RESEARCH ARTICLE

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Comparative Studies of Resveratrol, Oxyresveratrol and Dihydrooxyresveratrol on Doxorubicin-Treated Lung Cancer Cells

Waraporn Yahayo[1], Veerachai Pongkittiphan[2], Roongtawan Supabphol[1], Somrudee Saiyudthong[1]*

Abstract

Objectives: This research aims to comparatively investigate the capability of resveratrol (RES) and RES analogues, oxyresveratrol (Oxy-RES) and dihydrooxyresveratrol (DHoxy-RES), to potentiate doxorubicin (DOX) effects against lung carcinoma epithelial cells. **Methods:** All experiments were performed on lung carcinoma cell lines (A549) with DOX combination between DOX and RES or RES analogues. Cell viability or growth inhibitory effect was assessed by MTT assay and genes associated with survival and metastasis were monitored by real-time polymerase chain reaction (RT-PCR). **Results:** DOX obviously demonstrated cytotoxic and anti-metastatic activities against A549 cells. Expression of gene-associated with both activities was potentiated by RES and RES analogues. Oxy-RES showed highest capability to potentiate DOX effects. DHoxy-RES showed nearly no effect to DOX activities. **Conclusions:** These results provided an important basis of DOX combination with RES analogues, especially Oxy-RES, for better therapeutic effect. Further studies in human should be performed on exploring combination of DOX and Oxy-RES.

Keywords: Chemotherapy- *p53*- *SIRT1*- MMPs- CAMs

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Introduction

Lung cancer is one of the most common cancer among male and female in worldwide for several decades. It is classified as the leading cause of cancer-related mortality with complex and multiple etiologic factors, especially industrialization and environmental pollution. Majority of lung cancer cases are due to smoking and consuming tobacco products [1-3]. Thoracic surgery has been developed for more modern techniques and considered as the standard care for patients with early stage of lung cancer. Video-assisted thoracoscopic surgery is state of the art for lung resections with less invasive approach. Common treatments of lung cancer include surgery, radiation therapy, systemic therapies, or a combination of these treatments. Surgical resection might be effective for early-stage and non-metastatic lung cancer. Systemic treatment means to use the drug or substance that spreads throughout and targets the entire body. Most systemic therapy is called chemotherapy. The important aim of chemotherapy is to completely inhibit and destroy original and metastatic cancer mass, including to cause the least possible damage to healthy cells. The advances in systemic therapy have been developed from molecularly targeted therapeutics, checkpoint inhibitors, anti-angiogenic agents, and immunotherapy. These are expected for highly selective target to effect only cancer cells, but not to the healthy cells [4-6].

Chemotherapy can be used as the curative treatment to inhibit cancer cells reproducing, prevent cellular growth and metastasis. It is frequently combined with surgery or radiation to get more effective results. However, chemotherapy can produce common and serious side effects. Nausea and vomiting are the most common side effects found after treatment. Cancer cells are grown with very rapid and uncontrolled division rate. The mechanism of current chemotherapy is to suppress cancer cell cycle and eradicate cancerous mass. Besides cancer cells, it can also damage healthy cells with rapid-growing rate, such as cells in bone marrow, digestive tract, reproductive system, and hair follicles. These cells are very important for human body, especially cells forming in the bone marrow which affect to the function of blood and immune system. These lead to the medical non-adherence and cause the failure of cancer treatment [7-9].

Doxorubicin (DOX), launched under the trade name Adriamycin, is widely recognized as an effective cytotoxic medication via intravenous route for a various types of malignant tumors including lung cancer. It can be used as a first line drug or in combination with surgery, radiation, or

*[1]Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand. [2]KMITL research and innovation services, King Mongkut's Institute of Technology Ladkrabang, Bangkok 100520, Thailand. *For Correspondence:*

some other anticancer agents. Cellular level mechanisms of DOX are reported to act via the multiple targets [10-12]. The serious obstacle of DOX is not only cardiotoxicity but also drug resistance. Drug resistance is the important limitation for chemotherapy. Cellular mechanisms of DOX resistance in lung cancer were studied and factors caused drug resistance were reported [13-15]. Prevention of DOX resistance and/or side effect has been searched to enhance DOX efficacy. A new type of nanomicelles loaded with doxorubicin and curcumin was used to attenuate DOX resistance in lung cancer cell line, A549/Adr cells [16]. Many plant extracts have also been employed to enhance DOX efficacy and decrease its drug resistance.

Sufficient evidence both in clinical and preclinical studies has confirmed DOX-induced cardiotoxicity in myocardial cells via oxidative stress and apoptosis with the reduction of SIRT1 level. SIRT1 activation plays the important key role for the regulation of multiple cellular functions. It protects sestrin-2 (SESN2) from ubiquitination, the sufficient SESN2, in turn, activate adenosine monophosphate-activated protein kinase alpha (AMPKα) and results in nuclear factor erythroid 2-related factor 2 (NRF2) to attenuate oxidative stress from DOX [17-19].

Resveratrol (3, 5, 4′-trihydroxystilbene, RES), the very well-known natural non-flavonoid polyphenol, is abundantly found in grapes, apples, berries, plums, rhubarb, peanut, etc. It is one of the most researched natural polyphenolic stilbenoid as a promising compound for both nutritional and therapeutic purposes. Several *in vitro* an *in vivo* studies have been reported its beneficial effects against oxidative stress, glycation, inflammation, age-related changes or aging, and many types of cancer. The cardioprotective and neuroprotective activities have been mentioned frequently. The very popular function often mention is powerful SIRT1 activator [20, 21].

Accumulated evidence of RES also refers to therapeutic benefit for many kinds of cancer. The combination of low concentration DOX and RES could show the additive effect on antitumor activity on bladder cancer cells when compared to RES or DOX alone [22].

