Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Jul 30; 10(A):1300-1309.
https://doi.org/10.3889/oamjms.2022.10603
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pathology*

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

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Edited by: Sinisa Stojanoski
Citation: El Wahed SA, Hassabou NF, Hamouda MA. Anticancer Potential of Hesperidin against HEp-2 Laryngeal Carcinoma Cell Line in Comparison to Doxorubicin. Open Access Maced J Med Sci. 2022 Jul 30; 10(A):1300-1309. https://doi.org/10.3889/oamjms.2022.10603
Keywords: Hesperidin; Doxorubicin; HEp-2; Apoptosis; Cell cycle arrest
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Received: 03-Jul-2022
Revised: 19-Jul-2022
Accepted: 20-Jul-2022

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Doxorubicin (DOX) is a drug that is frequently used to treat a variety of cancers. Unfortunately, in many situations, it is ineffective, and raising the dosage is restricted 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 suppresses cancer cell replicating by triggering apoptosis and cell cycle arrest.

AIM: The study's goal was to investigate anticarcinogenic effects of Hesp in comparison with DOX against HEp-2 laryngeal carcinoma cell line.

MATERIALS AND 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. Furthermore, the cell cycle arrest and apoptotic induction were assessed.

RESULTS: Hesp exerted anti-proliferative effects against HEp-2 cells which increase in time dependent manner. Gene profile analysis revealed highly statistically significant decrease of anti-apoptotic Bcl-2 expression and highly statistically significant increase of tumor suppressor gene p53 expression ($p < 0.01$ and $p < 0.0001$, respectively) for both tested drugs.

CONCLUSIONS: Hesp proved potential anticancer effects with reducing cancer cell viability in HEp-2 cell line through cell cycle arrest and apoptotic mechanism. It could be used as a prodrug or coadjuvant 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, the incidence of HNSCCs is expected to rise by 30% by 2030, reaching 1.08 million new cases per year [1], [2], [3]. Laryngeal squamous cell carcinoma (LSCC) is one of the commonest prevalent entities of HNSCC (25–30% of all diagnosed cases) as a result, it is the most common cancer site of the aero-digestive tract [4].

Despite advances in cancer therapeutic interventions, about 40–50% of patients with HNSCC are treated at an advanced stage, with a low overall survival rate and high mortality rates, due to metastasis to distant organs and limited therapeutic strategies that traditionally include chemotherapy, radiotherapy, and surgery [5].

Chemotherapy effectiveness is jeopardized attributable to acquired and inherent chemotherapeutic resistance in tumor cells. Even though chemotherapy medications have been used to treat a wide range of human cancers, their efficacy and adverse 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 tumor cell resistance. As a result, several therapeutic approaches have been investigated, with naturally obtained substances with recognized 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 lines, including breast cancer, prostate cancer, bladder cancer, and hepatocellular carcinoma [11], [12], [13].

Due to the potential antioxidant effects of bioflavonoids such as Hesp, there has been a lot of research on their usage in cancer therapy lately. Recent literature from many research groups has emphasized Hesp's potent antioxidant action and its role as an anticancer drug [14], [15].

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-hazardous to normal cells [16], [17].

Its involvement in preventing transformation and development of cancer has been reported in several preclinical investigations, where it acts through several cellular signaling pathways. Hesp could influence a wide range of molecular targets involved in tumor cell division, survival, and apoptotic mechanisms [14].

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. As a result, the purpose of this research was to explore the anticancer potential 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 obtained 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%. A stock solution of 10 mg/mL was made by dissolving the powder in dimethyl sulfoxide (DMSO). Both Hesp powder and DMSO were purchased from Sigma Aldrich (USA).

Cell culture

Human laryngeal carcinoma (HEp-2) cell line was derived from American Type Culture Collection (ATCC, USA), all procedures were carried out in the Tissue Culture Laboratory of Scientific Research Center. The Research Ethics Committee at the Faculty of Dentistry gave its approval to the study protocol and carried out in conformity with the principles of the Helsinki Declaration.

HEp-2 cell line was divided into five groups to be examined at time interval of 24 and 48 h, 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 routinely cultured using DMEM (Invitrogen/Life Technologies, USA) and 10% fetal calf serum (FCS), 2% sodium bicarbonate, and 2% streptomycin penicillin were added in T25 flasks. All of reagents and chemicals were purchased from Invitrogen and Sigma.

