AMERICAN UNIVERSITY OF BEIRUT

SYNERGIZED ANTICANCER ACTIVITY OF HONEYBEE ROYAL JELLY AND THYMOQUINONE AGAINST AGGRESSIVE HUMAN BREAST CANCER

by
MAYA MAJED MOUBARAK

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AN ABSTRACT OF THE THESIS OF

Maya Majed Moubarak for Master of Science
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Title: Synergized Anticancer Activity of Honeybee Royal Jelly and Thymoquinone Against Aggressive Human Breast Cancer

Thymoquinone, the main constituent of *Nigella sativa* seeds and royal jelly (RJ), the honeybee secretion fed to queens, are effective against cancer. The anticancer activity of the combination of TQ and RJ against breast cancer is still unknown. Here, we investigated the effects of TQ alone, RJ alone, and combinations on the viability and cell cycle regulation in MDA-MB-231 human metastatic breast cancer cells and determined the cell death mechanism. Our study is the first to report prominent anticancer synergistic effects of the combination of TQ and RJ against MDA-MB-231 breast cancer cells. TQ alone inhibited cell viability in a dose-dependent manner at concentrations below and above the IC_{50}, which was established at 19 µM. A dose of 15 µM of TQ caused a significant increase in the pre-G1 population, while a more pronounced effect was observed in response to TQ and RJ combination. Royal jelly exhibited relatively nontoxic effects against MDA-MB-231 cells and FHS 74 Int small intestinal cells at concentrations below 5 µg/ml. High doses of RJ (200 µg/ml) had greater toxicity against MDA-MB-231 cells. Interestingly, the combination of both compounds synergistically inhibited cell viability and cell death was most pronounced in response to 15 µM TQ and 5 µg/ml RJ. Immunofluorescent staining showed that TQ was the main inducer of caspase 3-dependent apoptosis when applied alone and in combination with RJ. In contrast, no significant regulation of Ki67 expression was observed, indicating that the decrease in cell viability was due to apoptosis induction rather than to the inhibition of cell proliferation. In summary, RJ synergizes with TQ to inhibit the viability of MDA-MB-231 metastatic breast cancer cells and thus could confer an advantage for cancer therapy.

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ABBREVIATIONS

%: percent
+: positive
-: negative
µM: micromolar
µg/ml: microgram per milliliter
µl: microliter
mg: milligram
°C: degrees Celsius
min: minutes
h: hour
AGP: alpha -1 acid glycoprotein
ALT: alternative lengthening of telomeres
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
BCS: breast-conserving surgery
BCT: breast-conserving therapy
BPA: bisphenol A
BRCA1/2: breast cancer associated gene 1 and 2
BSA: bovine serum albumin
CAM: cell adhesion molecule
CCAs: cancer chemotherapeutic agents
CHF: congestive heart failure
CSCs: cancer stem cells
CT: chemotherapy
CVD: cardiovascular diseases
CXCR4: chemokine receptor type 4
DecDA: 1,10-decanedioic acid
DFI: disease-free interval
DMSO: Dimethyl sulfoxide
DNA: deoxyribonucleic acid
ECM: extra cellular matrix
eEF-2K: epidermal factor 2 kinase
EGF: epidermal growth factor
EMT: epithelial to mesenchymal transition
ER: estrogen receptor
FGF: fibroblast growth factor
GF: growth factor
HDAC: histone deacetylase
HER2: human epidermal receptor 2
HSV-1: herpes simplex virus type 1
IC50: half maximal inhibitory concentration
IDC: Invasive ductal carcinoma
IFN-γ: Interferon gamma
IL-1/2/6: interleukin 1, 2 and 6

ILC: invasive lobular carcinoma
LD50: Lethal Dose, 50%
LPS: lipopolysaccharide
LQTS: long QT syndrome
MAPK: mitogen-activated protein kinase
MMP-1/3: matrix metalloproteinases
MRJP: major royal jelly proteins
MRSA: methicillin-resistant Staphylococcus aureus
MTT: 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC: neoadjuvant chemotherapy
NaCl: Sodium Chloride
NAT: N-acetyl transferase
N-CAM: neural cell adhesion molecule
NF-κB: nuclear factor kappa light chain enhancer of activated B cells
NO: nitric oxide
NSCs: neural stem cells
OS: overall survival
P/S: penicillin–streptomycin
PARP: poly (ADP-ribose) polymerase
PBS: Phosphate Buffered Saline
PGE2: prostaglandin E2
PI: propidium iodide
PR: progesterone receptor
PSA: prostatic-specific antigen
PTEN: phosphatase and tensin homolog
RJ: royal jelly
RJPs: royal jelly peptides
ROS: reactive oxygen species
SEA: sebacic acid
SEM: Standard error of the mean
SERM: estrogen receptor modulator
TAM: tamoxifen
THQ: thymohydroquinone
TNBCs: triple-negative breast cancers
TQ: thymoquinone
VEFG: vascular endothelial growth factor
WBI: whole-breast irradiation
WHO: world health organization
γH2AX: phosphorylated histone
10-HDA: 10-Hydroxy-2decanoic acid
2-DecDA: 2-decene-1,10-dioic acid
3,10-HDecDA: 3,10-dihydroxydecanedioic acid
3-HHDA: 3-hydroxydecanoic acid
8-HOC: 8-hydroxy octanoic acid
9-HAD: 9-hydroxy-2-decenoic acid

CHAPTER I

INTRODUCTION

A. Global burden of cancer: current and future

According to the World Health Organization (WHO), cancer is the second leading cause of death worldwide, causing 9.6 million deaths, or one in six deaths, among 18.1 million cases in 2018 globally. For both sexes collectively, the most frequently diagnosed cancer is lung cancer (11.6% of all cases), followed by female breast 11.6%), colorectal cancers (10.2%) and prostate cancer (7.1%). Lung cancer is considered the primary cause of cancer deaths (18.4% of all deaths), followed by colorectal (9.2%), stomach and prostate cancers (8.2%) (Bray et al., 2018; WHO, 2018). Lung and breast cancer account for 2.09 million cases causing 1.76 million and 627,000 deaths, respectively (WHO, 2018).

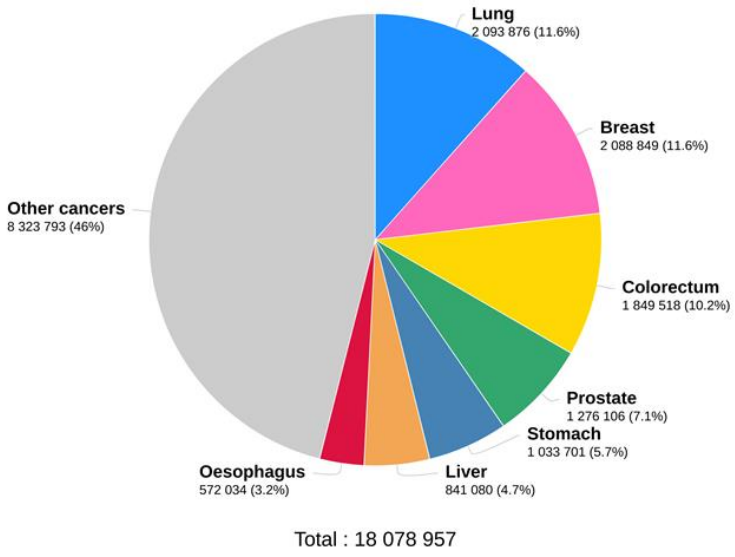


Figure 1. Estimated number of new cases in 2018, worldwide, both sexes, all ages. (As adapted from IARC, WHO 2018, Cancer Today).

In 2003, WHO declared that 10 million new cancer cases are diagnosed each year worldwide, and the number will rise to 20 million in the year 2020 unless preventive action is taken to scale down cancer rates. Among the United States population, 1,806,590 new cancer cases and 606,520 cancer deaths are predicted in 2020 by the American Cancer Society (Siegel RL, 2020). The World Health Organization predicted that the global burden of cancer would double to about 29–37 million new cancer cases by 2040. Their report estimated the trend of future cancer cases by which breast cancer will account for 2,778,850 cases, while lung cancer will account for 3,299,640 cases worldwide in 2040 (WHO, 2020b).

B. Cancer management and prevention

New cases are emerging, and deaths continue to rise because of increasing life expectancy and epidemiological and demographic changes. The successful fight against infections has helped to decrease mortality from infectious diseases over the past 60 years, while the burden of non-communicable diseases and cancer has increased. Also, the prevalence of risk factors is proportional to the incidence of cancer among populations and strongly affects the incidence of certain types of cancers. For example, the smoking epidemic is the primary cause of lung cancer incidence in countries with the prevalence of tobacco. In addition to the effect of risk factors, social and economic inequalities such as education, diet, gender, and environment affect cancer prevalence. For instance, preventable cancers are more likely to occur in economically disadvantaged populations due to late diagnosis, poor prognosis, and

inadequate access to treatment. Early detection and screening serve to decrease the burden of cancer, especially of types that could not be prevented. Cancer treatment has progressed over the past years, and include surgery, radiotherapy, and systemic therapies (e.g., chemotherapy, immunotherapy, endocrine therapy). Despite this tremendous improvement in therapeutic approaches, management of cancer should take place through awareness, vaccination against oncoviruses, and implementation of health care programs that are accessible for all people of all ages and particularly to disadvantaged groups (WHO, 2020c).

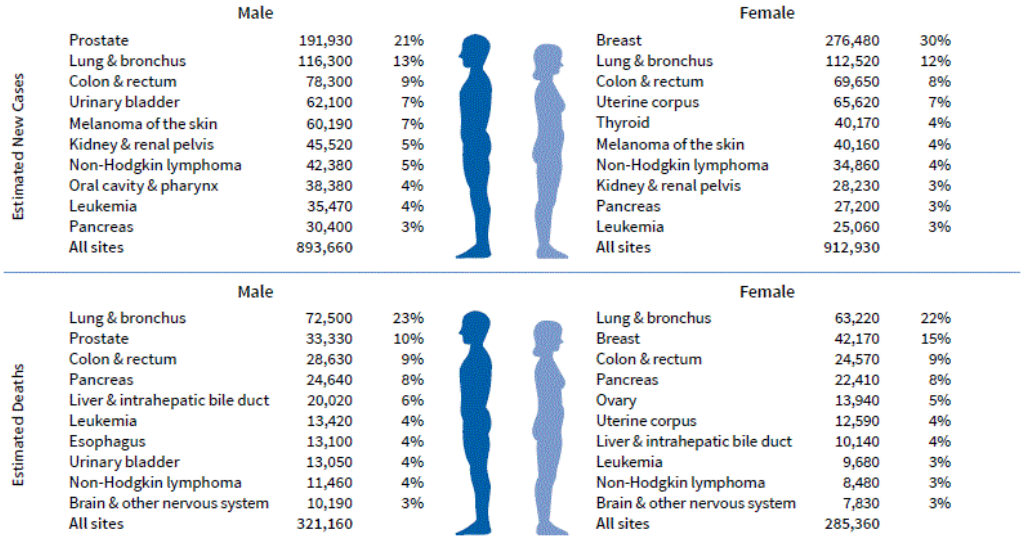


Figure 2. Leading sites of new cancer cases and deaths – 2020 estimates.
Deaths estimates among the United States population rounded to the nearest ten excluding basal cell and squamous cell skin cancers and in situ carcinoma except for urinary bladder. Estimates do not include Puerto Rico or other US territories. The ranking is based on modeled projections and may differ from the most recent observed data. (As adapted from American Cancer Society, Cancer Facts & Figures 2020).

C. Carcinogenesis

Carcinogenesis is known to be a multistep process that reflects accumulated genetic mutations causing the transition from normal to malignant cells. It consists of four steps: initiation, promotion, malignant conversion, and tumor progression. Tumor development is initiated upon the irreversible damage in the cell genome due to errors in DNA replication, depurination of DNA, or an intrinsic attack by reactive oxygen species (Bertram, 2000; Loeb and Cheng, 1990). Initiated cells are then promoted to clonally expand into a larger population of cells that are at risk of further genetic changes and malignant conversion (Marks et al., 2007). Tumor progression then takes place as tumor cells express a malignant phenotype and acquire more aggressive characteristics, including the ability of rapid proliferation, local invasion, and metastasis (Nowell, 1986).

Cancer research has generated a rich and complex body of knowledge revealing distinct types of cancer and subtypes classified as carcinomas, sarcomas, and leukemias, or lymphomas. Carcinomas account for nearly 90% of human cancers and are characterized by malignancies of epithelial cells. Sarcomas are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue. Leukemias and lymphomas constitute 8% of human malignancies coming from the blood-forming cells and cells of the immune system (Cooper, 2000).

D. Cancer hallmarks

Most types of human tumors share typical hallmarks that enable their growth, including self-sufficiency in growth signals, evasion of apoptosis, limitless replicative potential, angiogenesis, tissue invasion capacity and metastatic dissemination (Hanahan and Weinberg, 2000).

1. Self sufficiency in growth factors

Normal cells rely on transmembrane transmitted growth signals acquired from their niche to initiate cell proliferation. However, cancer cells produce their growth factors (GF) to which they are responsive in an autocrine loop of positive feedback. In turn, overexpression of tyrosine kinase GF receptors renders cancer cells to become hyperresponsive and proliferate in response to ambient levels of GF that usually are insufficient to trigger cell proliferation (Hanahan and Weinberg, 2000). Also, cancer cells favor the switch of their extracellular matrix receptors into pro-growth integrins linking the cells to the extracellular matrix (ECM) and transducing signals into cell cytoplasm to regulate their motility and cell cycle activation (Giancotti and Ruoslahti, 1999; Lukashev and Werb, 1998). Pro-growth integrins and ligand-activated GF receptors activate the SOS-Ras-Raf-MAP kinase pathway. However, in 25% of human cancers, Ras proteins are structurally modified and are present in a confirmation that induces mitogenic interaction independent of their standard upstream regulators (Aplin et al., 1998; Giancotti and Ruoslahti, 1999; Medema and Bos, 1993).

2. Insensitivity to antigrowth signals

Antiproliferative signals inhibit proliferation by forcing the cells to enter either the quiescent (G0) state or postmitotic state triggering cell differentiation (Hanahan and Weinberg, 2000). At the molecular level, in a hypo-phosphorylated state, Retinoblastoma protein (pRb) sequesters and alters the conformation of E2F transcription factors, which in turn blocks the expression of genes responsible for G1-S cell cycle transition. Disruption of the pRb signaling circuit in different types of cancer induces cancer cell proliferation and renders them insensitive to antigrowth signals that operate along with the G1 phase of the cell cycle (Fynan and Reiss, 1993; Moses et al., 1990; Weinberg, 1995). Besides their insensitivity to cytostatic antigrowth signals, cancer cells avoid terminal differentiation. Overexpression of the c-Myc oncoprotein in many tumors favors Myc–Max regulatory signals promoting cell growth over differentiation (Foley and Eisenman, 1999; Hanahan and Weinberg, 2000).

3. Evading apoptosis

Programmed cell death is triggered in response to DNA damage, thus signaling imbalance caused by oncogene action, lack of survival factors, or hypoxia (Evan and Littlewood, 1998). Apoptotic signals converge on the mitochondria, which in turn, activates intracellular proteases such as Caspases 8 and 9 to execute the death program (Green and Reed, 1998; Wang and Youle, 2009). The p53 suppressor gene is mutated in almost 50% of human tumors. Mutated p53 protein accounts for the acquired resistance to apoptosis as it loses its pro-apoptotic function in sensing damaged DNA that triggers cell death in normal cells (Harris, 1996). Additionally, the PI3 kinase–AKT/PKB pathway, when activated by extracellular factors such as IL2 or IGF-1/2, intracellular

RAS protein, or loss of PTEN suppressor gene, transmits antiapoptotic signals to oppose cell death (Cantley and Neel, 1999; Downward, 1998; Evan and Littlewood, 1998).

4. Limitless replicative potential

Most types of tumor cells acquire the limitless replicative potential that is essential for *in vivo* tumor progression (Hayflick, 1997). The replicative potential is a result of telomere maintenance in malignant cells. The majority of malignant cells upregulate the expression of the telomerase enzyme, which adds hexanucleotide repeats at telomere ends, avoiding telomere erosion during replication (Bryan and Cech, 1999). Besides, malignant cells activate a mechanism named alternative lengthening of telomeres (ALT) that sustains the telomeres through recombination-based intrachromosomal exchanges of sequence information (Bryan et al., 1995).

5. Sustained angiogenesis

An angiogenic switch is evident in tumors as tumor cells acquire the capability to maintain their growth. Shifting the balance between angiogenic regulators is mediated by favoring the upregulation of VEFG or FGF gene expression while downregulating that of angiogenic inhibitors such as thrombospondin-1 or β-interferon (Hanahan and Folkman, 1996; Singh et al., 1995; Volpert et al., 1997). Loss of p53 function in human tumors triggers thrombospondin-1 level to decrease while ras oncogene activation or loss of VHL tumor suppressor gene mediates the elevation in VEGF expression (Dameron et al., 1994; Maxwell et al., 1999; Rak et al., 1995). Angiogenesis is also regulated through proteases liberating ECM stores and bFGF,

whereas plasmin, a pro-angiogenic component of the clotting system, cleaves itself into angiostatin as an angiogenesis inhibitor (Gately et al., 1997; Whitelock et al., 1996).

6. Tissue invasion and metastasis

A general parameter of tumor cellular invasive phenotype is the altered cadherins and integrins responsible for cell-cell adhesion and cell-ECM connections, respectively. E-cadherins lose their inhibitory functions in tumor invasion and metastasis as they are altered in most epithelial cancers (Aplin et al., 1998; Christofori and Semb, 1999). Similarly, CAMs belonging to immunoglobulin superfamily as N-CAM give up their adhesive ability rendering them to become poorly adhesive members in different types of cancer (Johnson, 1991). However, carcinoma cells facilitate their invasion capability as they shift integrin expression favoring those that bind to degraded stromal components over intact ECM of healthy tissues (Lukashev and Werb, 1998; Varner and Cheresh, 1996). Another parameter of the invasiveness and metastatic capability is extracellular proteases activation, whose expression is elevated opposing that of protease inhibitors. Shifting the balance towards matrix-degrading proteases facilitates cancer cell invasion into the nearby stroma, blood vessel walls, and through regular epithelial layers (Stetler-Stevenson, 1999; Werb, 1997).

E. Breast cancer

1. Breast architect and tumor subtypes

The human breast is a multilobed structure composed of lobes, and each lobe constitutes multiple lobules. Each lobule connects to a terminal interlobular duct connected to an outlet duct to the nipple. Breast lobules and ducts are lined with one layer of luminal epithelial cells surrounded by transversely oriented myoepithelial cells attached to a basement membrane, which separates the duct from its surrounding stroma. The stroma comprises a variety of discrete cells, including fibroblasts, immune cells, adipocytes, and organized structures as blood vessels (Bertos and Park, 2011). Invasive ductal carcinoma (IDC) is the most frequent type of breast tumor, followed by invasive lobular carcinoma (ILC). Together they make up 90% of breast cancers while the remaining 10% is caused by particular types of none-ILC/none-IDC tumors (Bertos and Park, 2011; Li et al., 2005; Weigelt et al., 2010).

Breast cancer is the most common cancer among women, accounting for 2.1 million cases each year, and is the cause of the majority of cancer-related deaths among women (WHO, 2020a). Specific markers, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2), are associated with different breast tumors subtypes defining their prognosis and possible therapy. Differential expression of hormonal biomarkers yields specific tumor subtypes, namely ER+ (ER+/HER2–), HER2+ (ER–/HER2+), triple-negative (TN; ER–/PR–/HER2–), and triple positive (ER+/PR+/HER2+) breast cancers (Bertos and Park, 2011). ER status identifies tumors that may respond to anti-estrogen (endocrine) therapeutics that target ER-dependent signaling, such as ER antagonists or aromatase inhibitors (Jordan and Brodie, 2007; Patel et al., 2007). PR status is generally correlated with ER status,

however specific types of endocrine therapy do not exert anti-cancer activity on ER+/PR+ breast cancers (Bartlett et al., 2011; Dowsett et al., 2008). Targeted therapies utilizing monoclonal antibodies are used to treat HER2+ cases as it disrupts HER2-dependent signaling and mediates antibody-dependent cytotoxicity (Clynes et al., 2000; Junttila et al., 2009). The level of gene expression accounts for the molecular heterogeneity of tumor subtypes. ER+ breast cancers are divided into two luminal subtypes (A and B) that differ in the expression of ER regulated genes (Bertos and Park, 2011). The absence of specific markers characterizes triple-negative breast cancers (TNBCs). Existing heterogeneity within this group has further subdivided it into subgroups that respond to chemotherapeutic regimens with differential expression of basal-specific, immunomodulatory, mesenchymal, mesenchymal stem-like, and androgen receptor-related genes (Bertos and Park, 2011; Lehmann et al., 2011).

2. Risk factors and cancer susceptibility

Several factors contribute to the incidence and pathogenesis of breast cancer. Aging is considered a fundamental factor as the frequency of ER (+) tumors diagnosed in women increases after menopause while it is less frequent in women below 45 years old (Ban and Godellas, 2014). Genetic predisposition increases in women, especially in those whose first-degree relatives develop breast cancer due to mutations in breast cancer-related genes such as BRCA1 and BRCA2 (Brewer et al., 2017; Sun et al., 2017). Other intrinsic risk factors that influence breast cancer incidence include reproductive and hormonal changes during maturation. For instance, every 1-year delay in menopause increases the risk of breast cancer by 3% while a delay in menarche, early pregnancy, and prolonged breastfeeding decreases the risk of breast cancer occurrence

(Ban and Godellas, 2014; Hsieh et al., 1990; Sun et al., 2017). Proliferative lesions in mammary glands possessing benign phenotype increases both the risk of occurrence of malignant lesions and breast cancer in women with a cancer-related family history (Hartmann et al., 2005).

Moreover, several extrinsic factors, such as lifestyle and dietary habits, correlate with breast cancer incidence. Diets rich in fats and processed products promote the neoplastic transformation in mammary gland cells during the postmenopausal period (Thiebaut et al., 2007). Low-fat diet in women treated for neoplastic disease post-menopause decreased the probability of neoplasm relapse after surgical procedure. Also, regular physical activity not only reduces the risk of breast cancer occurrence by 20-40% but also improves the immune system, fitness, and quality of life (Lynch et al., 2011; Saxe et al., 1999). Diets rich in Vitamin D or anti-oxidants reduce the relative risk of breast cancer by 20% (Bauer et al., 2013; Saxe et al., 1999). However, breast cancer risk increases as a result of smoking and alcohol consumption (Knight et al., 2017).

3. Cell culture model

Studying cancer development and tumor progression relies on the use of cellular models for the identification of drug effects and molecular players. Triple-negative breast cancer is an aggressive type of breast cancer with limited treatment options. To investigate the drug-induced effect, MDA-MB-231 human breast cancer cell line, a highly invasive and poorly differentiated triple-negative breast cancer has been employed in our study. Being one of the most commonly used breast cancer cell lines in medical research, MDA-MB-231 derives from pleural effusion in metastatic mammary adenocarcinomas (Cailleau et al., 1978). Absence of ER, PR, and HER2 expression

renders them nonresponsive to hormonal treatments (Chavez et al., 2010). However, these cells possess high invasive capacity and metastatic potential as they degrade the extracellular matrix of tissues and metastasize into the lung, bone, or brain-specific cancers (Kang et al., 2003; Minn et al., 2005; Palmieri et al., 2006). Specific gene expression alternations identify the target site to which MDA-MB-231 cells metastasize (Wright et al., 2016).

The MDA-MB231 cell line was initially classified as a basal cancer cell line due to the absence of hormone receptors. It is now grouped into claudin-low subtype as it exhibits low expression for claudin-3, claudin-4, and Ki67 proliferation marker, upgraded expression of epithelial-mesenchymal transition-related markers as well as low CD44+CD24- profile featuring mammary cancer stem cells (CSCs) phenotype (Holliday and Speirs, 2011).

4. Treatment of breast cancer

Although breast cancer is the most prevalent cancer among women worldwide, early diagnosis and improved treatments account for the increased survival rate. Conventional treatment of breast cancer is comprised of surgical procedures, radiotherapy, and systemic therapy, including chemotherapy, endocrine (hormonal) therapy, and targeted therapies.

Surgery for breast cancer has undergone significant progress over the past decade. A total mastectomy removes the entire breast, including breast parenchyma, nipple-areolar complex, and extra skin from the chest wall. However, skin-sparing mastectomy and nipple areolar-sparing mastectomy remain as options for the majority of patients willing to undergo breast reconstructive surgeries (Moo et al., 2018). Breast-

conserving therapy (BCT) has been established as an alternative to radical mastectomy. BCT involves the removal of the tumor (lumpectomy) followed by adjuvant whole-breast irradiation (WBI) to eliminate the residual microscopic disease that may remain in the breast even when negative margins are obtained (Holland et al., 1985). Otherwise, neoadjuvant chemotherapy (NAC) is used in women with massive tumors relative to breast size before surgical excision to facilitate breast conservation (Moo et al., 2018).

Radiotherapy is known to reduce local relapse; however, this does not lead to a reduction in mortality. Usually, distant micrometastasis is reduced upon systemic therapy followed by radiotherapy, which plays a curative role when applied to the locoregional sites preventing secondary dissemination (Joshi et al., 2007). Most breast cancer types are classified as non-inflamed or immune 'cold' tumors as they are likely unresponsive to immunotherapy. Stimulating the immune response could be possible after priming the tumor's microenvironment using radiation therapy as an *in situ*-cancer vaccine, especially in patients with high-risk of developing breast cancer (Joshi et al., 2007; Krombach et al., 2019). In order to avoid local recurrence, radiotherapy is applied to the regional lymph nodes and chest wall after mastectomy, while it is often given to the residual breast in patients undergoing breast-conserving surgery (BCS) (Joshi et al., 2007).

On the other hand, chemotherapy (CT) is recommended to treat ER-negative tumors, TNBC, HER2-positive breast cancers, and high-risk luminal tumors. Endocrine therapy is applied either to balance or to block hormones in hormone receptor (HR)-positive breast cancer (Michaels et al., 2016; Nounou et al., 2015). Targeted therapy is used to treat breast cancer cells overexpressing proteins aiding in their abnormal

growth. At present, targeting the HER2 protein is the most effective breast cancer therapy. Targeted therapies work by inhibiting cancer cells from receiving energy supplies and growth signals, as well as blocking their proper vascularization (Masoud and Pages, 2017).