RES prevents epithelial-mesenchymal transition (EMT) and improves the DOX-resistance of gastric cancer through the modulation of PTEN/Akt signaling pathway [23]. RES can repress hypoxia-induced resistance to doxorubicin found in breast cancer cells by decreasing HIF-1α protein expression [24].

One of the major challenges in cancer therapy is anticancer drug resistance which leads to recurrence after treatment including metastasis. Drug resistance is believed as a result of multiple activities of neoplastic cells through several known and unknown mechanisms [25-27]. Searching for new natural compounds to cure or synergist with standard anticancer agents is the extreme hope for cancer patients facing with the failure of cancer chemotherapy. Because of several evidence against a variety of cancer types, RES has been expected for the most promising approach to cancer therapy [28-33]. Structure-modification has been studied to implicate RES analogs for better pharmacological activities and further development as pharmacologic agents for clinical application [34-37]. Oxyresveratrol (Oxy-RES) and dihydrooxyresveratrol (DHoxy-RES) are the interesting analogues and further studied its potential for an anticancer agent. Oxy-RES possesses 2-fold higher antioxidant activity than RES. RES and Oxy-RES have been mentioned to inhibit the gene expression of cancer stem cell markers, these can make cancer stem cells turn to a hypoxia-associated tumor [38]. Oxy-RES has been reported for anti-liver cancer pharmacological targets through down regulation of (1) estrogen receptor 1 (ESR1) found in primary malignancy and during metastasis (2) epidermal growth factor receptor (EGFR) which is responsible for cell proliferation and signal transduction (3) vascular endothelial growth factor (VEGF) receptor 3 and VEGF-C which can lead to the inhibition of both angiogenesis and lymph node metastasis [39]. Oxy-RES has been demonstrated to inhibit human colon cancer cell migration [40], human bladder cancer cell growth [41] and human breast cancer cell cycle [42]. No evidence of DHoxy-RES to cancer cells has been found. Most of therapeutic studies have been explored about melanin hyperpigmentation i.e. inhibitors of melanogenesis [43, 44].

The current study focuses on the comparison of RES, Oxy-RES and DHoxy-RES to potentiate DOX treatment in lung cancer cells. If possible, it might develop a new strategy in combination of conventional chemotherapeutic drugs and RES or RES analogue to enhance the survival rate of lung cancer patients.

Materials and Methods

Chemicals and reagents

Cell culture medium and supplements were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide were purchased from Sigma-Aldrich (St Louis, USA). TRIzol reagent was ordered from Thermo Fisher Scientific, Inc., USA and SuperScriptTM Reverse Transcriptase kit was obtained from Invitrogen, USA.

Cell line and cell culture

Adenocarcinomic human alveolar basal epithelial cell line (A549) was gifted from The Institute of Biotecnology and Genetic Engineering, Chulalongkorn University, Thailand. Cells were maintained in Dulbecco′s Modified Eagle′s Medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were placed in an atmosphere of 95% air and 5% CO_2 at 37˚C.

Cell viability assay

MTT assay was performed to determine the cytotoxicity as described previously [45]. The cells were seeded in 24-well plates at a density of 7×10^4 cells/well in 24-well plates, cultured for 24 h and treated with DOX, DOX with RES, DOX with Oxy-RES and DOX with DHoxy-RES for 3 days. All experiments were performed in triplicate. At the end of treatment period, the culture medium was discarded and cells were incubated with 1 mg/ml MTT solution for 3 h. After then dimethyl sulfoxide was added

to each well. The absorbance of dark blue formazan product was measured at 570 nm using enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotex-synergy-HT). The cell viability was calculated as a percentage of the control (untreated cells).

Quantitative real-time polymerase chain reaction (qPCR)

After treatment with DOX, DOX with RES, DOX with Oxy-RES and DOX with DHoxy-RES for 3 days, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc., USA) in accordance with the manufacturer's instructions. The purity and integrity of total RNA was confirmed at 260/280 nm ratio. cDNA synthesis was performed from 1.0 µg of total RNA using a SuperScriptTM Reverse Transcriptase kit (Invitrogen, USA). Gene expression of *p53, MDR, SIRT1, MMP2, MMP9*, *E-cadherin* and integrin, was quantified in comparison with an internal control (GAPDH) by quantitative real-time PCR using CFX96 Touch Real-Time PCR Detection System (BIO-RAD, USA) (Table 1) [46].

Table 1. Primers Used for Real–Time PCR Amplification.

Genes	Primer sequence (5' → 3')		Product size (bp)
p53	Forward primer:	TTGCGTGTGGAGTATTTGGA	100
	Reverse primer:	AGTGGATGGTGGTACAGTCAG	
SIRT1	Forward primer:	ACTGAAAAACCCCCACGAAC	149
	Reverse primer:	GCTGCTTGGTCTAAAAGTGTGA	
MMP2	Forward primer:	CAGGAGGAGAAGGCTGTGTT	139
	Reverse primer:	AGTTAAAGGCGGCATCCAC	
MMP9	Forward primer:	AGTCCACCCTTGTGCTCTTC	119
	Reverse primer:	CGACTCTCCACGCATCTCTG	
E-cadherin	Forward primer:	AGCGTGTGTGACTGTGAAGG	138
	Reverse primer:	CAGCAAGAGCAGCAGAATC	
Integrin	Forward primer:	CGTAGCAAAGGAACAGCAGA	142
	Reverse primer:	GGTCAATGGGATAGTCTTCAGC	
GAPDH	Forward primer:	AGTCCACTGGCGTCTTCACC	119
	Reverse primer:	GTTCACACCCATGACGAACATG	

Statistical analysis

All experimental data are expressed as mean and standard deviation (SD) from at least three independent experiments. - One way ANOVA followed by Dunnett's test using SPSS IBM Singapore Pte Ltd (Registration No.1975-01566-C) was used to test the significant differences between groups and the significance was accepted at $p<0.05$.