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 added to the side of the flask opposite the cells to eliminate all residues of the serum that might limit the activity of trypsin to avoid dislodging the cells.

Trypsin EDTA solution (0.25%) 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 5 mL RPMI 1640 medium (Sigma Aldrich, USA) and 10% FCS were added to the flask and the cells were aspirated gently by pipetting across the monolayer-bearing surface many times. Finally, the obtained cell suspension was diluted to the proper seeding concentration in the culture flask by adding an adequate number of cells to a pre-measured volume of medium.

After many times, cells were subcultured in plastic 96 well plates for the viability assay. All cell culture procedures were completed at 37°C and the cells were incubated in a 5% CO_2 incubator with 100% humidity. Hesp and DOX were appended in the beginning of therapy 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 h).

Measurement of cell viability by microculture tetrazolium (MTT) – cytotoxicity assay

In 96-well cell plates, cells were plated at a concentration of (2×10^5 cell/ml) and incubated at 37°C

for 24 h in CO_2 incubator to achieve confluence before the MTT assay. MTT assay is a colorimetric monitoring method that determine cellular metabolic activity as evidence for cell viability.

The MTT reagent is converted to formazan, an insoluble crystalline substances with a deep purple hue, by NADPH-dependent oxidoreductase enzymes found in viable cells. Experimental media were removed, and cells were washed in PBS. Hesp and DOX were added separately to cell line with serial dilutions (1 mg/ml, 100 μg/ml, 10 μg/ml, 1 g/ml and 0.1 μg/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 h. A 2-h incubation time is usually sufficient, although it can be extended for cells with low densities or reduced metabolic activity. The medium was aspirated, and the formazan crystals are dissolved in 50 μl of DMSO each well. Plates were incubated in dark room for 30 min at 37°C and absorbance was measured at 570 nm for each well using FLx 800 Fluorescence Microplate Reader (Biotek, USA). The darker the solution, the greater the number of viable, metabolically active cells. The results were interpreted, and the following formula was used to obtain the cell viability percentage according to previously described method [18]: cell viability percentage (%) = Optical density (OD) of treated wells × 100/OD of control wells. The half maximal inhibitory concentration (IC_{50}) 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) as directed by the manufacturer. A Beckman dual wavelength spectrophotometer set to 260 nm was used to determine the concentration of extracted RNA. (Beckman Instruments, USA). The expression levels of apoptosis-related genes: p53 (F 5'- CCCCTCCTGGCCCCTGTCATCTTC-3' and R 5'-GCAGCGCCTCACAACCTCCGTCAT-3') and Bcl-2 (F 5'-CCTGTG GAT GAC TGA GTA CC-3'and R 5'-GAGACA GCC AGG AGA AAT CA-3') compared to the control housekeeping gene β-actin (F 5'-GTGACATCCACACCCAGAGG-3' and R 5'-ACAGGATGTCAAAACTGCCC-3') were determined using real-time PCR (RT-PCR). From each sample, 10 μg of total RNA was isolated and utilized for cDNA synthesis with a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Thermo Fischer Scientific, USA). The acquired cDNA was then amplified using the Sybr Green I PCR master kit and the Step 1 equipment (Applied Biosystems, Thermo Fischer Scientific, USA). The thermal cycling conditions for RT-PCR included a first denaturation stage at 95°C for 10 min for enzyme activation, followed by 40 cycles at an appropriate annealing temperature 15 s at 95°C, 20 s at 55°C and 30 s at 72°C for the amplification step. The changes in each target gene's expression were standardized against the mean critical threshold (CT) values of β-actin as housekeeping gene by the 2-ΔΔCT method.

Annexin-V/PI for flow cytometry

Apoptosis was analyzed quantitatively by Fluorescein isothiocyanate (FITC) technique by using ab139418_ Flow Cytometry Kit/BD Propidium Iodide (PI). Before adding the tested medicines, cells (1.5×10^5) were grown overnight in cell culture flasks of 25 cm^2. Cells were subsequently treated with Hesp and DOX IC_{50} values that had previously been determined. The positive control was made by incubating the control HEp-2 cell line for 30 min in media containing 200 ml H_2O_2. At 4°C, cells were collected and settled for at least 1 h then rinsed twice in PBS and resuspended in binding buffer. Cells were collected and resuspended after being washed twice with cold PBS then poured into a tube with addition of 5 μl of FITC conjugated Annexin-V (Annexin-V FITC) and 5 μl of PI were added to cell pellet and mixed well then incubated at room temperature for 15 min.

