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Research Article

HR-LCMS-MS ANALYSIS OF METHANOLIC EXTRACT OF COCONUT KERNEL AND ITS *IN VITRO* CYTOTOXIC EFFECT AGAINST HUMAN ORAL CANCER CELL LINE

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ABSTRACT

Cocos nucifera, a member of the palm tree family *Arecaceae* is considered to be rich in several biologically active components, which are not fully elucidated. The kernel is considered to be very much effective as an anti-inflammatory, anti-nociceptive, antioxidant, antifungal, antimicrobial, and antitumor agent. This study was designed to evaluate the chemical composition of the methanol extract of coconut kernel and its effect against the growth of human oral cancer cell lines, KB. The methanol extract of the coconut kernel (MCK) was prepared and subjected to HR-LCMS to evaluate the different compounds present in it. MCK was evaluated for its anti-proliferative activity and its effect on ROS and mitochondrial membrane health of KB cell lines. The results showed that the MCK contains several compounds, belonging to the class of phenolics, alkaloids, terpenoids, hydroquinones, vitamins, aminoglycosides, etc. The extract was also found to be cytotoxic `to the growth of KB cells, as well as induced apoptosis and increased the ROS levels mediated by the decrease in mitochondrial membrane potential. The result warrants the characterization of individual compounds and studies on the anti-cancer effects in both cell lines and in *in vivo* animal models be done.

Keywords: Coconut Kernel, HR-LCMS, ROS, Cytotoxicity

1. INTRODUCTION

Phytochemicals are plant-derived secondary metabolites that have several therapeutic applications [1]. Extracts obtained from plants consumed as food are rich in a wide range of chemicals that can contribute to different biological activities such as anti-tumor, anti-inflammatory, modulation of the biological response by supporting the immune system and protect cells from oxidative damage [2]. Leukoplakia, a proliferative oral mucosa is normally seen as white patch areas on the inner surface of the mouth which has the potential to become cancer [3]. Phytochemicals with reduced side effects are considered to be new alternatives for oral cancer prevention since; several phytochemicals have been tested against the skin and mammary carcinogenesis, only a few studies have been conducted in the prevention of oral carcinogenesis [4].

Cocos nucifera or coconut palm is one of the most useful palms, which is grown around the world in lowland tropical and subtropical habitats. It is called the fruit of life due to its numerous nutritional, health, and economic benefits [5]. The coconut is considered a "functional food" because it provides health benefits beyond its nutritional content [6]. It has been recognized as an entity with multiple uses with every component of being biologically active in one way or the other. Coconut water and kernel consist of carbohydrates, proteins, microminerals, nutrients, vitamins, phytohormones, enzymes, and growth-promoting factors that contribute to its bioactivity [7]. Besides, they possess antibacterial, antifungal, antiviral, antiparasitic, anti-dermatophytic, antioxidant, hepato-protective, and immunostimulant properties[8]. This study was designed to characterize the various biochemical compounds present in the methanol extract of coconut kernel and its cytotoxic effects on human oral cancer cells in vitro.

2. MATERIAL AND METHODS

2.1. Chemicals and Extract

The DxT variety of coconut kernel was crushed and subjected to remove the fats by extraction with

petroleum ether. The defatted kernel was subsequently extracted with 80% methanol. The resulting extract was dried, weighed, and stored at -20°C until further use.

2.2. High Resolution-Liquid Chromatography-Mass Spectrometer (HR-LCMS) Analysis

The filtered MCK was injected into to High-Resolution Liquid Chromatographic Mass Spectrometer (Model-G6550A; Agilent Technologies, USA). The acquisition method was set to be MS Min Range 60(m/z) and MS Max Range 1000 (m/z) with MS scan rate and MS/MS scan rate (spectra/sec) as 1. The mobile phase used was as follows Solvent A: 0.1 % formic acid in water and Solvent B: Acetonitrile. The injection volume was 5 µL and the flow rate was 0.3 mL/min. The other conditions were the obtained mass spectrum was compared with the known Database.

