Accepted author version posted online: 23 November 2018

Potential Protective Effects of Rice Seedling Extracts of a Malaysian Rice Variety, Biris, Against Doxorubicin-Induced Cytotoxicity

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Running title: Protective Effects of Rice Seedling Extracts

Abstract. Doxorubicin (DOX) is one of the most effective chemotherapeutic drugs for treating a wide range of neoplasms such as leukaemia, lymphoma and breast cancer; however, it is often related to cardiomyopathy. Currently, there is no established treatment for reducing the impact of cardiomyopathy without noticeable side effects. Thus, this study set out to investigate potential protective effects of rice seedlings extracts (RSE) against DOX-induced cytotoxicity using *in vitro* cell culture studies. The antioxidant capabilities of RSE were evaluated, and the results showed lower amounts of total phenolic content (TPC), but similar total flavonoid content (TFC) and trolox equivalent antioxidant capacity (TEAC), compared to wheatgrass seedlings extracts. A series of absorbance and fluorescence spectroscopy experiments indicated that RSE could hinder the formation of DOX-DNA complexes at the tested concentrations. Further, the viability of a rat cardiomyocyte cell line, H9c2(2-1), was tested after 24, 48 and 72h of DOX treatments in the presence of RSE, using a tetrazolium salt (MTS reagent) based cell proliferation assay. The results indicated significant protective effects of RSE against DOX-induced cytotoxicity. The nasopharyngeal carcinoma cell line, HK1, was used as a control to determine whether the efficacy of DOX is affected by the co-administration of RSE. The results indicated no negative effects on the efficacy of the drug. These multiple beneficial properties of RSE indicate its strong potential for development of a cardioprotective agent to compliment the DOX treatment in clinical settings.

Keywords: rice; antioxidant; cardio-protective; cytotoxicity; copper chlorophyllin; doxorubicin

Abbreviations: CHL: Copper chlorophyllin; DOX: Doxorubicin; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic Acid Equivalent; PENSTREP: Penicillin-Streptomycin; QE: Quercetin Equivalent; RSE: Rice Seedlings Extract; TEAC: Trolox Equivalent Antioxidant Capacity; TPC: Total Phenolic Content; TFC: Total Flavonoid Content

INTRODUCTION

Doxorubicin (DOX) remains one of the most effective anti-cancer drugs against leukaemia, lymphoma and breast cancer (Carvalho et al. 2009). It inhibits the activities of human IIA-type topoisomerase by forming a stable complex with DNA, which leads to double-strand breaks and a halt in the cell's transcriptional activity (Thorn et al. 2011). However, it also triggers the production of reactive oxygen species (ROS) in the submitochondrial particles of the cell. The combination of these effects, directly or indirectly, leads to elevated expression of mitogen-activated protein kinases (MAPK) and caspase-3 genes (Thorn et al. 2011), resulting in the onset of apoptosis in the cells. Similar cytotoxicity effects have been reported when *in vitro* cultured cardiomyocytes were treated with DOX, indicating that its cytotoxicity is not specific to cancer cells (Minotti et al. 2004; Wallace 2007). Hence reducing the availability of DOX or production of ROS could provide significant cytoprotective effects (Kalam and Marwick 2013). One of the suggested methods is the incorporation of antioxidant molecules during the treatment (Chegaev et al. 2013). Antioxidants are "molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage" (Maritim et al. 2003). The antioxidant capabilities of many plant extracts are mainly due to the phenolic and flavonoid compounds it contains, as they play important roles in neutralising free radicals, singlet oxygen, and peroxides molecules (Osawa 1994). Another method is to reduce the availability of free Fe^{3+} in the cell using a metal chelator such as Dexrazoxane, which acts to prevent any iron-based oxygen free-radical damage (Hochster 1998) and is a cardioprotective agent used in clinical settings with proven efficacy (Minotti et al. 2004). However, it may reduce the efficacy of DOX and increase the danger of secondary malignancy in younger cancer patients (Lipshultz et al. 2010; Tebbi et al. 2007; Vrooman et al. 2011). Hence its dosage has been limited (Salvatorelli et al. 2015) and no equivalent substitute has yet been found.

Copper Chlorophyllin (CHL) is a semi-synthetic analogue of chlorophyll with many health benefits. Most prominently, CHL has been found to contain anti-mutagenic properties against a number of mutagenic agents such as heterocyclic amines (Hernaez et al. 1997), benzopyrene (Reddy et al. 1999), aflatoxin (Egner et al. 2003), heavy metals (Garcıa-Rodrıguez et al. 2001) and ionising radiation (Kumar et al. 1999). CHL has also been reported to form complexes with certain aromatic mutagens (Hayatsu et al. 1999), reducing the abundance of mutagens in monomeric forms, thereby reducing their efficacy. Previous studies have described the 'interceptor' properties of CHL against DOX using absorbance and fluorescence spectroscopy (Pietrzak et al. 2003; 2006). Since chlorophyllin shares a high structural similarity to natural chlorophyll, plant extracts containing a high amount of chlorophyll could impart similar protective properties. In particular, wheatgrass (*Triticum aestivum* L) has been widely used as a source of natural chlorophyll, and aqueous extracts of wheat seedlings have been reported to yield significant health benefits (Ben-Arye et al. 2002; Hemalatha et al. 2012; Kulkarni et al. 2006). It has been shown that wheatgrass juice may provide significant cardioprotective effects against DOX-induced cardiomyopathy in male rats (Papasani et al. 2015). These studies suggest that leaf tissues from other cereal plants such as rice may also have similar properties. However, the protective effects of RSE have never been investigated in this setting. Hence this study set out to investigate the potential benefits of RSE against DOX-induced cytotoxicity in three separate ways: the antioxidant properties of RSE, in terms of TPC, TFC and DPPH radical scavenging capacity, the optimal concentration of RSE which may interfere with the formation DOX-DNA complex, and the protective effects of RSE towards cultured rat cardiomyocytes co-administered with DOX.

METHODS AND MATERIALS

Rice Seedling Cultivation

Rice grains of a Malaysian local variety named Biris were obtained from the Agriculture Research Department Sarawak (Malaysia). They were surface sterilised with 70% ethanol by submersion for 5 min, rinsed twice with distilled water, and placed under UV light for 10 mins in a biosafety cabinet (1300 Series 2A, Thermo Fisher Scientific, USA). They were then transferred into individual disposable plastic cups containing 20g of vermiculite: perlite (1:1) soaked in sterile distilled water and left to grow for 19 days in a growth chamber (POL-EKO 750, Poland), at a constant temperature of 30°C and 300µmol m^{-2} s^{-1} light throughout the day (16h) and 25°C throughout the night (8h). The relative humidity ranged from 50% to 80%, and all watering was conducted twice a day with distilled water.