The stained cells were diluted by adding 50 μl of RNase to digest RNA and analyzed by the flow cytometer. Analysis was performed by BD FACSCalibur™ Flow Cytometer (BD Biosciences, USA) 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 when apoptosis is induced. The basis of Annexin-V/PI staining is the capacity of the protein to adhere to PS in flow cytometry analysis. Unlabeled cells (viable cells), cells that have just bounded to Annexin-V FITC (early apoptotic cells), cells that have been stained with PI (necrotic cells), and cells that have both bound to Annexin-V FITC, and been labeled with PI (late apoptotic cells) were all differentiated as cell populations.

Cell cycle analysis

Flow cytometry was used to analyze cell cycle dispersion by measuring the percentages of DNA content of cells at G1, S and G2/M phases for Hesp, and DOX treated HEp-2 cells. The cell cycle phase distributions were determined using the Cycle TEST PLUS DNA Reagent Kit (Biovision, USA) and the percentage was analyzed using Modfit software (ModFit, Verity Software House, USA).

Histopathological examination of HEp-2 cells

Clean slides were used to distribute the 50 μl of both Hesp and DOX exposed cells (three for each

drug). Air-dried slides were methanol fixed, rehydrated in declining alcohol concentrations, and washed in distilled water for 5 min. The prepared slides were subjected to hematoxylin and eosin (H&E) staining protocol for further histopathological investigation to determine the existence of morphological apoptotic criteria. Ten microscopic fields of each slide were examined using conventional light microscope with power of magnification (×400) while pictures were captured with original power of magnification (×100).

Statistical analysis

All the collected data were analyzed using SPSS (Statistical Package for the Social Sciences) 26.0 software (IBM, Chicago). The mean values and standard deviation (SD) of total expressed genes were reported, and mean values between tested groups were compared using One-way analysis of variance and Tukey's *post hoc* test for pair-wise comparison. The difference was considered statistically significant when $p < 0.05$.

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 IC_{50} as shown in (Figure 1a-c) with IC_{50} value (29.9 μg/ml and 1.56 μg/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 h of influence and was represented as mean values of expression. Bcl-2 as anti-apoptotic gene showed highly statistically significant fold decrease ($p < 0.01$*). In the meantime, p53 tumor suppressor gene profile revealed an extremely statistically significant upregulation ($p < 0.0001$***) when HEp-2 cell line treated with Hesp and DOX after 48 h compared to control untreated cells (Table 1 and Figure 1d).

Table 1: Mean values of analyzed genes expression in Hesp and DOX treated HEp-2 cell line and control untreated cells at 24 and 48 h

Samples	Gene Profile Fold Changes			
	p53		Bcl2	
	24 h	48 h	24 h	48 h
Hesp/HEp-2	2.491	4.045	0.671	0.445
DOX/HEp-2	3.801	7.207	0.492	0.168
Cont./HEp-2	1	1	1	1

Induced apoptosis in HEp-2 cell line

In the present study, annexin-V FITC/PI assay was employed to verify the cell death induced by Hesp

