Original Article

# Polyphenolic extract from coconut kernel modulates apoptotic genes, reactive oxygen species production, and prevents proliferation of human colon cancer cell line

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## Abstract

**Background and Aim:** The modern diet along with sedentary lifestyle has led to an increasing mortality rate for colon cancer. Several dietary phytochemicals have been investigated for colon cancer therapy, so as to replace the synthetic drugs having adverse health side effects. The aim of the study was to evaluate the antiproliferative effect of polyphenol-rich fraction from coconut kernel ($CK_f$) on human colon cancer cell lines (HT-29).

**Methods:** The total flavonoids and polyphenols present in $CK_f$ were determined colorimetrically. The cytotoxic and apoptotic effect of $CK_f$ was determined using 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide assay, acridine orange/ethidium bromide staining, and 4',6-diamidino-2-phenylindole-2 staining. Levels of caspase-3 activity were measured colorimetrically. The expression levels of apoptotic genes BAK, BAX, BID and p53 were measured using real-time polymerase chain reaction. The effect of $CK_f$ in inducing reactive oxygen species (ROS) was studied using 2′,7′-dichlorofluorescein diacetate staining. Mitochondrial potential of HT-29 cells treated with $CK_f$ was determined by Rhodamine 123 staining.

**Results:** Experimental results showed that $CK_f$ contains significant amount of polyphenols. $CK_f$ showed cytotoxicity against HT-29 cells (Lethal Dose 50% of 8 µg/ml) by increasing the free radical concentration, caspase 3 enzyme levels, and decreasing the mitochondrial membrane potential in dose-dependent manner. The levels of p53 and BAX showed a major increase in a dose-dependent manner, while BAK gene levels showed a slight but significant increase.

**Conclusion:** These results clearly indicate that coconut kernel which contains cytotoxic phenols affect the growth of colon cancer cells by modulating the apoptotic machinery mediated through mitochondrial ROS production.

**Key words:** Apoptosis, coconut kernel, cytotoxicity, human colon cancer cell lines, polyphenols

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## INTRODUCTION

Colon cancer is a serious health problem with very high mortality rates, affecting both men and women worldwide. The modern diet with high red meat consumption and excessive alcohol use along with sedentary lifestyle has led to an increasing mortality rate for colon cancer.[1] Presently, there are several synthetic drugs available for the control of colon cancer.[2] However, while synthetic anticancer drugs prolong survival, they

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often have adverse health effects and nonspecificity. Based on this fact, several dietary phytochemicals have been investigated for colon cancer therapy. Studies on diet-based antioxidants have advanced at a full pace due to their capability of quenching reactive oxygen species (ROS) and prevent our body from various deadly diseases including cancer. These compounds are capable of inhibiting critical cell cycle molecules and inhibit proliferation and/or inducing apoptotic death in cancer cells.[3] Polyphenols, nontoxic secondary metabolites, have been shown to possess anticancer properties.[4] There is little convincing epidemiological evidence that intake of polyphenols/flavonoids is inversely related to the incidence of cancer. In contrast, numerous cell culture and animal models indicate potent anticarcinogenic activity by certain polyphenols, mediated through a range of mechanisms including antioxidant activity, enzyme modulation, gene expression, apoptosis, upregulation of gap junction communication, and P-glycoprotein activation.[5] These compounds act as key modulators of signaling pathways and are therefore considered ideal chemopreventive agents.[6]

Coconut and its products have been an important part of the diet among Indian population for time immemorial. There are various reports regarding the beneficial effect of the oil isolated from coconut by wet as well as dry process.[7,8] Unfortunately, the use of coconut oil for culinary purpose has initiated a burning debate over its health benefits. Although the debate is centered on the oil consumption, not many studies are conducted on the biological effects of the minor components present in coconut kernel. It was previously reported that the virgin coconut oil (VCO) contains polyphenolic compounds having significant antioxidant effect with an important role in the cardiovascular health.[9-11] Although the cardiovascular benefits of these polyphenols are documented, their anticancer properties are poorly understood. This study was designed to evaluate the antiproliferative effect of the polyphenols/flavonoids isolated from coconut kernel on human colon cancer cell lines.

## MATERIALS AND METHODS

### Chemicals and coconut

Coconut samples Dwarf x Tall variety was collected from Shornur Panchayat Krishi Bhavan Office, Palakkad, Kerala, India. Gallic acid, Folin–Ciocalteu reagent, sodium carbonate, aluminum chloride, potassium acetate, McCoy's-5A medium, fetal bovine serum (FBS), antibiotic and antimycotic solution, 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), acridine orange/ethidium bromide (AO/EB), trypan blue, 4',6-diamidino-2-phenylindole (DAPI), RDP Trio Reagent, and other cell culture reagents were purchased from Hi-Media Laboratories, India. 2',7'-dichlorofluorescin diacetate (DCFH-DA), quercetin, diethylpyrocarbonate, and N-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) were purchased from Sigma-Aldrich, USA. Annexin V Alexa Fluor 488 Kit was purchased from Invitrogen. Revert Aid First Strand cDNA Synthesis Kit from Thermo Scientific. Go Taq Green Master Mix was procured from Promega.

