Anticancer Potential of Hesperidin against HEP-2 Laryngeal Carcinoma Cell Line in Comparison to Doxorubicin

Sara Abd El Wahed
British University in Egypt

Nadia Fathy Hassabou (nadiahassabou.dent@o6u.edu.eg)
October 6 University

Mai Abdelhalim Hamouda
Ahram Canadian University

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Abstract

Background

Doxorubicin (DOX) is widely used in therapy of many carcinoma types. Unfortunately, it is not sufficiently effective in many cases and increasing the dosage of it is limited due to systemic toxicity. An important strategy to minimize the toxic effects of the above cited drug is to use co-adjuvant. A citrus flavonoid hesperidin (Hesp) has emerged as promising anticancer natural product and proved to be potent antioxidant agent. It inhibits cancer cell proliferation by inducing apoptosis and cell cycle arrest. In this context, the objective of this study was to investigate anticarcinogenic effects of Hesp in comparison with DOX against HEp-2 laryngeal carcinoma cell line.

Methods

Five groups of HEp-2 cell line were included, two groups were subjected to Hesp and the other two groups were subjected to DOX, which was used as a reference drug, in addition to a control untreated group. Expression of Bcl-2 and p53 genes was evaluated. Also, the cell cycle arrest and apoptotic effects were assessed.

Results

Hesp exerted anti-proliferative effects against HEp-2 cells which increase in time dependent manner. Gene profile analysis revealed highly significant decrease of anti-apoptotic Bcl-2 and highly significant increase of tumour suppressor gene P53 (p < 0.01 and p < 0.0001, respectively) for both tested drugs.

Conclusion

Hesp proved potential anticancer effects with reducing cancer cell viability in HEp-2 cell line via cell cycle arrest and apoptotic mechanism. It could be used as a pro-drug or co-adjuvant in treatment of oral cancer.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent malignancy that arise from the mucosal surface epithelium in the oral cavity, pharynx and larynx. In 2018, around 450,000 deaths and 890,000 new cases of HNSCCs were reported worldwide. According to Global Cancer Observatory (GLOBOCAN), the incidence of HNSCCs is expected to rise by 30 % by 2030, reaching 1.08 million new cases per year [1-3]. Laryngeal squamous cell carcinoma (LSCC) is one of the commonest prevalent entities of HNSCC (25–30 %) of all cases; as a result, it is the most common cancer site of the aero-digestive tract [4].
Despite advances in cancer therapeutic interventions, only 40 – 50 % of patients with HNSCC are treated at advanced stages with poor overall survival and significant mortality rates, owing to metastasis to distant organs and limited therapeutic strategies that traditionally include chemotherapy, radiotherapy and surgery. Chemotherapy effectiveness is jeopardised owing to acquired and inherent tumour cell resistance to chemotherapeutic agents. Although chemotherapeutic drugs have been widely utilised to treat a wide spectrum of human malignancies, their effectiveness and side effects remain a problematic issue. Doxorubicin (DOX) is one of these drugs, which is extensively used in cancer treatment but has limited utility in the treatment of oral cancer due to tumour cell resistance. As a result, several therapeutic approaches have been investigated, with naturally obtained substances with recognised anticarcinogenic activities being the most promising [5, 6].

Many epidemiological researches conducted in recent years have suggested that diets high in vegetables and fruits exert anticarcinogenic and apoptosis-inducing capabilities. This has been related to the existence of high quantities of phytochemicals, which can be an effective method for cancer prevention and therapy [7]. Antioxidant, anti-inflammatory, anti-atherosclerotic, antitumor and anti-metastatic effects of these natural phytochemicals may be attributed to the presence of flavonoids compounds [8].

Flavonoids may be found in abundance in fruits and vegetables as well as grains. The majority of Citrus species include significant amounts of flavonoids, carotenoids and limonoids in the form of aglycone or glycoside. The aglycone, narirutin and hesperitin are considered the most significant flavanones [9].

Hesperidin (Hesp) is an active bioflavonoid glycoside of hesperitin that may be found in orange peel and other citrus species that frequently utilized in Chinese herbal medicine [10]. Hesp has been shown to have a powerful anticancer impact on a variety of cancer cell line, including bladder cancer, prostate cancer, breast cancer and hepatocellular carcinoma [11-13]. Hesp is thought to have anticancer properties through inducing apoptosis and preventing invasion and metastasis. It is extremely notable that Hesp has been demonstrated to be non-toxic to normal cells [14, 15]. Its involvement in preventing malignant transformation and progression has been reported in several preclinical investigations, where it acts via several cellular signalling pathways. Indeed, Hesp could influence a wide range of molecular targets involved in tumour cell division, survival and apoptotic mechanisms [16].

