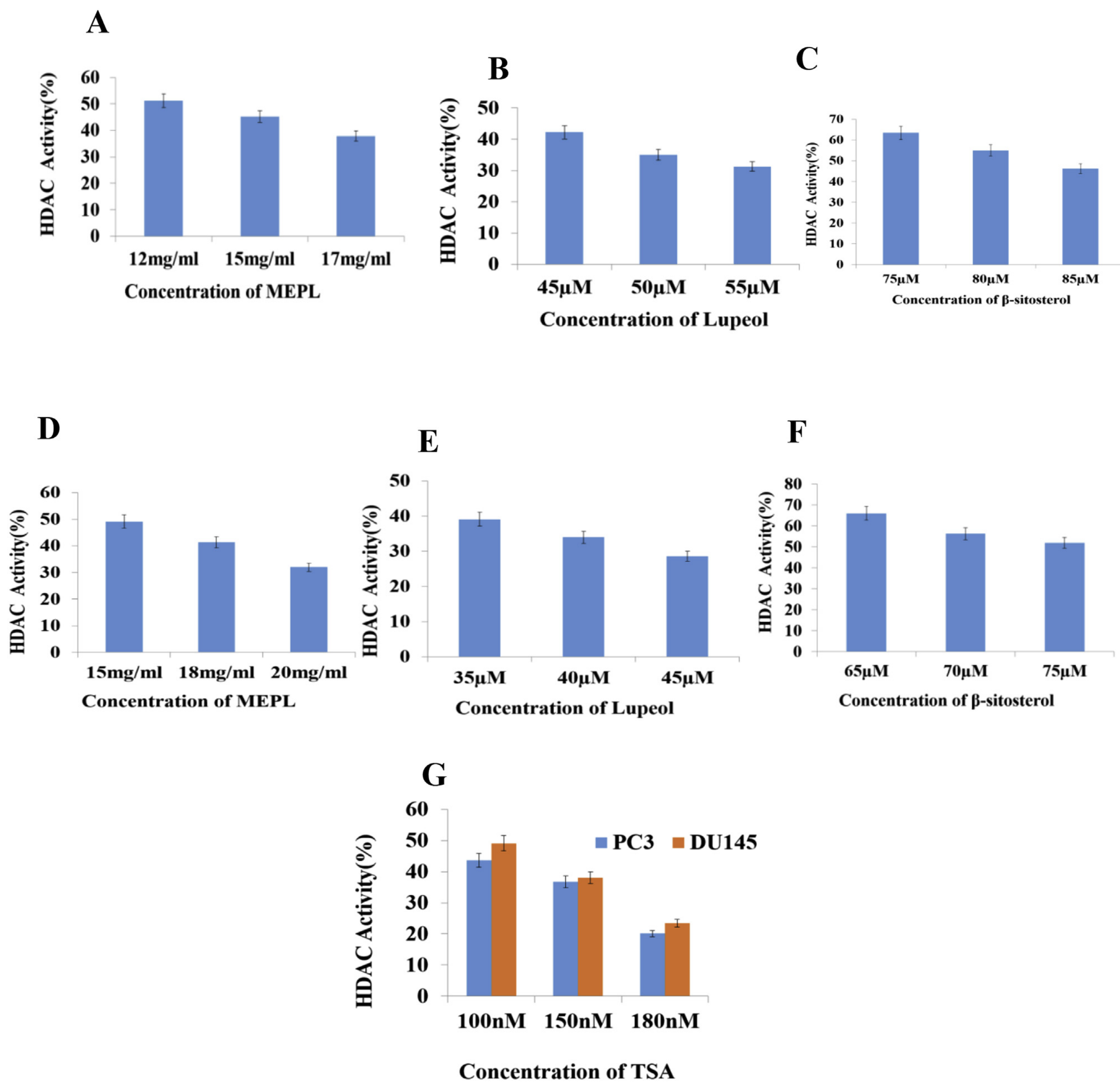

Fig. 9. MEPL, lupeol and β-sitosterol inhibits the HDAC activity in prostate cancer cells.
HDAC activity assay in (A) MEPL, (B) lupeol and (C) β-sitosterol treated PC-3 cell lines; HDAC activity Assay in (D) MEPL, (E) lupeol and (F) β-sitosterol treated DU-145 prostate cancer cell lines, (G) HDAC activity Assay of TSA in PC-3 and DU-145 prostate cancer cell lines. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$.

and 9F) prostate cancer cells respectively in a dose dependent manner. This decrease in HDAC activity is also consistent with decreased protein level expression of HDAC1 and HDAC2 in drug treated PC-3 (Fig. 8A) and DU-145 (Fig. 8B) cell lines as compared to respective untreated controls. TSA also exerts similar changes in HDAC activity in PC-3 and DU-145 prostate cancer cells (Fig. 9G).