Results

Cell viability assay

To determine whether RES and RES analogues can potentiate DOX effect to cancer cells, A549 cells were treated with culture media containing DOX only and DOX added to RES or RES analogues. As the concentration of doxorubicin increased, the inhibitory effect on the proliferation of human lung carcinoma, A549, was enhanced with lower absorbance value as shown in Figure 1. Dose-response curves (% cell survival and DOX concentration) of three independent sample preparations were used to evaluate average IC_{50} values. DOX exhibited anti-proliferative activity with IC_{50} values 13.07 ± 1.38 µM. DOX concentration of 1.0 and 2.5 µM exhibited the proliferative inhibition at 91.77 ± 0.19 and 84.86 ± 0.64 %, respectively. These two concentrations of DOX were further selected for the combination with RES or Oxy-RES or DHoxy-RES because the low cytotoxicity not more than 80 % can guarantee the good cellular health.

As indicated in Figure 2, % A549 survival exposed to the combination of DOX and 50 µM of RES, Oxy-RES or DHoxy-RES were lower than that exposed to DOX alone. Oxy-RES seemed to inhibit cell proliferation better than RES and DHoxy-RES.

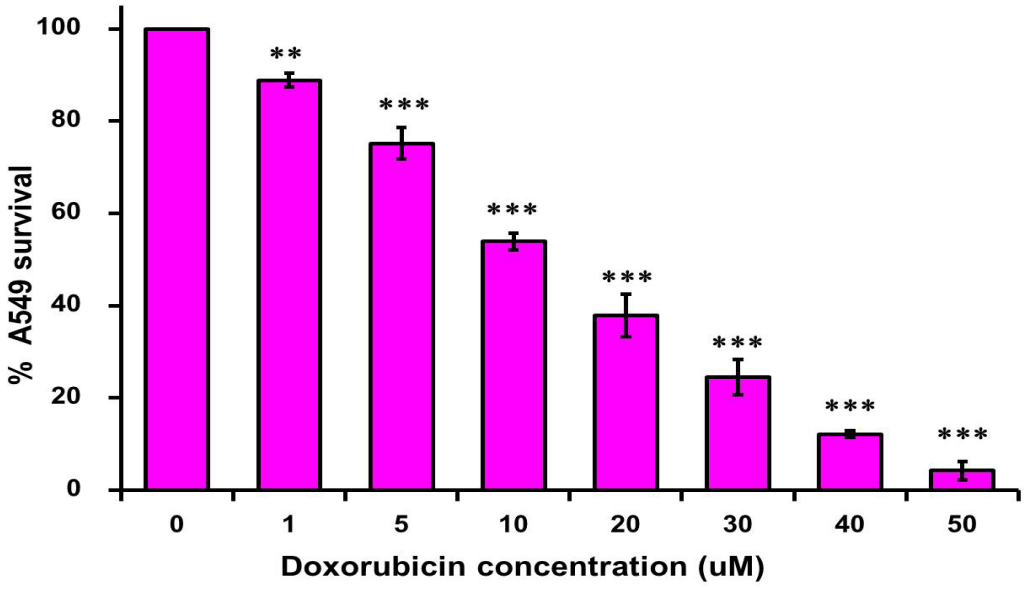

Figure 1. Doxorubicin Inhibited Proliferation of Human Lung Carcinoma, A549, in a Dose-Dependent Manner. Data were expressed in mean ± SD of three independent experiments. The overall survival of the A549 treated by DOX was significantly lower than that of control group (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

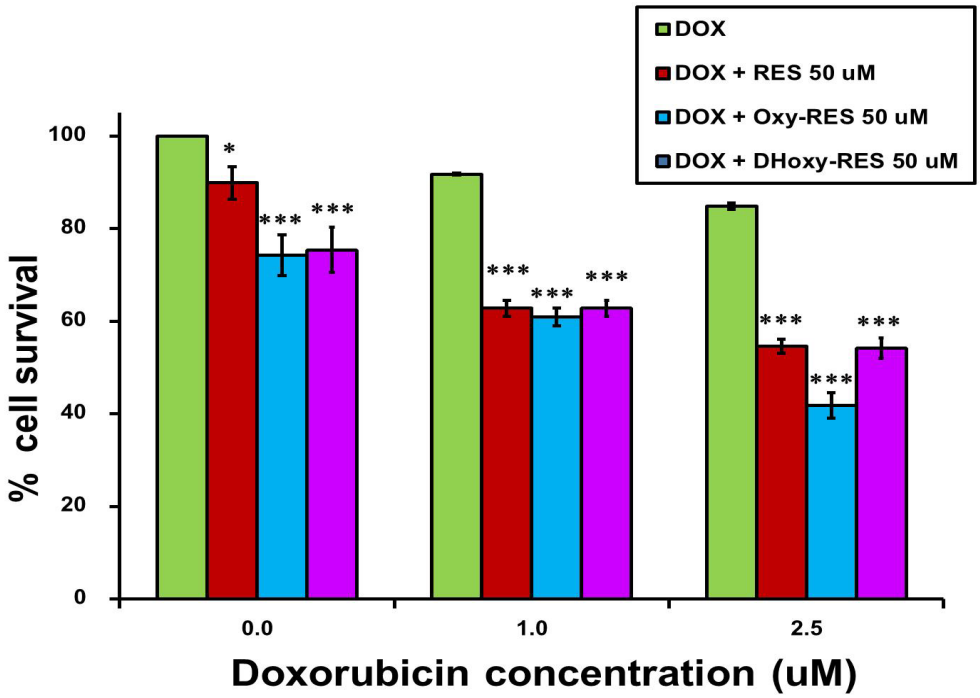

Figure 2.Proliferation Assay on A549 Following Exposure to 50 mM of RES, Oxy-RES or DHoxy-RES. Data were expressed in mean±SD of three independent experiments. All survival data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Effect of RES and RES analogue on DOX-treated A549

To evaluate the possible mechanisms of RES and RES analogues to DOX effect, gene expression were studied in A549 cells in the same condition as in proliferation assay. Expression of all genes in this study was assessed by quantitative real-time RT-PCR, normalized with GAPDH and expressed as mean of fold change compared to control condition. *P53* and *SIRT1* gene expression were selected for cell longevity studies.

