Effects of rhinacanthin-C in multidrug-resistance breast cancer cells

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ผลของไรนาแคนทิน-ซี ในเซลล์มะเร็งเต้านมที่ดื้อต่อยา

นายสุวิจักขณ์ ชัยสิทธิ์

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ไรนาแคนทิน-ซี (RN-C) เป็นสารในกลุ่ม naphthoquinone ester ที่ได้จากต้นทองพันชั่ง ซึ่งสารดังกล่าวมีฤทธิ์ทางเภสัชวิทยาหลากหลายรวมถึงฤทธิ์ต้านมะเร็ง มีการรายงานถึงความสามารถของสารไรนาแคนทิน-ซี ต่อการเพิ่มความเป็นพิษของยาด๊อกโซรูบิซินในเซลล์มะเร็งเต้านมโดยผ่านการยับยั้งการทำงานของโปรตีนขนส่งยา P-gp โดยตรง แต่อย่างไรก็ตาม ยังไม่มีการรายงานถึงความสามารถของสารไรนาแคนทิน-ซี ในการผันกลับการดื้อต่อยา ผ่านกลไกการลดการแสดงออกของโปรตีนขนส่งยา P-gp รวมไปถึงการกำจัดสารพิษ (detoxification) และการเพิ่มขึ้นของโปรตีนที่เกี่ยวข้องกับการตายแบบอะพอพโทซิส ดังนั้นในการศึกษานี้เพื่อพิสูจน์ผลของสารไรนาแคนทิน-ซี ในการผันกลับการดื้อต่อยา รวมถึงกลไกที่เกี่ยวข้องในเซลล์มะเร็งเต้านมที่ดื้อต่อยาด๊อกโซรูบิซิน ในการศึกษานี้พบว่าสารไรนาแคนทิน-ซี (ที่ความเข้มข้น 1 ไมโครโมลา ที่เวลา 48 ชั่วโมง) สามารถเพิ่มความเป็นพิษอย่างมีนัยสำคัญให้แก่ ยาแคมโตทีซิน ด๊อกโซรูบิซิน และอีโตโปไซด์ จากการศึกษาเห็นได้ชัดว่าการสูญเสียการทำงานของโปรตีนขนส่งยา P-gp ในเซลล์มะเร็งเต้านมที่ดื้อต่อยาด๊อกโซรูบิซินนั้น พบว่ายังสัมพันธ์กับการลดลงของระดับ *MDR1* mRNA และระดับโปรตีนของโปรตีนขนส่งยา P-gp หลังจากได้รับสารไรนาแคนทิน-ซีเป็นเวลา 24 ชั่วโมง นอกจากนี้ผลของสารไรนาแคนทิน-ซี ต่อการลดลงของการแสดงออกของโปรตีนขนส่งยา P-gp ซึ่งส่วนเกี่ยวข้องกับการลดลงผ่านวิถีสัญญาณ Akt/NF-**K**B และการแสดงออกของ YB-1 แต่พบว่าไม่สัมพันธ์กับวิถีสัญญาณของ MAPK/ERK1/2 ผลการศึกษายังแสดงให้เห็นว่าสารไรนาแคนทิน-ซี (ที่ความเข้มข้นที่สูงถึง 3 ไมโครโมลา ที่เวลา 24 ชั่วโมง) มีความสามารถเพิ่มระดับของ ROS ภายในเซลล์ และมีประสิทธิภาพทำให้ไฮโดรเจนเปอร์ออกไซด์สูงถึงระดับเป็นพิษ จึงถูกชักนำไปสู่การตายแบบอะพอพโทซิสในเซลล์มะเร็งเต้านมที่ดื้อต่อยาด๊อกโซรูบิซิน นอกจากนี้สารดังกล่าวสามารถยับยั้งการส่งวิถีสัญญาณผ่าน MAPKS ส่งผลให้ต่อการเปลี่ยนแปลงสัดส่วนของ Bax/Bcl-2 และลดการแสดงออกของโปรตีน PARP นอกจากนี้สารไรนาแคนทิน-ซี อาจเพิ่มความเครียดภายในเซลล์และถูกชักนำการตายแบบอะพอพโทซิส โดยการยับยั้งผ่านวิถีสัญญาณของ Akt/GSK-3β/Nrf2 ซึ่งมีผลต่อการควบคุมการลดลงของเอนไซม์ต้านอนุมูลอิสระและเอนไซม์การกำจัดสารพิษ เช่น NQO1 และ HO-1 ดังนั้นผลการวิจัยนี้แสดงให้เห็นว่าสารไรนาแคนทิน-ซี มีความสามารถในการผันกลับการดื้อต่อยาในเซลล์มะเร็งเต้านมที่ดื้อต่อยาด๊อกโซรูบิซิน ผ่านการควบคุมการแสดงออกของ *MDR1* และ P-gp โดยยับยั้งผ่านวิถีสัญญาณ Akt/NF-kB รวมถึงการแสดงออกของ YB-1 นอกจากนี้สารไรนาแคนทิน-ซี ยังสามารถชักนำให้เกิดการตายแบบอะพอพโทซิส โดยเป็นผลมาจากการเพิ่มขึ้นของระดับ ROS และลดการแสดงออกของโปรตีนที่เกี่ยวข้องกับการอยู่รอดของเซลล์ผ่านวิถีสัญญาณ MAPKs และ Akt/GSK-3β/Nrf2

สาขาวิชา	เภสัชวิทยา	ลายมือชื่อนิสิต .....
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KEYWORD: Apoptosis Breast cancer cells Cellular detoxification system Multidrug resistance Reversal effect P-glycoprotein Rhinacanthin-C

Suwichak Chaisit : Effects of rhinacanthin-C in multidrug-resistance breast cancer cells. Advisor: Assoc. Prof. SUREE JIANMONGKOL, Ph.D.

Rhinacanthin-C (RN-C) is a major bioactive naphthoquinone ester found in *Rhinacanthus nasutus* Kurz (Acanthaceae). This compound has potential therapeutic value as an anticancer agent. Rhinacanthin-C was reported to enhance doxorubicin cytotoxicity in breast cancer cells through direct inhibition of P-gp activity. However, its ability in reversing multidrug resistance (MDR) in cancer cells through the down-regulation of P-gp expression, alteration of detoxification system, and increase in apoptosis proteins remains unreported. Thus, this study was to investigate the potential MDR reversal effect of rhinacanthin-C and its underlying mechanisms in MCF-7/DOX. In this study, rhinacanthin-C (1 μM, 48 h) significantly enhanced cytotoxicity of camptothecin, doxorubicin, and etoposide. Apparently, the loss of P-gp function in the rhinacanthin-C-treated for 24- h in MCF-7/DOX cells was associated with the reduction of *MDR1* mRNA expression and P-gp protein levels. In addition, the down-regulation effect of rhinacanthin-C on P-gp expression involved with suppressions of the Akt/NF-kB signaling pathway and YB-1 expression, but not the MAPK/ERK1/2 signaling pathway. The result also demonstrated that rhinacanthin-C (up to 3 μM, 24-h) was able to produce intracellular ROS and potentiate H_2O_2 toxicity, leading to increased apoptosis in MCF-7/DOX cells. Moreover, this compound inhibited the MAPKs signaling, resulting in changes in the Bax/Bcl-2 ratios and a decrease in the expression of PARP protein. In addition, rhinacanthin-C might increase cellular stress and promote apoptosis via inhibition of the Akt/GSK-3β/Nrf2 pathway, leading to the down-regulation of antioxidant and detoxifying enzymes such as NQO1 and HO-1. Taken together, rhinacanthin-C displayed its MDR reversal effect in MCF-7/DOX cells through down-regulation of *MDR1* and P-gp expression by inhibiting Akt/NF-kB signaling pathway and YB-1 expression. In addition, rhincanthin-C was able to induce apoptosis through ROS production and suppression of the cell survival systems mediated by the MAPKs and Akt/GSK-3β/Nrf2 signaling pathways.

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LIST OF ABBREVIATIONS

ABC	= Adenosine triphosphate binding cassette
Akt	= Protein kinase B
ANOVA	= Analysis of variance
ATCC	= American Type Culture Collection
ATP	= Adenosine triphosphate
Bax	= BCL2-associated X
Bcl-2	= B-cell lymphoma 2
BSA	= Bovine serum albumin
°C	= Degree Celsius
cm^2	= Squared centimeter
Calcein-AM	= Calcein acetoxymethyl ester
CAM	= (S)- (+)-Camptothecin
cDNA	= Complementary DNA
$CHCl_3$	= Chloroform
CDCFH-DA	= 2′,7′-Dichlorofluorescin diacetate
CO_2	= Carbon dioxide
CsA	= Cyclosporine A
CT	= Cycle threshold
DOX	= Doxorubicin
DMSO	= Dimethyl sulfoxide
DNA	= Deoxyribonucleic acid
EDTA	= Ethylene diamine tetraacetic acid
EGTA	= Ethylene glycol-bis(2-aminoethylether)-N,N,N′
ETO	= Etoposide
ERK1/2	= Extracellular signal-regulated kinase 1/2
FBS	= Fetal bovine serum
GAPDH	= Glyceraldehyde 3-phosphate dehydrogenase

GSK-3β	= Glycogen synthase kinase 3 beta
HBSS	= Hanks' balanced salt
h	= hour
H_2O_2	= Hydrogen peroxide
HO-1	= Heme oxygenase 1
IC50	= Inhibitory Concentration 50
I**K**B-α	= Inhibitor of kappa-light-chain-enhancer of activated B cells-alpha
JNK	= C-Jun N-terminal kinase
MAPK	= Mitogen-activated protein kinase
MCF-7	= Human breast adenocarcinoma cell line
MCF-7/DOX	= Human breast adenocarcinoma doxorubicin resistant cell line
MDR	= Multidrug Resistance
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
mg/ml	= milligram per milliliter
ml	= milliliter
mM	= millimolar
Na_3VO_4	= Sodium orthovanadate
NaCl	= Sodium chloride
NaF	= Sodium fluoride
NF-**K**B	= Nuclear Factor kappa B
NQO1	= NAD (P) H dehydrogenase [quinone] 1
Nrf2	= Nuclear factor erythroid 2-related factor 2
PARP	= Poly (ADP-ribose) polymerase
PBS	= Phosphate buffered saline
PMSF	= Phenylmethylsulfonyl fluoride
PVDF	= Polyvinylidine fluoride

p	= Phosphorylation
p-38MAPK	= p38 mitogen-activated protein kinases
P-gp	= P-glycoprotein
qRT-PCR	= quantitative reverse transcription polymerase chain reaction
RF	= Reversal ratio
RI	= Resistance index
RN-C	= Rhinacanthin-C
ROS	= Reactive oxygen species
RPMI-1640	= Roswell Park Memorial Institute -1640 medium
SDS	= Sodium dodecyl sulfate
SEM	= Standard Error of Mean
TBS-T	= Tris Buffered Saline - Tween 20
Tris-HCl	=Tris hydrochloride
V =	= Voltage
YB-1	= Y box binding protein 1
μg	= microgram
μl	= microliter
μM	=micromolar

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Breast cancer is the most common cancer in women, counting for almost one third of all malignancies in females (American cancer society, 2020; Jemal et al., 2017). It is the one leading cause of cancer death worldwide. It was estimated that approximately 276,480 new cases and around 42,170 breast cancer deaths could be expected in 2020 (American cancer society, 2020). In 2018, the National Cancer Institute (NCI) of Thailand estimated that breast cancer was the most newly diagnosed cancer in a woman at the incidence of around 26.40 % of all cancer cases (Imsamram et al., 2015). Chemotherapy has been frequently used as a standard treatment of breast cancer. Examples of cytotoxicity agents in breast cancer chemotherapy include doxorubicin, docetaxel, vinblastine and mitoxantrone (Pan et al., 2016). Despite the large repertoire of therapies available the success of cancer therapy is still unlikely to achieve. A major factor contributing to the failure of chemotherapy in particular breast cancer is multidrug resistance (Holohan et al., 2013; Mansoori et al., 2017). Hence, any chemicals that can overcome drug resistance or enhance the efficacy of cytotoxic agents would be clinical significance.

The failure of cancer treatment often occurs because of either intrinsic or acquired multidrug resistance (MDR) of the tumor toward chemotherapy (Bukowski et al., 2020; Holohan et al., 2013). Drug resistance toward cancer chemotherapy is generally non-specific to one group of cytotoxic agents. It can occur toward a wide broad of structurally and functionally unrelated drugs even after repeated exposure to a single agent (Li et al., 2016; Gottesman et al., 2002; Ullah, 2008). This phenomenon is known as multidrug resistance (MDR). The underlying mechanisms of MDR development may involve either “drug efflux transporter” or “non-drug efflux

transporter". Examples of these mechanisms are overexpression of energy-dependent efflux proteins, decrease in drug uptake, modification of cell cycle checkpoints, defects in the regulation of genes controlling apoptosis, enhanced intracellular drug detoxification and alterations in DNA repair (Li et al., 2017).

The mechanism of MDR involves active "drug efflux transport" of ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp/MDR1), multidrug resistance-associated proteins subfamily (MRPs) and breast cancer resistance protein (BCRP) (Gottesman, 2002; Mansoori et al., 2017; Pakunlu et al., 2004). P-glycoprotein encoded by *MDR1* has attracted great interest because of its crucial role in MDR development in various cancers. High expression levels of P-gp in the doxorubicin-resistant cells with MDR phenotype, including MCF-7/DOX cells, have been linked to hyperactivation of either MAPK/ERK1/2 or PI3K/Akt/NF-**K** B signaling pathways (Nedeljković and Damjanović 2019; Yndestad et al., 2017; Zheng, 2017). Activation of these signaling pathways increases the activities of certain transcriptional factors related to cellular survival and adaptive responses, particularly nuclear factor-**K**B (NF-**K**B), Y-box binding protein 1 (YB-1) and activator protein 1 (AP-1) (Maurya et al., 2017; Muthusamy et al., 2019; Wang et al., 2016), and has been reported to involve MDR1 transcription, leading to the development of MDR phenomenon (Maurya et al., 2017; Sui et al., 2012). Numerous plant-based compounds have demonstrated their suppressive actions on the MAPK or Akt/NF-**K** B pathways in drug-resistant cancer cells, resulting in decreased P-gp expression and increased chemosensitivity of these cells to cytotoxic agents (Hamed et al., 2019; Muthusamy et al., 2019; Wang et al., 2016). For example, procyanidin and asiatic acid were able to reverse MDR via inhibition of P-gp function and expression in cancer cell models through the suppression of NF-**K**B activity and MAPK/ERK1/2 pathway (Cheng et al., 2018; Zhao et al., 2013). In addition, osthole and cepharanthine were reported to overcome P-gp-

mediated MDR in cancer cells through the inhibition of the PI3K/Akt signaling pathway (Huang et al., 2017; Wang et al., 2016).

The “non-efflux transporter”-related MDR mechanisms involve the up-regulation of the cellular detoxifying enzyme system and alteration of cell apoptosis responses (Bukowski et al., 2020; Green and Kroemer, 2005; Jaiswal, 2004; Zhong et al., 2013). It has been reported that overexpression of Nrf2, a key regulator of cellular redox homeostasis, enhanced resistance in breast cancer cells (Jaramillo and Zhang, 2013; Wang et al., 2008; Zhong et al., 2013). Expression of Nrf2 is related to increasing several cellular detoxifying enzymes such as glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), NAD(P)H dehydrogenase quinone 1 (NQO1), and heme oxygenase-1 (HO-1) (Choi et al., 2016; Panieri and Saso, 2019). Subsequently, the higher concentrations of cytotoxic drugs are needed (DeNicola et al., 2011; Manov et al., 2007). The MAPKs and PI3K/Akt cascades are two major signaling pathways involved in cell survival mechanisms. Activation of MAPKs, including ERK1/2, JNK and p-38 subfamilies, has been linked to the reduction of stress-induced apoptosis as well as the development of chemo-resistance in cancer cells (Lee et al., 2020). The increased expression level of the anti-apoptotic protein Bcl-2 along with activation of the MAPKs pathway was demonstrated in doxorubicin-resistant K562, MCF-7 and BEL-7402 cancer cells (García-Aranda et al., 2018; Kalinina et al., 2006). Moreover, activation of PI3K/Akt cascade was reported to enhance drug resistance in breast cancer cells via GSK-3β-mediated increase in transcriptional activity of Nrf2, a key regulator of expression of cellular detoxifying and antioxidant enzymes such as NQO1 and HO-1 (Basak et al., 2017). Any phytochemicals targeting these signaling pathways can likely enhance the effectiveness of standard chemotherapeutic regimens in cancer treatment.

Rhinacanthus nasutus Kur. (Acanthaceae) has been used in Thai traditional medicine for the treatment of eczema, skin diseases, tuberculosis, hepatitis, hypertension, and various parts of cancer (Siripong et al., 2006b). Rhinacanthin-C, a major naphthoquinone ester from the leaves and roots of this plant has been shown to possess exhibited various pharmacological activities including anti-inflammatory, antifungal, antibacterial, antiviral, and anti-cytotoxic activities (Bukke et al., 2011). It was reported the rhinacanthin-C has been known to inhibit cell proliferation and induce apoptosis in HeLS3 and KKU-M156 cells, possibly via inhibition of the Akt/NF-kB or MAPK/ERK1/2 signaling pathway (Boueroy et al., 2018; Siripong et al., 2006a). Recently, rhinacanthin-C was reported its ability to directly inhibit the function of P-gp transporter in breast cancer MCF-7 cells, leading to an increase of intracellular DOX accumulation as well as cell death (Chaisit et al., 2017; Wongwanakul et al., 2013). In addition, rhinacanthin-C significant pharmacokinetic herb-drug interaction via inhibiting various efflux and influx drug transporters (i.e., P-gp, BCRP, OATP1B1, and OATP1B3) and phase I drug-metabolizing enzymes CYP isoforms (i.e., CYP2C8, CYP2C9, and CYP2C19) (Dunkoksung et al., 2019). However, the ability of rhinacanthin-C in reversing cancer cells MDR through modification of other cellular adaptive response mechanisms such as down-regulation of P-gp expression, alteration of detoxification system and increase apoptosis proteins remains unreported.

1.2 Hypothesis

Rhinacanthin-C was able to increase sensitivity of cytotoxic drugs in MCF-7/DOX cells. It was possible that MDR reversing properties of rhinacanthin-C involve with down-regulation of P-gp as well as with other "non-efflux transporter"-related mechanisms such as induction of cell apoptosis and inhibition of cellular detoxification system.

1.3 Objective

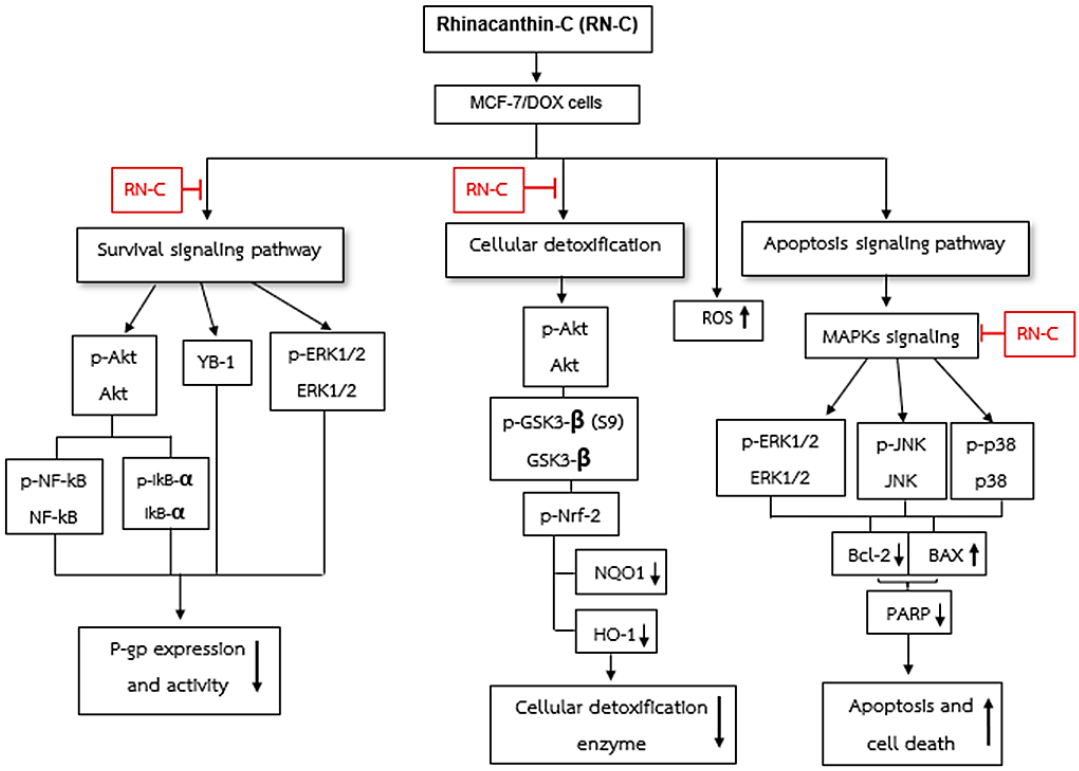
The objectives of this study were

1. To determine whether rhinacanthin-C enhanced the cytotoxicity of chemotherapeutic drugs through down-regulation of P-gp expression in MCF-7/DOX resistance cells.
2. To elucidate the signaling pathway involving in the down-regulation effect of rhinacanthin-C on P-gp expression in MCF-7/DOX resistance cells.
3. To investigate whether rhinacanthin-C could promote apoptosis through oxidative damages and alteration of MAPKs signaling pathway.
4. To examine the effects of rhinacanthin-C on expression of antioxidant and detoxifying enzymes such as NQO1 and HO-1 the involvement of the Akt/GSK-3β signaling pathway.

