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Morphological and molecular effects of phenolic extract from coconut kernel on human prostate cancer cell growth *in vitro*

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Abstract. Coconut is an indispensable ingredient in the diet and traditional medicine of individuals belonging to the Indian subcontinent. Coconut is of high nutritional value owing to the presence of all essential dietary components, *viz*, saturated fatty acids, arginine rich proteins, fibre and minor components like vitamin E, phytosterols, polyphenols and flavonoids. The polyphenolic content present in coconut kernel is of particular interest due to their numerous reported beneficial effects such as reduction of oxidative stress, combating cancer and in modulating anti-inflammatory pathways. Therefore, in the present study the cytotoxic effect of the polyphenol rich fraction from coconut kernel (CK_f) was evaluated in human prostate cancer (DU-145) cells. Individual components present in CK_f was determined by LC-MS analysis. It showed that CK_f contained several bioactive molecules which have potential anticancer activity *viz*, coumaric acid, myristin, chlorogenic acid and triterpenoid methyl esters. The cytotoxic effect of CK_f at various concentrations (2.5–20μg/ml) on DU-145 was assessed using MTT assay, AO/EB staining, mitochondrial superoxide/ROS production and changes in intracellular calcium levels, 24 hrs post treatment. Changes in the cell morphology and nucleus were observed using Scanning Electron Microscopy and Confocal microscopy. ROS and mitochondrial superoxide levels was evaluated using DCHF-DA and MitoSOX staining respectively. The impact of ROS on changes in cellular calcium levels was also studied using Fura-2-AM. LDH leakage from CK_f treated and control cells were observed colorimetrically. Further, PCR analysis was done to detect changes in mitochondria associated apoptotic gene expression. It was also observed that CK_f treatment increased the expression of pro-apoptotic genes - Bax, Bid, Bak and p53 in a dose-dependent manner. Based on the above results, it can be concluded that CK_f may be used as a part of a dietary regime for controlling the progression of prostate cancer.

Keywords: Coconut kernel, polyphenols, ROS, apoptosis, prostate cancer

1. Introduction

Prostate cancer is the most common non-cutaneous cancer in men. Several scientific studies have examined the relationship between prostate cancer and dietary antioxidants; however, the results of these studies are

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inconsistent [1–7]. Many dietary components may play a role in the development and progression of prostate cancer [8]. Prostate cancer incidence and mortality differ between countries and between men of different race or ethnicity [9–11], which may partly due to the differences, in dietary patterns [12]. Many plants and oil seeds contain significant amount of unexplored antioxidants and biologically active compounds which show promising anticancer activity [13–20]. Plant foods, especially fruits and vegetables provide a multitude of antioxidants and phytochemicals which have a demonstrable beneficial effect on prostate cancer. Plants have been used in traditional medicines for the treatment of several diseases including cancer and diabetes. In recent years, the interest in oriental medicinal herbs based pharmaceuticals/neutraceuticals has aroused the interest of scientific community as complementary/and or alternative medicines [21]. Several chemotherapeutic drugs derived from plants are used directly as chemotherapeutic agents, such as vinblastine, taxol, camptothecin and podophyllotoxin or for the parent compound for more potent medicines for cancer management [22].

Coconut, a nature's boon has been influencing the human health for many years in the past and will continue in the future too [23]. Though the saturated fats present in the coconut oil is been blamed for increasing the cholesterol levels in our body, there are few unnoticed components present in it which may prove beneficial to the mankind. Apart from fat, coconut contains water rich in amino acids, especially arginine and glutamic acid, minerals, growth factors and anti diabetic proteins [24]. Other minor components like phytosterols, polyphenols, flavonoids, tocopherols and organic components are also present [25, 26]. This study was designed to evaluate the polyphenol/flavonoids components present in the methanolic fraction and their effect on preventing the growth of prostate cancer cells in *in vitro* conditions.

2. Materials and methods

2.1. Materials and reagents

Dwarf × Tall (DxT) variety coconuts were collected from Shornur Panchayat Krishi Bhavan Office, Palakkad, Kerala, India. Minimal Essential Medium (MEM), Fetal bovine serum (FBS), Antibiotic and Antimycotic solution (100X), MTT (3;(4,5;Dimethylthiazol;2yl);2,5;diphenyl tetrazolium bromide), Acridine Orange (AO) (*N,N,N',N*';Tetramethylacridine;3;6;diamine), Ethidium bromide (EB) (3,8;Diamino;5;ethyl;6; phenylphenanthridinium bromide), Trypan blue, DAPI (4',6;Diamidino;2;phenylindole), RDP Trio Reagent and Lactate dehydrogenase assay kit (EZ count^{TM}) was purchased from Hi;Media Laboratories, India. DCFH;DA (2',7';dichlorofluorescin diacetate) was purchased from Sigma-Aldrich, USA. Synthesized oligos for PCR was purchased from Integrated DNA technologies, USA. Revert Aid First Strand cDNA Synthesis Kit was purchased from Thermo Fischer Scientific, USA. Go Taq Green Master Mix was procured from Promega, USA. MitoSOX and Fura-2-AM was procured from Invitrogen, Life Technologies, USA.

2.2. Extraction of polyphenol/flavonoid fraction from coconut kernel

Coconut kernel was removed and defatted with petroleum ether (60°–80°C) using a Soxhlet apparatus. The residue obtained after defatting was dried, weighed and exhaustively extracted using 80% methanol. Methanolic extract (CK_f) thus obtained was dried in rotary evaporator, weighed and used for further experiments [27].

2.3. LC-MS analysis of CK_f

Identification of the bioactive compounds present in CK_f was analyzed with Waters Xevo G2Q-TOFmassspectrometer with a Waters Acquity H class Ultra Performance Liquid Chromatography (UPLC) system equipped with ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm × 1.7 μm; Waters, Milford, MA, USA) at 30°C. 10 μl of the sample was injected for a total run time of 10 min at a flow rate of 0.3 ml/ min using 0.1%

formic acid in water (A) and methanol (B) in gradient program as the mobile phase. The sample was ionized by electrospray ionization (ESI) in negative mode with a capillary voltage at 2.5 kV, sample cone voltage at 30 V and nitrogen was set at 900 L/h and the temperature at 350°C. The cone gas, nitrogen, was set at a flow rate of 50 L/h, and the source temperature was set at 135°C. A full scan analysis ranging from 50 to 1000 m/z was performed. A lock spray ionization source is present along with the waters Q-TOF equipment that performs on line calibration using leucine-enkephalin ([M+H] + m/z 556.2771) for providing accurate and reproducible molecular masses of parent and product ions. The elemental composition was determined by Mass Lynx V 4.1 software.

