EVALUATION OF IN VITRO ANTIOXIDANT AND ANTI-PROLIFERATIVE EFFECT OF DEFATTED EXTRACT OF COCOS NUCIFERA KERNEL ON HUMAN BREAST CANCER CELL LINE

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ABSTRACT

Objective: Biologically active antioxidant dietary components have attracted public interest as effective alternative for the successful treatment and prevention of several types of cancer. The present study was designed to evaluate the in vitro antioxidant effects and potential benefit of using coconut kernel extract in preventing the proliferation of human breast cancer cell lines MCF-7. STUDY DESIGN: Defatted extract from coconut kernel (CKf) was obtained using methanol by soxhlet method. Radical scavenging and prevention of human LDL oxidation by CKf was done in vitro. The cytotoxic effect on MCF-7 cells were evaluated using MTT assay after treatment with different concentrations of CKf (12.5 - 100 mg/mL) for 24 hrs. Cell apoptosis and nuclear changes were determined using acridine orange/Ethidium bromide and DAPI staining. Intracellular production of reactive oxygen species (ROS) were studied using the fluorescent DCFH-DA staining. MitoSox Red fluorescent stain was used to evaluate the effect of CKf on the generation of superoxide radicals within the mitochondria of MCF-7 cells. Results: It was showed that CKf significantly scavenged the free radicals and prevented the chemically induced LDL oxidation. MCF-7 cell viability was found to be decreased in a dose dependent manner following treatment with the CKf. Cytotoxicity appeared to be absolute in all concentrations of CKf (>35mg/mL). AO-EB and DAPI staining confirmed that the treatment with CKf induced apoptosis and nuclear damage as distinctly visible in cells, as a result the nuclear/cellular damage and poor mitochondrial membrane potential (MMP) appears to be an indirect consequence of the excessive generation of reactive oxygen species (ROS) and superoxide radicals shown through DCFH-DA and MitoSox staining. Conclusions: These results proved that CKf have both significant antioxidant and anti-proliferative potential against MCF-7 cell line growth by inducing cell death through free radical production and nuclear damage.

Introduction

Plant derived natural formulations have contributed significantly to the discovery of a number of lead anti-cancer molecules, amongst which several are in clinical use at present. Noted examples include the discovery and widespread use of molecules such as vinca alkaloids, camptothecin and paclitaxel from its parent plants [1]. Breast cancer is the most common cancer amongst women in developed and less developed countries with an alarming mortality rate [2]. The treatment module commonly includes several rounds of chemotherapy and radiation besides surgical excision of the tumor mass. Chemotherapeutic agents however bring upon a number of side effects such as nausea, loss of appetite, premature menopause and drug resistance which not only impedes cancer treatment but also affects the quality of life of patients in general [3].

Few Research groups have reported the potential antiproliferative and apoptotic effects of plant/fruit derived polyphenolic rich extracts on colon cancer and gastric cell lines [4,5]. Modulation in the levels of antioxidant enzymes, generation of reactive oxygen species, differential gene expression and activation of cell death pathways might account for some of the mechanisms responsible for the antiproliferative-anti-tumor effects of such extracts. Plants have proved to be excellent sources of bioactive...
molecules since time immemorial. Many of these compounds have been developed as drugs or have also served as precursors for the design and synthesis of potent drugs [6].

Dietary phenols have come to be considered as potential therapeutics because of their increased consumption and ease of availability. These include compounds which form a part of our regular diet such as curcumin from Curcuma longa, resveratrol a compound found in grapes, mulberries and peanuts, sulphoraphane from broccoli, ginseng, garlic, yellow mustard oil and are a few of the several other pure compounds plant extracts have been patented for their significant anticancer effects [7]. Such findings add vigour to the rigorous research involved in identification and development of newer plant-based therapeutics as effective anti-tumor agents.

Coconut (Cocos nucifera) forms a significant part of the dietary intake in Asian populations where this plant is found in abundance. Coconut has been used in traditional folklore medicine to treat disorders such as skin infections, malnutrition, nausea, kidney stones, abscesses to name a few. Coconut oil is mainly made up of saturated fats and medium chain triglycerides such as lauric acid and capric acid [8]. Besides coconut, breast milk is the only source containing lauric acid in similar proportions and this suffices to explain the importance of the same in the development of a healthy immune system. Research has proved that medium chain triglycerides come with a host of benefits such as appetite suppression, prevention of atherosclerosis, immune enhancement and treating disorders associated with abnormalities in normal lipid metabolism [9]. Apart from the fatty acids, coconut kernel contains proteins, fibres and several other biologically active minor components [10]. It is in this light, that we have attempted to investigate the effects of a coconut kernel extract, rich in polyphenols whose anti-tumor potency remains unreported till date on breast cancer cell lines in vitro.