Despite all their therapeutic impact, conventional treatments of breast cancer exert additive side effects, which in turn affect both the disease-free interval (DFI) and overall survival (OS) (Bovelli et al., 2010). For instance, systemic therapy disrupts key processes in normal cells such as wound healing, immune response, and coagulation cascade (Teven et al., 2017). Most chemotherapeutic drugs lead to dose-related cardiotoxicity and increase the risk of cardiovascular diseases (CVD) as it causes chronic cardiovascular complications, including congestive heart failure (CHF), long QT syndrome (LQTS) and bradycardia (Bodai and Tuso, 2015). In addition to mucositis, systemic therapy often yields bone marrow, neuronal and renal toxicities (Plenderleith, 1990). Similarly, breast radiation may result in cardiac injury in addition to secondary malignancies in tissues with proximity to the breast, such as the lung and esophagus (Brownlee et al., 2018). Hormonal blockade using tamoxifen, a selective estrogen receptor modulator (SERM), yields side effects like those that accompany the onset of menopause such as hot flashes, mood swings, depression, and vaginal dryness. Also, it increases the risk of thromboembolic complications and endometrial cancer (Bodai and Tuso, 2015).

Almost all effective treatments for cancer have the potential to yield various side effects. Particularly anti-cancer chemotherapeutic agents (CCAs) could produce lethal toxicity as they eradicate normal cells owing to the lack of tumor specificity, high dose requirement, and systemic toxicity. Therefore, extensive research is conducted on

alternative treatments utilizing natural, relatively non-toxic compounds with less toxicity and high therapeutic potential.

In line with the progress in the field of optimum nutrition, plants are advertised not only as a primary source of food but also as curative medicines. The interest in phytomedicine resurged due to the easy accessibility of natural resources, low cost, and less harmful effects as compared to synthetic medicines (Yimer et al., 2019).

Nigella sativa L. (Ranunculaceae), commonly known as the black seed, is one of the most treasured nutrient-rich herbs. Historically, the black seed is used to treat various ailments, including airway disorders, chronic headache, diabetes, inflammation, hypertension, and digestive tract problems. In addition to its traditional medicinal uses, black seed possesses remarkable pharmacological activities. It is composed of 20-85% protein, 38.20% fat, 7-94% fiber, and 31.94% total carbohydrates, in addition to various amino acids as glutamate, arginine, aspartate, cysteine, and methionine. Fixed oil constitutes 26-34% of black seed composition, and essential oils make up 0.4%–2.5% of the fixed oil. The essential oils include trans-anethole, p-cymene, limonene, carvone, α-thujene, thymoquinone (TQ), thymohydroquinone (THQ), dithymoquinone, carvacrol, and β-Pinene (Yimer et al., 2019).

The black seed exhibits various pharmacological activities, including anti-oxidant, anti-diabetic, neuroprotective, anti-inflammatory, and anti-cancer activities. These activities are mainly attributed to its main bioactive constituent Thymoquinone (TQ) (Abulfadl et al., 2018; Amin and Hosseinzadeh, 2016; El Rabey et al., 2017; Schneider-Stock et al., 2014; Sultan et al., 2015).

F. Thymoquinone

Thymoquinone (TQ) is a yellow crystalline molecule (2-methyl-5-isopropyl-1,4-benzoquinone) having a quinone structure consisting of a para-substituted dione linked to a benzene ring with methyl and an isopropyl side chain groups added in positions 2 and 5, respectively (Yimer et al., 2019). Immunomodulatory, cytoprotective, hepatoprotective, and neuroprotective properties of TQ were reported. Besides, TQ showed potent anti-oxidant, anti-inflammatory and anti-cancer activities (Abulfadl et al., 2018; Aycan et al., 2014; Khader and Eckl, 2014; Mahmoud and Abdelrazek, 2019).

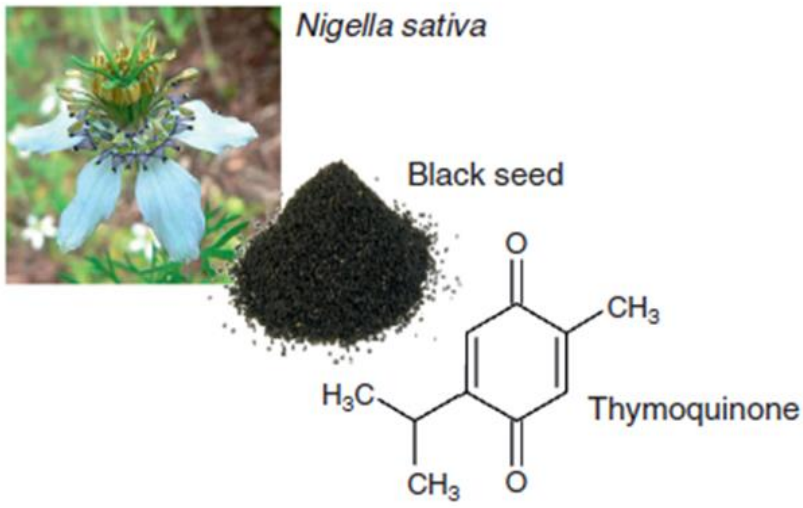


Figure 3. Thymoquinone (TQ) chemical structure.
(As adapted from Schneider-Stock et al., 2014. Drug Discovery Today, Vol. 19(1):18-30).

Thymoquinone (TQ) modulates almost all the hallmarks associated with most tumor types. Among the modulated hallmarks, sustained proliferative signaling is blocked via the downregulated mitogen-activated protein kinase (MAPK) and protein kinase B (AKT/PKB) signaling pathways (Arafa el et al., 2011; Das et al., 2012; Hussain et al., 2011; Yi et al., 2008). Growth inhibition and apoptosis were induced in response to TQ by the upregulation of the expression of the tumor suppressor p53 protein and its transcriptional target p21 in addition to reactive oxygen species (ROS) generation, thus overriding cell death resistance (Arafa el et al., 2011; Dergarabetian et al., 2013; Gali-Muhtasib et al., 2004b; Hussain et al., 2011; Kaseb et al., 2007). Moreover, TQ inhibits telomere attrition during DNA replication (Gurung et al., 2010), and reduces the invasive and metastatic capacity of cancer cells (Gali-Muhtasib et al., 2008b; Jafri et al., 2010; Kolli-Bouhafs et al., 2012) in addition to having both anti-angiogenic and anti-inflammatory potentials (Chehl et al., 2009; Sethi et al., 2008; Yi et al., 2008).

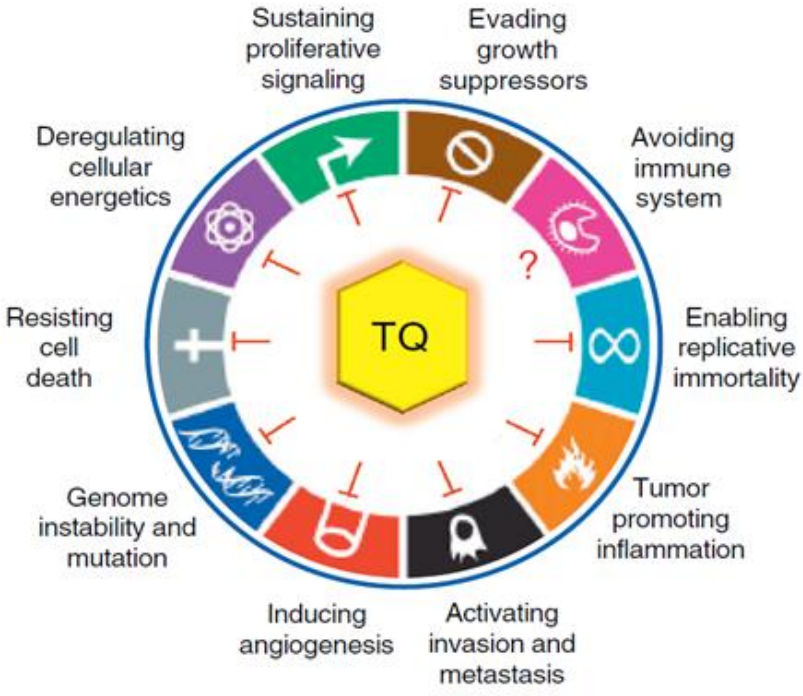


Figure 4. Thymoquinone inhibits the hallmarks of cancer.
(As adapted from Schneider-Stock et al., 2014. Drug Discovery Today, Vol. 19(1):18-30).

Thymoquinone is an effective anti-cancer molecule in various types of cancers, among which are brain, bone, liver, colon, lung, prostate, kidney, bladder, skin in addition to ovarian, cervical, and breast cancer (Imran et al., 2018). TQ abrogates the toxic effects of several chemotherapeutic agents, highlighting its cytoprotective effects (Schneider-Stock et al., 2014). Therefore, being selective to tumor cells and cytoprotective to normal tissues is an attractive feature that distinguishes TQ from amongst plant-derived compounds.

1. Thymoquinone: a therapeutic molecule against breast cancer

TQ, in combination with piperine, lowered VEGF expression and enhanced serum INF-γ levels yielding angiogenesis inhibition, apoptosis induction, and shifting the immune response toward T helper1 responses in EMT6 epithelial breast cancer cell line (Talib, 2017). Similarly, TQ in combination with resveratrol (RES), decreased VEGF expression, elevated IFN-γ in addition to inducing necrosis, enhancing apoptosis, and decreasing tumor size (Alobaedi et al., 2017). TQ alone or in combination with tamoxifen (TAM) induced apoptosis in both MDA-MB-231 and MCF-7 human breast cancer cells (Ganji-Harsini et al., 2016). TQ also suppressed the growth, viability, and invasion of MDA-MB 231 and MCF7 cell lines through the inhibition of Akt phosphorylation leading to DNA damage and activation of the mitochondrial pro-apoptotic pathway (Attoub et al., 2013). TQ's anti-neoplastic and pro-apoptotic activities against MDA-MB-468 and T-47D cells were mediated through the regulation of Akt signaling pathway and induction of G1 cell cycle arrest (Rajput et al., 2013).

In TNBC cells, TQ interrupted cell cycle progression by targeting various proteins, including cyclin E, cyclin D1, and p27 proteins resulting in cell cycle arrest at G1 to S phase. In addition, TQ inhibited the histone deacetylase (HDAC), targeted p21 and Maspin, induced Bax, downregulated the Bcl-2 and upregulated the expression of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and tensin homolog (PTEN) (Barkat et al., 2018). Using *in vivo* and *in vitro* approaches, Sakalar et al. (2016) showed that the treatment of TNBC with TQ resulted in elevated expression of Brca1, p21, and Hic1 tumor suppressor genes, EGF and VEGF growth factors, Caspase-3, Caspase-7, Caspase-12 and poly (ADP-ribose) polymerase (PARP) and reduced phosphorylated p65 and Akt1 proteins. Another study conducted by Sutton *et*

al. (2014), reported the activation of Caspases- 8 and 9, Poly(ADP-ribose) polymerase cleavage, increased γH2AX as well as reduced Akt phosphorylation and X-linked inhibitor of apoptosis expression in TNBC cell lines with mutant p53 in response to TQ treatment .

Overexpression of the eukaryotic epidermal factor 2 kinase (eEF-2K) in TNBC accounts for its aggressive phenotype and resistance to chemotherapy (Hamurcu et al., 2016). Concomitantly with its anti-metastatic activity, TQ reduced the expression of TWIST1 and its target protein N-cadherin in MDA-MB-435 and BT549 cell lines and increased E-cadherin expression in the 4T1 breast cancer cell line. Furthermore, TQ inhibited TNBC cancer initiation, progression and metastasis by targeting the mediators of epithelial to mesenchymal transition (EMT) (Khan et al., 2015).

The anti-tumor activities of TQ against breast carcinoma have been extensively tested in animal models. In 2013, an elegant study conducted by Woo *et al.* (2013), documented the antiproliferative and pro-apoptotic effects of TQ in nude mice xenografted with MDA-MB231 human breast cancer cell line. This study showed induction of p38 phosphorylation, production of ROS, suppression of tumor growth, inhibition of XIAP, survivin, Bcl-xL and Bcl-2 gene expression. Additionally, lowered Ki67 expression and a significant augmentation in anti-oxidant enzymes, including superoxide dismutase, catalase, and glutathione in mouse liver tissues were also observed.

Overexpression of chemokine receptor type 4 (CXCR4) is often associated with poor prognosis and breast cancer metastasis. Thymoquinone's anti-metastatic effect against bone metastasis was further investigated by Shanmugam *et al.* (2018) in breast cancer mouse and chick chorioallantoic membrane assay models. Their study showed

that TQ negatively regulated both nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and CXCR4 expression. Consequently, TQ-mediated inhibition of NF-κB activation cascade suppressed breast to bone metastasis as well as the migration and invasion capacity of MDA-MB 231 TNBC cells.

In summary, TQ exerts anti-cancer activities through different mechanisms against many types of cancers, particularly against breast cancer. Being inexpensive, abundant, tumor-selective, and easily extracted facilitates its clinical translation as a relatively non-toxic natural compound with significant therapeutic value.

2. Limitations of clinical translation of thymoquinone

Clinal trials utilizing TQ as a therapeutic agent in humans is limited due to its chemical properties and reduced membrane permeability. Being hydrophobic reduces its solubility and bioavailability in biological mediums. TQ proved to be a relatively safe compound, particularly when given orally to experimental animals. In mice, the LD50 of TQ was 104.7 mg/kg after intraperitoneal injection and 870.9 mg/kg after oral ingestion, while 57.5 mg/kg and 794.3 mg/kg were the LD50 values in rats after intraperitoneal and oral ingestion, respectively (Al-Ali et al., 2008). The oral administration of TQ facilitates its biotransformation by DT-diaphorase, a quinine reductase phase II liver enzyme, which catalyzes its reduction into hydroquinone (Nagi and Almakki, 2009). In a phase I clinical trial, TQ administration at high doses ranging between 75mg/day to 2600mg/day was safe and well-tolerated in adult humans with solid tumors or hematological malignancies who had failed or relapsed from standard therapy. Unfortunately, the poor solubility and limited bioavailability of TQ could

explain its failure to exert any therapeutic effects on the studied malignancies (Al-Amri and MBBS, 2009).

Extensive binding to plasma proteins is another factor affecting TQ's anti-cancer effects *in vivo*. Pharmacokinetic analysis of TQ reported its low recovery from serum with nearly 95% and 99% of TQ bound to bovine serum albumin (BSA) and alpha -1 acid glycoprotein (AGP), respectively (El-Najjar et al., 2011). Moreover, TQ exhibits low bioavailability due to rapid elimination and relatively slower absorption following its administration *in vivo* (Alkharfy et al., 2015*)*. Besides, TQ is relatively unstable in basic pH and when exposed to light (Goyal et al., 2017).

G. Royal jelly

Bees, flying insects belonging to the order Hymenoptera that are closely related to wasps and ants, are known for their ecological role in pollination and nutritious commercial products including honey, beeswax, propolis, and royal jelly. Indeed, due to their potent bioactivities in apitherapy, bee products attracted the attention for their potential use in cancer therapy, particularly the naturally derived royal jelly (RJ).

Royal jelly (RJ) is a yellowish, creamy, and acidic material with a slightly sharp odor and taste that is secreted from the mandibular and hypopharyngeal glands of worker bees *Apis mellifera* (Hymenoptera, Apidae). Royal jelly is fed temporarily up to 3 days to the bee workers and drones, while it is the only food of the queen bee at her larval and adult life and is responsible for sex determination, ovary development, fertility and prolonged life span (Kamakura, 2011; Leung et al., 1997; Melliou and Chinou, 2005).

1. Royal jelly composition

The chemical composition of RJ varies depending on the honeybee species, physiological state of the colony, environmental conditions, and production period. Generally, crude RJ is composed of water (50-60%), nitrogen compounds (18%), sugars (15%), lipids (3-6%), mineral salts (1.5%), and traces of vitamins (Gismondi et al., 2017). Fructose is the most abundant carbohydrate in RJ, followed by glucose, sucrose, and other minor saccharides, including maltose, trehalose, melibiose, and ribose (Fratini et al., 2016; Gismondi et al., 2017).

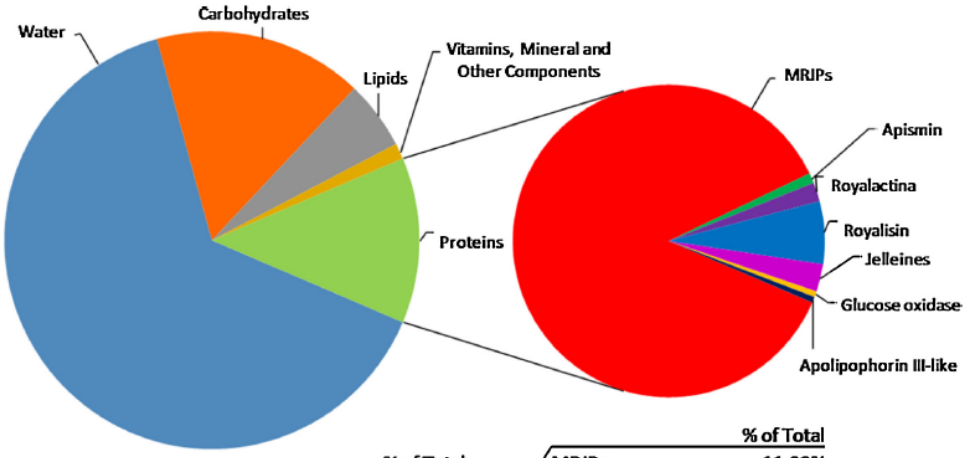
Among the nitrogen compounds, there are major royal jelly proteins (MRJP) constituting 80%–90% of the total protein content and other proteins as glucose oxidase α-glucosidase, α-amylase, 1-peroxiredoin, and glutathione S-transferase (Ahmad et al., 2020; Furusawa et al., 2008; Zhang et al., 2014). Also, the protein fraction contains a calcium-binding protein called regucalcin, a lipid-binding protein named apolipophorin-III, in addition to royalisin, phosphorylated icarapin (venom protein-II) and apolipophorin III-like (Ahmad et al., 2020; Fratini et al., 2016; Furusawa et al., 2008; Han et al., 2014; Schmitzova et al., 1998). Nine genes arranged in 60-kb tandem array encode the major royal jelly proteins MRJPs (1–9) of molecular mass 49–87 kDa (Drapeau et al., 2006; Schmitzova et al., 1998). .MRJP-1, a weak acidic glycoprotein, accounts for 48% of water-soluble RJ proteins and is present either as a monomer (mono MRJP-1) or as an oligomer known as apisin, a polymer of apisimin used to determine the quality of RJ. Mainly, MRJPs are responsible for honeybee queen differentiation, growth, nutrition, and formation of bee products as pollen-pellet and pollen-bread (Fratini et al., 2016). This protein family provides the essential amino acids as nutritive components of RJ, such as arginine, leucine, isoleucine, histidine,

lysine, threonine, tryptophan, methionine, valine, and phenylalanine. For instance, valine and leucine are most prominent in MRJP-1, MRJP-2, and MRJP-4, while arginine and lysine are common in MRJP-3. However, MRJP-5 is rich in methionine and arginine, leucine is plentiful in MRJP-(6–8), and isoleucine is most abundant in MRJP-9 (Ahmad et al., 2020; Scarselli et al., 2005; Schmitzova et al., 1998).

Similar to proteins, anti-bacterial peptides as apisimin, apalbumin α, royalactin and jelleines (I-IV) in addition to apidaecin, defensin and hymenoptaecin are found in RJ and are responsible for its biological effects (Ahmad et al., 2020; Bulet and Stocklin, 2005; Fontana et al., 2004; Han et al., 2014).

The lipidome of RJ consists mainly of medium-chained (8-12 carbon atoms) free fatty acids that are either hydroxyl fatty acids or dicarboxylic acids. It also contains phenolic compounds (4–10%), waxes (5–6%), steroids (3–4%), and phospholipids (0.4–0.8%) (Ahmad et al., 2020; Li et al., 2013). 10-Hydroxy-2decanoic acid (10-HDA) is the main acid in the fatty acid fraction; it constitutes 0.75 to 3.39% of RJ contents and is mostly responsible for its biological activities. The presence of 10-HDA in RJ is unique and is not reported in any natural raw material or any other product of apiculture. Being chemically stable, 10-HDA became a standard marker for the quality and freshness of RJ (Ahmad et al., 2020). Other carboxylic acids in RJ include 10-hydroxydecenoic acid (10-HDDA), sebacic acid (SEA), gluconic acid and dicarboxylic acids (Ahmad et al., 2020; Fratini et al., 2016; Gismondi et al., 2017; Kocot et al., 2018; Terada et al., 2011). In addition to 8-hydroxy octanoic acid (8-HOC), 3,10-dihydroxydecanedioic acid (3,10-HDecDA), 1,10-decanedioic acid (DecDA), 9-hydroxy-2-decenoic acid (9-HDA), 3-hydroxydecanoic acid (3-HHDA), and 2-decene-1,10-dioic acid (2-DecDA) are found among RJ fatty acids (Ahmad et al., 2020; Isidorov et al., 2012).

Vitamin and mineral salts content of RJ is affected by the seasonal changes; B group vitamins are the most abundant. In contrast, vitamin PP, vitamin C, mineral salts, among which are sodium (Na), calcium (Ca), potassium (k), aluminum (Al), Magnesium (Mg) and Zinc (Zn), are a minority (Fratini et al., 2016).



	% of Total
Water	64.32%
Carbohydrates	16.32%
Lipids	5.32%
Vitamins, Mineral and Other Components	1.22%
Proteins	12.82%

	% of Total
MRJPs	11.09%
Apismin	0.13%
Royalactina	0.25%
Royalisin	0.83%
Jelleines	0.37%
Glucose oxidase	0.08%
Apolipophorin III-like	0.08%

Figure 5. Royal jelly detailed composition.
(As adapted from Fratini et al., 2016. Microbiological Research, Vol. 192:130–141).

2. Royal jelly biological properties

Various studies documented the biological and pharmaceutical properties of RJ mainly through its unique bioactive 10-HDA molecule and proteome fraction. Royal jelly contributes to the wound healing process in mice with picryl chloride-induced skin lesions through downregulating antigen-specific interferon-gamma (IFNγ) production and upregulating nitric oxide (NO) synthase
(Taniguchi et al., 2003). RJ also improved healing in diabetic mice (Kim et al., 2010), diabetic patients (Siavash et al., 2011) and hamsters with chemotherapy-induced oral mucositis (Suemaru et al., 2008). Healing activity is mediated by promoting fibroblast migration, keratinocyte proliferation, growth and migration as well the increased levels of sphingolipids, defensin-I and matrix metalloproteinase-9 secretion needed for skin regeneration and wound closure (Bucekova et al., 2017; Kim et al., 2010; Lin et al., 2019; Taniguchi et al., 2003).

In terms of anti-oxidant activity, the essential components of RJ are flavonoids, phenolic compounds, and anti-oxidant peptides. Anti-oxidant peptides found in RJ are characterized by hydroxyl, hydrogen peroxide, DPPH, and superoxide radical scavenging activity (Kocot et al., 2018; Liu et al., 2008). Anti-oxidant properties and free-radical scavenging capacity of RJ mediates RJ anti-diabetic activity against diabetes mellitus as it ameliorates insulin resistance (Pourmoradian et al., 2014; Shidfar et al., 2015). Similarly, RJ anti-oxidant activity is associated with various protective effects against cisplatin-induced renal and hepatotoxicity (Karadeniz et al., 2011), radiation-induced apoptosis in human peripheral blood leukocytes (Silici et al., 2011) and anti-cancer agent-induced toxicities (Abdel-Hafez et al., 2017; Yamauchi et al., 2014; Zargar et al., 2017).

The neuroprotective effect of RJ is manifested through 10-HDA, which inhibits the production of oligodendrocytes, astrocytes, and induces neuronal differentiation from neural stem cells (NSCs) (Hattori et al., 2007). In addition to the neuroprotective RJ peptides (RJPs) that could serve as a potential natural product to treat neurodegenerative Alzheimer's disease (Zhang et al., 2019).

Immunomodulatory and anti-inflammatory activity of RJ has been revealed in many *in vivo* studies. Kohno *et al.* (2004), showed that RJ with the aid of its MRJP3 inhibits the production of proinflammatory cytokines IL-1, IL-6, and TNF-alpha by the active macrophages in a dose-dependent manner. RJ administration attenuates the LPS-induced inflammation in microglial cells (BV-2) by suppressing the phosphorylation of p38, and c-jun NH2-terminal kinases (JNK) and by inhibiting the nuclear translocation of nuclear factor kappa B (NF-kB) and p-65 (You et al., 2018). Furthermore, RJ serves as a protective and therapeutic agent in autoimmune diseases and inflammatory disorders. Through its unique 10-HDA, RJ protects against rheumatoid arthritis by attenuating the action of matrix metalloproteinases (MMP-1, MMP-3), p38, and the c-Jun N-terminal kinases-activating protein-1 (JNK-AP-1) signaling pathway (Fratini et al., 2016). Also, it is worth noting that the 10-HDA molecule possessed better anti-inflammatory effects compared to the other fatty acids present in RJ lipidome (Chen et al., 2016). 10-HDA inhibited lipopolysaccharide (LPS)-induced inhibitor of kappa-B-zeta (IkB-z) and IL-6 production (You et al., 2018), in addition to inhibiting major inflammatory–mediators (Chen et al., 2016) thus contributing to protection against autoimmune and inflammatory diseases. Vucevic *et al.* (2007), confirmed the modulatory role of 10-HDA and 3,10-dihydroxy-decanoic acid (3,10-DDA) on

dendritic cell-mediated immune response *in vitro* as well as the inhibition of the antigen-specific immune response *in vivo*.

Royal jelly is effective against a broad spectrum of microorganisms and bacteria such as yeast, gram-positive and gram-negative bacteria, including *Bacillus subtilis, Staphylococcus aureus, Paenibacillus larvae, Escherichia coli, and Pseudomonas aeruginosa*. Surprisingly, RJ can fight many multidrug-resistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA), which helps overcome public health problems with the onset of the current antibiotic-resistance phenomenon. Studies also documented that RJ has anti-fungal activity and antiviral activity against several viruses, including herpes simplex virus type 1 (HSV-1) (Ahmad et al., 2020; Fratini et al., 2016; Gismondi et al., 2017; Hashemipour et al., 2014).

Moreover, RJ exhibits anti-hypercholesterolemic (Chiu et al., 2017; Kamakura et al., 2006), anti-hypertensive (Fan et al., 2016), anti-aging (Pyrzanowska et al., 2014; Zargar et al., 2017) and anti-cancer activities (Nakaya et al., 2007; Zhang et al., 2017).

