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ROS-DEPENDENT APOPTOTIC EFFECT OF *Washingtonia filifera* H. Wendl. EXTRACTS ON HUMAN BREAST CANCER CELL MCF-7

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ABSTRACT

Washingtonia filifera **has many health benefits, but its activity against cancer cell lines has not yet been explored. The present study assessed the cytotoxicity of different solvent extracts of *W. filifera* using MTT assay. Fluorescence microscopy was used to evaluate apoptosis and oxidative stress. Only two extracts showed dose- and time-dependent decrease in cell survival. The ethyl acetate extract of *W. filifera* (WFET) exhibited maximum cytotoxicity against HUVECs, MCF7, HCT116, and HepG2 cells with IC_{50} values of 21.7, 48.8, 85.8, and 100.6 µg mL^{-1}, respectively, 24 h post-treatment. Similarly, methanolic extract of *W. filifera* (WFME) showed maximum cytotoxicity against HUVECs (IC_{50}: 21.8 µg mL^{-1}) cells, followed by MCF-7 (73.3 µg mL^{-1}), HCT116 (98.5 µg mL^{-1}), and HepG2 (100 µg mL^{-1}). Light and florescent microscopy using 4′,6-diamidino-2-phenylindole, and acridine orange-ethidium bromide dyes showed apoptotic cell death. Intracellular reactive oxygen species (ROS) was evaluated using DCFH-DA dye. The WFET and WFME extracts showed increased intracellular ROS with increased concentrations of the two extracts using MCF7 cells. Overall, the WFME and WFET extracts could act as promising chemo-preventive agents against breast carcinoma cells.**

Keywords: Apoptosis, anticancer, extract, fluorescent microscopy, ROS, *Washingtonia filifera*

INTRODUCTION

Worldwide, 19.3 million cancer cases were reported in 2020 with around 10 million cancer deaths; while the global cancer burden is anticipated to be 28.4 million cases by 2040, thereby revealing an expected 47% rise (Sung *et al*., 2021). For cancer treatment, gene-targeted therapy, hormonal therapy, chemotherapy, radiotherapy, and surgery have extensively been used. The development of detecting the methods and therapies has played a vital role in increasing the survival rates (Blumen *et al*., 2016). Unfortunately, these therapies have many side-effects, such as urinary and bladder problems, anemia, appetite loss, bleeding, constipation, delirium, diarrhea, edema, fatigue, hair loss, lymphedema, issues of mouth and throat, nausea, vomiting, nerve problems, pain, skin and nail changes, sleep problems and many more (NIH, 2021). Moreover, reports reveal that the burden of cost, availability, and resistance to chemotherapeutic drugs is unexpectedly on rise (Blumen *et al*., 2016; Trogdon *et al*., 2017). A study on therapeutic efficacy of 88 approved anticancer drugs compared with 650 natural anticancer products has concluded that 25% of natural products have a therapeutic potential like anticancer drug potency level (Qin *et al*., 2012). Therefore, searching natural sources as an alternative, effective, safe, and non-toxic option to treat cancer is necessary.

When used scientifically, the natural products can benefit patients in many ways, such as improving drug bioavailability, increasing the drug-sensitizing pathways, and suppressing the drug-resistance pathways (Somasundaram *et al*., 2002; Frye *et al.*, 2004; Qiao *et al*., 2011).

Arecaceae (palm family) is a monophyletic group of about 2700 species with geographical distribution confined to the tropical and subtropical regions (Wilson *et al.*, 1990). Its economic importance is attributed to the edible fruits like date palm, coconut, areca nut, etc. Many plants of this family have anticancer, antimicrobial, and anti-oxidant potentials, and many are grown as ornamentals (Jeng *et al.*, 2003; Yong *et al.*, 2009). *Washingtonia filifera* H. Wendl. (California fan palm) does not produce dates but sweet edible fruits with brown seeds covered with thin pulp. The plant is reported to possess antioxidant (El-Sayed *et al*., 2006; Gaagaia *et al.*, 2019), anti-aging (Era *et al*., 2021), and anti-inflammatory (Hemmati *et al.*, 2015) activities. There are no reports on the anticancer activity of *W. filifera* extract; therefore, this study was aimed to evaluate the cytotoxicity potential of solvent extracts prepared from fruits against colon, liver, and breast human cancer cells.