Cancer is not totally curable, despite extensive treatment protocols and regimens for cancer patients ranging from surgery, chemotherapy to immunotherapy, and the standard treatment regime is linked with short- and long-term side consequences. In recent years, there has been a lot of investigation into the use of bioflavonoids, such as Hesp in cancer therapy due to their possible antioxidant properties. Hesp's powerful antioxidant activity and possible involvement as an anticancer agent have been highlighted in recent literature from several research groups [16, 17].

Up to date, there is limited evidence in literatures addressing the possible anticarcinogenic effects of Hesp in HNSCC, which has not been thoroughly investigated in LSCC. Therefore, this study was designed to examine the anticancer effects of Hesp in LSCC cell line and to compare these effects with those of DOX, which is the most widely used chemotherapeutic drug.
Materials And Methods

Chemicals and Drugs

ADRICIN® 25 mL vial (Active ingredients: DOX HCl 2 mg/mL) was purchased from EIMC United Pharmaceuticals Company (Cairo, Egypt), the drug was protected from light and stored at 4°C. Hesp powder (Hesperetin 7-rhamnoglucoside, 3’,5,7-Trihydroxy 4’-methoxyflavanone 7-rutinoside, Hesperitin-7-rutinoside) with purity ≥ 80%. The powder was dissolved in dimethyl sulphoxide (DMSO) to get a stock solution of 10 mg/mL. Both Hesp powder and DMSO were purchased from Sigma Aldrich (USA).

Cell Culture

Human laryngeal carcinoma (HEp-2) cell line were obtained from American Type Culture Collection (ATCC), all procedures were carried out in the Tissue Culture Laboratory of Scientific Research Center. The study protocol was approved by Research Ethics Committee at Faculty of Dentistry and conducted in accordance with the principles of the declaration of Helsinki. HEp-2 cell line was divided into 5 groups to be examined at time interval of 24 and 48 hrs, two groups were subjected to Hesp and the other two groups were subjected to DOX, which was used as a reference drug, in addition to a control group not exposed to Hesp or DOX. Cells were cultured using DMEM (Invitrogen/Life Technologies).

Cells were routinely cultured in RPMI 1640 medium (Gibico, USA) and supplemented with 10% fetal calf serum (FCS), 2% sodium bicarbonate and 2% streptomycin penicillin in T25 flasks. All of chemicals and reagents were purchased from Sigma and Invitrogen.

Feeding of the culture and changing of media were performed every 2 days. Culture flasks were examined periodically under the inverted phase contrast microscope to assure viability, sterility, and adequacy for cultured cells. When cells reached 70–80% confluence, they were subcultured as follows: The old culture medium was withdrawn, then PBS was applied to the side of the ask opposite to the cells to avoid dislodging them to remove all traces of the serum that would inhibit the action of trypsin.

Trypsin EDTA solution (0.25%) at 4 was added to the flask to cover the whole surface of the monolayer. The cells were continuously examined until they became rounded and started to be dispersed (detach from the surface of the flask) with no shaking of the flask to avoid clumping of the cells. Fresh RPMI 1640 medium (5mL) and 1 % FCS were added to the flask and the cells were aspirated gently by repeated pipetting over surface bearing the monolayer. Finally, the obtained cell suspension was diluted to the proper seeding concentration by adding appropriate volume of cells to a pre-measured volume of medium in the culture flask.

After many times, cells were subcultured in plastic 96 well plates for the viability assay. All cell culture procedures were carried out at 37°C and the cells were incubated in a 5% CO2 incubator with 100% humidity. Hesp and DOX were added on the first day of treatment to HEp-2 cell line and the percentage of viability of LSCC cells, cell cycle and apoptosis analysis were assessed at 2-time intervals (24 and 48 hrs).
Measurement of Cell Viability by MTT – Cytotoxicity Assay

Cells were plated at a concentration of \((2 \times 10^5 \text{ cell/ml})\) in 96-well cell plates and incubated at 37° C for 24 hrs in CO2 incubator to achieve confluence before the micro-culture tetrazolium (MTT) assay. MTT assay is a colorimetric monitoring method that measures cellular metabolic activity as evidence for cell viability.