3.9. Expression profile of pro-inflammatory cytokine genes in prostate cancer cell lines PC-3 and DU-145 and in THP1 acute monocytic leukemia cell line

Pro-inflammatory cytokines play an important role in mediating the innate immune response. Over production of inflammatory cytokines contribute to different inflammatory diseases that are associated with cancer. We have evaluated the effect of MEPL, lupeol and β-sitosterol drugs on expression of pro-inflammatory cytokines, such as IL-6, IL1-β, TNF-α and anti-inflammatory IL-10 in PC-3 and DU-145 cell lines and in THP1 cell line. We observed the reduced expression of IL-6, IL1-β, TNF-α and IL-10 in drug treated cells as compared to respective controls (Fig. 11).

4. Discussion

The importance of epigenetic dysregulation in progression of cancer has been explored well over last twenty years. Aberrant methylation of DNA is a key mechanism involved in DNA templated epigenetics and responsible for silencing of tumor suppressor gene associated with carcinogenesis (Kar et al., 2014; Patra et al., 2011; Piyathilake and Johanning, 2002). Besides DNA methylation, histone modifications (Histone acetylation/Deacetylation and Histone methylation/De-methylation) also play an important role in the epigenetic processes (Chi et al., 2010; Deb et al., 2015; Patra et al., 2001). Current data