### Extraction of the kernel

Kernel from coconut was removed and defatted with petroleum ether (60–80) using a Soxhlet apparatus. The residue obtained after defatting was dried, weighed, and exhaustively extracted using 80% methanol. Methanolic extract thus obtained was dried in rotary evaporator, weighed, and used for further experiments.

### Estimation of total polyphenols and flavonoids

Total polyphenols in fraction from coconut kernel ($CK_f$) were determined by Folin–Ciocalteu reagent.[12] Briefly, different concentrations of $CK_f$ and gallic acid standard were mixed with Folin–Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na2CO3 (4 mL, 1 M). The mixture was incubated at 45°C for 15 min. The absorbance was measured at 765 nm using a ultraviolet-visible (UV-VIS) spectrophotometer. The total polyphenols present were expressed as μg/mg extract. Aluminum chloride colorimetric method was used for the determination of total flavonoid content (Chang *et al*., 2002). Samples were mixed with 1.5 mL methanol, 0.1 mL of 10% aluminum chloride (in methanol), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was kept at room temperature for 30 min. The absorbance of reaction mixture was measured at 415 nm. The calibration curve was prepared using quercetin (1 mg/mL in methanol) as standard. The total flavonoids present were expressed as μg/mg extract.

### Ultraviolet and Fourier transform infrared spectroscopic analysis

For UV-VIS and Fourier transform infrared (FTIR) spectrophotometer analysis, $CK_f$ was centrifuged at 3000 rpm for 10 min and filtered through Whatman No. 1 filter paper. The sample was diluted to 1:10 using methanol. The extract was scanned in the wavelength ranging from 185 to 1400 nm using Shimadzu UV-VIS Spectrophotometer (Model UV-2600, ISR-2600 plus) and the characteristic peaks were detected. FTIR analysis was performed using Shimadzu IR prestige 21 Spectrophotometer system, which was used to detect the characteristic peaks and their functional groups (Sahaya *et al*. 2002). The peak values of the UV-VIS and FTIR were recorded. Each and every analysis was repeated three times for the spectrum confirmation.

### Cell culture

HT-29 cells were procured from the National Centre for Cell Science, Pune, Maharashtra, India. The cells were grown and maintained in a humidified incubator at 37°C under 5% $CO_2$ atmosphere in McCoy's-5A supplemented with 10% FBS.

### *In vitro* antiproliferative effect of fraction from coconut kernel

To determine the antiproliferative effect of $CK_f$, the MTT assay was performed as described earlier.[13] This method is based on the conversion of the tetrazolium salt (MTT) to colored formazan by viable, but not dead cells. HT-29 cells were treated with different concentrations of $CK_f$ (0.2–10 μg/ml) for 24 and 72 h. After the treatment period, the viability of HT-29 cells was determined by adding MTT to the cell cultures to reach a final concentration of 1 mg/mL. After a 2 h incubation at 37°C, the dark crystals formed were dissolved by adding to the wells containing an equal volume of extraction buffer (20% sodium dodecyl sulfate, 50% N, N-dimethylformamide, and pH 4.7). Subsequently, plates were incubated overnight at 37°C and optical densities at 570 nm were measured by transferring 100 μL aliquots to 96-well plates and 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 (%)
= (Acontrol − Adrug/Acontrol) × 100

### Apoptosis assays

To evaluate the apoptotic effect of $CK_f$ on HT-29 cells, the following experiments were conducted.

### Acridine orange/ethidium bromide staining

Apoptotic morphology was investigated by double staining with AO and EB as described earlier.[14] In the experiment, HT-29 cells were treated with different concentrations of $CK_f$ (12 and 16 μg/ml) for 24 h. After the treatment period, the coverslips with monolayer cells were inverted on a glass slide with 20 μL of AO/EB stain (100 μg/mL). Photographs were taken using a fluorescence microscope (Olympus Co., Japan).