P53 expression was then investigated in A549 exposed to DOX and the combination of DOX and 50 μM of RES, Oxy-RES or DHoxy-RES. *P53* up regulation induced by DOX was significantly increased by RES and Oxy-RES (Figure 3). Oxy-RES can better up regulate *p53* expression

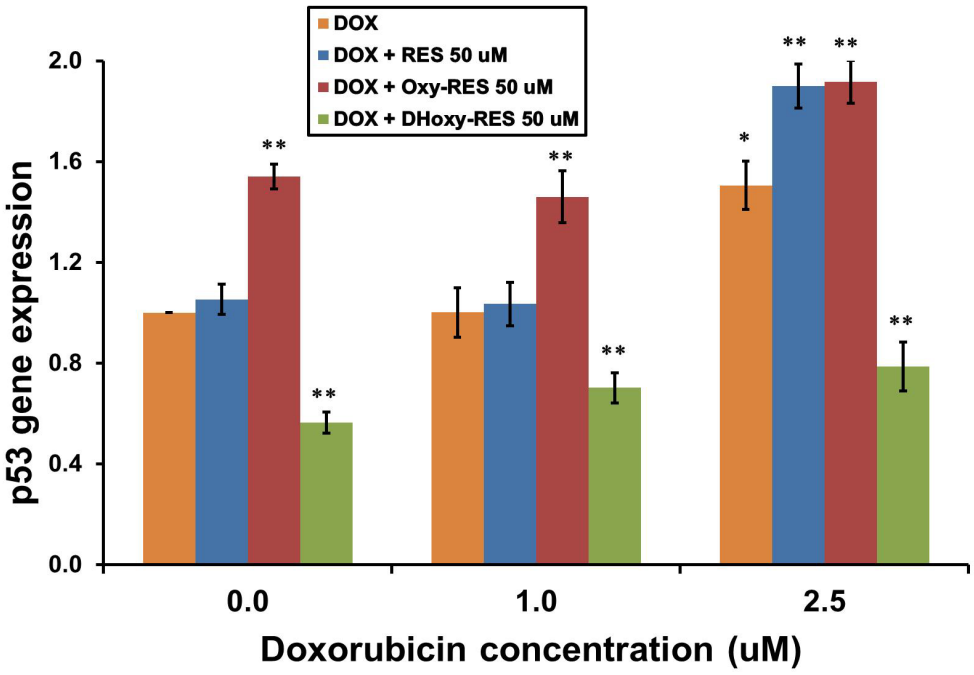

Figure 3. Effect of RES and RES Analogues on *p53* Gene Expression in A549 Treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

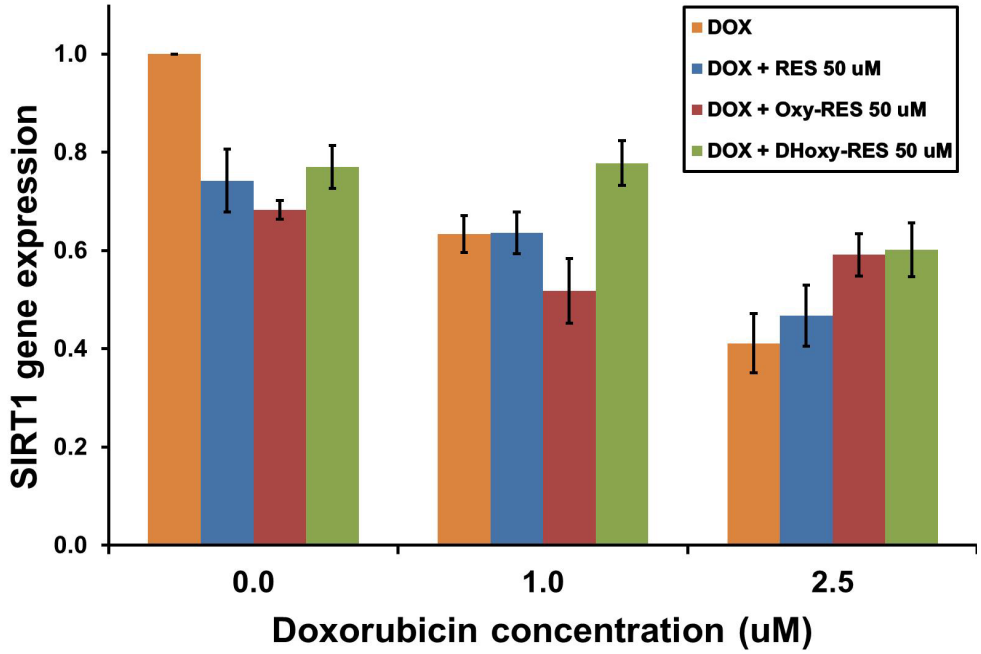

Figure 4. Effect of RES and RES Analogues on *SIRT1* Gene expression in A549 Treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and no significance was found.

than RES whereas DHoxy-RES down regulation *p53* expression was found in DOX + DHoxy-RES.

