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ORIGINAL ARTICLE

Methanolic Extract of *Costus pictus* D. DON Induces Cytotoxicity in Liver Hepatocellular Carcinoma Cells Mediated by Histone Deacetylase Inhibition

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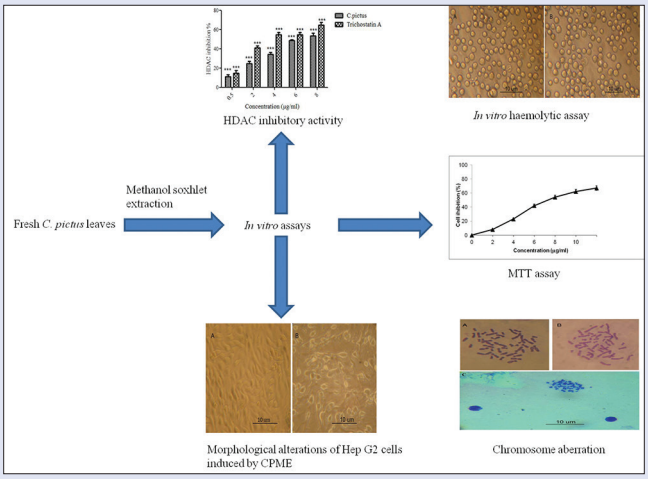
ABSTRACT

Background: Leaves of *Costus pictus* D. Don, (insulin plant) are used as dietary supplement for the treatment of diabetes. **Objective:** The antidiabetic activity of this plant is well documented, but its activity on different cell types and mechanism remains unknown. Thus, the present study evaluates the cytotoxicity of *C. pictus* methanolic extract (CPME) against various cancer and normal cells. **Materials and Methods:** Dried leaves of *C. pictus* were extracted using methanol and were subjected to histone deacetylase (HDAC) inhibition and toxicity studies. **Results:** The CPME displayed a selective toxicity toward tested cancer cells in a dose- and time-dependent manner. CPME exhibited significant cytotoxicity on Liver hepatocellular carcinoma cells (Hep G2) (half maximal inhibitory concentration IC_{50} = 6.7 µg/ml). Since CPME demonstrates both antidiabetic, anticancer activity, and HDAC enzyme play a detrimental role in both the complications, we have evaluated the CPME-induced HDAC regulation on Hep G2 cell lines. CPME showed a notable HDAC inhibition (55%). Furthermore, CPME did not show any genotoxicity or membrane instability at the tested concentrations. **Conclusion:** CPME demonstrates selective cytotoxicity toward tumor cells at a lower concentration through HDAC inhibition.

Key words: *Costus pictus* methanolic extract, cytotoxicity, liver hepatocellular carcinoma cells, histone deacetylase

SUMMARY

- *C. pictus* is used as munching supplementary food for the treatment of diabetes
- CPME selectively induces cytotoxicity in cancer cells leaving normal cells healthy
- Selective toxicity to cancer cells are attributed by the inhibition of HDAC enzyme
- CPME did not show any genotoxicity and membrane instability in blood cells
- CPME could be potential source of HDAC inhibitor.



Abbreviations used: A549: Human lung carcinoma cells, CPME: *Costus pictus* methanolic extract, DMEM: Dulbecco's modified eagle's medium, DMSO: Dimethyl sulfoxide, ELISA: Enzyme-linked immunosorbent assay, 5-FU: 5-Fluorouracil, Hep G2: Liver hepatocellular carcinoma cells, HEK-293: Human embryonic kidney cells, Hela: Human cervical carcinoma cells, HT-29: Human colorectal adenocarcinoma cells, HDAC: Histone deacetylase, IC_{50}: Half maximal inhibitory concentration, MCF-7: Human breast adenocarcinoma cells, MDA-MB-435S: Human breast cancer cells, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, NFF: Neonatal foreskin fibroblasts, PHA: Phytohemagglutinin, PBS: Phosphate buffer saline, RPMI-1640: Roswell Park Memorial Institute Medium.