### 4',6-diamidino-2-phenylindole-2 staining

Chromatin changes in $CK_f$-treated cell lines were studied by DAPI-HCl. This stain binds double-stranded DNA providing a blue fluorescence when viewed under UV light.[15] HT-29 cells were treated with different concentrations of $CK_f$ (12–16 μg/ml) for 24 h. Adherent cells grown on plates were rinsed thrice in phosphate-buffered saline (PBS) to completely remove the growth medium. Cells were fixed for 10 min in 3.7% formaldehyde and again rinsed thrice in PBS before permeabilization in 0.2% Triton-X-100 for 5 min. Cells were rinsed and incubated with DAPI (10 mg/mL in water stock; working solution - dilute the stock 1:5000 times) labeling solution for 5 min in the dark. The labeling solution was aspirated and cells were rinsed thrice in PBS, and morphology of the cells and the nuclei were observed using a fluorescence microscope (Olympus 1 × 51) with the DAPI filter. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation.

### Measurement of apoptotic, dead, and live cells by Tali-based cytometer

Apoptotic, dead, and live cells after 24 h $CK_f$ treatment were analyzed after Annexin-V Alexa Fluor 488 staining using Tali-based cytometer (Invitrogen). HT-29 cells were treated with different concentrations of $CK_f$ (4–16 μg/ml) for 24 h. After the treatment period, the cells were harvested with trypsin ethylenediaminetetraacetic acid, washed in PBS, and resuspended in Annexin binding buffer. To the suspension, Annexin V Alexa Fluor 488 was added and incubated at room temperature in dark for 20 min. The cells were centrifuged and resuspended in Annexin binding buffer and propidium iodide and incubated at room temperature in the dark for 1–5 min. Subsequently, the samples were loaded into an analysis slide and cell counting was done.

### Caspase 3 enzyme assay

HT-29 cells were treated with different concentrations of $CK_f$. After 24 h, cells were harvested and lysed using RIPA buffer. Cytosolic fraction was obtained, and the total protein was measured by Bradford method.[16] One hundred micrograms of cytosolic proteins in 100 μl caspase assay buffer (250 mM HEPES [pH 7.4], 25 mM triton ×100, 2dmM DTT) were incubated with the caspase 3 colorimetric substrate, DEVD-pNA. Plates were incubated at 37°C for 1 h. Release of free pNA, which absorbs at 405 nm was recorded.

### Reactive oxygen species measurement

Intracellular ROS levels were measured using a cell-permeable fluorescent probe, DCFH-DA.[17] In brief, HT 29 cells were seeded into 96-well culture plates at 1 × $10^4$ cells/well. After 24 h at 37°C and 5% $CO_2$, cells were treated with different doses of $CK_f$ (12–32 μg) for 24 h. Cells were then washed twice with PBS and incubated with fresh DCFH-DA (100 μM) in PBS for 30 min at 37°C in 5% $CO_2$. After that, cells were washed twice in PBS, and wells were filled with 100 μL PBS. Fluorescence images were taken using an inverted microscope (Olympus

1 × 51). The intensity of fluorescence was analyzed using Image J 1.48 software, National Institute of Health, USA.

### Analysis of mitochondrial membrane potential (ΔΨm)

To determine the effect of $CK_f$ on the electrical potential across the inner mitochondrial membrane, Rhodamine 123 (R-123), a lipophilic cationic indicator was used to label the mitochondria.[18] HT-29 cells were seeded into 96-well culture plates at 1 × $10^4$ cells/well and incubated for 24 h at 37°C and 5% $CO_2$. After 24 h, cells were treated with different doses of $CK_f$ (4–64 μg/ml) for 24 h. After 24 h, R-123 solution was added into the cell media to incubate for 20 min. Subsequently, the cells were washed with PBS twice, and the cellular images were taken at 525 nm using the fluorescence microscope (Olympus 1 × 51).

### Expression of apoptotic genes by real-time polymerase chain reaction

Total RNA was isolated from both control and treated HT-29 cells 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′-GAG AGG TCT TTT TCC GAG TGG-3′, Backward 5′-CCT TGA GCA CCA GTT TGC TG-3′); BAK (Forward 5′-GGG TCT ATG TTC CCC AGG AT-3′, Backward 5′-GCA GGG GTA GAG TTG AGC A-3′); p53 (Forward 5′-GGC CCA CTT CAC CGT ACT AA-3′, Backward 5′-GTG GTT TCA AGG CCA GAT GT-3′). GAPDH was used as an endogenous control. The polymerase chain reaction (PCR) products were analyzed on 1.5% agarose gel electrophoresis, and band intensity was detected using Gel Doc EZ imager (Biorad).

### Statistical analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed using SPSS 19 software (IBM SPSS software, USA) via a one-way analysis of variance followed by Duncan's multiple range test. $P < 0.05$ ($P < 0.05$) was considered statistically significant.