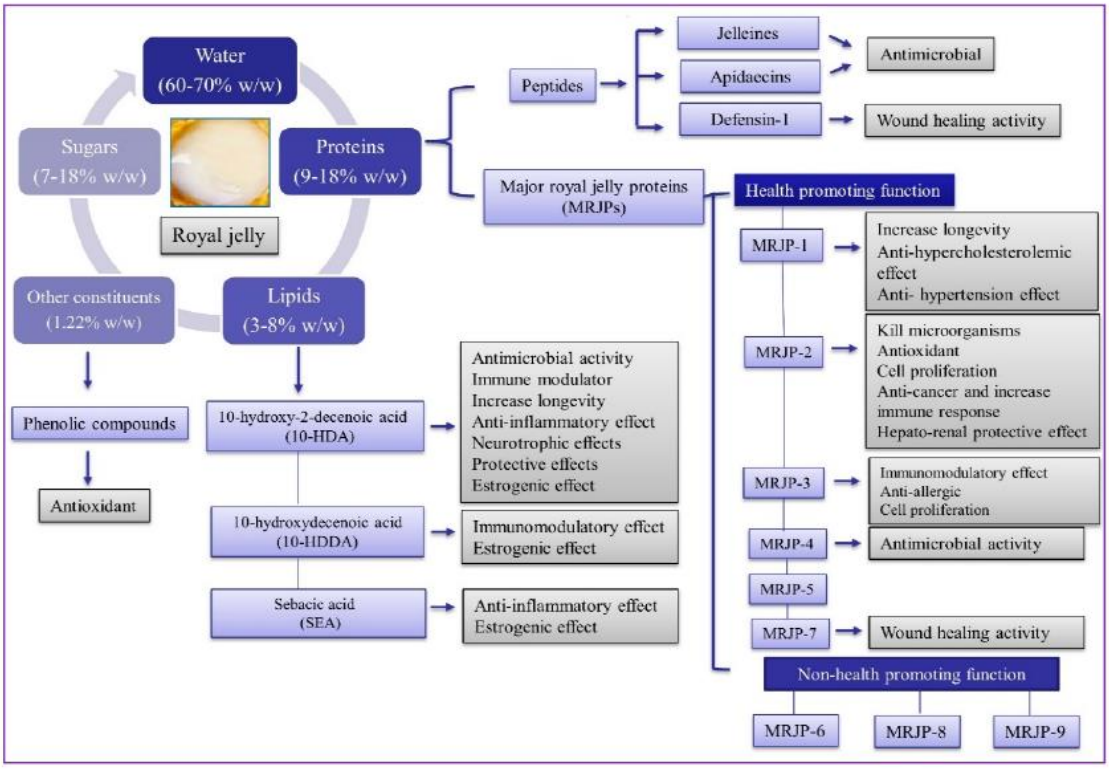


Figure 6. Main biological components in RJ and their functional activities. (As adapted from Ahmad et al., 2020. Int J Mol Sci, Vol. 21(2):382

3. Royal jelly pharmaceutical application in cancer

Among RJ's various biological activities, its possible anti-cancer activity deserves attention. Endogenous hormones are associated with carcinogenesis, tumor growth, and progression in a variety of cancers. It is well established that estradiol as well as Bisphenol A (BPA), a synthetic chemical widely present in plastics and food-related commercial products, causes the onset of many cancer types, including breast cancer (Ayyanan et al., 2011; Samavat and Kurzer, 2015; Vandenberg et al., 2007). In 2007, Nakaya *et al.* reported that RJ inhibits estradiol-induced cell proliferation of MCF-7 breast cancer cells in the presence of bisphenol by suppressing the estradiol-related signaling (Nakaya et al., 2007). Temozolomide cytotoxic effects were enhanced when combined with RJ against astrocytoma (DASC), human glioblastoma multiforme

(U87MG), and normal human astroglia (SVGp12) cancer cell lines (Borawska et al., 2014). Similarly, 10-HDA, together with HuIFN-αN3, inhibited the proliferation of human colorectal adenocarcinoma cells (CaCo-2) *in vitro*. However, this study showed the low antiproliferative activity of RJ monotherapy (Filipic et al., 2015).

In vivo studies have documented the effectiveness of RJ when used against a variety of cancer types. RJ oral administration before the inoculation of 4T1 breast cancer in mice models significantly reduced the volume of the mammary tumor while this inhibitory effect was lost when RJ was administered after tumor cell inoculation. Thus, RJ intake before tumor cell inoculation promotes RJ as an active prophylactic agent rather than a therapeutic agent (Zhang et al., 2017). Concerning its anti-tumor and immunomodulatory properties, RJ raised the survival rate to 85% of Ehrlich ascites tumor (EAT)-bearing mice in a dose-dependent manner. The authors of this study suggest that the improved prognosis could be related to the decreased Prostaglandin E2 (PGE2) levels detected in EAT-bearing mice after RJ treatment (Bincoletto et al., 2005). Also, RJ intake for three months ameliorated the quality of life in patients with benign prostate hyperplasia. Treatment with RJ reduced prostatic-specific antigen (PSA) in blood without any significant reduction in prostate volume or side effects (Pajovic et al., 2016).

The N-acetylation process is usually catalyzed by cytosolic N-acetyl transferase (NAT) and is closely related to the susceptibility of the bladder, breast, and colorectal cancers as it activates arylamine carcinogens (Yang et al., 2000). In contrast, treatment with RJ decreased the NAT activity, and the N-acetylation of 2-aminofluorene (2-AF) in human hepatocellular carcinoma derived (J5) cell line (Premratanachai and Chanchao, 2014).

A study conducted in 1987 by Tamura *et al.*, reported that RJ enhanced mice survival and inhibited the growth of Sarcoma-180 ascites tumor and Ehrlich ascites tumor (Tamura et al., 1987). Similarly, the anti-neoplastic activity of RJ was mediated through its lipophilic portion against the human neuroblastoma cell line (Gismondi et al., 2017). Despite being an exclusive bioactive component for RJ, 10-HDA anti-cancer activity was established nearly 60 years ago against leukemia and ascitic tumors (Townsend et al., 1959; Townsend et al., 1960). However, recent studies have shown that 10-HDA has an anti-proliferative activity in colon cancer, which was mediated by the regulation of inflammatory functions and oxidative stress (Townsend et al., 1960; Yang et al., 2018). Vascular endothelial growth factor (VEGF) is one of the pro-angiogenic proteins that regulate cancer neovascularization and endothelial cell proliferation, migration, survival, and permeability (Zachary, 2003). Treatment with 10-HDA prevented VEGF-induced migration, proliferation, and tube formation in human umbilical vein endothelial cells (HUVEC), as well as VEGF-induced angiogenesis, partly through inhibiting cell migration and proliferation (Izuta et al., 2009).

H. Hypothesis, rationale, and significance of study

Previous studies have documented the therapeutic potential of TQ alone or RJ alone against various kinds of solid and liquid tumors, including breast cancer. It is well established that triple-negative breast cancer is the most aggressive and chemo-resistant subtype of breast cancer.

To our knowledge, there are no studies that have investigated the combined effect of both natural compounds against breast cancer. Thus, we aimed to test the anti-cancer activity of a novel TQ and RJ combination *in vitro* against the MDA-MB231

human triple-negative breast cancer cell line. We hypothesized that TQ and RJ synergize to induce cell death in human breast cancer cells. In turn, this could override chemoresistance in triple-negative breast cancer since this combination could help improve TQ's bioavailability and delivery to cells, possibly through alleviating its extensive hydrophobicity and plasma protein binding capacity when combined with RJ. The rationale of our study stems from the need to develop a relatively non-toxic treatment with high therapeutic potential and relatively low cytotoxicity against human triple-negative breast cancer compared to the available breast cancer treatments.

Our experimental approach included investigating the effects of each compound alone or their combinations on the aggressive tumorigenic MDA-MB 231 cells and the non-tumorigenic FHs 74 Int small intestinal epithelial cell line. Using several concentrations of RJ ranging from 0.1 to 200 µg, we examined the effects of RJ on cell viability and established the safe doses at which the viability of the cells was not affected. We also established the concentration of TQ, at which 50% of the breast cancer cells were viable. Identification of TQ's IC_{50} value is crucial because a possible potentiated anti-cancer effect (i.e., synergistic or additive effects) between TQ and RJ is best detected at concentrations that are not highly cytotoxic to cells (i.e., less than 50% cell death). Furthermore, we investigated drug effects at the cellular level by determining whether each drug alone or their combinations induced apoptotic cell death.

Our study is the first to investigate the anti-cancer activity of a novel combination of TQ and RJ against aggressive human breast cancer cells. This project could result in marketing combinations of natural, relatively non-toxic compounds to treat breast cancer. It could also provide the cancer biology and therapy field with a

novel and effective drug combination that would serve the pharmaceutical industry and promote the clinical application of TQ as a potential therapeutic compound against triple-negative breast cancer patients in the coming future. The bio-guided chemical fractionation of RJ could lead to identifying the bioactive compounds that are responsible for enhancing TQ's anti-cancer effects.

CHAPTER II

MATERIALS & METHODS

A. Materials

MDA-MB-231 human breast cancer and FHs 74 Int human small intestinal cell lines were purchased from ATCC (Manassas, Virginia, USA). Dulbecco's Modified Eagle Medium (DMEM) and DMEM-F12 cell culture media were purchased from Lonza (Verviers, Belgium). Thymoquinone, trypsin–EDTA, Dulbecco's phosphate-buffered saline (PBS), horse serum, fetal bovine serum (FBS), penicillin-streptomycin (P/S), dimethyl sulfoxide (DMSO), MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypan blue and methanol were purchased from Sigma Aldrich (St Louis, Missouri, USA). Insulin used for FHs 74 Int cell line culture (Actrapid 100IU/ml) was purchased from the pharmacy at the American University of Beirut Medical Center. DAPI (4′,6-diamidino-2-phenylindole) stain was purchased from Abcam (Cambridge, UK). Rabbit Caspase-3 polyclonal antibody (9662) was -purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Goat anti-rabbit polyclonal secondary antibody, Alexa Fluor 568 (A11011) were purchased from Invitrogen, Thermo Fisher Scientific (California, USA). Rabbit Ki67 monoclonal primary antibody (Cell Marque 275R-15) and donkey anti-rabbit Cy3 secondary antibody (Jackson 711-165-152) were provided by Dr. Noel Ghanem lab at the Biology Department of the American University of Beirut. Crude royal jelly was purchased from the bee farm at Rashaya al-Wadi, Lebanon, located at 1200-1600m above the sea level. A variety of seasonal plants predominate at this altitude and contribute to the diet of

bees, among which are Brassicaceae (Nasturtium), Anacardiaceae (Rhus), Ulmaceae (Ulmus), Rosaceae (Rosa) and Apiaceae (Eryngium). Royal jelly was collected during summer season and stored at -20ºC.

B. Cell culture conditions

MDA-MB-231 and FHs 74 Int cell lines were cultured in their respective media in 2D monolayer conditions. FHs 74 Int cells were grown in DMEM cell culture media supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin (P/S with penicillin at 10,000 units and streptomycin at 10 mg/ml) and 10 µg/ml insulin. MDA-MB-231 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin and streptomycin. All cells were maintained in a humidified atmosphere of 5% CO2 at 37ºC.

C. Drug preparation and treatment

Directly before use, fresh stock of the purified synthetic compound Thymoquinone was prepared by dissolving 16.4 mg/mL of TQ crystals in 1 mL methanol. TQ stock was then diluted in respective media to obtain different TQ concentrations ranging from 1 to 100 µM used in cell treatment. RJ fresh stock was prepared by dissolving 20 mg in 200 µl DMSO mixed with 800 µl distilled water solution at 37ºC for 30 min. Intermediate concentrations of RJ ranging between 0.01 and 200 µg/ml were then prepared by serial dilutions from stock and used in cell treatment. In all experiments, treatment with TQ, RJ each alone or in combinations, was performed at 50% cell confluency. Treatment with TQ-RJ combination was done by

adding TQ and RJ, each alone in wells containing their respective media and incubating cells with this mixture at different concentrations for 24h.

D. MTT cell viability assay

Metabolically active cells can reduce the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye, a yellow tetrazole, into an insoluble formazan product of purple color. MTT assay was used to determine the inhibitory effect of TQ on the viability of the MDA-MB-231 breast cancer cell line. Cells were seeded in 96-well plates at a density of 10,000 cells/ well, then treated for 24 h after which the medium was removed, and the cells were incubated with 120 µl of MTT solution (5 mg/ml prepared in 1 x PBS) for 3 h at 37°C. Afterward, the solution containing the MTT dye was removed and replaced by 100 µl isopropanol to dissolve the formazan crystal. MTT optical density (OD) was then measured using a microplate reader enzyme-linked immuno-sorbent assay (ELISA) at 595 nm. Cellular viability was expressed as a percentage of metabolically active cells in treated conditions relative to control. Cell viability was reported as an average of 3 independent experiments, each condition in sextuplicate.

E. Trypan blue exclusion assay

The viability of both FHs 74 Int and MDA-MB-231 cells in response to the different drug treatments was determined using the trypan blue exclusion assay. FHs 74 Int cells were seeded in 24-well-plates at a density of 70,000 cells/well while MDA-MB-231 were seeded in 12-well plates at a density of 20,000 cells/well. A previous study in our lab showed that TQ was not toxic to FHs 74 Int human intestinal cells (El-Najjar et al., 2010), so we only investigated RJ toxicity in these cells. MDA-MB-231

cells were treated with RJ and TQ, each alone and in combinations. Following treatment of cells for 24 h, alive and dead cells were collected. Samples were centrifuged at 1300 rpm for 5 min. Then, pellets were resuspended in DMEM growth medium, and trypan blue was added to the cell suspension in a 1:1 ratio. Next, cells were counted using a hemocytometer under the Axiovert inverted microscope at 10x magnification. Cells stained blue were counted as dead, and results were expressed as a percentage of total cells. Cell viability was reported as an average of 3 independent experiments, each condition in duplicates.

F. Combination index analysis

The interaction between TQ and RJ was assessed using the Chou-Talalay plot (Fa-CI plot) was used (Chou and Talalay, 1984). Combination indices (CI) were calculated from the mean affected fraction (Fa) at each drug combination using CompuSyn software (CompuSyn, Inc. Paramus, NJ, USA). CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively.

G. Cell cycle analysis

MDA-MD-231 cells were seeded in 6-well plates at a density of 80,000 cells/well. Cells were treated with 0.1 µg/ml RJ and 15 µM TQ each alone. After 24 h of treatment, the cells were collected and washed twice with 1 x PBS, fixed in 70% ice-cold ethanol, and stored at −20°C for at least one day. Subsequently, cells were washed twice with 1 x PBS, incubated for 30 min at 37°C with 100 µl of propidium iodide solution (6 µl RNase, and 30 µl PI (1 mg/ml)/1 ml 1 x PBS). Supernatants were then transferred to flow tubes with 200 µl 1 x PBS added. Cell cycle analysis was performed using the FACS scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and

the Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to analyze the distribution of cells in the different phases of the cell cycle.

H. Immunofluorescence assay

MDA-MB-231 cells were plated on coverslips in 12-well plates at a density of 60,000 cells/well. The medium was then removed, and the cells were treated with either TQ, RJ, or combinations. After treatment, the cells were washed twice with 1 x PBS and fixed at room temperature for 20 min in 4% formaldehyde. The formaldehyde was then removed, and the cells were washed three times in 1 x PBS (10 min/wash) before permeabilization in 0.5% Triton solution for 5 min. After two successive 10 min washes in 1 x PBS, cells were blocked in blocking buffer with FBS for 1 h at room temperature. Apoptosis was assessed using the Caspase-3 antibody, which was subsequently diluted (1:500) in 3% BSA and incubated separately with the cells overnight at 4ºC. The primary antibody was removed the next day, and the cells were washed three times in 1 x PBS supplemented with 0.1% Tween 20 before incubation for 1 h with goat anti-rabbit secondary antibody diluted (1:200) in 3% BSA at room temperature. Finally, the secondary antibody was removed, and the cells were washed three times in 1 x PBS with 0.1% Tween 20 before staining the nuclei with DAPI and mounting on a glass slide. To evaluate cell proliferation, the same immunostaining protocol was followed for Ki67 immunofluorescence with minor modifications, including the preparation of Ki67 primary antibody and donkey anti-rabbit Cy3 secondary antibody solutions in blocking buffer with donkey serum at dilution 1:500 and 1:200 ratios, respectively. Also, cells were washed three times in 1 x PBS only after the removal of the primary and secondary antibodies.

Imaging and visualization were performed using the microscope Zeiss Axio. For cleaved Caspase, an equal number of representative images were taken for each slide in all conditions, and the percentage of apoptotic cells expressing cleaved Caspase 3 was then calculated. As for Ki67 biomarker, an equal number of representative images were taken for each slide per condition, and immunofluorescence intensity was measured by ZEN lite Digital Imaging Software to evaluate cell proliferation.

I. Statistical analysis

Unless otherwise stated, data are presented as mean ± standard error of the mean (SEM) of three independent experiments with statistical analysis performed by One Way Anova (non-parametric) Multiple Comparison Test on Graph Pad Prism V.7. Software. Statistical significance was set with a 95% confidence interval at $p < 0.05$.

J. Imaging

Cells were visualized and imaged by Axiovert inverted microscope from Zeiss at 10x magnification. Confocal images were taken on Confocal Microscope Zeiss LSM710 at 40X oil immersion magnification.

CHAPTER III

RESULTS

A. Royal jelly dissolution protocol

In order to examine RJ's anti-cancer effects alone and in combination with TQ *in vitro*, we first had to dissolve the fresh RJ supplied to our lab in the form of a solid extract from the bee farm at Rashaya al-Wadi, Lebanon. RJ is known to be a lipid-rich compound, so we attempted to dissolve it in distilled water using high temperatures. Good dissolution was obtained by gradually adding 2 ml of distilled water (dH_2O) to 100 mg RJ along with continuous heating for up to 65ºC for 45 min. Although this method yielded acceptable results in terms of clarity of the solution, subjecting RJ to such high temperatures for a prolonged period could denature its proteins affecting the activity of the extract. We then considered possible solvents that could be used for dissolving RJ since distilled water alone, if not heated, was not the optimal solvent. So, we considered dissolving RJ in 0.9% NaCl, an isotonic solution suitable for the cells when used for treatment. However, incomplete dissolution of RJ was achieved, as evidenced by the translucent turbidity and precipitation of the sample (Figure 7A). Therefore, neither distilled water without heating nor 0.9% NaCl proved to be optimal solvents for dissolving RJ.

Knowing that RJ contains both polar and non-polar compounds, we realized that using distilled water alone as a solvent will not completely dissolve the non-polar fatty acid components in RJ. Thus, we considered mixing the crude RJ with dimethyl sulfoxide (DMSO), a polar aprotic solvent that is miscible with dH_2O and that is capable of dissolving hydrophilic and hydrophobic compounds (Cevallos et al., 2017;

Galvao et al., 2014). Mixing RJ in a solution of 0.9% NaCl and DMSO in (3:1) ratio yielded partial solubility, but a precipitate was obtained (Figure 7B). Next, we used distilled water instead, but in combination with DMSO at concentrations of less than 1% when added on cells to improve RJ dissolution. Different proportions of dH_2O to DMSO were used to determine the ratio that produces the best solubility. Complete solubility was only obtained upon dissolving 20 mg of RJ in a solution of 800 µl dH_2O and 200 µl DMSO solution at 37ºC for 30 min along with vortexing every 10 min. Therefore, this protocol was used to prepare fresh RJ stocks prior to every treatment. Fresh RJ stock was then used to prepare dilutions needed for experiments.

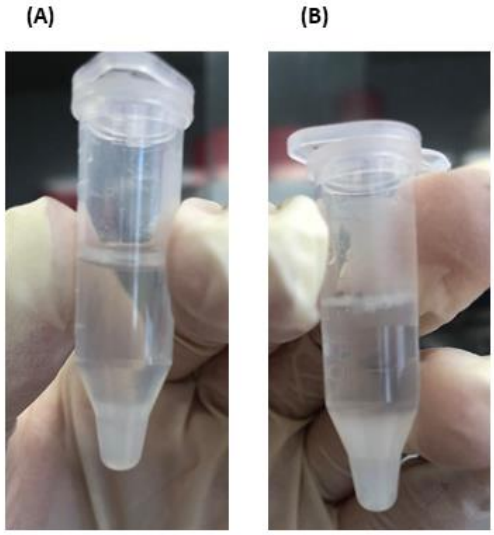


Figure 7. Royal jelly precipitation in 0.9% NaCl alone or in combination with DMSO.
The whitish precipitate is obtained upon dissolving RJ in 1 ml of 0.9% NaCl (A) and in 750 µl of 0.9% NaCl combined with 250 µl DMSO.

B. Royal jelly inhibitory effect on cell viability was dose dependent in non-tumorigenic FHs 74 int human small intestinal cells and metastatic MDA-MB-231 human breast cancer cell line

First, we determined whether the RJ extract exerted toxic effects against FHs 74 Int non-tumorigenic human intestinal cells. Second, we identified the range of inhibitory concentrations of RJ on MDA-MB-231 human breast cancer cells and determined its potential anti-cancer activity. FHs 74 Int and MDA-MB-231 cells were incubated in several concentrations of RJ and the effects on cell viability were determined after 24 h.

Relative to the control, RJ did not exert any significant inhibitory effects on the viability of FHs 74 Int cells at concentrations of 0.01µg/ml and 0.1µg/ml, while a reduction of 12% was obtained upon the treatment with doses of 1 µg/ml and 5 µg/ml. These results confirm the mild inhibitory effect exerted at low doses of RJ on non-tumorigenic cells. However, at higher doses ranging from 10 µg/ml to 200 µg/ml RJ, a more pronounced decrease of 20-24% in cell viability was observed, suggesting that high doses of RJ are toxic to FHs 74 Int non-tumorigenic cells (Figure 8A).

In MDA-MB-231 breast cancer cells, concentrations of RJ ranging from 0.01-1 µg/ml resulted in only a 10-13% decrease in cell viability. However, the viability of MDA-MB-231 cells decreased by 23-33% as RJ doses increased from 5 µg/ml to 100 µg/ml. The decrease in cell viability was most prominent at 200 µg/ml RJ (42% decrease), suggesting the greater toxicity of RJ to breast cancer cells (Figure 8B). Thus, the IC_{50} value was calculated to be 216 µg/ml in MDA-MB-231 cells while it was much higher (292 µg/ml) in the FHs 74 Int cell line.

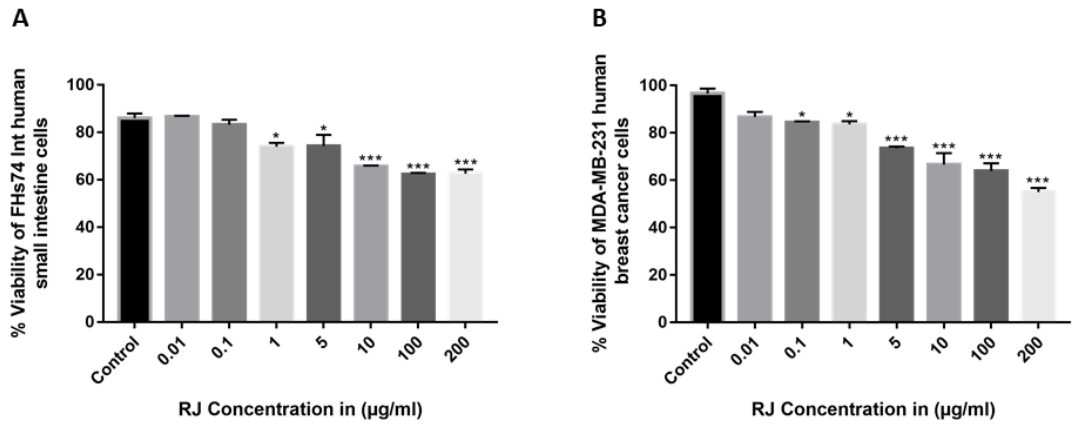


Figure 8. Royal jelly inhibitory effect in FHs 74 Int (A) and MDA-MB-231 (B) cell lines at doses ranging from 0.01 µg/ml to 200 µg/ml.
Trypan blue exclusion assay showing the percentage cell viability after 24 h of treatment with different RJ concentrations. Data shown are an average of 2 and 3 independent experiments for panels A and B, respectively expressed as mean ± SEM.

Based on these results showing that RJ doses below 1 µg/ml did not significantly affect cell viability of non-tumorigenic cell line in comparison to the control, and the fact that RJ doses higher the 5 µg/ml yielded significant toxicity, we used doses of RJ of 0.1 µg/ml as a minimum and 5 µg/ml as a maximum for all future experiments.

Using MTT assay, we then evaluated the effect of RJ at different concentrations on the viability of MDA-MB-231 cells. Surprisingly, cell viability increased by 10% in response to 0.1µg/ml RJ dose and a minimal decrease of 2% was observed upon the treatment with a high dose of 10 µg/ml RJ. Also, no remarkable cytotoxicity effects were observed even at very high doses of RJ (100 and 200 µg/ml), which resulted in only 1% and 5% decrease in MDA-MB-231 cell viability relative to the control (Figure 9B). This lack of cell toxicity by MTT assay did not correlate with microscopic images of cells that showed a very low number of viable cells in the presence of high concentrations of RJ (Figure 9A). These results indicated that RJ was interfering with the colorimetric absorbance measures of the MTT assay. Therefore, in future

experiments, we used the trypan blue exclusion assay in assessing cell viability in response to the treatment with RJ in combination with TQ.

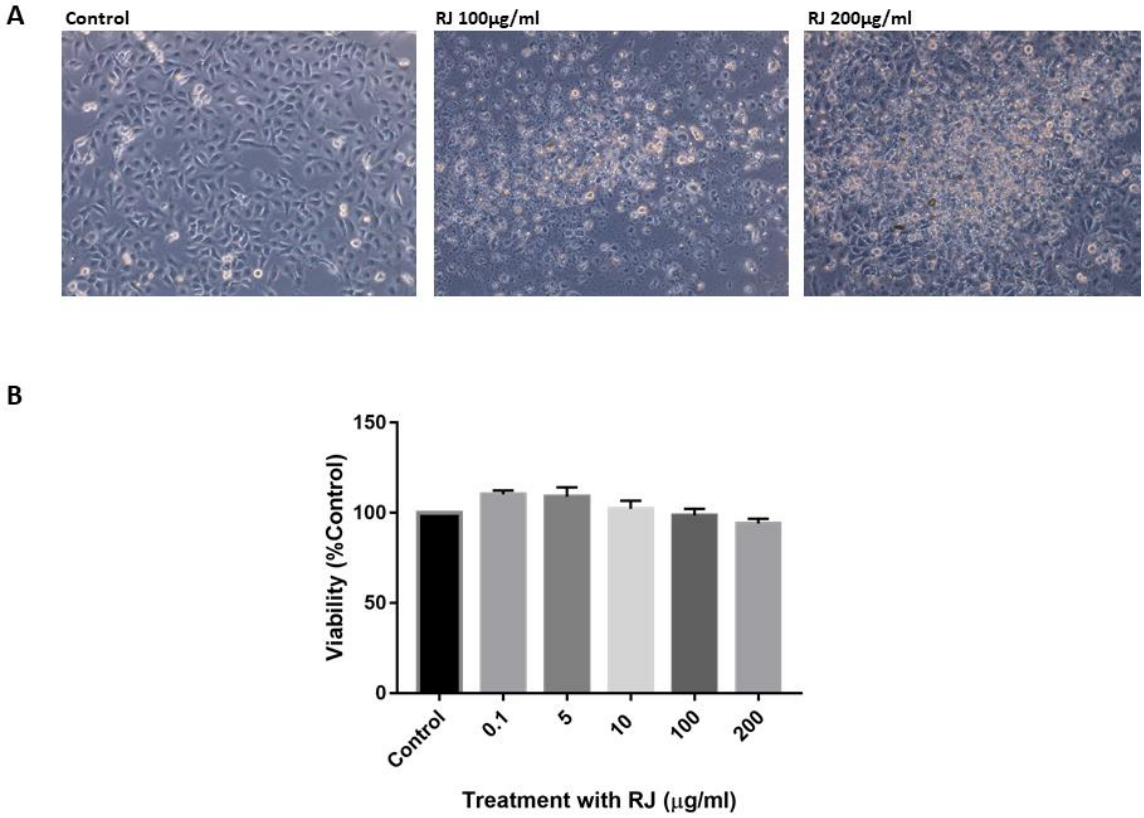


Figure 9. The absence of RJ inhibitory effect on MDA-MB-231 cells at high doses by MTT assay is evidence of interference of RJ with this colorimetric assay.
(A) Representative light microscopy images of MDA-MB-231 viability in response to RJ treatments. Cells were visualized by Axiovert inverted microscope from Zeiss at 10x magnification. (B) MTT assay showing the percentage viability of MDA-MB-231 cells after 24 h of treatment with different RJ concentrations. Cell viability was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data shown are an average of 1 experiment and are expressed as mean ± SEM.