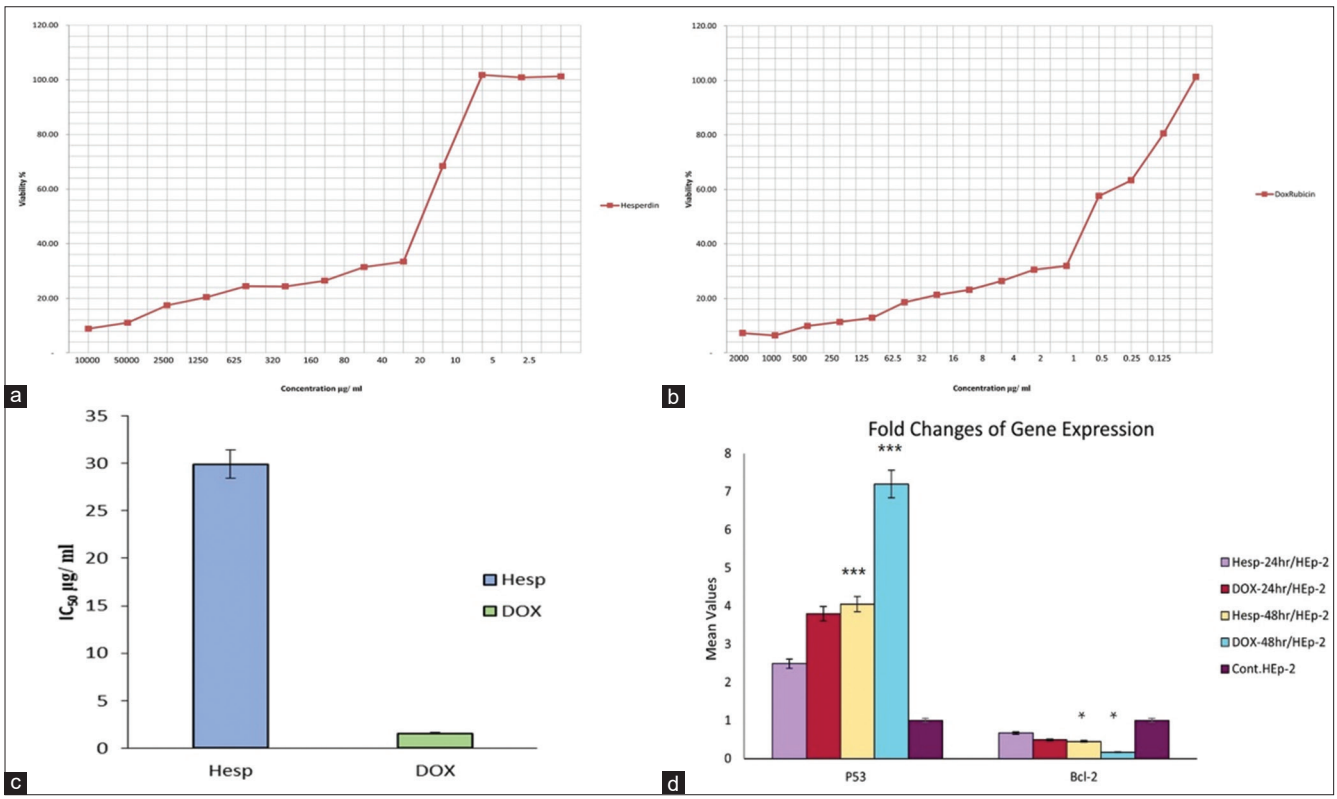

Figure 1: Plotted curves between the log concentration of Hesp (a) and DOX (b) versus the viability % of cancer cells. (c) Evaluation of inhibitory concentration (IC_{50}) of tested drugs. (d) Mean values of gene profile fold changes in all studied groups with error bars of standard deviation

and DOX through investigating apoptotic activity by screening 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$^+$), (Figure 2a-e).

The HEp-2 cell line's flow cytometry research revealed that the population of cells tended to transition from viable to apoptotic over time. Through the treatment with Hesp and DOX, the percentages of early apoptotic rates were (2.51 and 0.88), respectively, after 24 h and (1.89 and 1.76) after 48 h, proving statistically non-significant increase when compared with the apoptotic rate (0.69) of control untreated HEp-2 cells. In addition, extremely statistically significant differences ($p \leq 0.001$**) in late apoptotic rates percentages for DOX treated HEp-2 cells were noticed after 24 and 48 h 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 (Figure 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 h of treatment, a considerable rise in the percentages of arrested cells in the G2/M growth phase ($p < 0.001$**) was noted in Hesp treated group as compared to the control HEp-2 cells. In addition, cell cycle arrest was occurred in early S phase in DOX-48 h/HEp-2 cells (Table 2 and Figure 3a-f).

Table 2: Percentage of DNA content in control and treated HEp-2 cell lines during phases of cell cycle

Samples	DNA content				
	G0-G1%	S%	G2/M%	Pre-G1%	Comment
Hesp-24 h/HEp-2	44.38	35.99	19.63	13.27	Cell growth arrest at G2/M
DOX-24 h/HEp-2	44.23	49.83	5.94	19.51	Cell growth arrest at G2/M
Hesp-48 h/HEp-2	39.92	35.57	24.51	26.28	Cell growth arrest at G2/M
DOX-48 h/HEp-2	41.67	55.61	2.72	41.26	Cell growth arrest at G2/M and S
Cont./HEp-2	49.04	44.51	6.45	2.72	----------