Conversely, *SIRT1* expression in A549 maintained in the same condition as *p53* study was found down regulated in DOX. RES and Oxy-RES can down regulate DOX-induced *SIRT1* expression lower than DHoxy-RES (Figure 4).

A549 cells were treated in the same condition as previous studies, i.e. DOX and the combination of DOX and RES, Oxy-RES or DHoxy-RES 50 µM. To explore the ability of cancer cells to undergo migration and invasion, gene expression of MMPs involved in cancer metastasis, *MMP2* and *MMP9*, was explored by real-time polymerase chain reaction. Decreased expressions of MMP-2 and MMP-9 induced by DOX were shown in Figure 5 and Figure 6. Oxy-RES showed the best effect to potentiate

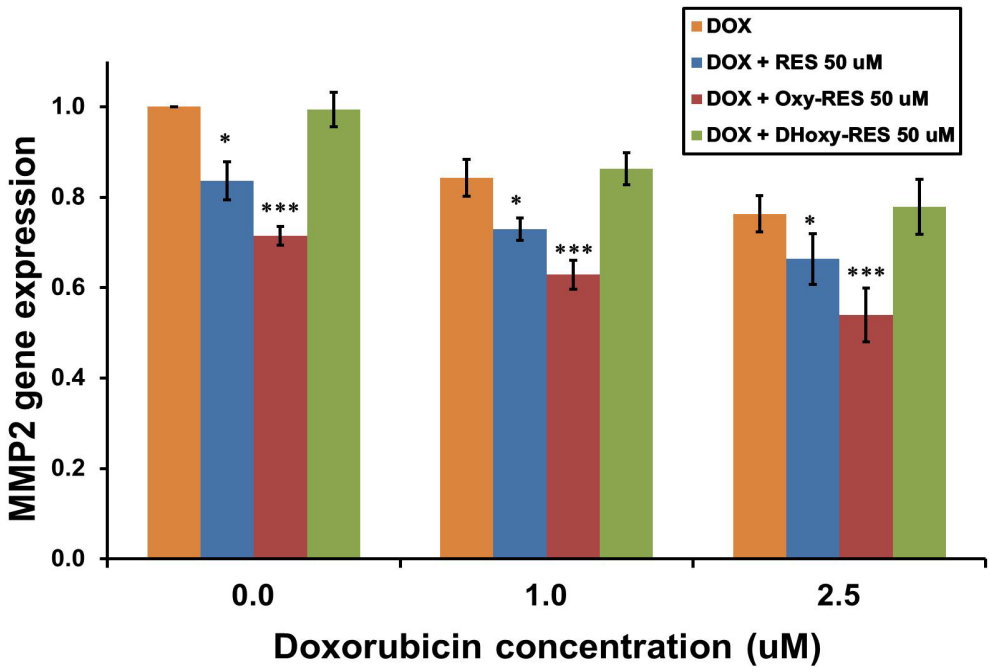

Figure 5. Effect of RES and RES Analogues on *MMP2* Gene Expression in A549 Treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$)).

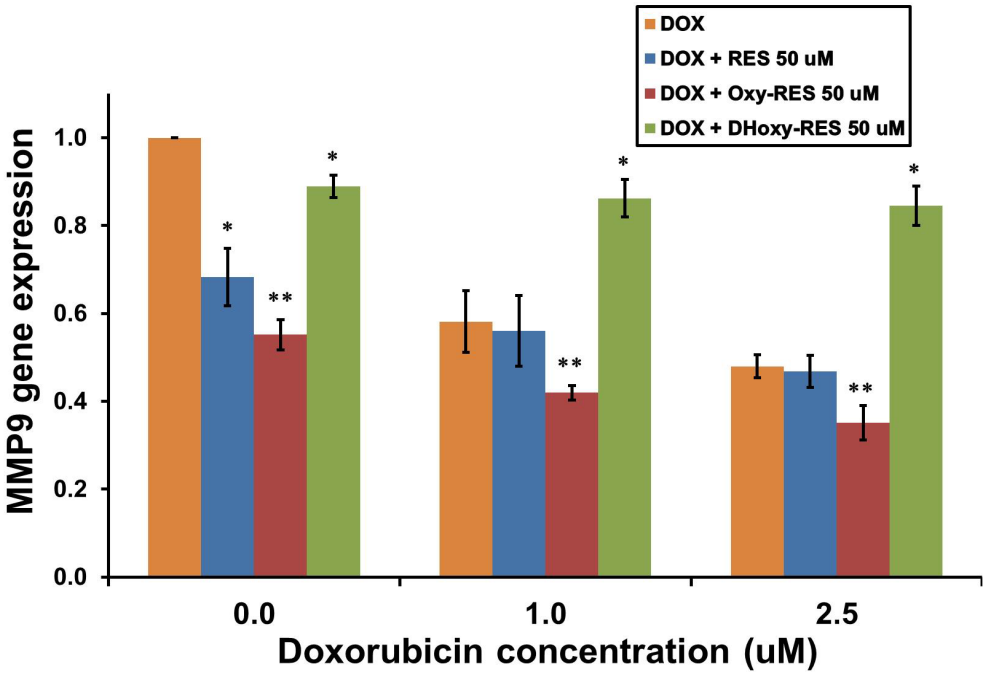

Figure 6. Effect of RES and RES Analogues on *MMP9* Gene Expression in A549 Treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

DOX-induced *MMP2* and *MMP9* down regulation. While DHoxy-RES had nearly no effect to expression of *MMP2* and *MMP9*. The effects to *MMP2* and *MMP9* expression might be used to explain the role of RES and RES analogues on metastatic process.