C. Thymoquinone exhibited anti-cancer activity in MDA-MB-231 cell line in a dose dependent manner by inducing cell death

To investigate the anti-cancer effects of TQ in human metastatic breast cancer, we first determined the range of inhibitory concentrations of TQ in MDA-MB-231 breast cancer cell line. We tested different doses of TQ, ranging from 1 µM to 100 µM, to establish TQ concentrations that could significantly affect the viability of breast cancer cells. After establishing the range of TQ inhibitory concentrations, we determined the IC_{50} value of TQ. Establishing IC_{50} is important since any possible anti-cancer synergy (or additive effects) is best detected at concentrations that are not highly cytotoxic to cells (i.e., less than 50% cell death).

Thymoquinone at concentrations of 1 µM and 10 µM did not exert any statistically significant toxicity on the MDA-MB-231 cell line relative to the control (Figure 10). As TQ concentrations increased, cell viability started to decrease remarkably, reaching 47% at 20 µM. Hence, nearly half of breast cancer cells seeded had died, suggesting that the IC_{50} value was slightly less than 20 µM. The decline in cell viability was more pronounced with increasing TQ concentrations yielding 80%, 90%, and 92% reduction upon the treatment with 30 µM, 50 µM, and 100 µM of TQ, respectively (Figure 10). Thus, TQ exhibited significant anti-cancer activity against MDA-MB-231 human breast cancer cells in a dose dependent manner.

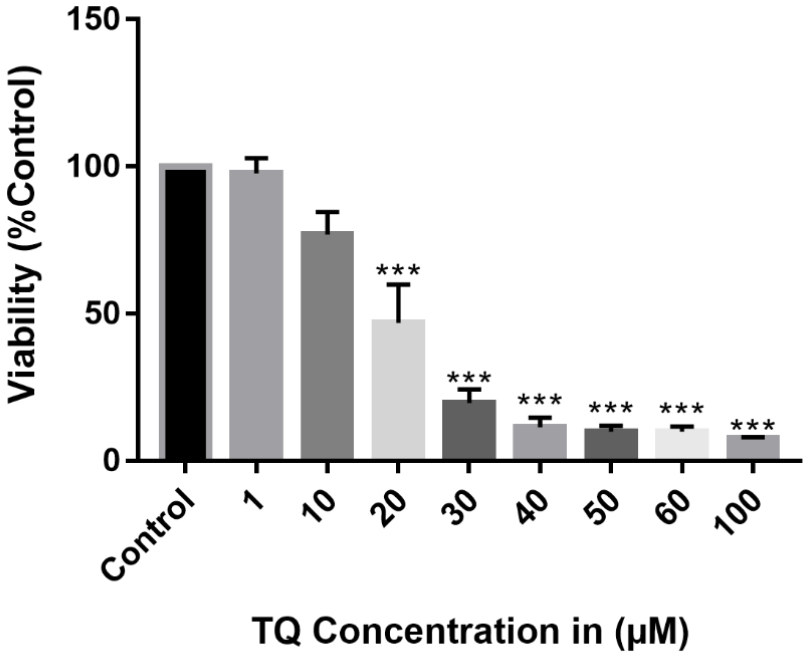


Figure 10. The effect of TQ on the viability of MDA-MB-231 cell line.
MTT assay showing the percentage viability of MDA-MB-231 cell line after 24 h of treatment with different TQ concentrations. Cell viability was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data shown are an average of 3 independent experiments expressed as mean ± SEM. Asterisks represent statistically significant results compared to the control, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Next, we determined the exact value of IC_{50} using a narrower scale of TQ concentrations ranging between 12.5 µM and 30 µM. Relative to the control, cell viability decreased significantly to 85% and 81% upon the treatment of MDA-MB-231 cells with 12.5 µM and 15 µM TQ, respectively. However, TQ exerted more significant toxicity at higher concentrations reducing cell viability to 66% at 17.5 µM TQ. As a result of this experiment, the IC_{50} value of TQ was established to be 19 µM (Figure 11).

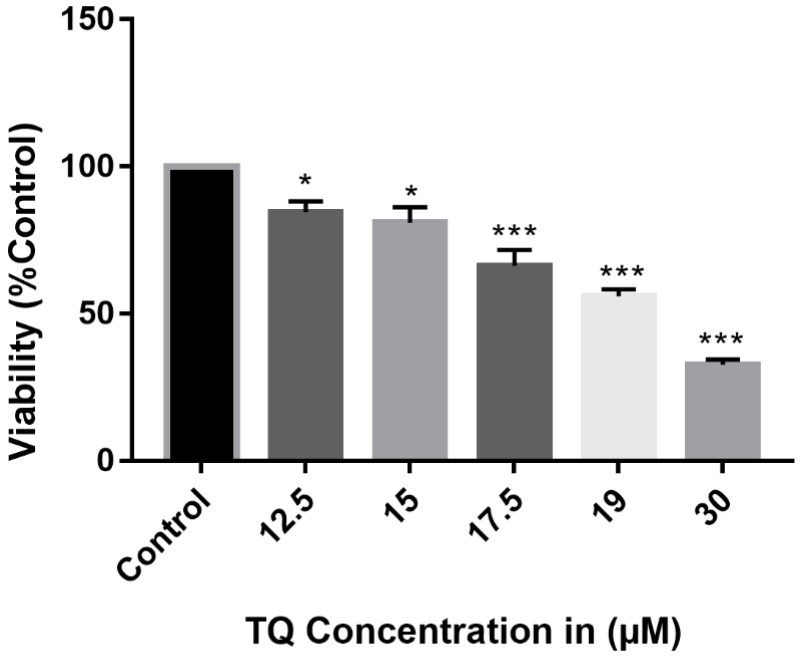


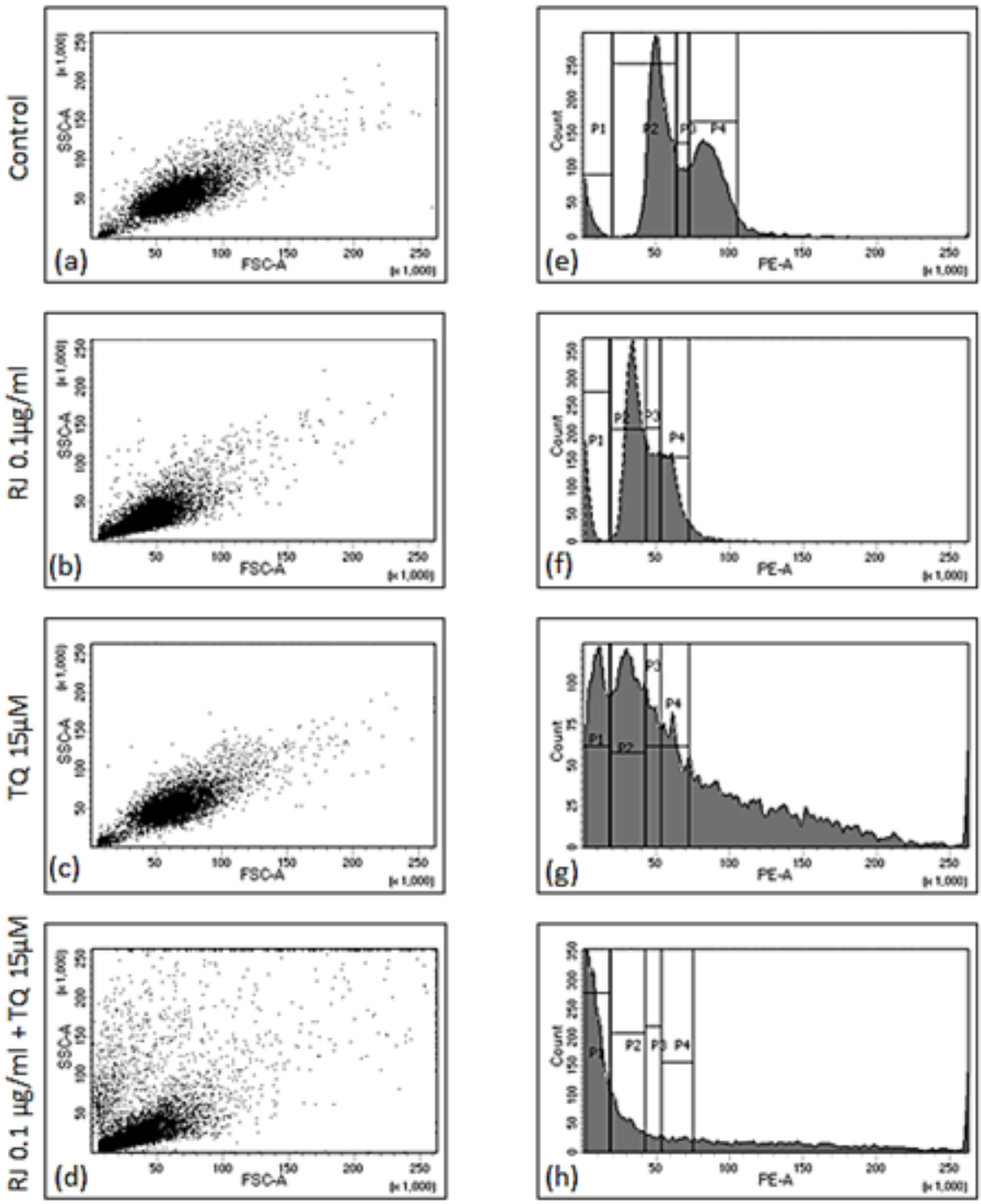
Figure 11. Half-maximal inhibitory concentrations of TQ on MDA-MB-231 human breast cancer cell line after 24 h of treatment.
MTT assay showing the half-maximal inhibitory concentration (IC_{50}) of TQ in MDA-MB-231 breast cancer cell line after 24 h of treatment with different TQ concentrations. Cell viability was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data shown are an average of 3 independent experiments expressed as mean ± SEM. Asterisks represent statistically significant results compared to the control, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

In summary, TQ at concentrations below and above its IC_{50} of 19 µM exerted a prominent anti-cancer effect by inducing cell death in MDA-MB-231 aggressive human breast cancer cells in a dose dependent manner.

D. Thymoquinone and royal jelly combination increases the sub-G1 population in MDA-MB-231 cells

To further confirm cell death and determine whether the inhibition of cell viability by RJ and TQ treatment of MDA-MB-231 cells was associated with changes in cell cycle regulation, we performed cell cycle analysis using propidium iodide (PI) DNA staining with flow cytometry. Cell death was enhanced in response to TQ alone and when TQ was combined with 0.1 µg/ml RJ (Figure 12A). In comparison with the control, the Pre-G1 population increased significantly to 23% and 34% upon the treatment with 15 µM TQ alone and when this dose of TQ was combined with 0.1 µg/ml RJ, respectively (Figure 12B). The increase in the Pre-G1 population was associated with a notable reduction in the G0/G1 and G2/M populations. As mentioned before, the treatment with 0.1 µg/ml RJ alone did not exert significant changes in cell viability (Figure 12 A, B), yet it caused a shift in the cell cycle histogram profile to the left (Figure 12A.f) without affecting the integrity of cells as shown in (Figure 10A.b). This shift could be due to a decreased PI intensity in MDA-MB-231 cells as a result of some form of interference with RJ, and this should be further explored in future experiments.

A


Control
RJ 0.1µg/ml
TQ 15µM
RJ 0.1 µg/ml + TQ 15µM
SSC-A
FSC-A
PE-A
Count
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
P1
P2
P3
P4

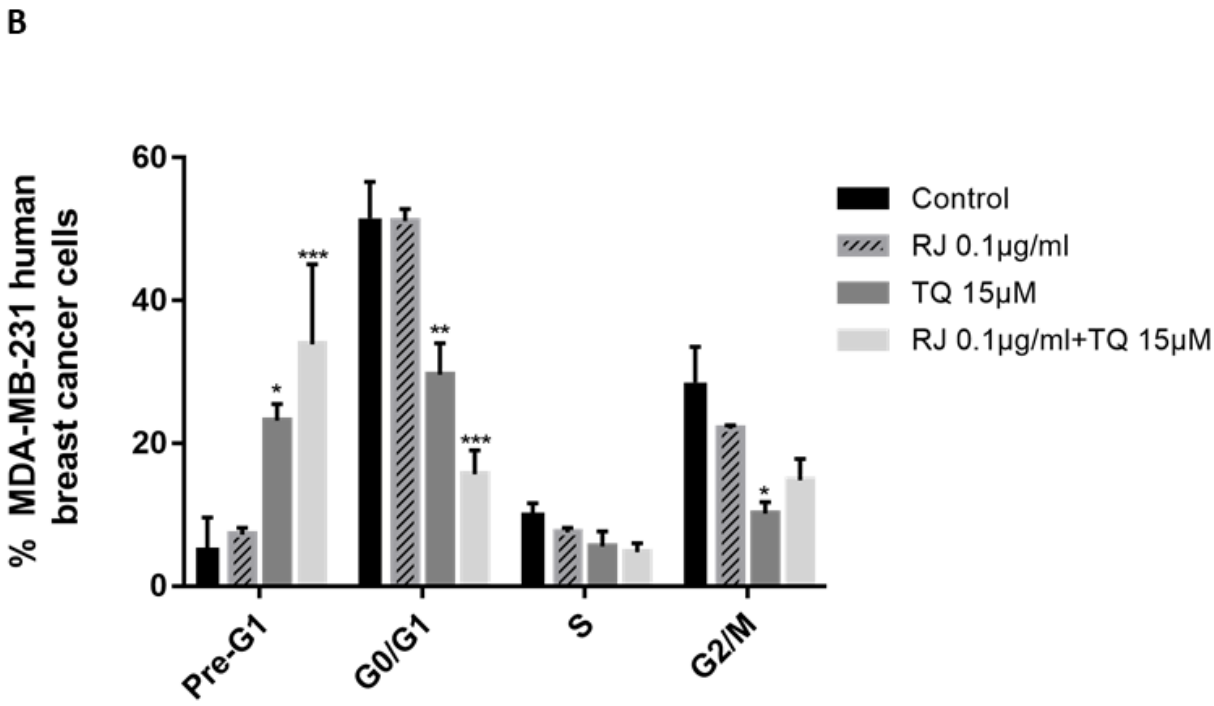


Figure 12. Cell death is enhanced by TQ alone and by the combination TQ and RJ. (Aa-d) Representative density plots showing cell distribution as a function of side scatter area (SSC-A) and forward scatter area (FSC-A). (Ae-h) Representative histograms showing the cell cycle distribution in the control and (B) post-treatment with 15 µM TQ and 0.1µg/ml RJ alone and in combination for 24 h. (C) Propidium iodide staining with flow cytometry showing the increase in Pre G1 upon treatment with TQ alone and the combination of TQ and RJ. Data shown are an average of 3 independent experiments expressed as mean ± SEM and analyzed by a two-way ANOVA test followed by multiple comparisons test. Asterisks represent statistically significant results compared to the control, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

E. The combination of royal jelly and Thymoquinone enhanced inhibition of the viability of MDA-MB-231 breast cancer cells than each compound alone

After establishing the anti-tumor effects of RJ alone and TQ alone against MDA-MB-231 cells, we attempted to study the effects of treatment with the combination of TQ and RJ. Thus, we investigated if there is a potentiated anti-cancer effect on human aggressive breast cancer using combinations of low concentrations of RJ and TQ. RJ alone at a dose of 0.1 µg/ml (2% inhibition) and TQ alone at concentrations of 1 µM (5% inhibition) did not cause any significant inhibition of the viability of MDA-MB-231 breast cancer cells confirming the low toxicity of these treatments. In addition, a decrease of 12% was reported at 5 µM TQ. The combination of 0.1 µg/ml of RJ with 1 µM of TQ did not cause significant reduction in cell viability (7% inhibition) while 0.1 µg/ml of RJ with 5 µM of TQ yielded the most notable decrease of 30% in cell viability in comparison to the control (Figure 13).

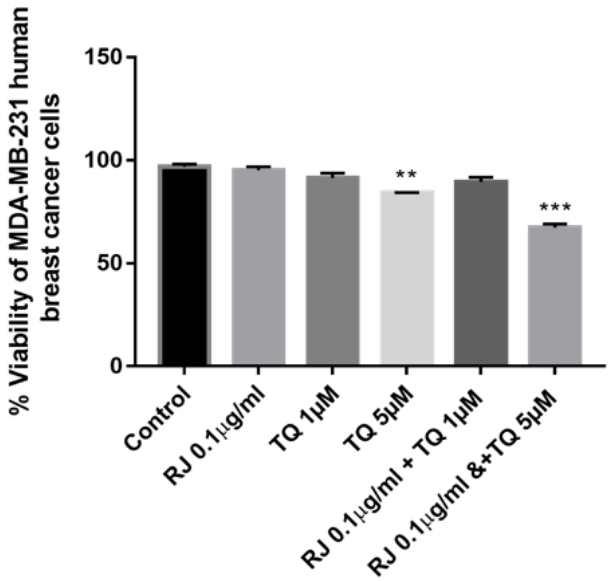
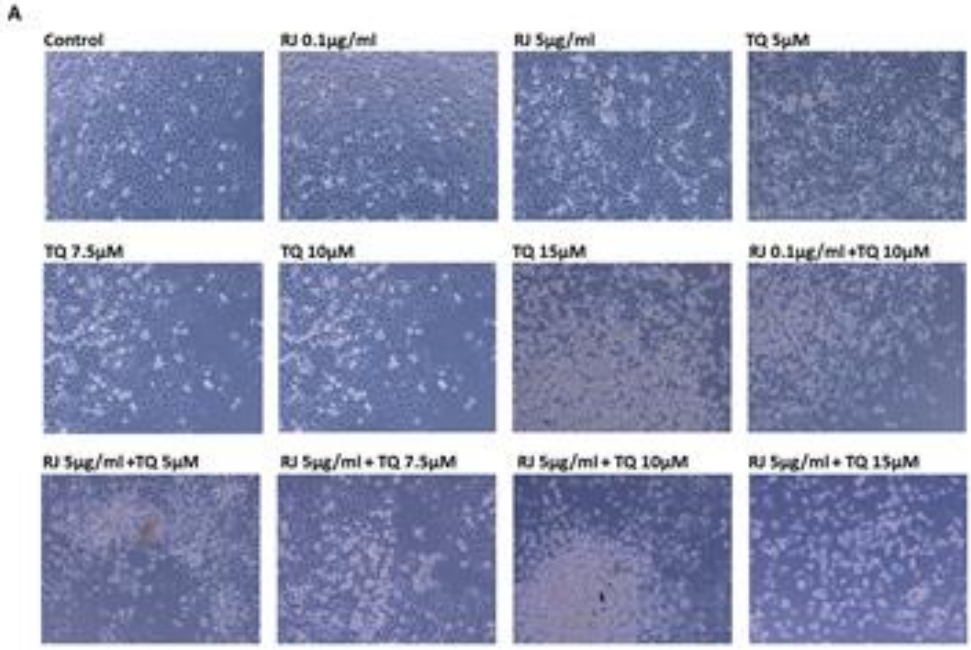
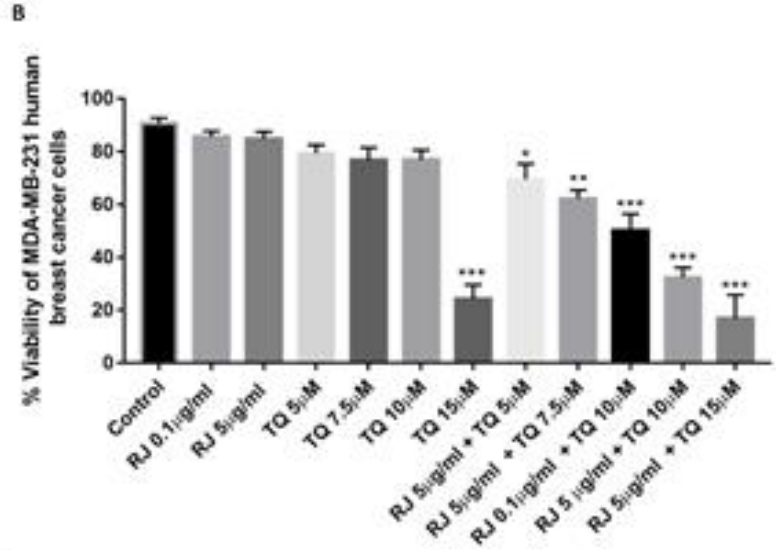


Figure 13. Royal jelly and TQ at low concentrations exerted low to moderate toxicity against MDA-MB-231 human breast cancer cell line.
Trypan blue exclusion assay showing the percentage viability of MDA-MB-231 cell line after 24 h of treatment with different concentrations of RJ and TQ, each alone or in

combination. Data shown are an average of 3 independent experiments expressed as mean ± SEM. Asterisks represent statistically significant results compared to the control, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Next, we determined the effects of the combination of increasing doses of both compounds (RJ and TQ) on cell viability. As shown in (Figure 15B), no significant reduction in MDA-MB-231 viability was detected upon the treatment with RJ alone at doses of 0.1 µg/ml and 5 µg/ml or with TQ alone at doses below 10 µM. However, a significant reduction in cell viability of approximately 66% was observed in response to 15 µM of TQ. In comparison with the control, the viability of MDA-MB-231 cells was significantly reduced in response to drug combinations. Treatment with 5 µg/ml RJ, when combined with 5 µM or 7.5 µM of TQ reduced cell viability by 21% and 29%, respectively. The anti-tumor effects were more pronounced upon treatment with higher TQ doses. A dose of 10 µM TQ in combination with 0.1 µg/ml RJ or 5 µg/ml RJ yielded a significant decrease in MDA-MB-231 cell viability by 40% and 58%, respectively. Treatment with a combination of 10 µM and 15 µM of TQ with 5 µg/ml RJ decreased cell viability by 58% and 74%, respectively confirming the more potent anti-cancer effects upon combination treatment with higher RJ doses (Figure 14A, B).


A
Control
RJ 0.1µg/ml
RJ 5µg/ml
TQ 5µM
TQ 7.5µM
TQ 10µM
TQ 15µM
RJ 0.1µg/ml +TQ 10µM
RJ 5µg/ml +TQ 5µM
RJ 5µg/ml + TQ 7.5µM
RJ 5µg/ml + TQ 10µM
RJ 5µg/ml + TQ 15µM


B
% Viability of MDA-MB-231 human breast cancer cells
100
80
60
40
20
0
Control
RJ 0.1µg/ml
RJ 5µg/ml
TQ 5µM
TQ 7.5µM
TQ 10µM
TQ 15µM
RJ 5µg/ml + TQ 5µM
RJ 5µg/ml + TQ 7.5µM
RJ 0.1µg/ml + TQ 10µM
RJ 5 µg/ml + TQ 10µM
RJ 5µg/ml + TQ 15µM

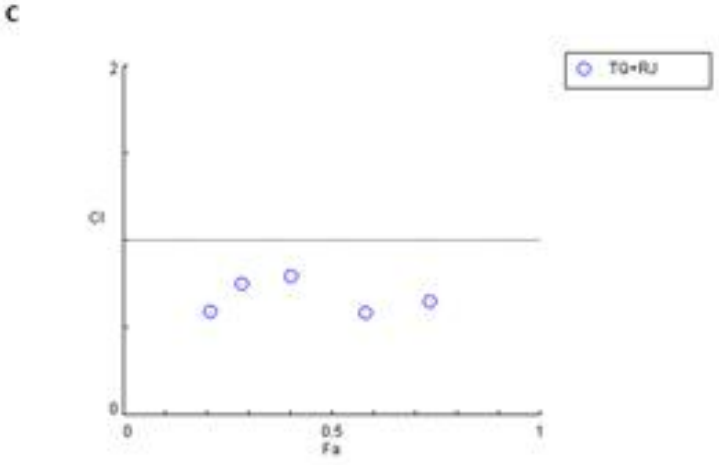

C
TQ+RJ
CI
2
0
0
0.5
1
Fa

Figure 14. Royal jelly and TQ combinations exerted synergistic effects on the viability of MDA-MB-231 human breast cancer cells.
(A) Representative light microscopy images of MDA-MB-231 viability in response to different treatments. Cells were visualized by Axiovert inverted microscope from Zeiss at 10x magnification. (B) Trypan blue exclusion assay showing the percentage viability of MDA-MB-231 cell line after 24 h of treatment with different concentrations of RJ, TQ, and combinations. Data shown are an average of 3 independent experiments expressed as mean ± SEM. Asterisks represent statistically significant results compared to the control, (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (C) Combination index (CI) values were plotted as a function of Fa values corresponding to the % cell death. The dotted line is the reference line, where CI value is equal to 1; circles in blue with a dot at the center represent CI values at different Fa.

Interestingly, the enhanced anti-tumor effect was best observed at combinations of 5 µg/ml RJ with 10 µM TQ. The inhibition of cell viability by this combination treatment (58% inhibition) was greater than the sum of inhibition observed by each compound alone (6% and 12% inhibition by TQ and RJ alone, respectively), suggesting a synergistic effect (Figure 14B). Combination indices were then calculated using CompuSyn software, confirming the synergistic interaction between both compounds in all the combinations tested with a CI value < 1 (Figure 14C). The most pronounced synergistic anti-tumor effect (CI=0.584) was revealed upon the combination of 5 µg/ml RJ with 10 µM TQ (Table 1).

Table 1. Drug doses and combination indices (CI) for TQ and RJ combinations against MDA-MB-231 cell line.
CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively.