Viable cells contain NADPH-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan which is an insoluble crystalline product with a deep purple color. Experimental media were removed, and cells were washed in PBS. Hesp and DOX were added separately to cell line with serial dilutions (1mg/ml, 100g/ml, 10g/ml, 1g/ml and 0.1g/ml). Cells were continuously examined under the inverted phase microscope, and then incubated with medium containing 0.5 mg/ml MTT at 37°C for 2–4 hrs. An incubation period of 2 hrs is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity. The medium was aspirated, and the formazan crystals are solubilized using 50 µl per well of DMSO. Plates were incubated in dark room for 30 min at 37°C and absorbance was measured at 570 nm for each well using a microplate reader (BioTek, Flx 800). The darker the solution, the greater the number of viable, metabolically active cells. The results were interpreted, and the cell viability percentage was calculated using the following formula according to previously described method [18]:

\[
\text{Cell viability percentage (\%)} = \frac{\text{Optical density (OD) of treated wells}}{\text{OD of control wells}} \times 100
\]

The half maximal inhibitory concentration (IC50) was estimated for Hesp and DOX using GraphPad Prism Software V.7 (CA, USA).

Expression of Apoptosis-Related Genes

Total RNA was extracted from control, Hesp and DOX exposed cell cultures, using RNeasy Mini Kit (Qiagen, USA) according to manufacturer’s instructions. The concentration of extracted RNA was evaluated using a Beckman dual wavelength spectrophotometer at 260 nm (Beckman Instruments, USA). The expression levels of apoptosis-related genes: p53 (F 5'- CCCCTCCTGGCCCTGCTCTTC-3' & R 5'-GCAGCGCTCACAACCTCCGTCAT-3') and bcl-2 (F 5'-CCTGTGGATGACTGATACCA-3' & R 5'-GAGACGCCAGAGAATCA-3') compared to the house keeping gene \(\beta\)-actin as a control (F 5'-GTGACATCCACACCAGGAGG-3' & R 5'-ACAGGATGTCAAAACTGCCC-3') were determined using real-time PCR (RT-PCR). From each sample 10 ng of the extracted total RNA was used for cDNA synthesis using high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Thermo Fischer Scientific, USA). The obtained cDNA was subsequently amplified using Sybr Green I PCR master kit using Step One apparatus (Applied Biosystems, Thermo Fischer Scientific). The thermal cycling conditions for RT-PCR comprised an initial denaturation step at 95°C for 10 min for enzyme activation, followed by 40 cycles at an appropriate annealing temperature 15 sec. at 95°C, 20 sec. at 55°C and 30 sec. at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of \(\beta\)-actin as housekeeping gene by the \(\Delta\)CT method.

Annexin-V/PI for Flow Cytometry
Quantitative assessment of apoptosis was performed by Fluorescein isothiocyanate (FITC) technique by using ab139418_Propidium Iodide (PI) Flow Cytometry Kit/BD. Cells were cultured overnight (1.5×10^5 cells) in 25 cm^2 cell culture flasks before addition of tested drugs. Cells were then treated with previously calculated IC50 value of Hesp and DOX.

The positive control was prepared by culturing the control HEp-2 cell line in medium containing 200 ml H_2O_2 for 30 min. Cells were collected and fixed for at least one hour at 4 and washed twice in PBS and re-suspended in binding buffer. Cells were collected, washed twice with cold PBS and re-suspended. Cells were transferred to a tube with addition of 5µl of FITC conjugated Annexin-V (Annexin-V FITC) and 5µl of PI were added to cell pellet and mixed well followed by incubation for 15 min at room temperature.

The stained cells were diluted by adding 50 l of RNase to digest RNA and analyzed by the flow cytometer. Analysis was performed by Becton Dicknison Fluorescence-activated cell sorting (BD-FACS Calibur) with data displayed as frequency histograms and dot plot. Apoptotic cells were detected initially by quantification of Annexin-V FITC binding to the expressed phosphatidyl serine (PS) which is released in the outer cell membrane upon induction of apoptosis. In flow cytometry analysis, Annexin-V/ PI staining is based on the ability of the protein Annexin-V to bind to PS. Four different populations of cells were differentiated: cells that were unlabeled(viable cells), those that have bound to Annexin-V FITC only (early apoptotic), those that have been stained with PI (necrotic) and those that have both bound to Annexin-V FITC and been labelled with PI (late apoptotic).