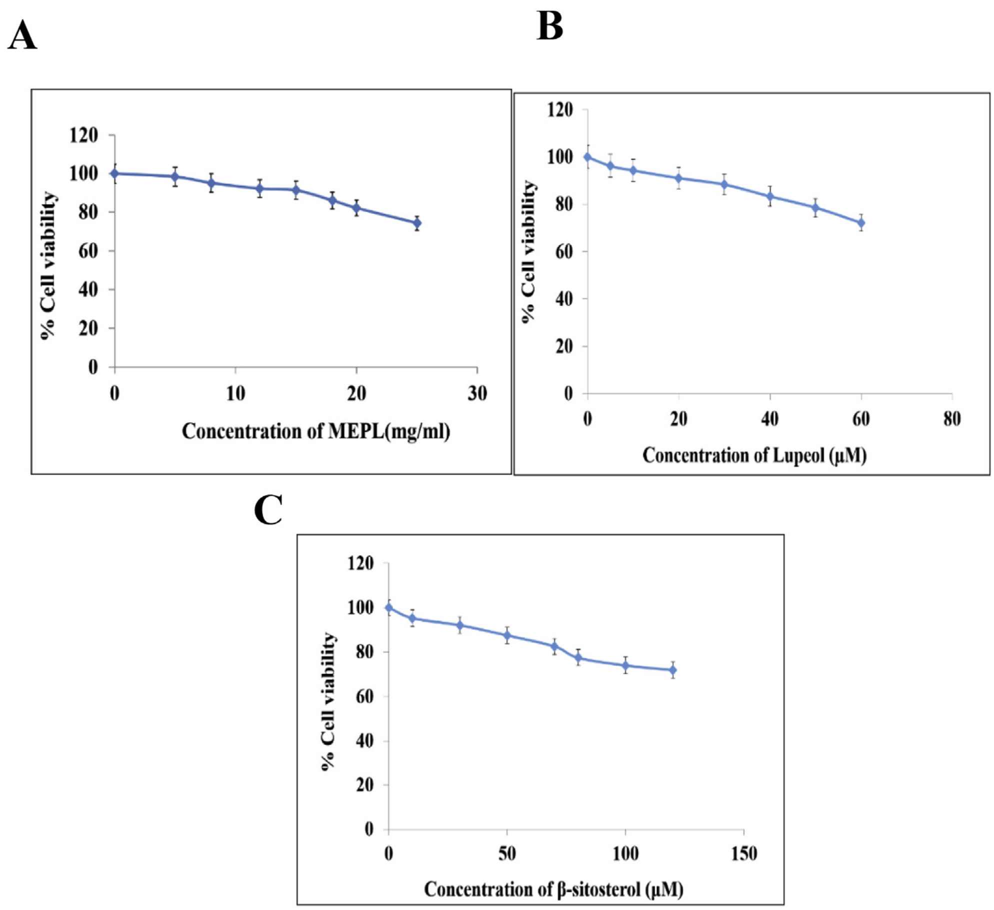

Fig. 10. Cytotoxicity activity of MEPL, Lupeol and β-sitosterol in THP1 cells.
Effect of MEPL (A), lupeol (B) and β-sitosterol (C) on viability and growth of TPH1 cell line.
Cells were treated with drugs at the respective concentrations as shown in the plot for 24 h and the IC_{30} values of MEPL, lupeol and β-sitosterol respectively at 25 mg/ml, 40 µM and 120 µM respectively were figured out. Data are represented as mean ± SD of three different experiments.

reveals that DNMT activity is much more in neoplastic cells which are associated with the increased rate of cell proliferation and tumorigenesis. Additionally, enhanced HDAC expression has been reported in many types of human cancers (Patra and Bettuzzi, 2007; Patra et al., 2008; Parbin et al., 2013). As epigenetic modifications are reversible processes, many of the DNMT and HDAC inhibitors are identified for the treatment of cancer (Chen et al., 2013; Parbin et al., 2016; Shilpi et al., 2015). But among the inhibitors several of them possesses high level of toxicity with less specificity of action and poor bioavailability, hence very poor therapeutic implications (Chen et al., 2012; Heightman, 2011; Rush et al., 2014). This led us to search for development of edible plant derived compound(s), which can be considered as promising drugs to target epigenetic machineries and processes that are at the forefront of malignant transformation and can open new avenues for cancer therapeutics. This provides a perfect opportunity for designing therapeutic and chemopreventive strategies suggesting the mechanism of interaction between various bioactive components from food products that will be helpful for novel drug discovery and development against cancer.

Emerging areas of research suggests that phytochemicals play an important role in the regulation of epigenetic modulators, thereby, reactivating the expression of silenced tumor suppressor gene(s). Many experimental and epidemiological evidences suggest that, consumption of whole or partially purified food extracts would not be so useful over a single-isolated constituent, which might be due to the existence of very low concentration of certain bioactive compounds in whole foods consumed at a time, its intestinal absorption and finally its bioavailability in the circulation. Therefore, intake of individual isolated compound would be suggested for therapeutic purpose. Here, we evaluated the anticancer and epigenetic regulatory activity of MEPL along with its active constituents i.e. lupeol and β-sitosterol on the regulation of principal epigenetic factors responsible for gene silencing along with the expression pattern of certain epigenetically silenced tumor suppressor genes in PC-3 and DU-145 prostate cancer cells. Previous studies have reported that 50% ethanolic leaf extract of *P. foetida* exhibit anti-inflammatory activity. Oral administration of aqueous leaf extract is also used in the treatment of arthritis and the methanolic leaf extract also possesses hepatoprotective activity (Kumar et al., 2014). There is a report in the literature, which suggested that 50% ethanolic extract of *P. foetida* possesses anticancer activity against human epidermal carcinoma of nasopharynx (De et al., 1994). However, after that no further work has been done on anticancer activity of *P. foetida* and till date the actual molecular mechanism of its anticancer activity was not explored. For the first time, the present study investigates the anticancer activity of MEPL by demonstrating its role as a modulator of epigenetic enzymes. We have taken 5-Aza-2′deoxycytidine and Trichostatin A (a well-known DNMT and HDAC inhibitors, respectively) as positive control. In this report, we investigated the anticancer and epigenetic regulatory activity of MEPL with its two active constituents, i.e. lupeol and β-sitosterol and elucidate the underlying molecular mechanism in human prostate cancer cells and tested the cytokine expression in human Leukemic monocyte THP1 cells.