Simple Solvent Extraction

Shoot tissues of 30 seedlings were harvested together at 19 days of seedling growth. The shoot tissues were weighted, submerged in liquid nitrogen and pulverised using a chilled mortar and pestle. Next, analytical grade absolute ethanol at 1:10 (g: mL) was added and the tissue suspension was placed in a chilled ultrasonic bath (B5510, Branson) for 60 min. It was then centrifuged at 6000 RPM at 4°C for 15 min and the supernatant collected to be used directly as crude rice seedling extract (RSE). The concentration of extract was expressed as “mg of fresh seedlings shoot/mL of solvent”. Three such batches were harvested, each batch was (consisting of 30 seedlings) used as one biological replicate.

Total Phenolic Content (TPC)

The TPC was determined using as per Singleton and Rossi (1965) with minor modifications. Briefly, 80µL of RSE, 200 µL of Folin-Ciocalteu reagent (diluted 10-fold) and 20 µL of 7.5% Na_2CO_3 were pipetted into the individual wells of a 96-well plate and kept in dark at 25ºC for 60 min. Absorbance at 765nm was measured using a microplate reader (Synergy HT, Biotek). The TPC was expressed as “mmol of gallic acid equivalent (GAE) per 100g of fresh rice seedlings shoot”.

Total Flavonoid Content (TFC)

The TFC was determined as per Zhishen et al. (1999) with minor modifications. In brief, 250µL of RSE, 1000µL of ultrapure water (Millipore) and 75µL of 5% sodium nitrite ($NaNO_2$) were added to the wells of a 96-well plate and held for 5 min at room temperature. Next, 150µL of 2% aluminium trichloride ($AlCl_3$) was added, and the plate kept at room temperature for another 6 min. Then, 500µL of 1M NaOH was added and the absorbance measured at 510nm as above. The TFC was expressed in units of “mmol of quercetin (QE) equivalent per 100g of fresh rice seedlings shoot”.

DPPH Scavenging Assay

The free radical scavenging ability of RSE was estimated using DPPH assay (Herald et al. 2012) with minor modifications. In brief, 200mM of DPPH solution was prepared using analytical grade absolute ethanol. Next, RSE and the positive control compound, Trolox (Sigma-Alrich, USA) were separately serially diluted with absolute ethanol to a range of 0.1mg/mL to 0.7mg/mL and 2mM to 0.01mM, respectively. Next, 100µL of the solutions were aliquoted into 96 wells microtiter plate and 100µL of DPPH added to each well. The plate was held in dark for 30 min at room temperature, and the absorbance at 517nm measured. The free radical scavenging capacity was estimated using the equation:

$$\text{DPPH free radical scavenging capacity} = \frac{(A0-A)}{A0} \times 100\%$$

(A0 = absorbance of control; A = absorbance of test sample)

Standard curve was constructed using serially diluted Trolox (2mM to 0.01mM). The Trolox equivalent antioxidant capacity (TEAC) was calculated based on the equation below and expressed as mmol Trolox/100g of fresh seedlings shoot.

$$\text{TEAC} = \frac{\text{mmol Trolox}}{\text{ml}} \text{ x } \frac{\text{ml}}{\text{mg seedlings shoot}} \text{ x } \frac{100\,000 \text{ mg seedlings shoot}}{100\text{g seedlings shoot}}$$

Auto-Aggregation Assay

Auto-aggregation is a process in which similar compounds bond as larger complexes at specific concentrations. This could result in peak-shifts in absorbance spectra, and thus should be avoided in all spectroscopy related assays. In this study, the absorbance spectrum of RSE was tested for auto-aggregation via absorbance titration in 50mM Tris-Cl (pH 7.4) at increasing concentrations (1.0 to 9.1mg/mL). This range was chosen as it includes the highest and lowest concentration of RSE used in this study. The absorbance spectra were measured from 300nm to 800nm using a Cary 300 spectrophotometer (Varian, Australia) and the absorbance peaks were determined using default parameters in the build in software.

Interactions Between RSE, DOX And DNA

Absorption spectral analysis was conducted based on published protocol (Pietrzak et al 2003; 2006). In summary, five separate solutions were prepared in 50mM Tris-Cl (pH 7.4). A control was set up with DOX diluted to a final concentration of 36.78μM. RSE was diluted to final concentrations of 1.25mg/mL (RSE1) and 2.5mg/mL (RSE2). RSE1-DOX mixture was prepared containing 1.25mg/mL of RSE and 36.78μM of DOX, and RSE2-DOX mixture contained 2.5mg/mL of RSE and 36.78μM of DOX. The absorbance (300nm to 800nm) of all solutions was measured spectrophotometrically as above. Next, each of the five solutions was individually added to a commercial DNA sample (G1521, Promega, USA) gradually to a final concentration of 56.2nM, and the absorbance recorded.

Fluorescence Quenching Effects Of RSE On DOX-DNA Complexation

Fluorescence spectral analysis was conducted as reported by Pietrzak et al. (2006) on a Cary Eclipse Fluorescence Spectrophotometer (Varian, Australia) in quartz cuvettes, using an excitation wavelength of 474nm with a 5nm excitation and 5nm emission widths. Control (DOX alone) samples were prepared by diluting DOX in 50mM Tris-Cl (pH 7.4) to a final concentration of 36.78μM and adding to DNA to obtain final DNA concentrations of 16.86nM to 56.20nM. The fluorescence spectra were measured after each addition. To determine the effects of fluorescence quenching on the DOX-DNA complex, two separate solutions, containing 36.78μM of DOX and 2.5mg/mL of RSE, or 36.78μM of DOX and 10μM of CHL, were prepared. They were individually added to the same amount of DNA samples as the control solutions, and the fluorescence spectra measured after each addition. Finally, the Stern-Volmer constant of samples was estimated as per Pietrzak et al. (2003) using the equation:

$$F_0 / F = 1 + K_{sv} [Q]$$

(F_0= fluorescence without quencher, F = fluorescence with quencher, K_{sv} = Stern-Volmer constant/drug-quenching rate constant, [Q] = molar concentration of quencher).

Mammalian Cell Culture

A cell line of *Rattus norvegicus* (rat) cardiomyocytes [H9c2(2-1), ATCC® CRL-1446] was used to test any cytotoxicity effects of DOX alone and a combination of DOX and RSE. The nasopharyngeal carcinoma cell line HK1 (kindly donated by Prof Lo Kwok Wai and Prof George Tsao) was used to investigate the combined effects of DOX and RSE. H9c2(2-1) was cultivated in DMEM containing 10% FBS and 100 units/mL PENSTREP. HK1 was cultivated in RPMI-1640 media containing 10% FBS and 100 units/mL PENSTREP. All media and antibiotics were obtained from Thermo Fisher Scientific (USA). All cells were incubated in a humidified mammalian cell incubator under 5% CO_2 at 37ºC. Sub-culturing was conducted when the cells reached 70 to 80% confluency and passage numbers were kept below 30 in all analyses.