**Cell Cycle Analysis**

Cell cycle distribution analysis was performed using flow cytometry to measure the percentages of DNA content of cells at G1, S and G2/M phases for Hesp and DOX treated HEp-2 cells. The Cycle TEST PLUS DNA Reagent Kit (Biovision, USA) was used to determine the cell cycle phase distributions and the percentage was analyzed using Modfit software (ModFit, Topsham, ME).

**Histopathological Studies of HEp-2 Cells**

Fifty microliters of both Hesp and DOX exposed cells were dispensed on clean slides (3 for each treatment). Slides were air-dried, methanol fixed, rehydrated in descending concentrations of alcohol, and washed in distilled water for 5 min. The prepared slides were subjected to hematoxylin and eosin (H & E) staining protocol for further histopathological examination to evaluate the presence of morphological criteria of apoptosis. Ten microscopic fields of each slide were examined using conventional light microscope with power of magnification (×400).

**Statistical Analysis**

All the collected data were analysed using SPSS (Statistical Package for Social Sciences) 26.0 software (IBM, Chicago). Scores of overall expressed genes were reported as mean values and standard deviation (SD), mean values between studied groups were compared using One-way analysis of variance (ANOVA) and Tukey's post hoc test for pairwise comparison. The difference was considered statistically significant when P value less than 0.05.
Results

MTT Cytotoxicity Assay

The cytotoxic effect of Hesp and DOX on HEp-2 cell line was evaluated by MTT assay and a curve was plotted between viability percentages and drug concentration for the calculated IC50 as shown in Fig. 1 (A – C) with IC50 value (29.9 ug/ml and 1.56 ug/ml, respectively).

Fold Changes in Gene Expression

The gene profile relevant to the cytotoxic and anti-proliferative effects of the drugs was increased in time dependent manner from 24 to 48 hrs of influence and was represented as mean values of expression. Bcl-2 as anti-apoptotic gene showed highly significant fold decrease (p ≤ 0.01). In the meantime, P53 tumour suppressor gene profile revealed a highly significant upregulation (p ≤ 0.0001) when HEp-2 cell line treated with Hesp and DOX compared to control untreated cells (Table 1, Fig. 1D).

Table 1
Mean values of studied genes expression in Hesp and DOX treated HEp-2 cell line and control untreated cells at 24 and 48 hrs.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Gene Profile Fold Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P53</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>Hesp / HEp-2</td>
<td>2.491</td>
</tr>
<tr>
<td>DOX / HEp-2</td>
<td>3.801</td>
</tr>
<tr>
<td>Cont. / HEp-2</td>
<td>1</td>
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</tbody>
</table>

Induced Apoptosis in HEp-2 Cell Line

In the current study, annexin-V FITC/PI assay was employed to verify the cell death induced by Hesp and DOX through investigating apoptotic activity by monitoring PS translocation. Four different populations of cells were distinguished by Annexin-V/ PI staining in our current findings: viable cells (Ann-V⁻/PI⁻), early apoptotic cells (Ann-V⁺/PI⁻), late apoptotic cells (Ann-V⁺/PI⁺) and necrotic cells (Ann-V⁻/PI⁺), (Fig. 2A - E).

The flow cytometry analysis of HEp-2 cell line showed that cell population tended to shift from viable to apoptotic which is time dependent. Through the treatment with Hesp and DOX the percentages of early apoptotic rates were (2.51 and 0.88), respectively after 24 hrs and (1.89 and 1.76) after 48 hrs, demonstrating non-significant increase when compared with the apoptotic rate (0.69) of control untreated HEp-2 cells. Furthermore, highly significant differences (p ≤ 0.001**) in the percentage of late apoptotic rates for DOX treated HEp-2 cells were observed after 24 and 48 hrs of incubation (12.24 and
28.42), respectively as compared to (3.5 and 8.15) of the Hesp treated HEp-2 cells in addition to (0.33) of the untreated control cells, (Fig. 2F).