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INTRODUCTION

Plant-based products are major components in the discovery of new therapeutic drugs and have received much attention as they are good source of biologically active substances including antioxidants, hypoglycemic, and hypolipidemic agents.[1] Plants of *Zingiberaceae* family are traditionally used for the management of various diseases.[2] *Costus pictus* D. Don, known as "spiral ginger" or insulin plant, is a medicinal herb originated in Mexico and introduced to India.[3] It is grown in gardens especially in the state of Kerala. It is used as a munching

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supplementary food for the management of diabetes in Kerala. The leaves and rhizomes of this plant possess biological activities such as antidiabetic, antibacterial, antioxidant, and cytotoxicity.[4-6]

Histone deacetylase (HDAC) plays an important role in epigenetic regulation of gene expression by catalyzing the removal of acetyl groups, stimulating chromatin condensation, and promoting transcription repression.[7] HDAC inhibitors are either natural products or synthetic compounds that inhibit Zn^{2+}-dependent HDAC enzymes.[8] Their efficacy and mode of action during cancer, diabetes, obesity, and inflammation are well documented.[9] Research has shown that there are increased levels of HDACs in tumor cells and inhibitors of these enzymes can be considered as a class of anticancer drugs.[10] HDAC inhibitors function through diverse mechanisms, including the promotion of cell cycle arrest, apoptosis and the inhibition of angiogenesis.[11] Furthermore, the HDACs regulate histone acetylation and transcriptional factors which are involved in glucose homeostasis and thus plays a crucial role in the regulation of glucose metabolism.[12] Several reports indicate that HDACs are involved in the pathogenesis of diabetes mellitus through regulating glucose homeostasis by a number of pathways.[13] HDACs have also been proven to decrease pancreatic insulin production and secretion.[14] Therefore, HDACs serve as a potential targets for the treatment of diabetes-related complications through the regulation of insulin resistance and secretion.

C. pictus methanolic extract (CPME) possess both anticancer and antidiabetic activity,[15,16] we presume that the CPME could be a possible HDAC regulator. Despite the preliminary reports on the toxicity of the extracts of *C. pictus,* molecular mechanism of CPME against various cancer cells have not been explored till date. Hence, the current study was carried out to evaluate the cytotoxic activity of methanolic extract of *C. pictus* against normal and tumor cell lines.

In addition, cytotoxic drugs induce various adverse effects resulting from interference with structures or processes essential for cell survival, proliferation, or function. These effects may involve the integrity of membrane, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division.[17] Thus, it is important to conduct toxicology studies in the drug discovery process to determine the safety and nonadverse effect of potential drug candidates. In this study, we have analyzed cytotoxic, membrane stability, and genotoxic effect of CPME on human blood cells. Overall, this study outlines the effect of CPME against various cancer and normal cells, HDAC inhibition, hemolysis, and genotoxicity.

MATERIALS AND METHODS

Plant material

Fresh *C. pictus* leaves were collected from Kerala Agricultural University, Mannuthy. A voucher specimen was submitted in VIT University herbal garden, Vellore (VIT/CP/G1). The leaves were shade dried; powdered and 100 g was extracted using 250 ml of methanol in soxhlet apparatus. The solvents were evaporated and the residue was dried using rotary evaporator (Super Fit, Rotavap, model, PBU-6, India). The dried extract was subjected to lyophilization (Lark, Penguin Classic Plus, India) and the powdered extract was used for further assays.

Cell culture

Vero (African green monkey kidney), human embryonic kidney (HEK)-293, human cervical carcinoma cells (Hela), human colorectal adenocarcinoma cells (HT-29), human lung carcinoma (A549), and neonatal foreskin fibroblasts (NFF) cell lines were obtained from the Human breast adenocarcinoma cells American type culture collection and human breast adenocarcinoma (MCF-7), Liver hepatocellular carcinoma cells (Hep G2), human breast cancer cell (MDA-MB-435S) lines were obtained from National Centre for Cell Science, Pune (India). The cell lines were maintained in Roswell Park Memorial Institute Medium (RPMI) 1640/DMEM/L-15 (Himedia/Gibco, Mumbai, India) medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 100 mg/L streptomycin and 100 IU/mL penicillin (Himedia, India), at 37°C in 5% CO_2.