1.4 Contribution of the study significance

The results from this study would provide preliminary data of whether rhinacanthin-C was able to increase sensitivity of the resistant MCF-7/DOX cells to cytotoxic anticancer drugs via the down-regulation of P-gp expression at the transcription level. This information would suggest the ability of rhinacanthin-C in overcoming drug resistance in breast cancer cells through promotion of oxidative stress and suppression of cellular detoxification system. This information would support the potential use of rhinacanthin-C in chemotherapeutic regimens for cancer treatment.

1.5 Conceptual framework



CHAPTER II
LITERATURE REVIEW

2.1 Breast cancer and treatments

Cancer is a cellular disorder characterized by the progressive accumulation of a mass of cell as a result of excessive cell proliferation more than cell death (Haber et al., 2011). Breast cancer is one of the most common cancers among women. The American Cancer Society estimated that approximately 276,480 new cases of breast cancer would be diagnosed in American women and 42,170 women could be expected in 2020 (American cancer society, 2020). In Thailand, according to National Cancer Institute, the incidence of breast cancer has ranked first with 31.40 % of all cancer cases in Thai women (Imsamran et al., 2015). Breast cancer therapies include five major types: surgery, radiotherapy, chemotherapy, targets therapy and immunotherapy. Chemotherapy is one of the common treatments for cancer with the use of cytotoxic agents that can kill cancer cells. It is sometimes the first or even the only choice to treat many cancers. Anti-cancer drugs available such as anthracyclines (doxorubicin, epirubicin), taxanes (docetaxel, paclitaxel) and mitoxantrone are frequently used in breast cancer patients (O'Shaughnessy, 2005; Xu et al., 2014).

Doxorubicin (DOX) an anthracycline derivative isolated from *Streptomyces peucetius* var. *caesius*. DOX is one of the most important anticancer drugs in the treatment of carcinomas of the breast, endometrium, ovary, testicle, thyroid, stomach, bladder, liver, and lung (Cutts et al., 2005; Kim et al., 2014). Three major cytotoxic mechanisms of DOX are (1) inhibition of topoisomerase II (2) DNA intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission; and (3) generation of semiquinone free radicals and oxygen free

radicals (Cutts et al., 2005; Suzuki et al., 2005). When doxorubicin is given as a single agent in cancer treatment, success rates in killing cancer cells are typically 40% to 80% (Faneyte et al., 2002). However, the usefulness of DOX-based treatment regimens is still limited because of dose-limiting toxicity and development of drug resistance (AbuHammad and Zihlif 2013; Ichikawa et al., 2014). The failure of doxorubicin treatment has been linked to the increased expression and activity of multidrug resistance (MDR) transporters, in particular the ABC drug efflux pumps including P-glycoprotein (P-gp), Multidrug resistance protein 2 (MRP2) and the increased cellular defense response through antioxidant action against oxidative stress and leading to blockage of apoptosis (Gottesman et al., 2002; Walker et al., 2004).

2.2 Drug resistance and multidrug resistance (MDR) in cancer

Cancer multidrug resistance is defined as the cross-resistance or insensitivity of cancer cells to either cytostatic or cytotoxic actions of various anticancer drugs that are structurally or functionally unrelated (Gottesman et al., 2002; Saraswathy and Gong 2013). In addition, these drugs may have different molecular targets (Johnston, 2005). Cancer drug resistance can be divided into two categories. (1) Intrinsic resistance is can be defined as a lack of tumor response to the expression of factors that interfere with therapy efficacy. (2) Acquired drug resistance is can be defined that tumor being drug initially sensitive become drug-resistant due to mutations and various adaptive responses during the process of chemotherapeutic treatments (Housman et al., 2014; Kumar et al., 2019). The resistance of tumor cells to chemotherapeutic agents lowers the effectiveness of anticancer drugs. Consequently, patients need to take higher doses of the agents or they need to change the anticancer drugs (Kartal et al., 2016; Longley and Johnston, 2005).

2.3 The mechanisms of drug resistance in cancer

Statistical data shows that over 90% of mortality of cancer patients is attributed to drug resistance (Bukowski et al., 2020). Mechanisms of MDR in cancer can be categorized into "drug efflux transporter" or "non-drug efflux transporter"-related resistance (Figure 1) (Kartal et al., 2016; Zheng, 2017). The drug efflux transporter resistance based MDR mechanisms may involve either direct inhibition of drug efflux function or expression (Borowski et al., 2005; Kumar et al., 2019; Ullah, 2009). The non-drug efflux transporter resistance MDR mechanisms may involve with several cellular adaptation pathways such as antioxidant and detoxification enzymes (e.g. CYP450, GST, HO-1, and NQO1) and the balance of apoptosis-related proteins, up-regulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, or the suppression of pro-apoptotic proteins such as BAX and BAK, mutations in the tumor suppressor gene p53, and inhibitors of apoptosis proteins (IAPs) such as survivin (Kartal et al., 2016; Ullah, 2009).

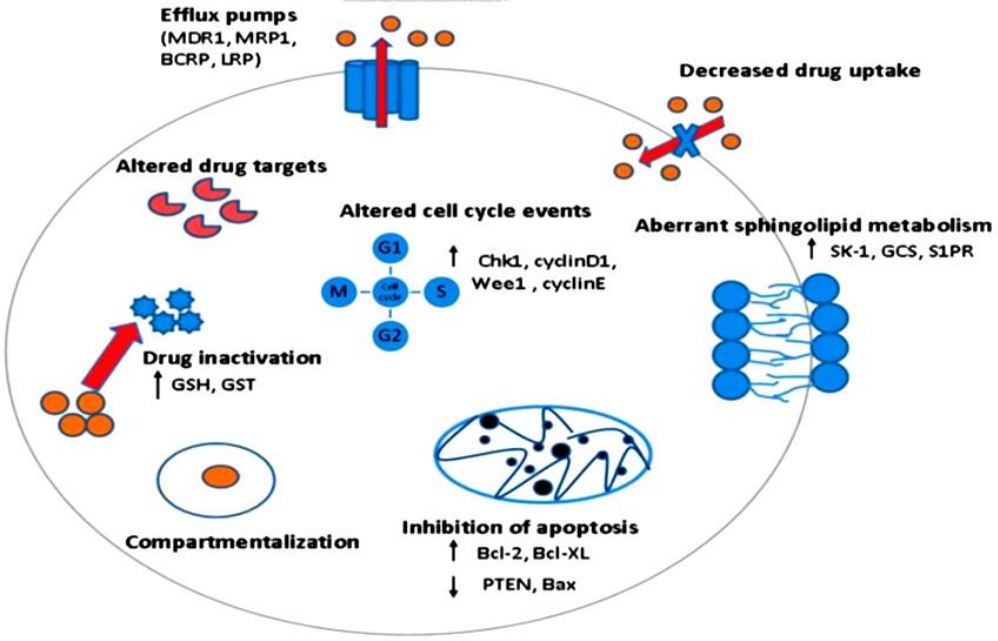


Figure 1. Cellular mechanisms of drug resistance in cancer cells (Kartal et al., 2016).

2.4 The "drug efflux transporter" resistance based MDR

Changing in either function or expression of the ATP-binding cassette (ABC) drug efflux transporters has been linked to MDR in cancer chemotherapeutic failure (Borst and Elferink, 2002; Wijdeven et al., 2016). The most studied ABC drug efflux transporters included P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance associated proteins subfamily (MRPs) (Aller et al., 2009; Chai et al., 2010). A wide range of lipophilic chemotherapeutical agents, such as anthracenes, anthracyclines, epipodophyllotoxins, taxanes, and vinca alkaloids, are known substrates of P-gp (Ambudkar et al., 2003; Li et al., 2016; Loo and Clarke, 2005; Ullah, 2009). Hence, the high expression level of these efflux transporter may prevent the intracellular accumulation of its anticancer drug substrates up to the cytotoxic threshold.

2.4.1. Role of P-gp expression in cancer cells

P-glycoprotein (P-gp) encoded by the *MDR1* gene (ABCB1) has 1280 amino acids being arranged in two homologous halves that are linked by a linker region. Each half of the pump begins with a transmembrane domain (TMD) containing six transmembrane (TM) segments and a hydrophilic region containing a nucleotide-binding domain (NBD) show in Figure 2.

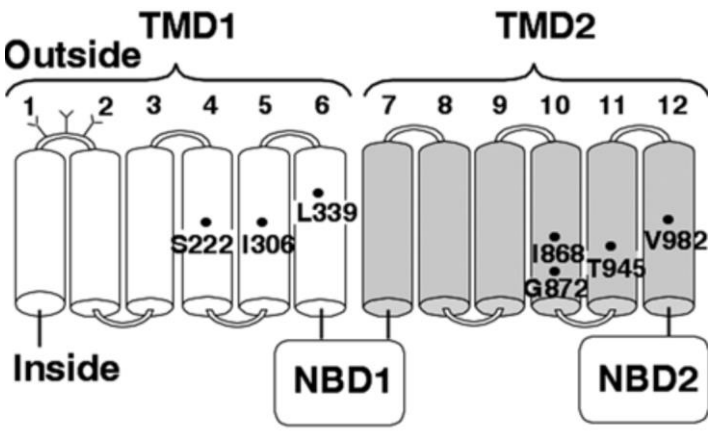


Figure 2. Illustration models of P-glycoprotein (P-gp) Loo and Clarke, 2005.

This transporter was first identified from its involvement with multidrug-resistance in cancer cells. Chemotherapeutic drugs including doxorubicin and vinblastine are known P-gp substrates with the ability to up-regulate the *MDR1* gene in cancer cells (Kumar et al., 2019; Martin et al., 2014; Xu et al., 2014). An increase of P-gp expression can cause less accumulation of the intracellular drug and the cells become resistant toward chemotherapy (Gottesman, 2002). Overexpression of P-gp has been linked to the up-regulation of the *MDR1* gene at either transcriptional level or translational processes in cancer cell lines (Gottesman, 2002).

Expression of *MDR1*/P-gp involves several signal transduction pathways in transcriptional level and post-translational modifications (Liem et al., 2002; Martin et al., 2014; Misra et al., 2005). Several transcriptional factors such as nuclear factor-**K**B (NF-**K** B) (Ronaldson et al., 2010; Kuo et al., 2002), Y-box binding protein-1 (YB-1) (Shen et al., 2011), activator protein-1 (AP-1) (Chen et al., 2014), and hypoxia-inducible factor-1 (HIF-1) (Ding et al., 2010) have been reported to bind to the promoter region of the MDR gene to initiate the transcription and expression of ABC transporters. Moreover, several signal transduction pathways have been involved in regulating the activities of those transcriptional factors, such as Mitogen-activated

protein kinase (MAPK) (Dhillon et al., 2007; Katayama et al., 2007; Shen et al., 2011; Yang et al., 2011), phosphatidylinositol 3-kinase (PI3K) (Barancík et al., 2001; Kuo et al., 2009; Liu et al., 2009), protein kinase C (PKC) (Liu et al., 2009; Rigor et al., 2010), and nuclear factor-**K**B (NF-**K**B) (Kim et al., 2011; O'Neill et al. 2011; Yao et al., 2014) pathways (Figure 3). Recently, the PI3K/Akt signal transduction pathway has been reported its involvement in MDR development (Lin et al., 2012). The PI3K/Akt signaling pathway is one of the most important signaling pathways in the control of multiple biological processes through phosphorylation of protein kinase Akt. For example, hyperactivated Akt markedly increased phosphorylates of downstream effector NF-**K**B, leading to increase *MDR1* expression in breast and ovarian cancer cells (Kim et al., 2011; Yang et al., 2012). Activation of the NF-kB pathway occurs in response to reactive oxygen species, inflammation, and chemotherapeutic drugs. It was demonstrated that, the phosphorylation of I**K** B by I**K** B kinase induced I**K** B ubiquitination and proteasome-mediated degradation, subsequently active NF-**K** B translocated into the nucleus and bound to MDR1 promoter region (Choi et al., 2008; Kim et al., 2011; Misra et al., 2005; Kuo et al., 2002; Shen et al., 2011). In addition, a transcription factor YB-1 is a biomarker for an unfavorable prognosis in breast cancer (Gimenez et al., 2004; Maurya et al., 2017; Saji et al., 2003). Overexpression and nuclear localization of YB-1 have been associated with the development to multidrug resistance of several cancers such as breast cancer, lung cancer, ovarian cancer, colorectal cancer, prostate cancer, and osteosarcoma (Maurya et al., 2017). It was likely that YB-1 was another important transcription factor involving P-gp expression. The translocation of YB-1 into the nucleus in response to stresses, hyperthermia, adenoviral infection, DNA damage and activation of signaling cascade in the Akt and MAPKs signaling pathways (Bansal et al., 2018; Li et al. 2018; Yang et al., 2001; Zhao et al., 2013). It was reported that doxorubicin can increase expression of *MDR1*/P-glycoprotein by activating MAPK/ERK1/2 pathway, then increasing YB-1

expression, and inducing DNA-binding promoter activity of YB-1 in resistant B-cell lymphoma (Shen et al., 2011). In addition, a blockade of the MEK-ERK-RSK signaling pathway was able to suppress P-gp expression in HT-15, SW620-14, MCF-7/MDR and MDA-MB-231/MDR cell lines (Katayama et al., 2007). It has been demonstrated that activation of the ERK1/2/MAPK and Akt/NF-**K**B pathways along with increased *MDR1* expression were observed in several doxorubicin resistant cells such as MCF-7/DOX (Das et al., 2011; Hien et al., 2010).

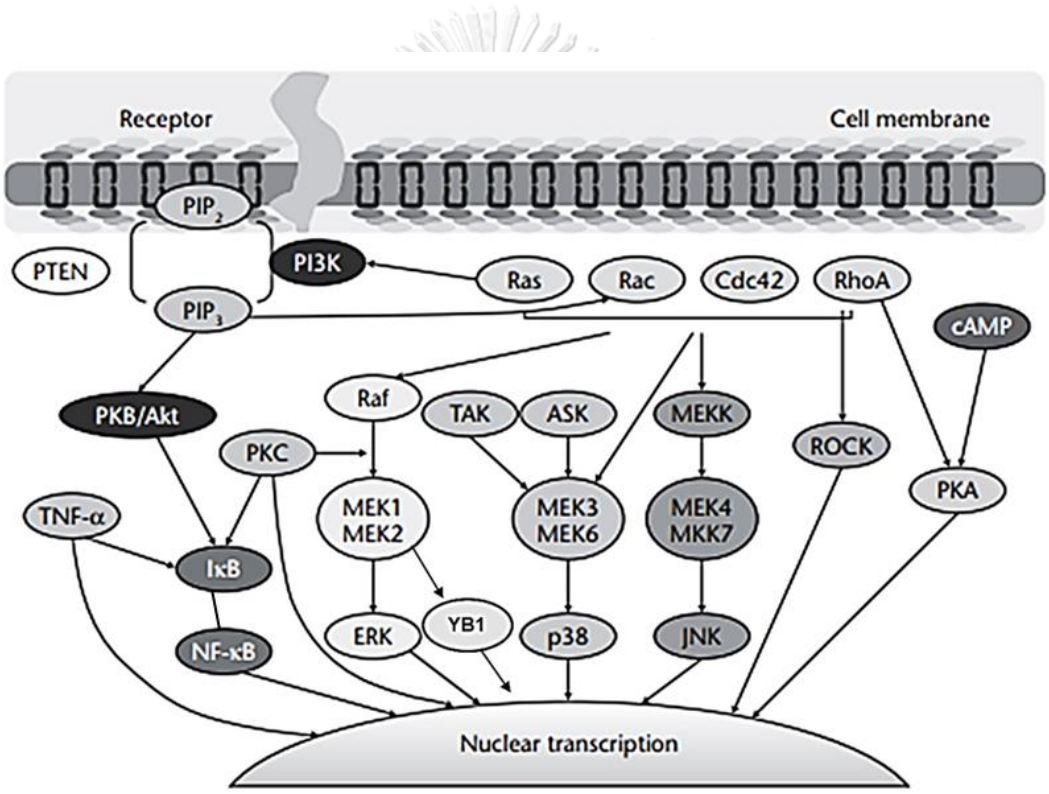


Figure 3. Signal transduction pathways and transcriptional mechanisms of MDR1/P-gp -mediated multiple drug resistance in human cancer cells (Sui et al., 2012).

2.5 The "non-drug efflux transporter" resistance based MDR

The "non-drug efflux transporter"-related mechanisms may involve with increasing cellular detoxifying enzyme systems such as glutathione S-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1), UDP glucuronosyltransferase (UGT), and heme oxygenase-1 (HO-1) (Kachalaki et al., 2016; Salazar et al., 2006). The cells resistant to alkylating agents such as cyclophosphamide, doxorubicin, melphalan and chlorambucil were found higher GSH levels than their sensitive counterparts (Krishna and Mayer, 2000). GST caused conjugation between GSH and alkylating agents resulting in the loss of cytotoxic activity detoxification of alkylating agents (Osbild et al., 2006). Moreover, signaling to cell death can be blocked by an increase in anti-apoptotic molecules and/or by a decrease or defective function of pro-apoptotic proteins. Alterations in the death receptor and mitochondrial pathway as well as aberrant expression pro-/anti-apoptosis proteins may lead to chemoresistance of cancer cells (Abrams et al., 2017; Danial and Korsmeyer, 2004).

2.5.1 Alteration of cellular detoxification systems

Nuclear factor erythroid 2-related factor 2 (Nrf2) is transcription factors, which regulates cell response to detoxification of reactive oxygen species (ROS) and xenobiotics, the repair of damaged proteins, the regulation of cell metabolism, as well as genes involved in cell survival and proliferation insults (Lee et al., 2020). Nrf2 is generally localized in the cytoplasm, where it is sequestered by its repressor Keap1. The Cul3 mediates the ubiquitination and subsequent 26S proteasome degradation of Nrf2. The effect of oxidative stressors or drugs on the Keap1 can trigger a conformational change in this protein, leading to Nrf2 stabilization (Figure 4) (Gómez et al., 2016). It has been demonstrated that Nrf2 in the Keap1-Nrf2-sMaf-ARE signaling pathway plays an essential role in the chemoresistance of cancer cells (Mahaffey et al., 2009; Vollrath et al., 2006). High expression Nrf2 levels have been linked to chemoresistance of cancer cell lines toward chemotherapeutic drugs such

as cisplatin, doxorubicin, and etoposide (DeNicola et al., 2011; Hayashi et al., 2003; Kim et al., 2008; Wang et al., 2008; Vollrath et al., 2006).

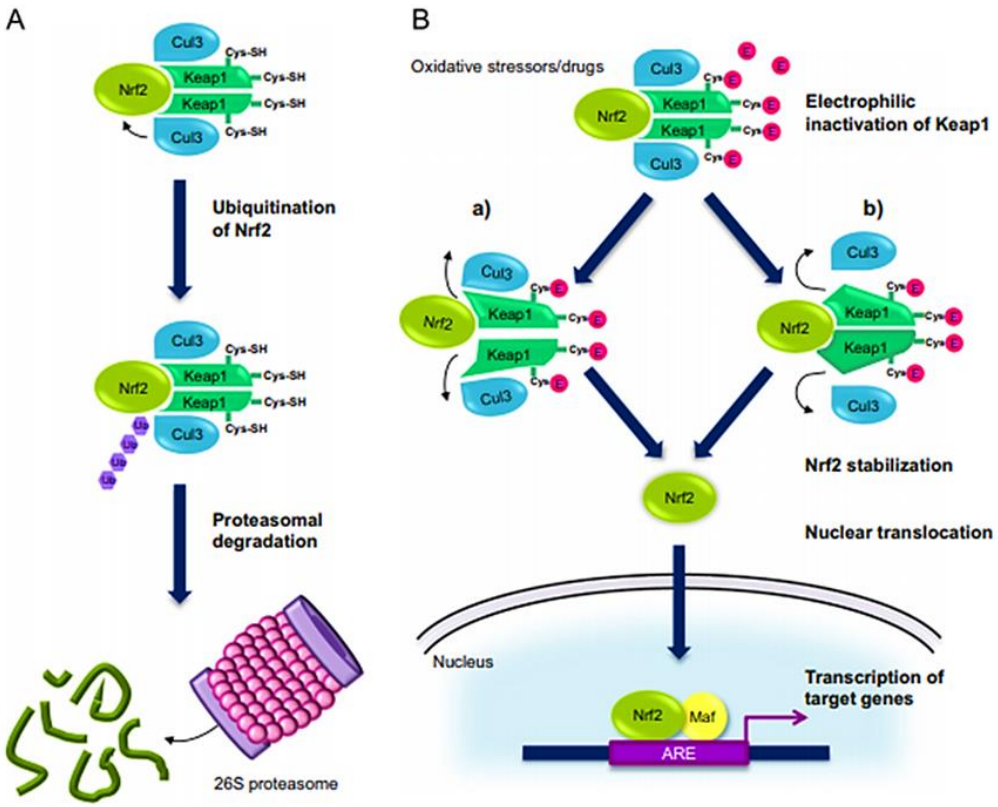


Figure 4. Regulation of Nrf2 in basal and oxidative stressors or drugs conditions in cancer cells (Gómez et al., 2016).