## RESULTS

Phytochemical analysis showed that $CK_f$ contained a significant amount of total polyphenols than total flavonoids [Figure 1a]. The UV-VIS spectrum [Figure 1b] of $CK_f$ was studied at a wavelength range of 185–1400 nm. A major band was recorded at 237 and 256 nm with high absorbance values. The spectra for phenolic compounds and flavonoids typically lie in the range of 230–290 nm. The result of UV-VIS spectroscopic analysis confirms the presence of flavonoids and polyphenols in $CK_f$. Previous reports showed that flavonoids, flavonol, rutin, fisetin, luteolin, and quercetin have an absorption maxima at 230–260 range. It was earlier reported that polyphenolic fraction isolated from VCO contains caffeic acid, p-coumaric acid, ferulic acid, and (±) catechin.[19] FTIR spectrum was performed to identify the functional groups present in $CK_f$ based on the peak values in the region of infrared region. FTIR studies enable the identification of the chemical constituents and elucidation of the structure of compounds.[20] A major band was observed between 4000 and 3000 $cm^{-1}$ which corresponds to the presence of OH group from phenols and alcohols. The bands 1745, 1849, and 2017 $cm^{-1}$ show the presence of phenyl ring. The peaks 1411 and 1450 correspond to C-H bonding. The peaks seen at 2835 and 292 $cm^{-1}$ correspond to the asymmetric stretch of CH2 groups, major bands were observed at 3438.6, 1637.4, 1404, and 1319.59 $cm^{-1}$ [Figure 1c]. These results further confirm the presence of polyphenol and flavonoid structures in $CK_f$. Preliminary phytochemical analysis and UV-VIS and FTIR spectrum showed the presence of significant amount of phenolic and flavonoid compounds.

Evaluation of $CK_f$ for potential anticancer activity on HT-29 cells was carried out by studying the growth inhibitory effects using the MTT assay for 24 and 72 h.

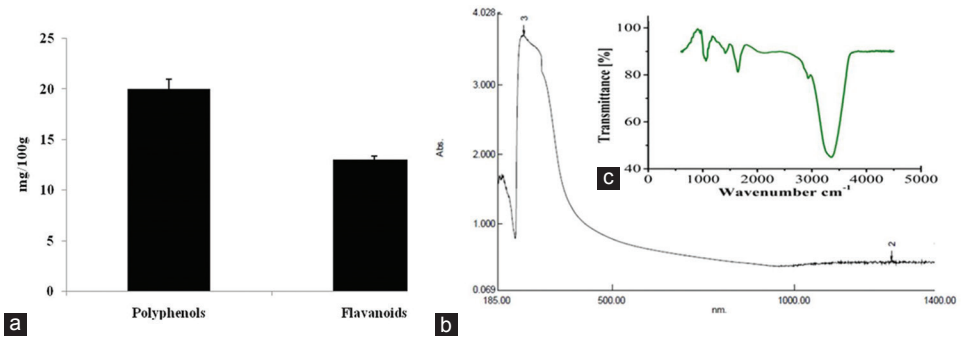


**Figure 1:** Composition analysis of fraction from coconut kernel. (a) Levels of total polyphenols and flavonoids in fraction from coconut kernel. (b) Ultraviolet-visible spectrum of fraction from coconut kernel in methanol (c) Fourier transform infrared spectroscopy spectrum of fraction from coconut kernel. Each values of phytochemical concentration are expressed as mean ± standard deviation of three individual experiments. Ultraviolet-visible and Fourier transform infrared spectroscopy spectrum images are representation of three separate experiments

It was found that $CK_f$ strongly inhibited the growth of HT-29 cells in a dose-dependent manner [Figure 2a]. *$CK_f$* showed more than 90% cell proliferation inhibition at the concentration of 100 mg/ml. Morphological analysis of $CK_f$ treated HT-29 using bright field showed that at higher concentrations (20 and 40 $\mu$g/ml) *$CK_f$* induced severe disintegrity on the cell structure. 7 $\mu$g/ml showed mild changes in the structure, whereas 10 $\mu$g/ml showed more disintegration compared to the control. The toxicity of 20 and 40 $\mu$g/ml was even prominent after 24 h of treatment compared to lower concentrations [Figure 2b]. The effect of $CK_f$ on cell viability on normal rat cardiomyocytes (H9c2) cell lines and human monocytes were tested and showed absolutely no toxicity (unpublished data). Our study revealed that $CK_f$ elicited significant cytotoxic effect in HT-29 colon cancer cells in a time- and concentration-dependent manner.