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 were fairly regular, with no signs of folding. Among control cells, only a few cells present cellular and nuclear pleomorphism (Figure 4). Hesp and DOX induced cell death in HEp-2 cell line by apoptosis. Morphologically, DNA fragmentation, chromatin condensation, cell shrinkage, and cell disintegration into minute pieces (apoptotic bodies)

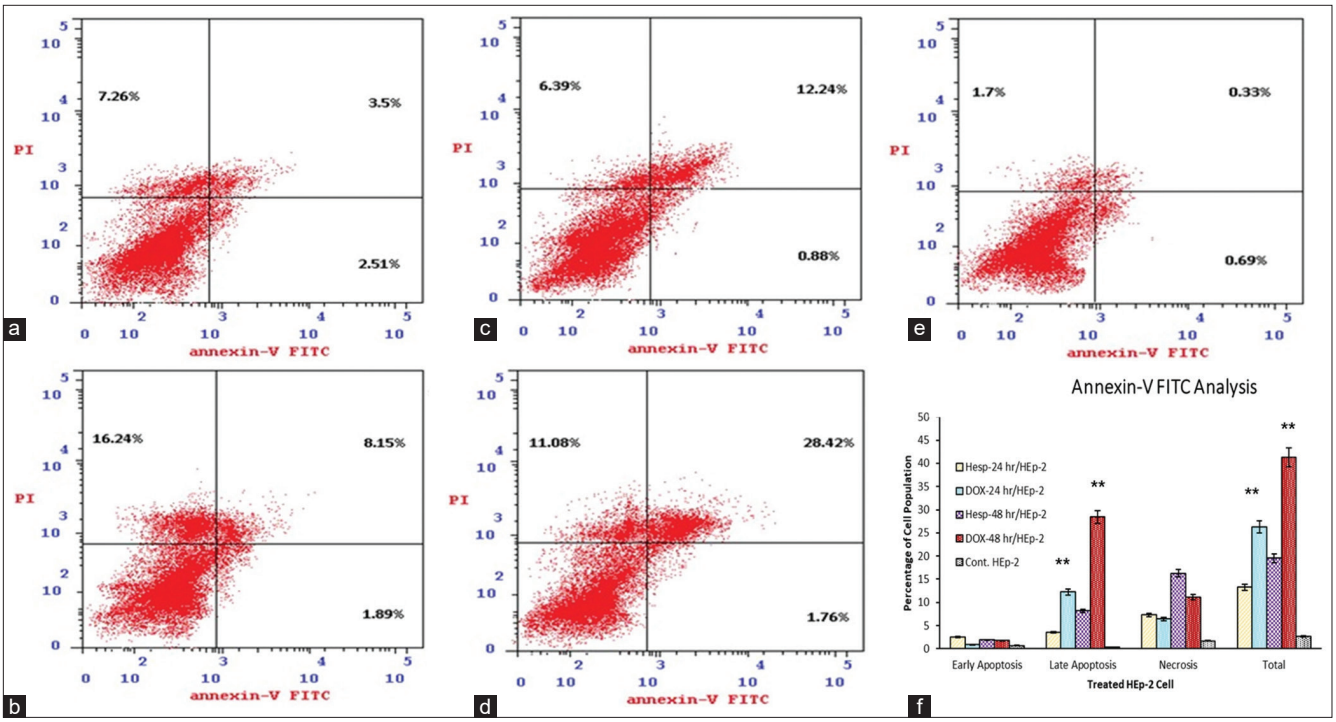

*Figure 2: Annexin V/PI double-staining assay by flow cytometry analysis: The Y-axis represents the PI-labeled population, the X-axis represents the labeled Annexin-V FITC positive cells. The lower left portion of the histogram shows HEp-2 viable cells (An-, PI-), the lower right portion (An+, PI) shows 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 h, 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 extremely significant increase ($p \leq 0.001$**) in late apoptosis of DOX-48 h/HEp-2 as compared to other studied groups*