Cell viability assays

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay

The cytotoxic activity of CPME was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Cancer and normal cells were incubated with 2, 4, 6, 8, and 10 µg/ml of CPME for 24 h at 37°C. The MTT assay was performed using cell Quanti-MTT cell viability assay kit (Bioassay Systems) and the procedure was followed as per the user manual. The optical density was measured at 570 nm for each well on a multiwell plate reader (Bio-Rad). The wells with only culture medium or treated with 0.1% of dimethyl sulfoxide served as control. The average of the blank controls were determined and subtracted from the absorbance values. The same protocol was followed for 5-fluorouracil (positive control). The assay was carried out in triplicates. The graph was plotted with cell viability against the concentrations of the compound. The half maximal inhibitory concentration (IC_{50}) values and their respective 95% confidence intervals were calculated using Bliss and Finney methods.

Morphological alterations

Hep G2 cells were treated with various concentrations (2, 4, 6, 8, and 10 µg/ml) of CPME and incubated for 24 h. The cell morphology was observed and photographed under an inverted microscope (Hund Wetzlar, Germany).

Histone deacetylase inhibition assay

HDAC activity was determined using EpiQuik™ HDAC Activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY, USA) according to manufacturer's instructions. Briefly, Hep G2 cells were seeded in 6-well plate. After 24 h, cells were treated with 0.5 µg/ml of CPME for 48 h. After treatment, nucleus was prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Briefly, cells were washed with ice-cold phosphate buffer saline (PBS) containing phosphatase inhibitors, resuspended with hypotonic buffer and detergent, and then centrifuged to obtain cytosolic fraction. Nuclear fractions were collected by suspending nuclear pellet with lysis buffer and centrifugation was again carried out. Biotinylated HDAC substrate was added to 8-well strip plate for 45 min. After washing the mixtures containing nuclear extract and HDAC assay buffer were added to the plate. After 1 h, the plate was washed, and the capture antibody was added for 1 h. After adding detection antibody and developing solution, the absorbance was recorded at 450 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc.) after adding stop solution. The known HDAC inhibitor trichostatin A (25 nM) (Sigma, India) was used as positive control.

In vitro hemolytic assay

Human erythrocytes were prepared from the peripheral blood (O^+) of healthy donor. The hemolytic assay was performed as described earlier.[18] The blood was used within 24 h after bleeding and washed three times in nine volumes of sterile 0.9% NaCl solution. After each washing, cells were centrifuged at 2000 rpm for 5 min and the supernatant was discarded. The final pellet was diluted 1/9 (v/v) in sterile Dulbecco's PBS, pH 7.0 containing 0.5 mM boric acid, and 1 mM calcium chloride. The different concentration of CPME (5, 10, 20, 40, 60, 80, and 100 µg/ml) was mixed

with 0.5 ml of normal erythrocyte suspension in small tubes and the final volume was made to 2 ml with 0.15 M saline. The mixture was incubated at 37°C for an hour and centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant was measured at 540 nm. The last well served as positive control containing 20 ml of 0.1% Triton X–100 in 0.85% saline.

Microscopic observation

The morphology of erythrocytes after treatment with CPME was also assessed by viewing the cells under light microscope (×100).