Using Tali-based cytometer, it was found that the number of apoptotic cells and dead cells were elevated with increased concentration of $CK_f$ [Figure 3]. In an attempt to elucidate the basic underlying mechanism of $CK_f$-induced colon cancer cell death, we examined the effect of $CK_f$ on cellular apoptotic using AO/EB and DAPI staining as well as the ROS production using DCF-DA staining. Staining cells with fluorescent dyes, including AO/EB are used in evaluating the nuclear morphology of apoptotic cells. In this study, apoptosis-inducing ability of $CK_f$ on HT-29 cells was analyzed by AO/EB staining. AO is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity. Two different concentrations were chosen based on the IC50 values determined by MTT assay, which were 12 and 16 $\mu$g/ml. As control, HT-29 cells alone were cultured in complete media and stained with AO/EB [Figure 4a]. The figure shows that 16 and 32 $\mu$g/ml $CK_f$ treatment significantly reduced the total number of live cells which appeared as red-colored cells corresponding to those undergone apoptosis after 72 h incubation. Cells stained green represent viable cells, whereas yellow staining represented early apoptotic cells and reddish or orange staining represents late apoptotic cells as shown in Figure 4. DAPI staining of HT-29 cells treated with 12 $\mu$g/ml $CK_f$ showed higher cell numbers, indicating lower cell death, changes in cellular morphology including chromatin condensation, membrane blebbing, and fragmented nuclei. Therefore, using the AO/EB staining procedure, the morphological features of a HT-29 cell line in apoptosis were dose-dependent, i.e., a stronger apoptosis signal was induced with higher concentrations of the respective extract.

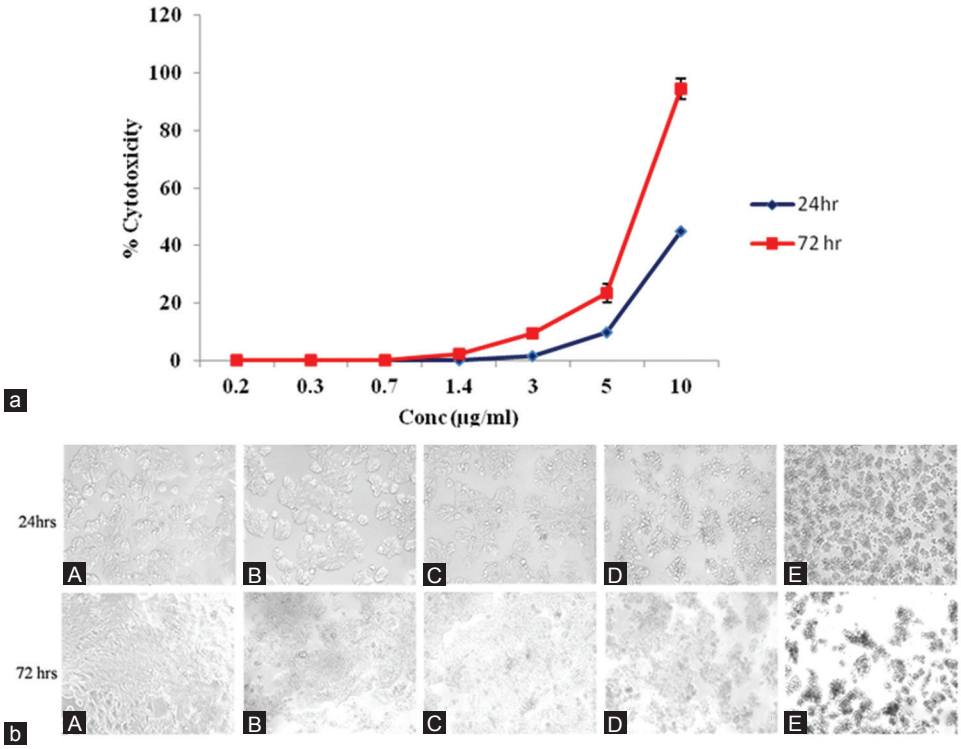


**Figure 2:** Cytotoxic effect of fraction from coconut kernel on HT-29 cells. (a) Antiproliferative effect of fraction from coconut kernel against HT-29 cell lines. Each value of cytotoxic effect is expressed as mean ± standard deviation of three individual experiments. (b) Typical morphological changes of HT-29 cells induced by different concentration of fraction from coconut kernel after 24 h and 72 h treatment. The images were taken using microscope at ×10. A: control, B: 4 µg/ml, C: 8 µg/ml, D: 16 µg/ml, E: 32 µg/ml, magnification ×10. The picture is the representation of three separate experiment