MATERIALS AND METHODS

Plant collection and extraction

Washingtonia filifera was collected from the King Saud University, Riyadh Campus (Saudi Arabia). The plant was deposited at the Bioproduct Research Chair Herbarium, King Saud University, Riyadh, under specimen accession No. BRC-040. The fresh fruits of plant were pulverized into a fine powder using a grinding mill (ARTC, China). The powdered material (60 g) was extracted with hexane (Hex), chloroform (CH), ethyl-acetate (EA), and 100% methanol (Me), respectively, for 12 h or until became colourless on Soxhlet apparatus. Later, the extracts were filtered (Whatman, England) and concentrated at 45°C using a vacuum evaporator (Heidolph, Germany). The stock solutions were prepared by dissolving the extracts in dimethylsulfoxide (DMSO).

Cell lines and cultures

Human breast cancer (MCF7), colorectal carcinoma (HCT116), hepatocellular carcinoma (HepG2), human umbilical vein endothelial cells (HUVECs) were obtained from the German Collection of Microorganisms and Cell Cultures (GmbH), Germany. All the cell lines were cultured in DMEM medium provided with fetal bovine serum 10% (v/v), streptomycin, and penicillin (100 U mL^{-1}). Cell plates were kept at 37°C and 5% CO_2 in a humidified atmosphere.

Cytotoxicity assay

For cytotoxicity assessment, all cell types were exposed to the increasing concentrations of *W. filifera* fruit extracts (5-100 µg mL^{-1}) and incubated for 24 h in a humidified incubator, followed by an MTT assay as previously assessed (Abutaha *et al.*, 2021).

Cell morphology

Cytomorphological changes caused by *W. filifera* fruit extracts in MCF7 cancer cells were observed and photographed using a light microscope (EVOS, USA) fitted with a camera. Cells were maintained and treated as reported in previous section.

Nuclear morphological

The treated and control cells were washed with PBS, fixed in ice-cold methanol, and stained with DAPI staining solution (1 mg mL^{-1}) (Life Technology, USA) and incubated at 25°C in dark for 10 min. The cells were observed for nuclear morphological changes using a fluorescence microscope (EVOS, USA) (Abutaha *et al.*, 2020).

Acridine orange (AO) and ethidium bromide (EB) dual staining

The treated and control cells were stained with AO/EB solution (1:1, v/v) and directly observed

using a fluorescence microscope (EVOS, USA) (Abutaha *et al.*, 2020).

H_2DCF-DA ROS detection assay

ROS assessment was carried out using MCF7 cells for control and treated cells following the method of Chen *et al*. 2013). Seeded cells were treated with *W. filifera* fruit extracts (100 µg mL^{-1}), and after 24 h post-treatment, 10 µM of H2DCF-DA was added, kept for 30 min in dark, and the wells were imaged.

Statistical analysis

All experimental data are expressed as mean ± SD from three replicas. Results were analyzed using origin software version 8.5.

RESULTS AND DISCUSSION

Cancer remains a global issue despite developing different therapeutic strategies in medical technology. The plants are well known for managing such chronic and non-chronic diseases (Forni *et al.*, 2019). Plant extracts and their isolated active compounds have been documented for their inhibitory action and induction of cancer cell death through several death pathways like apoptosis and/or necrosis (Shokrzadeh *et al.*, 2010; Aydemir *et al.*, 2015). *W. filifera* (Fig. 1) has been documented for its beneficial effects. However, the cytotoxic potential of *W. filifera* extract against cancer cell lines has not been reported as yet. The cellular toxicities of *W. filifera* fruits (WF) extracts [hexane (WFHE), chloroform (WFCH), ethyl acetate (WFET), and methanol (WFME)] were evaluated by MTT assay using colon, liver, and breast human cancer cell lines. Only two extracts showed dose- and time-dependent decrease in cell survival. The WFET exhibited maximum cytotoxicity against HUVECs, MCF7, HCT116, and HepG2, with IC_{50} values of 21.7, 48.8, 85.8 and 100.6 µg mL^{-1} 24 h post-treatment (Fig. 2 and 3). The lowest IC_{50} values among cancer cell lines