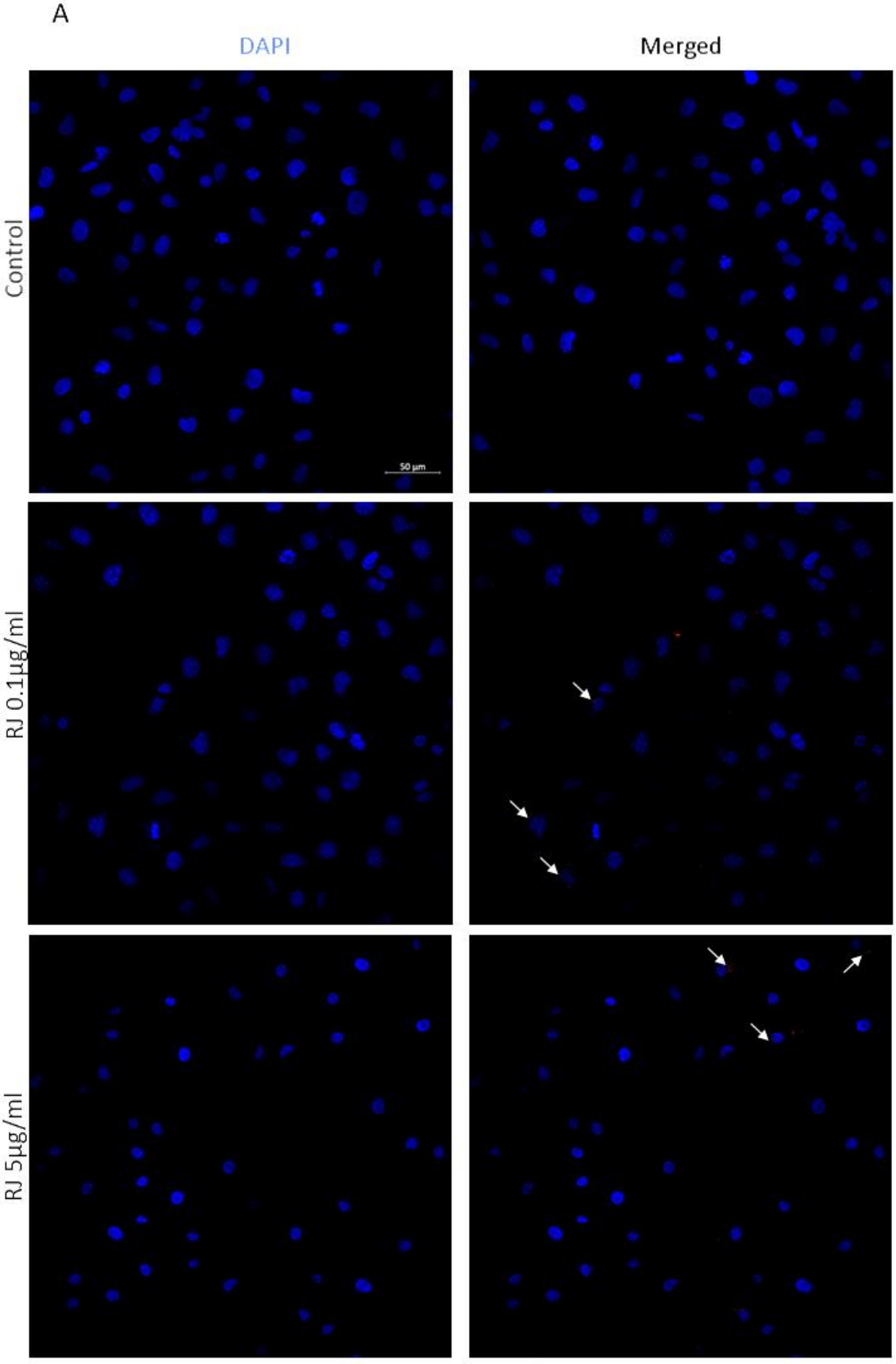
RJ (µg/ml)	TQ (µm)	CI	Interpretation
0.1	10	0.794	Synergism
5	5	0.592	Synergism
5	7.5	0.748	Synergism
5	10	0.584	Synergism
5	15	0.648	Synergism

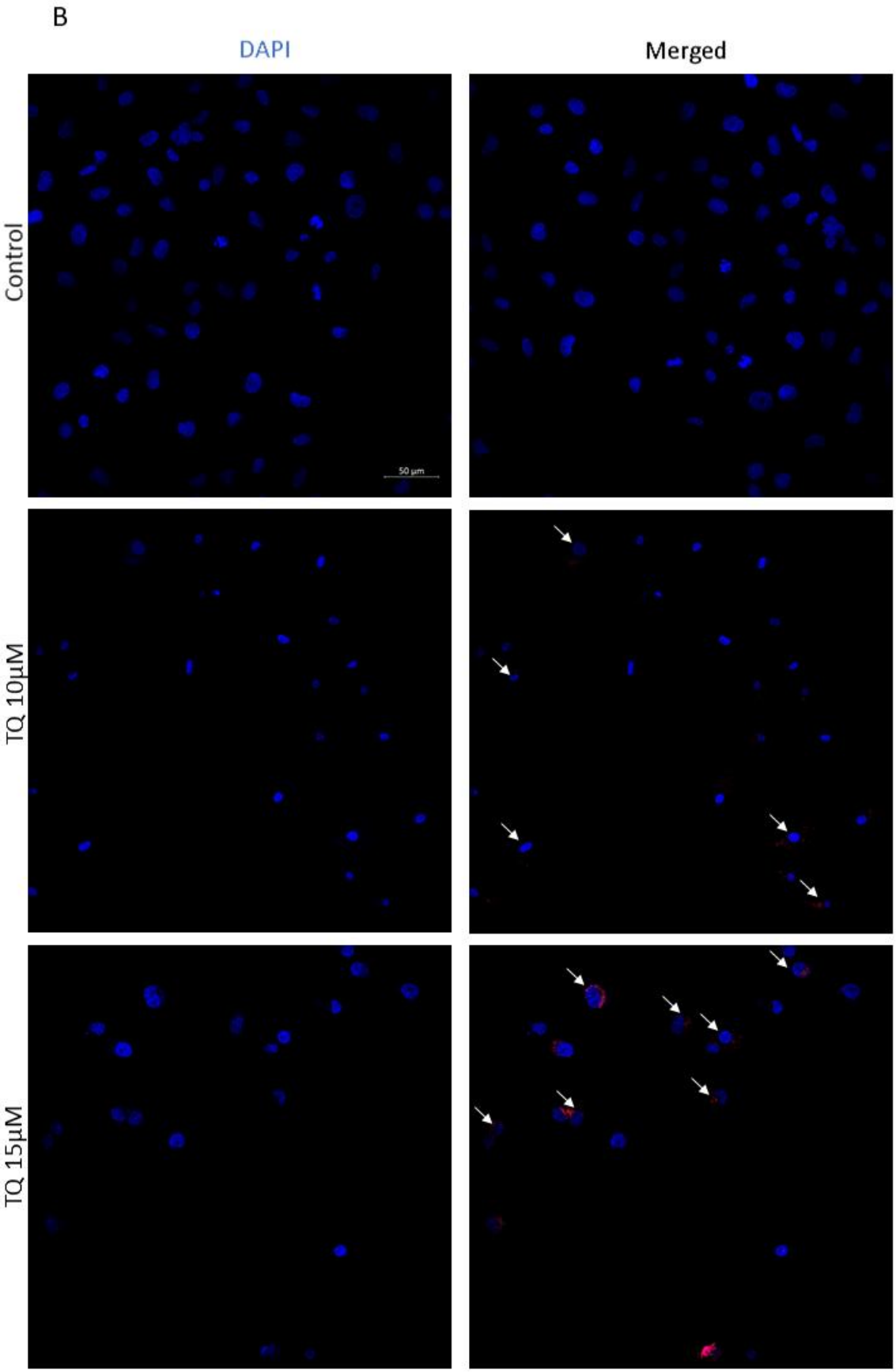
Therefore, RJ and TQ in combination act synergistically to induce dose dependent cell death effects.

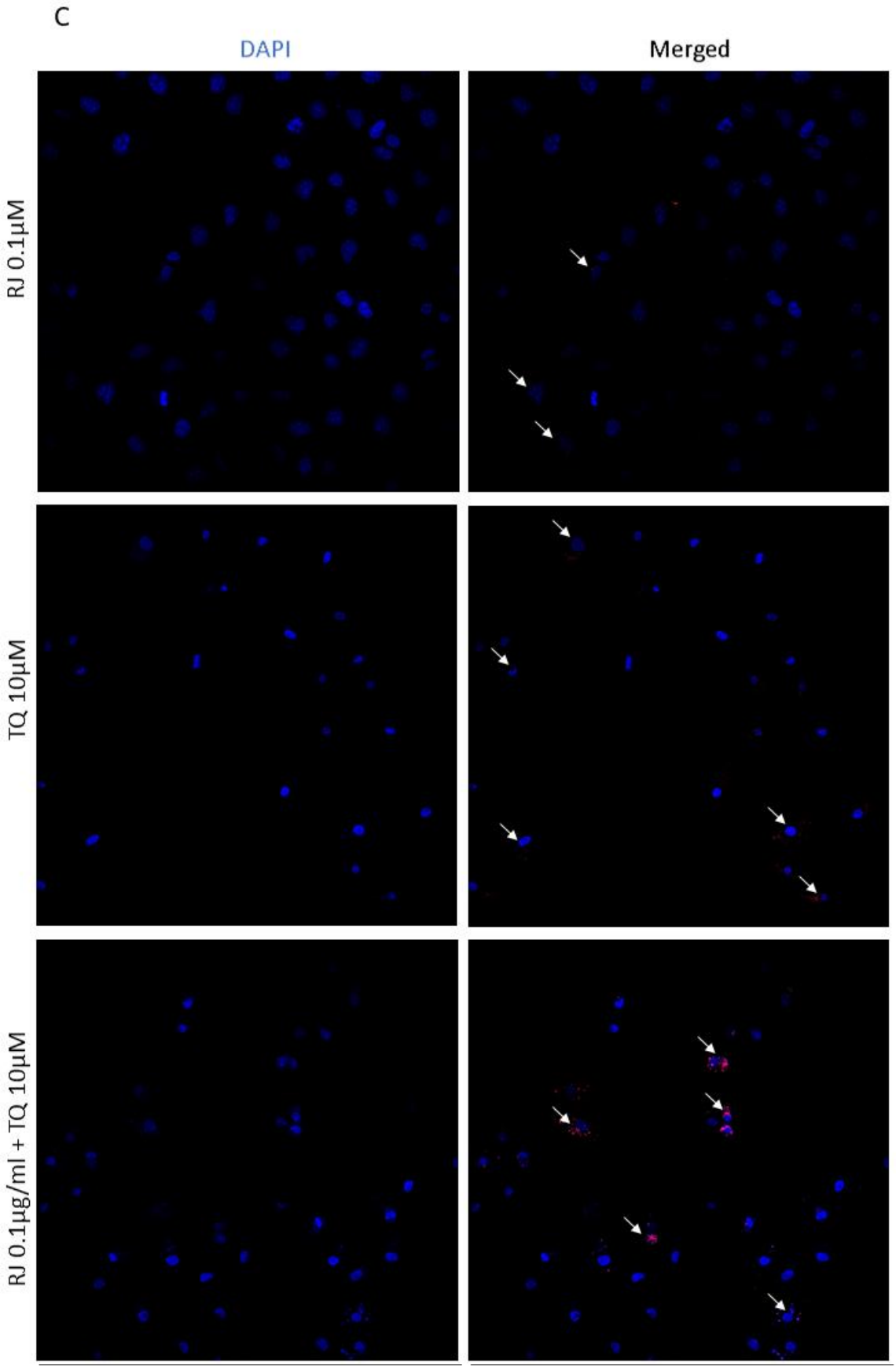
F. The combinations of Thymoquinone and royal jelly induced caspase 3-dependent apoptosis in MDA-MB-231 human breast cancer cells in a dose dependent manner

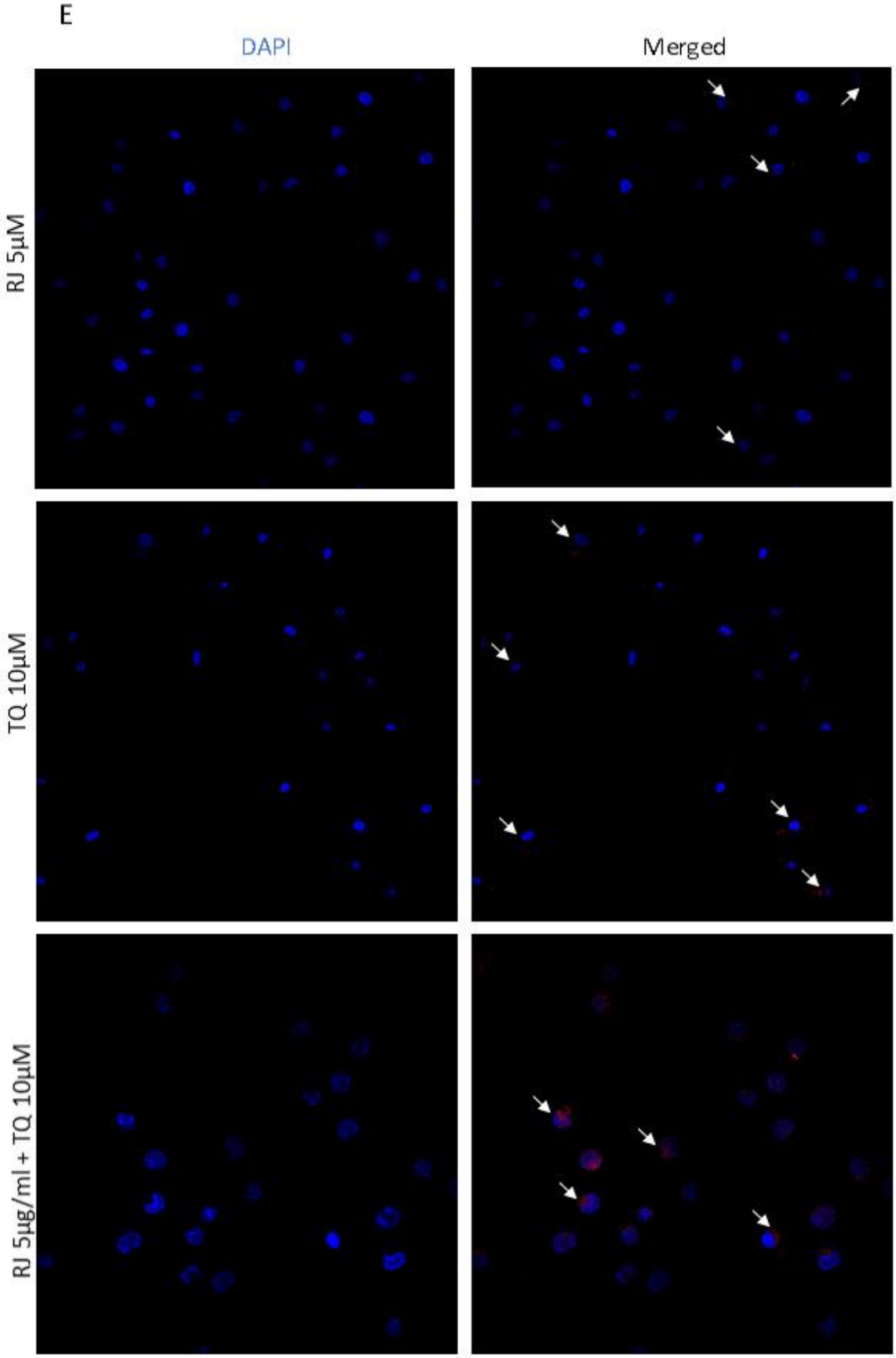
To identify the mechanism of action responsible for the enhanced cell death effect of TQ and RJ combination treatment, we assessed the apoptotic effects of each compound alone and their combinations in MDA-MB-231 cell line by examining the apoptotic bodies in nuclei of cells and by measuring the expression of cleaved caspase 3 in response to 24 h treatment using immunofluorescence staining of active caspase 3.

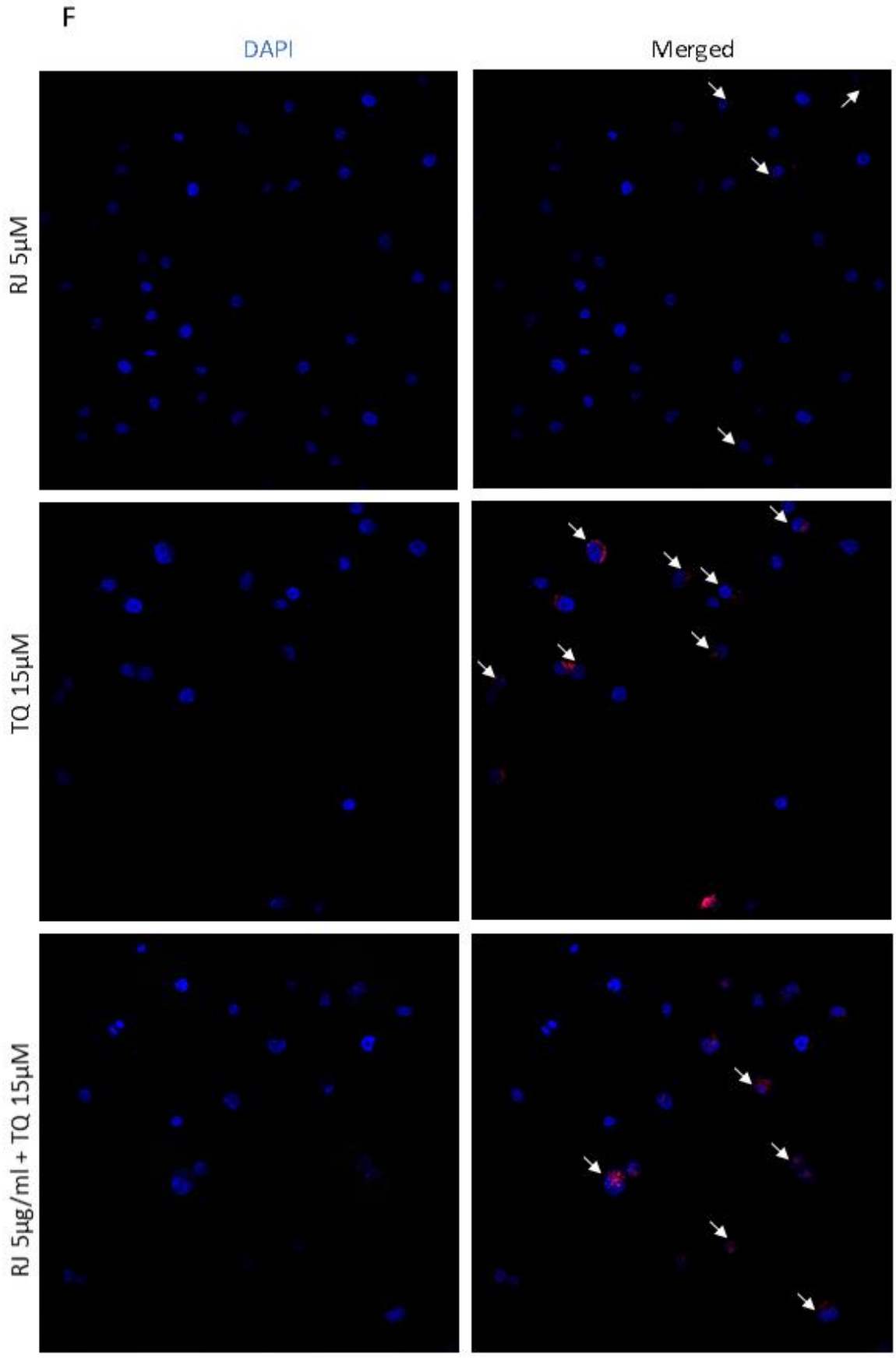
Relative to control, there was no significant increase in apoptosis levels in cells treated with 0.1 µg/ml or 5 µg/ml of RJ. However, active caspase-3 expression was significantly higher in response to treatment with 10 µM and 15 µM TQ alone, yielding a respective increase of 52% and 73% of caspase 3 expression in MDA-MB-231 cells (Figure 15G). Similar results were obtained upon treatment with 0.1 µg/ml RJ in combination with 10 µM and 15 µM TQ with 58% and 75% increase in caspase 3 expression, respectively. A more pronounced apoptotic effect was observed in response to treatment with 5µg/ml RJ in combination with 10 µM and 15 µM TQ as cleaved caspase 3 expression increased to 63% and 77%, respectively. The confocal micrographs confirmed the enhancement of apoptosis in response to the combination treatment, as evidenced by the increase in apoptotic nuclear bodies in MDA-MB-231 cells (Figure 15A-F).


A
DAPI
Merged
Control
RJ 0.1µg/ml
RJ 5µg/ml
50 µm


B
DAPI
Merged
Control
TQ 10µM
TQ 15µM
50 µm


C
DAPI
Merged
RJ 0.1µM
TQ 10µM
RJ 0.1µg/ml + TQ 10µM


E
DAPI
Merged
RJ 5µM
TQ 10µM
RJ 5µg/ml + TQ 10µM


F
DAPI
Merged
RJ 5µM
TQ 15µM
RJ 5µg/ml + TQ 15µM

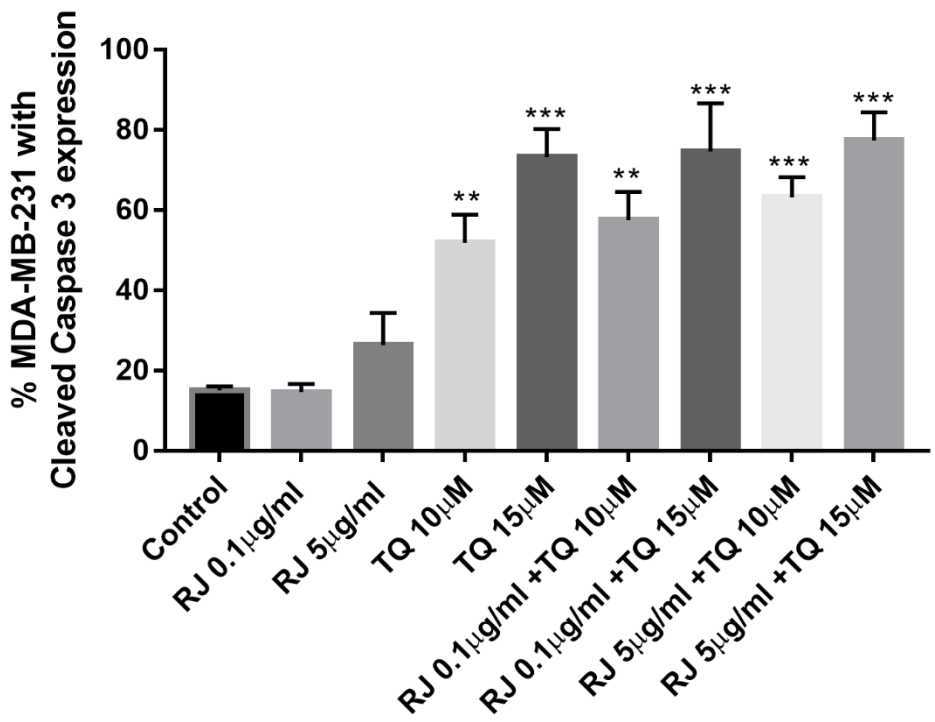
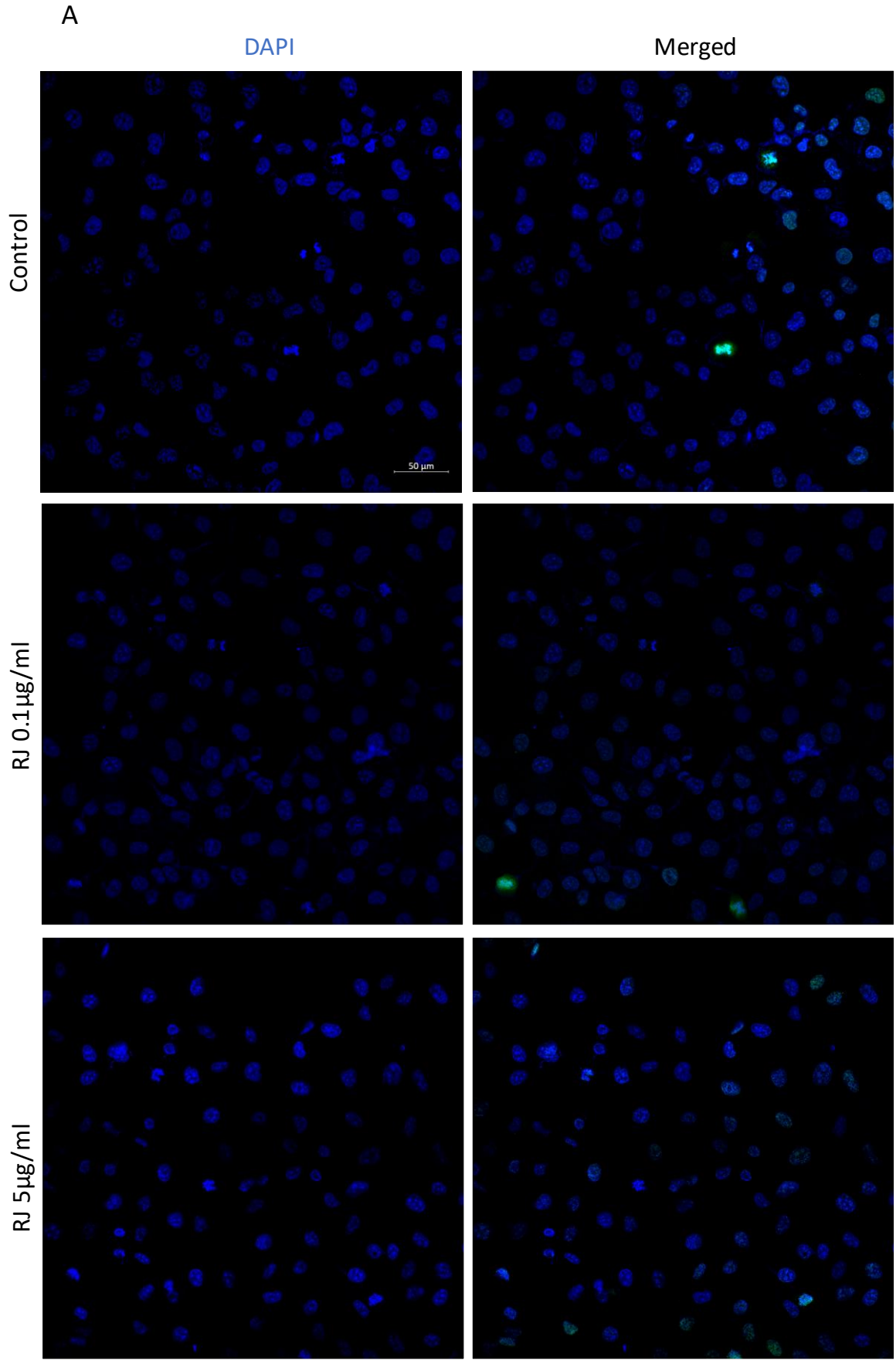