Figure 4b shows the activity of caspase 3 in control and $CK_f$-treated HT-29 cells. $CK_f$ treatment increased the activity of caspase 3 in a dose-dependent manner. Accumulation of intracellular ROS can induce cell towards apoptosis. We examined the effect of $CK_f$ on ROS generation in HT-29 cells. Treatment of cells with $CK_f$ generated ROS in a concentration-dependent manner as revealed by DCFH-DA staining as shown in Figure 5a: it was found that the concentration of 12 $\mu$g/ml produced more ROS (29.44 ± 1.47) compared to 16 and 32 $\mu$g/ml (23.01 ± 0.74 and 21.49 ± 0.6). This is due to the difference in total cell number. It was shown that the treatment of 16 and 32 $\mu$g/ml concentration of $CK_f$ do prevent the proliferation of HT-29 compared to lower concentrations. The fluorescence intensity for 166 and 32 $\mu$g/ml (23.01 ± 0.74 and 21.49 ± 0.6) was found to be similar but significantly higher compared to the control cells (10.49 ± 0.16) stained with DCFH-DA. The fluorescent dye rhodamine-123 (Rh-123) is a specific probe for the detection of changes in mitochondrial membrane potential of viable cells. The results of this study revealed that different doses of the $CK_f$ induced a potent and dose-dependent loss in $\Delta\Psi$m after 24 h treatment. Compared to the control, the CK cells displayed a decreased mitochondrial membrane potential. At a very high concentration of $CK_f$ (40 $\mu$g/ml), there were several granular-like structures, which may be due to fragmented mitochondria. Effect $CK_f$ on $\Delta\Psi$m is shown in Figure 5b.

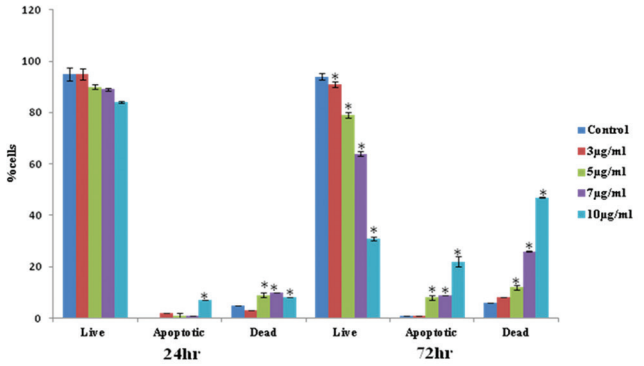


**Figure 3:** Percentage live, dead, and apoptotic cells treated with different concentrations of fraction from coconut kernel, 24 and 72 h treatment. Values are represented as mean ± standard deviation of three individual experiments. *Significant compare to control ($P < 0.05$)

Apoptosis, a process of controlled cell suicide is tightly controlled by several genes, which plays a key role in the pathogenesis of cancer. Therefore, real-time-PCR analyses were conducted to determine whether $CK_f$ modulates the expression levels of BAX (apoptotic promoter), BAK (Proapoptotic), and p53 in HT-29 cells as shown in Figure 6. Exposure of cells to increasing concentrations of $CK_f$ for 24 h resulted in a marked increase in the expression of proapoptotic genes, BAX, and p53 in concentration-dependent manner. Meanwhile, the expression of BAK gene required to

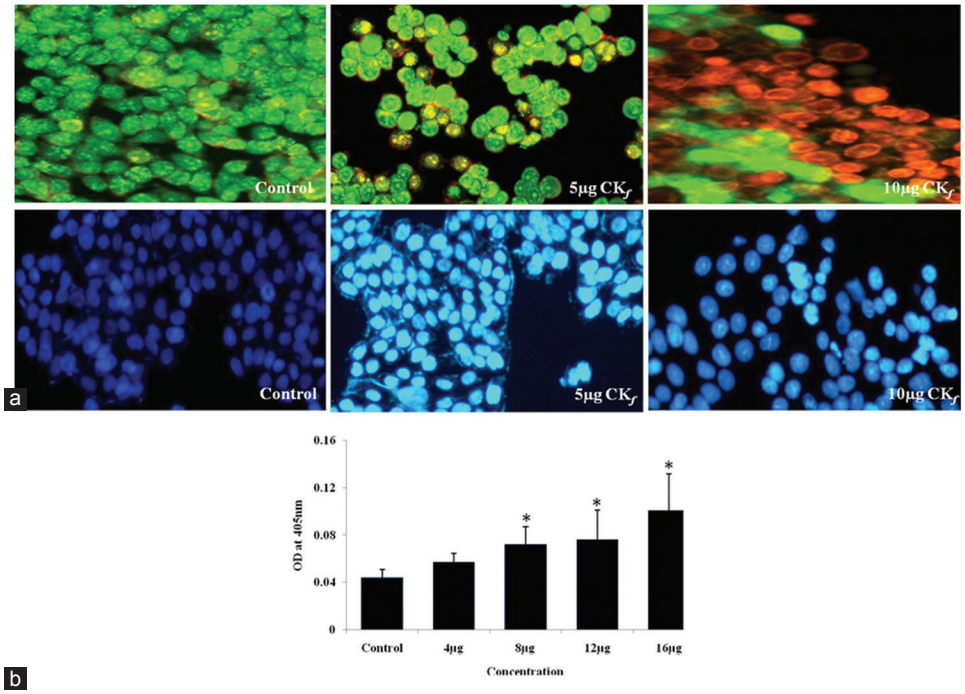


**Figure 4:** Apoptotic effect of fraction from coconut kernel on HT-29 cells. (a) acridine orange/ethidium bromide and 4',6-diamidino-2-phenylindole-2 staining of HT-29 treated with fraction from coconut kernel. Green color shows live cells, yellow color shows apoptotic cells, and red color shows dead cells. Magnification ×20. The picture is the representation of three separate experiments. (b) Activity of caspase 3 activity in HT-29 cells treated with fraction from coconut kernel. Values are expressed as mean ± standard deviation of three independent experiments. *Significant compare to control ($P < 0.05$)