2.3. Cytotoxicity analysis

2.3.1. Cell lines

KB cell lines were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown and maintained in a humidified incubator at 37^{O}C under 5% CO_2 atmosphere in McCoy's-5A, MEM, and DMEM supplemented with 10% FBS and 1x antibiotic and antimycotic solution. For the experimental purpose cells were plated in 96 well plates (at a density of 1 to 2.5x104 cells), 12 well plate (at a density of 6 to 8x104), 24 well plate (at a density of 4 to 6x10^4) and 6 well plates (at a density of 15 to 20x104). After 24 hrs incubation period to allow cell attachment, the cells were treated with fresh medium containing different concentrations of MCK dissolved in 10% DMSO and incubated for 24hrs. After 24 hrs, the cell viability and apoptotic process were observed by different staining methods.

2.3.2. MTT assay, AO-EB, and DAPI staining

After 24 hrs of treatment with MCK, KB cells were washed and treated with 200µL 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) solution and incubated for another 3 to 4hrs cells. The supernatant was removed and replaced with 200µL of DMSO to dissolve the resulting MTT formazan crystals followed by mixing and measuring the absorbance at 570nm. The optical density obtained was directly related to the viability of cells. The amount of formazan produced is directly proportional to the cell number in a range of cell lines [9]. For apoptotic analysis, Adherent cells grown on plates were rinsed thrice in PBS to completely remove the growth medium. Cells were fixed for ten minutes in 3.7% formaldehyde and again rinsed thrice in PBS before permeabilization in 0.2% Triton-X-100 for 5 minutes. The apoptotic analysis was evaluated by staining the cells with acridine orange ethidium bromide (AO/EB). Acridine orange 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 [10]. Nuclear damage or changes in chromatin if any caused by MCK treatment was observed by staining with 4,6-diamidino-2-phenylindole-2-HCl (DAPI), which binds to damaged dsDNA providing a blue fluorescence when viewed under ultraviolet light. Fixed cells were incubated with DAPI(10mg/mL) in water-Stock; working solution-dilute the stock 1:5000 times) labeling solution for 5 minutes in the dark. morphology of the nuclei of the cells was observed using a fluorescence microscope (Olympus) with the DAPI filter [11].

2.3.3. Rhodamine, DCHF-DA and Mitosox staining

Reactive oxygen species (ROS) and mitochondrial superoxide production were observed by staining the cells with cell-permeable DCHF-DA and Mitosox. MCK treated KB cells was treated with DCFH-DA (10mM-stock dissolved in absolute DMSO) at a final concentration of 10µM and incubate in the dark at 37°C for 30 minutes. Post-staining, plates were rinsed twice with PBS followed by fluorescence was read on a Thermo scientific Varioskan Flash Microplate Reader (λex-485nm λem-520nm) three times at 90-sec intervals and fluorescent images were taken on a fluorescent microscope (Olympus) using bandpass FITC filter (#2) [12]. MitoSOX Red mitochondrial superoxide indicator, a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells was used to observe the formation of superoxide in MCK treated KB cells. Gently wash the cells with PBS thrice and was observed using a fluorescence microscope (Olympus) with the superoxide filter. (λex/λem-510/580nm)/Filter #3.

The mitochondrial membrane potential of the MCK treated KB cells was evaluated by staining with Rhodamine 123. Cells, treated with various concentrations of were washed with PBS and fresh medium containing RH-123 solution (10ug/mL) was added and incubated in the dark at 37^{O}C for 20-30 min. Subsequently, the cells were washed twice with PBS and the cell images were taken using a fluorescence

microscope (Olympus) with the rhodamine 123 filter #2 [13].