Genotoxicity analysis using leukocytes

Genotoxicity of CPME was carried out as described earlier.[19] Briefly, 5 ml of blood was obtained from healthy volunteers using heparinized syringe. Leukocyte cultures were incubated along with CPME (5, 10, 20, 40, 60, 80, and 100 µg/ml). Vials containing 0.5 ml of blood was inoculated in 5 ml of RPMI-1640 medium containing 1.5 ml of FBS, 0.1 ml of phytohemagglutinin under aseptic condition. The cultures were then incubated for 72 h at 37°C. At the end of the incubation period, the dividing cells were arrested at metaphase by the addition of two drops of 0.1% colchicine solution to each culture vial. The cultures were incubated for 20 min at 37°C. Leukocytes were harvested after 20 min by centrifuging cell suspension at 2000 rpm to remove the culture medium, addition of hypotonic solution (KCl 0.075 M) at 37°C for 6–7 min and treated twice with Carnoy's fixative (methanol:glacial acetic acid, 3:1). Slides were then dried, stained with Giemsa and Observed under light microscope (Labomed) at 100×. Mitomycin C (1 µg/ml) was used as positive control and culture tubes without any extract were treated as negative control.

Micronuclei detection

The protocol for the micronuclei detection was adopted from Fenech.[20] For micronuclei detection, 0.5 ml of blood was added in 5 ml of RPMI-1640 medium. Five culture vials were made and incubated for 72 h at 37°C. The cultures were shaken and carbon dioxide was released once in 24 h. At 44th h, two drops of cytochalasin B were added to each vial, and the vials were kept for incubation. At the 71st h, various concentrations (5, 10, 20, 40, 60, 80, and 100 µg/ml) of CPME were added to each vial. The vials were kept for incubation for 1 h. After incubation, cells were harvested as per the standard protocol and stained using Giemsa. The cells were observed under light microscope (Labomed) at 100×.

RESULTS

Cytotoxic activity of *Costus pictus* methanolic extract on cancer and normal cells

The effect of CPME was examined on cancer (Hep G2, A549, HT-29, MCF-7, and Hela) and normal cells (MDA-MB-435S, Vero, HEK-293, and NFF) and their respective IC_{50} values are shown in [Table 1]. All the tested cancer cells were susceptible to the CPME. The effect of various concentrations of CPME on the tested cells exhibited concentration and time-dependent inhibition. The strongest cytotoxicity was observed on Hep G2 cells at a concentration of 10 µg/ml, and the IC_{50} value was found to be 6.7 µg/ml. Hence, further studies were performed using Hep G2 cells. About 30% cell death was observed when treated with 4 µg/ml of CPME for 24 h [Figure 1]. The percentage of cell death was doubled when incubated for 48 h at the same concentration. CPME also exhibited notable cytotoxicity against A549, HT-29, MCF-7, Hela cells and their IC_{50} values were 9.1, 13.9, 14.2, and 26.8 µg/ml, respectively. Comparatively, CPME showed less or no cytotoxicity toward the tested normal cells. All the results were compared with a positive control, 5-FU.

Morphological alteration of liver hepatocellular carcinoma cells induced by *Costus pictus* methanolic extract

Hep G2 cells were treated with various concentrations (2–10 µg/ml) of CPME. As shown in [Figure 2a], untreated cells appear epithelial and very few round cells are observed. Cells treated with 8 µg/ml of CPME showed morphological alterations including adhesion loss and sporadic distribution [Figure 2b]. These morphological changes were seen within 3 h after incubation with CPME and lasted throughout the whole period of treatment.

In vitro histone deacetylase inhibitory effect of *Costus pictus* methanolic extract on liver hepatocellular carcinoma cells

The present study elucidates HDAC inhibitory activity of CPME on Hep G2 cells [Figure 3]. The results indicate that the CPME significantly induces the HDAC inhibition on Hep G2 cells in a dose-dependent manner (0.5–8 µg/ml). Maximum HDAC inhibition (56%) was seen with 8 µg/ml of CPME whereas 68% of inhibition was observed with positive control trichostatin A. It should be noted that the IC_{50} value against Hep G2 cell is 6.7 µg/ml and induced a drastic morphological changes when observed at 8 µg/ml of CPME (IC_{50} = 7.1 µg/ml).