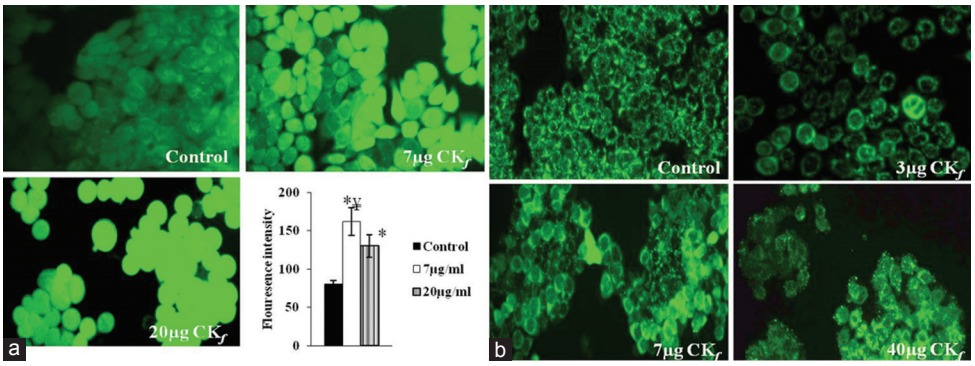


**Figure 5:** Reactive oxygen species production in HT-29 cells treated with fraction from coconut kernel. (a) Shows the fluorescence image of fraction from coconut kernel-treated cells after staining with 2′,7′-dichlorofluorescein diacetate. The experiment was repeated three times. *significant compared to control ($P < 0.05$), ¥significant compared to 20 µg, ($P < 0.05$). (b) Rhodamine 123 staining to determine the mitochondrial membrane potential. Images are representation of three separate experiments (×20)

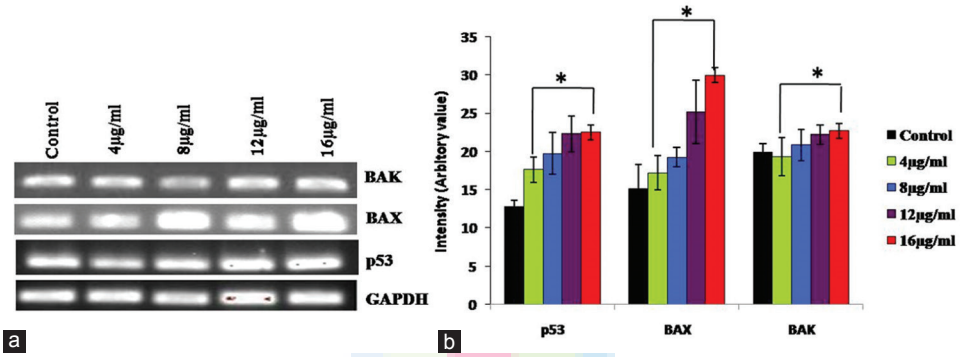


**Figure 6:** Expression of apoptosis induction gene in HT-29 cells treated with fraction from coconut kernel after 24 h of treatment. (a) Gel imaged of the polymerase chain reaction products (b) band intensities of each gene. The experiment was conducted 3 times

permeabilize the mitochondrial outer membrane during the mitochondrial (intrinsic) pathway of apoptotic cell death was also found to be significantly increased with increasing concentration.

## DISCUSSION

The present study evaluated the antiproliferative potential of the polyphenol-containing extract from coconut kernel on human colon cancer cell lines (HT-29). Preliminary phytochemical analysis showed that $CK_f$ contains polyphenolic compounds. Phenols and flavonoids are polyphenolic compounds that are distributed widely in the plant kingdom; they are especially abundant in fruits and vegetables. The beneficial health effects of flavonoids have been attributed to their free radical scavenging properties. In addition to their antioxidant properties, flavonoids have antiviral, antiallergic, anti-inflammatory, and antitumor activities.[21] There are insufficient and conflicting evidence regarding flavonoid intake and the prevention of colorectal neoplasms. Moreover, it is difficult to determine the flavonoid intake. Therefore, more studies are needed to clarify the association between flavonoids and colorectal neoplasms. While most of the researches on the identification of phenolic/flavonoid compounds in several diet and dietary components are extensively studied, similar studies on coconut kernel polyphenols are surprisingly meager. Although the antioxidant properties of the total polyphenols isolated from VCO were basically studied, this is the first report on the antiproliferative activity of polyphenol-rich extract from coconut kernel against colon cancer cells.