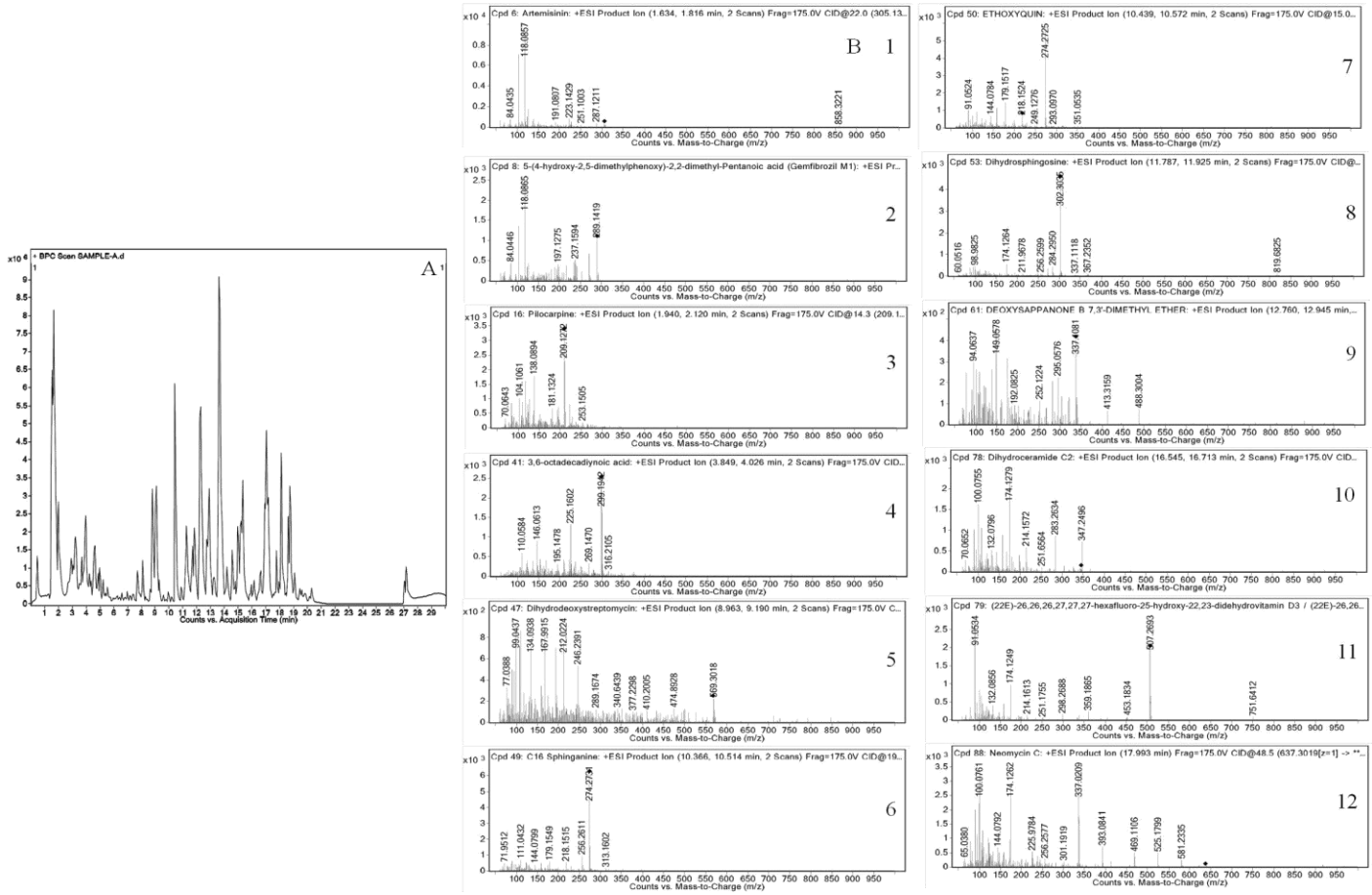
2.4. Statistical Analysis

Data was expressed as mean±SEM statistical significance were measured using one-way analysis of variance (ANOVA) using SPSS.