Furthermore, Nrf2 system mediated acquired resistance toward DOX in ovarian and breast cancer cells were related to a PI3K/Akt/Nrf2 signaling axis (Ganan-Gomez et al., 2013; Shim et al., 2009; Turpaev, 2013). Activation of the PI3K/Akt signaling pathway can also induce Nrf2 accumulation, either through an increase in Nrf2 transcription, nuclear accumulation, or inhibition of GSK3-β-TrCP-induced proteasomal degradation of Nrf2 (Jung et al., 2018). Nrf2 binds to ARE, which affects the transcription of various antioxidant and detoxification genes such as glutathione S-transferase (GST), NAD (P) H: quinone oxidoreductase 1 (NQO1), and heme

oxygenase-1 (HO-1) genes (Figure 5) (Salazar et al., 2006). Generally, the phosphorylation of a repressor GSK-3β at Ser 9 by Akt reduces Nrf2 phosphorylation so that Nrf2 can escape the ubiquitination and proteasomal degradation processes in the cytoplasm (Mcubrey et al., 2014). This protein suppresses various transcription factors implicated in oncogenes such as NF-kB, Snail, Notch, forkhead and CAAT-enhancer binding protein (C/EBP) (Mcubrey et al., 2014; Mishra et al., 2010; Walz et al., 2017). Thus GSK-3β can regulate transcription factors activity by multiple mechanisms. Lying downstream of the PI3K/Akt pathway, GSK-3β can be regulated by active Akt through phosphorylation at serine 9 position (Ser 9) (Chowdhry et al., 2013; Li et al., 2018; Ugolkov et al., 2016). Therefore, the regulation of Nrf2 signaling cascade through Akt/GSK-3β activation can be a potential therapeutic approach to enhance the efficacy of anticancer drugs and overcome MDR.

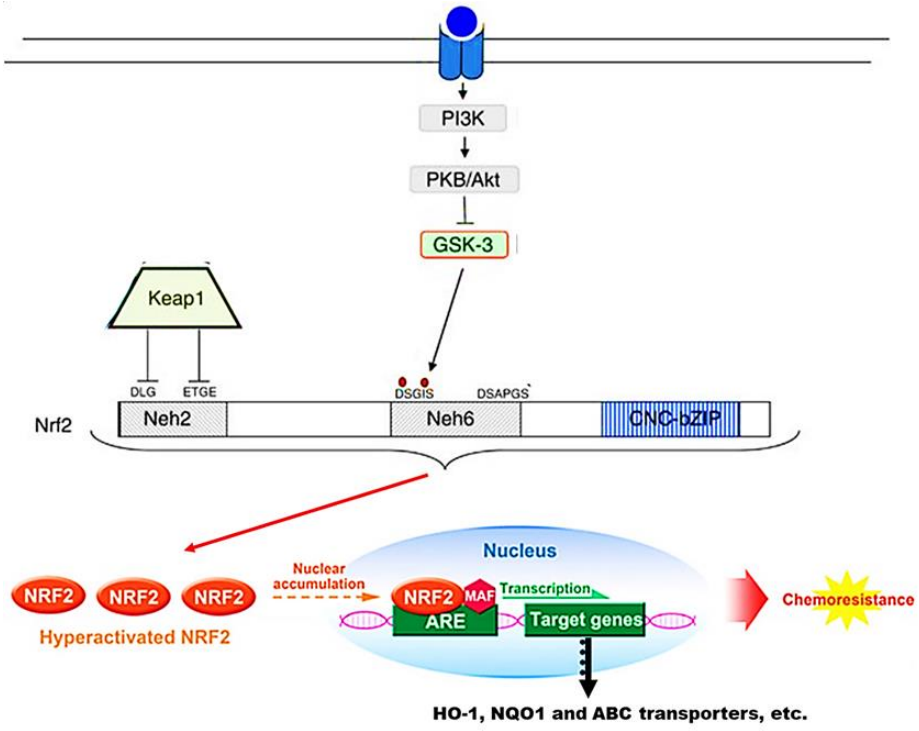


Figure 5. Activating the PI3K/AKT pathway and inhibits GSK-3β to activate Nrf2 and induce Nrf2 nuclear translocation and promote the transcription of various Nrf2 target genes (Chowdhry et al., 2013).

2.5.2 Inhibition of the cell death (blocking apoptosis pathway)

Failure to activate the process of programmed cell death or apoptosis is an important mode of drug resistance and survival in cancer cells (Figure 6) (Fulda, 2009) either by an increase in anti-apoptotic molecules and/or by a decrease of pro-apoptotic proteins (Pan et al., 2016). Apoptosis occurs through both intrinsic and extrinsic pathways (Ouyang et al., 2012). The extrinsic pathway is activated by binding of a ligand to death receptor on the cell surface, which subsequently activates caspase-8 and sequential downstream caspases. The intrinsic pathway centers on mitochondria and checkpoint proteins (pro-/-anti-apoptotic proteins). The pro-apoptotic molecules such as Bax cause mitochondria to release cytochrome c, leading to activation of downstream caspases (Danial et al., 2004; Holohan et al., 2013; Wang et al., 2015). In contrast, anti-apoptotic factors such as Bcl-2 and survival

pathway (Akt and MAPKs) prevent apoptosis initiation has been linked to the reduction of stress-induced apoptosis as well as the development of chemo-resistance in cancer cells. Bcl-2 located on mitochondria can inhibit cytochrome c release. High levels of Bcl-2 have been associated with aggressive cancer and/or chemoresistance (Fulda, 2009). Preclinical models have also established that the down-regulation of Bcl-2 protein leads to an increase in apoptosis and improved response to chemotherapy. It was demonstrated that doxorubicin-resistant cancer cells had higher expression levels of anti-apoptotic proteins and activation survival pathways such as ERK1/2, JNK and p-38/MAPK (Braicu et al., 2019; Brown and Attardi, 2005; Davis et al., 2003). Previous studies have shown that the mitogen activated protein kinase (MAPK) pathways are critical for converting diverse extracellular signals, including oxidative stress (ROS), to biological responses, and they modulate many cellular processes, such as cell proliferation, differentiation, and apoptosis. Numerous studies indicate that ROS regulate certain cellular processes, such as apoptosis, gene expression/mutations, and activation of cell signaling cascades (Braicu et al., 2019; Zhang et al., 2016). Following generation by cellular metabolism or induction by exogenous factors, ROS play a role in numerous reduction-oxidation reactions and signaling pathways that regulate cancer initialization and development, such as oxidative-stimulated MAPK pathway (Cui et al., 2018; Newsholme et al., 2016), PI3K/AKT pathway, nuclear factor kappa B (NF-**K**B) (Jin et al., 2014; Liu et al., 2009). The activation of MAPKs depends on the dual phosphorylation of threonine and tyrosine residues by upstream MAPK kinases (MEKs) and other MKKs. High activated MAPKs play key roles in activating transcription factors and downstream kinases, leading to the induction of immediate-early genes expression, and subsequent changes in other cellular processes on resistant chemotherapy (Lee et al., 2020; Peti and Page, 2013). MAPKs can divide into three subfamilies including extracellular signal-related kinases 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), and p-

38 MAP kinase (p-38MAPK) (Johnson et al., 2002). ERK1/2 behave mainly as mitogen-activated proliferation/differentiation factors (Tamura et al., 2002), whereas JNK and p-38MAPK are mainly stress-activated proteins related to apoptotic cell death (Braicu et al., 2019; Sui et al., 2012). The modulation of the three MAPK subfamilies is responsible for the cells fate, whether they undergo apoptosis or cells death. Moreover, the Bcl-2/Bax ratio was high in the breast cancer doxorubicin resistance (MCF-7/DOX) cells, as the Bcl-2 was up-regulated while the Bax level was almost unchanged (AbuHammad and Zihlif, 2013; Korsmeyer et al., 1993). The increased value of the Bcl-2/Bax ratio was associated with the suppression of apoptosis via activation of the p-38MAPK signaling pathway. (Chen et al., 2014; Guo et al., 2008; Li et al., 2017; Yang et al., 2011). In addition, a blockade of the MEK-ERK signaling pathway was able to suppress P-gp expression in HT-15, SW620-14, MCF-7/MDR and MDA-MB-231/MDR cell lines (Katayama et al., 2007). The discovery of responsible apoptosis escape mechanisms has a great potential for translational medicine since such defects in apoptosis molecules may serve as targets for the design of novel therapeutic strategies as well as molecular markers to predict treatment response and reverse chemotherapy resistance.

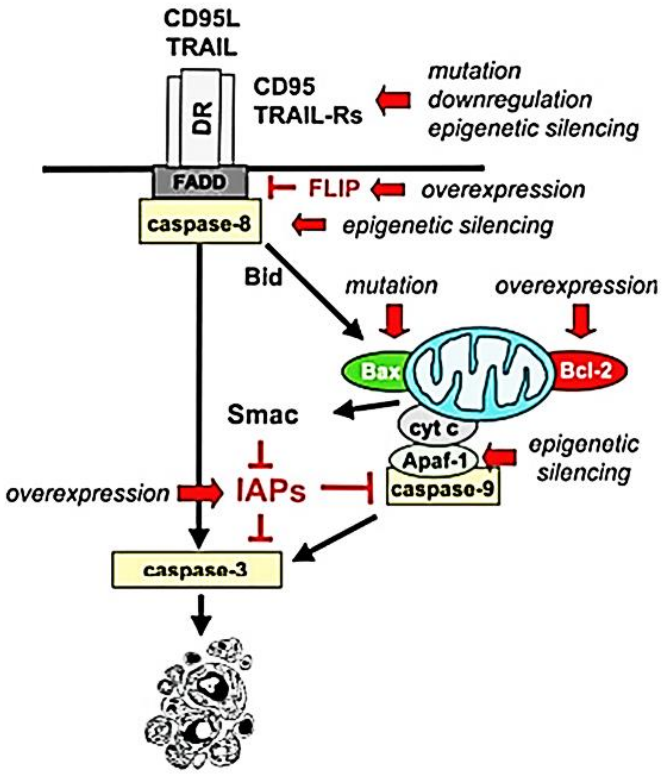


Figure 6. Defects in apoptosis pathways in human cancers (Fulda, 2009).

2.6 Phytochemicals and pharmacological activities

Rhinacanthus nasutus (Linn.) Kurz

Rhinacanthus nasutus (Linn.) Kurz (*R. nasutus*) (Figure 7) is one of the herbal medicines in the family Acanthaceae. It is so called in Thai "Thong phan chang". It widely distributes in Southeast Asia, South China, and India. This plant has been used in traditional medicine for the treatment of diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and several skin diseases (Siripong et al., 2006b).

Figure 7. Rhinacanthus nasutus *(Linn.) Kurz.*

R. nasutus plant is well known as the sources of flavonoids, steroids, terpenoids, anthraquinones, lignans and especially naphthoquinone analogs as major constituents. Rhinacanthins were naphthoquinones derivatives isolated from leaves and roots of *R. nasutus* plant (Bukke et al., 2011; Siripong et al., 2006a, b). Currently, there are 15 rhinacanthin derivatives identified (rhinacanthins A, B, C, D, G, H, I, J, K, L, M, N, O, P, and Q). Rhinacanthin-C (RN-C), a major naphthoquinone ester from the leaves and roots of this plant (Figure 8), has been shown to possess antiviral, anti-

inflammatory, antibacterial activity, anti-proliferative, cytotoxic activities, anti-tumor, immunomodulatory activity, antioxidant activity, and antiplatelet activity (Bukke et al., 2013; Gotoh et al., 2004; Punturee et al., 2004; Siripong et al., 2006b). These naphthoquinone compounds have gained major attention as new potential therapeutic agents for cancer.

The pharmacological effects of *R. nasutus* and rhinacanthin-C, in particular, anti-cancer and cytotoxic activities were reported in several in vitro cell culture studies. For examples, extracts of *R. nasutus* and rhinacanthin-C were found effective as cytotoxic substances in several cancerous cells including human epidermoid carcinoma (KB), human laryngeal carcinoma (Hep2), human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HepG2), human cervical carcinoma (SiHa), human amelanotic melanoma carcinoma (C-32), lewis lung carcinoma (LLC), human cervix adenocarcinoma (HeLa), MDR1 overexpressing subline of human cervical carcinoma (Hvr100-6), human prostatic cancer cell (PC-3), human lung carcinoma (A-549), human colon adenocarcinoma (HT-29), and leukemia (HL-60) cells and human bladder carcinoma (T-24) (Gotoh et al., 2004). Rhinacanthin-C was capable of inhibiting cell proliferation and induced apoptosis, of human cervical carcinoma (HeLS3) in concentration-and time-dependent manners. Is this the antitumor efficacy was interfering with cell cycle progression, inducing mediated by activation of caspase 3 activity as well as targeting DNA topoisomerase II of tumor cells (Siripong et al., 2006b). Furthermore, extract of *R. nasutus* containing rhinacanthin derivatives was shown to inhibit iNOS and COX-2 gene expressions and to suppress the release of nitric oxide (NO), PGE2 and TNF-**α** in LPS-activated RAW 264.7 cells (Nisarat et al., 2010). Rhinacanthin-C was able to suppress LPS-stimulated osteoclastogenesis and bone resorption in vitro and in vivo (Tomomura et al., 2015). The anti-osteoclastogenic action of rhinacanthin-C was linked to inhibition of RANKL-induced TRAF6-TAK1 association followed by activation of MAPKs/NF-**K**B (Tomomura

et al., 2015). Rhinacanthin-C showed anti-metastasis activities and protein expression levels of MMP-2 and uPA were mediated through decreasing FAK, p38-, ERK1/2-, JNK/MAPK pathway, downregulating the transcription factor NF-**K**B, leading to inhibition of the expression of MMP-2 and uPA against a human CCA (KKU-M156) cell line (Boueroy et al., 2018).

Rhinacanthin-C has reported its ability to reversibly inhibit the function P-gp and MRP2 in Caco-2 cells (Wongwanakul et al., 2013). In addition, rhinacanthin-C at non-cytotoxic concentration could potentiate doxorubicin cytotoxicity in MCF-7 cells by increasing intracellular doxorubicin concentration up to the cytotoxicity level time-and concentration-dependent manners and rhinacanthin-C significant pharmacokinetic herb-drug interaction via inhibiting various influx and efflux drug transporters (i.e., P-gp, BCRP, OATP1B1, and OATP1B3) and phase I drug-metabolizing enzymes CYP isoforms (i.e., CYP2C8, CYP2C9, and CYP2C19) (Chaisit et al., 2017; Dunkoksung et al., 2019). Currently, the effect of rhinacanthin-C in reversing cancer cells MDR was limit to direct inhibition of P-gp function, other potential mechanisms such as suppression of P-gp expression and other "non-drug efflux transporter" related mechanisms (e.g. induction apoptosis, suppression of detoxification system) the key defense mechanism against oxidative stress remains unreported.

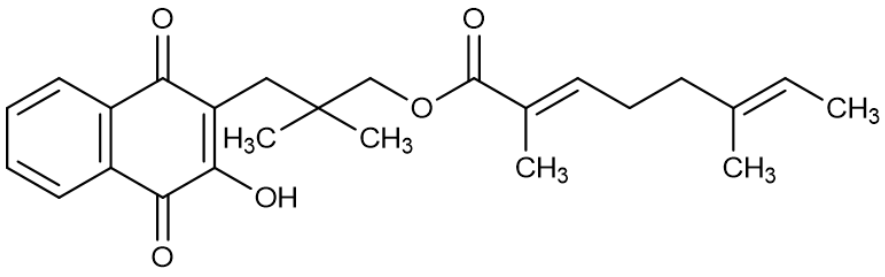


Figure 8. Chemical structure of rhinacanhin-C (RN-C).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Test compound

Rhinacanthin-C (RN-C) purity (> 94%) was provided by Dr. Pongpun Siripong, Natural Products Research Section, Research Division, National Cancer Institute (NCI), Bangkok, Thailand. This naphthoquinone ester was isolated from leaves and roots of *Rhinacanthus nasutus* Kurz., using the purification and identification processes as previously described in Siripong et al., 2006.

A 50 mM rhinacanthin-C stock solution was prepared in dimethyl sulfoxide (DMSO) 99.9% and kept at -20 °C until used. The final concentration of DMSO in each experiment was less than 0.1% (v/v). At this concentration, DMSO had no cytotoxic effect on either the parental MCF-7 or the doxorubicin-resistant (MCF-7/DOX) cells.

3.1.2 Chemicals and reagents

Calcein acetoxymethyl (Calcein-AM), (S)-(+)-camptothecin (CAM), etoposide (ETO), Hank's Balanced Salt Solution (HBSS), 2', 7'-dichlorofluorescin di-acetate (H_2DCFDA), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), glycerol, Hoechst 33342, sodium chloride (NaCl), sodium dodecyl, sulfate (SDS), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), tris hydrochloride (Tris-HCl), tritron-X 100, trypsin, tween-20, protease inhibitor cocktails and verapamil were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), hydrogen peroxide (H_2O_2) and LY294002 were purchased from Merck (Darmstadt, Germany). Doxorubicin hydrochloride (DOX) was purchased from Abcam (Cambridge, UK). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was

purchased from Invitrogen (Carlsbad, CA, USA). RPMI-1640 medium, TRIzol® reagent and L-glutamine were obtained from Gibco Life Technologies (Grand Island, NY, USA). The ImProm-II™ reverse transcription system was from Promega (Madison, WI, USA). Pierce™ BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The SsoFast™ EvaGreen® Supermix kit was from Bio-Rad (Berkeley, CA, USA). Mouse monoclonal anti-phosphorylated I**K**B-α (sc-8404), anti-I**K**B-α (sc-1643), anti-phosphorylated NF-**K**B (p65) (sc-136548), anti-NF-**K**B p65 (sc-8008), Nrf2 (sc-365949), anti-NQO1 (sc-32793), anti-HO-1 (sc-136960), anti-Bcl-2 (sc-7382), anti-BAX (sc-7480), anti-P-gp (sc-55510), anti-PARP (sc-8007) and anti-YB-1(sc-101198) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Mouse monoclonal anti-1 phosphorylated ERK, anti-ERK, rabbit monoclonal anti-phosphorylated Akt and anti-Akt anti-phosphorylated Nrf2, anti-phosphorylated JNK, anti-JNK, anti-phosphorylated GSK-3β, anti- GSK-3β, anti-phosphorylated p-38 and anti-p-38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit monoclonal anti-p-Nrf-2 was purchased from Boster Bio (Pleasanton, CA, USA). Mouse monoclonal anti-GAPDH and horseradish peroxidase-conjugated anti-(Mouse-IgG) antibody were purchased from Calbiochem (San Diego, CA, USA). Oligonucleotide primers were obtained from Integrated DNA Technologies (The Gemini Singapore Science Park II, Singapore). The SuperSignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA).

3.1.3 Experimental instruments

1. Analytical balance: Mettler Toledo, Switzerland
2. Autoclave: Hirayama, Saitama, Japan
3. Autopipette: Gilson, USA
4. Cell culture plate: 6-well, 24-well, and 96-well: Coring, New York, USA

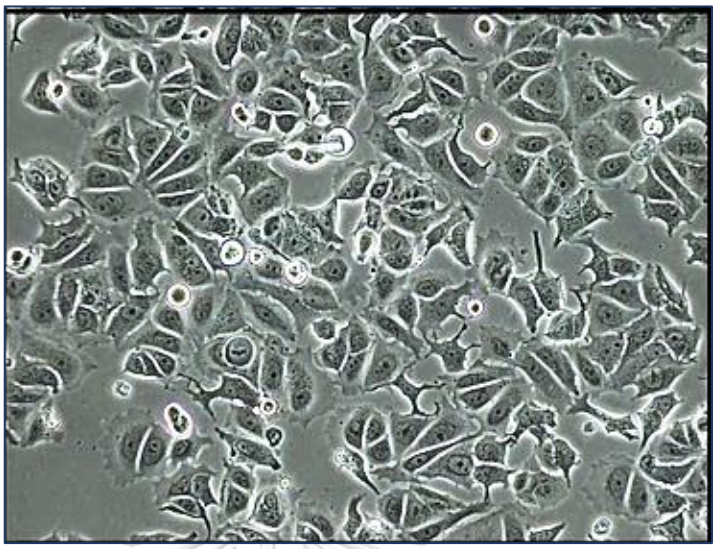
5. CFX96 Touch™ Real-Time PCR Detection System: Bio-rad, Hercules, CA, USA
6. Fluorescence microscope: BX-FLA, Olympus, Tokyo, Japan
7. GE ImageQuant LAS 4000: GE Healthcare Life-Sciences Ltd., Branch, Taiwan
8. Hot air oven: MEMMERT, Buchenbach, Germany
9. Humidified carbon dioxide incubator: Thermo Scientific Forma Series II, New York, USA
10. Image J software: NIH, Bethesda, MD, U.S.A
11. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany
12. Laminar air flow hood: BossTech, Bangkok, Thailand
13. Luminescence-image analyzer: ImageQuant™ LAS 4000, GE Healthcare Bio-sciences, Japan
14. Microplate reader: Wallac 1420 Perkin-Elmer Victor 3, Perkin Elmer lnc., Massachusetts, USA
15. Microplate reader: Spectra MAX M5, Molecular Devices, USA
16. NanoDrop™ 2000/2000c Spectrophotometers: Thermo Scientific, Wilmington, UK
17. OmniPAGE mini vertical systems: Cleaver Scientific, Warwickshire, UK
18. Orbital shaker: OS-20, Biosan, Riga, Latvia
19. pH meter: CG 842, Schott, Hofheim, Germany
20. Refrigerated centrifuge: Z 383K, Hermle Labortechink,Burladingen, Germany
21. Tissue culture flasks: Coring, New York, USA
22. Trans-blot® SD semi-dry transfer cell: Missouri, TX, USA
23. Vortex mixer: mode K550-GE. Scientific Industries, New York, USA
24. Water bath: WB22, Memmert, Germany

3.1.4 Cell culture

Two types of cell culture including MCF-7 (ATCC® HTB-22™) and doxorubicin-resistant subline MCF-7/DOX were used in this study (Figure 9). The cells were subculture at 80-90% confluence, using 0.25% trypsin solution containing 1 mM EDTA.