Aims and Objectives

1. Evaluate the antioxidant activity and in vitro preventive effect of LDL oxidation by methanol extract of coconut kernel
2. To study the cytotoxic, nuclear and morphological effects of methanol extract of coconut kernel on human breast cancer cell lines, MCF-7.

Materials and Methods

CHEMICALS AND PLANT MATERIAL:

Dulbecco’s modified Eagle's medium (DMEM), foetal bovine serum (FBS), antibiotic and antimycotic solution, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), acridine orange, ethidium bromide, trypan Blue, 4',6-diamidino-2-phenylindole (DAPI) and other cell culture reagents were purchased from Hi-Media Laboratories, India. Dichlorofluorescin diacetate (DCFH-DA) was purchased from Sigma Aldrich, USA. MitoSOX Red was purchased from Molecular Probes, Thermo Fisher Scientific. Samples of Dwarf x Tall (DxT) variety of coconuts were collected from an authorised nursery (Agrofert Private Nursery, Kottayam, Kerala, India).

DEFATTING AND EXTRACTION:

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 to obtain the extract (CKf). CKf thus obtained was dried in rotary evaporator, weighed and used for further experiments.

DPPH RADICAL SCAVENGING ASSAY:

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical- scavenging activity of CKf [11]. Different concentrations of the sample was added at an equal volume to methanolic solution of DPPH (0.135mM). After 30 min dark incubation at room temperature, the absorbance was recorded at 517 nm. The experiments were repeated for three times. Gallic acid was used as positive control. The percentage of inhibition (In%) was calculated using the formula, In% = [(Abs(Control) - Abs(Sample))/Abs (Control)] × 100. IC50 values, concentration of the sample required to scavenge 50% of DPPH was also determined.

FERRIC-REDUCING/ANTIOXIDANT POWER (FRAP) ASSAY:

The FRAP assay was carried out by the method described by Benzie & Strain [12], with slight modification. Briefly, 20 µL of extract was mixed with 180 µL of FRAP reagent (prepared freshly by mixing 300 mM acetate buffer pH - 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM FeCl3.6H2O in the volume ratio 10:1:1), kept for 6 min at room temperature and absorbance was measured at 595 nm. Appropriate blanks of extract were taken together with ascorbic acid and FeSO4 as standards. FRAP activity was calculated as ferrous equivalents (FE), the concentration of extracts or ascorbic acid which produced an absorbance value equal to that of 1mM FeSO4.

ABTS CATION FREE RADICAL-SCAVENGING ACTIVITY:

ABTS assay was done according to the procedure described by Re et al [13], with slight modifications. ABTS was dissolved in water to make a concentration of 7 mM. ABTS+ was generated by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the test samples, the ABTS+ stock solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. Test samples of 20 µL were allowed to react with 180µL of ABTS+ solution for 12 min in dark at room temperature. BHT was used as positive control. This activity was measured as the percentage ABTS+ scavenging calculated by the following formula: ABTS+ scavenging activity (%) = [(Ac-At)/Ac] × 100], where Ac is the absorbance value of the control and At is the absorbance value of the test samples.

ISOLATION OF HUMAN LDL:

Human LDL was isolated from the plasma of healthy individuals by precipitation method developed by Wieland & Seidel [14]. Sodium citrate buffer (0.064M pH 5.04) containing 50,000 U/l heparin was added to the plasma samples. After mixing with vortex mixer, a portion was removed for cholesterol determination and the remaining portion was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by

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centrifugation in a refrigerated bench top centrifuge at 1000 g for 10 min. The pellets were resuspended in 1ml of 0.1M sodium phosphate buffer (pH 7.4 containing 0.9% NaCl). Concentration of LDL was expressed as LDL protein measured by the method described by lowry et al [15].