3. RESULTS AND DISCUSSION

3.1. Identification of Bioactive Compounds of MCK

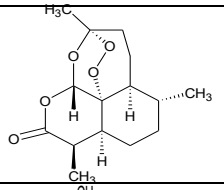
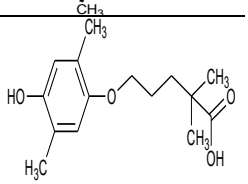
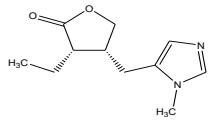
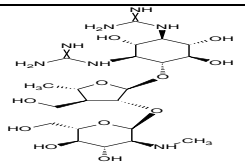
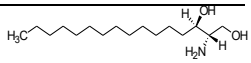
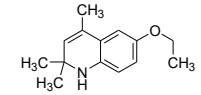
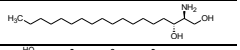
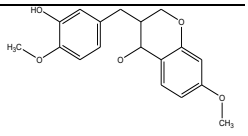
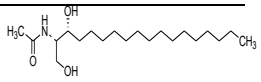
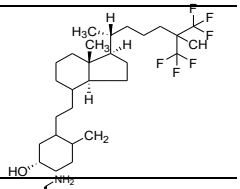
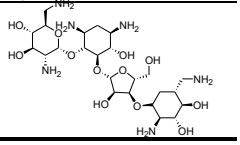
HR-LCMS analysis of MCK showed the presence of various phytochemical constituents. Both positive and negative mode spectrum was obtained. More number of bioactive compounds was found to be present in the positive mode spectrum. Fig 1 shows the positive mode chromatogram of MCK along with the MS spectrum. Table 1 shows the compounds identified in MCK, out of 23 compounds, 13 major bioactive compounds were confirmed based on their retention time, mass and molecular formula, and 10 were found to be unknown compounds, but abundant in the extract. Identified compounds in MCK were Artemisinin, 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid, Pilocarpine, 3,6-octadecadiynoic acid, Dihydrodeoxystreptomycin, C16 Sphinganine, Ethoxyquin, 2-R-amino-hexadecanoic acid, Dihydrosphingosine, Deoxysappanone B 7,3'-dimethyl ether, Dihydroceramide C2, (22E)-26,26,26,27,27,27-hexafluoro-25-hydroxy-22, 23-didehydrovitamin D3, Neomycin. These compounds belong to various classes of phytochemicals such as phenolics, alkaloids, terpenoids, hydroquinolones, vitamins, and aminoglycosides.



***A**-Total Ion chromatogram of MCK. **B**-MS/MS spectrum of each peaks. **1;** Artemisinin (mass 282.1443; RT 1.725 min), **2;**5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid (Mass 266.1496; RT 1.769 min), **3;** Pilocarpine (Mass 208.1201; RT 2.03 min), **4;**3,6-octadecadiynoic acid (Mass 276.2068; RT 3.938 min), **5**; Dihydrodeoxystreptomycin (Mass 567.287; RT 9.076 min), **6**; C16 Spinganine (Mass 217.147; RT 10.44 min),**7**; Ethoxyquin (Mass 173.1204; RT 10.505 min), **8;** Dihydrosphingosine (Mass 301.2975; RT 11.856 min), **9;** Deoxysappanone B 7,3'-dimethyl ether (Mass 314.1148; RT 12.852 min)**10;** Dihydroceramide C (Mass 2343.3075; RT 16.629 min)**11;** (22E)-26,26,26,27,27,27- dexafluoro-25-hydroxy-22,23- didehydrovitamin D3(Mass 506.2625; RT 16.851 min), **12;** Neomycin (Mass 614.3128; RT 17.993min)*