Our findings implicate that, in untreated PC-3 and DU-145 cells higher expression and activity of DNMTs is associated with higher rate of cellular proliferation and migration. MEPL as well as lupeol and β-sitosterol are found to downregulate the expression of DNMTs like DNMT1 (Fig. 8). Besides, total DNMT activity is also significantly reduced, suggesting the role of *P. foetida* in modulation of DNA methylation (Fig. 7). Synergistically, DNMTs and HDACs work cooperatively in regulation of gene transcription. As epigenetic mechanisms are reversible, use of epigenetic modulators for reversal of DNMT and HDAC activity can inhibit the progression of various diseases, including

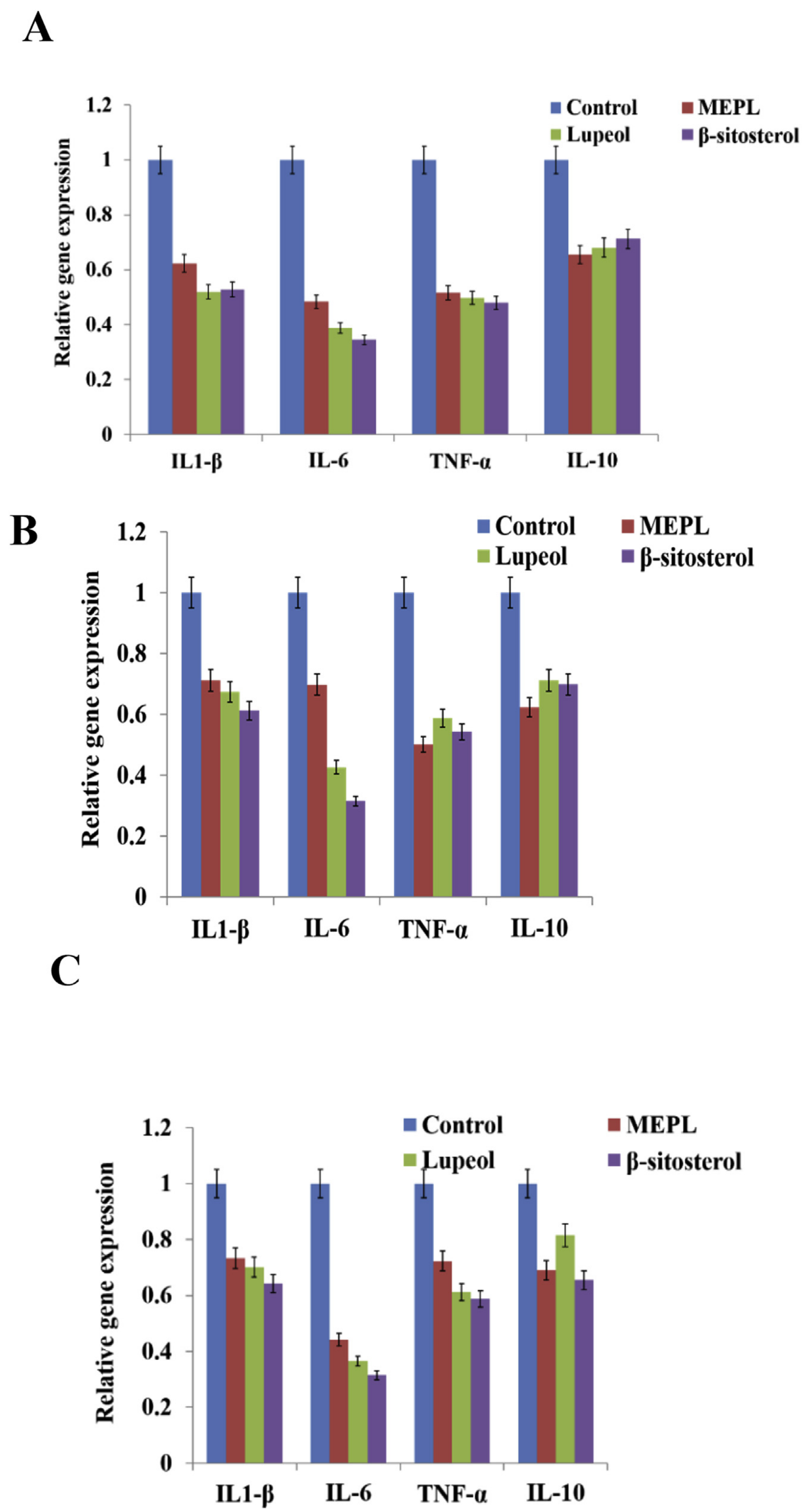

Fig. 11. qRT-PCR analysis of IL-β, IL-6. TNF-α and IL-10 in PC-3, DU-145 and THP1 cell lines.
Relative mRNA level expression of IL-1β, IL-6, TNF-α and IL-10 was measured by qRT-PCR:
(A) PC-3, (B) DU-145 and (C) THP1 cell line, respectively. Data are represented in terms of mean ± SD., n = three independent experiments, $p < 0.05$. It sharply indicates that expression of all the cytokines gone down after drug treatment, implicating immunomodulatory capability of *P. foetida* leaves extract.