- The MCF-7 cells, a human breast adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2.
- The doxorubicin-resistant subline MCF-7/DOX cells were in-house developed from the MCF-7 cells by culturing the cells in RPMI-1640 medium containing doxorubicin. The cells were further developed into the doxorubicin-resistant subline MCF-7/DOX cells by stepwise selection for resistance upon increasing concentrations of DOX up to 1.5 μM (Chaisit et al., 2017; Mealey et al., 2002). The MCF-7/DOX cells were maintained in RPMI-1640 completed medium containing 1.5 μM of DOX in order to keep their resistance DOX.

A.



B.

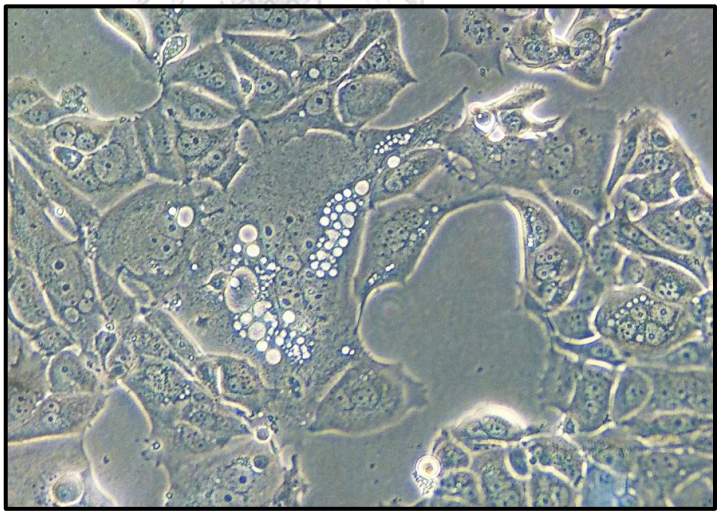


Figure 9. The morphology of the human breast adenocarcinoma cell line. (A) MCF-7 parental cell line. (B) MCF-7/DOX resistant cell line.

3.1.5 Drug treatments

One week before the experiments, the medium for MCF-7/DOX cells was replaced by DOX-free RPMI-1640 complete medium. On the experimental day, the treatments were divided into two parts:

Part I: Either non-toxic concentration of rhinacanthin-C (0.25, 0.5, and 1 μM) or verapamil (60 μM; a positive control) was added to MCF-7/DOX cells for 24 to 48 h. Then the cells were washed and harvested for further assessment of their chemosensitivity, P-gp function and expression (Figure 10).

Part II: The rhinacanthin-C (3, 16, and 28 μM) was add to MCF-7/DOX cells for either 1 h, 24 h or 48 h. The cells were then harvested and evaluated the promotion of oxidative stress-induced apoptosis and the involved MAPKs signaling pathway. In addition, the suppression of cellular detoxification system via the Akt/GSK-3β/Nrf2 signaling pathway was evaluated, as shown in Figure 10.

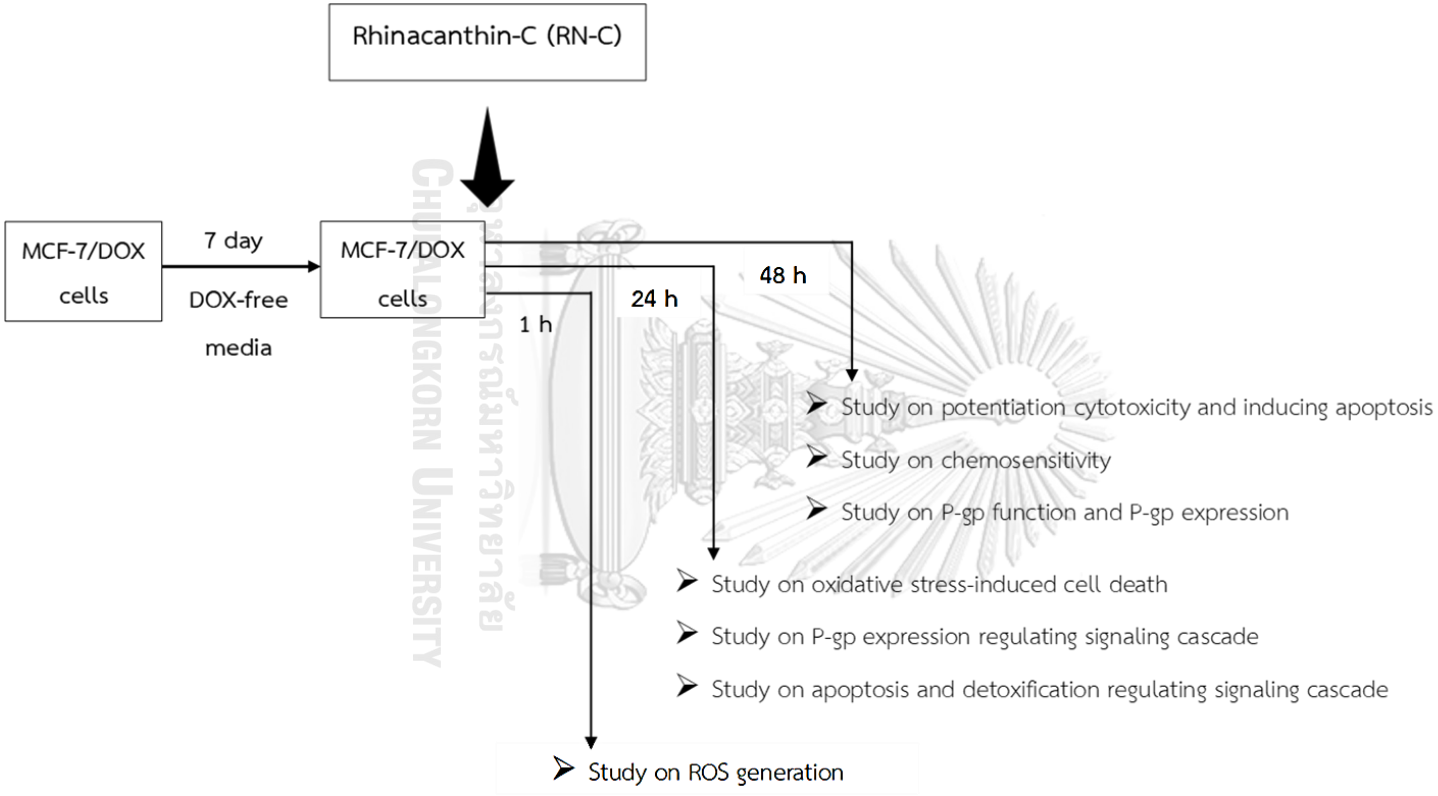


Figure 10. Schematic diagram of the study design for assessment of rhinacanthin-C-mediated downregulation of P-gp, suppression of cellular detoxifying enzyme and signaling pathway-induce apoptosis in MCF-7/DOX cells

3.2 Methods

3.2.1 Cell viability

Cell viability was determined by using an MTT assay (Carmichael et al., 1987). MTT is a yellow water-soluble tetrazolium dye that can be converted to a purple insoluble formazan using mitochondria reductase enzyme (Figure 11). Which can be measured spectrophotometrically with a microplate reader at 570 nm. The cells (either MCF-7 or MCF-7/DOX) were seeded onto 96 well-plates at a density of 5×10^3 cells per well. After 24 h, the cells were treated as follows:

1. Treating the MCF-7 and the MCF-7/DOX cells with various concentrations of rhinacanthin-C (0-30 μM) for 48 h at 37 °C in a humidified atmosphere of 5% CO_2.
2. Treating the MCF-7 and the MCF-7/DOX cells with various concentrations of doxorubicin (0-300 μM) for 48 h at 37 °C in a humidified atmosphere of 5% CO_2.
3. Treating the MCF-7/DOX cells were cultured in the presence or absence of rhinacanthin-C at non-toxic concentrations (0.25, 0.5, and 1 μM) for 48 h. Then, the cells were washed and were added various concentration of cytotoxic agents (i.e. camptothecin (0.01, 0.1, and 1 μM), doxorubicin (5, 50, and 100 μM) and etoposide (25, 250, and 750 μM) for 24 h, at 37 °C in a humidified atmosphere of 5% CO_2. Verapamil (60 μM) was used as a positive control.
4. Treating the MCF-7/DOX cells were incubated with H_2O_2 (50 μM) for 1 h before the addition of rhinacanthin-C (3, 16, and 28 μM) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2.

At the end of the treatment, the cells were incubated with MTT solution (0.5 mg/ml in serum-free RPMI-1640 without phenol red) for another 4 h, at 37 ºC. The intracellular formazan crystals in each well were dissolved with 100 µl DMSO. The absorbance was evaluated using in a microplate reader at a wavelength of 570 nm. Cell viability was calculated as the percentage of the control (untreated group).

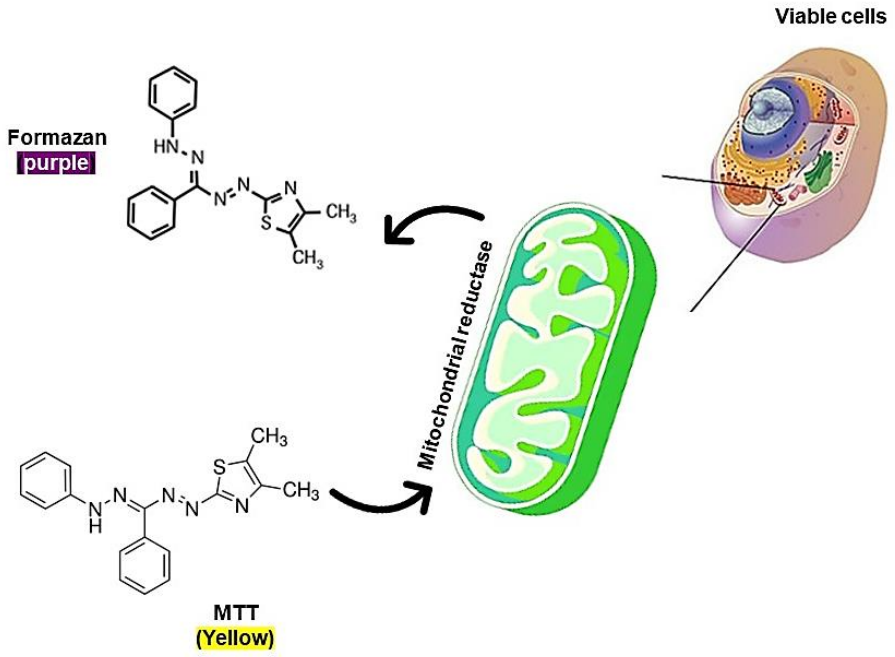


Figure 11. Principle of MTT assay.

3.2.2 Determination of P-gp functions

P-gp function was assessed by substrate uptake assay as described previously (Chaisit et al., 2017). Briefly, the cells were seeded onto 24-well plates (2.5×10^5 cells/well) overnight, and then incubated with calcein-AM (0.4 μM) in the dark for 60 min. At the end of the incubation period, cells were washed and lysed with 0.3 N NaOH and 1% Triton X-100. Fluorescence intensity of the calcein was determined at 485/535 nm (excitation/ emission wavelength) and normalized to the protein content in each sample. The amount of protein was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

3.2.3 Determination of reactive oxygen species (ROS) productions

Cellular ROS was measured by $DCFH_2$-DA assay. Dichloro-dihydro-fluorescein diacetate ($DCFH_2$-DA), a hydrophobic compound, can diffuse into the cells and is hydrolyzed by esterase to obtain 2', 7'- dichlorodihydrofluorescein (DCFH) which can further be oxidized by intracellular reactive oxygen species (ROS) to 2', 7'-dichlorofluorescein, the highly fluorescent product. The fluorescence intensity indicated the ROS level in the cell (Boonyong et al., 2017). In brief, the cells were grown at a density of 2×10^4 cells/well for 24 h. Then, the cells were treated with 100 μM DCFH-DA for 30 min prior to the addition of either rhinacanthin-C or a positive control hydrogen peroxide (H_2O_2; 250 μM) for 1 h. At the end of the treatment period, the cells were washed with ice-cold PBS and lysed with 1% Triton X-100. The fluorescence intensity of DCF was measured by a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA) at the excitation and emission wavelengths of 485 and 535 nm, respectively.

3.2.4 Apoptosis assay

Apoptotic cells were assessed using a fluorescent nuclear staining dye Hoechst 33342 (Treesuwan et al., 2018). MCF-7/DOX cells (8×10^3 cells/wells) were seeded in 96-wells plates overnight. Then, the cells were treated with rhinacanthin-C for 24 h and 48 h. Following the treatment, the cells were incubated with Hoechst 33342 (10 µg/ml) for 30 min in the dark. Then, the cells were washed three times with ice-cold PBS. The morphology of nuclear chromatin condensation and DNA fragmentation of apoptotic cells were visualized under a fluorescence microscope (20x, original magnification) (Nikon Inverted Microscope Eclipse Ti-U Ti-U/B, NY, USA) at the excitation/emission wavelengths of 350/461 nm. The apoptotic cells were counted and presented as a percentage of total cells.

3.2.5 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Expression levels of *MDR1* mRNA were determined by qRT-PCR technique. The cells were cultured at a seeding density of 1.0×10^6 cells/well and incubated overnight. Then, the cells were treated with rhinacanthin-C at 0.25, 0.5, and 1 µM and incubated for 48 h. After treatment, total RNA was extracted using TRIzol® Reagent kit, according to the manufacturer's instructions and kept at -80 °C. The amounts of RNA samples were quantified using a NanoDrop™ 2000/2000c spectrometer at the wavelengths 260 and 280 nm. In this study, (1 µg) of RNA was reversely transcribed to cDNA by ImProm-II™ reverse transcription system.

The qPCR amplification was performed using the SsoFast™ EvaGreen® Supermix kit. The sense (S) and antisense (AS) primers were *MDR1*, S: 5'-CCCATCATTGCAATAGCAGG-3' and AS: 5'-TGTTCAAACTTCTGCTCCTGA-3'; and *GAPDH*, S: 5'-AAGGTCGGAGTCAACGGATTTGGT-3' and AS: 5'-ATGGCATGGACTGTGGTCATGAGT-3'. The conditions of qPCR were as follows: an initial denaturing at 95 °C for 2 min, 40 cycles of denaturing at 95 °C for 5 s, annealing, and extension at 60 °C for 5 s. The

mRNA content was normalized to that of GAPDH (an internal control) and calculated using the Ct ($2^{-\Delta\Delta_{CT}}$) method (Boonyong et al., 2017).

3.2.6 Western blot analysis

Expression of target proteins in this study (e.g., P-gp and signaling proteins), expression of apoptosis marker protein and regulatory proteins apoptotic signaling (MAPKs; JNK, ERK1/2, and p-38) and cellular detoxifying enzyme and signaling proteins (Akt/GSK-3β and Nrf2 signaling pathway) were determined by western blot analysis.

Briefly, the MCF-7/DOX cells were treated with rhinacanthin-C at various concentrations for 24 h. After treatments, the cells were washed with PBS and lysed with the ice-cold lysis buffer [50 mM Tris-HCl pH 6.8, 150 mM NaCl, 20 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail] (Roche Diagnostics, Indianapolis, IN, USA). The cell lysates were then centrifuged at 12000 x g for 20 min at 4 °C and collected the supernatant for blotting analysis. Protein concentration were quantified using the Pierce BCA protein assay kit (Thermo Fisher Sciencetific, Waltham, MA) at 570 nm.

Proteins (30 µg) were added into sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and denatured at 95 °C for 5 min. The proteins were loaded onto a 7-15% SDS-polyacrylamide gel electrophoresis and separated at a constant voltage of 90 V for 3 h. Then, the proteins were electrically transferred to PVDF membrane using a constant voltage of 10 V for 1 h. The membranes were blocked with 5% skim milk or 5% BSA in TBS-T (25 mM Tris–HCl, pH 7.4, 125 mM sodium chloride, 0.05 % Tween 20) for 1 h at room temperature and then probed with primary antibodies against for P-gp, phospho-ERK1/2, ERK1/2, phospho-p-38, p-38, phospho-Akt, Akt, Bax, Bcl-2, phospho-I**K**B-α, I**K**B-α, phospho-GSK-3β, GSK-3β, phospho-NF-**K**B (p65), NF-**K**B (p65), phospho-Nrf2,

Nrf-2, NQO1, HO-1, PARP, YB-1 and GAPDH at 1:1,000 dilution with TBS-T containing 5% non-fat dry milk at 4 °C overnight. After washing with TBS-T, the membranes were incubated at room temperature with secondary conjugated secondary antibody (1:2000) in TBS-T for 1 h and then developed by using the Super Signal® West Pico chemiluminescent substrates. The membranes were further visualized by a GE ImageQuant™ LAS 4000 (GE Healthcare Life-Sciences Ltd., Branch, Taiwan). In this study, GAPDH were used as an internal standard. The density of bands corresponding to protein blotting with the antibody were quantified using Image J software (NIH) and normalized based on the amount of GAPDH.

3.2.7 Statistical analysis

All data are presented as the mean ± standard error of mean (SEM) from at least three independent experiments performed in duplicate or triplicate.

The degree of resistance toward DOX (or resistance index; RI) of MCF-7/DOX cells was estimated in the condition that both MCF-7 and MCF-7/DOX cells were treated with DOX for 48 h. The RI was calculated from the ratio between the IC_{50} value of doxorubicin on MCF-7/DOX cells vs the IC_{50} of doxorubicin on MCF-7 cells.

Moreover, the effect of rhinacanthin-C on reversal fold (RF) was calculated from the ratio between the IC_{50} value of each cytotoxic agent obtained from the rhinacanthin-C-treated (or verapamil-treated) cells and those from untreated cells. The RF value could be a measure of an increase in cell sensitivity toward cytotoxic agent treatment in the presence of rhinacanthin-C.

Statistical analyses were performed by either the Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's post hoc analysis was used to compare the significant differences between the two groups using SPSS statistics 21 software (IBM Corporation, USA). Differences were considered statistically significant at $*p < 0.05$.

CHAPTER IV

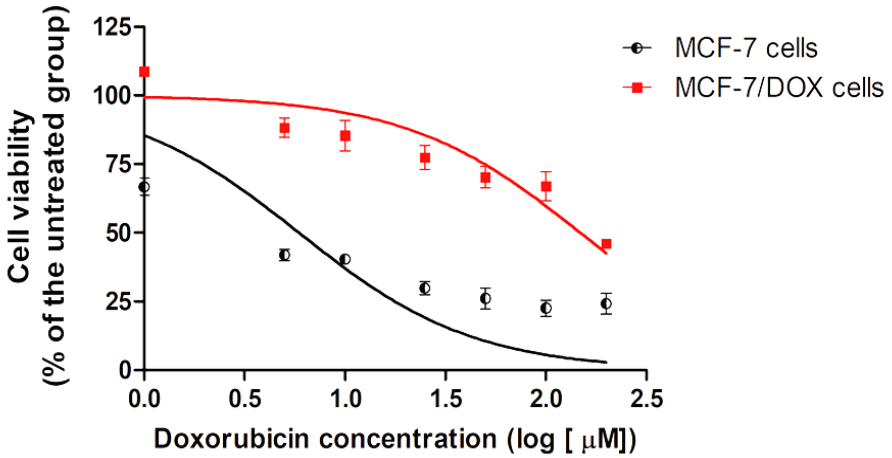
RESULTS

4.1 Part 1. Chemosensitivity enhancing effect of rhinacanthin-C (RN-C) on MCF-7/DOX cells and its involvement the mechanisms with P-gp down-regulation.

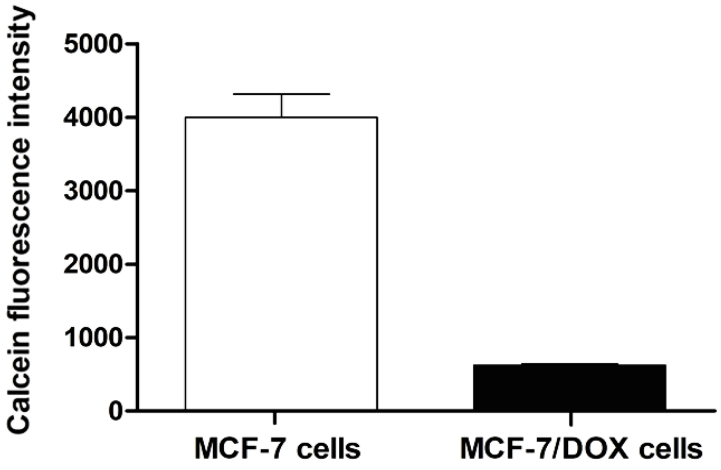
4.1.1 Expression of P-gp in the MCF-7/DOX cells

The DOX-resistant MCF-7 (MCF-7/DOX) cells were developed after long-term continuous exposure of the MCF-7 cells to doxorubicin. As shown in Figure 12A, the MCF-7/DOX cells were approximately 62-fold more resistant to DOX cytotoxicity than MCF-7 cells. The IC_{50} values of DOX in MCF-7/DOX cells and MCF-7 cells were 155.85 ± 1.04 μM and 2.52 ± 1.55 μM, respectively. Since DOX is a known P-gp substrate, we further assessed the function and expression of P-gp in the DOX-resistant MCF-7/DOX cells compared to parental DOX-sensitive MCF-7 cells. The activity of P-gp as an efflux pump was demonstrated by using calcein-AM, a specific P-gp substrate, in a substrate uptake assay. As shown in Figure 12B, the intracellular accumulation of calcein in the MCF-7/DOX cells was approximately 6.32-fold less than that in the sensitive parental cells. These findings were well correlated to the observed higher P-gp expression levels in the resistant cells compared to those of MCF-7 cells (Figure 12C and D). Hence, higher degree of resistance to DOX of the MCF-7/DOX cells could be attributed to higher expression levels of P-gp in these cells.