Table 1: Doses inducing 50% cell inhibition (IC50) by *Costus pictus* methanolic extract and positive control (5-fluorouracil) on cancer and normal cells

Cell lines	IC_{50} (µg/ml)	5-FU
Hep G2	6.7	1.35
A549	9.1	1.20
HT-29	13.9	0.23
MCF-7	14.2	0.58
Hela	26.8	1.93
MDA-MB-435S	17.2	1.27
Vero	ND	3.67
HEK-293	ND	2.77
NFF	ND	4.04

NFF: Neonatal foreskin fibroblasts; ND: Not determined; HEK-293: Human embryonic kidney; 5-FU: 5-Fluorouracil; Hep G2: Liver hepatocellular carcinoma; A549: Human lung carcinoma; HT-29: Human colorectal adenocarcinoma; MCF-7: Human breast adenocarcinoma; Hela: Human cervical carcinoma; MDA-MB-435S: Human breast cancer; Vero: African green monkey kidney

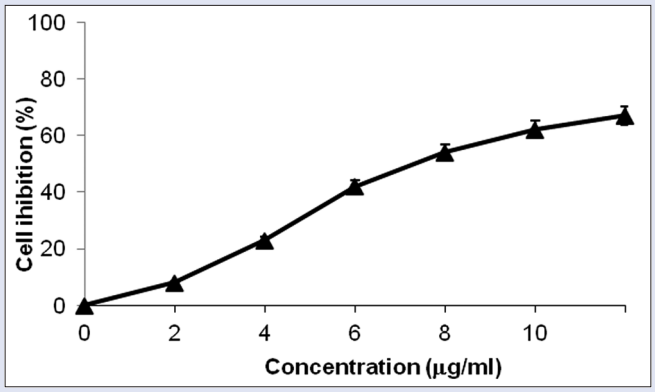

Figure 1: Cytotoxic activity of *Costus pictus* methanolic extract on liver hepatocellular carcinoma cells. Cells were treated with various concentrations (2, 4, 6, 8, and 10 µg/ml) of *Costus pictus* methanolic extract. The experiments are carried out in triplicates and results are expressed as mean ± standard deviation

In vitro hemolytic activity

In vitro hemolytic activity of CPME was evaluated using normal human erythrocytes. The CPME exhibited weak or no activity on the lysis of erythrocytes. Both untreated and treated erythrocytes exhibited round, biconcave disc shape morphology [Figure 4]. Microscopic examination revealed that human erythrocytes treated with 50 µg/ml of CPME showed no significant morphological changes as compared to control (PBS).

Chromosome aberration

The metaphase analysis of human leukocytes exposed to various concentrations of CPME (5–100 µg/ml) was evaluated *in vitro* and the results are shown in [Figure 5]. CPME showed insignificant changes in the chromosome aberrations in comparison to the positive control (mitomycin C). Relatively, higher frequencies of breaks and gaps were observed in the positive control. Further, there were no micronuclei formation observed in the CPME-treated leukocytes.

DISCUSSION

Bioactive compounds derived from plants have played significant role in drug discovery and development. Phytochemicals from the medicinal plants are developed for the treatment of many human ailments.[21] Plant extracts have been used as traditional herbal remedies to treat diseases such as cancer and diabetes.[22,23] The phytochemical screening of *C. pictus* showed the presence of saponin, glycosides, flavonoids, phytosterols, and carbohydrates in detectable amount.[24] *C. pictus* extracts have demonstrated antibacterial, antioxidant, antidiabetic, cytotoxic, and antiglycation activities.[25-28] It was also been reported that *C. pictus* contain several bioactive compound producing genes.[29] However, there are no extensive studies on the anticancer property of this plant.