E-cadherin and integrin are the cell adhesion molecules (CAMs) functioned both in physiological and pathological roles. DOX-reduced *E-cadherin* and *integrin* were shown in Figure 7 and Figure 8 respectively. DOX-reduced *E-cadherin* and *integrin* were more potentiated by Oxy-RES than RES. DHoxy-RES had nearly no effect to *E-cadherin* and *integrin* expression.

Discussion

DOX was approved for chemotherapy medication and

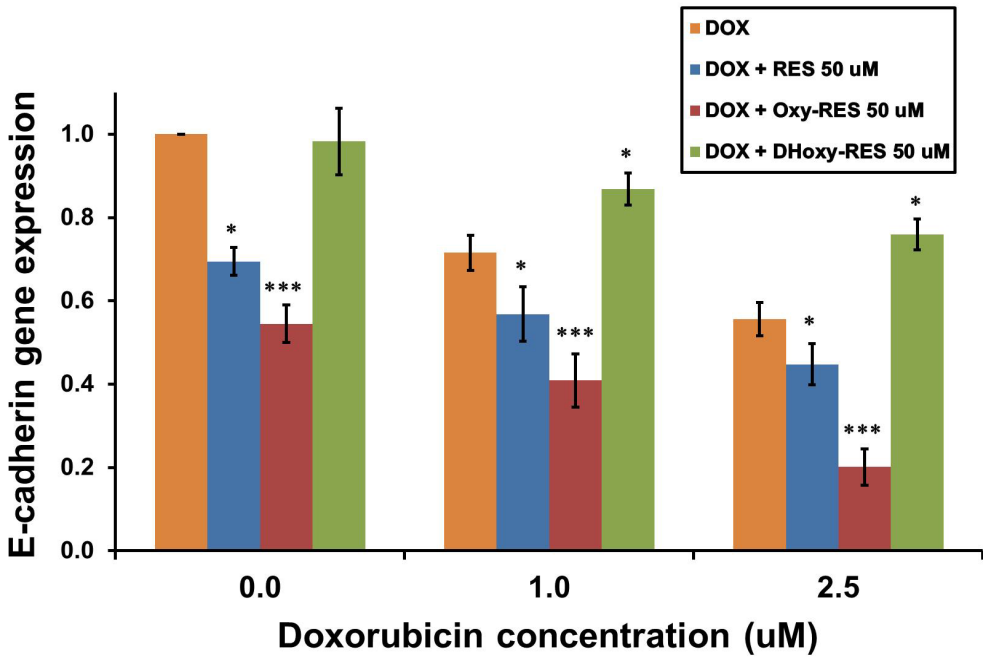

Figure 7. Effect of RES and RES analogues on *E-cadherin* Gene Expression in A549 Treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

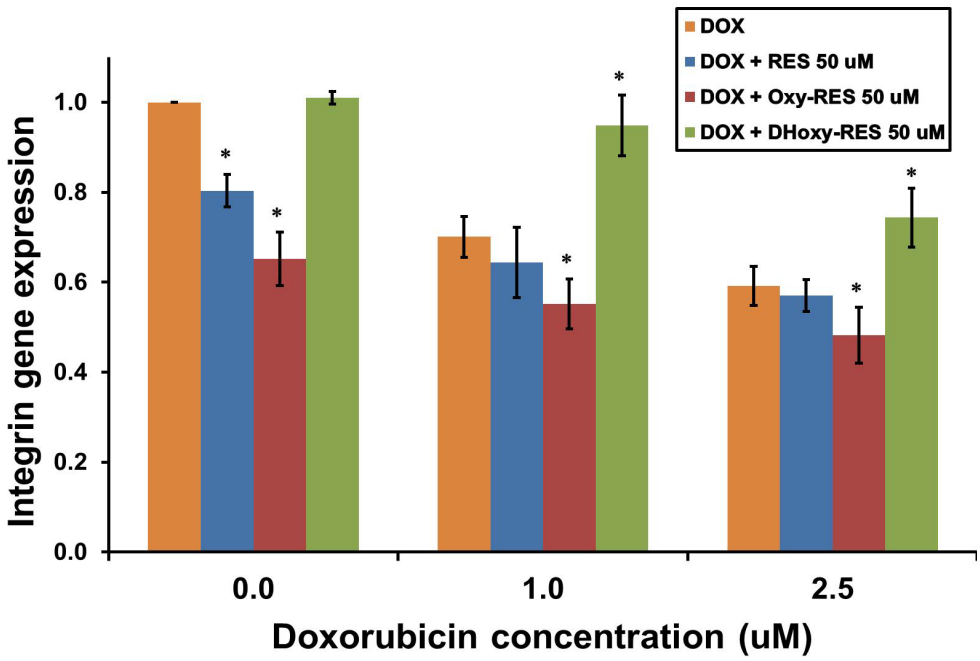

Figure 8. Effect of RES and RES Analogues on Integrin Gene Expression in A549 treated with DOX. Data are expressed in mean ± SD of three independent experiments. All expression data were compared to that of control, untreated cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

currently used to treat many kinds of cancer. In this study, DOX showed a toxic effect on A549 as in other malignant cells. To select the suitable DOX concentration for further experiments, DOX was firstly assessed cell viability by tetrazolium-based colorimetric assay, MTT assay. IC50 13.07 + 1.38 µM was obtained from proliferative assay. The overview of DOX cytotoxicity, 0-50 µM, was shown in Figure 1. Very low concentration of DOX in early part of Figure 1, 1.0 and 2.5 µM, possessed only 10-15 % inhibition to proliferative activity were chosen in order to