2.4. Cell culture

Human prostate cancer cell line (DU-145) was procured from National Centre for Cell Science (NCCS), Pune, India. The cells were grown and maintained in a humidified incubator at 37°C, 5% CO2 atmosphere in MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics.

2.5. In vitro cytotoxic effect of CK_f on DU cells: MTT assay

To determine the anti-proliferative effect of CK_f, the MTT assay was performed as described earlier [28]. This method is based on conversion of the tetrazolium salt (MTT) to colored formazan by viable, but not dead, cells. DU-145 cells were treated with different concentrations of CK_f (0.2 to 10μg/ml) for 24 h. After the treatment period, the viability of DU-145 cells was determined by adding MTT to the cell cultures to reach a final concentration of 1 mg/mL. After 4 h incubation at 37°C, the dark crystals formed were dissolved by adding to the wells an equal volume of DMSO and the plates were incubated overnight at 37°C and optical densities at 570 nm was measured using a plate reader (Varioskan Flash Microplate reader, Thermo scientific) with a corresponding filter. Data are presented as a percentage of the value obtained from cells incubated in fresh medium only. All experiments were performed in triplicate. The inhibition rate was calculated as follows: Growth inhibition rate(%) = $(A_{control}\text{-}A_{drug}/A_{control}) \times 100$.

2.6. Determination of apoptosis: AO-EB double staining

To visualize the effect of CK_f on apoptosis, the cells were stained using fluorescent Acridine orange-ethidium bromide [29]. DU-145 cells were seeded at a density of 3×10^4 cells/well and treated with CK_f at varying concentrations between 1–10 μg/ml for a period of 24 h at 37°C, 5% CO_2. Following incubation, the wells were washed with PBS and AO-EB stain (100μg/ml each) was added at a ratio of 1:1. The plates were incubated in the dark for 30 min at 37°C. Finally cells were examined at 20X magnification using an Olympus inverted fluorescence microscope.

2.7. DAPI staining: Confocal Raman spectra

DU-145 cells were grown and maintained in a humidified incubator at 37°C, 5% CO_2 atmosphere in MEM supplemented with 10% FBS and 100X Antibiotic and Antimycotic solution. For the experimental purpose, cells were seeded onto 12-well plate with cover slip (at a density of 4 to 7×10^4 cells/ml). After 24 h incubation period to allow cell attachment, the cells were treated with various concentrations of CK_f, (2.5, 7, 10 μg/ml) and incubated for 24 h. After incubation, cells were washed with PBS and fixed with 10% formaldehyde for 5–10 min. Fixed cells were then washed twice with PBS and permeabilised with Triton-X 100 for 5 min and then subsequently washed 2-3 times in PBS. Cells were then stained with DAPI for 10–15 min, washed twice and mounted onto a slide with mounting solution. Confocal images were taken using NIKON AIR Microscope. Both the bright field and fluorescent images (DAPI) were taken in a 60X magnification.

2.8. SEM analysis

DU-145 cells were grown and maintained in a humidified incubator at 37°C under 5% CO_2 atmosphere in MEM supplemented with 10% FBS and 100X Antibiotic-Antimycotic solution. For experimental purposes, cells were seeded onto coverslips in 12-well plates at a density of 4–7 $\times$ 10^4 cells. After a 24 h incubation period to allow for cell attachment, the cells were treated with various concentrations of CK_f (2.5, 7, 10 μg/ml) and incubated for 24 h. Cells were then washed with PBS and fixed with 10% formaldehyde for 5–10 min. Fixed cells were washed twice with PBS and then air dried before the samples were processed for SEM analysis.

2.9. FTIR; ATR analysis

DU-145 cells (2 $\times$ 10^4 cells/well) were treated with different concentrations of CK_f (2.5, 7, 10 μg/ml) for 24 h. After the incubation period, cells were centrifuged at 1000 rpm for 5 min to remove medium and then fixed in 70% ethanol (EtOH) for 1 h. After centrifugation and washing 2 more times using 70% EtOH, the concentrated cells were applied to aluminium foil and air-dried. ATR-FTIR spectral measurements were performed using a Shimadzu ATR attachment containing a diamond crystal internal reflective element [30].

2.10. Ca^{2+} signalling and mitochondrial membrane potential

The effect of CK_f on alterations in intracellular calcium levels were evaluated in DU-145 cells using the ratiometric probe Fura-2-AM. DU-145 cells (2 $\times$ 10^4cells/well) were treated with different concentrations of CK_f (2.5, 7, 10 μg/ml) for 24 h. After the treatment period, culture medium in the wells was replaced with Krebs buffer (1 mM $CaCl_2$; 132 mM NaCl; 4 mM KCl; 1.2 mM Na_2HPO_4; 1.4 mM $MgCl_2$; 6 mM Glucose;10 mM HEPES, pH 7.4), supplemented with 1 mg/ml bovine serum albumin (BSA) and 5M Fura-2-AM, and incubated at 37°C in the dark for 40 mins. Cells were rinsed twice with Kreb's buffer following the incubation period and fresh buffer supplemented with BSA minus the probe was added to the wells. The cells were then observed by fluorescence microscopy using the appropriate band-pass filter. (Olympus 1 $\times$ 51) [31].

The electrical potential across the inner mitochondrial membrane of CK_f treated DU-145 cells was determined using Rhodamine-123 (R-123), a lipophilic, cationic indicator. DU-145 cells (2 $\times$ 10^4cells/well in 96-well plates) were treated with different concentrations of CK_f for 24 h. After the treatment, cells were rinsed with PBS and fresh media containing R-123 solution (10μg/ml) was added to the treated wells and the plates were incubated in the dark at 37°C for 20–30 min. Subsequently, the cells were washed twice with PBS and the cell images were taken using a fluorescence microscope (Olympus 1$\times$51) [32].

2.11. Levels of ROS: DCFH;DA staining

To determine whether CK_f treatment increases the generation of excessive intracellular ROS in DU-145 cells, which may contribute towards cytotoxicity were examined using a fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) [33]. 96-well plates seeded with DU-145 cells were treated with different concentrations of CK_f (2.5, 7, 10 μg/ml) for 24 h. Treated cells were then washed with phosphate buffered saline (PBS) and incubated with fresh DCFH-DA (100 μM) in PBS for 30 min at 37°C, 5% CO_2. After incubation, fresh medium without FBS was added to the wells and fluorescence images were taken using an inverted microscope (Olympus 1 $\times$ 51).