Cytotoxicity Effects Of RSE, DOX, Ethanol And CHL

The H9c2(2-1) cells, grown as described above, were cultivated at 7 x 10^3 cells per well, while HK1 were cultivated at 1 x 10^4 cells per well in a 96-well plate. The cells were incubated under 5% CO_2 at 37°C for 24h to become semi-confluent (70 to 80%). Next, the RSE was dissolved at 100mg of seedlings (shoot tissue) per 100mL of absolute ethanol. After dilution with the media, 1mg/mL RSE contained 1% ethanol in the mixture. For treating H9c2(2-1), RSE and DOX were serially diluted with serum-free DMEM to final concentrations of 10.0 to 7.8 x 10^{-2} mg/mL (RSE) and 172.0 to 13.5µM (DOX). For treating HK1, serum-free RPMI was used to serially dilute RSE final concentrations of 10.0 to 7.8 x 10^{-2} mg/mL, DOX to 10.36 to 0.08µM, ethanol to 10% to 1% (v/v) and Copper Chlorophyllin (CHL) from 20 to 1.0µM. For treating H9c2(2-1), similarly diluted ethanol and CHL were used. Finally, the diluted ethanol/DOX/RSE/CHL solutions were added to cells, and the cells incubated for 24h under 5% CO_2 at 37ºC. The cell viability was determined using a tetrazolium salt (MTS reagent) based cell proliferation kit (Cat no G3582, Promega, USA). In brief, after the 24h treatment period, the media were drained, the cells were rinsed with phosphate buffered saline (pH 7.4) (Thermo Fisher Scientific, USA), replenished with 100µL of respective serum-free medium and mixed with 20µL of MTS reagent. Next, the cells were incubated under 5% CO_2 at 37°C for another 4h and the absorbance at 490nm was measured using the microplate reader. The cytotoxicity of RSE and/or DOX was determined by identifying their LD_{50} (Median Lethal Dose). Each essay was conducted in three technical triplicates, while assay involving RSE was run with three independently extract RSE solution.

Protective Effects Of RSE On DOX-Induced Cytotoxicity

The H9c2(2-1) were treated with 36.78µM DOX and 2.5mg/mL RSE in serum-free DMEM, while HK1 cells were treated with 1.0µM DOX and 2.5mg/mL RSE in serum-free RPMI. As a positive control for the measurement of the 'interceptor' properties, 10µM of CHL was added to H9c2(2-1) containing 36.78µM of DOX, and to HK1 containing 1µM of DOX. All reagents were first diluted in ethanol prior to the induction process to maintain a constant ethanol concentration at 2.5% (v/v) in all testing condition. The cells were further incubated for 24, 48 and 72h before determining cell viability assay kit as above. Each essay was repeated three times as described in section above and any morphological changes to the cells were visualized and photographed using a phase contrast microscope and a camera. The significances level of each comparison was calculated using GraphPad software on a standard student t-test model (Unpaired, two tailed).

RESULTS AND DISCUSSION

Biochemical Assessments Of RSE

The present study investigated the antioxidant properties of the rice seedling extracts (RSE). The results per 100g of fresh rice seedlings shoot are summarised in Table 1. Briefly, the TPC, TFC and TEAC of RSE were 0.058mmol GAE/100g, 0.536 mmol QE/100g, and 0.464 mmol TE/100g, respectively. The results indicate that the TPC content of RSE is approximately 10 times less than that of wheatgrass extracts (0.700 mmol GAE/100g), and its DPPH scavenging capacity is about three times less (0.464 vs. 1.4 mmol TEAC/100g), but they are similar in TFC levels (0.536 vs 0.50 mmol QE/100g). The comparison of nutritional content in rice seedlings and wheatgrass has been summarised in a previous study (Chomchan et al. 2016). They have reported that "Ricegrass juice contained great level of phenolic acid molecules. Even if rice grass juice gave less statistically ability on antioxidant activities, they still had comparable levels of antioxidant activities". A separate study (Khanthapoka et al. 2015) has also demonstrated the antioxidant and DNA protection capacity of juice extracted from rice seedlings. Due to the differences in sample preparations, the results cannot be directly compared to the present study. Nevertheless, the present study has shown that RSE contains significant antioxidant capacity and might contribute towards a reduction in the activities of cell's oxidative damage pathways. However, further investigations are needed for testing this hypothesis, as the protective effects might not be a direct, or only, result of the antioxidant capacity.

Auto-aggregation test was conducted to check whether the absorbance spectra of RSE changed due to aggregation or complexation of components. The spectra were found to remain relatively constant within the concentration range tested (Supplementary material Fig. S1). By examining the peak maxima, the extinction coefficient of RSE at the optimal absorbance wavelength (679nm) was calculated to be 25.47 ± 0.17mL cm^{-1} g^{-1} for all concentrations, with no peak-shifts observed. Thus, any changes in absorbance spectra that could be due to the auto-aggregation within the RSE were considered negligible in the rest of the study.

The mixing of DNA with RSE or CHL did not show any changes in the absorbance and fluorescence spectra (data not shown), suggesting little interaction between them, if any. However, the absorption spectra of DOX mixed with RSE (1.25 to 2.50 mg/mL) illustrated characteristic changes (Fig. 1) compared to those of solutions containing RSE or DOX alone at identical concentrations. The results suggest that the two components may form a complex when mixed. A similar pattern has been reported in a study on complexation of CHL and DOX (Pietrzak et al. 2006); specifically, the absorption peak at 474nm of a solution containing DOX-RSE was red-shifted compared to DOX alone. However, further studies such as NMR or Raman scattering spectroscopy are needed to validate (or negate) the hypothesis of complexation.