COPPER-MEDIATED LDL OXIDATION:

To evaluate the ability of CKf to inhibit copper (Cu) mediated LDL oxidation, tiobarbituric acid substances (TBARS) production was monitored. The LDL samples were pretreated with various concentration of CKf and LDL oxidation was initiated by adding copper sulphate at a final concentration of 10 μM/mL in a volume of 1 mL. After 24 hr of incubation, oxidation was terminated by the addition of 33mM EDTA. TBARS were measured in each tubes by the method of Ohkawa et al. [16].

ELECTROPHORETIC MOBILITY OF OXIDIZED LDL:

After pretreatment of LDL with different concentrations of CKf, oxidation was initiated by adding copper sulphate (10 μM/mL). After 24hrs of incubation, the oxidized and treated LDL was denatured with 10% SDS, 50% glycerol and 2% 2-mercaptoethanol at 37°C for 5 minutes. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE, 10% gradient) was performed to detect LDL fragmentation. The electrophoresis was processed at 90V for 2 hours. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue and visualised [17].

CELL CULTURE:

MCF-7 cells were procured from National Centre for Cell Science (NCCS), Pune India. The cells were grown and maintained in a humidified incubator at 37°C under 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum (FBS).

MTT ASSAY:

The antiproliferative effect of CKf on breast cancer cell lines were performed according to the method of Marks et al. [18]. Briefly, cells were seeded at a density of 3x104 cells/well in a 96 well plate overnight, following which CKf at different concentrations (12.5-200mg/mL) was added to the well in triplicates and incubated for a period of 24 hours. Post incubation, the wells were washed with PBS and MTT was added at a concentration of 5mg/mL and the plates were incubated for 4 hours. Following which the supernatant was discarded and the formazan crystals formed were solubilised using absolute dimethyl sulphoxide (DMSO). Absorbance was measured at 590nm using a multimode microplate reader (Thermoscientific Varioskan Flash Microplate Reader). Growth inhibitory efet of CKf was calculated as follows. Growth inhibition rate=(Acontrol-Adrug/ Acontrol)×100. IC50 values were calculated by plotting the OD readings versus the drug concentrations using EasyPlot 2.8 software.

ACRIDINE ORANGE/ETHIDUIM BROMIDE (AO/EB) STAINING:

The effects of varying concentrations of CKf on cell integrity and morphology was visualised by the fluorescent acridine orange-ethidium bromide staining method [19]. MCF-7 cells were seeded in 96well plates at a density of 3x104 cells/well and the cells were treated with CKf at varying concentrations between 12.5-50 mg/ml for a period of 24 hours at 37°C with 5%CO2. 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 minutes at 37°C. Wells were examined at 20x magnification using an Olympus inverted fluorescence microscopewith a FITC filter.

DAPSTAINING:

Nuclear damage observed as changes in nuclear shape, size, nuclear fragmentation and chromatin changes were studied by the fluorescent 4,6-diamidino-2-phenylindole-2- HCl (DAPI) staining method. DAPI binds to double stranded DNA and fluoresce brightly on being excited by ultraviolet light [20]. MCF-7 cells were treated with different concentrations of CKf (12.5-100mg/ml) for 24 hours in a 96 well plate. Wells were rinsed thrice in PBS to completely remove the growth medium and 37% formaldehyde solution was added to fix the cells. The fixative was rinsed off with PBS ten minutes post incubation and 0.2% Triton-X-100 solution was added to permeablize the cells. Cells were rinsed with PBS after five minutes and labelled with DAPI at a concentration of 1μg/ml and kept for 5-10 minutes in dark. The labeling solution was then aspirated and cells were rinsed thrice in PBS and the morphology of the cell nuclei was observed using a fluorescemicroscope (Olympus 1x51) with the DAPI filter.

INTRACELLULAR ROS MEASUREMENT: DCFH-DASTAINING:

Generation of excessive intracellular ROS following treatment of cells with extract was examined using a fluorescent probe, 2′,7′- dichlorofluorescein diacetate (DCFH-DA) [21]. 96 well plates seeded with MCF-7 cells were allowed to become confluent and incubated with different concentrations of CKf (35 and 50mg/ml) for 24 hours. 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 in 5% CO2. After incubation, fresh medium without FBS was added to the wells and fluorescent images were taken using an inverted microscope (Olympus 1x51).