Cell Cycle Analysis

Cell cycle arrest at G2/M phase was observed post treatment in HEp-2 cells with Hesp and DOX as they were examined by flow cytometry. After 48 hrs of treatment with both drugs, a significant increase (p ≤ 0.001) in the percentages of cells arrested at the G2/M growth phase was observed as compared to the control HEp-2 cells. In addition, cell cycle arrest was occurred in early S phase in DOX-48 hrs/HEp-2 cells (Table 2, Fig. 3A - F).

<table>
<thead>
<tr>
<th>Samples</th>
<th>DNA content</th>
<th>G0-G1%</th>
<th>S%</th>
<th>G2/M%</th>
<th>Pre-G1%</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesp-24hrs/HEp-2</td>
<td></td>
<td>44.38</td>
<td>35.99</td>
<td>19.63</td>
<td>13.27</td>
<td>Cell growth arrest at G2/M</td>
</tr>
<tr>
<td>DOX-24hrs/HEp-2</td>
<td></td>
<td>44.23</td>
<td>49.83</td>
<td>5.94</td>
<td>19.51</td>
<td>Cell growth arrest at G2/M</td>
</tr>
<tr>
<td>DOX-48hrs/HEp-2</td>
<td></td>
<td>41.67</td>
<td>55.61</td>
<td>2.72</td>
<td>41.26</td>
<td>Cell growth arrest at G2/M and S</td>
</tr>
<tr>
<td>Cont./HEp-2</td>
<td></td>
<td>49.04</td>
<td>44.51</td>
<td>6.45</td>
<td>2.72</td>
<td>—</td>
</tr>
</tbody>
</table>

Histopathological Results

Microscopic observation of H & E stained treated cells showed morphological differences in comparison to control HEp-2 that showed almost regular rounded tumor cells, hyperchromatic nuclei and the cellular outline was almost regular without evidence of any folding. Only a few cells among control cells present cellular and nuclear pleomorphism (Fig. 4). Hesp and DOX induced cell death in HEp-2 cell line by apoptosis. Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and collapse of the cell into minute fragments (apoptotic bodies). Our microscopic findings showed increase in the number of apoptotic cells corresponding to the time of incubation for both tested drugs. Furthermore, DOX/HEp-2 cells showed numerous apoptotic bodies, irregular cell membrane of neoplastic and necrotic cell with mixed euchromatin and heterochromatin in comparison to Hesp treated HEp-2 cells (Fig. 5A - H).

Discussion

Cancer research has achieved amazing advances in our basic knowledge and approach to cancer biology and therapy during the previous decade. According to recent findings, apoptosis has a critical role in the
development of therapeutics agents and cancer elimination. The ultimate objective is to encourage the demise of cancer cells without inflicting undue harm to healthy normal cells [19].

One of the most critical variables that causes cancer is oxidative stress, which is a well-studied occurrence that leads to the circumstances that contribute to tumour initiation and progression. DOX is widely used drug in cancer treatment corresponding to its extensive antitumor activity and cytotoxicity that caused by generation of oxidative stress and interfering with the glutathione redox cycling. DOX has the ability to react with nucleic acids and proteins thus it was suggested to target cells which are rapidly dividing, disrupting cell growth, mitotic activity and differentiation [20]. Previous studies reported that DOX therapy causes a rise in H$_2$O$_2$ concentration, which triggers apoptosis by inducing oxidative DNA damage. Furthermore, free radical-mediated oxidative stress, which activates both intrinsic and extrinsic pathways, has been proposed as a possible mechanism driving drug-induced apoptosis [21].

Cancer chemoprevention is now widely recognised as a promising strategy that relies on natural dietary products and synthetic substances to provide not only protection against oxidative reactions, but also important preventive mechanisms such as cell proliferation, apoptosis, and epigenetic process modulation [22]. Using edible phytochemicals is regarded as one of the most essential cancer therapy techniques. Free radical scavengers found in plants include phenolic chemicals, flavonoids, and flavonols, which have antioxidant and anticancer properties. These compounds’ active ingredients can be separated and employed as antioxidants to prevent and cure free radical damage. Hesp is significant citrus bioflavonoid with a diverse variety of pharmacological effects [13, 23]. In present work, we critically review the Hesp against human LSCC with emphasis on its anticancer effects in comparison to widely used DOX.

The results of current work showed cytotoxic and anti-proliferative effects of applied Hesp and DOX on cancer cells achieved from HEp-2 cell line that significantly increase with time and confirmed that treatment with Hesp as a natural dietary product inhibit cancer cell proliferation.