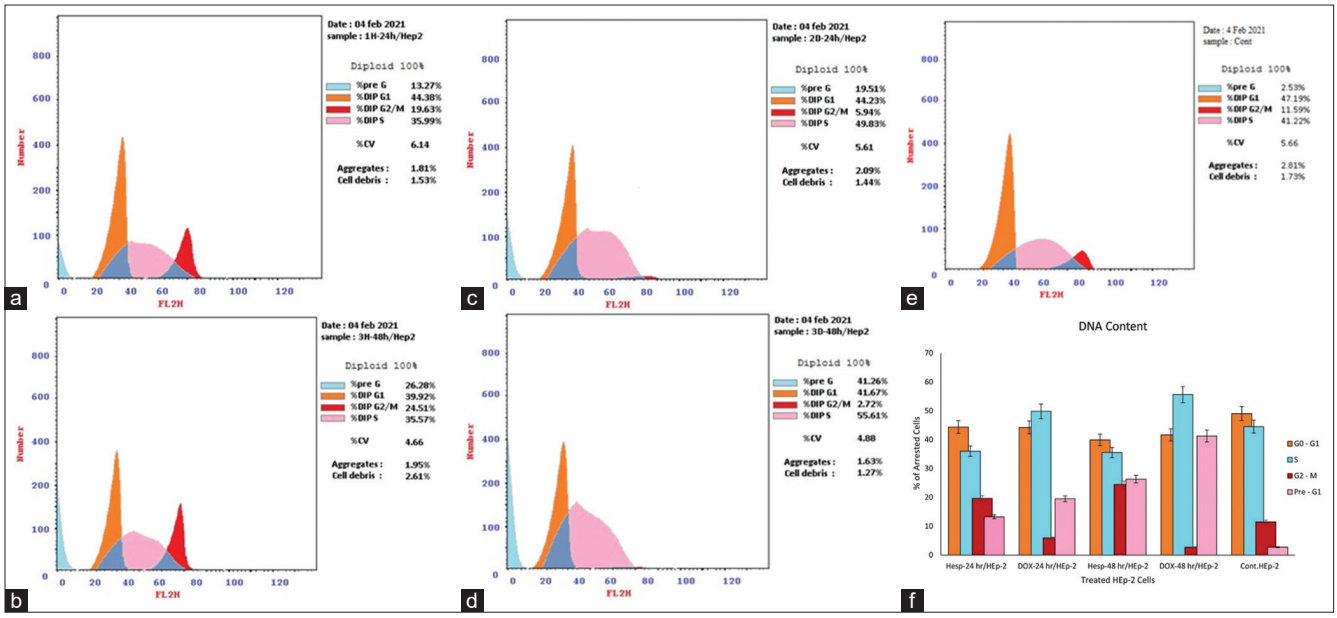

Figure 3: Flow cytometric analysis for cell cycle distribution in Hesp/HEp-2 (a and b) and DOX /HEp-2 (c and d) at 24 and 48 h respectively, as well as control group (e). Representative flow cytometry error bars graph of DNA content for untreated and treated HEp-2 groups (f)

are all features of apoptosis. Our microscopic findings revealed increase in the number of apoptotic cells as the incubation period for both medications increased. Furthermore, DOX/HEp-2 cells showed more apoptotic bodies, uneven cell membrane of cancer cells with mixed heterochromatin, and euchromatin in comparison to Hesp treated HEp-2 cells. It was observed that total number of necrotic cells in Hesp treated cells was much higher than that of DOX treated cell (Figure 5a-h).

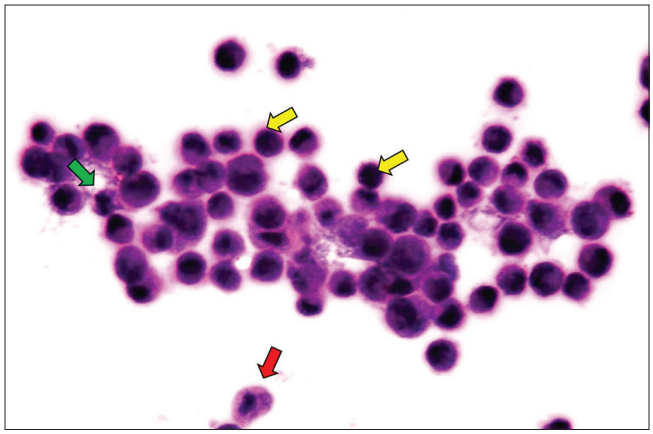

Figure 4: Control untreated HEp-2 showed regular rounded tumor cells with hyperchromatic nuclei and regular cellular outline (yellow arrows), cellular pleomorphism (red arrow) and nuclear pleomorphism (green arrow), (H&E, original magnification ×100)

Discussion

Cancer research has achieved amazing advances in our fundamental understanding and new perspective on cancer biology and therapy during the previous decade. According to recent findings, apoptosis has a significant influence 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].