2.12. Determination of mitochondrial superoxide production: MitoSOX staining

DU-145 cells (2 $\times$ 10^4) were plated on 96-well plates and incubated overnight to allow for cell attachment. Following treatment with different concentrations of CK_f (2.5, 7, 10 μg/ml), cells were labelled with MitoSOX

Red (396μM) in complete medium for 20 mins at 37°C. Post incubation, the cell medium was replaced with fresh medium without the dye and cells were incubated for another 20 mins at 37°C. Images were taken by an inverted microscope using either 10/20X objective lens MitoSOX was excited by laser at 514 nm [34].

2.13. LDH leakage assay

Membrane damage in DU-145 cells on treatment with CK_f was detected using the EZcount^{TM} LDH Cell Assay Kit, Hi-Media in a one-step reaction. The enzyme in the LDH reagent uses NADH to reduce the dye to a coloured product which can be measured colorimetrically. Briefly, DU-145 cells (2×10^4) were seeded on a 96-well plate and incubated overnight for adherence. Post adherence, cells were treated with different concentrations of CK_f (2.5, 7, 10 μg/ml) for 24 hrs. After the incubation period, lysis solution was added to each well and incubated at 37°C. Post-lysis the LDH reagent was added to all the wells and the plate was incubated at room temperature for 10 min. Reaction was terminated with the addition of a stop solution. Absorbance was read at 580 nm as a main wavelength and at 630 nm for the reference wavelength.

2.14. Expression of apoptotic genes by polymerase chain reaction

Total RNA was isolated from DU-145 cells non treated and treated with different concentration of CK_f (2.5, 7, 10 μg/ml) for 24 hrs using RDP Trio Reagent, and cDNA was synthesized using Revert Aid First Stand cDNA synthesis Kit, stored at –20°C. Expression of apoptotic genes, Bax, Bid, Bak, and p53 was performed using the following primers: Bax (Forward 5'; GAGAGGTCTTTTTCCGAGTGG; 3', Reverse 5'; CCTTGAGCACCAGTTTGCTG; 3'), Bak (Forward 5'; GGGTCTATGTTCCCCAGGAT; 3', Reverse 5'; GCAGGGGTAGAGTTGAGCA; 3'); p53 (Forward 5'; GGCCCACTTCACCGTACTAA; 3', Reverse 5'; GTGGTTTCAAGGCCAGATGT; 3') Bid (Forward 5'; CCCACACTGGTGAGACAACT; 3'; Reverse 5'; GTCGTTCTCCATGTCCCTA; 3'). GAPDH was used as an endogenous control. The polymerase chain reaction (PCR) products were analyzed on a 1.5% agarose gel electrophoresis, and band intensity was detected using Gel Doc-EZ imager (BIO-RAD).

2.15. Statistical analysis

Experimental results are expressed as mean ± S.D of three independent experiments. One-way ANOVA using SPSS-19, IBM Technologies Software was used for statistical analysis, followed by Duncan's multiple comparison test to assess the significance between groups. Value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. CK_f shows the presence of biologically active components

Earlier studies in our laboratory showed that CK_f contained polyphenols and flavonoid class of compounds. Further evaluation using LC-MS showed several peaks. Assessment of each peak and the mass obtained was compared with available literature for confirmation of the compounds. Results showed the presence of coumaric acid, caffeic acid, trihydroxy flavone derivatives, chlorogenic acid, myricetin derivative, tetramer of 5-dihydrobenzoic acid, hydroxy cinnamic acid derivative and titerpene methyl esters in CK_f (Fig. 1).

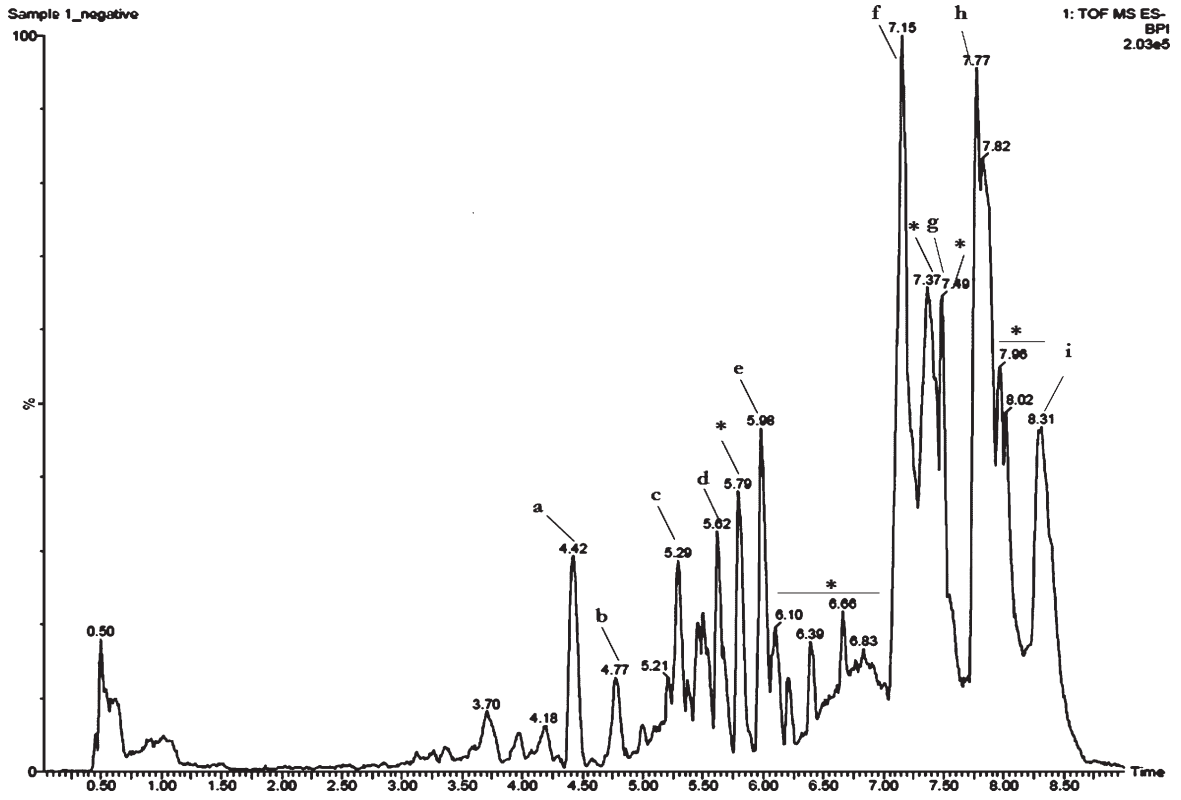

Fig. 1. Total ion chromatogram (TIC) of CK_f. a-Coumaric acid, b-Caffeic acid, c-Naringenin, d-Naringenin derivative, e-Chlorogenic acid, f-myricetin derivative, g-Tetramer of 5-dihydrobenzoic acid, h-Hydroxy cinnamic acid derivative, i-Titerpene methyl esters. *Unidentified.