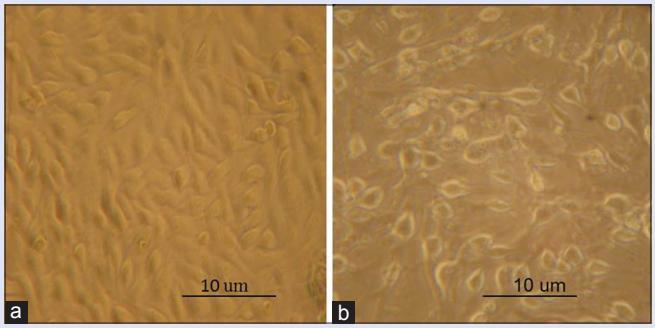

Figure 2: Morphological changes of liver hepatocellular carcinoma cells under a light microscope. (a) Control liver hepatocellular carcinoma cells. (b) Liver hepatocellular carcinoma cells after exposure to *Costus pictus* methanolic extract (8 µg/ml) for 24 h

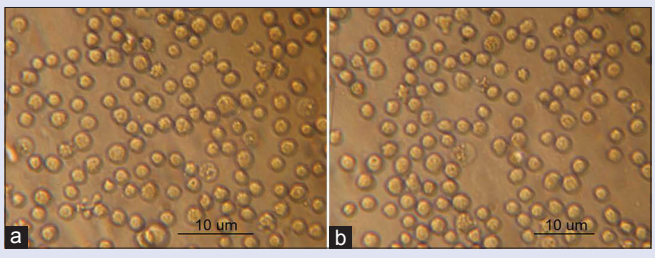

Figure 4: Morphological changes of (a) control untreated erythrocytes. (b) Erythrocytes after exposure to *Costus pictus* methanolic extract (50 µg/ml)

Epigenetic regulation of gene expression is recognized as new approach to treat diseases such as cancer and diabetes. In particular, HDAC inhibitors provides a significant role as anticancer agents.[30] The HDAC expression is upregulated during various clinical conditions such as cancer, diabetes, and inflammation. Recent studies state that the HDACs regulate glucose homeostasis and can be targeted for the treatment of diabetic microvascular complications.[31] Further, HDAC inhibitors are shown to promote β-cell development, proliferation, differentiation, and function. These inhibitors also positively affect the late diabetic-related complications. Several studies have addressed the isolation of HDAC inhibitors from various plants and microbes to be used as therapeutic agents for cancer and diabetes treatment. However, the inhibitors that have cleared phase III human trials are very limited. *C. pictus* possess both antidiabetic and cytotoxic activities.[32,33] With proven antidiabetic activity, *C. pictus* extracts can be potential HDAC modulators. In light of its important pharmacological activities, the objective of present study is to establish its anticancer efficiency and its HDAC inhibition. To determine whether the antidiabetic and anticancer activity of

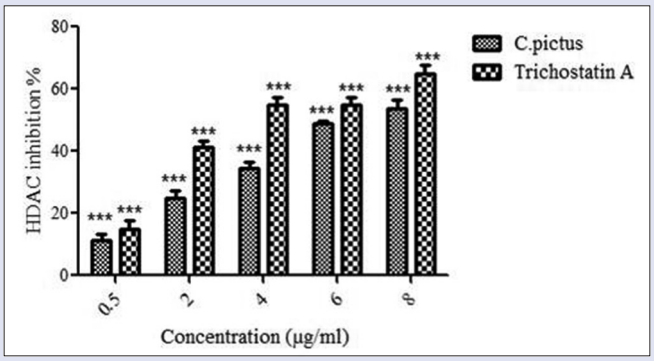

Figure 3: Histone deacetylase inhibition studies using *Costus pictus* methanolic extract and trichostatin A (positive control). Results were expressed as relative histone deacetylase activity with respect to positive control. Each value represents the mean ± standard deviation, performed in triplicates. Asterisks (*) denote significant differences vs control ($P < 0.0001$)

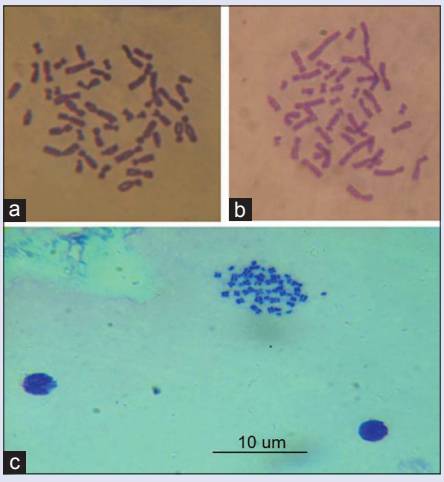

Figure 5: Effect of *Costus pictus* methanolic extract on leukocyte chromosomes. (a) A regular sized pair of intact chromosomes was observed with negative control. (b) Metaphase stages of human leukocytes after treatment of *Costus pictus* methanolic extract (50 µg/ml). (c) Positive control, mitomycin C (1 µg/ml)