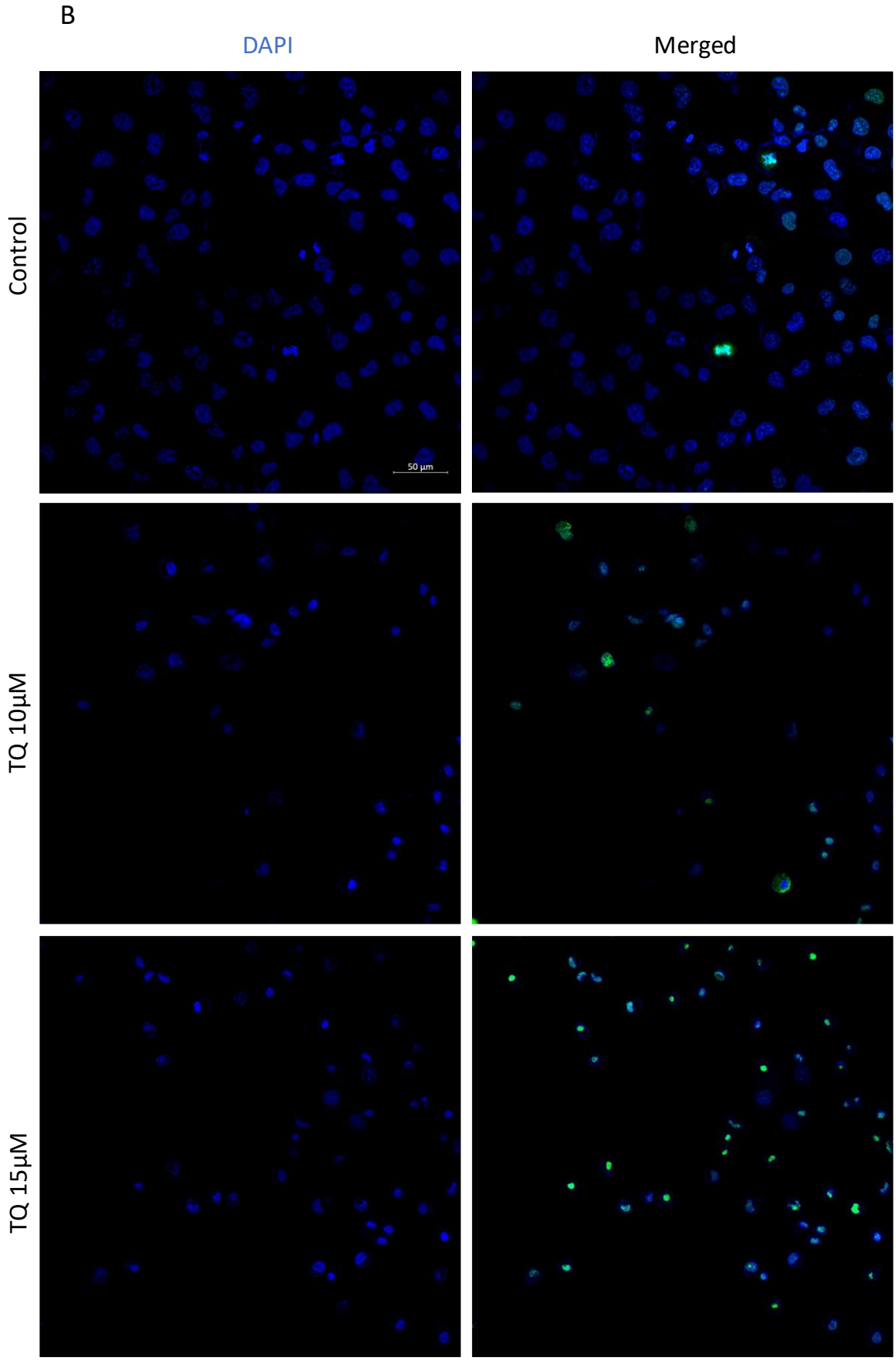
Figure 15. Royal jelly and TQ combinations induce Caspase 3 cleavage in MDA-MB-231 human breast cancer cell line.
(A-F) Immunofluorescence micrographs of cleaved caspase 3 expression at 24 h after treatment. Red indicates cleaved caspase-3 expression and blue indicates nuclei counter stained by DAPI. Arrows indicate apoptotic nuclei. Nuclei were visualized by confocal Zeiss Axio microscope, 40X oil immersion with scale bar = 50 μM. (G) Quantification of cleaved caspase 3 in MDA-MB-231 cells at 24 h of treatment with different concentrations of RJ, TQ, and RJ-TQ combinations. Data shown are an average of 3 independent experiments expressed as mean ± SEM. Asterisks represent statistically significant results, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

In summary, we showed that TQ alone exerted a more pronounced apoptotic effect than RJ alone in MDA-MB-231 human breast cancer cells, and the cell death effect was potentiated when both compounds were combined.

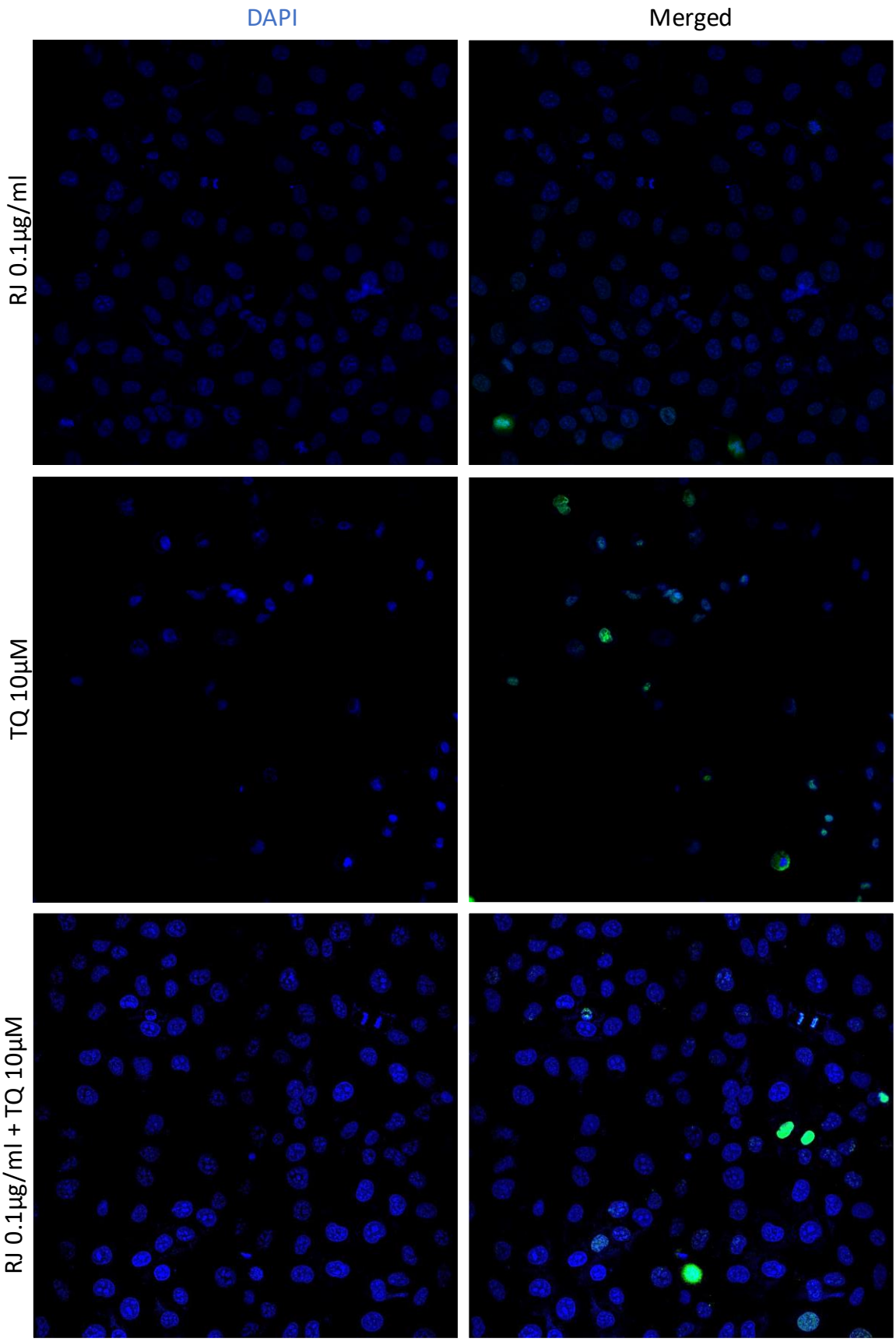
G. Royal jelly and Thymoquinone or combinations did not alter Ki67 expression in MDA-MB-231 human breast cancer cell line

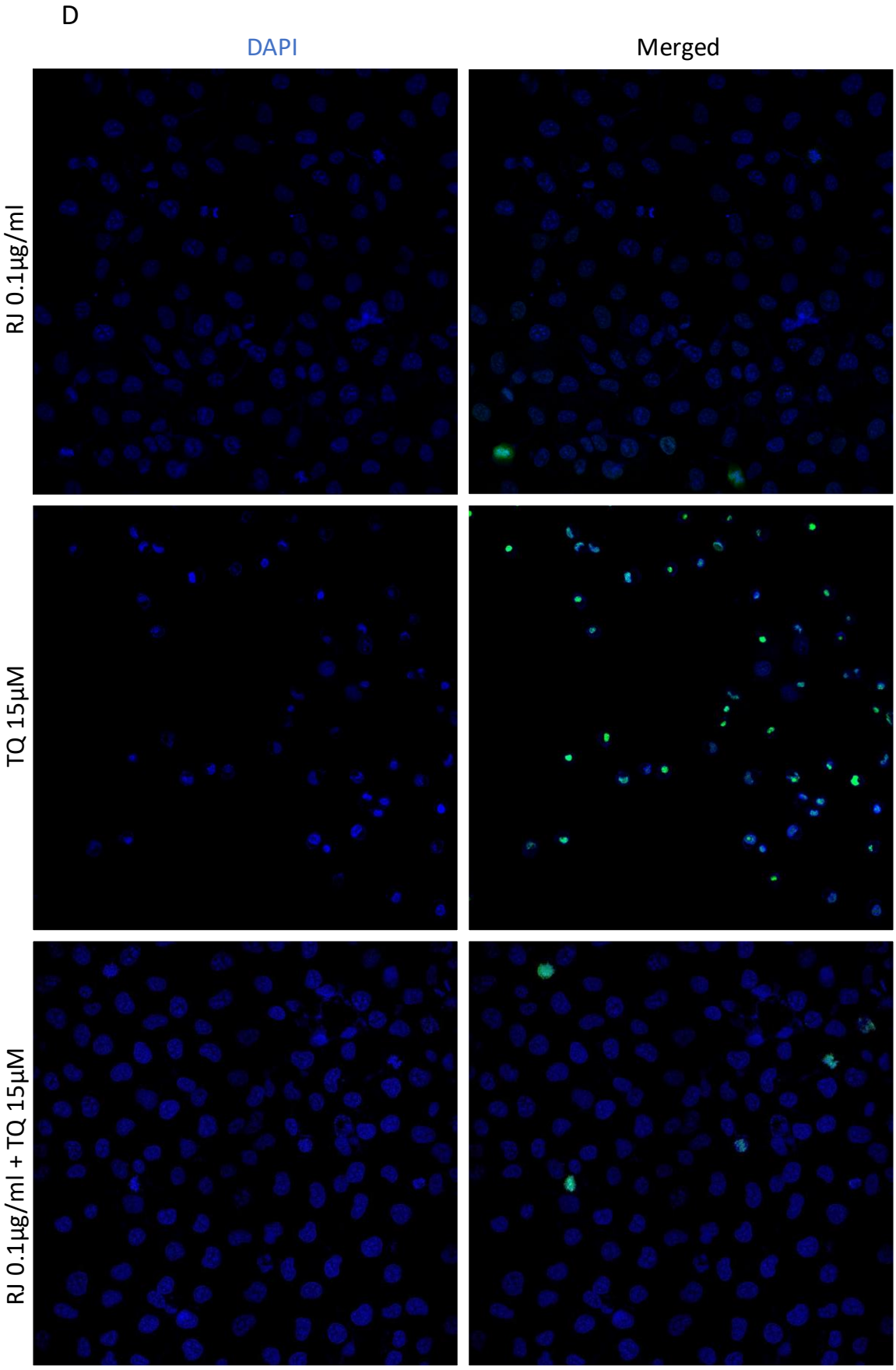
After identifying that apoptosis is the cell death mechanism when combining TQ and RJ, we investigated whether the proliferation of cells was affected in response to TQ alone, RJ alone, and combinations. Cell proliferation was evaluated by measuring the intensity of Ki67 fluorescence, a sensitive and specific proliferation biomarker after 24 h of treatment. Immunofluorescence staining showed a modest decrease of 6% and 10% in Ki67 expression upon treatment with 0.1 µg/ml and 5 µg/ml RJ, respectively, in comparison to the control (Figure 16G). A similar response was observed upon the treatment with 10 and 15 µM TQ alone, causing Ki67 intensity to drop by 7% under both conditions. Moreover, a minimal decrease in Ki67 expression (6-9%) was observed in response to all doses of RJ and TQ combinations as compared to the control. This relatively low anti-proliferative effect was confirmed by confocal imaging showing the modest change in the nuclear expression of Ki67 in response to the different treatments in MDA-MB-231 cells (Figure 16A-F).

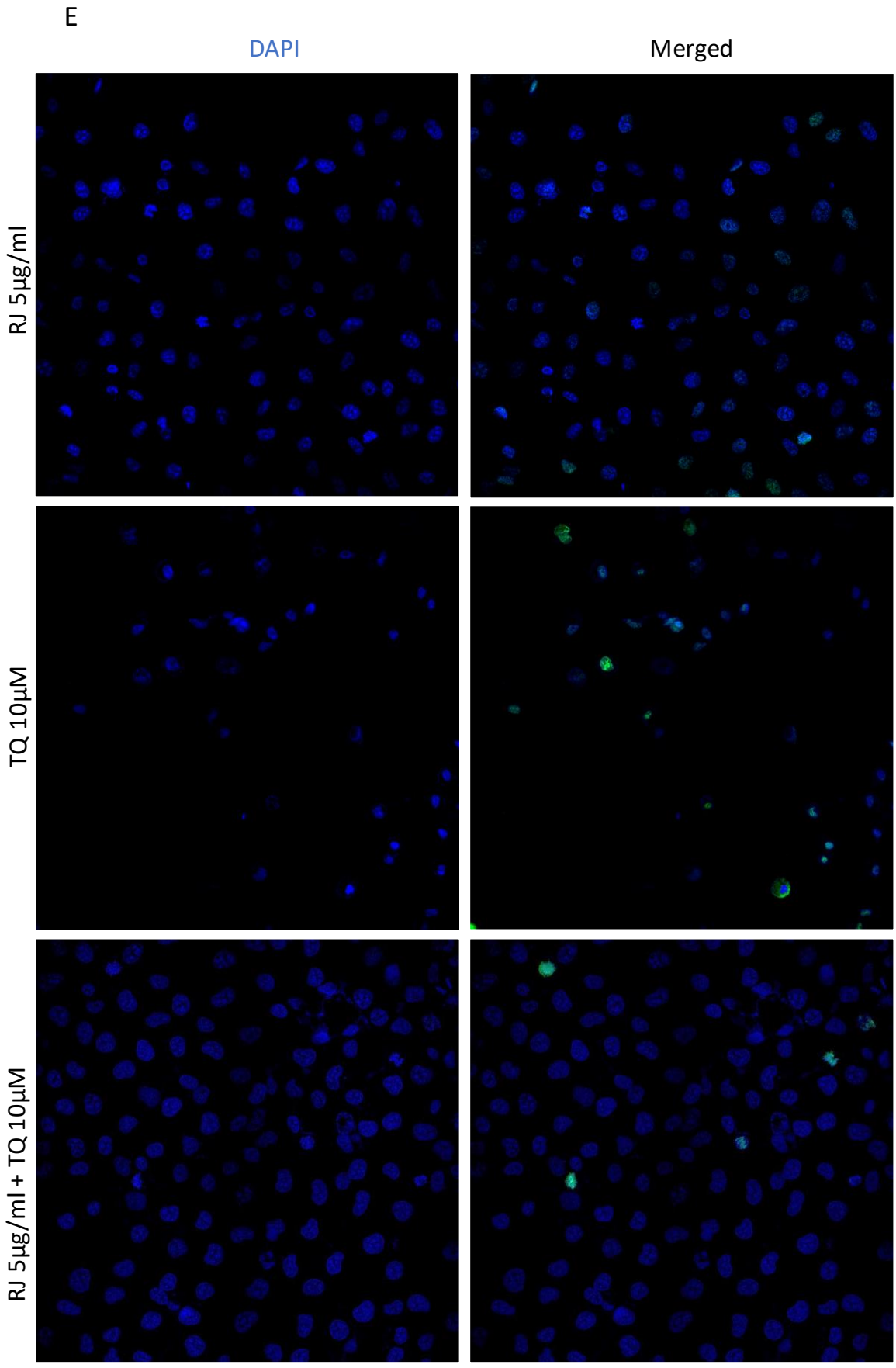

A
DAPI
Merged
Control
RJ 0.1µg/ml
RJ 5µg/ml
50 µm


B
DAPI
Merged
Control
TQ 10µM
TQ 15µM
50 µm

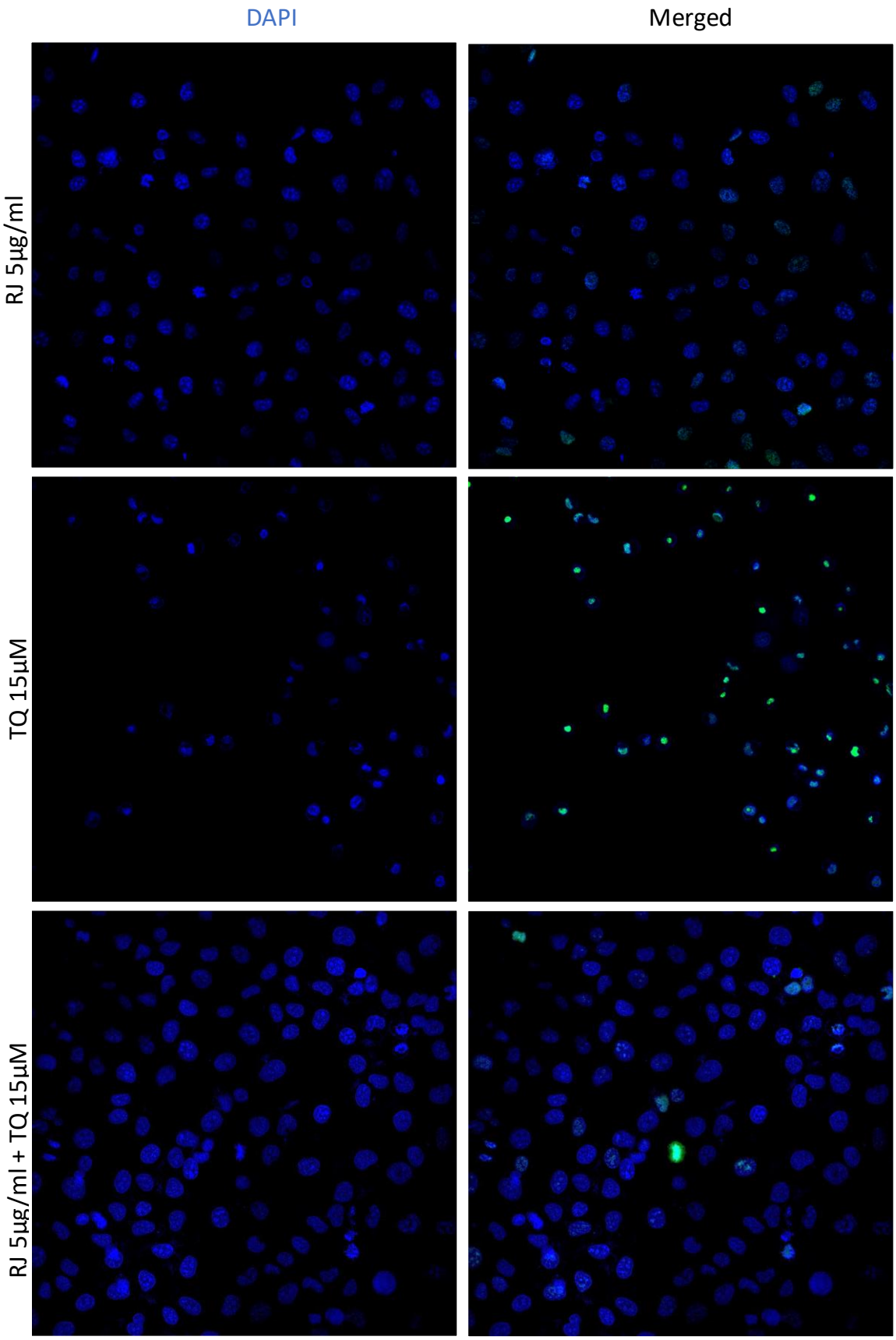
C




D
DAPI
Merged
RJ 0.1µg/ml
TQ 15µM
RJ 0.1µg/ml + TQ 15µM


E
DAPI
Merged
RJ 5µg/ml
TQ 10µM
RJ 5µg/ml + TQ 10µM

F



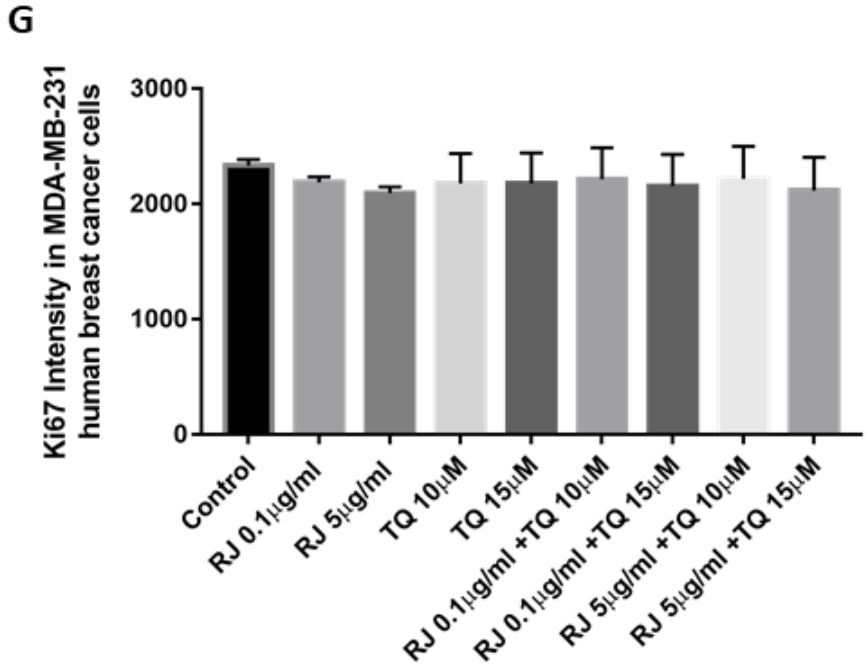


Figure 16. Effect of RJ, TQ and combinations on Ki67 expression in MDA-MB-231 human breast cancer cells.
(A-F) Immunofluorescence micrographs of Ki67 expression at 24 h after treatment. Green indicates Ki67 expression and blue indicates nuclei counterstained by DAPI. Nuclei were visualized by confocal Zeiss Axio microscope, 40X oil immersion with scale bar = 50 μM. (G) Quantification of the levels of Ki67 intensity in MDA-MB-231 cells at 24 h of treatment with different concentrations of RJ, TQ, and combinations. Data are expressed as mean ± SEM of 2 independent experiments.

Therefore, RJ alone and TQ alone or their combinations exerted a relatively low anti-proliferative effect in human triple-negative breast cancer.

CHAPTER IV
DISCUSSION

Breast cancer, the most common cancer among women, is identified as a heterogeneous disease arising from the differential expression of hormonal receptors along with the genomic and intratumoral heterogeneity (Bertos and Park, 2011). Female breast cancer ranks second among the most frequently diagnosed cancer types worldwide (Bray et al., 2018), causing the majority of cancer-related deaths among women (WHO, 2020a). Several internal and external factors including, aging (Ban and Godellas, 2014), genetic susceptibility (Brewer et al., 2017; Sun et al., 2017), lifestyle (Thiebaut et al., 2007) in addition to early diagnosis, preventative screening and treatment availability (WHO, 2020c), affect cancer prognosis and survival rates. Despite the tremendous improvement in the therapeutic approaches, conventional treatments of breast cancer, including systemic therapy, exert organ-specific toxicity along with various side effects (Bodai and Tuso, 2015; Teven et al., 2017). The interest in alternative treatments relying on relatively non-toxic and cost-effective natural resources has surged over the past decades, particularly from medicinal plants and honeybee products. Thymoquinone, the principal constituent of *Nigella sativa* essential oil, and the nutritious honeybee secretions of RJ were shown to have potent anti-cancer activities against many types of cancers, including breast cancer (Ahmad et al., 2020; Khader and Eckl, 2014). Triple-negative breast cancer (TNBC) is well known to be an aggressive breast cancer with poor prognosis because of its ability to resist chemotherapy and metastasize (Nedeljkovic and Damjanovic, 2019; Yao et al., 2019). Therefore, identifying new therapeutic approaches with an emphasis on natural compounds is essential to overcome resistance in TNBC and chemotherapy-induced

cytotoxicity. Royal jelly exhibits various biological and pharmaceutical properties against many types of cancer. However, few studies investigated its anti-cancer potential against breast cancer (Nakaya et al., 2007; Okic-Djordjevic et al., 2013). Although TQ's anti-cancer activity has been well documented in various models, combining it with RJ and assessing the efficacy of this combination against breast cancer is still unknown. Our study is the first to investigate the anti-cancer activity of both TQ and RJ alone and in combination against the triple-negative MDA-MB-231 human metastatic breast cancer cell line.

Royal jelly, secreted from the mandibular and hypopharyngeal glands of worker bees, comes in the form of fresh solid extract. Its complex composition in addition to the presence of proteins, fatty acids, and amino acids (Gismondi et al., 2017) makes it hard to dissolve prior to cell treatment. Our study generated an optimum dissolution protocol for RJ supplied by the Rashaya al Wadi bee farm after several attempts that yielded partial dissolution.