DOX has the ability to react with nucleic acids and proteins thus it was suggested to target the rapidly dividing cells, interrupting mitotic activity, cell growth, and differentiation [20].

Cancer chemoprevention is now widely recognized as an auspicious strategy that relies on natural and synthetic dietary ingredients to provide not only protection from oxidative reactions, but also crucial preventative processes such as apoptosis, cell proliferation, and epigenetic process modification [21]. 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. Hesp is significant citrus bioflavonoid with a diverse variety of pharmacological effects [13], [22].

Apoptosis is a biological process that is genetically programmed and increases the commencement of tumor genesis when subjugated to inhibition. It is regulated through the synergistic action of opposing groups of genes that encode pro- and anti-apoptotic proteins. The Bcl-2 family composed of three groups according to structure and function. Bcl-2 gene supports cell survival through inhibition of apoptosis, whereas the Bax gene causes cell death by promoting apoptosis. In addition, BH-3 is a regulatory protein which preserved as a domain that inhibits anti-apoptotic

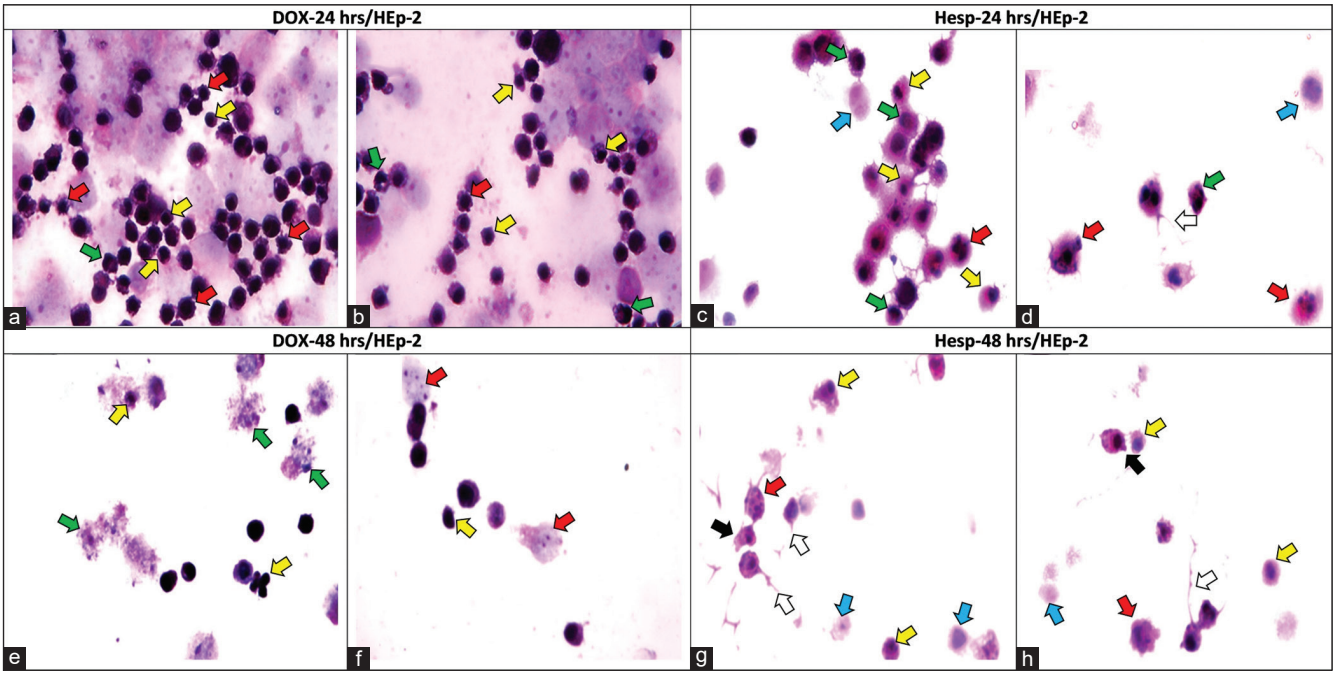

Figure 5: Photomicrograph of treated cells: (a and b) DOX-24 h/HEp-2 showing apoptotic cells with shrunken nuclei (Yellow arrows), uneven irregular cell membranes (Red arrows) and condensation of chromatin at periphery (Green arrows). (c and d) Hesp-24 h/HEp-2 showing shrunken apoptotic cells with shrunken nuclei (Yellow arrows), peripheral chromatin condensation (Green arrows), necrotic cells (Blue arrows), echinoid spikes (White arrows) and nuclear fragmentation (Red arrows). (e and f) DOX-48 h/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 and h) Hesp-48 h/HEp-2 showing shrunken apoptotic cells with shrunken nuclei (Yellow arrows), membrane blebbing (Black arrows), necrotic cells (Blue arrows), echinoid spikes (White arrows) and nuclear fragmentation (Red arrow) (H&E, original magnification ×100)