Fig. 1: *Washingtonia filifera* tree in King Saud University, Riyadh Campus (KSA)

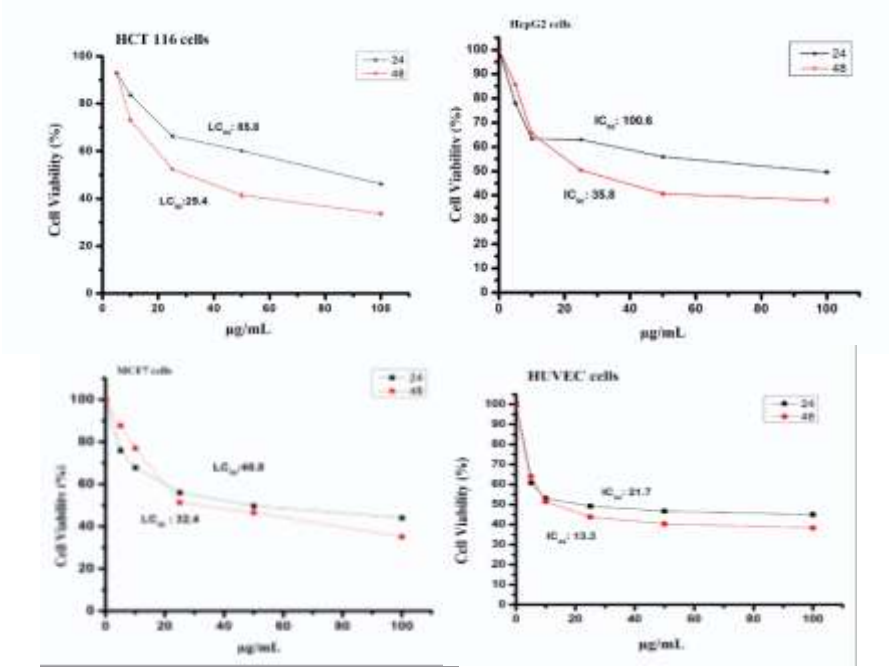

Fig. 2: Cytotoxic effects of ethyl acetate extract of *Washingtonia filifera* fruits (WFET) on HCT 116, HepG2, MCF-7, and one normal cell (HUVEC) after 24 and 48 h of exposure. The vehicle control was 0.01% DMSO. The data present as mean ± SD.