Examination of drug toxicity using *in vitro* and *in vivo* models is a major prerequisite before any clinical application. Few are the studies investigating *in vitro* toxicity of RJ in breast cancer with none addressing TNBC so far. For this purpose, we assessed RJ toxicity and anti-cancer activity in FHs 74 Int non-tumorigenic human intestinal cells and MDA-MB-231 tumorigenic human metastatic breast cancer cell lines. Our results showed that RJ exhibited minimal toxicity on FHs 74 Int cell line at doses below 5 µg/ml, while a more pronounced inhibitory effect was observed at higher doses with a clear saturation effect obtained at doses equal or greater than 100 µg/ml. This indicates that RJ, unless at high doses, is relatively nontoxic to the non-tumorigenic human small intestinal cell line. In line with the previously published

studies (Nakaya et al., 2007; Zhang et al., 2017), our findings demonstrate that RJ has anti-cancer effects against breast cancer. RJ exerted low to mild dose-dependent inhibitory effects on the viability of MDA-MB-231cell line at doses below 5 µg/ml. Cell death was more pronounced in MDA-MB-231 cell line at 200 µg/ml RJ, suggesting the greater toxicity of RJ to breast cancer cells with the IC_{50} estimated to be 1.4 fold greater in FHs 74 Int cell line compared to that in MDA-MB-231 cell line.

Assessing RJ toxicity using MTT assay yielded false-negative results evidenced by the higher viability of treated MDA-MB-231 cells in comparison to control at low doses of RJ and the absence of an inhibitory effect in response to very high RJ doses. These results strongly contradict the microscopy images which showed a significant decrease in the viability of MDA-MB-231 cells in response to the increasing doses of RJ. This suggests a possible interference of RJ with the colorimetric absorbance measures of the MTT assay. The overestimation of MTT readings could be explained by a possible non-enzymatic reduction of MTT tetrazolium dye. Chemical reducing compounds, including phenols, antioxidants such as ascorbic acid, vitamin E, vitamin A, N-acetylcysteine, thiol-containing compounds are known to interfere with MTT readings (Bruggisser et al., 2002; Chakrabarti et al., 2000; Maioli et al., 2009; Natarajan et al., 2000). Knowing that RJ constitutes phenols and vitamin proportions, including vitamin C, a direct reduction of MTT tetrazolium may explain MTT overestimation. Another scenario could be the possible overlap of RJ spectra with formazan absorbance, which in turn needs further investigation in a cell-free system to ensure such overlap and the extent of subsequent interference. Therefore, due to RJ interference in MTT results, the trypan blue exclusion assay was used to investigate the inhibitory effect of RJ alone or in combination with TQ on MDA-MB-231 cell viability.

Thymoquinone has been shown to possess potent anti-cancer activities against various cancer types, including colon cancer with minimal cytotoxic effects on normal intestinal cells (El-Najjar et al., 2010). In the context of breast cancer, previous studies reported TQ-mediated induction of apoptosis, growth inhibition, in addition to suppression of viability and invasion of MDA-MB-231 and MCF7 cell lines mainly through the inhibition of Akt phosphorylation (Attoub et al., 2013; Ganji-Harsini et al., 2016). In line with these studies, we further confirmed TQ's anti-cancer activity in MDA-MB-231 cell line as evidenced by the dose-dependent cell death effects at concentrations below and above the IC_{50} value of TQ, which was established at 19 µM using MTT assay.

Cell cycle analysis using propidium iodide staining was performed to further confirm cell death and to examine whether TQ and RJ alone or in combination affect the cell cycle progression of breast cancer cells. In accordance with our findings using trypan blue exclusion assay, RJ alone at a dose of 0.1 µg/ml did not exert significant changes in cell viability compared to the control. Consistent with previous studies reporting the inhibitory activity of TQ (El-Najjar et al., 2010; Gali-Muhtasib et al., 2004a; Gali-Muhtasib et al., 2008a; Khalife et al., 2016), our study reports 4 fold increase in the Pre-G1 cell population along with a 2 and 3 fold decrease in G0/G1 and G2/M cell populations of MDA-MB-231 cells in response to 15 µM of TQ, respectively, further confirming TQ-mediated cell death. Interestingly, combining 15 µM TQ with 0.1 µg/ml RJ yielded a more pronounced cell death effect evidenced by the 6 fold increase in the Pre-G1 population. These results indicate that the cell death effect is enhanced upon the combination of TQ with RJ compared to single treatments with each compound alone. Treatment with RJ shifted the flow cytometry histogram as a

result of the decreased PI intensity without affecting the size and complexity of MDA-MB-231 cells compared to untreated cells. These results indicate that RJ at 0.1 µg/ml does not induce cell death, but rather it interferes with the PI staining. The decreased PI intensity could be explained by inhibiting the intercalation of PI to the deoxyribonucleic acid (DNA) in breast cancer cells by reducing its binding affinity to DNA bases. Further investigation is needed to confirm the direct and/or indirect inhibitory effect of RJ on PI intercalation.

Combination therapy is usually used to enhance the therapeutic response and overcome any possible drug resistance in cancer patients (Palmer and Sorger, 2017). Enhanced anti-cancer activity was reported in response to the combination of TQ with other plant extracts like piperine or chemical drugs such as Tamoxifen and Doxorubicin (Fatfat et al., 2019; Ganji-Harsini et al., 2016; Talib, 2017). Our study is the first to evaluate the combined anti-cancer activity of TQ and RJ against TNBC. We documented an enhanced anti-cancer activity of TQ when combined with RJ against MDA-MB-231 cell line. Cell death was enhanced in response to different combinations as compared to the treatment with each drug alone. Combinations at relatively low concentrations of both natural compounds (0.1 µg/ml RJ and 5 µM TQ) augmented cell death which was further amplified by 3 and 5 folds in response to the combination of 5 µg/ml of RJ with 10µM and 15 µM of TQ, respectively. An apparent synergistic interaction with CI<1 provided further evidence for the augmented reduction in cell viability in response to the combinations relative to the single-drug treatments. The synergistic effect in MDA-MB-231 cells was best detected upon the combination of 5 µg/ml RJ with 10 µM TQ. These results are consistent with the previous studies that reported the synergism of TQ in combination with different agents including, melatonin

(Odeh et al., 2018) and piperine (Talib, 2017) against breast cancer, diosgenin on squamous cell carcinoma (Das et al., 2012), docetaxel against prostate cancer (Dirican et al., 2015) in addition to arsenic and interferon-alpha against human T-cell leukemia/lymphoma (Houssein et al., 2020).

Evading apoptosis is one of the main cancer hallmarks that aids in the survival of cancer cells. Natural extracts or combination therapies capable of inducing apoptosis are considered as effective therapeutic candidates against cancer progression. To understand the underlying mechanism of the observed reduction in the viability of metastatic breast cancer cells upon the different treatments, we investigated apoptosis induction as a possible mechanism of cell death. Enhanced induction of apoptosis was evidenced by the increase in caspase 3 cleavage in response to the increasing TQ doses alone or in combination with RJ. On the other hand, treatment with RJ alone did not induce any significant apoptotic effect compared to the control. Apoptotic cell death was increased by 4 folds in response to the combination of 10 µM TQ with 0.1 µg/ml and 5 µg/ml RJ while a 5 fold increase was obtained upon combining 15 µM TQ with both RJ doses. Our results indicate that TQ is the main inducer of apoptosis, although an augmented apoptotic response was observed upon the combination with RJ, indicating that RJ could modestly potentiate the anti-cancer activity of TQ in TNBC. Our findings are consistent with previous studies showing induction of apoptosis upon treatment with TQ alone and in combination with other agents against cancer cells (Gali-Muhtasib et al., 2008b; Houssein et al., 2020; Roepke et al., 2007; Talib, 2017). In line with previously published data (Gali-Muhtasib et al., 2008b), our study shows the absence of a TQ mediated anti-proliferative effect against breast cancer cells. Minimal decrease of Ki67 intensity in MDA-MB-231 cells was obtained irrespective of the doses

used of both TQ and RJ each alone or in combination. Therefore, cell death of TNBC cells was not due to the inhibition of proliferation but rather to apoptosis as evidenced by the enhanced caspase 3 cleavage.

In conclusion, RJ and TQ being relatively non-toxic to normal cells, exhibit anti-cancer activity against human metastatic breast cancer. RJ alone exerts low cytotoxic effects except at high doses in MDA-MB-231 cells. On the other hand, TQ exerts a potent anti-tumor activity as it mediates TNBC cell death at doses below and above its IC_{50}. Both compounds, in combination, act synergistically to inhibit TNBC. Our findings demonstrate the potent pro-apoptotic activity of TQ compared to that of RJ against MDA-MB-231 cell line. Although their combination relatively enhances the apoptotic cell death, TQ acts as the main inducer of apoptosis mediating cell death by inducing caspase 3 dependent apoptosis in a dose-dependent manner. The combination of these two natural compounds deserves further investigation to identify the exact cell death mechanism responsible for the synergistic activity of these compounds. Also, future studies will identify RJ's bioactive components responsible for its anti-cancer activity alone and in combination with TQ, further investigate their mechanism of action in terms of cell invasion, migration and *in vivo* tumor development.

BIBLIOGRAPHY

Abdel-Hafez, S.M.N., R.A. Rifaai, and W.Y. Abdelzaher. 2017. Possible protective effect of royal jelly against cyclophosphamide induced prostatic damage in male albino rats; a biochemical, histological and immuno-histo-chemical study. *Biomed Pharmacother*. 90:15-23.

Abulfadl, Y.S., N.N. El-Maraghy, A.A.E. Ahmed, S. Nofal, and O.A. Badary. 2018. Protective effects of thymoquinone on D-galactose and aluminum chloride induced neurotoxicity in rats: biochemical, histological and behavioral changes. *Neurol Res*. 40:324-333.

Ahmad, S., M.G. Campos, F. Fratini, S.Z. Altaye, and J. Li. 2020. New Insights into the Biological and Pharmaceutical Properties of Royal Jelly. *Int J Mol Sci*. 21.

Al-Ali, A., A.A. Alkhawajah, M.A. Randhawa, and N.A. Shaikh. 2008. Oral and intraperitoneal LD50 of thymoquinone, an active principle of Nigella sativa, in mice and rats. *J Ayub Med Coll Abbottabad*. 20:25-27.

Al-Amri, M., A. M., and P. MBBS, A. O. B. 2009. Phase I Safety and Clinical Activity Study of Thymoquinone in Patients with Advanced Refractory Malignant Disease. *Shiraz E-Med J*. 10:107-111.

Alkharfy, K.M., A. Ahmad, R.M. Khan, and W.M. Al-Shagha. 2015. Pharmacokinetic plasma behaviors of intravenous and oral bioavailability of thymoquinone in a rabbit model. *Eur J Drug Metab Pharmacokinet*. 40:319-323.

Alobaedi, O.H., W.H. Talib, and I.A. Basheti. 2017. Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer. *Asian Pac J Trop Med*. 10:400-408.

Amin, B., and H. Hosseinzadeh. 2016. Black Cumin (Nigella sativa) and Its Active Constituent, Thymoquinone: An Overview on the Analgesic and Anti-inflammatory Effects. *Planta Med*. 82:8-16.

Aplin, A.E., A. Howe, S.K. Alahari, and R.L. Juliano. 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev*. 50:197-263.

Arafa el, S.A., Q. Zhu, Z.I. Shah, G. Wani, B.M. Barakat, I. Racoma, M.A. El-Mahdy, and A.A. Wani. 2011. Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *Mutat Res*. 706:28-35.

Attoub, S., O. Sperandio, H. Raza, K. Arafat, S. Al-Salam, M.A. Al Sultan, M. Al Safi, T. Takahashi, and A. Adem. 2013. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo. *Fundam Clin Pharmacol*. 27:557-569.

Aycan, I.O., A. Tufek, O. Tokgoz, O. Evliyaoglu, U. Firat, G.O. Kavak, H. Turgut, and M.U. Yuksel. 2014. Thymoquinone treatment against acetaminophen-induced hepatotoxicity in rats. *Int J Surg*. 12:213-218.

Ayyanan, A., O. Laribi, S. Schuepbach-Mallepell, C. Schrick, M. Gutierrez, T. Tanos, G. Lefebvre, J. Rougemont, O. Yalcin-Ozuysal, and C. Brisken. 2011. Perinatal exposure to bisphenol a increases adult mammary gland progesterone response and cell number. *Mol Endocrinol*. 25:1915-1923.

Ban, K.A., and C.V. Godellas. 2014. Epidemiology of breast cancer. *Surg Oncol Clin N Am*. 23:409-422.

Barkat, M.A., Harshita, J. Ahmad, M.A. Khan, S. Beg, and F.J. Ahmad. 2018. Insights into the Targeting Potential of Thymoquinone for Therapeutic Intervention Against Triple-negative Breast Cancer. *Curr Drug Targets*. 19:70-80.

Bartlett, J.M., C.L. Brookes, T. Robson, C.J. van de Velde, L.J. Billingham, F.M. Campbell, M. Grant, A. Hasenburg, E.T. Hille, C. Kay, D.G. Kieback, H. Putter, C. Markopoulos, E.M. Kranenbarg, E.A. Mallon, L. Dirix, C. Seynaeve, and D. Rea. 2011. Estrogen receptor and progesterone receptor as predictive biomarkers of response to endocrine therapy: a prospectively powered pathology study in the Tamoxifen and Exemestane Adjuvant Multinational trial. *J Clin Oncol*. 29:1531-1538.

Bauer, S.R., S.E. Hankinson, E.R. Bertone-Johnson, and E.L. Ding. 2013. Plasma vitamin D levels, menopause, and risk of breast cancer: dose-response meta-analysis of prospective studies. *Medicine (Baltimore)*. 92:123-131.

Bertos, N.R., and M. Park. 2011. Breast cancer - one term, many entities? *J Clin Invest*. 121:3789-3796.

Bertram, J.S. 2000. The molecular biology of cancer. *Mol Aspects Med*. 21:167-223.

Bincoletto, C., S. Eberlin, C.A. Figueiredo, M.B. Luengo, and M.L. Queiroz. 2005. Effects produced by Royal Jelly on haematopoiesis: relation with host resistance against Ehrlich ascites tumour challenge. *Int Immunopharmacol*. 5:679-688.

Bodai, B.I., and P. Tuso. 2015. Breast cancer survivorship: a comprehensive review of long-term medical issues and lifestyle recommendations. *Perm J*. 19:48-79.

Borawska, M.H., R. Markiewicz-Zukowska, S.K. Naliwajko, J. Moskwa, E. Bartosiuk, K. Socha, A. Surazynski, J. Kochanowicz, and Z. Mariak. 2014. The interaction of bee products with temozolomide in human diffuse astrocytoma, glioblastoma multiforme and astroglia cell lines. *Nutr Cancer*. 66:1247-1256.

Bovelli, D., G. Plataniotis, F. Roila, and E.G.W. Group. 2010. Cardiotoxicity of chemotherapeutic agents and radiotherapy-related heart disease: ESMO Clinical Practice Guidelines. *Ann Oncol*. 21 Suppl 5:v277-282.

Bray, F., J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, and A. Jemal. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 68:394-424.

Brewer, H.R., M.E. Jones, M.J. Schoemaker, A. Ashworth, and A.J. Swerdlow. 2017. Family history and risk of breast cancer: an analysis accounting for family structure. *Breast Cancer Res Treat*. 165:193-200.

Brownlee, Z., R. Garg, M. Listo, P. Zavitsanos, D.E. Wazer, and K.E. Huber. 2018. Late complications of radiation therapy for breast cancer: evolution in techniques and risk over time. *Gland Surg*. 7:371-378.

Bruggisser, R., K. von Daeniken, G. Jundt, W. Schaffner, and H. Tullberg-Reinert. 2002. Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. *Planta Med*. 68:445-448.

Bryan, T.M., and T.R. Cech. 1999. Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol*. 11:318-324.

Bryan, T.M., A. Englezou, J. Gupta, S. Bacchetti, and R.R. Reddel. 1995. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J*. 14:4240-4248.

Bucekova, M., M. Sojka, I. Valachova, S. Martinotti, E. Ranzato, Z. Szep, V. Majtan, J. Klaudiny, and J. Majtan. 2017. Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation in vitro and in vivo. *Sci Rep*. 7:7340.

Bulet, P., and R. Stocklin. 2005. Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept Lett*. 12:3-11.

Cailleau, R., M. Olive, and Q.V. Cruciger. 1978. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro*. 14:911-915.

Cancer, I.A.f.R.o. 2018. Cancer Today. Vol. 2020.

Cantley, L.C., and B.G. Neel. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A*. 96:4240-4245.

Cevallos, A.M., J. Herrera, I. Lopez-Villasenor, and R. Hernandez. 2017. Differential Effects of Two Widely Used Solvents, DMSO and Ethanol, on the Growth and Recovery of Trypanosoma cruzi Epimastigotes in Culture. *Korean J Parasitol*. 55:81-84.

Chakrabarti, R., S. Kundu, S. Kumar, and R. Chakrabarti. 2000. Vitamin A as an enzyme that catalyzes the reduction of MTT to formazan by vitamin C. *J Cell Biochem*. 80:133-138.

Chavez, K.J., S.V. Garimella, and S. Lipkowitz. 2010. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast Dis*. 32:35-48.

Chehl, N., G. Chipitsyna, Q. Gong, C.J. Yeo, and H.A. Arafat. 2009. Anti-inflammatory effects of the Nigella sativa seed extract, thymoquinone, in pancreatic cancer cells. *HPB (Oxford)*. 11:373-381.

Chen, Y.F., K. Wang, Y.Z. Zhang, Y.F. Zheng, and F.L. Hu. 2016. In Vitro Anti-Inflammatory Effects of Three Fatty Acids from Royal Jelly. *Mediators Inflamm*. 2016:3583684.

Chiu, H.F., B.K. Chen, Y.Y. Lu, Y.C. Han, Y.C. Shen, K. Venkatakrishnan, O. Golovinskaia, and C.K. Wang. 2017. Hypocholesterolemic efficacy of royal jelly in healthy mild hypercholesterolemic adults. *Pharm Biol*. 55:497-502.

Chou, T.C., and P. Talalay. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 22:27-55.

Christofori, G., and H. Semb. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci*. 24:73-76.

Clynes, R.A., T.L. Towers, L.G. Presta, and J.V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med*. 6:443-446.

Cooper, G.M. 2000. The Cell: A Molecular Approach. 2nd edition. Washington, D.C. : ASM Press ; Sunderland, Mass. : Sinauer Associates, ©2000.

Dameron, K.M., O.V. Volpert, M.A. Tainsky, and N. Bouck. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science*. 265:1582-1584.

Das, S., K.K. Dey, G. Dey, I. Pal, A. Majumder, S. MaitiChoudhury, S.C. kundu, and M. Mandal. 2012. Antineoplastic and apoptotic potential of traditional medicines thymoquinone and diosgenin in squamous cell carcinoma. *PLoS One*. 7:e46641.

Dergarabetian, E.M., K.I. Ghattass, S.B. El-Sitt, R.M. Al-Mismar, C.O. El-Baba, W.S. Itani, N.M. Melhem, H.A. El-Hajj, A.A. Bazarbachi, R. Schneider-Stock, and H.U. Gali-Muhtasib. 2013. Thymoquinone induces apoptosis in malignant T-cells via generation of ROS. *Front Biosci (Elite Ed)*. 5:706-719.

Dirican, A., H. Atmaca, E. Bozkurt, C. Erten, B. Karaca, and R. Uslu. 2015. Novel combination of docetaxel and thymoquinone induces synergistic cytotoxicity and apoptosis in DU-145 human prostate cancer cells by modulating PI3K-AKT pathway. *Clin Transl Oncol*. 17:145-151.

Downward, J. 1998. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol*. 10:262-267.

Dowsett, M., C. Allred, J. Knox, E. Quinn, J. Salter, C. Wale, J. Cuzick, J. Houghton, N. Williams, E. Mallon, H. Bishop, I. Ellis, D. Larsimont, H. Sasano, P. Carder, A.L. Cussac, F. Knox, V. Speirs, J. Forbes, and A. Buzdar. 2008. Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status with recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial. *J Clin Oncol*. 26:1059-1065.

Drapeau, M.D., S. Albert, R. Kucharski, C. Prusko, and R. Maleszka. 2006. Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. *Genome Res*. 16:1385-1394.

El-Najjar, N., M. Chatila, H. Moukadem, H. Vuorela, M. Ocker, M. Gandesiri, R. Schneider-Stock, and H. Gali-Muhtasib. 2010. Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. *Apoptosis*. 15:183-195.

El-Najjar, N., R.A. Ketola, T. Nissila, T. Mauriala, M. Antopolsky, J. Janis, H. Gali-Muhtasib, A. Urtti, and H. Vuorela. 2011. Impact of protein binding on the analytical detectability and anticancer activity of thymoquinone. *J Chem Biol*. 4:97-107.

El Rabey, H.A., M.N. Al-Seeni, and A.S. Bakhashwain. 2017. The Antidiabetic Activity of Nigella sativa and Propolis on Streptozotocin-Induced Diabetes and Diabetic Nephropathy in Male Rats. *Evid Based Complement Alternat Med*. 2017:5439645.

Evan, G., and T. Littlewood. 1998. A matter of life and cell death. *Science*. 281:1317-1322.

Fan, P., B. Han, M. Feng, Y. Fang, L. Zhang, H. Hu, Y. Hao, Y. Qi, X. Zhang, and J. Li. 2016. Functional and Proteomic Investigations Reveal Major Royal Jelly Protein 1 Associated with Anti-hypertension Activity in Mouse Vascular Smooth Muscle Cells. *Sci Rep*. 6:30230.

Fatfat, M., I. Fakhoury, Z. Habli, R. Mismar, and H. Gali-Muhtasib. 2019. Thymoquinone enhances the anticancer activity of doxorubicin against adult T-cell leukemia in vitro and in vivo through ROS-dependent mechanisms. *Life Sci*. 232:116628.

Filipic, B., L. Gradisnik, K. Rihar, E. Soos, A. Pereyra, and J. Potokar. 2015. The influence of royal jelly and human interferon-alpha (HuIFN-alphaN3) on proliferation, glutathione level and lipid peroxidation in human colorectal adenocarcinoma cells in vitro. *Arh Hig Rada Toksikol*. 66:269-274.

Foley, K.P., and R.N. Eisenman. 1999. Two MAD tails: what the recent knockouts of Mad1 and Mxi1 tell us about the MYC/MAX/MAD network. *Biochim Biophys Acta*. 1423:M37-47.

Fontana, R., M.A. Mendes, B.M. de Souza, K. Konno, L.M. Cesar, O. Malaspina, and M.S. Palma. 2004. Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (Apis mellifera). *Peptides*. 25:919-928.

Fratini, F., G. Cilia, S. Mancini, and A. Felicioli. 2016. Royal Jelly: An ancient remedy with remarkable antibacterial properties. *Microbiol Res*. 192:130-141.

Furusawa, T., R. Rakwal, H.W. Nam, J. Shibato, G.K. Agrawal, Y.S. Kim, Y. Ogawa, Y. Yoshida, Y. Kouzuma, Y. Masuo, and M. Yonekura. 2008. Comprehensive royal jelly (RJ) proteomics using one- and two-dimensional proteomics platforms reveals novel RJ proteins and potential phospho/glycoproteins. *J Proteome Res*. 7:3194-3229.

Fynan, T.M., and M. Reiss. 1993. Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog*. 4:493-540.

Gali-Muhtasib, H., M. Diab-Assaf, C. Boltze, J. Al-Hmaira, R. Hartig, A. Roessner, and R. Schneider-Stock. 2004a. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. *Int J Oncol*. 25:857-866.

Gali-Muhtasib, H., D. Kuester, C. Mawrin, K. Bajbouj, A. Diestel, M. Ocker, C. Habold, C. Foltzer-Jourdainne, P. Schoenfeld, B. Peters, M. Diab-Assaf, U. Pommrich, W. Itani, H. Lippert, A. Roessner, and R. Schneider-Stock. 2008a. Thymoquinone triggers inactivation of the stress response pathway sensor CHEK1 and contributes to apoptosis in colorectal cancer cells. *Cancer Res*. 68:5609-5618.

Gali-Muhtasib, H., M. Ocker, D. Kuester, S. Krueger, Z. El-Hajj, A. Diestel, M. Evert, N. El-Najjar, B. Peters, A. Jurjus, A. Roessner, and R. Schneider-Stock. 2008b. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med*. 12:330-342.

Gali-Muhtasib, H.U., W.G. Abou Kheir, L.A. Kheir, N. Darwiche, and P.A. Crooks. 2004b. Molecular pathway for thymoquinone-induced cell-cycle arrest and apoptosis in neoplastic keratinocytes. *Anticancer Drugs*. 15:389-399.

Galvao, J., B. Davis, M. Tilley, E. Normando, M.R. Duchen, and M.F. Cordeiro. 2014. Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J*. 28:1317-1330.

Ganji-Harsini, S., M. Khazaei, Z. Rashidi, and A. Ghanbari. 2016. Thymoquinone Could Increase The Efficacy of Tamoxifen Induced Apoptosis in Human Breast Cancer Cells: An In Vitro Study. *Cell J*. 18:245-254.

Gately, S., P. Twardowski, M.S. Stack, D.L. Cundiff, D. Grella, F.J. Castellino, J. Enghild, H.C. Kwaan, F. Lee, R.A. Kramer, O. Volpert, N. Bouck, and G.A. Soff. 1997. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Proc Natl Acad Sci U S A*. 94:10868-10872.

Giancotti, F.G., and E. Ruoslahti. 1999. Integrin signaling. *Science*. 285:1028-1032.

Gismondi, A., E. Trionfera, L. Canuti, G. Di Marco, and A. Canini. 2017. Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells. *Oncol Rep*. 38:1833-1844.

Goyal, S.N., C.P. Prajapati, P.R. Gore, C.R. Patil, U.B. Mahajan, C. Sharma, S.P. Talla, and S.K. Ojha. 2017. Therapeutic Potential and Pharmaceutical Development of Thymoquinone: A Multitargeted Molecule of Natural Origin. *Front Pharmacol*. 8:656.

Green, D.R., and J.C. Reed. 1998. Mitochondria and apoptosis. *Science*. 281:1309-1312.

Gurung, R.L., S.N. Lim, A.K. Khaw, J.F. Soon, K. Shenoy, S. Mohamed Ali, M. Jayapal, S. Sethu, R. Baskar, and M.P. Hande. 2010. Thymoquinone induces telomere shortening, DNA damage and apoptosis in human glioblastoma cells. *PLoS One*. 5:e12124.

Hamurcu, Z., A. Ashour, N. Kahraman, and B. Ozpolat. 2016. FOXM1 regulates expression of eukaryotic elongation factor 2 kinase and promotes proliferation, invasion and tumorgenesis of human triple negative breast cancer cells. *Oncotarget*. 7:16619-16635.

Han, B., Y. Fang, M. Feng, X. Lu, X. Huo, L. Meng, B. Wu, and J. Li. 2014. In-depth phosphoproteomic analysis of royal jelly derived from western and eastern honeybee species. *J Proteome Res*. 13:5928-5943.

Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86:353-364.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Harris, C.C. 1996. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis*. 17:1187-1198.

Hartmann, L.C., T.A. Sellers, M.H. Frost, W.L. Lingle, A.C. Degnim, K. Ghosh, R.A. Vierkant, S.D. Maloney, V.S. Pankratz, D.W. Hillman, V.J. Suman, J. Johnson, C. Blake, T. Tlsty, C.M. Vachon, L.J. Melton, 3rd, and D.W. Visscher. 2005. Benign breast disease and the risk of breast cancer. *N Engl J Med*. 353:229-237.

