The in vitro and in vivo antitumor effects of Dracaena cinnabari resin extract on oral cancer


Abstract

Objective: To study the potential for apoptosis induction of Dracaena cinnabari Balf. f methanolic extract (DCBME) on tongue squamous cell carcinoma cell line, H103. We evaluated the chemopreventive activity of DCBME against 4-nitroquinolone-1-oxide (4NQO)-induced tongue carcinogenesis in rat.

Design: Phase contrast microscopy, acridine orange/propidium iodide (AO/PI) analysis of cells under fluorescence microscope, annexin-V flow-cytometry, DNA fragmentation, mitochondrial membrane potential, and caspase 3/7, 8 and 9 assays were performed. In vivo study, the rats were given 4NQO in their drinking water. The tongue was subjected to histopathological study to evaluate the incidence of squamous cell carcinoma (SCC).

Results: DCBME showed cytotoxic effect on H103 cells in a dose- and time-dependent manner. Furthermore, DCBME showed low cytotoxic effect on a normal cell line. In H103 cells, it caused cell morphology changes, S and G2/M-phase cell cycle arrest, significant reduction of cell migration and induced apoptosis through the intrinsic (mitochondrial) pathway. The incidence of SCC was 85.7% in the induced cancer and vehicle groups while in rats treated with DCBME at 100, 500 and 1000 mg/kg was 57.1%, 28.6% and 14.3%, respectively.

Conclusions: (DCBME)-apoptosis induction reported in this work can be exploited as a potential antitumor agent with applications in medicinal treatments of tongue SCC.

Keywords: Dracaena cinnabari Balf. f. Tongue squamous cell carcinoma Cytotoxicity Apoptosis Histopathological

1. Introduction

Squamous cell carcinoma (SCC) is the most frequent malignant tumour of the head and neck region (90%). Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by incidence throughout the world. Roughly 650,000 new cases of HNSCC and 350,000 fatalities are reported annually worldwide (Ferlay et al., 2010). The oral cavity is the world. Roughly 650,000 new cases of HNSCC and 350,000 fatalities are reported annually worldwide (Ferlay et al., 2010). The oral cavity is the world.
key role in the discovery as well as development of new antican-der agents, representing an excellent source of biologically active com-pounds. Most chemotherapeutic medications used in cancer therapy were derived from natural products, or are semisynthetic analogues or synthetic compounds that were synthesized according to natural-pro-duct pharmacophores (Cragg, Grothaus, & Newman, 2009). Treatments with conventional anticaner drugs do not always induce a positive effect (Sato et al., 2013). Inherent and acquired resistance to the drugs and toxic responses of patients to these medicines restrict their applica-tions in cancer treatment (Xie et al., 2008). Therefore, effective chemother-apy drugs against cancer development are highly desirable and of demand. Dracaena cinnabari Balf.f. also known as dragon’s blood tree, is endemic to the island of Socotra, Yemen. The name Dracaena is derived from the Greek word ‘drakaina’ which means female dragon (Stearn, 1997). The resin of Dracaena cinnabari is named dragon’s blood as that is a reddish substance exuded by the dragon trees (González et al., 2004).

In the past, Dragon’s blood was traditionally used by early Greeks, Romans, as well as, Arabs mainly due to its therapeutic characteristics. In Socotra Island, people belonging to the city of Moomy have utilized Dracaena cinnabari as a sort of “cure-all” to treat and cure different wound healing, fevers, diarrhea, dysentery illnesses as well as ulcers of the oral cavity, throat, stomach, and intestines (Gupta, Bleakley, & et al., 2004). Moreover, it was also useful to cure blood derived from Dracaena cinnabari Romans, as well as, Arabs mainly due to its therapeutic characteristics. In the 20th century, the resin of Dracaena cinnabari was also used to treat overloow of courses (menses), fluxes, dysentery, spitting of blood and as a fixative for the loose teeth (Gerard, 2015). Moreover, it was also useful to cure gonorrhea, cessation of urine, minor burns as well as watery eyes. Ex-ternal application of Dragon’s blood could stop bleeding, improve wound healing, and it is largely used for several mucosal or skin dis-orders (Wang et al., 2010). Its cytotoxic effect on carcinoma cells of human bladder has been reported (Al-Fatimi, Friedrich, & Jenet-Siems, 2005). Nevertheless, the mechanism of action of Dracaena cinnabari on oral cancer cell lines as well as the anticaner effects has not been studied.

There are various types of cell death including apoptosis and ne-crosis, which can be differentiated by means of biochemical and ultrastructural criteria (Farber, 1994). The programmed cell death or apoptosis induction is the most effective approach in preventing the development of cancer and can be controlled by two well-defined pathways, intrinsic and extrinsic (Chowdhury, Tharakan, & Bhat, 2006; Li et al., 2012). On the other hand, necrosis is a passive process of death in which the injured cells demonstrated no direct contribution to the process of cell death (Eastman, 1993).

The present study aims to investigate the cytotoxic activity, the morphological changes and cell death mode induced by Dracaena cin-nabari Balf. f. methanolic extract (DCBME) on tongue squamous cell carcinoma cell line, H103. Besides, the effects of DCBME on the cell cycle and cell migration of the H103 cell line were also analyzed. In addition, we investigated the in vivo chemopreventive activity of sys-temically administered DCBME against 4-nitroquinoline-1-oxide (4-NQO)-induced tongue carcinogenesis in rats through histopathological analysis.

2. Materials and methods

2.1. Extract preparation

The collected Dracaena cinnabari from the island of Socotra in Yemen was identified and authenticated by the Environmental Protection Authority of Yemen, Ministry of Water and Environment, Republic of Yemen. The powdered form of Dracaena cinnabari’s resin (50 g) was soaked with methanol (MeOH) (3 × 500 mL). Subsequently, the filtered resultant was dried to produce 28.0 g of the extract. Finally, 10 mg/ml stock solution of Dracaena cinnabari crude extract was pre-pared in DMSO (Vivantis, USA) and stored at –20 °C in aliquots for future use (Alabsi, Li, Paterson, Ali-Saeed, & Moharam, 2016; Al-Affi, Alabsi, Bakri, & Ramanathan, 2018).