MEASUREMENT OF MITOCHONDRIAL SUPEROXIDE PRODUCTION: MITOSOXASSAY:

MCF-7 cells were allowed to become confluent and incubated with different concentrations of CKf (35 and 50mg/ml) for 24 hours. Cells treated with the CKf were loaded with the fluorogenic mitochondrial superoxide probe MitoSOX Red (5μM) for 10 minutes at 37°C. Cells were washed twice with complete medium and images were taken using and inverted microscope (Olympus 1x51) and the appropriate bandpass filter [22].

MITOCHONDRIAL MEMBRANE POTENTIAL ANALYSIS: RHODAMINE 123 STAINING:

To determine the effect of CKf on the mitochondrial electric potential across the inner membrane, rhodamine 123 (R-123), a lipophilic cationic indicator was used to label mitochondria [7]. MCF-7 cells were seeded into 96-well culture plates at 1 x 104 cells/well and incubated for 24 h at 37°C and 5% CO2. After 24h, cells were treated with different doses of CKf (35 and 50mg/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 1x51).

STATISTICAL ANALYSES:

All the data are expressed as mean ± standard deviation of three independent experiments. Statistical comparison was performed using SPSS 19 software via a one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT). P values of less than 0.05 (p< 0.05) was considered as significant.

Results

ANTIOXIDANT EFFECT:

Methanolic extract (CKf) showed significant DPPH radical scavenging effect in a concentration dependent manner, with IC50 value of about 1.26 µg/µl. ABTS method shows greater antiradical and antioxidant activities in CKf extract. This result may be due to the presence of total phenolic content or phenolic compounds. DXT kernel extract (CKf) showed significant cation free radical scavenging effect in a concentration dependent manner, with IC50 value of about 0.27 µg/µl (Fig 1).

Figure 1: In vitro radical scavenging activity of CKf: DPPH and ABTS assay

Values are expressed as mean ± SD of three independent experiments

PREVENTION OF IN VITRO LDL OXIDATION:

Pre-treatment of human LDL with CKf and subsequent exposure to CuSO4 showed lower levels of TBARS, indicative of lesser oxidative damage compared to oxidized LDL control. This effect of CKf was comparable to the result obtained by using Vitamin C as positive control. Based on the electrophoretic mobility assay, it was found that LDL pre treated with higher concentration of CKf showed profound protective effect on oxidation compared with the mobility of oxidized LDL (Fig 2).

Figure 2: Effect of CKf on preventing Cu2+ induced LDL oxidation in vitro

Results are expressed as mean ± SD of three separate experiments. A: TBARS production in native, oxidized and CKf pre-treated LDL; B: Electrophoretic mobility of oxidized and treated LDL. *Significant compared to control (p<0.05), $- Significant compared to oxidized LDL (p<0.05).

CYTOTOXIC EFFECT OF CKF EXTRACT ON MCF-7 CELL LINES:

The cytotoxic effect of CKf on MCF-7 cell lines 24 hours after treatment was evaluated using MTT assay. A broad range of concentrations starting from 12-100mg/ml of CKf was used for the experiments. It was observed that cytotoxicity increased with increasing concentration of the CKf in comparison to the control. The percentage of cell death was observed to increase at a steady pace till about 25mg/ml after which the cytotoxicity appears to be relatively absolute in all concentrations >25 mg/ml (Fig 3).

Figure 3: Cytotoxic effect of CKf on breast cancer cell lines (MCF-7) in vitro

Values are expressed as mean ± SD of three separate experiments, A: MTT assay to evaluate the cytotoxic effect of different concentrations of CKf against MCF-7 cells; B: light microscopic images of MCF-7 cells treated with varying concentrations of the extract (24h) (Magnification 20x)

EFFECT OF CKF ON CELL MORPHOLOGY AND VIABILITY OBSERVED BY AO-EB FLUORESCENT STAINING:

Effect of CKf on cell health was assessed by observing its impact on cell membrane integrity and viability using the acridine orange-ethidium bromide dual staining method. Acridine orange uniformly permeates all cells and stains them green, whereas
ethidium bromide is only taken up by cells whose membrane integrity has been compromised. Fig 4 distinctly shows CKF treated cells with brightly fluorescing ethidium bromide in the nucleus and periphery which is indicative of loss of membrane integrity and early apoptosis. Moreover, the evident decrease in cell number with increasing concentrations of CKf is indicative of its ability to prevent the proliferation of breast cancer cells.

