Cytotoxicity of Doxorubicin (Adriamycin), Cyclophosphamide, alpha and gamma tocopherol towards breast and granulosa tumor-derived cell lines *in vitro*

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DECLARATION

I certify that this thesis does not contain any material previously submitted for any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due references has been made in the text.

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ABSTRACT

The combination chemotherapy of doxorubicin (DOX) and cyclophosphamide (CYC) (AC) is the most commonly used regime for treating breast cancer, but it can cause premature ovary failure and infertility in premenopausal breast cancer survivors. AC is cytotoxic to tumor cells and each causes DNA fragmentation, ROS generation and apoptosis. Tocopherols are antioxidants which can reduce the damage caused by ROS generation. **The objectives**: to examine if DOX (5, 10 and 25uM), HCYC (0.5, 1 and 2.5uM), alpha tocopherol (A-TOC) (50, 75 and 100uM) or gamma tocopherol (G-TOC) (50, 75 and 100uM) alone will cause dose-dependent cytotoxicity in MCF-7 breast cancer cells and KGN granulosa cells; if the combination of AC or AC plus tocopherols will be more cytotoxic than either chemotherapeutic alone; if the EC25, EC50 and EC75 values of DOX, HCYC, A-TOC and G-TOC cause a dose-dependent increase in apoptosis, and if the combination of EC25 value of DOX and EC25 value of HCYC will be cytotoxic more than 50% of MCF-7 cells and if the combination of EC25 values of DOX, EC25 value of HCYC and A-TOC or G-TOC will be cytotoxic more than 75% of MCF-7 cells. **The methods**: applied the crystal violet (CV) and MTT cytotoxicity assays to examine different doses of DOX, HCYC, A-TOC and G-TOC and their combinations at four different exposure periods which were 24hr exposure, followed by 24hr or 48hr culture, and 72hr continuous exposure, in MCF-7 and KGN cell lines; use the CV assay to determine the EC25, EC50 and EC75 values of DOX, HCYC, A-TOC and G-TOC after 24hr exposure or 24hr exposure with 24hr culture in MCF-7 cells; use Annexin V and PI staining to examine MCF-7 cells apoptosis after exposure to different doses of chemotherapeutics and tocopherols, and their combinations corresponding to their EC25, EC50 and EC75 values. **The result**: in both the CV and MTT assays all tested concentrations of HCYC and A-TOC were not cytotoxic, but DOX (5, 10 and 25uM) was significantly cytotoxic to MCF-7 and KGN cells. G-TOC was

cytotoxic to MCF-7 cells but not to KGN cells. The combinations of AC or AC with tocopherols did not have synergistic cytotoxicity towards MCF-7 and KGN cells except when a lower initial seeding density (10,000 cells/well) was used. In these conditions the combination of AC had a synergism effect in the MTT assay after 24hr exposure to MCF-7 cells, and the combination of AC or the combination of AC with alpha or gamma tocopherols had synergistic cytotoxicity to KGN cells. The EC50 value of DOX was 3.63uM after 24hr exposure and 3.24uM after 24hr exposure with 24hr culture. The EC50 value of HCYC was 63.69uM after 24hr exposure and 54.65uM after 24hr exposure with 24hr culture. The EC50 value of G-TOC was 105.3uM after 24hr exposure and 78.13uM after 24hr exposure with 24hr culture. The EC50 value could not be determined for A-TOC as the highest concentration tested, 500uM, was still not cytotoxic to MCF-7 cells. DOX, HCYC, A-TOC and G-TOC and their combinations did not cause MCF-7cell apoptosis. In the single experiment replicate that could be complicated in the time available.

KEYWORDS

Breast cancer, Doxorubicin, Cyclophosphamide, vitamin E, alpha tocopherol, gamma tocopherol, Apoptosis, ROS, MCF7, KGN, Crystal Violet assay, MTT

ABBREVIATIONS

µl	Microliter
µg	Microgram
%	Percentage
A	Adriamycin
AC	Doxorubicin (Adriamycin) and Cyclophosphamide
ACF	Doxorubicin, Cyclophosphamide and 5-Fluorouracil
AMH	Anti mullerian hormone
ANOVA	Analysis of Variance
A-TOC	Alpha Tocopherol
ATCC	American Type Culture Collection
BLE	Bleomycin
℃	Degree Celsius
CMF	Cyclophosphamide, Methotrexate and 5-Fluorouracil
CV	Crystal Violet
CYC	Cyclophosphamide
DCIS	Ductal Carcinoma in Situ
DMEM/F12	Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
EC25	25% Effect Concentration
EC50	50% Effect Concentration
EC75	75% Effect Concentration
ER	Estrogen Receptor
EtOH	Ethanol
FBS	Fetal Bovine Serum
g	Gram
G-TOC	Gamma Tocopherol
HCL	Hydrochloric Acid
HCYC	4-Hydroxycyclophosphamide
HER2	Human Epidermal Growth Factor Receptor 2
H_2O_2	Hydrogen Peroxide
hr	Hour
IC50	50% inhibitory concentration
IDC	Invasive Ductal Carcinoma
ILCs	Invasive Lobular Carcinoma
ITS	Insulin-Transferrin-Selenium
IV	Intravenously
L	Litre
LCIS	Lobular Carcinoma in Situ
MAPK	Mitogen-activated Protein Kinase
MF	Methotrexate and 5-Fluorouracil