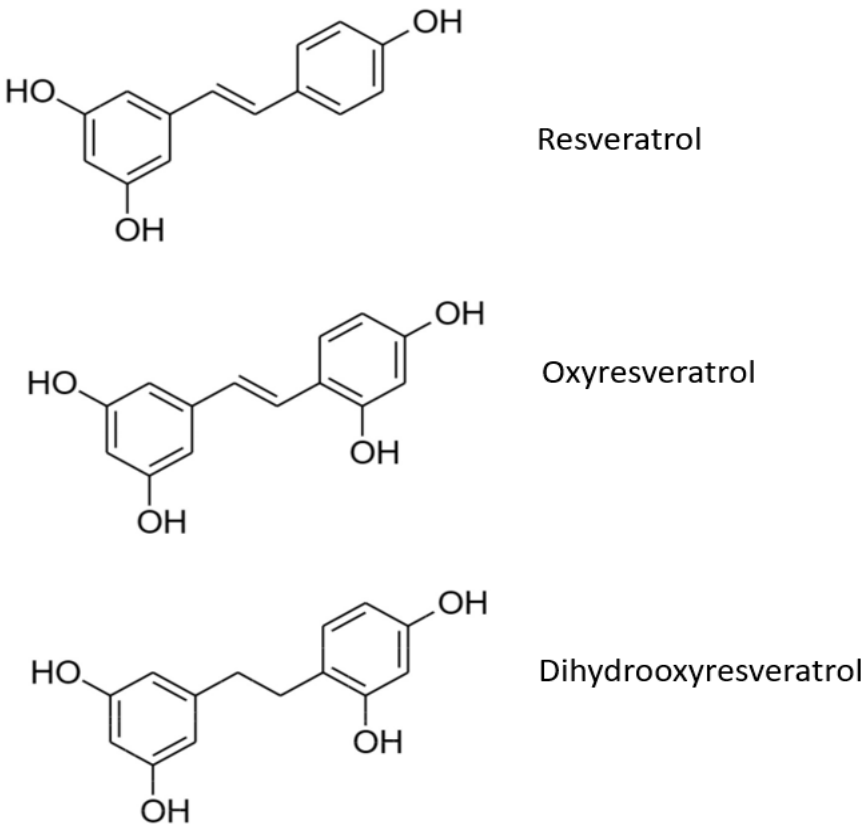

Figure 9. Chemical Structure of Resveratrol, Oxyresveratrol and Dihydrooxyresveratrol.

confirm the healthy cancer cells for further experiments to study real effects of RES or RES analogues. Cytotoxicity of DOX 1.0 and 2.5 μM without RES or RES analogues was shown in Figure 2.

Lower cell survival was found when RES, Oxy-RES or DHoxy-RES was combined with DOX 1.0 and 2.5 μM as shown in Figure 2 with statistical significance ($p < 0.001$). 2.5 μM DOX showed lower cell survival than 1.0 μM DOX. Oxy-RES can better potentiate anti-proliferative effect of DOX than RES and DHoxy-RES. To compare the anti-proliferative potentiation of RES, Oxy-RES and DHoxy-RES on A549 exposed to DOX, gene expressions associated with longer lifespan, *p53* and *SIRT1*, were studied.

P53 gene plays a crucial role in preventing cancer formation via the cell cycle regulation, apoptosis, and genomic stability via several mechanisms. It is the most frequently mutated gene in human cancer. Most chemotherapeutic agents functions by activation of *p53*, resulting in cytotoxicity, cardiotoxicity and cardiomyocyte apoptosis included. DOX induced apoptosis in H9c2 cells. DOX induced apoptosis of cardiomyocytes, H9c2 cells, via *p53* upregulated modulator of apoptosis [47, 48]. In Figure 3, compared to a DOX alone or simultaneous RES (or RES analogue) exposure, *p53* expression was significantly increased in DOX + RES group and the highest increase was found in DOX + Oxy-RES group. On the contrary, DOX + DHoxy-RES group obviously decreased *p53* expression.

Normally, p53 can induce apoptosis by means of intrinsic and extrinsic pathways. Sirtuin 1 protein, class III histone deacetylase proteins, has been reported to deacetylate and thereby deactivate p53 protein. It intracellularly increased basal autophagic activity and lifespan extension. Autophagy is a catabolic process to remove unnecessary or dysfunctional components through a lysosome-dependent regulated mechanism. Sirtuins and autophagy can promote cellular longevity, cytoprotection and then lead to protect individual organisms from age-associated diseases. However, *SIRT1* overexpression has been found in several cancers including lung cancer [49]. For parallel explanation with *p53*, *SIRT1* gene expression was studied together as shown in Figure 4. DOX showed the down regulation of *SIRT1* expression. Moreover, it was interesting that RES and RES analogues can potentiate DOX effect at 1 μM to lower *SIRT1* expression than DOX alone, Oxy-RES > RES > DHoxy-RES. The lowering of *SIRT1* expression occurred concomitant with the increasing of *p53* expression. These could promote cellular apoptosis and might be useful for cancer therapy both primary and secondary metastatic mass.