Fig.1: HR-LC MS/MS spectrum of MCK

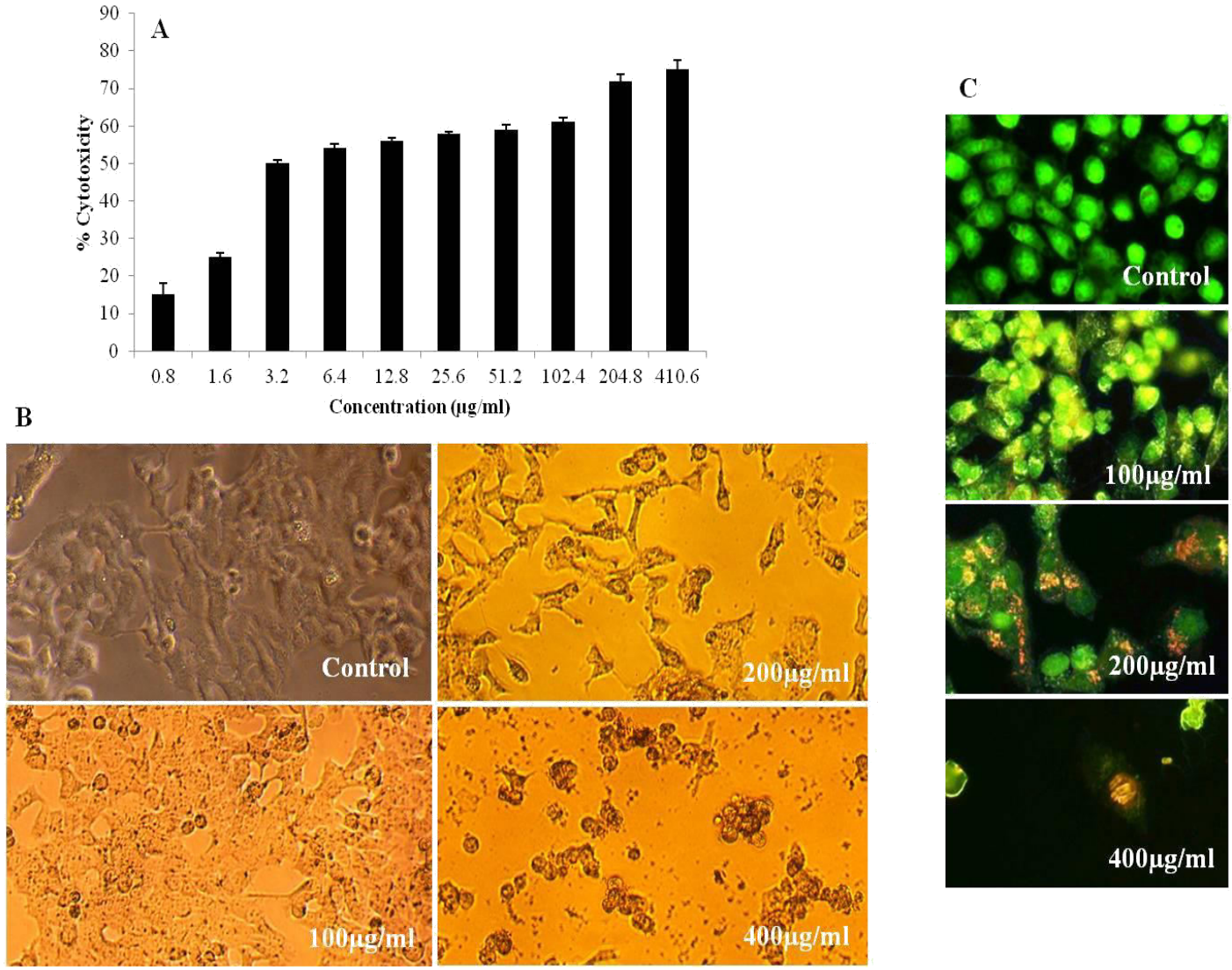
Table 1: Compounds identified in MCK using HR-LC MS MS

Sl. No.	Compound name	RT	Mass	Chemical Formula	DB Diff (ppm)	Class	Reported Activity with reference	Structure of the compound
1	Artemisinin	1.725	282.1443	$C_{15}H_{22}O_5$	8.53	Sesquiter -penoid	Antimalarial activity, antibacterial, antifungal, antileishmanial, and antitumor agent [15]	
2	5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid	1.769	266.1496	$C_{15}H_{22}O_4$	8.12	Fibric acid	Antioxidant and antiatherogenic activity [28]	
3	Pilocarpine	2.03	208.1201	$C_{11}H_{16}N_2O_2$	5.31	Alkaloid	Antimicrobial activity [29]	
4	3,6-octadecadiynoic acid276.2068	3.938	276.2068	$C_{18}H_{28}O_2$	7.85	Methyl ester	Anti-asthma, Neurons protective, antiinflammatory and hepato-protective property [18]	
5	Dihydrodeoxy streptomycin	9.076	567.287	$C_{21}H_{41}N_7O_{11}$	-1.16	Aminogl-ycoside	Antimicrobial activity [14]	
6	C16 Spinganine	10.44	217.147	$C_{16}H_{35}NO_2$	1.59	Lipid	Antitumor activity [16]	
7	Ethoxyquin	10.505	173.1204	$C_{14}H_{19}NO$	-1.47	Hydroqui -nolones	Antioxidant activity [17]	
8	Dihydrosphingosine	11.856	301.2975	$C_{18}H_{39}NO_2$	1.83	Lipid	Antibacterial [30]	
9	Deoxysappanone B 7,3'-dimethyl ether	12.852	314.1148	$C_{18}H_{18}O_5$	2.05	Phenolic comp.	Anti neuroinflammatory and neuroprotective property [31]	
10	Dihydroceramide C2	16.629	343.3075	$C_{20}H_{41}NO_3$	3.23	Lipid	Antioxidant and antitumor [32, 33]	
11	(22E)-26,26,26,27,27,27-hexafluoro-25-hydroxy-22,23-didehydrovitamin	16.851	506.2625	$C_{27}H_{36}F_6O_2$	-1.06	Sterol lipids	Regulates metabolism, [19] Anticancer activity[20]	
12	Neomycin614.3128	17.993	614.3128	$C_{23}H_{46}N_6O_{13}$	-0.79	Aminogl-ycoside	Antioxidant and anti lipid peroxidation activity [34]	