Apoptosis is a genetically programmed process which increases the commencement of tumorgenesis when subjected to inhibition. It relates to the production of two synergistically working genes that encode anti- and pro- apoptotic proteins. The Bcl-2 gene supports cell survival, whereas the Bax gene causes cell death. The mutation of the p53 tumour suppressor gene is one of the most critical events in carcinogenesis. Apoptosis is avoided by cells with a mutated p53 gene. Aside from cell-cycle control, mounting evidence shows that caspase family proteases play a key role in the apoptosis process. Caspases are essential enzymes that govern the apoptosis pathway [24].

In present study, a highly significant upregulation in the expression of p53 tumour suppressor gene was inversely related to a reduction in the expression of Bcl-2 antiapoptotic gene when HEp-2 cell line treated with Hesp and DOX indicating apoptosis and anti-proliferative potential in time dependant manner. These findings were similar to observations reported from previous work which investigated Hesp as additive therapy to DOX in treatment of mice inoculated with solid Ehrlich carcinoma [25]. Also, it was found that
Hesp restores the tumour suppressor genes p53 and p21 level thus inhibit the cell cycle proteins in lung cancer [26].

In addition, the current study proved apoptotic morphological alterations, such as cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation and apoptotic bodies that were seen in the HEp-2 treated cells. These obtained findings were in accordance with earlier studies that were conducted to evaluate antitumor and apoptotic properties of Hesp that loaded on gold nanoparticles in human breast cancer cell line [27].

Current data revealed a significant increase in the percentages of cells that arrested at G2/M growth phase in both Hesp and DOX treated HEp-2 cells. In addition, it was observed that cell cycle arrest was occurred in early S phase in DOX-48 hrs/HEp-2 cells. It was reported that abnormalities in the G2/M arrest checkpoint allow a damaged cell to escape mitosis and undergo apoptosis, enhancing the therapeutic cytotoxic effects [28].

Our findings were in same line with the previous studies that shown anti-cancer effects of Hesp in different malignancies, emphasizing its molecular mechanism of action. Hesp acts as an anti-cancer agent by promoting apoptosis in malignant cells such as liver cancer and bladder cancer cells via NF-B, MAPK, and PI3K/AKT pathways. Moreover, it inhibits the expression of MMP and epithelial-mesenchymal transition related proteins, suppressing cell migration and invasion, as well as being an anti-inflammatory agent [16]. Hesperidin has been reported to induce paraptosis which is a distinct mode of cell death in liver cancer [29]. Natarajan et al. evaluated apoptotic activity of Hesp in human mammary carcinoma cell line, they showed that Hesp significantly triggered the shrinking of cells, vacuolation, formation of plasma membrane blebs and cell detachment. Also, they reported that anti-cancer effect was also indicated by DNA fragmentation, accumulation of p53 and stimulation of caspase 3 proteins [30].

Regarding to our reported observations of Hesp, the study of Wuditwai et al. was in accordance where they revealed that treatment of two types of oral cancer cell line with Hesp resulted in significant reductions of cell viability in both dose and time dependent manners [10]. Furthermore, our results were in harmony with previous studies that indicated Hesp may suppress human Burkitt's lymphoma and cause apoptosis by inhibiting both constitutive and DOX-induced NF-kappaB activation [31]. Citrus Hesp has also been shown to promote apoptosis in human pre-B NALM-6 leukemic cells by activating p53 and blocking NF-kappaB signalling pathways [32]. In addition, in vivo mouse metastasis model research, metastasis of HEp-2 laryngeal cancer cells to the lungs and livers was suppressed in response to Hesp. In vitro assays, it was observed that Hesp significantly inhibit secretion of angiopoietin-1 which is an angiogenic enhancer and increase annexin-V as an apoptotic indicator at relatively low levels which came in the same context of our findings [33].

In a two-stage induced skin carcinogenesis model in Swiss albino mice, the chemopreventive potential of Hesp was investigated with treatment after cancer initiation reducing the tumor incidence in a dose dependent manner. The chemopreventive action of Hesp appears to be attributable to the suppression of
carcinogenesis’ initiation and promotion stages. The majority of chemopreventive drugs in vivo trials showed a substantial decrease in tumour development [34].