3.2. CK_f is cytotoxic to DU-145 cells

Prostate cancer has the highest mortality rate of all cancers with 94% of patients dying within years of diagnosis. This cancer grows quickly with no effective treatments available, underscoring the importance of finding new therapies. Therefore, the identification and development of alternative medicines for the treatment and prevention of prostate cancer is needed. Dietary sources are a promising source of new therapeutic options, Coconut kernel and oil extracted from it is rich in phytochemicals including phytosterols and polyphenols has many significant medical effects. Though coconut kernel has anti-diabetic and anti-atherosclerotic effect [35–37], there are no published studies on its anti-cancer activity. Therefore, in the present study, we examined the effect of CK_f on human prostate cancer cells, DU-145 *in vitro*. CK_f is a methanolic extract containing polyphenols/flavonoids isolated from DxT variety of coconut. The anti-cancer effect of CK_f on decreasing the growth rate of cancer cells has been scrutinized at molecular level through following up the expression of relevant mitochondria related genes.The morphological changes inclusive of damaged cell membranes and cell shrinkage, which are features of apoptosis have also been evaluated. Anti-cancer activities of CK_f on DU-145 cells at different concentrations were tested using MTT assay. The results showed a significant cytotoxicity in a dose-dependent manner. CK_f at 10 μg/ml inhibited growth by 70%, while 5-fluorouracil, an anti-cancer drug could induce cell death at 50% at a similar concentration (Fig. 2). This suggests that CK_f is promising in preventing the growth of the prostate

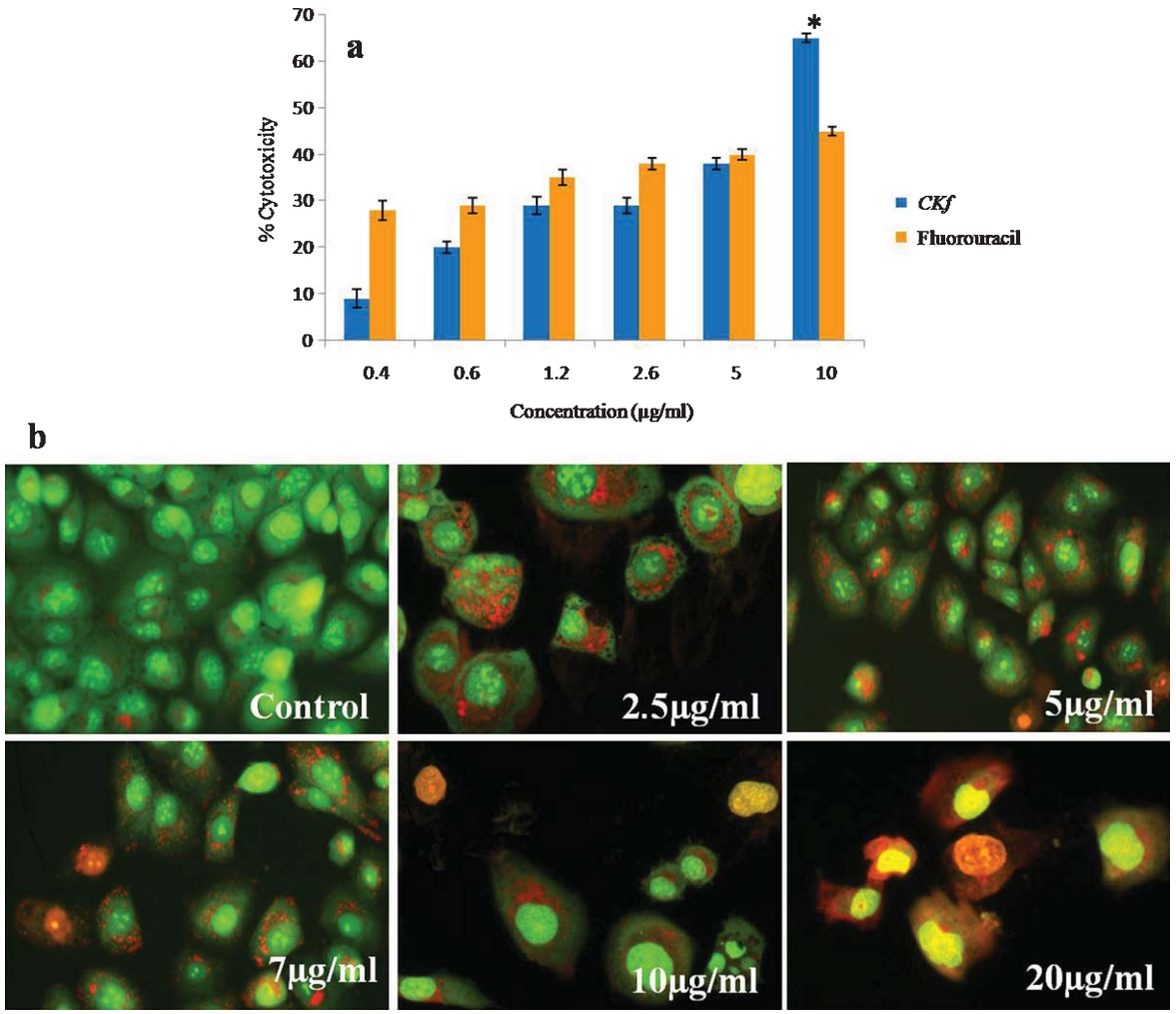

Fig. 2. Cytotoxicy analysis of CK_f on DU-145 cells. a) MTT assay; *statistically significant compared to the higher dose of fluorouracil ($p < 0.05$). b) Acridine orange ethidium bromide staining on DU-145 cells for evaluating apoptosis. Values are expressed as Mean ± SD of three independent experiments.

cancer cell line under study. Therefore, further studies were done to analyze various processes involved in the CK_f mediated cell death.

3.3. CK_f induces apoptosis of DU-145 cells

The results from AO/EB double staining are shown in Fig. 2. From the data it is clear that in 7, 10, 20 µg/mL of CK_f treated DU-145 cells, the number of viable cells decreased tremendously. Besides, some cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. However, the number of cells stained was larger at higher concentrations of CK_f. This indicates that most of the cells were not undergoing cell death which occurs primarily through apoptosis. This effect was comparable with the standard drug 5-fluorouracil.