Among the compounds identified in MCK, Artemisinin, Pilocarpine, Dihydrosphingosine, Dihydrodeoxystreptomycin, C16 Sphinganine, and Dihydroceramide C2 have antibacterial, antimalarial and antitumor activity [14-16]. 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid, Ethoxyquin, and Neomycin have antioxidant activity [17]. 3,6-octadecadienoic acid and Deoxysappanone B 7,3'-dimethyl ether are having neuron protective, and anti-inflammatory activity [18]. (22E)-26,26,26,27,27,27-hexafluoro-25-hydroxy-22,23-didehydrovitamin D3 regulates the calcium absorption and also reported to have antitumor activity [19, 20].

3.2. MCK induces apoptosis and increases ROS in KB cells and reduce reduces mitochondrial membrane potential

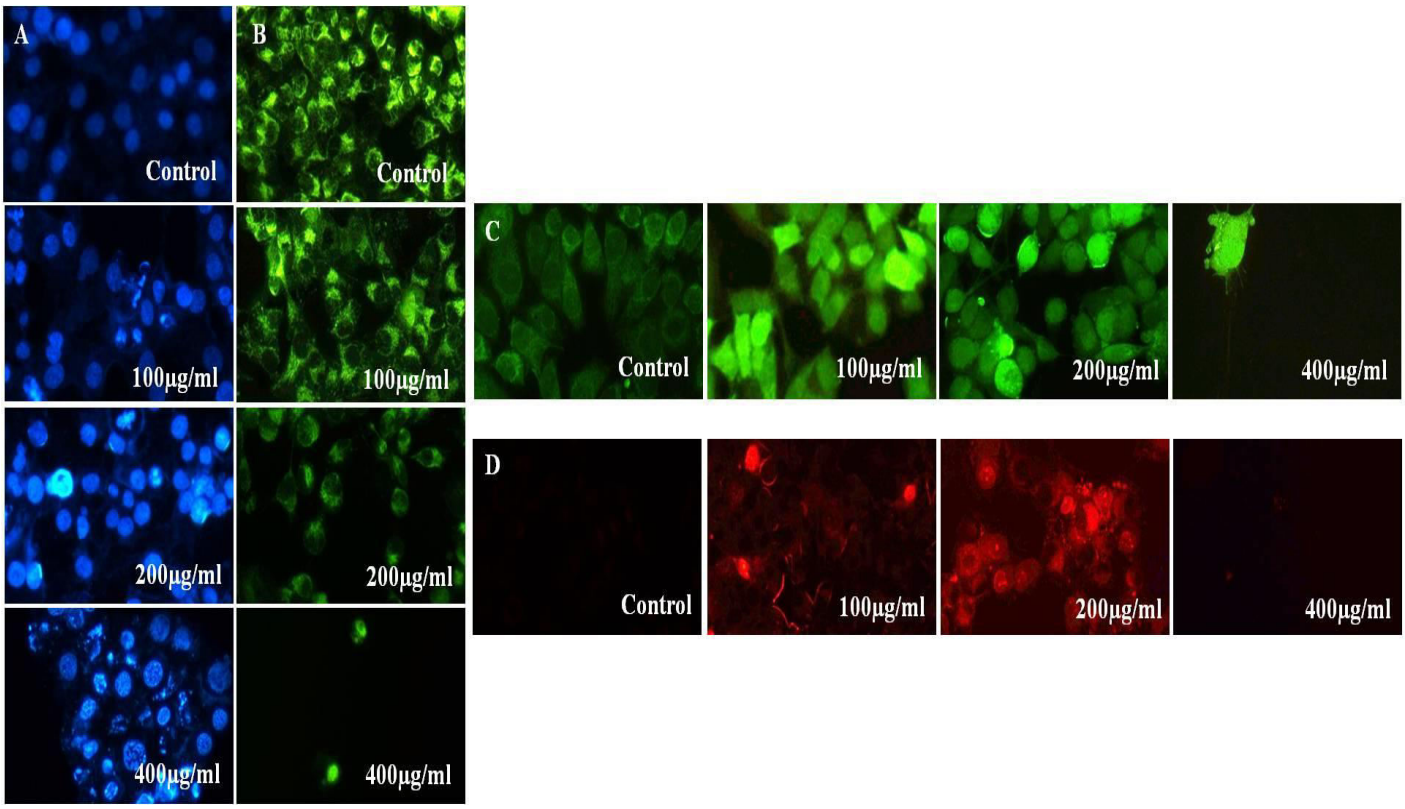
MTT assay showed that MCK prevented the proliferation of KB cells in a dose-dependent manner (Fig 2). AOEB and DAPI staining showed that MCK induces apoptotic changes and the nucleus showed chromatin damages.