Titration of DOX with DNA in the presence of RSE or CHL illustrated the quenching effects of the latter two against DOX-DNA complexation, through a decrease of fluorescence. RSE, at a final concentration of 2.5mg/mL, showed significant quenching effects (Fig. 2). The dose-dependent quenching effects of RSE were weaker compared to CHL at 10μM but displayed a similar pattern to it. The quenching effects of RSE are similar to those reported in another study (Pietrzak et al. 2006). The Stern-Volmer quenching coefficient (K_{sv}) was calculated in the presence of quencher (Agudelo et al. 2013). The K_{sv} for RSE was (4.62 ± 0.06) x 10^{-2} mg^{-1}, while that for CHL was (3.78 ± 0.03) x 10^{2} mg^{-1}. Even though the apparent K_{sv} for CHL is four orders of magnitude greater than that of RSE, it should be noted that the concentration of RSE is expressed as fresh seedling weight (shoot tissue), while CHL is expressed as concentration of the pure compound. CHL could also contain impurities such as metal ions, depending on the manufacturer, that could result in inconsistency of results. In addition, natural

chlorophyll, when excited by light, produces significant fluorescence (Satoh and Butler 1978) that could interfere with the results. In this study, the concentration of RSE was kept to a maximum of 2.5mg/mL to minimize interference of any compounds with excitation wavelengths of 400 to 500nm. At this concentration, no visible fluorescence was observed (data not shown). The results thus strongly suggest that in aqueous settings, certain compounds in RSE may form complexes with DOX, indicating the potential of RSE to reduce association of DOX with DNA and hence side effects of DOX. The presence of RSE-DOX complexes was demonstrated by the signature spectral shift of the absorption patterns during the mixing of RSE and DOX, accompanied by quenching of fluorescence of the DNA-DOX complex. However, the mechanisms of complex formation need to be investigated further. It also needs to be noted that RSE could contain a number of different components, some of which may specific to the rice variety tested, hence the work may not represent the results for all rice varieties.

Protective Effects Of RSE Towards DOX-Induced Cytotoxicity

The effects of RSE on DOX-induced cytotoxicity were investigated using mammalian cell culture assay in two ways. Any cytotoxicity effects of ethanol, DOX, RSE and CHL on the rat H9c2(2-1) cardiomyocytes and nasopharyngeal carcinoma HK1 cells were tested by determining their individual medial lethal dose (LD_{50}). Ethanol (the solvent for RSE preparation) was used as control for testing the background activity, and CHL was used as assumed positive control for cytoprotection (or negative control for cytotoxicity), due to its status as approved health supplement (EC 1994). Next, DOX was co-administered with either RSE or CHL. The cells were incubated for 24, 48 or 72h. The viability of cells was tested using commercial cell proliferation assay (Cat no G3582, Promega, USA).

The LD_{50} of ethanol, RSE and DOX for H9c2(2-1) were 5.771 ± 0.415%, 5.914 ± 1.182mg/mL, and 32.473 ± 3.808μM, respectively (Table 2). For HK1, these were 3.804 ± 0.971%, 4.065 ± 0.617mg/mL, and 0.969 ± 0.093μM, respectively. CHL showed no cytotoxicity in both cell lines at all concentrations tested (data not shown), which was expected, as CHL has been approved as a health supplement more than two decades ago (EC 1994). The cytotoxicity of DOX towards cardiomyocytes was dose-dependent (LD_{50} of 32.473μM). The changes in morphology and the LD_{50} were similar to previously reported data (IC_{50} of 30.1μM) (Maillet et al. 2016). The LD_{50} of RSE was similar to that of ethanol, suggesting RSE has no enhanced cytotoxicity (Fig. 3). The cell viabilities remained at >80% when cells were treated with either 2.5mg/mL of RSE or 10μM of CHL (Fig. 3). According to International Organization of Standardization (ISO), “cellular response with cell viability that falls within 70% and above is considered non-cytotoxic” (Wallin and Arscott 1998). Thus, these can be considered as safe working concentration of the RSE and CHL samples tested.

For testing of cytoprotective effects, the induction concentration of DOX for H9c2(2-1) was set at 36.78μM, as it was the optimum concentration to form a complex with RSE or CHL, based on the results of the fluorescence and absorption spectroscopy assay (Fig. 1 and Fig. 2) and it was close to its LD_{50} for H9c2(2-1) (Fig 3). The induction concentration of DOX for HK1 was set at 1μM as it was close to its LD_{50} for HK1. The induction concentration of CHL was chosen as 10μM, as it had demonstrated the ability to hinder the formation of DOX-DNA complex in the fluorescence spectroscopy assay, and no interference in the MTS assay (data not shown). H9c2(2-1) cells co-administered 36.78μM DOX and 2.5mg/mL of RSE showed significant ($P<0.05$) increase in cell viability, compared to cells administered without RSE or CHL (Fig. 4). H9c2(2-1) co-administered with 36.78μM DOX and 10μM of CHL also showed a significant increase in cell viability ($P<0.05$). HK1 co-administered 1μM DOX and 2.5mg/mL of RSE did not show any significant increase in viability when compared to cells induced with DOX only (with ethanol). However, HK1 co-administered with 1μM of DOX and 10μM of CHL showed a highly significant ($P < 0.001$) increase in cell viability after the treatment. The full data of cell viability have been attached separately

(Supplementary material, Table S1). Both HK1 and H9c2(2-1) cell treated with DOX had showed distinct morphological changes following 24 hours treatment period. H9c2(2-1) lost its original elongated spindle shaped-like structure and got detached from the bottom plate (Hescheler et al. 1991; Priya et al. 2017). Meanwhile, HK1 lost its original squamous epithelial appearance and had aggregated into clumps after the treatment (Huang et al. 1980) (Supplementary material, Fig. S2 and Fig. S3). The addition of CHL in the HK1 cell line during the treatment has shown clear improvement to the viability of the cells, but the effects were less obvious in the treatment of H9c2(2-1) cells.

In summary, both RSE and CHL demonstrate the ability to increase the cell viability after co-administered with DOX in H9c2(2-1) cells at the tested concentration. The addition of CHL during co-administration with DOX does not only significantly increase the cell viability of HK1 after the treatment period but have completely neutralized the toxicity of DOX in HK1 at the tested concentrations (cell viability > 70%, Fig. 4). The results demonstrate that although CHL may provide better protection in H9c2(2-1), it had undesirable effects of significantly reducing the efficacy of the anti-neoplastic drug DOX towards HK1. On the other hand, the introduction of RSE during the DOX treatment resulted in a significant increase in cell viability in H9c2(2-1) but did not significantly decrease the efficacy of the drug towards HK1 in the tested condition. Since the binding constant of RSE towards DOX was much lower than CHL, it is speculated that the reduction in DOX concentration (36.78μM vs. 1μM) in the HK1 assay had made the formation of RSE-DOX complex unlikely. This hypothesis, however, could not be directly tested, as the fluorescence intensity of DOX at 1μM was below the limit of detection of the equipment (data not shown).

The likely interaction between DOX and components of RSE noted in this study could be further investigated using techniques such as NMR, FT-IR or Raman scattering. The RSE could also be fractionated and the protective effects of different fractions could be evaluated by *in vitro* cell culture studies and/or animal models. The beneficial compounds could be purified and characterised using LC-MS-MS and MALDI-TOF Mass Spectrometry for development into a cardioprotective agents that could compliment DOX treatment in the clinical settings.