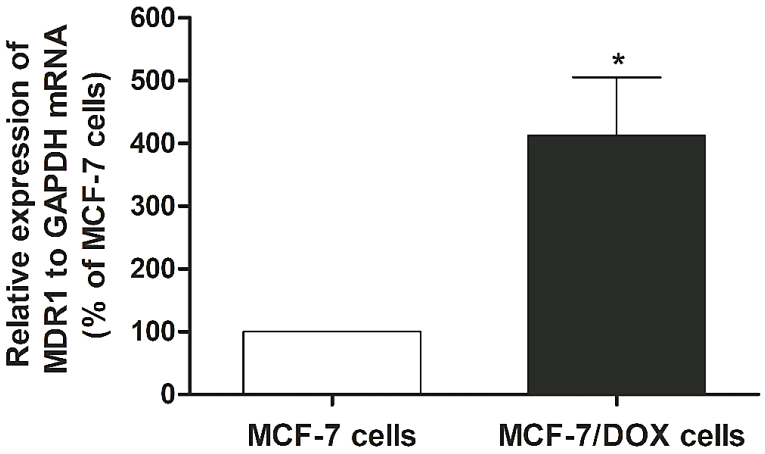
A.


Cell viability
(% of the untreated group)
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
Doxorubicin concentration (log [μM])
MCF-7 cells
MCF-7/DOX cells

B.


Calcein fluorescence intensity
5000
4000
3000
2000
1000
0
MCF-7 cells
MCF-7/DOX cells

C.



D.

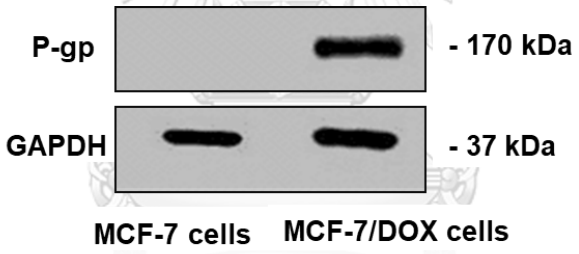


Figure 12. Chemosensitivity, basal activity and expression of P-gp in MCF-7 and MCF-7/DOX cells. (A) Cytotoxicity of doxorubicin (DOX) after 48-h exposure as measured by MTT assay. Data are expressed as the percentage of the untreated control. (B) Basal activity of P-gp as assessed by calcein-AM uptake assay. The bar graphs represent intracellular accumulation of calcein. (C) Basal expression of *MDR1* mRNA as analyzed by qRT-PCR. The bar graphs represent extents of MDR1 mRNA in relative to those of GADPH and are expressed as the percentage of MCF-7 cells. (D) Immunoblots of P-gp and GAPDH (an internal control). Data are means ± SEM (n = 4). *$p < 0.05$ compared with the MCF-7 group.

4.1.2 Cytotoxicity of rhinacanthin-C in breast cancer cells

The effects of rhinacanthin-C on cell viability were determined by an MTT assay. The cells were treated with various concentrations of rhinacanthin-C (0-30 μM) for 48 h. As show in Figure 13, rhinacanthin-C caused concentrations-dependent cytotoxicity on the MCF-7 cells after 48-h treatment. In addition, the cytotoxicity of both MCF-7 and MCF-7/DOX cells was affected by the time-and-concentration dependents. The IC_{50} values of rhinacanthin-C approximately 12.63 ± 1.31 μM and 11.22 ± 1.41 μM respectively, when the treatment period increased from 48 h. rhinacanthin-C at the concentrations up to 1.5 μM was not toxic to the cells after 24-h exposure.

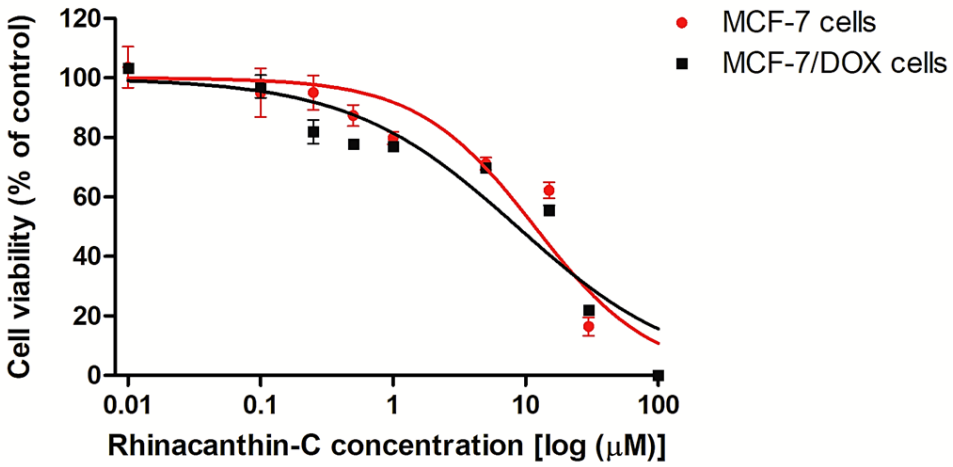


Figure 13. Cytotoxicity of rhinacanthin-C (RN-C) against both MCF-7 and MCF-7/DOX cells after 48 h treatment. Data are presented as the mean ± SEM (N=4). Compare with the control group.

4.1.3 MDR reversal effect of rhinacanthin-C in MCF-7/DOX cells

The MDR reversal effect of rhinacanthin-C was determined after the MCF-7/DOX cells were exposed to non-cytotoxic concentrations of this compound for 48 h. Our results showed that the rhinacanthin-C-treated in MCF-7/DOX cells were more sensitive to camptothecin, doxorubicin, and etoposide than the untreated MCF-7/DOX cells, and the MDR reversal effect of rhinacanthin-C was concentrations dependent. After exposure to 1 μM rhinacantin-C, the IC_{50} values of these cytotoxic drugs in the resistant cells significantly decreased by approximately 1.97 to 2.6-fold (Table 1). Interestingly, rhinacanthin-C (1 μM) elicited greater MDR reversal effect than the positive control verapamil (60 μM).

Table 1. Chemosensitivity shift of MCF-7/DOX cells after 48-h exposure to rhinacanthin-C or verapamil.

Groups	Camptothecin		Etoposide		Doxorubicin	
	IC_{50} (μM)	Reversal fold	IC_{50} (μM)	Reversal fold	IC_{50} (μM)	Reversal fold
Untreated (Control)	0.99 ± 1.06	-	893.40 ± 1.87	-	155.84 ± 1.04	-
Rhinacanthin-C 0.25 μM	0.81 ± 1.54	1.22	737.66 ± 2.55	1.21	102.77 ± 1.51*	1.51
Rhinacanthin-C 0.5 μM	0.92 ± 0.29	1.07	727.68 ± 1.85	1.22	89.79 ± 2.57*	1.73
Rhinacanthin-C 1.0 μM	0.47 ± 0.37*	2.10	452.31 ± 0.65*	1.97	59.61 ± 1.10*	2.61
Verapamil 60 μM	0.60 ± 0.98*	1.65	801.99 ± 2.76	1.11	81.59 ± 0.40*	1.91

The cells were treated with either rhinacanthin–C or verapamil for 48 h prior to determination of cell viability in the presence of a cytotoxic agent (i.e. camptothecin, doxorubicin and etoposide) at various concentrations.

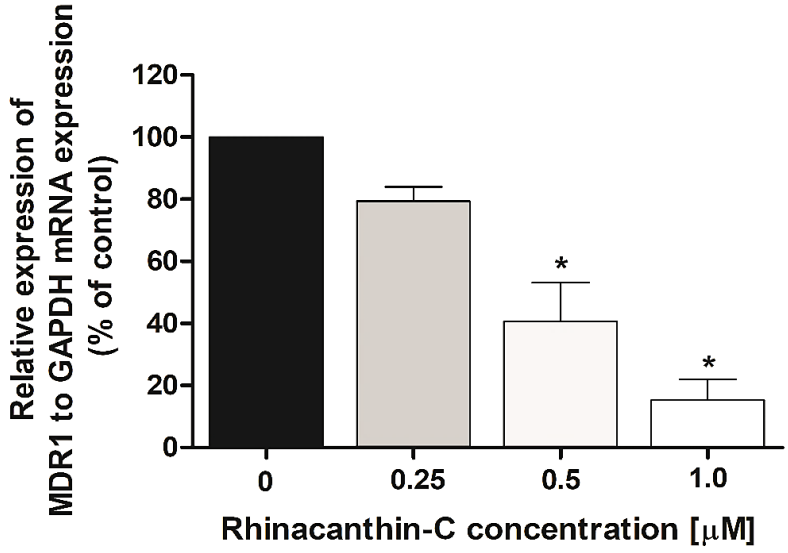
Reversal fold (RF) was calculated from the ratio between the IC_{50} value of each cytotoxic agent obtained from the rhinacanthin-C-treated (or verapamil-treated) cells and those from untreated cells.

Data are expressed as means ± S.E.M. (n = 4). *$p < 0.05$ compared with the untreated group.

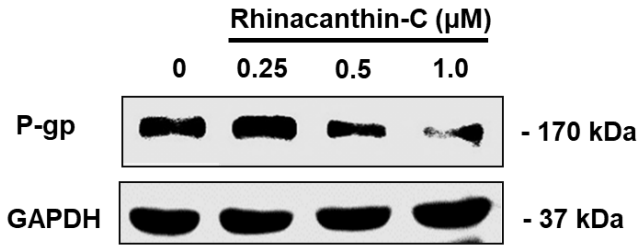
4.1.4 Effects of rhinacanthin-C on the expression of *MDR1* mRNA and P-gp

Camptothecin, doxorubicin and etoposide are known P-gp substrates. We determined whether rhinacanthin-C could reverse the resistance to these cytotoxic compounds via the reduction of P-gp expression in MCF-7/DOX cells after 48-h exposure. As shown in Figure 14A-C, rhinacanthin-C could reduce the expression levels of *MDR1* mRNA and P-gp protein in concentration-dependent manner. Compared to those of the untreated control, extents of *MDR1* mRNA and P-gp protein in the MCF-7/DOX cells after 48-h exposure to 1 μM rhinacanthin-C were significantly decreased by 84.8-fold and 35.5-fold, respectively.

A.



B.



C.

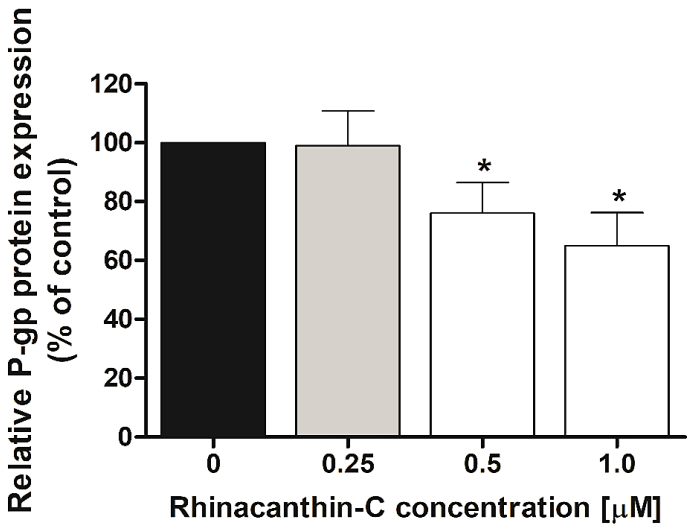


Figure 14. Expression of *MDR1* mRNA and P-gp protein in MCF-7/DOX cells after 48-h treatment with rhinacanthin-C at various concentrations. (A) *MDR1* mRNA, (B) immunoblots of P-gp and GAPDH (an internal control), and (C) their densitometrical analysis. Data are means ± SEM (n = 4). *$p < 0.05$ compared with control (untreated group).

In addition, these findings were well correlated to the decreased P-gp activity in the rhinacanthin-C-treated cells, as measured by substrate accumulation assay (Figure 15). Upon increasing the concentration of rhinacanthin-C from 0.25 to 1 μM, intracellular calcein in the rhinacanthin-C-treated cells increased significantly in concentration-dependent manner. Our results suggested that the rhinacanthin-C-treated cells had lower P-gp activity than the untreated cells. The level of calcein accumulated within the cells exposed to 1 μM rhinacanthin-C for 48-h increased by 3.4-fold compared to that of the control untreated group. In addition, this suppressive effect on P-gp function was apparently greater than that of verapamil, a known suppressive agent for P-gp expression used as a positive control in this study.

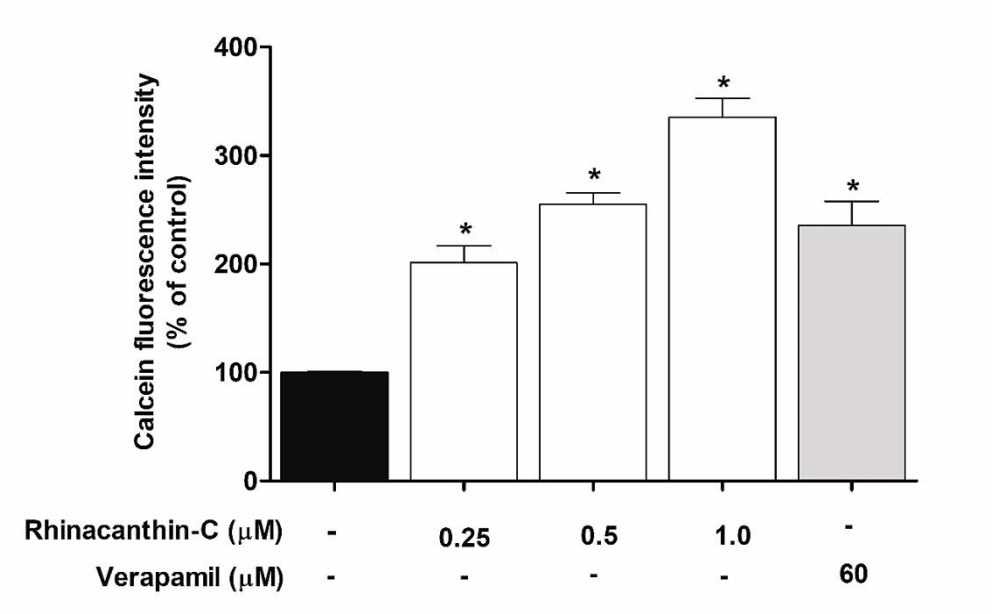


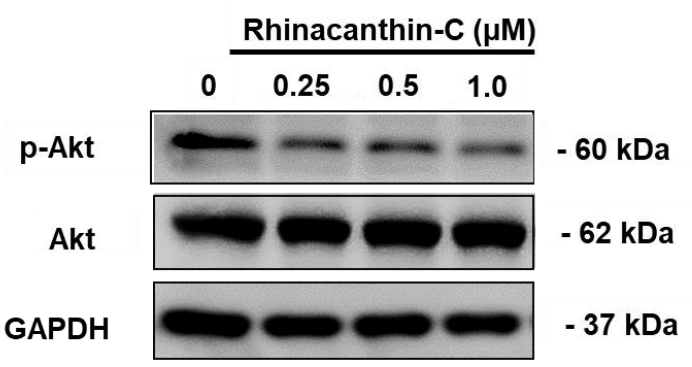
Figure 15. Intracellular accumulation of calcein in MCF-7/DOX cells after 48-h treatment with rhinacanthin-C or verapamil (a positive control). Each bar represents mean ± SEM (n = 4). *$p < 0.05$ compared with control (untreated group).

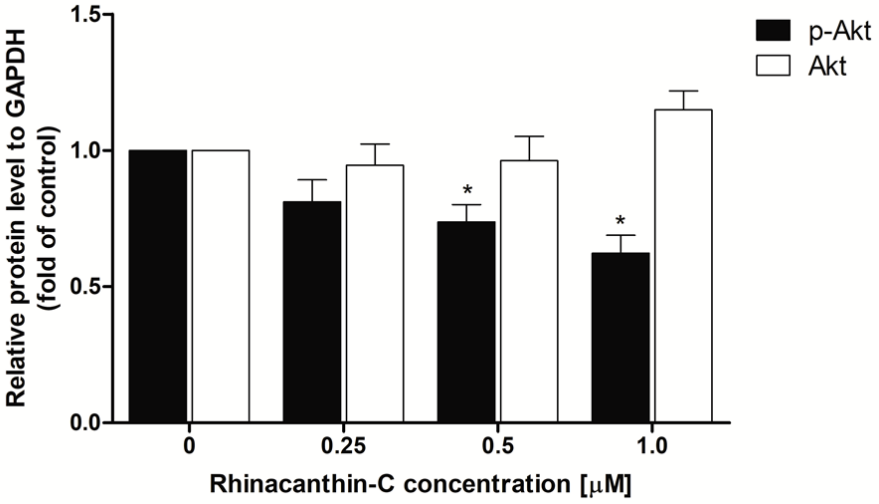
4.1.5 Effects of rhinacanthin-C on the PI3K/Akt/NF-κB signaling pathway and YB-1 expression.

To determine whether the PI3K/Akt/NF-κB signaling pathway was involved in rhinacanthin-C-mediated *MDR1*/P-gp downregulation, we measured protein expression levels of Akt and NF-κB and their phosphorylated forms in the MCF-7/DOX cells after 24-h treatment. Compared with the untreated group, the levels of phosphorylated Akt decreased significantly in the cells treated with 0.5 and 1 μM of rhinacanthin-C (Figure 16A). In addition, rhinacanthin-C could inhibit the activity of NF-κB, the well-known downstream effector of Akt signaling cascade. As shown in Figure 16B, rhinacanthin-C at 0.5 and 1 μM significantly reduced the phosphorylation of NF-κB and IκB-α after treatment for 24 h. Furthermore, the effects of rhinacanthin-C on the activities of Akt/NF-κB signaling pathway were concentration dependent.

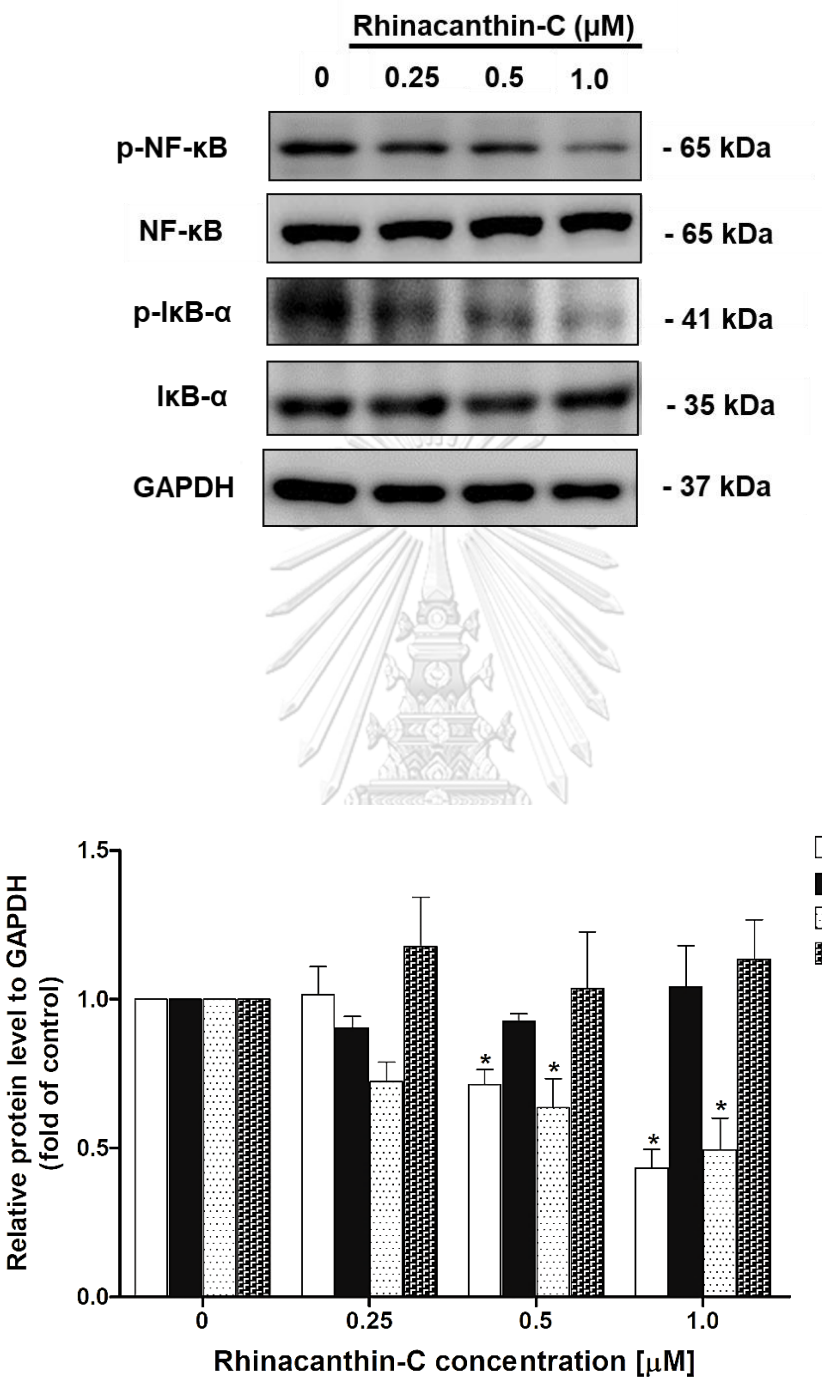
In addition, the NF-κB signaling pathway, we further determined the effect of rhinacanthin-C on YB-1 promoter in the MCF-7/DOX cells after 24-h treatment. YB-1 is another downstream transcription factor in the PI3K/Akt signaling pathway which regulates chemosensitivity and P-gp expression in cancer cells (Cheng et al., 2018; Maurya et al., 2017). Compared with the untreated group, rhinacanthin-C at 1 μM could significantly reduce YB-1 expression level (Figure 16C).