MK5	Mitogen-activated Protein Kinase -activated Protein Kinase 5
ml	Millilitre
MOA	Mechanism of Action
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NPXs	NAD(P)H Oxidases
O^-	Singlet Oxide
O_2^-	Peroxide Anions
OD	Optical Density
OH^-	Hydroxyl Radicals
P	Cisplatin
PBS	Phosphate Buffer Saline
PD	Paget Disease
PI	Propidium Iodide
PR	Progesterone Receptor
PS	Phosphatidylserine
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
T	Docetaxel
TAC	Docetaxel, Doxorubicin and Cyclophosphamide
TOC	Tocopherol
TOP2	Topoisomerase II
uM	Micromole per Litre
v/v	Volume/Volume

CHAPTER 1: Introduction and Literature Review

1. Introduction and Literature Review

Breast cancer is the most common cancer in women in the world (Ghoncheh et al., 2015), and it is commonly treated with a combined regime of Doxorubicin (Adriamycin) and Cyclophosphamide (AC) (Fisher et al., 2004, Senchukova et al., 2015), which cause premature ovary failure and infertility in premenopausal breast cancer survivors (Ezoe et al., 2014, Gurgen et al., 2013, Roti et al., 2012). This study is part of a large project to determine if co-administration of tocopherols with AC will reduce the adverse side-effects on fertility whilst maintaining anti-cancer activity.

1.1 Incidence of breast cancer

More than 1.3 million new cases of breast cancer are diagnosed world-wide each year (Ghoncheh et al., 2015). In 2007 about 178,480 women had breast cancer in the USA, and premenopausal women comprised one-third of these (Anders et al., 2008). In recent decades, the cases of premenopausal breast cancer in Asia have increased to 53% of all breast cancer patients (Youlden et al., 2014). The global incidence of breast cancer is around 12% in women aged 20–34 years (Hickey et al., 2009). In the USA, breast cancer survival rates have increased (Yu et al., 2010), particularly in premenopausal women (Comish et al., 2014, Ezoe et al., 2014).

There are many types of breast cancers (Table 1.1), which can be divided into subtypes based on molecular criteria; estrogen receptor (ER)-positive and ER-negative breast cancers, progesterone receptor (PR) cancers and human epidermal growth factor receptor 2 positive and negative (HER2) breast cancers (Anderson et al., 2011, Kahan et al., 2005, Senchukova et al., 2015).

Table 1.1. Types of breast cancer

Types	Incidence	Characteristic
1. Non-invasive (in situ) Breast cancer		
a) Ductal Carcinoma in Situ (DCIS)	81% of pre-invasive carcinomas (Rosen et al., 1980).	Can be cured, not life-threatening, and a mammogram is the best way to find DCIS early (Fisher et al., 2001, Rosen et al., 1980).
b) Lobular Carcinoma in Situ (LCIS)	19% of pre-invasive carcinomas (Rosen et al., 1980)..	LCIS is not a true cancer and does not directly progress to cancer; common in premenopausal women (Carolin et al., 2002).
2. Invasive Breast Cancer		
a. Invasive Ductal Carcinoma (IDC)	85% to 90% of invasive carcinomas are IDC (Carlson et al., 2011).	It starts in a milk duct of the breast, grows through the wall of the duct, and spreads into the fatty tissue of the breast (Yoder et al., 2007).
b. Invasive Lobular Carcinoma (ILCs)	Approximately 5–15% of breast cancers are ILC (Rakha et al., 2008).	ILC is associated with older age, larger tumour size, lower grade, positive expression of steroid receptors, and lack of expression of the P53 and HER2 genes (Rakha et al., 2008).
3. Locally Advanced Breast Cancer	About 5% to 15% of new breast cancer cases in the USA (Esteva and Hortobagyi, 1999). About	It has a large tumor (>5 cm) and/or involves tissues around the breast such as the skin, muscle, ribs or

	10–20% of the 13,000 cases of breast cancer in Australia each year (AIHW, 2012).	chest wall (El Saghir et al., 2008, Esteva and Hortobagyi, 1999).
4. Paget disease (PD) of the nipple	Approximately 1-4% of all cases of breast cancer (Gunhan-Bilgen and Oktay, 2006, Kothari et al., 2002).	PD starts in the ducts of the nipple then spreads to the nipple surface and the areola, which cause redness, itching, scaling, and burning on the skin of the nipple (Gunhan-Bilgen and Oktay, 2006, Kanitakis, 2007, Kothari et al., 2002). The majority of PD patients have invasive breast cancer and 40-45% have DCIS (Ling et al., 2013).