CK_f was found to be less toxic towards normal cells, as tested in rat cardiomyocytes, H9c2 cells (*unpublished report*).

3.4. CK_f induces morphological and nuclear changes in DU-145 cells

Morphological changes in DU-145 cells exposed to different concentrations of CK_f (2–10μg/ml) for 24 h was observed by SEM (Fig. 3). Contrary to untreated DU-145 cells, which possessed a regular characteristic shape, SEM revealed cell volume shrinkage, membrane blebbing, and membrane cracks in cells treated with CK_f. Confocal images of DU-145 cells stained with DAPI showed a reduction in nuclear size, blebbing, apoptotic body formation and chromatin condensation (Fig. 4). Inhibition of proliferation and/or induction of apoptosis in cancer cells are the most important effect of many anti-cancer agents [38]. In the present work, it was found that CK_f induced apoptosis in DU-145 cells. FTIR analysis of DU-145 cells treated with CK_f showed differential changes in the lipid/acyl chain region (3500–3600 cm^{-1}), nucleic acid region (1500–2000 cm^{-1}) and amide regions (2000–3000 cm^{-1}). These changes indicate the nuclear and cell membrane damage to treated cells (Fig. 4).

3.5. CK_f induces a decrease in mitochondrial membrane potential

One of the most important hallmarks of apoptosis is the depolarisation of the mitochondrial membrane. In addition, mitochondria are the main source of ROS in the cells [39, 40]. Thus, we tested the biological effect

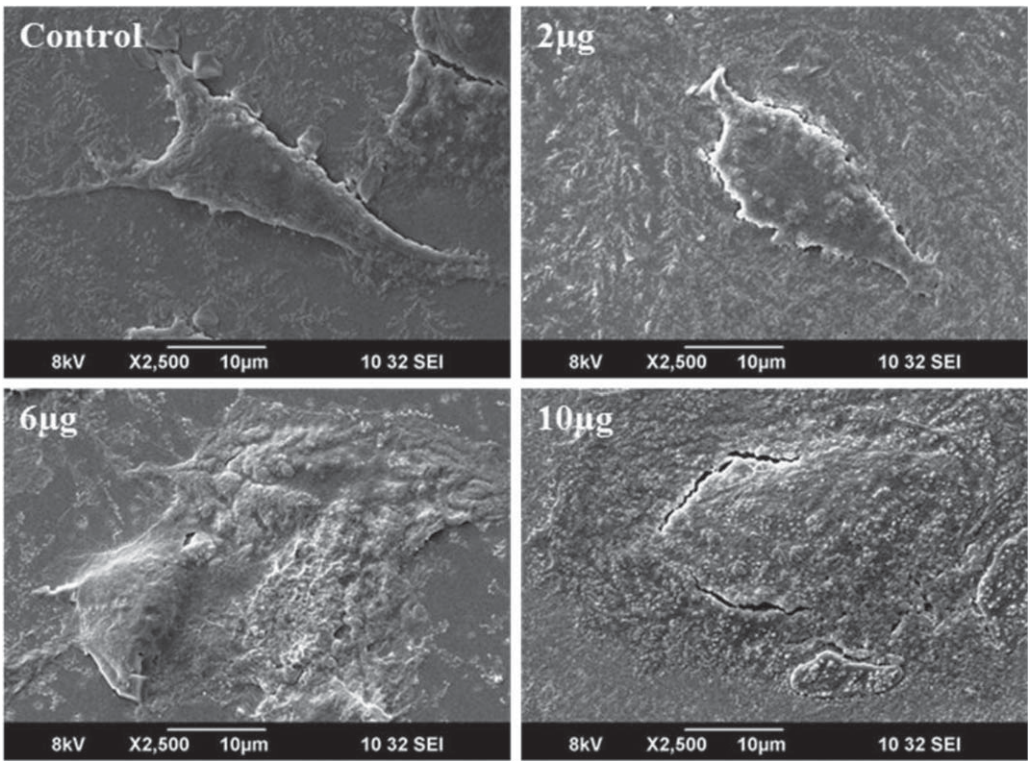

Fig. 3. SEM analysis for morphological changes of control and CK_f treated DU-145 cells. All the experiments were done in triplicate.