A.


Rhinacanthin-C (μM)
0
0.25
0.5
1.0
p-Akt
- 60 kDa
Akt
- 62 kDa
GAPDH
- 37 kDa


Relative protein level to GAPDH
(fold of control)
1.5
1.0
0.5
0.0
0
0.25
0.5
1.0
Rhinacanthin-C concentration [μM]
p-Akt
Akt
*
*

B.


Rhinacanthin-C (μM)
0
0.25
0.5
1.0
p-NF-κB
- 65 kDa
NF-κB
- 65 kDa
p-IκB-α
- 41 kDa
IκB-α
- 35 kDa
GAPDH
- 37 kDa
Relative protein level to GAPDH (fold of control)
1.5
1.0
0.5
0.0
p-NFκB
NFκB
p-IκB-α
IκB-α
*
0
0.25
0.5
1.0
Rhinacanthin-C concentration [μM]

C.

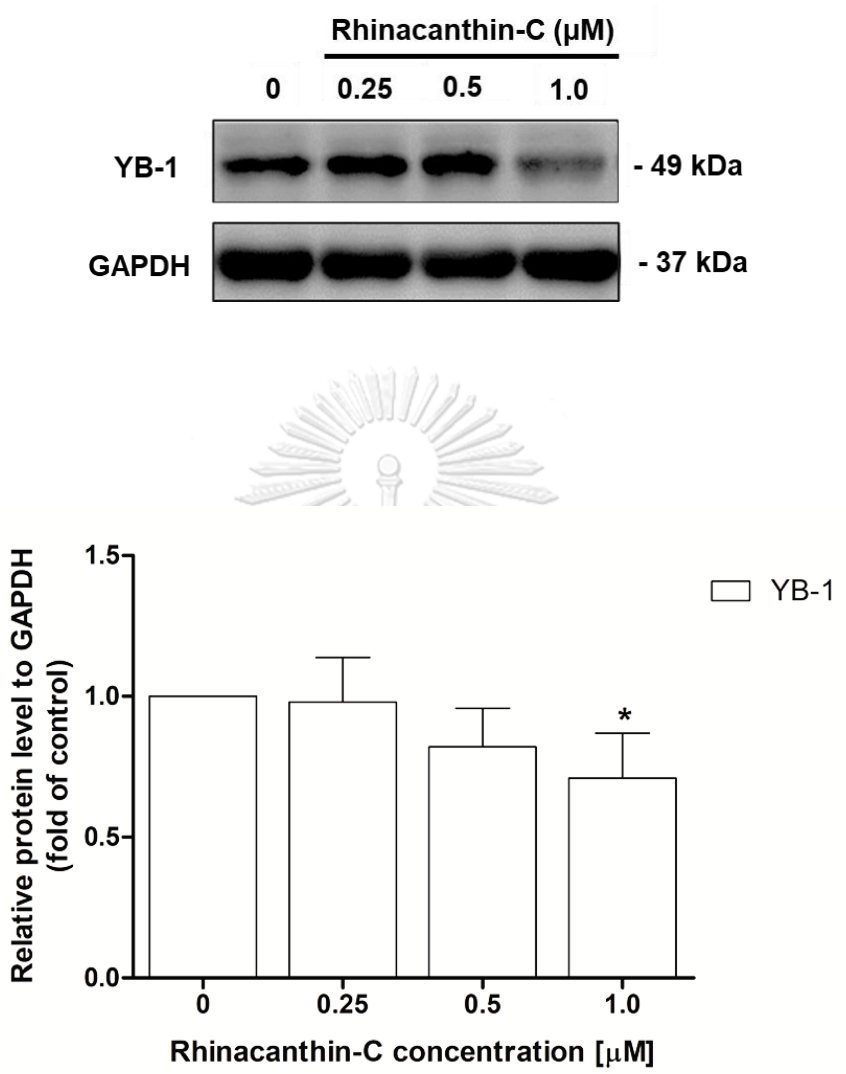


Figure 16. Immunoblots and their densitometrical analysis of (A) Akt and its phosphorylated form, (B) NF-**K**B, I**K**B-**α** and their phosphorylated forms, (C) YB-1 in MCF-7/DOX cells after 24-h treatment with rhinacanthin-C at various concentrations. Each bar represents mean ± SEM (n = 4). **p* < 0.05 compared with control (untreated group).

We also confirmed that suppression of the PI3K/Akt signaling pathway led to inhibition of NF-**K**B and YB-1 activities in the MCF-7/DOX cells. Treatment of these cells with a known Akt inhibitor, LY294002 (10 μM), for 1 h caused significant the reduction of phosphorylated Akt (p-Akt), phosphorylated NF-**K**B (p-NF-**K**B) and YB-1 protein levels, compared to the untreated group (Figure 17). Subsequent incubation of LY294002-treated cells with rhinacanthin-C (1 μM) for another 24 h had no additional effects on the expression levels of p-Akt, p-NF-**K**B and YB-1, compared to the LY294002-treated group (Figure 17). These findings suggested that the suppressive effect of rhinacanthin-C on NF-**K**B and YB-1 is associated with the inhibition of the PI3K/Akt pathway.

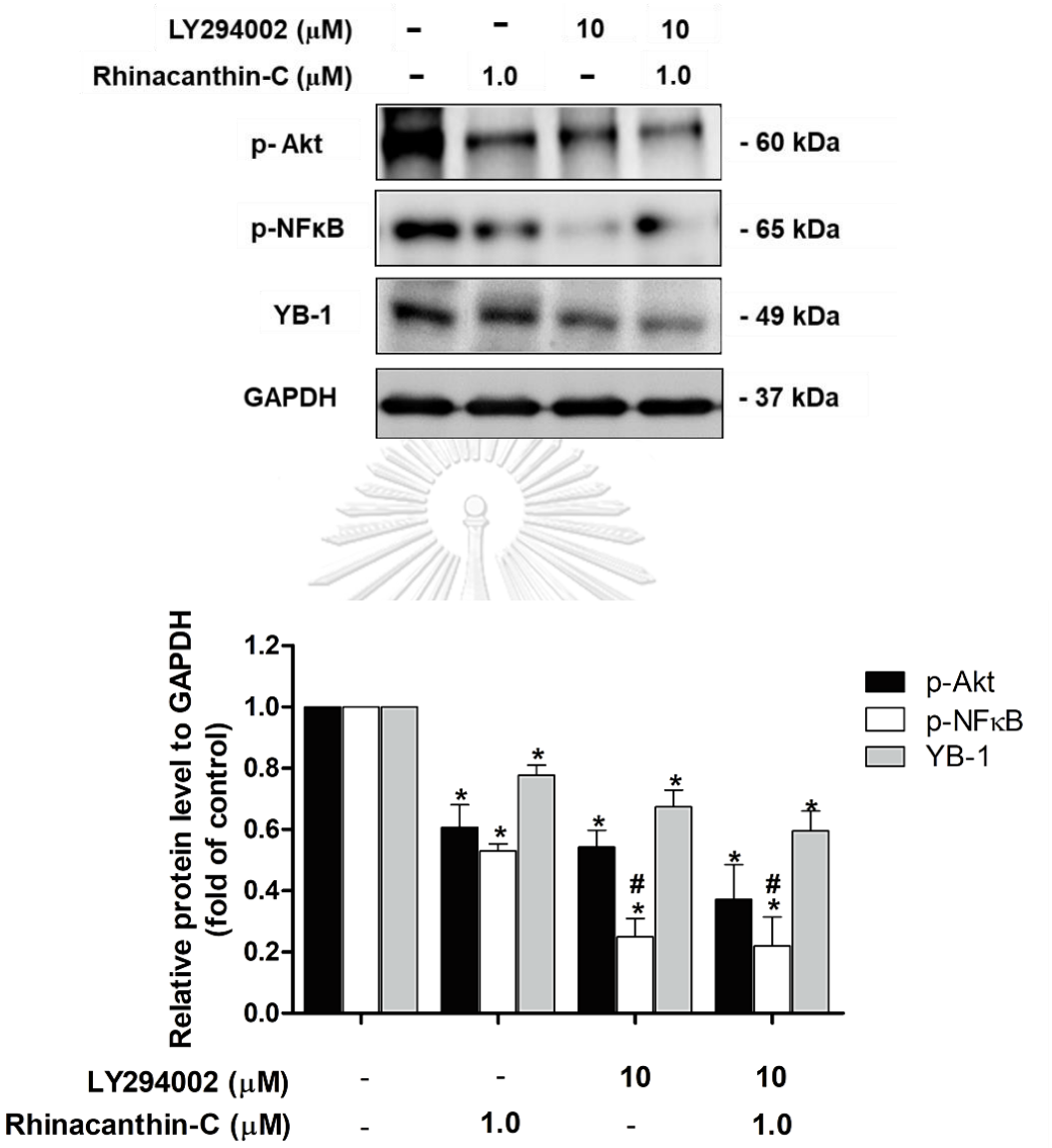


Figure 17. Expression of phosphorylated Akt, phosphorylated NF-**K**B and YB-1 in the LY294002-treated MCF-7/DOX cells after 24-h exposure to rhinacanthin-C. Each bar represents mean ± SEM (n = 4). *$p < 0.05$ compared with control (untreated group); $^{\#}p < 0.05$ compared with rhinacanthin-C-treated group in the absence of LY294002 (n = 4).

4.1.6 Effects of rhinacanthin-C on the MAPK/ERK1/2 pathway

The MAPK/ERK1/2 pathway is linked to survival mechanisms and drug resistance in MDR cells (Lee et al., 2020). We investigated whether rhinacanthin-C interfered with the MAPK/ERK1/2 pathway in the MCF-7/DOX cells after 24-h treatment. As shown in Figure 18, rhinacanthin-C at the concentrations up to 1 μM had no effect on protein expression levels of either ERK1/2 or its phosphorylated form (p-ERK1/2), compared with the untreated group. These findings suggested that the MDR reversal effects of rhinacanthin-C (up to 1 μM) in the MCF-7/DOX cells did not involve the MAPK/ERK1/2 pathway.

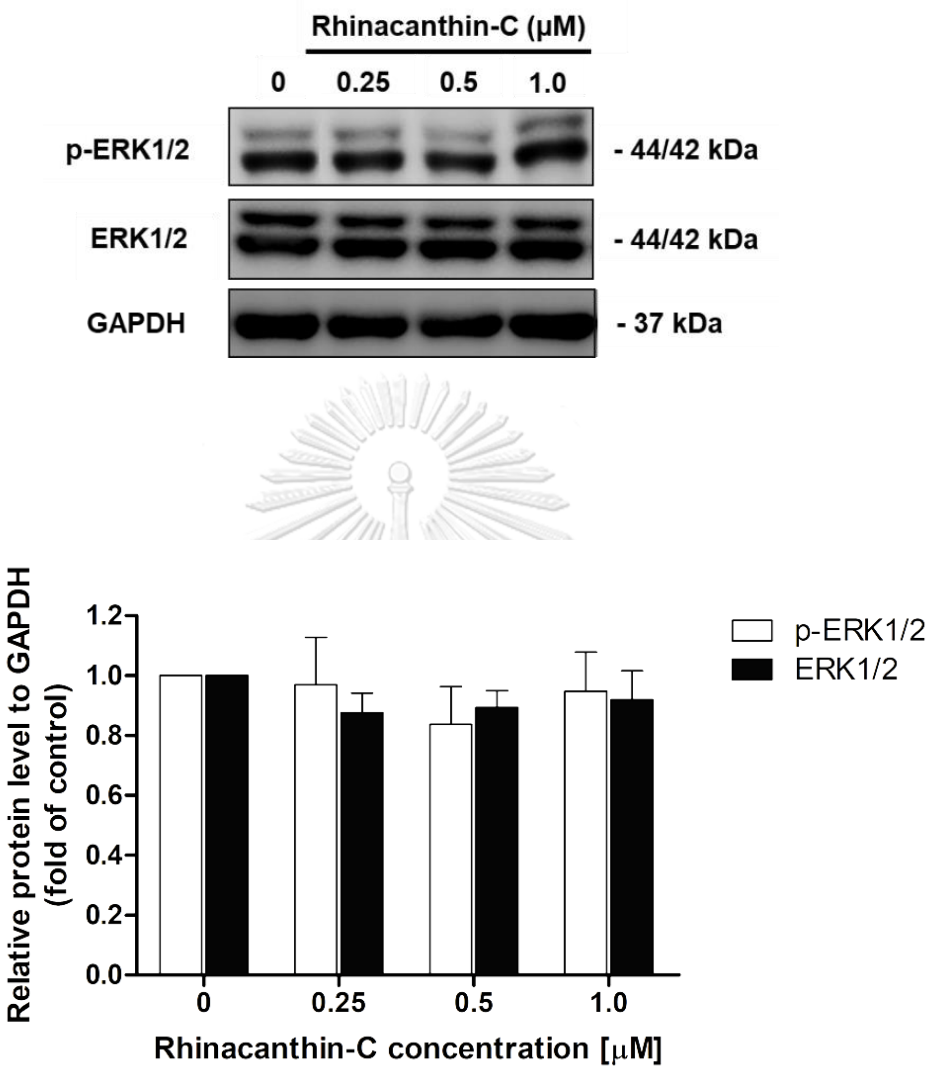


Figure 18. Immunoblots and their densitometrical analysis of ERK1/2 and phosphorylated ERK1/2 in MCF-7/DOX cells after 24-h treatment with rhinacanthin-C at various concentrations. Each bar represents mean ± SEM (n = 4). *$p < 0.05$ compared with control (untreated group).

4.2 Part 2. The ability of rhinacanthin-C to overcome drug resistance the MCF-7/DOX cells and It was could promotion of apoptosis through oxidative damage and suppression of the cellular detoxification system.

4.2.1 Effect of rhinacanthin-C on oxidative stress-induced cell death

At 24-h treatment, neither rhinacanthin-C (3, 16 and 28 μM) nor H_2O_2 (at 50 μM) was toxic to MCF-7/DOX cells, as evidenced by > 80% cell viability. The combination of rhinacanthin-C and H_2O_2 remarkably increased cytotoxicity in MCF-7/DOX cells when compared with each compound alone (Figure 19). The increasing the concentrations of rhinacanthin-C from 3 μM to 28 μM in the combinatorial regimens resulted in reduction of cell viability from 62.85 to 38.55%.

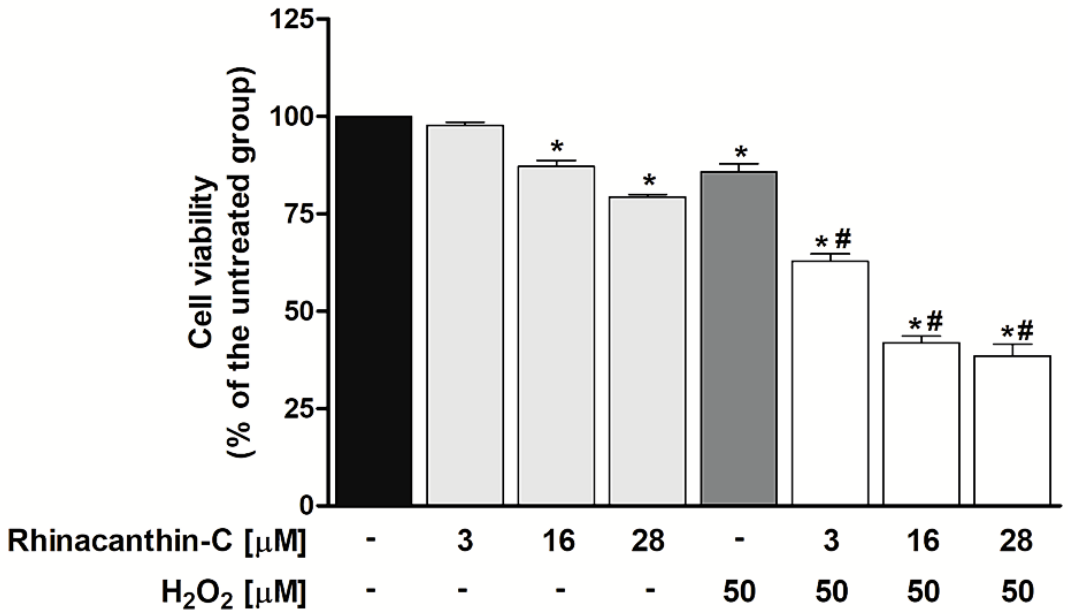


Figure 19. Effect of rhinacanthin-C on oxidative stress-induced cell death in MCF-7/DOX cells. The cells were treated with various concentrations of rhinacanthin-C was the absence and presence of H_2O_2 (50 μM) after exposing 24-h determined the cell viability was measured by MTT assay. Each value represents the means ± SEM (n = 3-4) *$p < 0.05$; compared to untreated group, $^{\#}p < 0.05$; compared with H_2O_2 alone.

4.2.2 Effect of rhinacanthin-C on intracellular ROS in MCF-7/DOX cells

An increase of ROS generation was observed in MCF-7/DOX cells after 1-h exposure of rhinacanthin-C, as measured by the $DCFH_2$-DA assay (Figure 20). At the concentration of 3 μM, rhinacanthin-C significantly increased cellular ROS by 3.1-fold, as compared to untreated control group. Upon increasing the concentration of rhinacanthin-C to 16 and 28 μM, the levels of ROS reduced to 1.7- fold higher than that of the untreated group (Figure 20). Nevertheless, these findings suggested that rhinacanthin-C was able to induce oxidative stress in the MCF-7/DOX cells through ROS production.

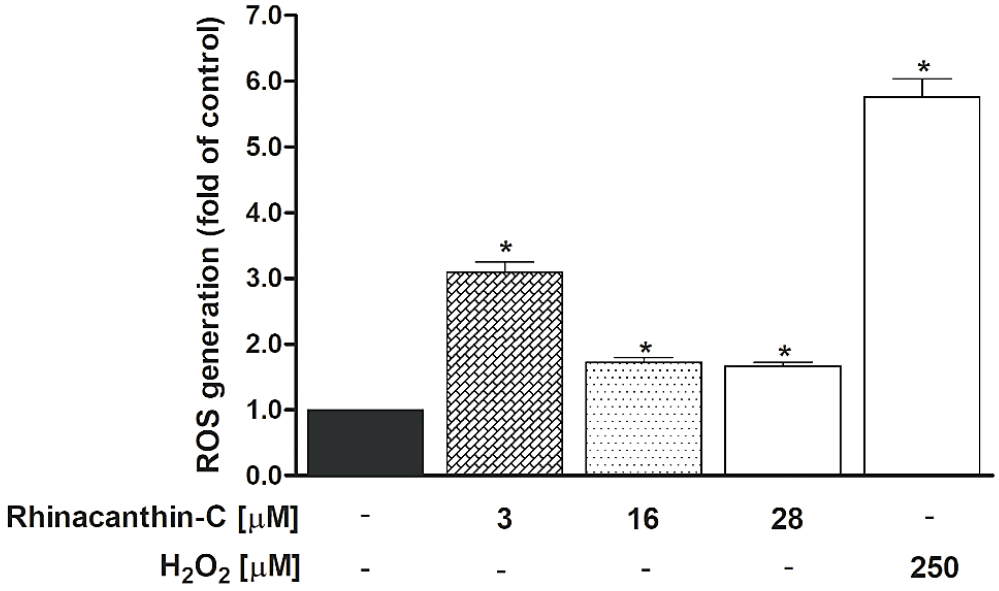
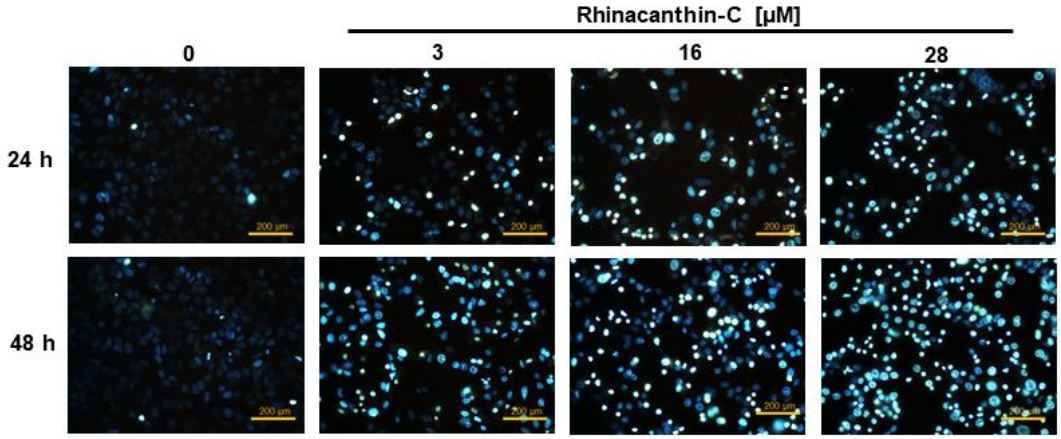


Figure 20. Effect of rhinacanthin-C on cellular ROS level after 1 h treatment was detected by $DCFH_2$-DA fluorescent dye. Hydrogen peroxide (H_2O_2; 250 μM) was used as a positive control group. Each value represents the means ± SEM (n = 3-4) *p < 0.05; compared to untreated group.