Hashemipour, M.A., Z. Tavakolineghad, S.A. Arabzadeh, Z. Iranmanesh, and S.A. Nassab. 2014. Antiviral Activities of Honey, Royal Jelly, and Acyclovir Against HSV-1. *Wounds*. 26:47-54.

Hattori, N., H. Nomoto, H. Fukumitsu, S. Mishima, and S. Furukawa. 2007. Royal jelly and its unique fatty acid, 10-hydroxy-trans-2-decenoic acid, promote neurogenesis by neural stem/progenitor cells in vitro. *Biomed Res*. 28:261-266.

Hayflick, L. 1997. Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc)*. 62:1180-1190.

Holland, R., S.H. Veling, M. Mravunac, and J.H. Hendriks. 1985. Histologic multifocality of Tis, T1-2 breast carcinomas. Implications for clinical trials of breast-conserving surgery. *Cancer*. 56:979-990.

Holliday, D.L., and V. Speirs. 2011. Choosing the right cell line for breast cancer research. *Breast Cancer Res*. 13:215.

Houssein, M., M. Fatfat, Z. Habli, N. Ghazal, S. Moodad, H. Khalife, M. Khalil, and H. Gali-Muhtasib. 2020. Thymoquinone synergizes with arsenic and interferon alpha to target human T-cell leukemia/lymphoma. *Life Sci*. 251:117639.

Hsieh, C.C., D. Trichopoulos, K. Katsouyanni, and S. Yuasa. 1990. Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: associations and interactions in an international case-control study. *Int J Cancer*. 46:796-800.

Hussain, A.R., M. Ahmed, S. Ahmed, P. Manogaran, L.C. Platanias, S.N. Alvi, K.S. Al-Kuraya, and S. Uddin. 2011. Thymoquinone suppresses growth and induces

apoptosis via generation of reactive oxygen species in primary effusion lymphoma. *Free Radic Biol Med*. 50:978-987.

Imran, M., A. Rauf, I.A. Khan, M. Shahbaz, T.B. Qaisrani, S. Fatmawati, T. Abu-Izneid, A. Imran, K.U. Rahman, and T.A. Gondal. 2018. Thymoquinone: A novel strategy to combat cancer: A review. *Biomed Pharmacother*. 106:390-402.

Isidorov, V.A., S. Bakier, and I. Grzech. 2012. Gas chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. *J Chromatogr B Analyt Technol Biomed Life Sci*. 885-886:109-116.

Izuta, H., Y. Chikaraishi, M. Shimazawa, S. Mishima, and H. Hara. 2009. 10-Hydroxy-2-decenoic acid, a major fatty acid from royal jelly, inhibits VEGF-induced angiogenesis in human umbilical vein endothelial cells. *Evid Based Complement Alternat Med*. 6:489-494.

Jafri, S.H., J. Glass, R. Shi, S. Zhang, M. Prince, and H. Kleiner-Hancock. 2010. Thymoquinone and cisplatin as a therapeutic combination in lung cancer: In vitro and in vivo. *J Exp Clin Cancer Res*. 29:87.

Johnson, J.P. 1991. Cell adhesion molecules of the immunoglobulin supergene family and their role in malignant transformation and progression to metastatic disease. *Cancer Metastasis Rev*. 10:11-22.

Jordan, V.C., and A.M. Brodie. 2007. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids*. 72:7-25.

Joshi, S.C., F.A. Khan, I. Pant, and A. Shukla. 2007. Role of radiotherapy in early breast cancer: an overview. *Int J Health Sci (Qassim)*. 1:259-264.

Junttila, T.T., R.W. Akita, K. Parsons, C. Fields, G.D. Lewis Phillips, L.S. Friedman, D. Sampath, and M.X. Sliwkowski. 2009. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell*. 15:429-440.

Kamakura, M. 2011. Royalactin induces queen differentiation in honeybees. *Nature*. 473:478-483.

Kamakura, M., T. Moriyama, and T. Sakaki. 2006. Changes in hepatic gene expression associated with the hypocholesterolaemic activity of royal jelly. *J Pharm Pharmacol*. 58:1683-1689.

Kang, Y., P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordon-Cardo, T.A. Guise, and J. Massague. 2003. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 3:537-549.

Karadeniz, A., N. Simsek, E. Karakus, S. Yildirim, A. Kara, I. Can, F. Kisa, H. Emre, and M. Turkeli. 2011. Royal jelly modulates oxidative stress and apoptosis in liver and kidneys of rats treated with cisplatin. *Oxid Med Cell Longev*. 2011:981793.

Kaseb, A.O., K. Chinnakannu, D. Chen, A. Sivanandam, S. Tejwani, M. Menon, Q.P. Dou, and G.P. Reddy. 2007. Androgen receptor and E2F-1 targeted thymoquinone therapy for hormone-refractory prostate cancer. *Cancer Res*. 67:7782-7788.

Khader, M., and P.M. Eckl. 2014. Thymoquinone: an emerging natural drug with a wide range of medical applications. *Iran J Basic Med Sci*. 17:950-957.

Khalife, R., M.H. Hodroj, R. Fakhoury, and S. Rizk. 2016. Thymoquinone from Nigella sativa Seeds Promotes the Antitumor Activity of Noncytotoxic Doses of Topotecan in Human Colorectal Cancer Cells in Vitro. *Planta Med*. 82:312-321.

Khan, M.A., M. Tania, C. Wei, Z. Mei, S. Fu, J. Cheng, J. Xu, and J. Fu. 2015. Thymoquinone inhibits cancer metastasis by downregulating TWIST1 expression to reduce epithelial to mesenchymal transition. *Oncotarget*. 6:19580-19591.

Kim, J., Y. Kim, H. Yun, H. Park, S.Y. Kim, K.G. Lee, S.M. Han, and Y. Cho. 2010. Royal jelly enhances migration of human dermal fibroblasts and alters the levels of cholesterol and sphinganine in an in vitro wound healing model. *Nutr Res Pract*. 4:362-368.

Knight, J.A., J. Fan, K.E. Malone, E.M. John, C.F. Lynch, R. Langballe, L. Bernstein, R.E. Shore, J.D. Brooks, A.S. Reiner, M. Woods, X. Liang, J.L. Bernstein, and W.S.C. Group. 2017. Alcohol consumption and cigarette smoking in combination: A predictor of contralateral breast cancer risk in the WECARE study. *Int J Cancer*. 141:916-924.

Kocot, J., M. Kielczykowska, D. Luchowska-Kocot, J. Kurzepa, and I. Musik. 2018. Antioxidant Potential of Propolis, Bee Pollen, and Royal Jelly: Possible Medical Application. *Oxid Med Cell Longev*. 2018:7074209.

Kohno, K., I. Okamoto, O. Sano, N. Arai, K. Iwaki, M. Ikeda, and M. Kurimoto. 2004. Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci Biotechnol Biochem*. 68:138-145.

Kolli-Bouhafs, K., A. Boukhari, A. Abusnina, E. Velot, J.P. Gies, C. Lugnier, and P. Ronde. 2012. Thymoquinone reduces migration and invasion of human glioblastoma cells associated with FAK, MMP-2 and MMP-9 down-regulation. *Invest New Drugs*. 30:2121-2131.

Krombach, J., R. Hennel, N. Brix, M. Orth, U. Schoetz, A. Ernst, J. Schuster, G. Zuchtriegel, C.A. Reichel, S. Bierschenk, M. Sperandio, T. Vogl, S. Unkel, C. Belka, and K. Lauber. 2019. Priming anti-tumor immunity by radiotherapy: Dying tumor cell-derived DAMPs trigger endothelial cell activation and recruitment of myeloid cells. *Oncoimmunology*. 8:e1523097.

Lehmann, B.D., J.A. Bauer, X. Chen, M.E. Sanders, A.B. Chakravarthy, Y. Shyr, and J.A. Pietenpol. 2011. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 121:2750-2767.

Leung, R., A. Ho, J. Chan, D. Choy, and C.K. Lai. 1997. Royal jelly consumption and hypersensitivity in the community. *Clin Exp Allergy*. 27:333-336.

Li, C.I., D.J. Uribe, and J.R. Daling. 2005. Clinical characteristics of different histologic types of breast cancer. *Br J Cancer*. 93:1046-1052.

Li, X., C. Huang, and Y. Xue. 2013. Contribution of lipids in honeybee (Apis mellifera) royal jelly to health. *J Med Food*. 16:96-102.

Lin, Y., Q. Shao, M. Zhang, C. Lu, J. Fleming, and S. Su. 2019. Royal jelly-derived proteins enhance proliferation and migration of human epidermal keratinocytes in an in vitro scratch wound model. *BMC Complement Altern Med*. 19:175.

Liu, J.R., Y.C. Yang, L.S. Shi, and C.C. Peng. 2008. Antioxidant properties of royal jelly associated with larval age and time of harvest. *J Agric Food Chem*. 56:11447-11452.

Loeb, L.A., and K.C. Cheng. 1990. Errors in DNA synthesis: a source of spontaneous mutations. *Mutat Res*. 238:297-304.

Lukashev, M.E., and Z. Werb. 1998. ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol*. 8:437-441.

Lynch, B.M., H.K. Neilson, and C.M. Friedenreich. 2011. Physical activity and breast cancer prevention. *Recent Results Cancer Res*. 186:13-42.

Mahmoud, Y.K., and H.M.A. Abdelrazek. 2019. Cancer: Thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy. *Biomed Pharmacother*. 115:108783.

Maioli, E., C. Torricelli, V. Fortino, F. Carlucci, V. Tommassini, and A. Pacini. 2009. Critical appraisal of the MTT assay in the presence of rottlerin and uncouplers. *Biol Proced Online*. 11:227-240.

Marks, F., G. Furstenberger, and K. Muller-Decker. 2007. Tumor promotion as a target of cancer prevention. *Recent Results Cancer Res*. 174:37-47.

Masoud, V., and G. Pages. 2017. Targeted therapies in breast cancer: New challenges to fight against resistance. *World J Clin Oncol*. 8:120-134.

Maxwell, P.H., M.S. Wiesener, G.W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher, and P.J. Ratcliffe. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*. 399:271-275.

Medema, R.H., and J.L. Bos. 1993. The role of p21ras in receptor tyrosine kinase signaling. *Crit Rev Oncog*. 4:615-661.

Melliou, E., and I. Chinou. 2005. Chemistry and bioactivity of royal jelly from Greece. *J Agric Food Chem*. 53:8987-8992.

Michaels, A.Y., A.R. Keraliya, S.H. Tirumani, A.B. Shinagare, and N.H. Ramaiya. 2016. Systemic treatment in breast cancer: a primer for radiologists. *Insights Imaging*. 7:131-144.

Minn, A.J., G.P. Gupta, P.M. Siegel, P.D. Bos, W. Shu, D.D. Giri, A. Viale, A.B. Olshen, W.L. Gerald, and J. Massague. 2005. Genes that mediate breast cancer metastasis to lung. *Nature*. 436:518-524.

Moo, T.A., R. Sanford, C. Dang, and M. Morrow. 2018. Overview of Breast Cancer Therapy. *PET Clin*. 13:339-354.

Moses, H.L., E.Y. Yang, and J.A. Pietenpol. 1990. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell*. 63:245-247.

Nagi, M.N., and H.A. Almakki. 2009. Thymoquinone supplementation induces quinone reductase and glutathione transferase in mice liver: possible role in protection against chemical carcinogenesis and toxicity. *Phytother Res*. 23:1295-1298.

Nakaya, M., H. Onda, K. Sasaki, A. Yukiyoshi, H. Tachibana, and K. Yamada. 2007. Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells. *Biosci Biotechnol Biochem*. 71:253-255.

Natarajan, M., S. Mohan, B.R. Martinez, M.L. Meltz, and T.S. Herman. 2000. Antioxidant compounds interfere with the 3. *Cancer Detect Prev*. 24:405-414.

Nedeljkovic, M., and A. Damjanovic. 2019. Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. *Cells*. 8.

Nounou, M.I., F. ElAmrawy, N. Ahmed, K. Abdelraouf, S. Goda, and H. Syed-Sha-Qhattal. 2015. Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies. *Breast Cancer (Auckl)*. 9:17-34.

Nowell, P.C. 1986. Mechanisms of tumor progression. *Cancer Res*. 46:2203-2207.

Odeh, L.H., W.H. Talib, and I.A. Basheti. 2018. Synergistic effect of thymoquinone and melatonin against breast cancer implanted in mice. *J Cancer Res Ther*. 14:S324-S330.

Okic-Djordjevic, I., D. Trivanovic, J. Krstic, A. Jaukovic, S. Mojsilovic, J.F. Santibanez, M. Terzic, D. Vesovic, and D. Bugarski. 2013. GE132+Natural: Novel promising dietetic supplement with antiproliferative influence on prostate, colon, and breast cancer cells. *J BUON*. 18:504-510.

Pajovic, B., N. Radojevic, A. Dimitrovski, S. Tomovic, and M. Vukovic. 2016. The therapeutic potential of royal jelly in benign prostatic hyperplasia. Comparison with contemporary literature. *Aging Male*. 19:192-196.

Palmer, A.C., and P.K. Sorger. 2017. Combination Cancer Therapy Can Confer Benefit via Patient-to-Patient Variability without Drug Additivity or Synergy. *Cell*. 171:1678-1691 e1613.

Palmieri, D., Q.R. Smith, P.R. Lockman, J. Bronder, B. Gril, A.F. Chambers, R.J. Weil, and P.S. Steeg. 2006. Brain metastases of breast cancer. *Breast Dis*. 26:139-147.

Patel, R.R., C.G. Sharma, and V.C. Jordan. 2007. Optimizing the antihormonal treatment and prevention of breast cancer. *Breast Cancer*. 14:113-122.

Plenderleith, I.H. 1990. Treating the treatment: toxicity of cancer chemotherapy. *Can Fam Physician*. 36:1827-1830.

Pourmoradian, S., R. Mahdavi, M. Mobasseri, E. Faramarzi, and M. Mobasseri. 2014. Effects of royal jelly supplementation on glycemic control and oxidative stress factors in type 2 diabetic female: a randomized clinical trial. *Chin J Integr Med*. 20:347-352.

Premratanachai, P., and C. Chanchao. 2014. Review of the anticancer activities of bee products. *Asian Pac J Trop Biomed*. 4:337-344.

Pyrzanowska, J., A. Piechal, K. Blecharz-Klin, I. Joniec-Maciejak, K. Graikou, I. Chinou, and E. Widy-Tyszkiewicz. 2014. Long-term administration of Greek Royal Jelly improves spatial memory and influences the concentration of brain neurotransmitters in naturally aged Wistar male rats. *J Ethnopharmacol*. 155:343-351.

Rajput, S., B.N. Kumar, K.K. Dey, I. Pal, A. Parekh, and M. Mandal. 2013. Molecular targeting of Akt by thymoquinone promotes G(1) arrest through translation inhibition of cyclin D1 and induces apoptosis in breast cancer cells. *Life Sci*. 93:783-790.

Rak, J., J. Filmus, G. Finkenzeller, S. Grugel, D. Marme, and R.S. Kerbel. 1995. Oncogenes as inducers of tumor angiogenesis. *Cancer Metastasis Rev*. 14:263-277.

Roepke, M., A. Diestel, K. Bajbouj, D. Walluscheck, P. Schonfeld, A. Roessner, R. Schneider-Stock, and H. Gali-Muhtasib. 2007. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Cancer Biol Ther*. 6:160-169.

Sakalar, C., K. Izgi, B. Iskender, S. Sezen, H. Aksu, M. Cakir, B. Kurt, A. Turan, and H. Canatan. 2016. The combination of thymoquinone and paclitaxel shows anti-tumor activity through the interplay with apoptosis network in triple-negative breast cancer. *Tumour Biol*. 37:4467-4477.

Samavat, H., and M.S. Kurzer. 2015. Estrogen metabolism and breast cancer. *Cancer Lett*. 356:231-243.

Saxe, G.A., C.L. Rock, M.S. Wicha, and D. Schottenfeld. 1999. Diet and risk for breast cancer recurrence and survival. *Breast Cancer Res Treat*. 53:241-253.

Scarselli, R., E. Donadio, M.G. Giuffrida, D. Fortunato, A. Conti, E. Balestreri, R. Felicioli, M. Pinzauti, A.G. Sabatini, and A. Felicioli. 2005. Towards royal jelly proteome. *Proteomics*. 5:769-776.

Schmitzova, J., J. Klaudiny, S. Albert, W. Schroder, W. Schreckengost, J. Hanes, J. Judova, and J. Simuth. 1998. A family of major royal jelly proteins of the honeybee Apis mellifera L. *Cell Mol Life Sci*. 54:1020-1030.

Schneider-Stock, R., I.H. Fakhoury, A.M. Zaki, C.O. El-Baba, and H.U. Gali-Muhtasib. 2014. Thymoquinone: fifty years of success in the battle against cancer models. *Drug Discov Today*. 19:18-30.

Sethi, G., K.S. Ahn, and B.B. Aggarwal. 2008. Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. *Mol Cancer Res*. 6:1059-1070.

Shanmugam, M.K., K.S. Ahn, A. Hsu, C.C. Woo, Y. Yuan, K.H.B. Tan, A. Chinnathambi, T.A. Alahmadi, S.A. Alharbi, A.P.F. Koh, F. Arfuso, R.Y. Huang, L.H.K. Lim, G. Sethi, and A.P. Kumar. 2018. Thymoquinone Inhibits Bone Metastasis of Breast Cancer Cells Through Abrogation of the CXCR4 Signaling Axis. *Front Pharmacol*. 9:1294.

Shidfar, F., S. Jazayeri, S.N. Mousavi, M. Malek, A.F. Hosseini, and B. Khoshpey. 2015. Does Supplementation with Royal Jelly Improve Oxidative Stress and Insulin Resistance in Type 2 Diabetic Patients? *Iran J Public Health*. 44:797-803.

Siavash, M., S. Shokri, S. Haghighi, M. Mohammadi, M.A. Shahtalebi, and Z. Farajzadehgan. 2011. The efficacy of topical Royal Jelly on diabetic foot ulcers healing: A case series. *J Res Med Sci*. 16:904-909.

Siegel RL, M.K., Jemal A. 2020. Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians*.

Silici, S., O. Ekmekcioglu, M. Kanbur, and K. Deniz. 2011. The protective effect of royal jelly against cisplatin-induced renal oxidative stress in rats. *World J Urol*. 29:127-132.

Singh, R.K., M. Gutman, C.D. Bucana, R. Sanchez, N. Llansa, and I.J. Fidler. 1995. Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci U S A*. 92:4562-4566.

Stetler-Stevenson, W.G. 1999. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest*. 103:1237-1241.

Suemaru, K., R. Cui, B. Li, S. Watanabe, K. Okihara, K. Hashimoto, H. Yamada, and H. Araki. 2008. Topical application of royal jelly has a healing effect for 5-fluorouracil-induced experimental oral mucositis in hamsters. *Methods Find Exp Clin Pharmacol*. 30:103-106.

Sultan, M.T., M.S. Butt, R. Karim, W. Ahmed, U. Kaka, S. Ahmad, S. Dewanjee, H.Z. Jaafar, and M. Zia-Ul-Haq. 2015. Nigella sativa fixed and essential oil modulates glutathione redox enzymes in potassium bromate induced oxidative stress. *BMC Complement Altern Med*. 15:330.

Sun, Y.S., Z. Zhao, Z.N. Yang, F. Xu, H.J. Lu, Z.Y. Zhu, W. Shi, J. Jiang, P.P. Yao, and H.P. Zhu. 2017. Risk Factors and Preventions of Breast Cancer. *Int J Biol Sci*. 13:1387-1397.

Sutton, K.M., A.L. Greenshields, and D.W. Hoskin. 2014. Thymoquinone, a bioactive component of black caraway seeds, causes G1 phase cell cycle arrest and

apoptosis in triple-negative breast cancer cells with mutant p53. *Nutr Cancer*. 66:408-418.
Talib, W.H. 2017. Regressions of Breast Carcinoma Syngraft Following Treatment with Piperine in Combination with Thymoquinone. *Sci Pharm*. 85.
Tamura, T., A. Fujii, and N. Kuboyama. 1987. [Antitumor effects of royal jelly (RJ)]. *Nihon Yakurigaku Zasshi*. 89:73-80.
Taniguchi, Y., K. Kohno, S. Inoue, S. Koya-Miyata, I. Okamoto, N. Arai, K. Iwaki, M. Ikeda, and M. Kurimoto. 2003. Oral administration of royal jelly inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice. *Int Immunopharmacol*. 3:1313-1324.
Terada, Y., M. Narukawa, and T. Watanabe. 2011. Specific hydroxy fatty acids in royal jelly activate TRPA1. *J Agric Food Chem*. 59:2627-2635.
Teven, C.M., D.B. Schmid, M. Sisco, J. Ward, and M.A. Howard. 2017. Systemic Therapy for Early-Stage Breast Cancer: What the Plastic Surgeon Should Know. *Eplasty*. 17:e7.
Thiebaut, A.C., V. Kipnis, S.C. Chang, A.F. Subar, F.E. Thompson, P.S. Rosenberg, A.R. Hollenbeck, M. Leitzmann, and A. Schatzkin. 2007. Dietary fat and postmenopausal invasive breast cancer in the National Institutes of Health-AARP Diet and Health Study cohort. *J Natl Cancer Inst*. 99:451-462.
Townsend, G.F., J.F. Morgan, and B. Hazlett. 1959. Activity of 10-hydroxydecenoic acid from royal jelly against experimental leukaemia and ascitic tumours. *Nature*. 183:1270-1271.
Townsend, G.F., J.F. Morgan, S. Tolnai, B. Hazlett, H.J. Morton, and R.W. Shuel. 1960. Studies on the in vitro antitumor activity of fatty acids. I. 10-Hydroxy-2-decenoic acid from royal jelly. *Cancer Res*. 20:503-510.
Vandenberg, L.N., R. Hauser, M. Marcus, N. Olea, and W.V. Welshons. 2007. Human exposure to bisphenol A (BPA). *Reprod Toxicol*. 24:139-177.
Varner, J.A., and D.A. Cheresh. 1996. Integrins and cancer. *Curr Opin Cell Biol*. 8:724-730.
Volpert, O.V., K.M. Dameron, and N. Bouck. 1997. Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene*. 14:1495-1502.
Vucevic, D., E. Melliou, S. Vasilijic, S. Gasic, P. Ivanovski, I. Chinou, and M. Colic. 2007. Fatty acids isolated from royal jelly modulate dendritic cell-mediated immune response in vitro. *Int Immunopharmacol*. 7:1211-1220.
Wang, C., and R.J. Youle. 2009. The role of mitochondria in apoptosis*. *Annu Rev Genet*. 43:95-118.
Weigelt, B., F.C. Geyer, and J.S. Reis-Filho. 2010. Histological types of breast cancer: how special are they? *Mol Oncol*. 4:192-208.
Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell*. 81:323-330.
Werb, Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology. *Cell*. 91:439-442.
Whitelock, J.M., A.D. Murdoch, R.V. Iozzo, and P.A. Underwood. 1996. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. *J Biol Chem*. 271:10079-10086.
WHO, W.H.O. 2018. Newsroom,Key facts, Cancer.

WHO, W.H.O. 2020a. Cancer,Cancer prevention,Early Diagnosis and Screening, Breast Cancer.
WHO, W.H.O. 2020b. WHO-CancerReport-2020-Global Profile. World Health Organization.
WHO, W.H.O. 2020c. WHO report on cancer: setting priorities, investing wisely and providing care for all.
Woo, C.C., A. Hsu, A.P. Kumar, G. Sethi, and K.H. Tan. 2013. Thymoquinone inhibits tumor growth and induces apoptosis in a breast cancer xenograft mouse model: the role of p38 MAPK and ROS. *PLoS One*. 8:e75356.
Wright, L.E., P.D. Ottewell, N. Rucci, O. Peyruchaud, G.M. Pagnotti, A. Chiechi, J.T. Buijs, and J.A. Sterling. 2016. Murine models of breast cancer bone metastasis. *Bonekey Rep*. 5:804.
Yamauchi, K., Y. Kogashiwa, Y. Moro, and N. Kohno. 2014. The effect of topical application of royal jelly on chemoradiotherapy-induced mucositis in head and neck cancer: a preliminary study. *Int J Otolaryngol*. 2014:974967.
Yang, X., T. Takeshita, and K. Morimoto. 2000. N-Acetyltransferase polymorphism and human cancer risk. *Environ Health Prev Med*. 4:165-173.
Yang, Y.C., W.M. Chou, D.A. Widowati, I.P. Lin, and C.C. Peng. 2018. 10-hydroxy-2-decenoic acid of royal jelly exhibits bactericide and anti-inflammatory activity in human colon cancer cells. *BMC Complement Altern Med*. 18:202.
Yao, Y., Y. Chu, B. Xu, Q. Hu, and Q. Song. 2019. Risk factors for distant metastasis of patients with primary triple-negative breast cancer. *Biosci Rep*. 39.
Yi, T., S.G. Cho, Z. Yi, X. Pang, M. Rodriguez, Y. Wang, G. Sethi, B.B. Aggarwal, and M. Liu. 2008. Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways. *Mol Cancer Ther*. 7:1789-1796.
Yimer, E.M., K.B. Tuem, A. Karim, N. Ur-Rehman, and F. Anwar. 2019. Nigella sativa L. (Black Cumin): A Promising Natural Remedy for Wide Range of Illnesses. *Evid Based Complement Alternat Med*. 2019:1528635.
You, M.M., Y.F. Chen, Y.M. Pan, Y.C. Liu, J. Tu, K. Wang, and F.L. Hu. 2018. Royal Jelly Attenuates LPS-Induced Inflammation in BV-2 Microglial Cells through Modulating NF-kappaB and p38/JNK Signaling Pathways. *Mediators Inflamm*. 2018:7834381.
Zachary, I. 2003. VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem Soc Trans*. 31:1171-1177.
Zargar, H.R., A.A. Hemmati, M. Ghafourian, A. Arzi, A. Rezaie, and S.A. Javad-Moosavi. 2017. Long-term treatment with royal jelly improves bleomycin-induced pulmonary fibrosis in rats. *Can J Physiol Pharmacol*. 95:23-31.
Zhang, L., B. Han, R. Li, X. Lu, A. Nie, L. Guo, Y. Fang, M. Feng, and J. Li. 2014. Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly. *BMC Genomics*. 15:135.
Zhang, S., Q. Shao, H. Geng, and S. Su. 2017. The effect of royal jelly on the growth of breast cancer in mice. *Oncol Lett*. 14:7615-7621.
Zhang, X., Y. Yu, P. Sun, Z. Fan, W. Zhang, and C. Feng. 2019. Royal jelly peptides: potential inhibitors of beta-secretase in N2a/APP695swe cells. *Sci Rep*. 9:168.