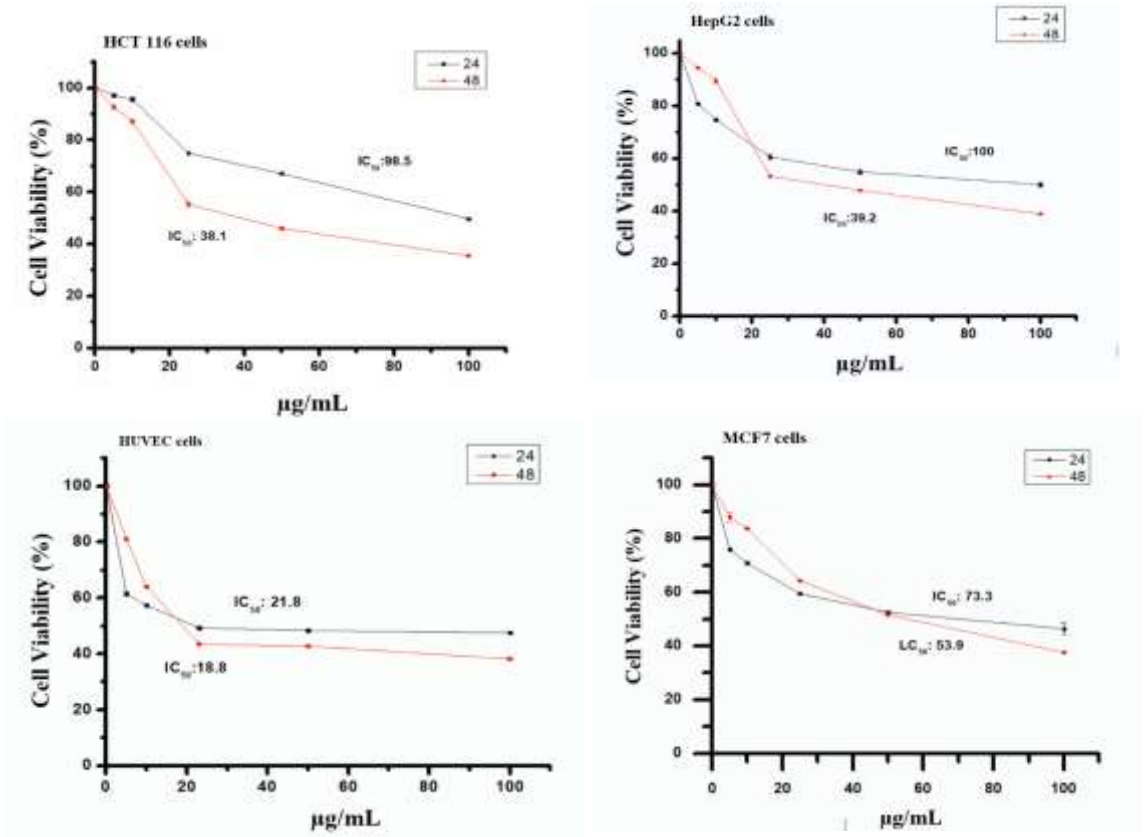

Fig. 3: Cytotoxic effects of methanol extract of *Washingtonia filifera* fruits (WFME) on HCT 116, HepG2, MCF-7, and one normal cell (HUVEC) after 24 and 48 h exposure. The vehicle control was 0.01%DMSO. The data are presented as mean ± SD from three replicas

evaluated were recorded for MCF7 (IC_{50}: 48.8 µg mL^{-1}) cells 24 h after treatment and HCT116 (IC_{50}: 29.4 µg mL^{-1}) 48 h after treatment. Similarly, the methanol extract showed maximum cytotoxicity against HUVECs (IC_{50}: 21.8 µg mL^{-1}) cells, followed by MCF7 (53.9 µg mL^{-1}), HCT116 (98.5 µg mL^{-1}), and HepG2 (100 µg mL^{-1}). WFET extract displayed superior cytotoxicity activity amongst all extracts tested. Therefore, all subsequent assays were investigated on MCF7 cell lines, showing the highest WFET extract sensitivity.

Several plant species of family Arecaceae reportedly induce cytotoxicity in different cancer cell lines. For example, the fruit and bark extract *Hyphaene thebaica* (Arecaceae) had anticancer activities against acute myeloid leukemia (Soare *et al.*, 1997), fibroblast cells (BJ-1), lung carcinoma (A549), and breast adenocarcinoma (MCF-7) (Fayad *et al.*, 2015). Similarly, crude pit extracts of different varieties (Khalas, Abu Maan, Mabroom and Ajwa) of *P. dactylifera* (Arecaceae) have antiproliferative effects on triple-negative breast cancer MDA-MB-231 cells (Khattak *et al.*, 2020; Khan *et al*., 2021). In addition, wild date palm fruits (*Phoenix sylvestris*) were reported for their anticancer activity against hepatocellular carcinoma (Lamia and Mukti, 2021).

Anticancer compounds that can induce apoptosis are effective strategies for cancer treatment (Sawicka *et al*., 2012). Thus, the candidate anticancer compounds should exhibit apoptosis induction in cancer cells (Surh, 2003). In order to assess the role of apoptosis in growth inhibition by WFET and WFME extracts, the morphological changes in MCF7 cells were monitored by phase-contrast and florescent (DAPI and AO/EB staining) microscopy. Many reports have used these assays to assess the induction of cell death via apoptosis (Rajavel *et al.*, 2017; Suganya *et al.*, 2019). Treated MCF7 cells resulted in low cell confluence, membrane blebbing, and floating cells (Fig. 4). DMSO-treated cells (0.1%) were attached to the plates with more than 90% confluence under the same conditions as treated cells. MCF-7 cells incubated with WFET and WFME exhibited