**Figure 4: Analysis of changes in cell morphology and viability of MCF-7 cells treated with different concentrations of CKf**

The images were taken using a fluorescent microscope and is the representation of three independent experiments (Magnification 20x)

**CKf INDUCED CELL DEATH MIGHT BE A CONSEQUENCE OF NUCLEAR DAMAGE:**

To identify the probable mechanisms involved in CKf induced cytotoxicity, cells were stained with a nucleic acid stain DAPI which selectively binds to the AT rich regions in the minor grooves of DNA. Bound dye exhibits a higher fluorescence in contrast to a non-DNA bound dye. DAPI labels fixed cells quite easily. Fig 5 clearly shows that CKF treated cells have different size, nuclear fragmentation patterns and chromatin condensation compared to non treated control cells. This indicates that nuclear damage precedes cell death following treatment with CKF extract.

**Figure 5: Analysis of nuclear damage and chromatin changes in MCF-7 cells treated with different concentrations of CKf**

The images were taken using a fluorescent microscope and is the representation of three independent experiments (Magnification 20x)

**CKF REDUCED THE MITOCHONDRIAL MEMBRANE POTENTIAL OF MCF-7 CELLS:**

The effect of CKf on the mitochondrial potential was studied using a cationic fluorochrome rhodamine 123 dye, which bind specifically to the mitochondria of living cells. Dead cells exhibit either uniform, strong fluorescence or show a patchy labelling pattern suggesting swollen mitochondria [23]. The results showed that CKf treatment showed a dose dependent decrease in the mitochondrial membrane potential compared to the control cells which showed a very high fluorescence (Fig 5).

**Figure 6: Analysis on the effect of CKF treatment on the ROS, superoxide production and mitochondrial membrane potential in MCF-7 cells**

The images were taken using a fluorescent microscope and is the representation of three independent experiments (Magnification 20x)

**TREATMENT OF CELLS WITH CKF EXTRACT IS ACCOMPANIED BY A NOTICEABLE INCREASE IN INTRACELLULAR ROS AND MITOCHONDRIAL SUPEROXIDE LEVELS:**

Fig 5 indicates that the levels of intracellular ROS have risen tremendously in cells treated with the CKF in comparison to the basal ROS levels in the untreated control cells. Mean while the mitochondrial superoxide production in CKF treated MCF-7 cells were also found to be increased significantly compared to control cells. This observation indicates that drug induced-ROS/superoxide generation in cells might contribute to the cytotoxic effect of the extract on breast cancer cell lines in vitro.

**Discussion**

The present study aims at evaluating the antioxidant and preventive effect of LDL oxidation as well as the antiproliferative and/or anti-tumorogenic properties of CKF on MCF-7 in vitro. Previous studies in our laboratory showed that CKF contained significant amount of polyphenols and trace amount of flavonoids (Unpublished data). CKF showed significant radical scavenging
activity a evidenced from the clearance of DPPH and ABTS free radicals in in vitro system. Likewise the in vitro LDL oxidation prevention by CKf was evaluated with Cu2+ ions. Oxidation of LDL by Cu2+ is considered a more aggressive system that transmits through Cu2+ binding, causing continuous free radical formation with reduction of lipoprotein antioxidants [24]. It was found that CKf had inhibitory effect on chemical induced oxidation as evidenced by the lower levels of TBARS and reduced electrophoretic mobility of LDL. The free radical scavenging and antioxidant activities of phenolics are dependent upon the arrangement of functional groups about the nuclear structure. Both the number and configuration of H-donating hydroxyl groups are the main structural features influencing the antioxidant capacity of phenolics [25].

MCF 7 cells were initially tested over a broad range of concentrations ranging between 12.5-100mg/ml to evaluate the cytotoxicity of CKf and based on the results, a few selected concentrations (12.5, 25, 35, 50 and 100mg/ml) were selected for further experiments. The results showed a decrease in cell viability with increasing concentrations of the extract and cytotoxicity reached a maximum (close to 100 percent) at concentrations >35mg/ml. Cytotoxic observations at the mentioned range is supplemented by the fluorescent images depicting nuclear damage, cell size variation and generation of ROS following CKf treatment.