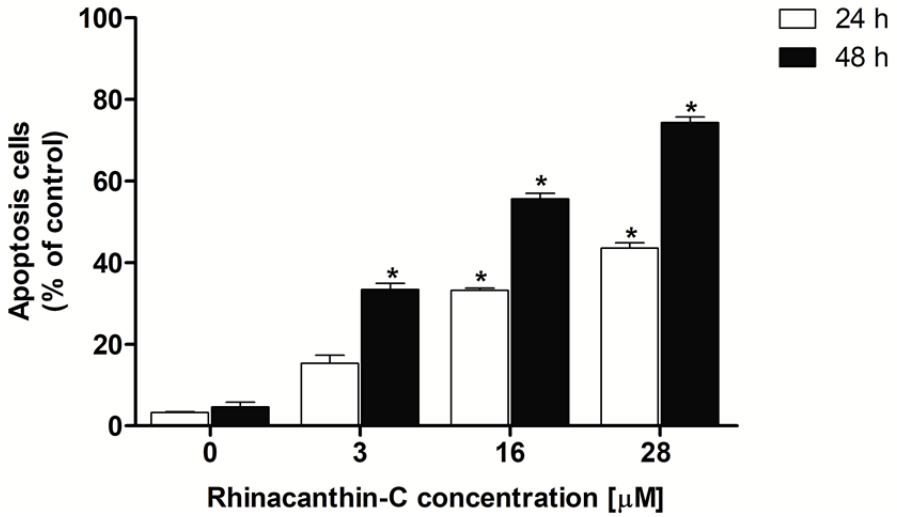
4.2.3 Effects of rhinacanthin-C on apoptosis induction and expression of Bcl-2 and PARP proteins

As shown in Figure 21A, a significant number of MCF-7/DOX cells underwent apoptosis after exposure to rhinacanthin-C for 24 and 48 h, as measured by Hoechst 33342 staining assay. The induction of apoptosis by rhinacanthin-C was concentrations- and time-dependent. After 24-h exposure to rhinacanthin-C, the percentages of apoptotic MCF-7/DOX cells increased from 15.3% to 43.6% when the concentration was increased from 3 μM to 28 μM (Figure 21B). Moreover, when the treatment period was extended to 48 h, the percentages of apoptotic cells increased by approximately 1.7- to 2.2-fold.

A

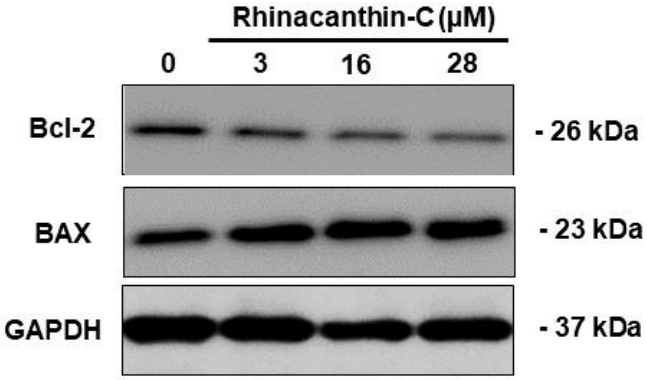

Rhinacanthin-C [μM]
0
3
16
28
24 h
48 h

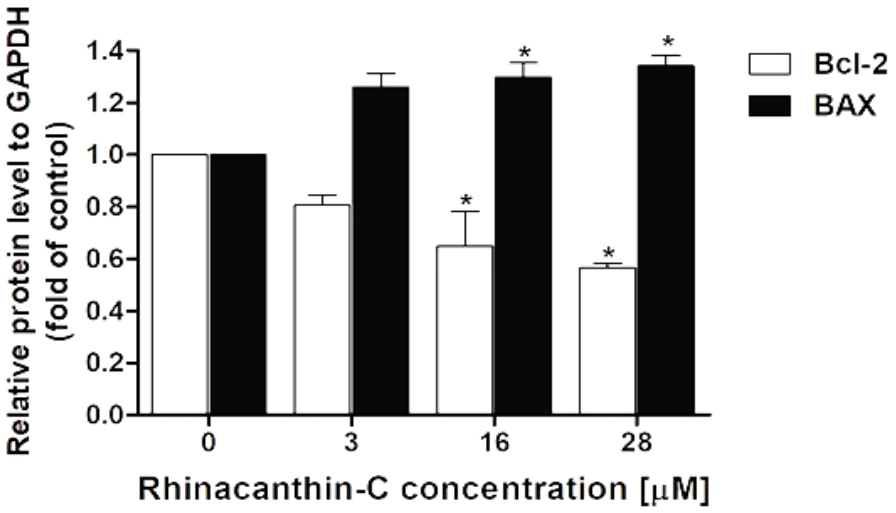
B


24 h
48 h
Apoptosis cells
(% of control)
100
80
60
40
20
0
0
3
16
28
Rhinacanthin-C concentration [μM]

Expression levels of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 proteins, along with PARP protein, in MCF-7/DOX cells were determined after 24-h treatment with rhinacanthin-C at 3 different concentrations. Our western blot analyses showed that rhinacanthin-C significantly increased the Bax/Bcl-2 ratio and suppressed PARP expression in concentration-dependent manner (Figure 21C and D). The Bax/Bcl-2 ratio in the cells treated with rhinacanthin-C at 28 μM was approximately 2.4-fold higher than the untreated control group. Meanwhile, extent of poly (ADP-ribose) polymerase (PARP) was reduced by 70% from that of the untreated control.

C


Rhinacanthin-C (μM)
0
3
16
28
Bcl-2
- 26 kDa
BAX
- 23 kDa
GAPDH
- 37 kDa


Relative protein level to GAPDH
(fold of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
16
28
Rhinacanthin-C concentration [μM]
Bcl-2
BAX
*

D

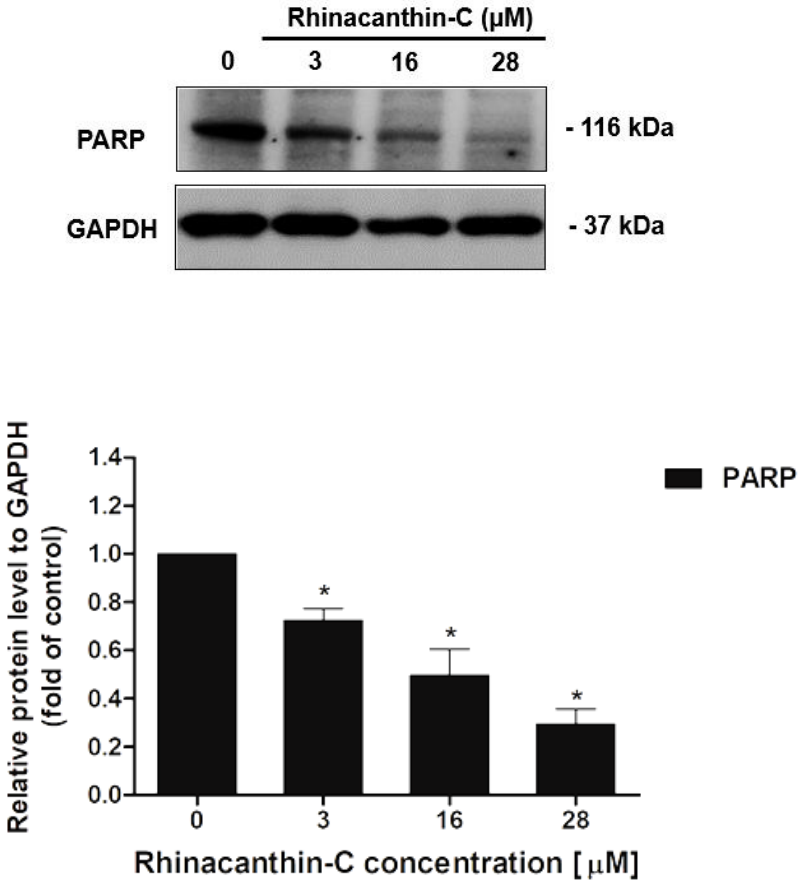
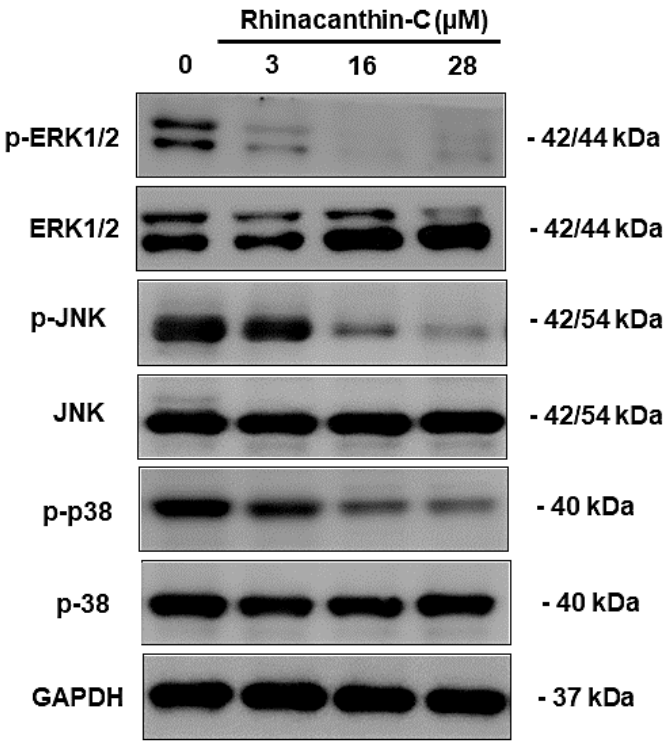


Figure 21. Effect of rhinacanthin-C induced apoptosis cell death and expression of apoptosis-related proteins in MCF-7/DOX cells. (A) Nuclear morphology of MCF-7/DOX cells treated with rhinacanthin-C at 3, 16, and 28 μM for 24 and 48 h. Cells were stained with Hoechst 33342 and visualized under a fluorescence microscope (20x magnification; scale bar = 200 μm). The cells with apoptotic nuclei, condensed and/or fragmented nuclei. (B) The statistical fluorescence data of percentage of apoptotic cells. (C) The expression of Bcl-2 and Bax protein and (D) PARP-mediated response induce cell death after exposed to rhinacanthin-C for 24-h were determined by immunoblotting using specific antibodies. GAPDH was used as a loading control. Each value represents the means ± SEM (n = 3-4) *$p < 0.05$; compared to control group.

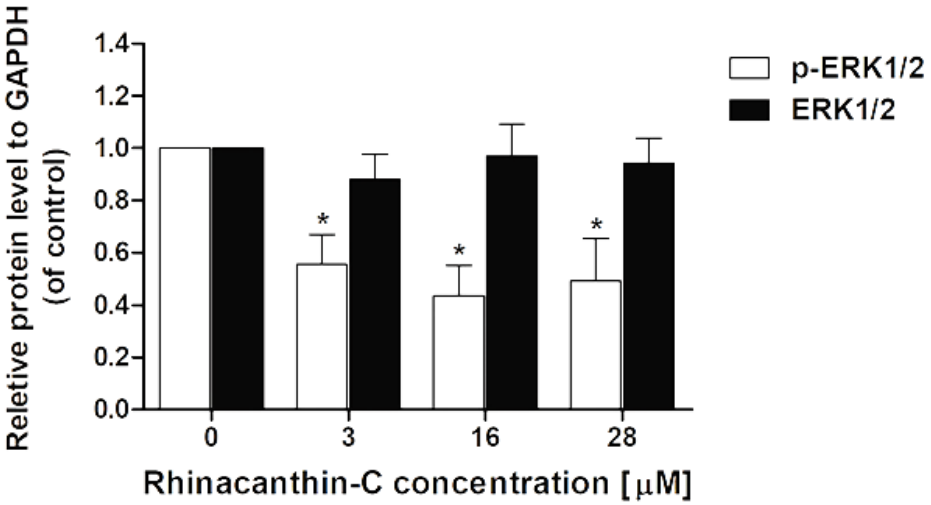
4.2.4 Effects of rhinacanthin-C on the MAPKs signaling pathway

We further investigated the effects of rhinacanthin-C on the expression levels of phosphorylated signaling proteins in the MAPKs pathway including ERK1/2, JNK and p-38. The MAPKs signaling pathway has been associated with cell stress response, cell survival and apoptosis mechanisms (Lee et al., 2020). Upon treatment with rhinacanthin-C for 24 h, expression levels of the phosphorylated ERK1/2 (p-ERK1/2), JNK (p-JNK) and p-38 (p-p38) proteins in the MCF-7/DOX cells were significantly lower than those of the untreated control group (Figure 22A - D). Thus, low concentrations (3-16 μM) of rhinacanthin-C were able to suppress MAPKs activities in the MCF-7/DOX cells after 24-h treatment period.

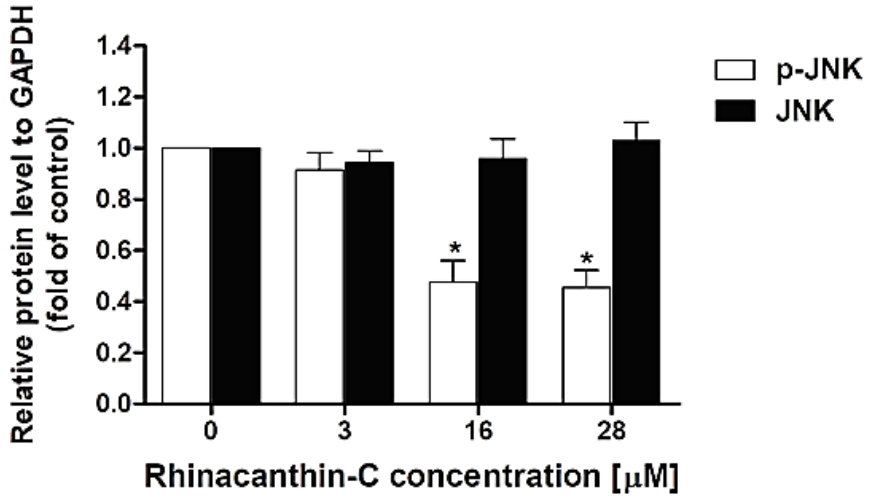
A


Rhinacanthin-C (μM)
0
3
16
28
p-ERK1/2
- 42/44 kDa
ERK1/2
- 42/44 kDa
p-JNK
- 42/54 kDa
JNK
- 42/54 kDa
p-p38
- 40 kDa
p-38
- 40 kDa
GAPDH
- 37 kDa

B


Reletive protein level to GAPDH
(of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
0
3
16
28
Rhinacanthin-C concentration [μM]
p-ERK1/2
ERK1/2

C



D

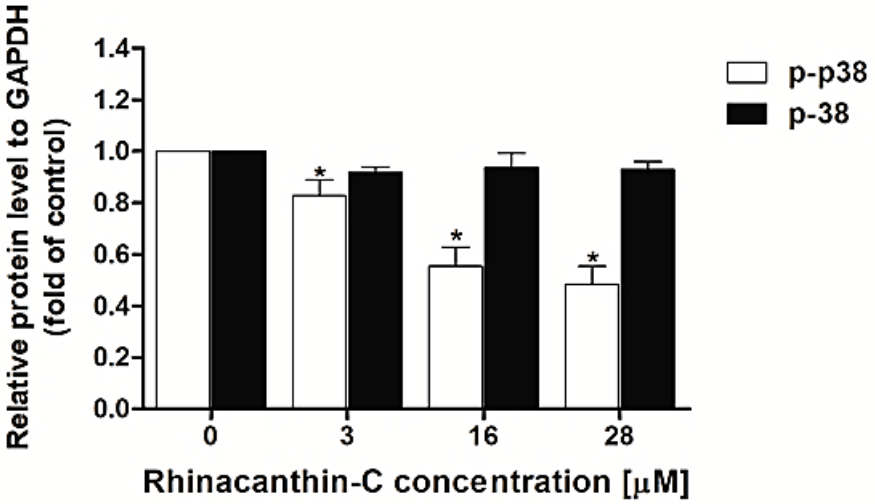
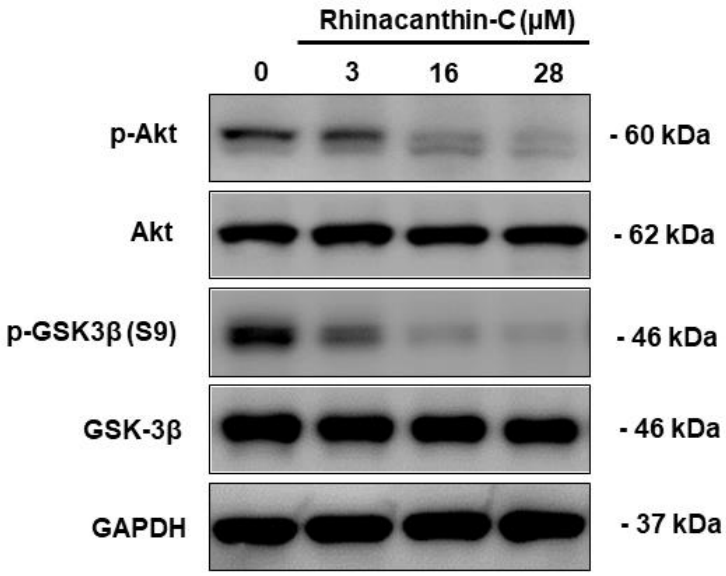


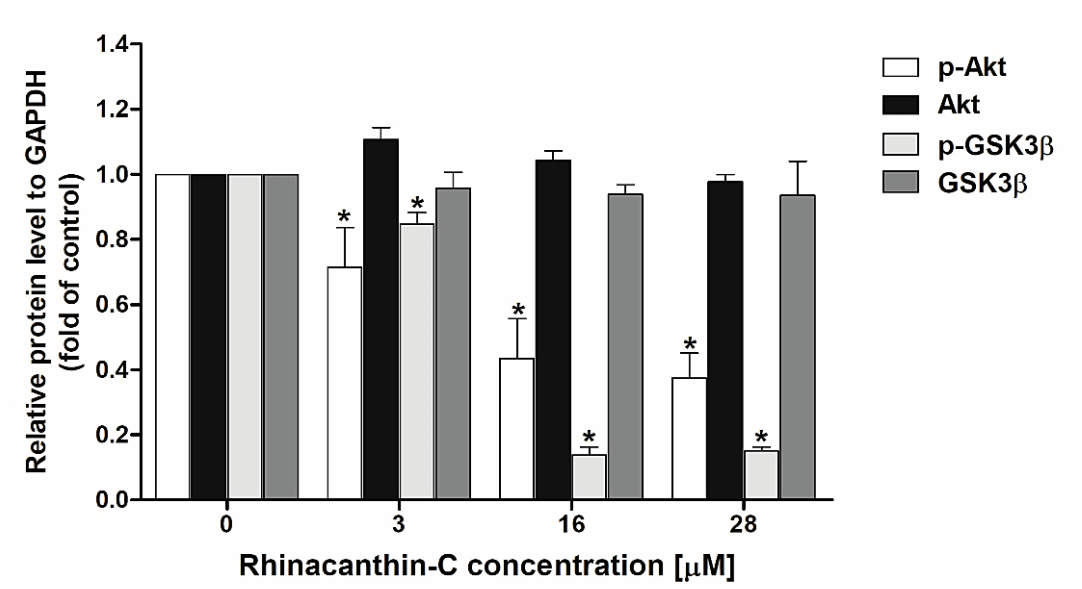
Figure 22. Immunoblots and their densitometrical analysis the effect of rhinacanthin-C inhibited the MAPKs pathway. (A) ERK1/2, (B) JNK, (C) p-38, and it their phosphorylated forms in MCF-7/DOX cells after 24-h treatment with rhinacanthin-C (3, 16, and 28 μM). The relative expression of protein was normalized by GAPDH (internal control). Each bar represents mean ± SEM (n = 4). *$p < 0.05$ compared with control.

4.2.5 Effects of rhinacanthin-C on the Akt/GSK-3β/Nrf2 pathway

In this study, we also determined the effects of rhinacanthin-C on cellular detoxification processes through the Akt/GSK-3β/Nrf2 pathway. It has been reported that the Akt/GSK-3β/Nrf2 cascade is involved in chemo-resistance through induction of several antioxidant and detoxifying enzymes such as NQO1 and HO-1 in response to stresses (Jung et al., 2018; Panieri and Saso, 2019). As shown in Figure 23A and B, even at a low concentration of 3 μM, rhinacanthin-C could significantly reduce Akt and phosphorylation of GSK-3β in the MCF-7/DOX cells after 24-h treatment period. The levels of phosphorylated forms of both proteins were markedly decreased when the concentration of rhinacanthin-C had been increased to 16 μM. Moreover, our results demonstrated that phosphorylated Nrf2 protein, the well-known downstream effector of the Akt/GSK-3β signaling cascade, was expressed at the lower degree in rhinacanthin-C-treated cells than in the untreated control group.