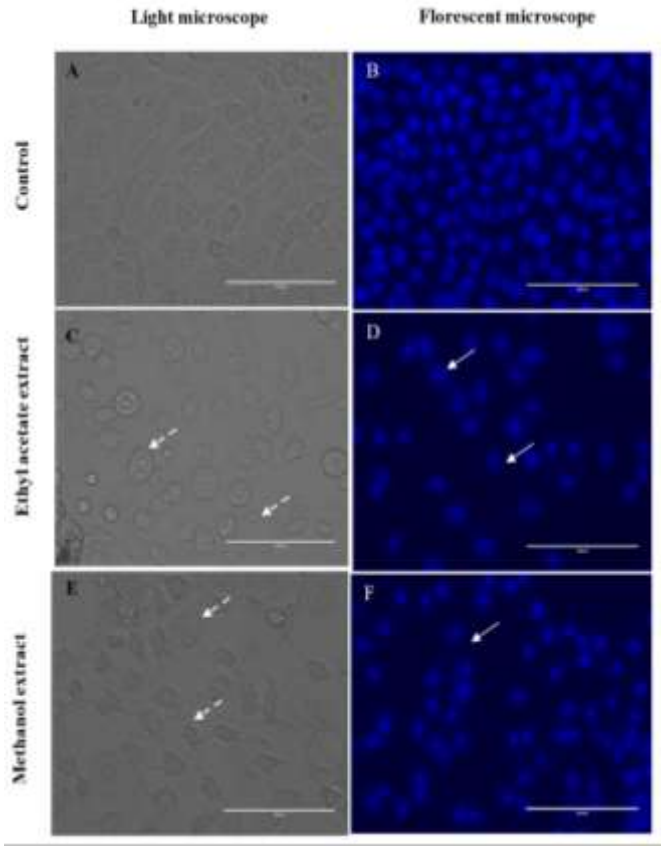

Fig. 4: Morphological changes in MCF7 treated with WFEA and WFME extracts (IC_{50}) of *W. filifera* fruits. Cells were observed by using a phase-contrast microscope. A: control, C and E: cells treated with WFEA and WFME extract, respectively; B: control, D, and F fluorescent micrographs of DAPI-stained MCF-7 cells post-treatment with WFEA (D) and WFME (F) extracts, respectively.

cytoplasmic and nuclear shrinkage, apoptotic body formation, and chromatin condensation (Fig. 4). These events represent the classical features of apoptosis (Elmore, 2007). However, in control, the stained nuclei were round and evenly stained with DAPI. The results of AO/EB staining of MCF7 cells treated with WFET and WFME extracts are shown in Fig. 5. AO is a cell-permeable dye and stains nuclear DNA in both live and dead cells, whereas EB is a dye that only stains nuclear DNA in cells that have lost the integrity of its membrane (Ahmad *et al.*, 2007). After AO/EB staining, the viable cells evenly stained green. Early apoptotic cells stained greenish-yellow or displayed green fragments, late apoptotic cells stained orange or displayed orange fragments, and necrotic cells showed orange to red fluorescing nuclei with no indication of chromatin fragmentation. In MCF7 cells, the treatment with WFET and WFME (IC_{50}) extracts showed live cells with initial stages of apoptotic nuclei appearing as indicated by green chromatin, which was highly condensed or fragmented with some cells undergoing cell death. Both WFET and WFME extracts effectively induced cell death in MCF7 cells, and the mode of cell death was apoptosis, as indicated by highly fragmented and condensed DNA.

The ROS level in treated cells was assessed by using DCFH-DA stain using fluorescence microscopy. In present study, both WFME and WFET extracts showed increased intracellular ROS with increasing concentrations of the extracts using MCF7 cells (Fig. 6). The control of intracellular ROS levels is critical in preserving cellular homeostasis, and thus, different ROS levels can result in diverse cellular responses.