proteins and activates pro-apoptotic proteins. The mutation of the p53 tumor suppressor gene is one of the most critical events in carcinogenesis. Apoptosis is avoided by cells with a mutated p53 gene [23].

In the 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 present study revealed anti-proliferative and cytotoxic effects of applied Hesp and DOX on cancer cells obtained from HEp-2 cell line that dramatically increased with time confirming that treatment with Hesp as a natural dietary product inhibit cancer cell proliferation.

In the present work, a highly significant upregulation in the expression of p53 tumor 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 dependent manner. These findings were similar to observations reported from the previous work which investigated Hesp as adjunct therapy to DOX in treatment of mice with solid Ehrlich carcinoma implantation [24]. Furthermore, it was found that Hesp restores the tumor suppressor gene p53 level thus inhibit the cell cycle proteins in lung cancer [25].

The present 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 h/HEp-2 cells. It was reported that abnormalities in the G2/M permit a damaged cell to escape mitosis and enter apoptosis, enhancing the therapeutic cytotoxic consequences [26].

Our findings were in same line with the previous studies that shown anti-cancer effects of Hesp in many cancers, with focus on its molecular mechanism of action. Hesp promotes apoptosis in malignant cells such as bladder and liver cancer cells via the NF-κB, MAPK, and PI3K/AKT pathways, acting as an anti-cancer agent [14], [27].

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 lines (HN6 and HN15) with Hesp resulted in significant reductions of cell viability in both dose and time dependent manners [10]. Furthermore, our results were in consistence with prior research findings that indicated Hesp may suppress Burkitt's lymphoma and cause apoptosis by preventing both constitutive and DOX-induced activation of NF-κB [28]. Citrus Hesp has also been shown to promote apoptosis in human leukemic cells by activating p53 and blocking NF-κB signaling pathways [29]. 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 [30]. *In vitro* assays, it was observed that Hesp significantly increases annexin-V as an apoptotic

indicator at relatively low levels which came in the same context of our findings [30].

Furthermore, the present study proved apoptotic morphological alterations, such as cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, echinoid spikes, necrotic cells, and apoptotic bodies that were seen in the HEp-2 treated cells. In histopathological findings, some necrotic cells were found in Hesp treated cells more than DOX treated cell after 24 and 48 h and this was in accordance to the results of annexin-V FITC/PI assay. These results were consistent with previous research on anticancer and apoptotic characteristics of Hesp that loaded on gold nanoparticles in cell line of breast cancer [31].

Natarajan *et al*. evaluated apoptotic activity of Hesp in human mammary carcinoma cell line, they showed that Hesp significantly triggered the cell detachment, shrinking, vacuolations, and formation of blebs in plasma membrane. Furthermore, they reported that DNA fragmentation and p53 accumulation all suggested an anti-cancer impact [32].

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 tumor development [33].

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 minimize therapeutic adverse effects, increasing its efficiency in combating cancer cells. Therefore, further studies in the future using Hesp in conjunction with chemotherapeutic drugs are recommended. Last but not least, this study highlights the need of using non-toxic and cost-effective natural product for cancer chemoprevention and therapy.

Declarations

Ethics approval and consent to participate

The study protocol was approved by Research Ethics Committee at Faculty of Dentistry, October 6 University with approval number (RECO6U/15-2020). The study was conducted in accordance with the principles of the declaration of Helsinki. Consent to participate is not applicable, this research does not contain any studies with human or animal subjects, only LSCC cell line (HEp-2) obtained from ATCC which complied with all relevant regulations.

Availability of data and material

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Authors' contributions

All authors contributed to the study conception and design. SAW and MAH performed material preparation and data collection. NFH analyzed data identified by flow cytometry and interpretation of morphological investigation of apoptosis. 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.

Acknowledgment

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

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