Cessation of cancer cell proliferation and metastatic prevention are the ultimate goals for cancer therapy. Metastasis is a multi-step process divided conceptually into three stages: migration into the adjacent tissue, transendothelial migration, and remote proliferation as secondary metastatic tumor. Matrix metalloproteinases (MMPs) are a family of Ca^{2+}-dependent Zn^{2+}-containing endopeptidases used to degrade almost every component of extracellular matrix (ECM) led to ECM remodeling in several physiological processes (e.g. angiogenesis, tissue morphogenesis, menstruation, etc.) and pathological processes especially many kinds of cancer (e.g. tumor angiogenesis and metastasis). In a variety of cancers, capability of MMPs to degrade ECM proteins leads to promote cancer cell migration, invasion and metastasis [50, 51]. Normally, MMP2 and MMP9 are classified in gelatinase family that mainly digest collagen IV in basement membrane and then promotes cancer cell migration and invasion to finally form secondary cancer mass in the distant organs. Tumoral MMP2 and MMP9 are reported as the promising markers for predicting the prognosis in cancer patients with breast cancer [52] including breast cancer cell line, MDA-MB-231 [53]. In this study, Oxy-RES can obviously aggravate DOX-induced *MMP2* and *MMP9* down regulation more than RES. Oxy-RES made *MMP2* and *MMP9* more down regulation. Lower *MMP2* and *MMP9* expression levels should be associated with better metastatic inhibition. Based on previous cancer studies about the correlation between high *MMP2* and *MMP9* and metastasis, Oxy-RES can induce DOX to achieve higher metastatic inhibition than DOX alone and DOX with RES or DHoxy -RES. Synergistic effect of suppression of *MMP2* and *MMP9* might be possible to increase DOX activity as found in combination of brazilein and DOX [54, 55]. This should be the benefit of RES analog, especially Oxy-RES, for cancer therapy both primary and metastatic mass as found in *p53* and *SIRT1* expressions.

CAMs are a subset of cell surface proteins that are involved in the adhesion between cell-cell and cell-ECM. E-cadherin is the calcium-dependent cell-cell adhesion protein molecule that plays crucial roles in homophilic interaction of epithelial cells to maintain epithelial tissue integrity. Tumor progression is often found with the loss of E-cadherin expression and/or function. Loss of *E-cadherin* promotes tumor progression via cancer cell dissociation from the primary tumor mass and facilitates cancer cells to invade surrounding tissues and migrate to remote tissues. However, many metastases still contain high levels of E-cadherin. *E-cadherin* expression can contribute to either tumor-suppressing or tumor-promoting processes [47-49]. In this study, DOX decreased *E-cadherin* expression in dose-dependent manner. Oxy-RES can obviously potentiate DOX-reduced *E-cadherin* expression as shown in Figure 7. It might be possible that the reduced E-cadherin affects to reduce cell attachment during secondary or tertiary metastatic mass. DHoxy-RES had nearly no effect to DOX-reduced *E-cadherin* expression

Integrin is in a large family of CAMs with heterophilic binding between cell and ECM to stabilize cell attachment to ECM. This is a basic requirement for a multicellular formation. Integrins play important roles inside the cells via the regulation of cellular growth, proliferation, apoptosis, migration, tissue repair, signaling, including all processes critical to inflammation, infection, and angiogenesis [50, 51]. DOX decreased integrin expression in dose-dependent manner and was anticipated to inhibit cell attachment to ECM in metastatic tumor. In Figure 8,

Oxy-RES can clearly potentiate DOX-reduced integrin expression as found in *E-cadherin* expression. The same result of DHoxy-RES effect to *integrin* expression was also obtained as *E-cadherin* expression.

Chemical structure of RES and RES analogs as shown in Figure 9 can be used to explain the structure-activity relationship. For the previous reports, the binding of RES with lipid membranes is non-specific, its hydroxyl groups interact with the head groups of phospholipids. It was found that RES molecules rapidly diffuse to localize under the head polar groups of dipalmitoylphosphatidylcholine (DPPC). However, if RES was compared with cholesterol, RES binding position with DPPC bilayer locate more towards the membrane surface. It is due to the hydroxyl group which is found one and three groups in cholesterol and RES, respectively. RES molecule function has been reported that the hydroxyl groups of resveratrol interact with the head groups of phospholipids with hydrogen bond formation [52-54]. This should be the reason of higher activity to cancer cells of Oxy-RES than RES. Moreover, the higher water solubility of Oxy-RES (more than 0.1 mg/ml) than RES (0.05 mg/ml) [55] (manufacturer instruction) can imply to higher bioavailability leading to higher therapeutic activity. For DHoxy-RES, its molecular structure with single bond at the center of molecule may be the cause of less contact to polar head of phospholipid and lead to low antimetastatic activity to cancer cells.

After all data were taken together, DOX demonstrated cytotoxic and anti-metastatic activities against A549 cells. Cytotoxicity was supported by up regulating *p53* and down regulating *SIRT1*. Antimetastasis was found in DOX treatment via down regulating *MMP2* and *MMP9* to prevent ECM digestion, and then cancer cells will not dissociate from primary tumor mass including hinder the cell establishment in the remote area as metastatic mass. Antimetastasis was also support by down regulating *E-cadherin* and *integrin* to prevent cell adhesion in the distant area for metastatic mass formation. These activities of DOX were enhanced by RES and RES analogs. Besides, Oxy-RES can more potentiate or increase DOX effect than RES due to one more hydroxyl group while DHoxy-RES had nearly no effect to DOX activities.

In conclusions, importantly, Oxy-RES may potentially be combined with chemotherapeutic agents, particularly DOX, to inhibit cancer cell proliferation, migration, and invasion. This is the great advantage for alternative choice in cancer therapy. Oxy-RES can be high yield extracted from heartwood of *Artocarpus lakoocha* Roxb., Thai traditional drug 'Puag-Haad', which is not difficult to obtain in Thailand [56]. It would be cost effectiveness of primary health care in combination between Oxy-RES and DOX for cancer treatment in the future.

Author Contribution Statement

S Saiyudthong and R Supabphol were co-responsible for the overall experimental design and supervision of the experiments, grant application and preparation of the final article. W. Yahayo performed most of the experiments and data analysis. V Pongkittiphan took responsibility for chemical structure drawing and discussion in chemical structure idea. All authors have read and agreed the final version of the manuscript.

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Conflicts of Interest

The authors declare that there is no potential conflict of interest.

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