CONCLUSIONS

The results of the study indicate that fresh seedling extracts of a Malaysian local rice variety, Biris, contain a significant amount of antioxidants and also interfere with complexation of DNA with a major anthracycline anticancer drug, doxorubicin (DOX). Furthermore, RSE imparts significant cytoprotective effects to rat cardiomyocytes treated DOX and does not compromise the efficacy of DOX towards nasopharyngeal carcinoma cells. The properties of RSE can be further exploited for its development as a natural cardioprotective agent to complement the DOX treatment.

ACKNOWLEDGEMENTS

We are most grateful to Professor Lo Kwok Wai (Department of Anatomical and Cellular pathology, Chinese University of Hong Kong) and Professor George Tsao (School of medicine, University of Hong Kong) for providing the HK1 cell line. The work was supported by a Swinburne University of Technology Competitive Grant (StraG Grant 2-5605).

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Table 1. Antioxidant contents of RSE.

Assay	RSE*	Wheatgrass*	Ricegrass**
TPC (mmol GAE/100 g)	0.058 ± 0.009	0.7	2.6
TFC (mmol QE/100g)	0.536 ± 0.011	0.5	N/A
DPPH (mmol TEAC/100g)	0.464 ± 0.098	1.4	8.8

Units expressed as *per 100g of fresh rice seedlings in this work and wheatgrass (Kulkarni et al. 2006) and **per 100g of dried extracts of rice seedling (Khanthapoka et al. 2015). [1]mean ± standard deviation of three biological replicates.

Table 2. Median Lethal Doses (LD_{50}) of solvent, RSE, DOX and CHL for H9c2(2-1) and HK1 cells.

Sample	LD_{50} on H9c2(2-1)	LD_{50} on HK1
Solvent control (Ethanol, %v/v)	5.771 ± 0.415	3.804 ± 0.971
RSE (mg/mL)	5.914 ± 1.182	4.065 ± 0.617
DOX (μM)	32.473 ± 3.808	0.969± 0.093
Negative Control (CHL, μM)	N/A	N/A

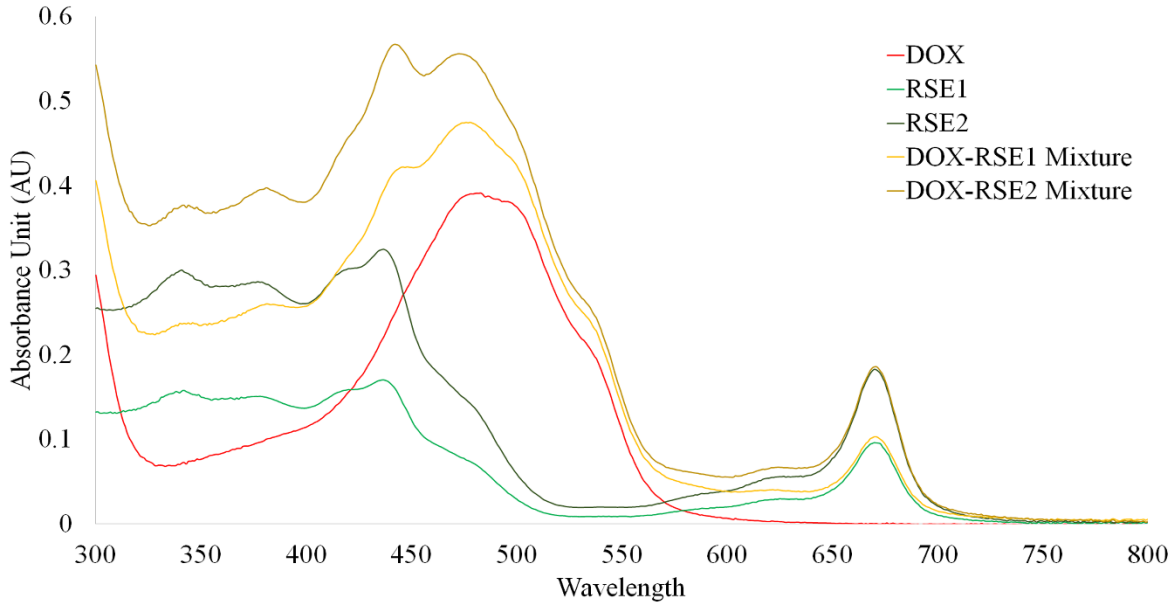

Figure 1. Absorbance spectra of DOX and RSE individually and in mixtures.

DOX: 36.78μM; RSE1: 1.25mg/mL; RSE2: 2.50mg/mL; DOX-RSE1 Mixture: 1.25mg/mL; + 36.78μM; DOX-RSE2 Mixture: 2.5mg/mL + 36.78μM