A: MTT assay for evaluating cytotoxicity. Each value of is expressed as mean ± SD of three individual experiments. B: Picture shows the morphological changes in KB cells after treating with MCK. C: AO-EB staining of KB cells after treating with MCK. Concentrations are expressed as polyphenol equivalance/extract. Pictures are representation of three separate experiments.

Fig. 2:Antiproliferative effect of different concentration of MCK against KB cells

Results also showed that MCK increased the production of ROS and superoxide radicals as indicated by the higher fluorescence, as well as reduced the mitochondrial membrane potential in a dose-dependent manner (Fig 3).



A: DAPI staining B: Mitochondrial membrane potential C: DCHF-DA staining D: Mitosox staining for superoxide radical. Concentrations are expressed as polyphenol equivalance/extract. Pictures are representation of three separate experiments.

Fig. 3: Nulear changes, ROS production and mitochondrial membrane potential of KB cells treated with different concentration of MCK

Reports show that a combination of chemotherapeutic agents with naturally occurring dietary supplements is a preferred modality for cancer treatment than single-agent that might reduce systemic toxicity of the chemotherapeutic agents. Previously, in vitro studies carried out with the extract of coconut husk fiber have proven its antiproliferative activity against erythroleukaemia cell lines (K562) [21]. The results of the present study showed that MCK is cytotoxic to human oral cancer cell lines as it induced morphological, detachment, shrunken, and dispersed cells when compared to that of control cells. AO-EB staining showed that MCK treatment induced apoptotic changes in the cells and DAPI staining indicated the shrinkage of the cell membrane, enlargement of cell size, and nuclear blebbing.

The generation of reactive oxygen species as a consequence of treatment with the extract was evaluated using DCFH-DA and MitoSOX staining methods. Mitochondria play a major role in the regulation of cell death and proliferation [22]. Intracellular ROS generation and dissipation of the mitochondrial membrane potential (MMP) are the characteristic features of mitochondrial apoptosis [23-25]. Several studies have previously reported that ROS are mediators of intracellular signaling cascades in various types of cancer cells and these results in the activation of the programmed cell death mechanism [26]. The results suggest that the generation of excessive ROS either in the cytoplasm/mitochondria of KB cells treated with the MCK as indicated by the green fluorescence. This might be a major player in modulating cell death and hence the antiproliferative effect of the extract. Here, a decrease in mitochondrial membrane potential was induced by MCK in KB cells in a concentration-dependent manner. This could suggest that the MCK may induce cell death in KB cells through the over production of oxidative radical-mediated apoptosis involving mitochondrial membrane depolarization.

In recent years, several antioxidants such as vitamin E, flavonoids, and polyphenols have been exploited for their actual or supposed beneficial effect against oxidative stress [27]. In MitoSOX Red staining, KB cells treated with MCK for 24 h exhibited an increase in red fluorescence, indicative of superoxide production

when compared to control cells. This result confirms the increased red fluorescence represents mostly mitochondrial generated superoxide. Hence the compounds present in MCK can act as redox-sensitive compounds by inducing the pro-oxidant effect thereby enhancing the ROS and superoxide production leading to apoptosis.

4. CONCLUSIONS

Observations from subsequent experiments suggest that coconut kernel can be considered as a potential source of compounds that have a cytotoxic effect on cancer cells. Studies on a suitable animal model of cancer and development of a formulation that selectively targets a cancerous population of cells will aid in improving therapeutic efficacy and the clinical outcome of a cancer diagnosis.

5. ACKNOWLEDGMENTS

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