ROS act as signaling molecules (at low level), can induce cell damage/ death at high levels (Redza-Dutordoir and Averill-Bates, 2016) and plays a critical role in apoptosis (Chu *et al*., 2017). This role is a double-edged sword because ROS can induce cell transformation through mutations during DNA replication. ROS plays a vital role in the oxidation of macromolecules and inducing signaling pathways, cell cycle arrest, oxidative stress, aging, mtDNA mutations, and apoptosis (Kromer *et al.*, 2007; Juan *et al*., 2008; Moon *et al*., 2010; Chu *et al*., 2017). WFET and WFME enhanced oxidative stress in MCF-7 cells (Fig. 6). Interestingly, the increase in ROS generation reportedly is associated with cell growth inhibition, morphological changes, DNA fragmentation. The induction of oxidative stress by WFET and WFME may contribute to the apoptosis of MCF-7 cells. It has also been reported that the treatment of MDA-MB-231 (Marvibaigi *et al*., 2016) and MCF-6 cells (Al-Oqail, 2021) with *Scurrula ferruginea* and *Krameria lappacea* extracts, respectively, produced excessive ROS, leading to oxidative and apoptotic cell death.

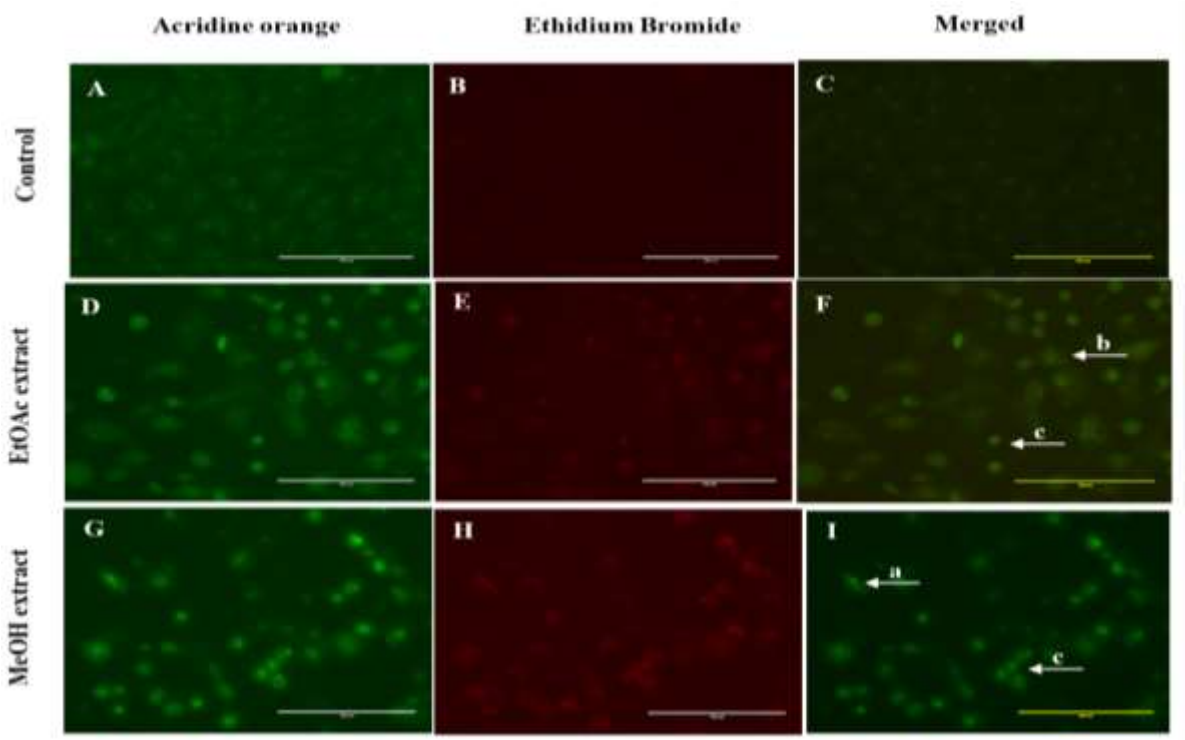

Fig. 5: AO/EtBr-stained images of MCF7 cells treated with IC_{50} of WFEA (D, E and F) and WFME (G, H, and I) extracts. a, b and c represent control cells treated with 0.01% DMSO were a) fragmented nucleus, b) membrane blebbing, c) apoptotic cells