cancer. Here, in our study, the effect of MEPL as well as lupeol and β-sitosterol on expression of HDAC was observed to be down regulated, which is also supported by reduced HDAC activity (Figs. 8 and 9). This result implies that, *P. foetida* exhibit anticancer activity by regulating the epigenetic process, through modulation of both DNA methylation and histone acetylation. This can be explained as; phytochemicals play an important role in regulation of epigenetic changes by reactivating the expression of certain tumor suppressor genes by hypomethylation of DNA via inhibition of DNMTs. In agreement with this, the effect of MEPL, lupeol and β-sitosterol on the expression of CDH1, a well-known tumor suppressor gene was observed to be upregulated in treated cells, compared to untreated. Reduced expression of CDH1 is associated with progression of cancer (Fig. 6). These observations suggest the inhibitory activity of *P. foetida* on cancer progression by suppression of cellular migration through induction of CDH1 expression. (see also, Sengupta et al., 2016). In addition, *P. foetida* was observed to inhibit cellular migration showing less motility towards wound region compared to untreated, suggesting its role in inhibition of cancer metastasis (Fig. 4A, B and 4C). Generally, induction of apoptosis is a characteristic feature of anticancer drugs. Thus, to further confirm the role of *P. foetida* on anticancer effect, its role on apoptosis was analysed in human prostate cancer cell lines. *P. foetida* along with lupeol and β-sitosterol exhibit cytotoxic effect in PC-3 and DU-145 prostate cancer cells compared to normal HaCaT cells by induction of apoptosis thereby inhibiting cell proliferation (Figs. 2 and 3). Induction of apoptosis is also further substantiated by formation of condensed chromatin in MEPL as well as lupeol and β-sitosterol treated cells. Besides, the formation of condensed chromatin is also supported by the fact that MEPL as well as lupeol and β-sitosterol downregulated the expression of anti-apoptotic protein Bcl2, whereas upregulated the expression of pro-apoptotic protein Bax, clearly suggesting the molecular mechanism of apoptosis induced by *P. foetida* in PC-3 and DU-145 prostate cancer cells (Fig. 5).

The relevance of inflammatory pathway(s) in cancer progression can be well understood from the study of cancer risk in long term exposure to nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin. NSAIDs exerts activity by inhibiting the cyclo-oxygenases (COX-1 and -2), inducer of inflammatory reactions, and reduces the risk of colon cancer. TNF-α, is a pro-inflammatory cytokine and acts as key downstream mediator of inflammation (Page et al., 2018). Hence, in inflammation mediated cancer development TNF-α acts as a tumor promoter. Thus, blocking these pathways which have significant therapeutic efficiency in other inflammatory diseases may have applications in cancer therapy. Here, we found MEPL as well as lupeol and β-sitosterol to inhibit the expression of pro-inflammatory cytokines in both prostate cancer cells as well as in THP1 cell line (Fig. 11). Notably, reduction of anti-inflammatory IL-10 expression was less in comparison to pro-inflammatory cytokines.

Considering all the results and observations, it is clear that, *P. foetida* (precisely the methanolic extract, MEPL), lupeol and β-sitosterol inhibits prostate cancer cell growth, migration and induce cell death by apoptosis. Upregulation of tumor suppressor gene CDH1, down regulation and inhibition of chromatin modifying enzymes DNMT1 and HDACs and down regulation of pro-inflammatory cytokines are associated with this scenario. We suggest, extensive studies to trace why cytokine genes gone down regulation after application of *P. foetida* products? What are the mechanisms of DNMT and HDACs inactivation? Albeit all those restrictions, we may conclude, edible plant-derived compounds could be considered for curing malignant transformation and can open new avenues for cancer therapeutics provoking immune response and epigenetic modulations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.016.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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