2.2. Cisplatin preparation

Cisplatin 50 mg Freeze-dried yellowish crystalline powder, soluble in physiological saline solution, purchased from (Axon scientific sdn Bhd) was used. Preparation: 50 mg of the powder was diluted in 50 ml of saline solution 0.9%, making up a final concentration of 1 mg/ml (Freitas et al., 2009). In clinical practice, administration of cisplatin 100 mg/m² once every 3 weeks parallel with radiotherapy is a com-monly recommended schedule for head and neck SCC treatment. Chem-otherapy protocol schedule was achieved by administration of cis-platin in week 1, 4 and 7 (cisplatin 100 mg/m²) over a period of 2 to 3 days every 3 weekly Chemotherapy (Geeta et al., 2006; Tsan et al., 2012). In the present study, the dose of Cisplatin was decided based on the clinical doses converted into animal doses using surface area and weight factors. Therefore, Cisplatin 3 mg/kg body weight was ad-ministered in week 9, 12 and 15 over a period of 2 to 3 days every 3 weeks.

2.3. Cell lines and culture conditions

The Human oral squamous cell carcinoma cell lines H103, H314, H376 and H357 (Table 1) and Normal Human Oral Fibroblast cells were provided by Prof. Ian Charles Paterson, Department of Oral and Craniofacial sciences, Faculty of Dentistry, University of Malaya. Briefly, the human oral squamous cell carcinoma cell lines were cultured in culture flasks containing DMEM/F-12 medium (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS). Normal human oral fibroblast (NHOF) cells were cultured in DMEM (GIBCO, USA) supplemented with 20% FBS (JR Scientific, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

2.4. Cell viability assay (MTT cytotoxic assay)

Human oral squamous cell carcinoma and NHOF cell lines inhibition by DCBME was determined using microtitration cytotoxicity. 100 µL complete medium were transferred into a 96-well culture plate (Nunclon™, Denmark) and 100 µL of DCBME were added into the wells to make the concentration of 30 µg/mL. In other words, cells were treated with DCBME at a concentration range of 30 µg/mL- 0 µg/mL for 72 h. Untreated cells served as negative controls. Afterwards, 100 µL of 5 × 10⁵ cells were added to reach the ultimate volume of 200 µL. The culture plate was incubated at 37 °C with 5% CO₂. After 72 h, 20 µL of MTT (5 mg/ml) in PBS solution was transferred to each well and then the culture plate was more incubated for 4 h. After removal of MTT medium, 100 µL of dimethyl sulfoxide (DMSO) was dispensed to the wells to solubilize the crystals. Consequently, the OD was quantified using an ELISA plate reader at 570 nm. The graphs of the percentage of viable cells versus DCBME concentration (µg/mL) were charted. IC₅₀ was determined at the dose that caused 50% drop in cell viability as

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cytotoxicity against NHOF cells and selectivity index of DCBME. Data are presented as mean ± SEM from three independent experiments (n = 3).</th>
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<tbody>
<tr>
<td>Cell Lines</td>
<td>IC₅₀ ± SEM (µg/mL)</td>
</tr>
<tr>
<td>H103</td>
<td>5.5 ± 0.05</td>
</tr>
<tr>
<td>H314</td>
<td>17.4 ± 1.56</td>
</tr>
<tr>
<td>H376</td>
<td>17.2 ± 0.16</td>
</tr>
<tr>
<td>H357</td>
<td>9.0 ± 1.63</td>
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a IC₅₀ on NHOF cells / IC₅₀ on HNSCC cells.

b Not Detected (as the IC₅₀ values on NHOF > 30 µg/mL).
compared to controls. Moreover, in order to investigate whether the cytotoxic effect was selective against cancer cells, DCBME were screened on NHOF cells using MTT assay and IC_{50} and the selectivity index (SI) values were calculated. The SI value is defined as the ratio of cytotoxicity on NHOF cells to the selected OSCC cells.

2.5. Cell proliferation assay

Briefly, H103 cells were plated at a concentration of 1 × 10^5 cells/ml in a cell culture microplate (Nunclon™, Denmark) overnight. The cells were then exposed to DCBME in complete medium with three distinct doses of crude extract (IC_{25}, IC_{50} and IC_{75}) for 24, 48 and 72 h. In this assay, four replicates were performed for each concentration and control samples included cells without treatment. After the designated time period, the MTT assay was performed. Absorbance was assessed at 575 nm using an ELISA microplate reader (Tecan, Männedorf, Switzerland) and the wavelength of 650 nm was applied as a reference.

2.6. BrdU proliferation assay

BrdU proliferation assay for treated and untreated H103 cells (1 × 10^5 cells/mL) was carried out using BrdU Cell Proliferation assay kit according to manufactures instructions (CHEMICON, USA). H103 cells were treated at different concentration (IC_{25}, IC_{50} and IC_{75}) of DCBME. Then the plates were incubated in an atmosphere of 5% CO_{2} at 37 °C for 24, 48 and 72 h. After incubation periods, the cells were washed with PBS twice. The plates were read using ELISA reader. The OD of samples was plotted against time to determine the growth rates of cells in a given value.

2.7. Detection of apoptosis: morphological appraisal

2.7.1. Phase contrast microscopy

H103 cells were seeded into a 6-well plate (Nunclon™, Nunc) at a concentration of 1 × 10^5 cell/ml. The cells were then treated with DCBME at the IC_{50} value for 24, 48 and 72 h. After the incubation period at 37 °C with 5% CO_{2}, morphological alterations were analysed using a phase contrast microscope.

2.7.2. Fluorescent microscopy (acidine orange and propidium iodide staining)

H103 cells were plated at 1 × 10^5 cell/ml and then treated with DCBME at the IC_{50} value. The plates were incubated at 37 °C for 24, 48 and 72 h. After the incubation period, the cells were spun down at 600 × g for 10 min. The supernatant liquid was removed and thrown out. Subsequently, the equal volume of fluorescent dyes, containing 10 μg/ml acidine orange (AO) (DNA/RNA fluorochrome) (Invitrogen, USA) with 10 μg/ml of propidium iodide (PI) (DNA fluorochrome) (Invitrogen, USA), dissolved in PBS was added into the pellet. This was followed by dropping the cells suspension and staining solution onto a clean glass slide and covered with a cover slip. Finally, the slide was monitored under a UV-fluorescence microscope for 30 min before the fluorescence started to fade.