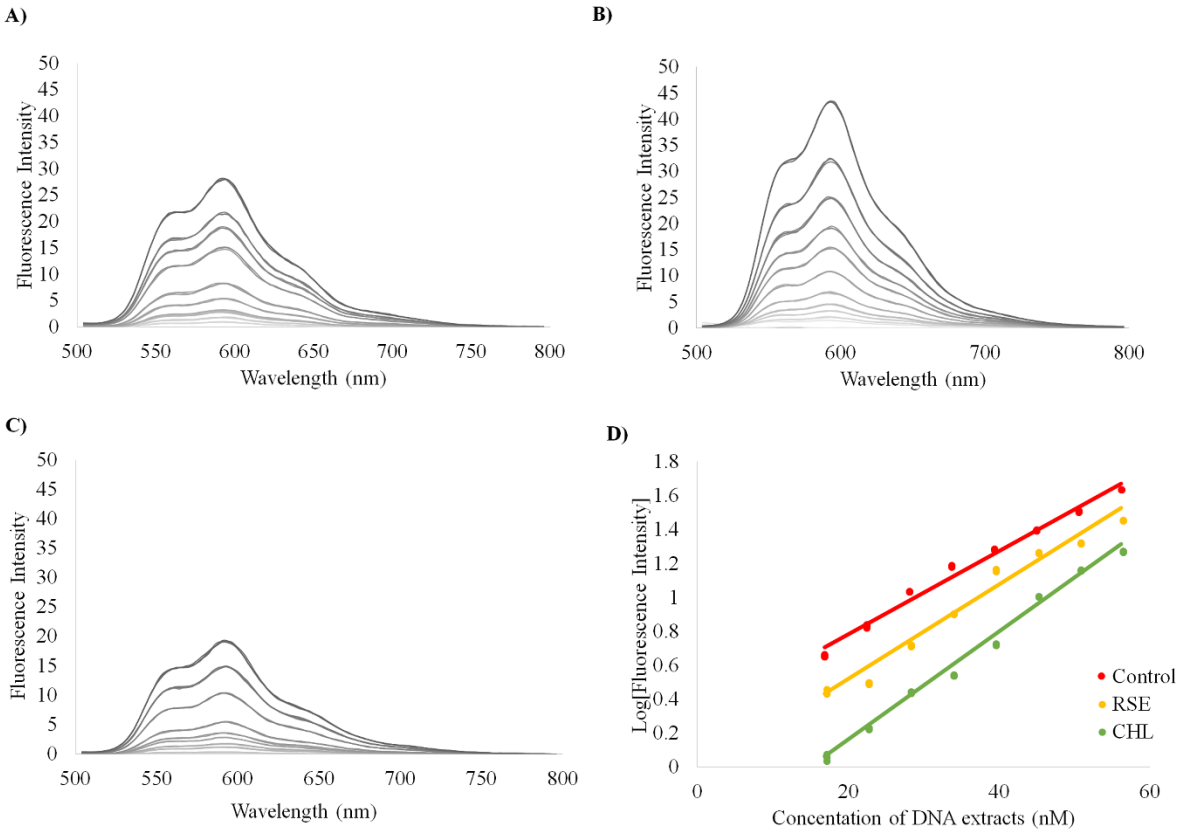

Figure 2. Fluorescence spectra of DOX, CHL and RSE alone and mixed with DNA.

(A) Control (36.78 μM DOX only); (B) 2.5mg/mL RSE and 36.78 μM DOX; (C) 10 μM CHL and 36.78 μM DOX; (D) Changes in fluorescence intensities of DOX-DNA at 592nm in the presence of RSE (2.5mg/mL) or CHL (10μM). DNA concentrations used: 16.86nM to 56.2nM.

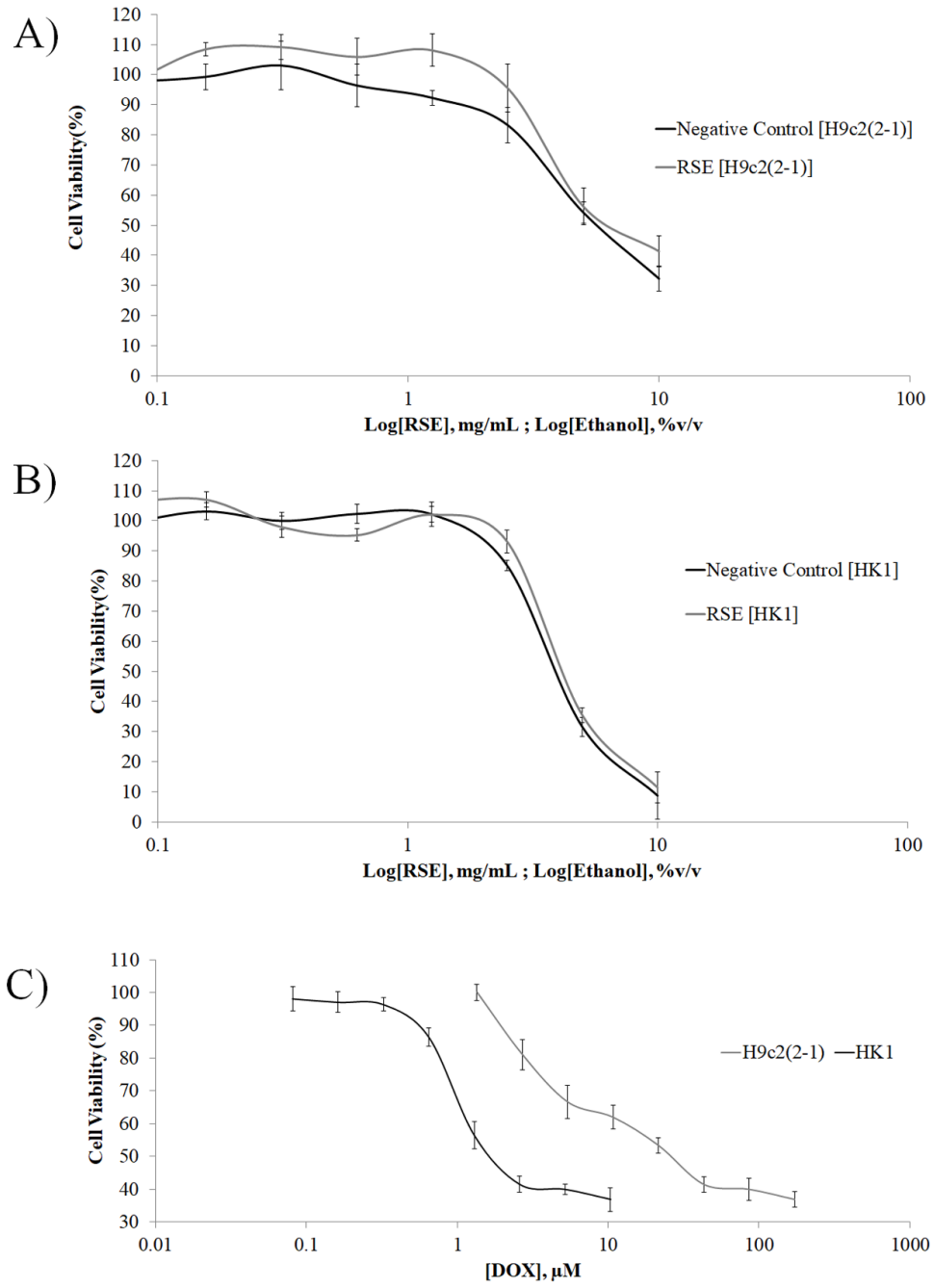

Figure 3. Cell viabilities following treatment with RSE, ethanol or DOX. Data represent the mean ± standard variation of 3 biological replicates.

(A) Cell viabilities of H9c2(2-1) after treatment with ethanol or RSE; (B) Cell viabilities of HK1 after treatment with ethanol or RSE; (C) Cell viabilities of H9c2(2-1) and HK1 after treatment with DOX. All treatments were for 24h. In A and B, the numerical value of the concentration of RSE (mg/mL) is identical to the numerical value of concentration of ethanol (%v/v) and the x-axis was merged.