Colorectal cancers are common cancers and leading causes of cancer deaths worldwide. Colorectal cancer is widely considered to be an environmental disease, due to ill-defined cultural, social, and lifestyle factors. Colorectal cancer may be one for which modifiable causes may be readily identified and theoretically preventable.[22] Because the alimentary tract can interact directly with dietary components, stomach and colorectal cancer may be closely related to dietary intake. Since majority of all chronic diseases are lifestyle related, both human epidemiologic and animal studies have drawn an inverse relationship between consumption of plant-derived components and risk of carcinogenesis in different types of cancer.[23] Plant-derived compounds suppress chronic diseases mediated by inflammation, hyperproliferation, and transformation. Thus, they may

ultimately suppress angiogenesis and metastasis by blocking the cell cycle in tumoral cells, counteracting the dysregulation of proliferation and also synergize with chemotherapeutic drugs, thereby reducing the dose of treatment and toxicity.[24] In this study, it is clear that $CK_f$, containing polyphenolic compounds induced cell death in a dose-dependent manner. The number of apoptotic and dead cells were also found to be increased in a time- and dose-dependent manner.

Two basic pathways involved in apoptosis are intrinsic (mitochondrial) and extrinsic (death receptor) pathways.[25] Caspase-3/7 is one of the effector caspase that is involved in the final execution of dying cells, whereas caspase-9 is an initiator caspase that is involved in the intrinsic pathway. Caspase-3 is a frequently activated apoptotic death protease, catalyzing mitochondrial-dependent or independent cleavage of many key cellular proteins. To understand the mechanism of action induced by the extract, caspase-3 was evaluated. The results showed that with increasing concentration, the caspase 3 levels were found to be increasing which shows that $CK_f$ is influencing the caspase pathway in preventing the proliferation of these cell lines.

One of the triggers of apoptosis mediated by mitochondrial dysfunction is the accumulation of intracellular ROS. There are several biological molecules isolated from dietary components which are reported to induce ROS-dependent apoptosis in cancer cells.[26] Studies in our laboratory have shown that the extract posses significant antioxidant activity in *in vitro* conditions (Unpublished data). Studies showed that several antioxidant molecules isolated from dietary components induce apoptosis and autophagy in cancer cells which are mediated via ROS production.[27] Antioxidants are reported to exert different biological activities in cancer cells and in nontransformed cells. Antioxidants effectively induce apoptosis in HT-29 cells via increased ROS production.[28] In the present study, CK*f* treatment dose-dependently increased ROS production with subsequent reduction in mitochondrial membrane potential.

Earlier studies have shown that BAX, a proapoptotic gene, induces apoptosis by increasing the activity of caspase 3 mediated through p53.[29] 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.[30] It is clear from our experiments that the mRNA levels of BAX and p53 are significantly increased with increased concentration of $CK_f$; meanwhile, the caspase 3 levels were also elevated in dose-dependent manner which indicate the role of the BAX-p53-caspase 3 axis in the apoptotic mechanism of $CK_f$. The loss of mitochondrial membrane potential and subsequent increase in ROS was further supported by the increase in mRNA levels of BAK gene since BAK and BAX form large oligomeric pores in the mitochondrial outer membrane during apoptosis.[31] It was reported that polyphenols induce intracellular oxidative stress and DNA damage with subsequent activation of kinases (MAPK, ATM, and DNA-PK) responsible for p53 phosphorylation leading to the activation of several other key molecules which activate cell death cascade in cancer cells.[32]

### Limitations of the study

The polyphenol-rich extract derived from coconut kernel demonstrates a dose-dependent cytotoxic effect on HT-29 colon cancer cell line in our experimental observations. However, the study lacks in its ability to attribute the toxic effect of the extract to any one candidate molecule or a class of compounds. Structural analysis and component validation of the extract will strengthen our findings and add clarity to the identity of the active cytotoxic compound(s).

## CONCLUSION

The results derived from the present investigation showed that $CK_f$ exerts apoptosis in HT-29 human colon cancer cells. A treatment period of 24 h seems to be necessary to achieve an apoptosis-inducing effect. The mechanism of the apoptotic effect involves mitochondria and BAX-p53-caspase-3-mediated pathway. All these results provide valuable preliminary mechanistic insight to the antiproliferative effects of $CK_f$. These encouraging preliminary data may facilitate the development of novel chemotherapeutic food based on coconut for the effective management of colon cancer.

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### Conflicts of interest

There are no conflicts of interest.

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