A


Rhinacanthin-C (μM)
0
3
16
28
p-Akt
- 60 kDa
Akt
- 62 kDa
p-GSK3β (S9)
- 46 kDa
GSK-3β
- 46 kDa
GAPDH
- 37 kDa


Relative protein level to GAPDH (fold of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
16
28
Rhinacanthin-C concentration [μM]
p-Akt
Akt
p-GSK3β
GSK3β
*

B

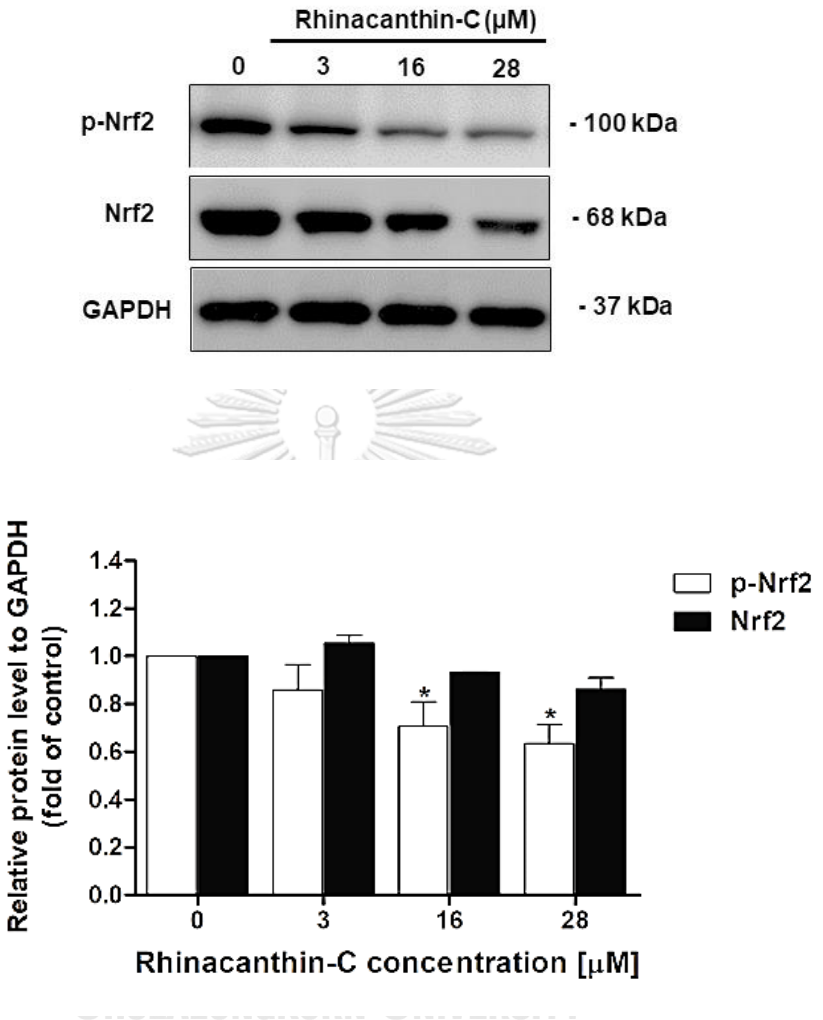


Figure 23. Effect of rhinacanthin-C on Akt/GSK-3β/Nrf2 signaling cascade in MCF-7/DOX cells. (A) Immunoblots and their densitometrical analysis of Akt, GSK-3β and it their phosphorylated forms in MCF-7/DOX cells after 24-h treatment with rhinacanthin-C at various concentrations. (B) After cells were treated with different concentrations of rhinacanthin-C for 24 h, the Nrf2 and phosphorylation of Nrf2 were detected by immunoblot and their densitometrical analysis. The relative expression of protein was normalized by GAPDH (internal control). Each bar represents mean ± SEM (n = 4). *$p < 0.05$ compared with control.

4.2.6 Effects of rhinacanthin-C on expression levels of NQO1 and HO-1

Activity of transcription factor Nrf2 is essential in regulating expression of antioxidant and detoxifying enzymes such as NQO1 and HO-1 (Basak et al., 2017; Choi et al., 2016; Li et al., 2016). Figure 24, shows that, after 24-h exposure, rhinacanthin-C at 16 and 28 μM significantly reduced the extents of HO-1 and NQO1 expression in the MCF-7/DOX cells by approximately 37% and 54%, respectively, compared with the untreated control group.

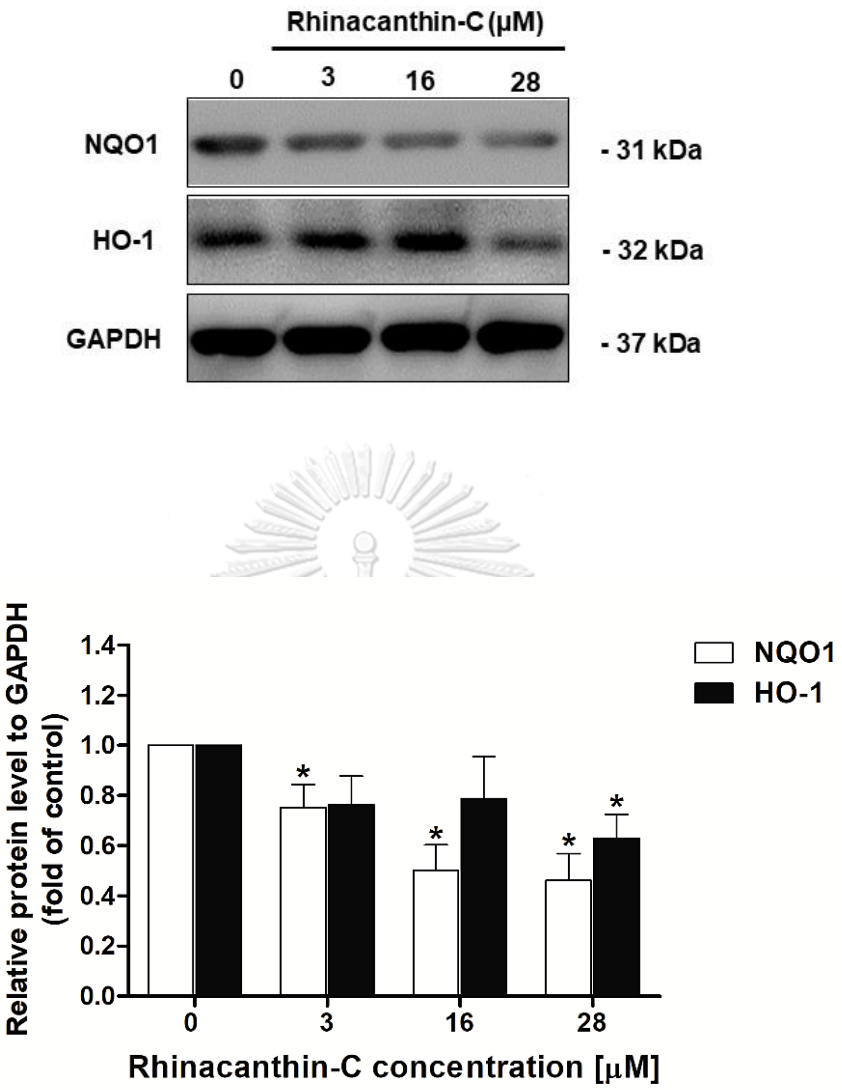


Figure 24. Effect of rhinacanthin-C on expression levels of NQO1 and HO-1 in MCF-7/DOX cells. After treated with rhinacanthin-C for 24 h and the expression of antioxidant and detoxifying enzymes NQO1 and HO-1 were detected by immunoblot and their densitometrical analysis. GAPDH (an internal control). Data are represented as means ± SD (n = 4) and significant differences are indicated as $*p < 0.05$ compared with control (untreated group).

CHAPTER V
DISCUSSIONS AND CONCLUSION

Doxorubicin is an anthracycline cytotoxic agent commonly used for patients with breast cancer (Housman et al., 2014). The uses of doxorubicin can be limited due to its serious adverse effects and development of multidrug resistance later on. Several studies reported that the overexpression of P-gp-mediated MDR is associated with the loss of chemotherapeutic efficacy in various types of cancer such as breast cancer, ovarian cancer and leukemia (Kumar et al., 2019; Nedeljković and Damjanović, 2019; Wang et al., 2016). Numerous researches in the last decade have focused on the search for novel MDR reversing agents with the ability to suppress P-gp activities. Two classical approaches in reducing P-gp-mediated drug extrusion are direct inhibition of P-gp activity and reduction of MDR1 expression levels in cancer cells (Hamed et al., 2019). Phytochemicals such as cepharanthine, curcumin, procyanidin and rhinacanthin-C have been evaluated for their potential MDR reversal effects thought inhibited the Akt/NF-**K** B and MAPKs signaling pathway, leading to downregulate the P-gp expression in MDR cancer cells (Abdallah et al., 2015; Chaisit et al., 2017; Zhao et al., 2013). Several of these natural compounds are ingredients of herbal medicine or health products with a long history of use.

Rhinacanthin-C is a major bioactive constituent of *Rhinacanthus nasutus*, a medicinal plant native to Thailand as well as South and Southeast Asia countries (Gotoh et al., 2004). Previously, we demonstrated that at its low non-cytotoxic concentrations, rhinacanthin-C could be a promising chemosensitizer through direct inhibition of P-gp function (Chaisit et al., 2017). In the present study, we revealed for the first time that rhinacanthin-C could improve cancer cell sensitivity to anti-cancer drugs via the downregulation of P-gp expression. In this study, DOX-resistant MCF-7 cells (MCF-7/DOX) with MDR1 phenotype were developed after long term exposure

to DOX, similar to those reported in literatures (Christowitz et al., 2019; Mealey et al., 2002). These MCF-7/DOX cells was exhibited increased levels of P-gp mRNA, expressed protein and activity. MTT assay showed that, at concentrations lower than 1 μM, rhinacanthin-C was not cytotoxic to MCF-7/DOX cells after 48-h exposure. However, the compound could enhance the cytotoxicity of "P-gp substrate" anti-cancer drugs i.e. camptothecin, doxorubicin and etoposide. This observed MDR reversal effect can be strongly correlated to the reduction of P-gp function in the cells, resulting from treatment with rhinacanthin-C. It should be noted that in this study the direct inhibitory effect of rhinacanthin-C against P-gp activity was excluded, since the compound was washed out prior to calcein-AM uptake assay. Loss of P-gp function in the rhinacanthin-C-treated MCF-7/DOX cells was also confirmed by a significant reduction of *MDR1* expression at both mRNA and protein levels. These findings indicated that rhinacanthin-C could downregulate the expression of P-gp at transcription levels and reverse P-gp-mediated MDR.

To further investigate the mechanisms by which rhinacanthin-C suppressed *MDR1* expression, we primarily focused on the PI3K/Akt and MAPKs signaling pathways. These two signaling pathways have been linked to the regulation of *MDR1* gene expression in various types of cancer cells such as breast and ovarian cancer (Yndestad et al., 2017; Zhou et al., 2019). It has been demonstrated that activation of the Akt or MAPK signaling cascades leads to increase activity and nuclear translocation of active transcription factor NF-kB and YB-1 (Maurya et al., 2017; Zheng, 2017), which bind to the promoter region of *MDR1* gene to initiate the transcription and expression of P-gp (Cheng et al., 2018; Hamed et al., 2019). Also, hyperactivation of Akt/NF-**K** B or MAPK/ERK1/2pathway along with transcription factors NF-kB and YB-1 and increased *MDR1* expression were observed in several resistant cancer cells with MDR phenotype such as doxorubicin-resistant or paclitaxel-resistant MCF-7 cells (Christowitz et al., 2019; Zhang et. al., 2015).

Inhibition of the PI3K/Akt or MAPK/ERK1/2 signaling pathway can result in the reduction of P-gp activity and promote of chemosensitivity to anti-cancer drugs (Cheng et al., 2018; Muthusamy et al., 2019; Satonaka et al., 2017). In this study, we found that 24-h treatment with rhinacanthin-C reduced the levels of p-Akt, p-IkB-**α**, p-NF-kB, and YB-1 in MCF-7/DOX cells in a concentrations-dependent manner, whereas the p-ERK1/2 level remained unaffected. Hence, suppression of the PI3K/Akt, but not MAPK/ERK1/2, the signaling pathway was associated with inhibition of NF-**K**B and YB-1 activities in the resistant MCF-7/DOX cells. It is very likely that rhinacanthin-C downregulated P-gp expression at the transcription level by inhibiting the PI3K/Akt/NF-kB signaling pathway and YB-1 expression. Apparently, the inhibitory effects of rhinacanthin-C on the PI3K/Akt/NF-kB pathway and YB-1 expression were similar to those of specific PI3K/Akt signaling pathway inhibitor LY294002.

Several studies have been reported that cancer cells can develop multi-drug resistance (MDR) and survive against cellular stresses or chemical threats through various mechanisms such as altered apoptotic pathway and disturbed cellular detoxification processes (Dharmaraja, 2017; Kartal-Yandim et al., 2016). Previous studies demonstrated that rhinacanthin-C exerted its cytotoxic action and inhibited cell proliferation in several cancer cells (Siripong et al., 2006a; Siripong et al., 2006b; Wu et al., 1988). The results demonstrated that rhinacanthin-C was able to induce cytotoxicity against doxorubicin-resistant MCF-7 cells with a high expression level of P-gp to a comparable degree as doxorubicin-sensitive MCF-7 cells after 48 h exposure. These results supported our previous findings that, despite its intrinsic ability to inhibit P-gp function, rhinacanthin-C was not a substrate of P-gp (Chaisit et al., 2017; Wongwanakul et al., 2013). This compound was approximately 14-fold more potent than doxorubicin in reducing the viability of the MCF-7/DOX cells. In this study, the ability of rhinacanthin-C to overcome drug resistance in breast cancer cells through the promotion of oxidative stress and suppression of cellular

detoxification system, leading to increased apoptotic cell death. Also, rhinacanthin-C (at 3, 16 and 28 μM) was able to produce intracellular ROS and potentiate H_2O_2 toxicity. Evidently, rhinacanthin-C was able to induce apoptotic cell death with its intrinsic ability to suppress detoxification processes of the doxorubicin-resistant (MCF-7/DOX) cells.

Various approaches to overcoming MDR in cancer therapy have been introduced in order to effectively improve chemotherapeutic outcome. In addition to suppression of P-gp activities, induction of apoptosis particularly to the resistant cancerous cells could be an effective MDR reversal strategy (Elshimali et al., 2018; Kartal-Yandim et al.,2016). In this study, we further demonstrated the effectiveness of rhinacanthin-C to induce apoptosis of the MCF-7/DOX cells within 24 and 48 h after exposure. The increase in apoptotic cell death could result from an increase in the ratio of apoptotic checkpoint proteins Bax/Bcl-2 as well as a decrease in the expression of PARP, a DNA repair protein. The anti-apoptotic Bcl-2 family proteins have been known to be highly expressed in cancer cells with MDR phenotype as an adaptive response to sustain their viability and give rise to chemotherapy resistance (Abuhammad & Zihlif, M., 2013; Kalinina et al., 2016). An increase of the Bax/Bcl-2 ratio subsequently results in activation of caspase activity, an increase in DNA damage and apoptotic cell death (Chen et al., 2018; De et al., 2014; García-Aranda et al., 2018). Rhinacanthin-C has been reported to cause G2/M cell-cycle arrest and caspase-3 activation in HeLaS3 cells, resulting in apoptosis (Siripong et al., 2006a).

Suppression of the detoxifying processes related to MAPKs and Akt/GSK-3β/Nrf2 signaling pathways might contribute to rhinacanthin-C-mediated apoptosis. In response to cell stress signal, activation of MAPKs including ERK1/2, JNK and p-38 is essential for cell growth, proliferation and differentiation, leading to a decrease in apoptotic cell death (Lee et al, 2020; Yuan et al., 2016). However, hyperactivation of

the MAPKs pathway has been associated with the development of MDR in cancer cells (Chen et al., 2014; Lee et al., 2020). Our results showed that rhinacanthin-C was able to inhibit the sustained activation of the MAPKs signaling pathway, as evidenced by the reduction of phosphorylated forms of ERK1/2, JNK and p-38 in MCF-7/DOX cells after 24-h exposure, followed by the significant increase in the number of apoptotic cells. Concurrently, rhinacanthin-C significantly reduced the amount of phosphorylated Akt, GSK-3β and Nrf2 in the rhinacanthin-C treated MCF-7/DOX cells. Moreover, expression levels of HO-1 and NQO1 in these cells were significantly decreased after treated rhinacanthin-C for 24 h. These findings suggested that the rhinacanthin-C was able to inhibit the sustained activation of Akt/GSK-3β/Nrf2 signaling pathway and MAPKs pathway in the MCF-7/DOX cells. Nrf2 is an essential transcription factor that fights oxidative stress at cellular level by regulating the expression of antioxidant and detoxifying enzymes such as HO-1 and NQO1 (Basak et al., 2017; Choi et al., 2016; Jung et al., 2018). The phosphorylation of a repressor GSK-3β at Ser 9 by Akt reduces Nrf2 phosphorylation so that Nrf2 can escape the ubiquitination and proteasomal degradation processes in the cytoplasm (Basak et al., 2017; Panieri & Saso, 2019). Hence, Nrf2 translocated into the nucleus and its transcriptional activity increases, making the cells resistant to stress and apoptotic signals (Kumar et al., 2014; Panieri & Saso, 2019). Similar to the MAPKs pathway, high Akt/GSK-3β/Nrf2 basal activities with overexpression of HO-1 and NQO1 were detected in MDR cancer cells such as MCF-7/DOX, BEL-7402/ADM and A549/DDP cells (Gao et al., 2013; Xia et al., 2015; Zhong, 2013). Our results indicated that rhinacanthin-C could suppress the expression of antioxidant and detoxifying enzymes HO-1 and NQO1 via inhibition of the Akt/GSK-3β/Nrf2 pathway. The collective inhibitory effect of rhinacanthin-C on cellular defense mechanisms through the MAPKs and Akt/GSK-3β/Nrf2 signaling cascades contributed, in part, to promote stress-mediated apoptosis in the MCF-7/DOX cells. The potential effects of this

naphthoquinone on other redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and hypoxia-inducible factor 1 (HIF-1), as well as other survival pathways should be investigated further.

In conclusion, this study demonstrated that rhinacanthin-C at non-cytotoxic concentrations could down-regulate the expression of P-gp at the transcription level in the MCF-7/DOX cells, leading to its MDR reversal effect. Rhinacanthin-C-mediated reduction of *MDR1* mRNA may involve the inhibition of the PI3K/Akt, but not MAPK/ERK1/2 signaling pathway. In addition, rhinacanthin-C was able to induce apoptosis in the MCF-7/DOX cells through ROS production and inhibition of the MAPKs signaling affected the ratio of checkpoint proteins Bax/Bcl-2 in favor to apoptosis induction. Furthermore, rhinacanthin-C could increase cellular stress and promote apoptosis via inhibition of the Akt/GSK-3β/Nrf2 pathway, leading to the downregulation of antioxidant and detoxifying enzymes NQO1 and HO-1. Taken together, the ability of rhinacanthin-C to overcome MDR mechanism against MCF-7DOX cells death might be involved with their downregulation of P-gp, promote apoptosis and cellular detoxifying signaling pathways, as shown in Figure 24.

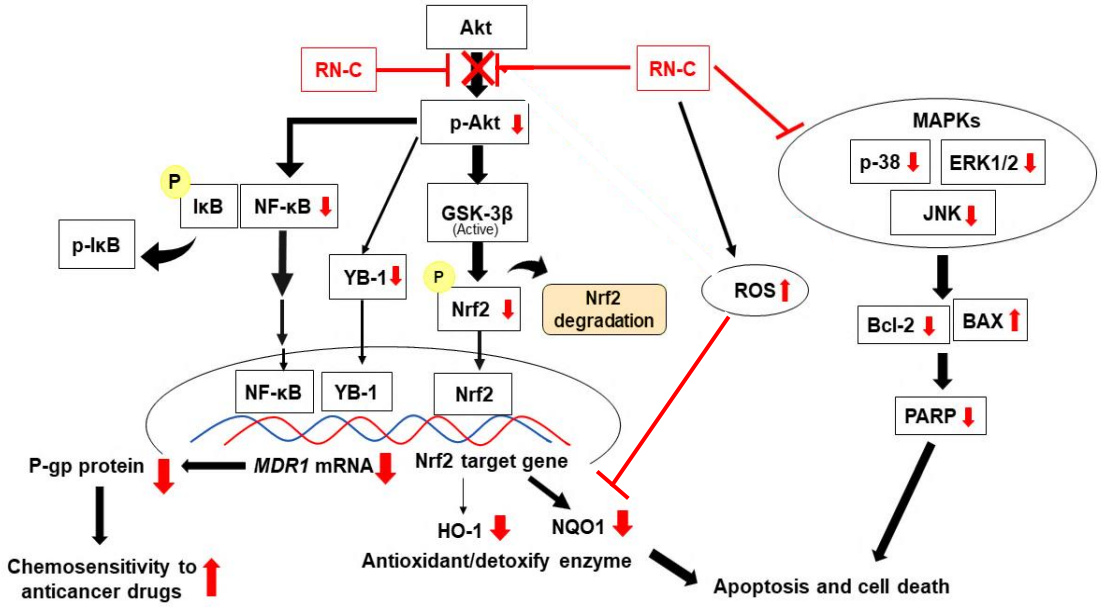


Figure 24. The overcoming MDR mechanisms of rhinacanthin-C (RN-C) in MCF-7DOX cells.

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