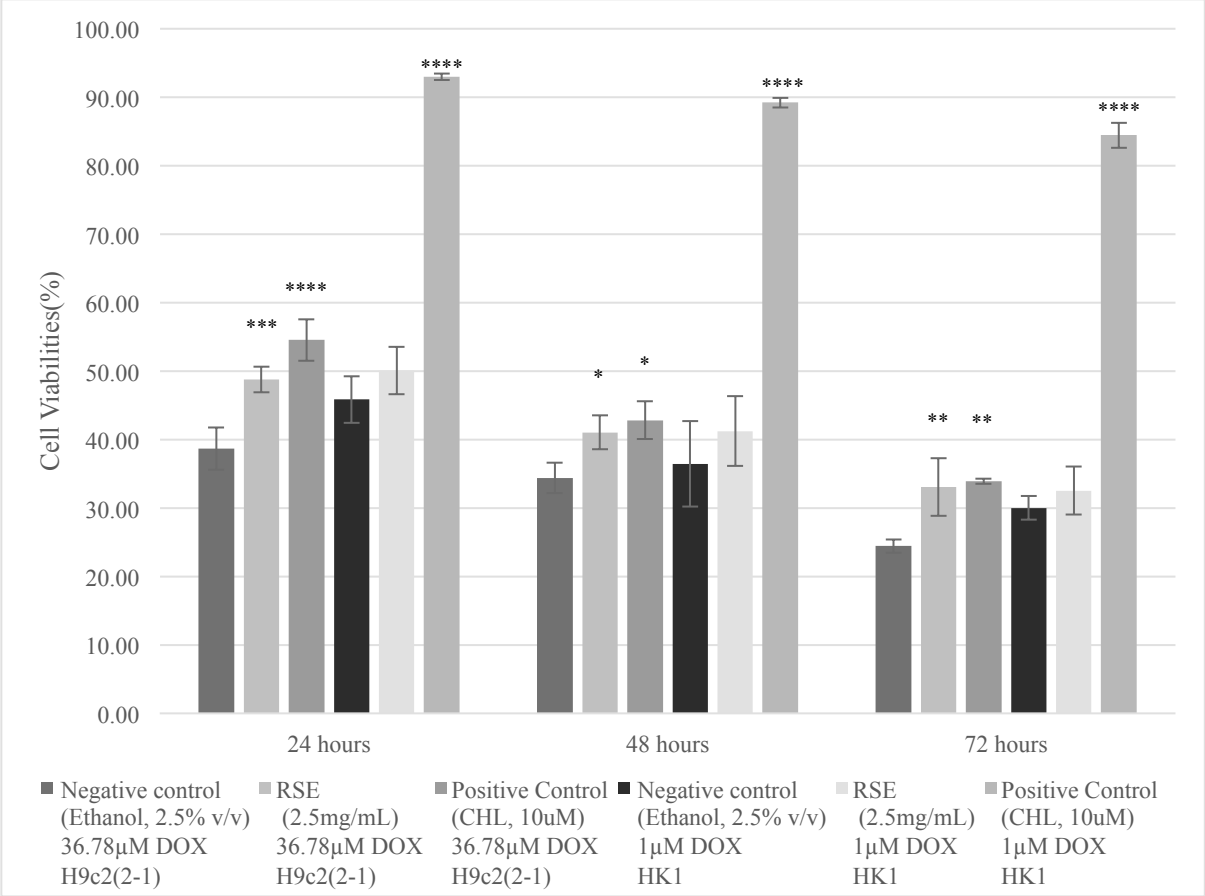

Figure 4. The cell viabilities following treatment with DOX, DOX+RSE and DOX+CHL.

Data represent the mean ± standard variation of 3 biological replicates. Asterisks denotated significances level between sample and negative control (*: p<0.05; **: P<0.01; ***: P<0.005; ****: P<0.001).

APPENDIX

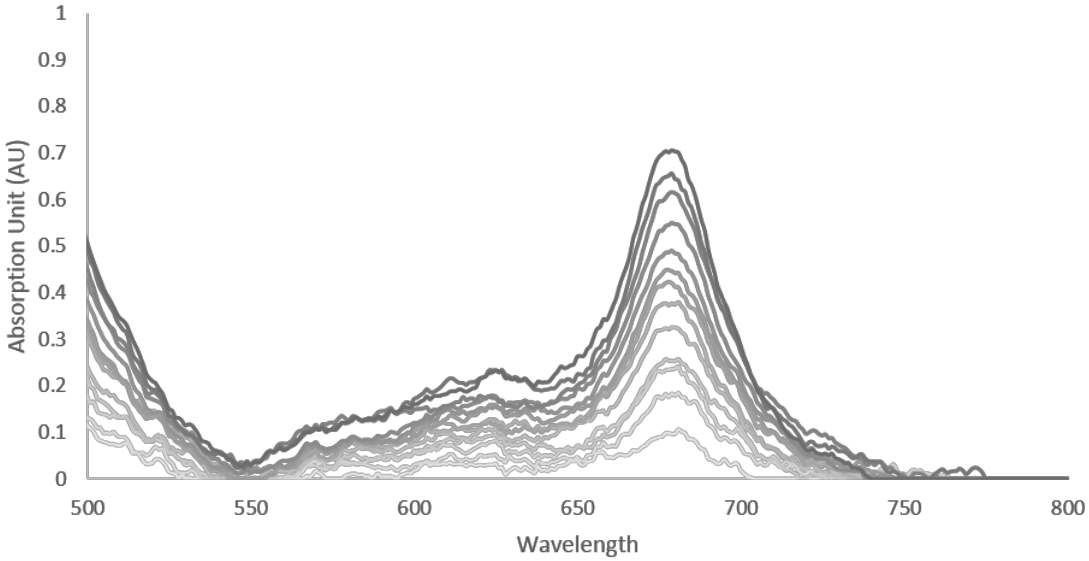

Figure S1. Absorption spectra of RSE (1.0 to 9.1mg/mL) in 50mM Tris buffer (pH 7.4).