C. pictus is due to HDAC modulation, the HDAC inhibition study was performed using Hep G2 cells. The results show a reduced HDAC activity on treatment with CPME. The CPME showed a maximum of 56% inhibition (8 µg/ml) and IC_{50} of 7.1 µg/ml. The known HDAC inhibitor, trichostatin A displayed a strong inhibition (67%) at 25 nM concentration. Several studies have reported that HDAC inhibitors potentially inhibit cell proliferation and induce apoptosis in tumor cells.[34] Comparatively, HDACs are found to be upregulated in cancer cells, and any HDAC inhibitors should selectively inhibit the cancer cell proliferation.[35] Our results demonstrate that CPME is more toxic to cancer cells leaving normal cells less or nontoxic. This inhibitory effect of plant extract could be attributed to a direct ionic interaction of the active ingredients of plant with the active zinc site of HDAC enzyme since classical HDAC's are zinc-dependent enzymes with a highly conserved Zinc domain.[36] As reported by Lundh *et al.*, the HDAC inhibitors potentially inhibit the Zn^{2+}-dependent HDACs in animal models of diabetes mellitus.[37] Reports indicate isolation of novel HDAC Inhibitors from n-hexane fractions of *Zingiber zerumbet*.[38] Further, the plant extracts and/or synthetic compounds are reported to have anticancer and antidiabetic property through HDAC inhibition.[39,40]

Cytotoxic potential of CPME was determined by MTT assay against various cancerous and normal cells. CPME exhibits cytotoxic potential against all the tested cancer cell lines under *in vitro* conditions. CPME showed highest cytotoxic activity in Hep G2 cells (IC_{50} = 6.7 µg/ml). The alterations in the cellular morphology of Hep G2 cells exposed to CPME showed detachment of cells with increasing concentration. The CPME also induced significant changes in the morphology of Hep G2 at higher concentration (8 µg/ml). An IC_{50} value below 20 µg/ml was observed for Hep G2, A549, HT-29, and MCF-7 cells which falls within NCI guidelines,[41] and thus considered to have anticancer potential. It should also be noted that the CPME selectively inhibits the proliferation of cancer cells leaving normal cells less toxic. The above data demonstrate the potential anticancer activity of CPME.

In the present study, we have also determined the effect of CPME in normal blood cells. The hemolytic assay was performed using erythrocytes, and the results show that CPME did not possess any blood cell lysis property. The CPME was found to be nontoxic even at 50 µg/ml concentration. Erythrocytes represent a good model for the study of membrane stability, and the assessment of membrane stability is important during preclinical drug discovery process.[42] The mechanical stability of the erythrocyte membrane is a good indicator of the effect of various bioactive compounds and CPME do not cause any membrane damage to erythrocytes. These data suggest that the cytotoxicity of CPME is not due to cell membrane disruption or lytic property. Furthermore, the effect of CPME was evaluated using leukocytes for genotoxic property. The results show that tested dosages of CPME were nontoxic against human leukocytes *in vitro* and hence the cytotoxicity of CPME is not due to genotoxicity. CPME (50–200 µg/ml) did not induce any chromosomal gaps and breaks, and no micronucleus formation was observed in CPME-treated leukocytes.

CONCLUSION

The present study indicates that the CPME selectively targets the cancer cell via HDAC inhibition. It also highlights that the anticancer and antidiabetic activity of CPME is due HDAC inhibition and requires further studies at molecular level. The isolation and characterization of HDAC inhibitor form *C. pictus* are in progress in our laboratory.

Acknowledgement

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Conflicts of interest

There are no conflicts of interest.

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