2.8. Detection of apoptosis

2.8.1. DNA fragmentation analysis

H103 cells were seeded at 5 × 10^5 cells/mL in 6-well plates and then treated with DCBME at the IC_{50} and IC_{75} values for 24, 48, and 72 h. The floating and harvested cells were rinsed with ice cold phosphate-buffered saline. DNA was then extracted from both untreated and DCBME-treated cells using Vivantis GF-1 Nucleic Acid Extraction kit based on the manufacturer’s protocol. The extracted DNA was subjected to electrophoresis on a 1.5% agarose gel and it was run at 50 V for 1 h. Fragments of DNA, including multimers of 180–200 base pairs, were visualized by staining the gel with ethidium bromide and observing the gel using a gel documentation system with UV light.

2.8.2. Flow cytometry annexin V/PI double staining

The detection of early apoptosis for untreated and DCBME-treated H103 cells was conducted using Annexin V and Apo 2.7-PE kit (BD Bioscience-Pharmingen, USA). H103 cells were seeded into a 6-wells plate at a concentration of 1 × 10^6 cells/ml. The cells were then treated with DCBME at the IC_{50} value. The plates were then incubated at 37 °C for 12, 24 and 48 h. This was followed by centrifugation at 1000 × g for 10 min. The supernatant was removed, and the pellet was rinsed with cold PBS. The cell pellets were then washed and resuspended in 200 μl of 1X binding buffer. Five microlitres of Annexin V and 10 μl of propidium iodide were next added. Following the incubation at room temperature for 5–15 min in the absence of light, 500 μl of binding buffer was added. Lastly, the cells characteristics were examined by flow cytometry and the outcomes were analyzed using Summit v4.3 software.

2.9. Determination of the mechanism of apoptosis

2.9.1. Mitochondrial membrane potential (ΔΨm) assay

The mechanism of apoptosis was investigated using the Mitochondrial Membrane Potential Kit (Abcam, Cambridge, UK). H103 cells were plated in a 96-well plate with dark walls and glass clear bottom at 1 × 10^5 cells/ml and a final volume of 90 μl. Approximately 10 μl of 10X (10 × IC_{50}) DCBME was applied as a treatment for 24 h. Following the incubation of H103 cells with 50 μl of JC-10 dye-loading solution at 37 °C for 30 min, 50 μl of Assay Buffer B was added into the dye loading plate. Finally, the fluorescence intensities for both J-aggregates and monomeric forms of JC-10 were evaluated at Ex/Em = 490/525 nm and 490/590 nm using the ELISA microplate reader.

2.9.2. Analysis of activities of caspases 3/7, 8 and 9

The activity of Caspase-3/7, 8 and 9 was examined using Caspase-Glo 3, 7, 8 and 9 Assay Kit (Promega, USA) according to the manufacturer’s instruction. Caspase-3/7, 8 and 9 substrates in a reagent optimized for caspase activity, luciferase activity and cell lysis have been provided in the kit. Briefly, H103 cells were seeded at 50,000 cells/mL and treated at the IC_{50} value of DCBME for 24 h. Afterwards, 100 μl of the caspase-Glo reagent was added to each well and the plate was incubated for 1 h. Ultimately, the caspase activity was evaluated using the ELISA plate reader.

2.9.3. Analysis of cellular DNA content using propidium iodide

H103 cells were grown in 6-well plates at the concentration of 5 × 10^5 cells/ml. The cells were then treated at the IC_{50} value of DCBME for 24 and 48 h. The wells with no DCBME was considered as control. Subsequently, the cells were centrifuged, and fixed by cold 66% ethanol. This was followed by incubation at 4 °C overnight. Afterwards, the cells were rinsed with PBS. Eventually, the cell pellets were stained in freshly prepared PBS buffer containing 1 mg/ml propidium iodide (PI) and 110,000 U/ml RNase A. The cells were then analysed using a flow cytometer (Beckman Coulter, USA). The emitted florescence from PI-stained cells was collected in FL2 channel using 488 nm laser illumination. G1, S and G2/M cell cycle phase percentages were analysed by ModFit LT software.

2.10. Inhibition of cancer cell migration

H103 cells were pre-treated with IC_{50} value of DCBME for 24 h and were harvested and seeded in a μ-Dish within an IBIDI culture insert (IBIDI GmbH) that consisted of two reservoirs separated by a 500 μm-thick wall. Approximately, 5 × 10^5 cells/ml of untreated and DCBME treated cells with the volume of 70 μl were added into the two reservoirs of the same insert and stored at 37 °C overnight. Media from the wells was discarded and replaced with 70 μl of media containing...
mitomycin-C (10 μg/mL) (Sigma Aldrich) and further incubated at 37 °C for 2 h. Following the incubation period, the insert was gently removed forming a gap of ~500 μm. Finally, the μ-Dish was filled with complete media and wound closure or cell migration was observed for every 3 h using a phase-contrast microscope (Leica DMI3000B, Wetzlar, Germany) at 50× magnifications. Phase-contrast images were taken from the same scratch area. WimScratch software was applied to analyze the pictures. The results were reported as percentages of scratched areas.

2.11. Animals studies and tumour induction

Prior to starting the in vivo study, the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University of Malaya, Malaysia studied and approved the animal protocol (Ethics No. 2014-02-14/OBBS/R/NAA). In the present study, a specific criteria was considered to maintain the Humane endpoint of all animals as described elsewhere (McCormick et al., 2015) which involved: loss of the body weight for more than 15% in one week; loss of the body weight in a steady and constant manner; sufficient signals that a study rat may additionally no longer continue to exist until the next schedule of inspection; and protracted unhealthy appearances such as a hunched posture or rough coat.

Male Sprague Dawley (SD) rats (6–8 weeks) weigh between (160–200 g) had been kept at 24 °C in a climate-controlled environment with 12 h light/dark cycle. All animals have been acclimatized to laboratory environments for 7 days before the experiments.