**Conclusions**

Chemopreventive activity and prospective usefulness of Hesp as a promising anticancer agent have been proved in reducing cancer cell viability in HEp-2 cell line. Furthermore, it has the potential to minimise therapeutic adverse effects induced by oxidative stress, making it more efficient in combating cancer cells. Therefore, further studies in future using Hesp in conjunction with chemotherapeutic drugs are recommended. Last but not least, this study highlights the need of using nontoxic and cost-effective natural product for cancer chemoprevention and therapy.

**Declarations**

**Competing Interest**

Authors declare no conflict of interest.

**Ethics approval**

The study protocol was approved by Research Ethics Committee at Faculty of Dentistry with approval number (RECO6U/15-2020). The study was conducted in accordance with the principles of the declaration of Helsinki.

**Consent to Participate**

Not applicable: This research does not contain any studies with human or animal subjects, only LSCC cell line (HEp-2) obtained from American Type Culture Collection which complied with all relevant regulations.

**Consent for Publication**

Not applicable

**Availability of Data and Material**

All data generated or analysed during this study are included in this article.

Further enquiries can be directed to the corresponding author.

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Authors’ Contributions

All authors contributed to the study conception and design. SAW and MAH performed material preparation and data collection. Analysis of data identified by flow cytometry and interpretation of morphological investigation of apoptosis performed by NFH. The first draft was written by NFH and all authors commented on previous version of the manuscript. All authors have reviewed and approved the final manuscript.

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Authors acknowledge sincerely the support given by Nawah Scientific Research Center during all procedures of tissue culturing.

References


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**Figures**

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)
Figure 1

Plotted curves between the log concentration of Hesp (A) and DOX (B) versus the viability % of cancer cells. (C) Evaluation of inhibitory concentration (IC50) of tested drugs. (D) Mean values of fold changes of gene expression in all studied group with error bars of standard deviation.

Figure 2

Annexin V/PI double-staining assay by flow cytometry analysis: The Y-axis represents the PI-labeled population, the X-axis represents the labeled Annexin-V FITC positive cells. The lower left portion of the histogram shows HEp-2 viable cells (An-, PI-), the lower right portion (An+, PI) shows early apoptotic cells. The upper right portion (An+, PI+) shows late apoptotic cells. While the upper left portion (An-/PI+) demonstrates the percentage of necrotic cells. A and B: Hesp/HEp-2 treated cells. C and D: DOX/HEp-2 treated cells 24 and 48 hrs, respectively. E: Control HEp-2 cell line. F: Error bars represent SD of measurements performed in relation to percentages of arrested cell populations in treated HEp-2 cell line demonstrating a highly significant increase ($p \leq 0.001^{\alpha}$) in late apoptosis of DOX-48 hrs/HEp-2 as compared to other studied groups.
Figure 3

Flow cytometric analysis for cell cycle distribution in Hesp/HEp-2 (A & B) and DOX /HEp-2 (C & D) at 24 and 48 hrs respectively, as well as control group (E). Representative flow cytometry error bars graph of DNA content for untreated and treated HEp-2 groups (F)
Figure 4

Control untreated HEp-2 showed regular rounded tumor cells with hyperchromatic nuclei and regular cellular outline (yellow arrows), cellular pleomorphism (red arrow) and nuclear pleomorphism (green arrow), (H & E, original magnification x100).
Figure 5

Photomicrograph of treated cells: (A & B) DOX-24 hrs/HEp-2 showing shrunken apoptotic cells with shrunken nuclei (Yellow arrows), irregular cell membranes (Red arrows) and peripheral condensation of chromatin (Green arrows). (C & D) Hesp-24 hrs/HEp-2 showing shrunken apoptotic cells and shrunken nuclei (Yellow arrows), peripheral condensation of chromatin (Green arrows) and nuclear fragmentation (Red arrows). (E & F) DOX-48 hrs/HEp-2 showing shrunken apoptotic cells with shrunken nuclei (Yellow arrows), apoptotic bodies (Green arrows) and necrotic cells with ruptured cell membrane (Red arrows). (G & H) Hesp-48 hrs/HEp-2 showing shrunken apoptotic cells with shrunken nuclei (Yellow arrows), membrane blebbing (Black arrows), nuclear fragmentation (Red arrow) (H & E, original magnification x100)

Supplementary Files

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- Highlights.docx