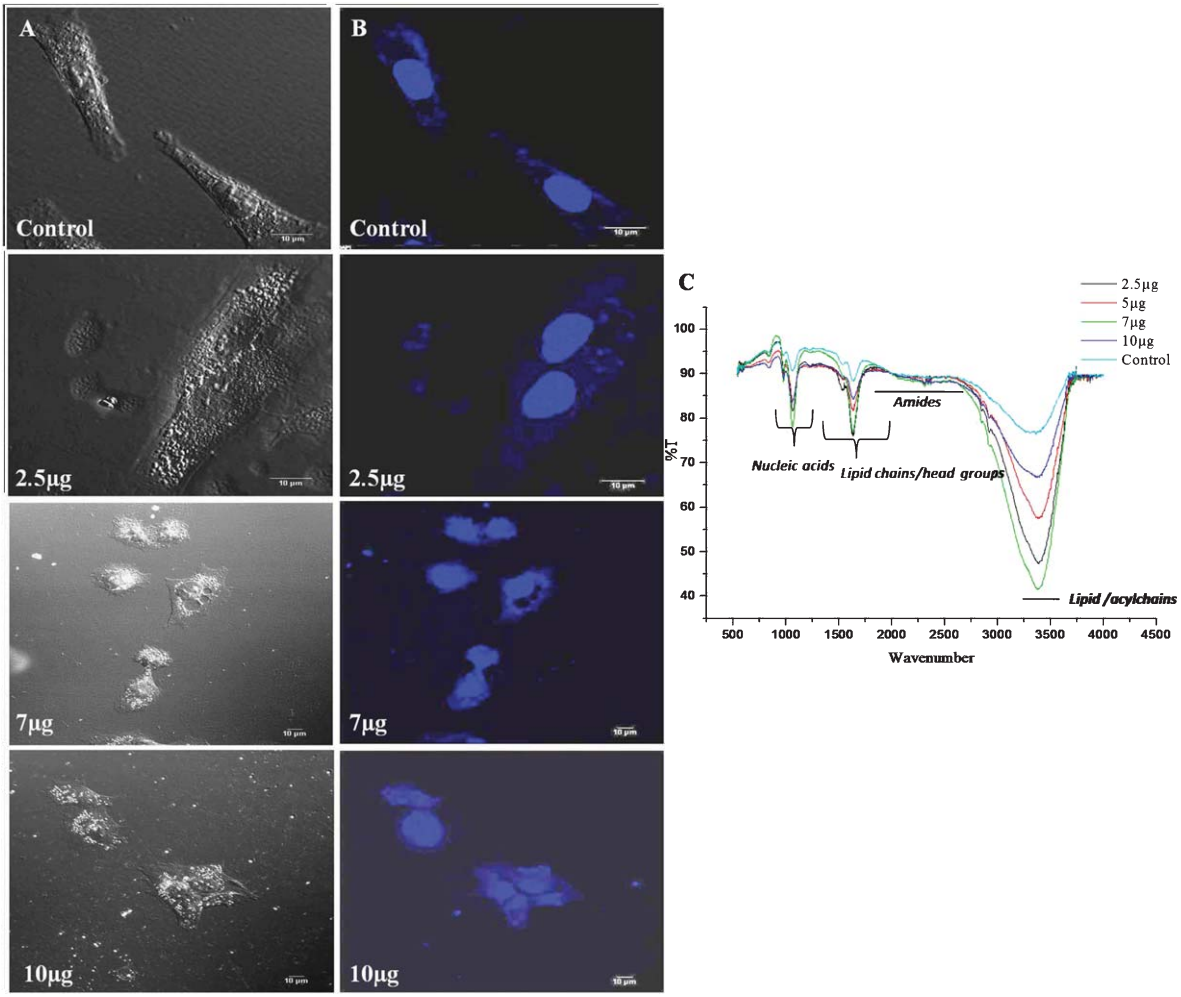

Fig. 4. Confocal microscopy and FTIR analysis of control and CK_f treated DU-145 cells. A: Normal confocal image B: Confocal image of DAPI stained cells. C: FTIR analysis of control and CK_f treated DU-145 cells. All the experiments were done in triplicate.

of CK_f on the mitochondrial membrane potential at different concentration for 24 h. The results showed that 7 and 10 µg/ml of CK_f significantly reduced the mitochondrial membrane potential (Fig. 5).

3.6. CK_f did not affect intracellular calcium levels in DU-145 cells

Since Ca^{2+} signalling is involved in apoptosis, the modulation of intracellular Ca^{2+} levels in CK_f treated DU-145 cells were recorded. It was found that there was no significant increase in intracellular Ca^{2+} was detected after CK_f stimulation. This indicates that CK_f induces cell death through a mechanism independent of calcium (Fig. 5). Mitochondria are central players in cellular Ca^{2+} signalling by buffering cellular Ca^{2+} signals [41]. It is widely known that Ca^{2+} displays growth-inhibiting and differentiation-promoting activities in a variety of normal and malignant cells [42]. In the present experiment, intracellular Ca^{2+} did not show any significant change in CK_f treated DU-145 cells. From these results it may be speculated that CK_f is not of any effect on

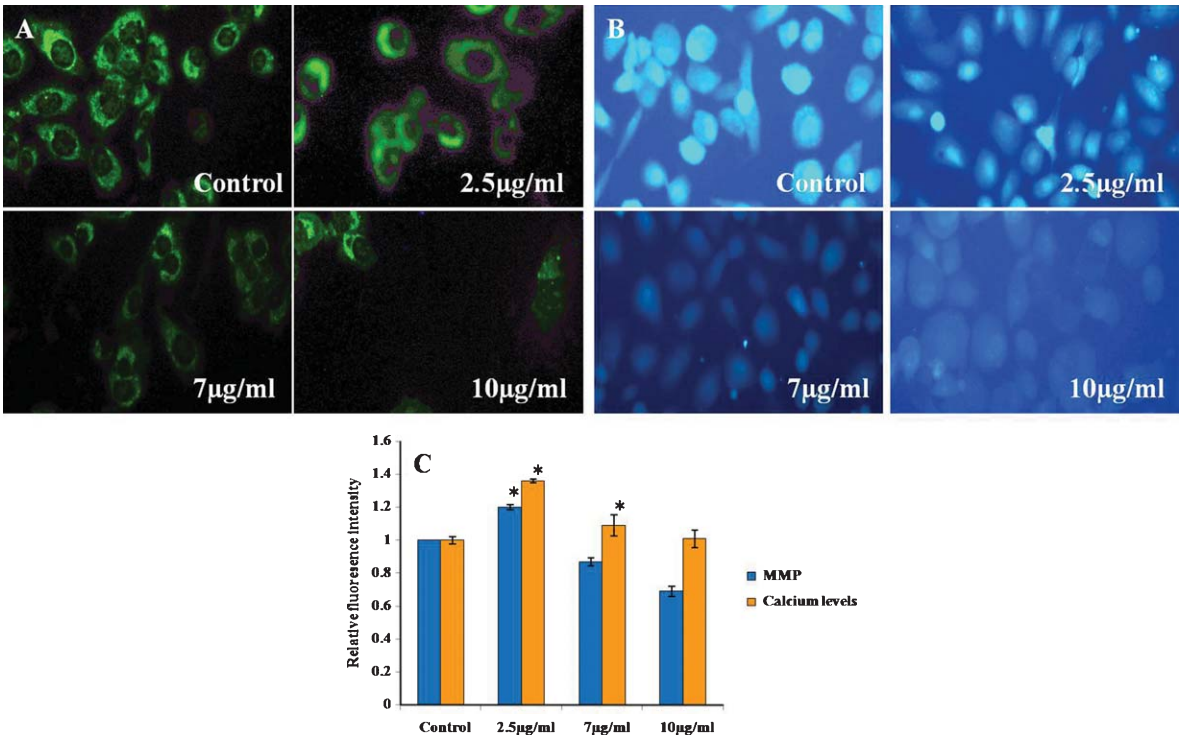

Fig. 5. Calcium handling and MMP of CK_f treated DU-145 cells. A) MMP B) Fura-2-AM staining, C: Relative fluorescence intensity of Rhodamine-123 and Fura-2-AM stained cells. Values are expressed as Mean ± SD of three independent experiments. *Statistically significant compared to control ($p < 0.05$).

endoplasmic reticulum (ER), which is the major source of intracellular calcium mobilisation or there may not be any effect on the influx of extracellular Ca^{2+}. These results suggest that CK_f exerts a selective biological effect on mitochondria and ER physiology. Though Ca^{2+} shows to be essential for inducing apoptosis, CK_f induced apoptosis may be Ca^{2+} independent.