Rats were supplied with ad libitum rat chow and drinking water accompanied with 4-nitroquinoline 1-oxide (4NQO) (Sigma Aldrich) to a final concentration of 20 ppm (0.02 g/l) to produce hyperplastic and neoplastic lesions in the tongue of the rats as mentioned by Tanaka and Ishigamori (2011). Freshly formed 4NQO supplemented water was distributed to rats in bottles wrapped with aluminum foil to prevent potential photodegradation of the carcinogen. The rats were received drinking water containing 4NQO for 8 weeks that changed twice a week during the administration period.

2.12. Experimental design

The experiment of this study was designed to determine the effects of DCBME during the post-initiation phase of 4NQO-induced tongue carcinogenesis in rats. In the current study, the dose levels of DCBME used was selected in line with the results of the oral toxicity tests that performed in our research laboratory (Al-Affif et al., 2018). On this basis, doses of DCBME 100 mg/kg (low), 500 mg/kg (medium) and 1000 mg/kg (high) body weight were selected for the current study and given orally once per day for 10 consecutive weeks.

A total of 70 male SD rats were used in this study (10 groups of seven rats per each). Group 1 was provided with RO water ad libitum and served as normal control group. These rats left untreated, but their body weights were recorded at the end of study (week 22) to be compared with the experimental rats that given 4NQO. The remaining 63 male SD rats were given 4NQO (20 ppm) for 8 weeks in their drinking water ad libitum; after 8 weeks, rats received drinking water without added 4NQO. Then, the rats were randomized into nine groups of 7 rats per each. From 9–22 weeks, Group 2 served as induced cancer group. Group 3 was given 10% DMSO (orally) and served as a vehicle. In group 4, Cisplatin 3 mg/kg (axon scientific sdn Bhd) (a widely used chemotherapy agent for oral cancer) was administered i.p to the rats. DC extract 100 mg/kg, DC extract 500 mg/kg and DC extract 1000 mg/kg were administered orally to group 5, 6 and 7, respectively. Cisplatin 3 mg/kg and DC extract at doses of 100, 500 and 1000 mg/kg were given as a combination to group 8, 9 and 10, respectively, (i.e. DC extract was administered orally 2 h before the Cisplatin 3 mg/kg i.p) as mentioned in previous study (Patel & Damle, 2013). The Cisplatin 3 mg/kg was administered once every three weeks, whereas the DC extract was given orally/day. Oral administration to the rats was done by gastric intubation using force-feeding needle size 18 G (Harvard Apparatus, INC) starting 1 week after cessation of 4NQO exposure and maintained on this treatment for 10 consecutive weeks as described elsewhere (Tanaka et al., 1998; Zhang et al., 2013). The experiment was terminated after week 22 (Fig. 1). All animals were sacrificed to assess the incidences of pre-neoplastic and neoplastic lesions in the tongue.

At the termination of the experiment; general anesthesia has been performed to the surviving rats through intraperitoneonal injection of 80 mg/kg of ketamine 100 mg/ml + 7 mg/kg of xylazine 100 mg/ml (Troy laboratories PTY. Limited, Australia) then rats were euthanized by cervical dislocation. A restricted gross necropsy was performed that centered on the tongue and oral cavity of all the rats. The tongue was properly excised from each rat, then bisected longitudinally, fixed with 10% buffered formalin, processed routinely and embedded in paraffin wax for histopathological investigation.

2.13. Histopathological analysis (H&E)

The half of the tongue tissue sample was placed in a container containing 50 mL 10% buffer formalin and fixed for 24 h. The histopathological analysis in this study was achieved using a light microscope (Nikon E500). An expert pathologist who had been unaware of the experimental groups to which each section belonged carried out the evaluation. Finally, the tongue sections were analysed and graded as hyperplasia, dysplasia and squamous cell carcinoma per animal as described by Ribeiro et al. (2007) and El-Rouby (2011).
2.14. Measurement reliability

Re-evaluation of all samples was done two times using intra-examiner reliability in seven days interval but without the knowledge of any information about the previous reading. Cohen’s kappa (k) has been done to ascertain in cases where there was an agreement between the two readings of the incidence of OSCC. There was a substantial agreement between the two readings, \( k = 0.777, p = 0.000 \).

2.15. Statistical analysis

Data were presented as mean ± SEM. The statistical differences between treated and control groups were determined using one-way analysis of variance (ANOVA), Independent-sample t-test or Mann-Whitney U test for different assays where \( P < 0.05 \) was considered to be statistically significant. The measured values were expressed as mean ± standard deviation. Body weights gain were compared by one-way analysis of variance (ANOVA), with post-hoc comparisons made using Dunnett’s test. Comparison of the histopathological incidence (hyperplasia, dysplasia and squamous cell carcinoma) was done using descriptive statistics and chi-square test.

3. Results

3.1. Dracaena cinnabari Balf. f. methanolic extract (DCBME) inhibits growth of human oral squamous cell carcinoma cell lines (OSCC)

The MTT cytotoxicity assay clearly showed the cytotoxic effect of DCBME on the human OSCC cell lines. The lowest dose of DCBME that killed 50% (IC50) of OSCC cells was observed with H103 compared to untreated (control) cells after 72 h of treatment. The IC50 was determined to be 5.5 \( \mu \text{g/mL} \) (Table 1). In addition, the DCBME extract also exhibited low cytotoxic effect on NHOF normal cells compared to H103 cells (> 30 \( \mu \text{g/mL} \)).

3.2. DCBME inhibits proliferation of H103 cells

As shown in Fig. 2, untreated H103 cells demonstrated a rise in OD from 24 to 72 h after incubation. However, treated cells after 24, 48 and 72 h, displayed a decline in OD from IC25 to IC75. In other words, the optical density of DCBME-treated H103 cells was lower than the control in all tested concentrations. Besides, the exposure of the cells to DCBME decreased the H103 cell proliferation in a concentration-dependent as well as time-dependent manner.