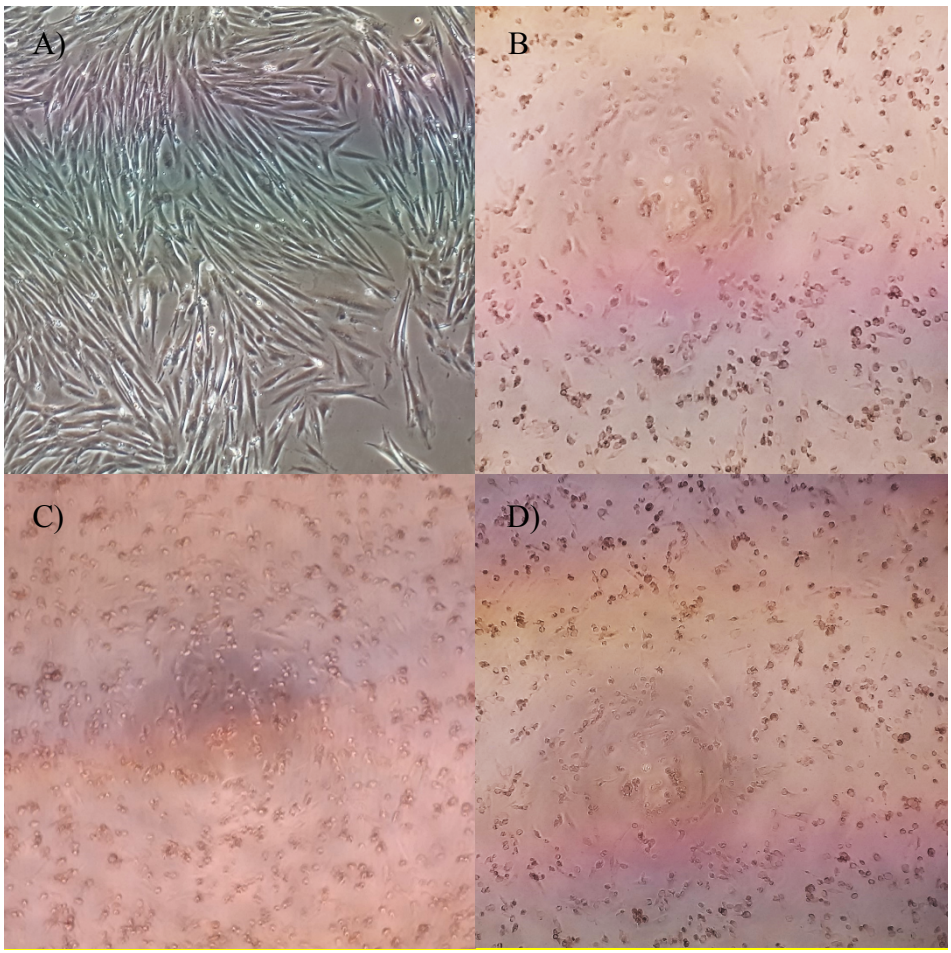

Figure S2. Morphological changes on H9c2 (2-1) at 24 hours following co-treatment of DOX with either 2.5mg/ml RSE or 10μM CHL.

A) Negative control consisting of 2.5% ethanol solution only; B) Cells treated with 2.5% ethanol and 36.68μM DOX solution; C) Cells treated with 36.68μM DOX and 2.5mg/ml RSE solution (Dissolved in ethanol); D) Cells treated with 36.68μM DOX and 10μM CHL solution (Dissolved in ethanol).

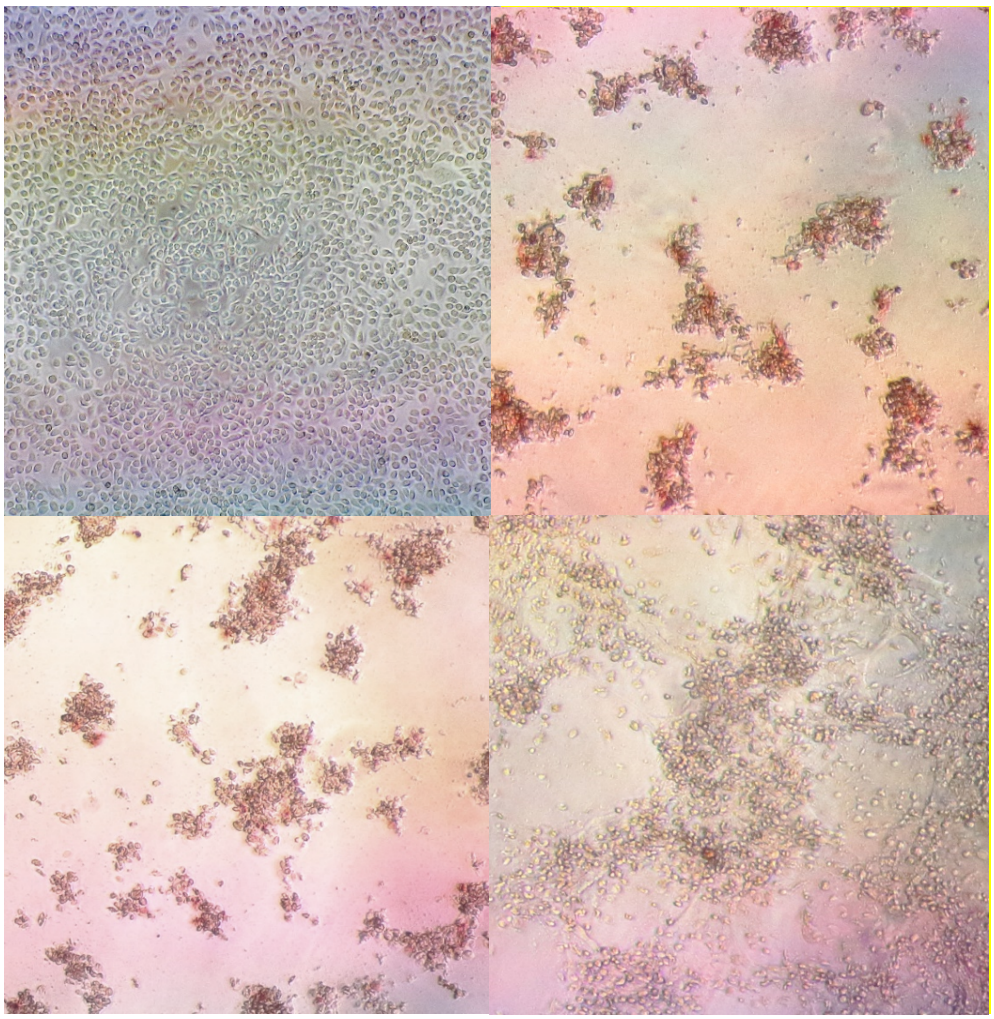

Figure S3. Morphological changes on HK1 at 24 hours following co-treatment of DOX with either 2.5mg/ml RSE or 10μM CHL.

A) Negative control consisting of 2.5% ethanol solution only; B) Cells treated with 2.5% ethanol and 36.68μM DOX solution; C) Cells treated with 36.68μM DOX and 2.5mg/ml RSE solution (Dissolved in ethanol); D) Cells treated with 36.68μM DOX and 10μM CHL solution (Dissolved in ethanol).

Table S1. Cell viabilities of H9c2(2-1) and HK1 following treatment with DOX, RSE or CHL.

	24 hours	48 hours	72 hours
Negative control (Ethanol, 2.5% v/v) 36.78μM DOX H9c2(2-1)	39 ± 3.1%	34 ± 2.2%	24 ± 1%
RSE (2.5mg/mL) 36.78μM DOX H9c2(2-1)	49 ± 1.9%	41 ± 2.5%	33 ± 4.2%
Positive Control (CHL, 10uM) 36.78μM DOX H9c2(2-1)	55 ± 3%	43 ± 2.8%	34 ± 0.4%
Negative control (Ethanol, 2.5% v/v) 1μM DOX HK1	46 ± 3.4%	36 ± 6.3%	30 ± 1.7%
RSE (2.5mg/mL) 1μM DOX HK1	50 ± 3.5%	41 ± 5.1%	33 ± 3.5%
Positive Control (CHL, 10uM) 1μM DOX HK1	93 ± 0.5%	89 ± 0.7%	84 ± 1.9%