3.7. CK_f triggers an increase in reactive oxygen species production: Lowers antioxidant master switch, Nrf-2 gene expression

Considering the results shown in Fig. 6, we examined whether CK_f could trigger intracellular ROS increase and mitochondrial superoxide production using DCFH-DA as a specific ROS probe and MitoSOX as superoxide probe. In fact, 5, 7 and 10 µg/ml of CK_f induced a significant increase in ROS levels and superoxide production in 24 h. Nrf-2, a transcription factor acts as antioxidant master switch inducing several antioxidant enzymes to protect cells from harmful oxidants. Our experimental results showed that 10 µg/ml of CK_f showed maximum amount of ROS produced and reduced the levels of Nrf-2 significantly compared to control and lower concentrations, indicative of severe intracellular stress. Changes in the intracellular level of ROS have been reported to play an important role in the early step of apoptosis, anticipating loss of the mitochondrial membrane potential and release of the apoptotic-inducing factors and enhanced expressions of mitochondria associated apoptotic gene [43]. Studies using several cells lines have proved that chemically induced ROS production is responsible for apoptosis in different types of cancer [44–49]. In our cell model, DU-145 cells, CK_f significantly increased

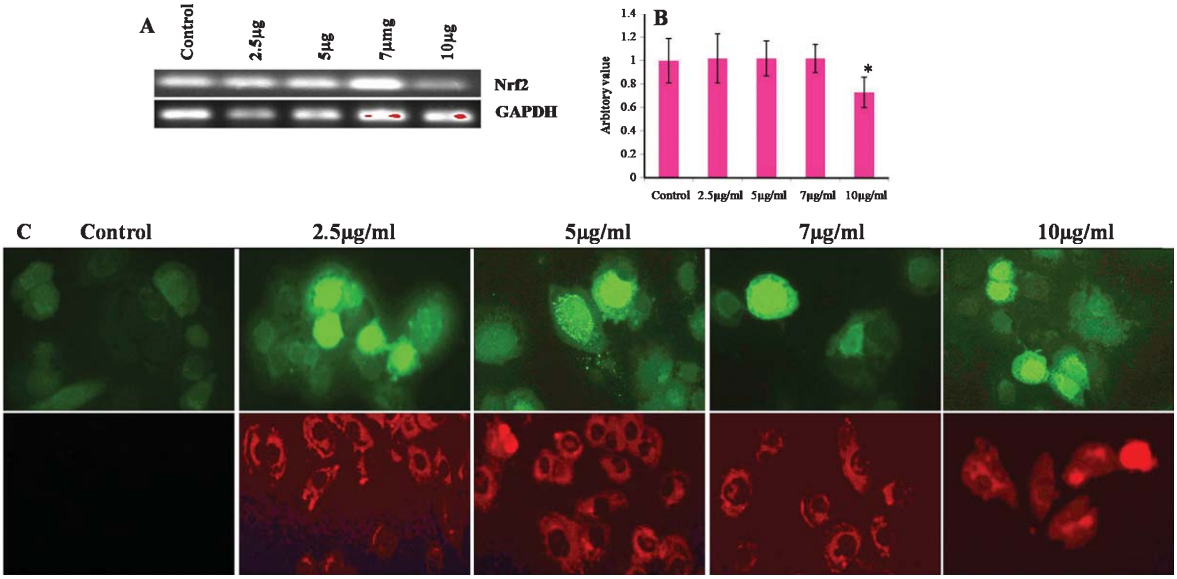

Fig. 6. Effect of different concentrations of CK_f on ROS and mitochondrial superoxide production and *Nrf-2* gene expression in control and treated DU-145 cells. A: Gel image of the *Nrf-2* gene expression in control and CK_f treated DU-145 cells B: Band intensities of each gene. C: Panel above shows the florescence image of CK_f treated DU-145 cells after staining with DCFH-DA, Panel below shows the fluorescence image of CK_f treated DU-145 cells after staining with MitoSOX. * Statistically significant compared to control ($p < 0.05$). All the experiments were repeated three times.

ROS and mitochondrial superoxide production in a dose-dependent manner with a decrease in mitochondrial membrane potential. Mitochondrial trans-membrane potential is often used as an indicator of metabolic health as well as cellular viability. Any disruption to the membrane potential is detrimental to cell growth and function which may result in apoptotic phenomenon [50, 51]. The present study underlines that CK_f induce cell death in DU-145 cells in the same manner involving mitochondria and ROS.

3.8. CK_f treated cells showed leakage of LDH

Figure 7 shows the effect of CK_f on LDH leakage in DU-145 cells. The LDH leakage assay is a simple, reliable and fast cytotoxicity assay based on the measurement of lactate dehydrogenase activity in the extracellular medium. LDH, a cytoplasmic enzyme is released when the cell membrane is damaged [52]. The cell membrane damage of DU-145 cells after the treatment with CK_f extract was measured by the release of LDH. The control cells and lower concentrations of CK_f showed lower levels of LDH in the extracellular medium, while a concentration of 10 µg/ml showed significantly higher levels of LDL in the extracellular medium. This observation was supported by the confocal and SEM images.

3.9. CK_f modulates mitochondria associated gene expression in DU-145 cells

Reverse transcriptase PCR analysis was done to evaluate the expression of pro-apoptotic genes, Bax, *Bak*, Bid as well as *p53* analysed to determine the role of mitochondria in CK_f induced cell death in DU-145 cells. *Bax*, *Bak* and *p53* were found to be increased in all treated cells compared to control cells. The expression of *Bid* showed differential changes in control and treated DU-145 cells. *Bid* expression of 7µg treated cells were

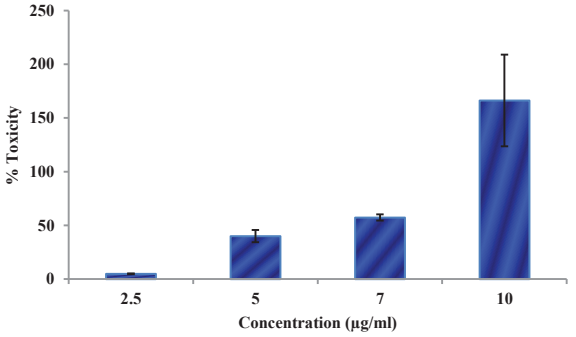

Fig. 7. LDH Leakage levels in control and CK_f treated DU-145 cells. Values are expressed as Mean ± SD of three independent experiments. * Statistically significant compared to control cells ($p < 0.05$).