![Fig. 2. MTT proliferation assay. The effects of IC25, IC50 and IC75 of DCBME on the proliferation of H103 cells after 24, 48 and 72 h. Untreated control cells exhibited an increase in OD from 24 to 72 h after incubation. In contrast, DCBME showed a proliferation inhibition effect in a concentration-dependent manner in treated cells. In addition, the cell proliferation was decreased in H103 cells in a time-dependent fashion.](image)

3.3. BrdU cell proliferation assay

Cell proliferation of H103 cells based on the DNA synthesis phase were investigated using BrdU cell proliferation assay. The results obtained showed that there was a decrease in optical density (OD) of H103 cells after treated with DCBME in a concentration and time-dependent manner (Fig. 3). As shown as in Fig. 3, untreated H103 cells exhibited an increase in OD from 24 h to 72 h compared to H103 cells treated with IC25, IC50 and IC75.

3.4. DCBME causes apoptotic morphological changes in H103 cells

As presented in Fig. 4A, the morphological observation of untreated cells under Phase Contrast Microscopy indicated that the cells preserved their original morphology and were also adhered to the plates. In contrast, the number of DCBME-treated cells decreased considerably in comparison with the untreated cells. Besides, the treated cells displayed typical apoptotic features including shrinkage of cells, cell blebbing and creation of apoptotic bodies. Apoptotic cells also ended in other types of morphological alterations such as losing contact with neighbouring cells and some cells were even detached from the plates. Fluorometric cell viability assays showed normal viable cells with undamaged DNA and nucleus, which are permeable to acridine orange, presented a round and uniformly green nuclei revealing that the cells are healthy. On the other hand, apoptotic cells with disrupted plasma membrane integrity and condensed chromatin displayed several green-coloured nuclei and apoptotic blebs. In addition, the DNA of the necrotic cells (permeable to propidium iodide) was stained orange to red. As shown in Fig. 4A, H103 cell lines treated with DCBME demonstrated characteristic changes and different signs of programmed cell death. Furthermore, the number of necrotic cells was generally very low in the treated population which revealed the cell death mode was apoptosis.

3.5. DCBME induces DNA fragmentation in H103 cells

Degradation of chromosomal DNA into small internucleosomal fragments, as a conventional characteristic of apoptosis, was clearly detected in DCBME-treated H103 cells after 24, 48 and 72 h in both doses of extract. In contrast, the DNA of control cells demonstrated no fragmentation pattern (Fig. 5).

3.6. DCBME induces early apoptosis in H103 cells

The results showed the percentage of viable cells in DCBME-treated H103 cells decreased dramatically in comparison to the control cells. In contrast, the percentage of early apoptotic (R6) in which cells were Annexin V+/PI− and late apoptotic/secondary necrotic (R4) in which cells were Annexin V+/PI+ increased significantly in a time-dependent manner (Fig. 6). The proportion of apoptotic cells augmented from 2.03% in control cells to 10.09% in DCBME-treated cells after 24 h. Hence, this study corroborated that the main mode of induced cell death in DCBME-treated H103 cells was apoptosis.

3.7. DCBME stimulates intrinsic apoptotic pathway in H103 cells through mitochondrial depolarization and activation of caspases 3, 7 and 9

The mitochondrial membrane potential changes, induced by DCBME in H103 cells, were examined with JC-10 dye. The dye is capable of entering into the mitochondrial matrix and converting the colour from red to green when the ΔΨm is decreased. The accumulation of JC-10 occurred in the mitochondrial matrix of live cells and it led to the selective production of red fluorescent aggregates. In contrast, in apoptotic and necrotic cells, JC-10 diffused out of mitochondria, was converted to monomeric form and stained the cells in green florescence. The florescent intensities for both forms of JC-10 were measured at specific wavelengths and shown in Fig. 7A. H103 cells demonstrated a
substantial decline in fluorescence intensity [ratio of 590/525 nm or red/green] following 24 h treatment with DCBME, which revealed the collapse of the mitochondrial membrane potential. As presented in Fig. 7A, the fluorescence intensity in H103 cells after 24 h treatment with DCBME was 23.08% as compared to the control cells. Furthermore, Fig. 7B showed a significant increment in caspase 3/7 and 9 activities in DCBME-treated cells compared to the control. In addition, caspase 8 activity was not influenced in all treated cell lines. Hence, the outcomes suggest that DCBME induced apoptosis in H103 cells through the intrinsic pathway.

3.8. DCBME effect on the cell cycle and cellular DNA content of H103 cells

As shown in Fig. 8, H103 cells demonstrated a striking accumulation of cells in both S and G2/M phases suggesting that DCBME induced both S and G2/M phase cell cycle arrest after 24 and 48 h treatment. Based on the proposed results, it is suggested that the inhibitory influence of DCBME on the proliferation of H103 cells has been because of the blockade of cell cycle progression in the S and/or G2/M phases.

3.9. DCBME inhibits migration of H103 cells

As indicated in Fig. 9, the migration capability of treated H103 cells
was significantly reduced in comparison with the control cells. Therefore, the current results suggest that DCBME inhibit the migration of H103 cells in vitro.

3.10. Effect of DCBME on mean body weight gain

Mean body weights gain for all groups at the end of the study are indicated in Fig. 10. A one-way analysis of variance ANOVA test presented a significant difference between the groups (p = 0.000).

The mean body weight was decreased with a statistically significant difference presented in all groups compared to the normal control group, except for both groups of DCBME at 500 and 1000 mg/kg. Mean body weights gain in normal control and DCBME treated groups at 500 and 1000 mg/kg were comparable throughout the study as p > 0.05 (Table 2).

3.11. Effect of DCBME on the incidence of pre-neoplasms and neoplasms

Anticancer activity of DCBME was confirmed as a significantly decreasing in the incidence of tongue SCC (Table 3). In both groups (induced cancer and vehicle), the incidence of tongue SCC caused by 4NQO was 85.7% while in rats treated with DCBME at 100, 500 and 1000 mg/kg was 57.1%, 28.6% and 14.3%, respectively, which has been combined with an obvious reduction in the incidence of tongue SCC, mainly in the group that received DCBME at 1000 mg/kg. Whereas, the incidence of tongue SCC in the Cisplatin and combination groups at 100 and 500 mg/kg was 14.3% per each. Finally, in the combination group of DCBME 1000 mg/kg + Cisplatin no incidence of tongue SCC was detected as the incidence was 0%, offering the best protection to the rats exposed to 4NQO. The Chi square test that used to assess the incidence of hyperplasia, dysplasia and SCC demonstrated a statistically significance difference between groups (Chi.Sq = 29.442; p = 0.021). An adjusted residuals and a Bonferroni correction were performed to check the significant differences after the chi-square test (Garcia-Perez & Nunez-Anton, 2003). Therefore, the alpha value has been changed from 0.05 to 0.0019. There were no significant differences have been detected between the groups. However, the difference that was nearing significance with a P value of 0.0027 was presented at SCC of the induced cancer and vehicle groups.