Coconut occurs in abundance in the tropics and nearly one third of the world’s population use coconut as part of their diet in some form or the other. Though the use of coconut oil has been a matter of debate with regards to its cardiovascular implications since decades, research has shown that virgin coconut oil possess sufficient amounts of medium chain fatty acids, polyphenols, tocopherols and free radical scavengers which not only improve the antioxidant status, but also reduces free radical induced protein oxidative damage and exerts a general hypolipidemic effect on treated subjects despite having lower concentrations of unsaturated fatty acids which are thought to be heart friendly [26-28]. Coconut oil and palm oil are rich in medium chain fatty acids such as lauric acid, capric acid and myristic acid. The antimicrobial activities of MCFA have been documented to some extent, however the anti-proliferative and apoptosis inducing effects of such MCFA’s are yet to be studied extensively [29]. One such report indicates that lauric acid significantly reduces cell viability on two human leukemic cell lines-Jurkat and Raji cells in vitro [30]. Conversely, reports stating the non-inhibitory effect of saturated fatty acids such as lauric acid on the proliferation of human pancreatic cell lines have also been reported [31]. However, it remains to be assessed if whether the cytotoxic effect of the polyphenol extract on human breast cancer cell lines is the result of the high polyphenol content or due to the presence of saturated fatty acids.

In our study we demonstrated the effects of the polyphenol rich extract derived from the coconut kernel on the growth and viability of the breast cancer cell lines MCF-7. Our observations lead us to hypothesize that the extract induced oxidative stress plays a significant role in the decreasing cell viability. It is well known that the basal levels of intracellular ROS is significantly higher in cancer cells as compared to the normal cells which is what drives the growth, progression and other metabolic activities of these cells. However, the pro-carcinogenic effect of this basal ROS level can propel a cell towards apoptosis once a threshold ROS limit has been exceeded [32,33]. From our observations as depicted in Fig 4 the cells treated with varying concentrations of extract display a higher threshold fluorescence in contrast to the control cells which hints at the pro-oxidant potential of the CKf extract in general. Generally ROS generated as a part of regular cell metabolism is balanced by the intracellular glutathione and thioredoxin antioxidant systems. Mitochondria are also a significant contributor to cell ROS through the flow of electrons in the electron transport chain. Considering the prospects of oxidative stress induced cell death also prompts us to consider the possibility of compromised cellular antioxidant defenses which could be the cause of cytotoxicity [34]. Studies by Duchler & Stepnik, have attributed the cytotoxic effects of a combinatorial treatment of three polyphenolic compounds on leukemic cells to reduced glutathione levels which asserts that the cell redox regulation cannot be overlooked [35]. A common dietary polyphenol, curcumin has also been reported to increase oxidative stress and nuclear as well as mitochondrial DNA damage human hepatoma cell line, HepG2 [36].

Interestingly, sanguanarine a plant derived alkaloid has being reported to cause cell death in colon cancer cell lines independent of the status of the guardian p53 and reports the induction of DNA double stranded breaks as a result of apoptosis [37]. What makes this observation interesting is the fact that the destruction of cell morphology and cell viability in the previous study and current study is linked by similar observations in terms of cytotoxicity at similar drug exposure times (<25mg/ml). Prolonged incubation of cells at 24 and 72 hours with CKf showed a precipitous decline in cell viability and retention of regular cell morphology after the said time intervals (data not shown).

Our hypothesis is strengthened through the nuclear stained images as shown in Fig 4 which clearly indicates the formation of apoptotic bodies and nuclear fragmentation in concentrations as low as 12.5mg/ml which is indicative of late stage apoptosis. The mitochondrial specific ROS probe labelled MCF-7 cells (Fig 6) showed an increased fluorescence at concentrations >35mg/ml indicating the loss of mitochondrial membrane integrity at higher doses of the extract. Compromised mitochondrial membrane potential caused by excessive stress can initiate the signalling cascade of apoptosis which could possibly account for the CKf induced cytotoxicity [38,39].

**Summary and Conclusions**

These results propel us to envision the benefits that could come about in identifying the bioactive components involved in exerting cytotoxic effects on cancer cell lines in vitro. Our observations fall within the limits of certain barriers which
remains unexplored. It is also needed to evaluate the effect of these extracts at the same concentrations on normal cells in vitro and much more needs to be done in terms of identifying the active ingredient(s) and in evaluating the effects of treatment on gene expression before a drug-effect mechanism can be explained. However, our results indicate that increased oxidative stress, compromised mitochondrial membrane integrity and nuclear damage are major events in CKf induced cytotoxicity.

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