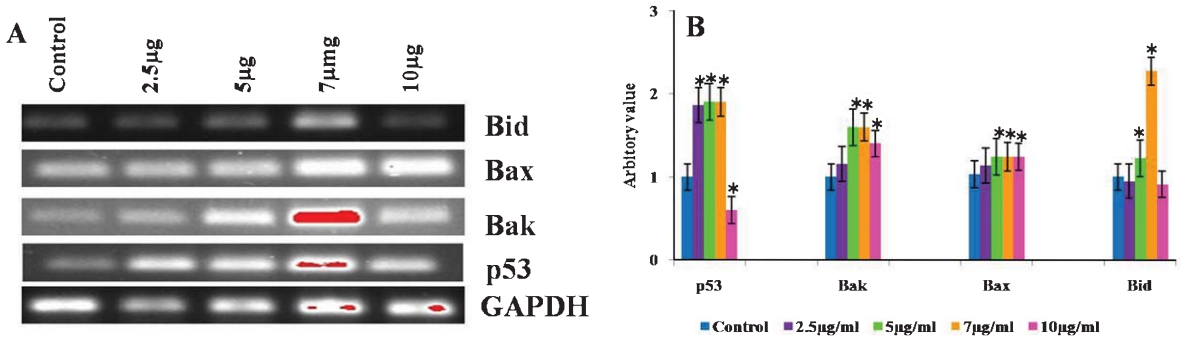

Fig. 8. Effect of CK_f of different mitochondria associated gene expression in control and treated DU-145 cells. The experiment was repeated three times. A: Gel imaged of the PCR products B: Band intensities of each gene.

found to be higher, while 5 µg dose showed slight increase. At higher concentrations, *Bid* levels in DU-145 cells were found to be lowered (Fig. 8). *Bax* and *Bak*, pro-apoptotic genes necessary for the execution of the apoptotic program. Several works have emphasized the role of *Bax* in both cancer progression and/or resistance to chemo- or radiotherapy-induced apoptosis in human and in animal models [53]. Studies have shown that *Bax*, a pro-apoptotic gene, induces apoptosis by increasing the activity of Caspase-3 mediated through *p53*. The activation of *Bax* results in mitochondrial disruption and subsequent release of Cytochrome-c through the outer mitochondrial membrane into the cytosol. Inside the cytosol, Cytochrome-c associates with Apoptotic Protease Activating Factor-1 and activates Caspase-9 which, in turn, triggers the activation of Caspase-3 [54]. It is clear from our experiments that the mRNA levels of *Bax* and *p53* are significantly increased with increased concentration of CK_f indicating the role of mitochondria in cell death.

CK_f was showed to contain several biologically active components as revealed by the LC-MS data. LC-MS data showed that CK_f contained myricetin, hydroxycinnamic acids, coumaric acid, caffeic acid, chlorogenic acid, and triterpene methyl esters etc as active components. The presence of caffeic acid, *p*-coumaric acid, ferulic acid, and (±) catechin was already reported in virgin coconut oil, but the spectrum of polyphenolic compounds in kernel has not been isolated or studied so far [55]. p-Coumaric acid is the abundant isomer of cinnamic acid and also widely found in edible plants such as peanuts, tomatoes, carrots etc. p-Coumaric acid is reported to have antitumor and anti-mutagenic activities [56, 57]. It was shown that p-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway [58]. Myricetin is

a flavanol found in various berries, herbs, and walnuts. Previous studies have demonstrated that myricetin has anti-cancer effects against several types of cancer, including hepatocarcinoma, skin carcinoma, and pancreatic cancer [59, 60]. Myricetin increased the BCL2-associated X protein/B-cell lymphoma 2 ratio and induced the release of apoptosis-inducing factor from mitochondria of colon cancer cells [61]. Chlorogenic acid is shown to have significant anti-tumor activity by affecting changes in the gene expression by significantly upregulating the responsive genes (CaN, NFATC2, NFATC2ip, and NFATC3) involved in immune pathways as well as the IL-2R and IFN-γ to promote activation and proliferation of T cells, macrophages, and NK cells, thus enhancing their surveillance and killing abilities, further suppressing the growth rate of tumor cells [62]. Accumulated data show that triterpenoids exhibit a broad spectrum of anti-cancer properties, including anti-proliferative, anti-metastatic and anti-angiogenic activities mediated through androgen receptor, nuclear factor-kappa B, activator protein-1, p53 and 14-3-3 [63].

In the light of our observations with *Cocos nucifera* and previous reports which link the presence of dietary polyphenols with reduced oxidative stress and improved health benefits, it wouldn't be wrong to suggest that there exists a need for the identification, development and promotion of polyphenol enriched foods as an essential part of our daily diet. This proposal however does raise some very significant questions. One of them being the criterion for selection of these super-foods on the basis of their antioxidant potential. Oxidant scavenging is a broad term and all bioactive molecules wouldn't be equally effective in scavenging the various types of free radicals generated which includes superoxides, singlet oxygen, hydroxyl radical and peroxynitrites. Previous study reported the significance of $ORAC_{MR5,}$ a battery of tests which determine the antioxidant potential of various foods and the differences encountered in processed vs unprocessed samples [64, 65]. Such food profiles might be helpful when assigning a polyphenol rich diet to counter oxidative stress and related ailments. Again, several studies using single phytochemicals in oxidant-scavenging studies generally use concentrations which cells will never be exposed to when the compound is administered orally or otherwise to a human being. Systemic studies in live animal models cannot be considered as completely foolproof alternatives to a human body due to the existing differences in drug absorption, metabolism and scavenging properties. On the positive side, it is entirely possible that the beneficial effect exerted by one component on a system might be enhanced or rendered much more effective when multiple plant components are involved. Phytochemicals are also being evaluated as candidates useful against self-renewal of cancer stem cells when looking at possible anticancer strategies [66].

4. Conclusion

Polyphenols containing fraction from coconut kernel (CK_f) was found to prevent the growth of human prostate cancer cells, DU-145 *in vitro* through the ROS-mitochondria mediated apoptosis. The cytotoxic activity of the extract could be attributed to a combinatorial effect of multiple polyphenolic compounds which have been identified and their possible role in altering cellular redox levels. *In vitro* experiments prove without a doubt that the extract has a significant impact on mitochondrial viability and in the expression of relevant genes. These results suggest that CK_f can be considered as a part of the dietary plan in using nutraceuticals when preventing prostate cancer. The discussion of our present work can be enhanced by evaluating the impact of administration of CK_f on a suitable tumor bearing animal model. Effect of polyphenols on the tumor-cell antioxidant system will be determined through western blot experiments and consequently the mode of cell death will be elucidated.

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Conflict of interest

The authors declare no conflict of interests.

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