The descriptive analysis presented that the gross lesions associated with 4NQO-induced rat oral cancer model were located on the posterior region of the tongue (on the dorsal surface of the tongue).

On microscopic investigation, tongues of rats in the normal control group exhibited normal oral mucosa of the tongue with surface...
keratinized stratified squamous epithelium with normal architecture and normal sizes and orientation of the papillae with underlying connective tissue and skeletal muscle bundle (Fig. 11A).

Microscopic investigation demonstrated different histopathological alterations such as hyperplasia, dysplasia and SCC in all groups that provided with 4NOQ (20 ppm) in their drinking water. The incidence of tongue SCC was recognized by the presence of discontinuation of the basement membrane and submucosal invasion of epithelial tumor cells as islands, nests and sheets. The epithelial tumor cells exhibit nuclear and cellular pleomorphism, hyperchromatic nuclei, and modified nucleus: cytoplasmic ratio and keratin pearls in connective tissue. These alterations were definitely seen in both induced cancer and vehicle groups (Fig. 11B and C).

4. Discussion

Dragon’s blood is an extreme red resin, which has been applied as a well-known traditional medication since ancient times by numerous nations (Gupta et al., 2008). Antibacterial, antiviral, analgesic, anti-inflammatory and antioxidative activities of several compounds isolated from Dragon’s blood have recently been demonstrated and they have presented some proof for its effectiveness in traditional use (Cui, Guo, Dong, & Xiao, 2011). Antimicrobial activity of Dracaena cinnabari resin from Socotra against several types of bacteria has been reported (Kumar, Chauhan, Padh, & Rajani, 2006; Mothana & Lindequist, 2005). Furthermore, Mothana, Mentel, Reiss, and Lindequist (2006) declared antiviral property of Dracaena cinnabari resin against Herpes simplex virus as well as Human influenza virus (Mothana et al., 2006). Vachalkova et al. (1994) disclosed the carcinogenicity of three homoiso-flavonoids and four flavonoids isolated from Dracaena cinnabari resin (Vachalkova, Novotny, Nejedlikova, & Suchy, 1995). Besides, Al-Fatimi et al. (2005) claimed a remarkable cytotoxic effect of Dracaena cinnabari resin towards human ECV-304 cell line with an IC50 value of 8.9 μg/mL. (Al-Fatimi et al., 2005). Antioxidant property of three homoiso-flavonoids isolated from Dracaena cinnabari resin was reported by Juránek, Suchý, Stará, Mašterová, and Grancicova (1993) (Juránek et al., 1993). The assessment of the anti-inflammatory and analgesic activities of Dracaena cinnabari resin revealed that the extract showed no central analgesic effect but had peripheral analgesic and anti-inflammatory properties, which supported the traditional applications of this plant in curing different illness associated with pain and inflammation (Alwashli, Al-Sobarry, Cherrah, & Alaoui, 2012).

Cancer of the oral cavity is often used to describe a number of cancers that start in the region of the mouth. Based on the American Head and Neck Society (AHNS), the most common site for oral cavity cancer in the United States is the tongue. In other areas of the world different sites are more frequently affected. In the current study H103 and H357 cell lines derived from the tongue, and H314 and H376 cell lines derived from the floor of mouth were used as representatives of oral cancer and the cytotoxic activity of Dracaena cinnabari was investigated on those cell lines. As shown in Table 1, Dracaena cinnabari Balf. f. methanolic extract (DCBME) demonstrated significant cytotoxic effect on the H103 cell line with an IC50 value of 5.5 μg/mL. The IC50 value of H103 cell lines was the lowest amongst the selected cancer cell lines and therefore, H103 which is tongue Squamous Cell Carcinoma cell line was selected for further study. On the other hand, DCBME exhibited low cytotoxic activity on normal cell line (> 30 μg/mL). These selective cytotoxic effects associated with DCBME is essential, because currently existing anticancer medications can target both cancer and healthy cells, resulting in severe side effects. Thus, these results invite further investigation of the apoptotic activity of DCBME and its efficacy as an antitumor drug for tongue Squamous Cell Carcinoma cell line (H103) cells therapy.

Phase contrast inverted microscope, AO/PI analysis of cells under fluorescence microscope, DNA fragmentation assay and annexin V-FITC/PI staining were performed to elucidate the cell death mode induced by DCBME in human H103 cell line. Morphological analysis by applying phase contrast and fluorescence microscope is one of the efficient methods to determine apoptosis (Doonan & Cotter, 2008). In this study, obvious morphological changes of apoptosis such as loss of cellular adhesion to their neighbours, membrane blebbing, chromatin condensation as well as creation of apoptotic bodies were identified in DCBME-treated cells.

DNA fragmentation is an important distinguishing biochemical characteristic of apoptosis. DNA fragments with integer multiples of 180–200 bp units result from the effect of cellular endonucleases on genomic DNA. These endonucleases cleave the DNA at the internucleosomal regions leading to the creation of a ladder-like pattern on agarose gel electrophoresis (Wyllie, 1980; Zhang & Xu, 2002). In DCBME-treated H103 cell line, the standard ladder-like pattern of nuclear DNA was detected which supports the apoptosis-inducing ability of the methanolic crude extract. Annexin V-FITC is normally utilised in conjunction with propidium iodide to characterize apoptotic cells before the loss of cell membrane integrity. Translocation of phosphatidylserine (PS) from the inner to the outer leafllet of the plasma membrane or externalization of PS to the cell surface occurs in apoptotic cells (Ali et al., 2011). Flow cytometric analysis of apoptosis by Annexin V-FITC/PI corroborated that DCBME induces apoptosis in human H103.