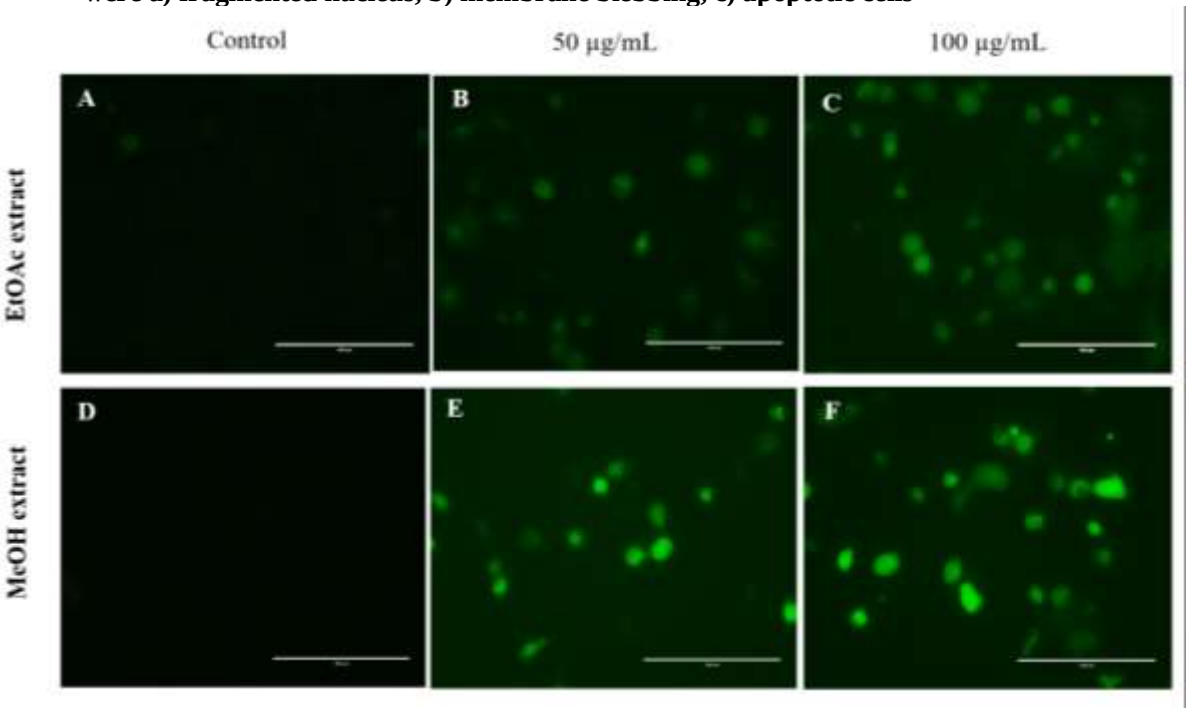

Fig. 6: Intracellular ROS level detected by fluorescence microscope using DCFH-DA. The cells were treated with IC_{50} (B) and $2IC_{50}$ (C) of WFEA. Similarly, cells were treated with IC_{50} (E) and $2IC_{50}$ (F) of WFME extracts. A and D were the controls incubated with DMSO (0.01%). All cells were incubated with 10 µM DCFH-DA. After 30 min of incubation, the MCF-7 cells were washed and examined by fluorescence microscope (40X).

The extracts showed no selective toxicity against endothelial non- tumor cells (HUVEC); however, anticancer drugs are known to induce toxicity to normal cells. The future challenge will be to discover new anticancer drugs that specifically induce apoptosis of cancer cells but not that of normal cells, or the discovery of compounds able to protect the normal cells against anticancer drugs but with no protective effect on cancer cells to keep the efficacy of treatment intact (Mailloux *et al*., 2001).

Conclusion: This study demonstrated the cytotoxic potential of various fruit extracts *W. filifera viz*. WFHE, WFCH, WFET, and WFME against human carcinoma cell lines. The results indicated that the WFET and WFME caused apoptotic potential as examined by MTT assay and morphological analysis. Moreover, results revealed that WFET and WFME induced MCF-7 cell death via the elevated ROS level. However, further purification and modification of the active compounds are required to enhance the activity.

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Conflict of interest: The authors have declared that they have no conflicts of interest.

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