Fig. 7. Mitochondrial membrane potential and Caspases activation assays (A) Histogram showing disruption of mitochondrial membrane potential (ΔΨm) in treated H103 cells with DCBME. H103 cells exhibited a significant reduction of fluorescence intensity [ratio (590/525) or red/green] after 24 h treatment with DCBME. (B) Histograms showing Caspase 3/7, 8 and 9 activity in H103 cells after 24 h treatment with DCBME. There was a significant increment in caspase 3/7 and 9 activity in treated cells with IC50 value of DCBME compared to the control cells. Besides, caspase 8 activity was not influenced in all treated cell lines. Data were shown as mean ± SEM. Statistically significant differences between control and treated cells was set at * P < 0.05 & ** P < 0.01.
Apoptosis is a programmed cell death that occurs via diverse internal or external stimuli. These signals are monitored by two discrete pathways, intrinsic and extrinsic (Chowdhury et al., 2006; Li et al., 2012). The intrinsic (mitochondria-mediated) pathway occurs in response to stimuli such as DNA damage, hypoxia, a defective cell cycle, loss of survival factors, or other sorts of intense cell stress in addition to chemotherapeutic medicines (Kaufmann & Earnshaw, 2000). The intrinsic pathway is firmly controlled by pro-apoptotic members that are activated by various apoptotic stimuli, resulting in oligomerization as well as insertion into the mitochondrial outer membrane to the release of apoptogenic factors like cytochrome c (Ferri & Kroemer, 2001). Afterwards, Cytochrome c binds and activates apoptosis procaspase-9, forming a supramolecular complex called the apoptosome (Chinnaiyan, 1999; Hill, Adrian, Duriez, Creagh, & Martin, 2004). Consequently, the apoptosome activates caspase-9 via autocatalysis, which leads to the activation of executioner caspase-3, -6 and – 7 resulting in cell death (Green & Reed, 1998). On the other hand, the activated caspase-8 directly cleaves and activates caspase 3 and caspase 7 through the extrinsic pathway and this ultimately ends in apoptosis (Cullen & Martin, 2009). In this study, significant growth was observed in the activity of caspase 3/7 and 9 in Dracaena cinnabari-treated cells compared to the control cells. Dracaena cinnabari also induced mitochondrial membrane potential changes in H103 cells. The results obtained from both assays showed that Dracaena cinnabari caused apoptosis in H103 cells via the intrinsic/mitochondrial pathway, but not the extrinsic/death receptor pathway.

Numerous research has revealed that the anti-proliferative properties associated with cytotoxic agents are generally related to cell cycle arrest at distinct phases and result in apoptosis induction (Evan & Vousden, 2001). In other words, cell cycle arrest is an essential mechanism of many antitumor drugs such as Cisplatin, Doxorubicin,
Camptothecin, and 5-fluorouracil (Pieme et al., 2013). In this study, the outcome of cell cycle distribution analysis confirmed that DCBME arrested the H103 cell line at both S and G2/M phases. This arrest at S phase of H103 cells suggests that DNA synthesis was interrupted, stopping the cell cycle progression at S phase and ultimately inducing apoptosis. Moreover, the observed G2/M phase arrest implied that the cells could not commence mitosis before repairing their damaged DNA after replication (Cuddihy & O’Connell, 2003).

Tumour metastasis, i.e., the dissemination of tumour cells to distant organs of the body from its primary site, is the leading cause of cancer-related morbidity and mortality (Chambers, Groom, & MacDonald, 2002). The fatality related to metastasized tumours, accounted for 90% of all cancer deaths (Gupta & Massagué, 2006). In the present study, cell migration was examined using wound scratch assay and the migratory activity of H103 cells was inhibited significantly after treatment with DCBME compared to the control group.

Several characteristics of oral cavity make it a significant site for medical efforts in most cancer prevention studies. Key risk factors for
OSCC are well-established that can be used to recognize high-risk persons who are most probably to gain benefit from a chemopreventive intervention (McCormick et al., 2015). Cisplatin is one of the main standard anticancer drugs, which still has an essential role in cancer chemotherapy (Ravindra, Bhiwgade, Kulkarni, Rataboli, & Dhume, 2010). The prevalent component of chemotherapy for the treatment of solid organ cancers was cisplatin that first described by Michele Peyrone as early as in 1845 (Kim, Kim, Kim, & Chang, 2015). Cisplatin and other platinum drugs are the common drugs in the contemporary medical oncology, which considered to have a major impact in controlling tumors of the ovary, testes, head and neck and other cancers (Kelland, 2007). However, the dose toxicities related to the platinum therapy has offered a serious concern where cisplatin can affect organs such as nervous system and kidneys among the clinically established platinum compound (Hartmann & Lipp, 2003; Hartmann, Kollmannsberger, Kanz, & Bikemeyer, 1999). For this reason, in the present study a combination of DCBME with cisplatin have been evaluated.

Administration of 4NQO triggered a significant body weight reduction in experimental rats due to the intra oral cancer development associated with malnutrition that caused by lack of appetite, not able to eat, elevated metabolic rate (Thandavamoorthy et al., 2014). Patient with cancer cachexia commonly experience body weight reduction which usually play an important role in patient morbidity and mortality (Fearon & Preston, 1990). In the present study, 4NQO induced a significant reduction of body weight gain among the induced cancer and vehicle groups which have been managed by treatment with DCBME with or without a combination of cisplatin not with cisplatin alone. Herein, the result based on the body weight gain revealed that the combination of DCBME (DCBME 500 mg/kg) with cisplatin reduce the toxic effect of cisplatin by increasing the body weight as nearly as that in normal control group. This result is in a like manner with study performed by Patel and Damle (2013), who demonstrated that loss of body weight caused by cisplatin could be controlled by treatment with combination of telmisartan with cisplatin.

The current study was carried out to assess the null hypothesis that carcinogenesis in the oral cavity cannot be delayed or inhibited by pharmacologic activation of medicinal plant. To address this null

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence pre-neoplasms and neoplasms (%)</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced cancer</td>
<td>0/7 (0)</td>
<td>1/7 (14.3)</td>
<td>6/7 (85.7)</td>
<td>0.021*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0/7 (0)</td>
<td>1/7 (14.3)</td>
<td>6/7 (85.7)</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2/7 (28.6)</td>
<td>4/7 (57.1)</td>
<td>1/7 (14.3)</td>
<td></td>
</tr>
<tr>
<td>DCBME 100 mg/kg</td>
<td>0/7 (0)</td>
<td>3/7 (42.9)</td>
<td>4/7 (57.1)</td>
<td></td>
</tr>
<tr>
<td>DCBME 100 mg/kg + Cisplatin</td>
<td>2/7 (28.6)</td>
<td>4/7 (57.1)</td>
<td>1/7 (14.3)</td>
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<tr>
<td>DCBME 500 mg/kg</td>
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<td>0/7 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*a p value less than 0.05, (p < 0.05) significant value.

Fig. 11. Photomicrographs (A) normal control group showing normal oral mucosa with papillae having surface keratinized epithelium, underlying connective tissue and skeletal muscle bundles. Following oral carcinogenesis induced by 4NQO and treated with DCBME resin methanol extract with/without cisplatin or cisplatin. (B) Induced cancer - SCC. (C) Vehicle - SCC. (D) Cisplatin - Dysplasia. (E) DCBME 100 mg/kg - Dysplasia. (F) DCBME 100 mg/kg + Cisplatin - Hyperplasia. (G) DCBME 500 mg/kg - Dysplasia. (H) DCBME 500 mg/kg + Cisplatin – Hyperplasia. (I) DCBME 1000 mg/kg- Hyperplasia. (J) DCBME 1000 mg/kg + Cisplatin- Hyperplasia. H & E stain, 100× magnification.
hypothesis, in vivo study was achieved to evaluate the anticancer potential of DCBME. In this study, 4-Nitroquinoline 1-oxide (4NQO) was used to induce oral cancer in rat, 4NQO is a potent chemical carcinogen. This material has been previously used in some studies to induce oral cancer in the tongue of the rat (Lee & Choi, 2011; McCormick et al., 2015; Patel & Damle, 2013; Ribeiro et al., 2007).

4NQO produces histopathological changes in oral tongue of the rats vary from normal epithelium, hyperplasia, premalignant dysplasia, and carcinoma in situ to invasive SCC similar to those in humans (Ribeiro, Salvadori, da Silva, Darros, & Marques, 2004). The results of the current study revealed that none of the animals in the normal control group developed any visible lesions of tongue epithelium and histopathological alterations throughout the termination of the study. However, in the present study, the carcinogenic agent (4NQO, 20 ppm) that given for the rats for 8 weeks induced typical precancerous signs and morphological changes on the tongue epithelium after 22 weeks from the start of carcinogen administration. None of the rats in both groups (induced cancer and vehicle) demonstrated with hyperplasia. Undoubtedly, there were sever epithelial dysplasia and SCC of well-differentiated type among the rats of induced cancer and vehicle groups. However, the sever epithelial dysplasia and SCC where controlled by DCBME especially at doses of DCBME 500 and DCBME 1000 mg/kg with or without cisplatin and with cisplatin alone. Combination of DCBME (DCBME 500 mg/kg and DCBME 1000 mg/kg) with cisplatin showed better improvement in histology of tongue. The combination of DCBME 1000 mg/kg + cisplatin reduced the incidence of SCC to 0%. Similarly, in previous study, the combination of telmisartan with cisplatin reduced 4NQO induced invasive SCC in the tongue (Patel & Damle, 2013). The histopathological grade was generally SCC of well-differentiated type. The tumors propagated into the submucosa and underlying muscle layer, creating small nests with typical keratin pearl formation (Zhao et al., 2014). In like manner, a study achieved to assess the effect of a etodolac (Cox-2 inhibitor) on 4NQO-induced oral tongue carcinogenesis of the rat, revealed that the histopathological changes as dysplasia, papilloma and SCC were produced at incidences of 21.4, 7.2 and 100%, respectively, while hyperplasia has not been recognized. Nevertheless, the 4NQO (20–30 ppm) was given in the drinking water for 2 months (8 weeks 2) as well as the experiment was accomplished at 28 weeks (Yamamoto et al., 2004). In another study, when the rats provided with 4NQO at 20 ppm supplemented with drinking water for 10 weeks, the incidence of invasive OSCC in the rat tongue was extremely reproducible which was 83% and 75% in the two experiments exhibited invasive oral cancers at 26 weeks following the 4NQO administration (Peng, Li, Johnson, Torres, & McCormick, 2015). Generally, based on the dose and duration of 4NQO administration, various dysplastic and neoplastic lesions with morphological and molecular changes in the oro-esophageal epithelium that simulate those taking place in human oral epithelial pre-neoplastic and neoplastic lesions are produced (Tang, Knudsen, Bemis, Tickoo, & Gudas, 2004).

5. Conclusion

This study showed that Dracaena cinnabari Balf. f methanolic extract was able to inhibit cell proliferation, reduce cell migration, cause cell cycle arrest and triggered apoptosis in human H103 cells. DCBME was shown to induce apoptosis in H103 cells via the intrinsic pathway. Anticancer activity was expressed as a statistically significant increase in body weight gain. The present study reported that DCBME especially at doses of DCBME 500 mg/kg and 1000 mg/kg confer a significant protection against carcinogenesis in a well-studied rat model for OSCC. These outcomes suggest that Dracaena cinnabari may function as a promising experimental antitumor agent for oral squamous cell carcinoma cells therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was financially supported by UMRG Grant (RG422/ 12HTM), University of Malaya, Malaysia.

Authors’ contributions

Each author participated sufficiently in taking public responsibility for appropriate portions of the content.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University of Malaya, Malaysia (Ethics No. 2014-02-14/OBBS/R/NAA).

Informed consent

For this type of study, formal consent is not required.

Limitation

In traditional cell culture, the oxygen level that cells experience is dramatically higher (~20%) than in vivo conditions (10–13%). On the basis of this consideration, virtually all routine cell cultures are currently being performed under hypoxic conditions that may not reflect in vivo physiology. In addition, detail exploration of the mechanism of action of DBME against oral cancer needs to be done by quantifying the reactive oxygen species (ROS) level in the cells.

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References