It has been proposed that ER-positive and ER-negative are the main types of breast cancers, and that ER expression is the best characterize to direct breast cancer treatment (Kahan et al., 2005). The incidence of ER-negative breast cancers in the USA decreased by 11.2% from 2009 to 2016 (Anderson et al., 2011), but the incidence of ER- positive breast cancers is predicted to remain at high levels in the USA (Anderson et al., 2011). ER-positive breast cancers, approximately 75% of all breast cancers (Flageng et al., 2015), are treated with the combined chemotherapeutic regime of doxorubicin and cyclophosphamide (AC).

1.2 Breast cancer and mortality

The survival rates for breast cancer patients are affected by age, economy, culture and the availability of breast cancer scanning programs. In developed countries such as the USA, the mortality rate is about 20%, whereas it is around 60% in

developing countries (Ghoncheh et al., 2015). About 522,000 breast cancer patients died during 2012 in the whole world, but mortality rates varied according to location (Table 1.2) (Ghoncheh et al., 2015, Youlden et al., 2014). Mortality rates have declined by about 2% each year in Australia and New Zealand during the last decade, but in China mortality rates have increased (Youlden et al., 2014).

Table 1.2. Breast cancer mortality rates in the Asia-Pacific region during 2012

Region	Mortality rate
China	41%
Indonesia	17%
Australia and New Zealand	16%
Japan	12%

Data taken from Youlden et al (2014).

Mortality rates vary according to the type of breast cancer (Table 1.3) and HER2 positive breast cancer patients have the lowest survival rates (Spanheimer et al., 2013). One clinical trial which compared treatment with methotrexate and 5-fluorouracil (MF), and with cyclophosphamide and MF (CMF) and with AC, found that the survival rates were different according to the ages of the patients (Fisher et al., 2004). In this clinical trial, 17.1% were premenopausal women. Survival rates were higher when treated with AC or CMF than with MF at any age (Fisher et al., 2004). The survival rates of the AC group were 11% and 5% in premenopausal and postmenopausal women, respectively (Fisher et al., 2004).

Table 1.3 Mortality rate based on the type of breast cancer

Types of Breast Cancer	ER+/ HER2-	ER+/ HER2+	ER-/ HER2+	ER-/ HER2-
Patients	39 (54%)	4 (6%)	10 (14%)	19 (26%)
Mortality Percentage	2 (5%)	1 (25%)	3 (30%)	2 (11%)

72 patients response to chemotherapy, the combination of doxorubicin, cyclophosphamide and paclitaxel, followed by surgery. Data taken from (Spanheimer et al., 2013).

1.3 Current treatment of breast cancer

There are many treatment methods for breast cancer including radiotherapy, surgery, endocrine therapy and chemotherapy (Yuan et al., 2011). Chemotherapy is a common approach to treating many types of breast cancers (Fisher et al., 2004, Hikino et al., 2006), and there are many types: Cisplatin (P), Docetaxel (T), Bleomycin (BLE), Doxorubicin (DOX) (previously known as Adriamycin (A)), cyclophosphamide (CYC), Methotrexate and 5-fluorouracil (MF).

These are usually used in combination; commonly MF, CMF, AC, ACF or TAC (Fisher et al., 2004, Hikino et al., 2006, Kasapovic et al., 2010). DOX is widely used for treating breast cancers in combination with other anticancer drugs (Chegaev et al., 2013). It was first used in clinical trials in the 1960's and is still used to treat around 50% of breast cancers in premenopausal women (Roti et al., 2012).However, AC is the most common regime for treating breast cancer**,** and the concentrations of AC are administered according to the size and surface area of the patient; DOX ($60mg/m^2$) is infused intravenously (IV) first, followed by $600mg/m^2$ CYC; this is commonly repeated every 14 days for 4 cycles (John and Schwartz, 2016, Swenson et al., 2003). After the in *vivo* administration of doxorubicin $60\ mg/m^2$ and CYC $600\ mg/m^2$, the plasma concentrations were 1.8 ± 0.4uM and 0.02uM respectively in the 24hr period after administration (Struck et al., 1987, Swenson et al., 2003). In *vivo* CYC is hydrolyzed by hepatic cytochrome P450 enzymes to the pharmacologically active form, 4-hydroxycyclophosphamide (HCYC) (Madondo et al., 2016). Human primary-derived peripheral blood mononucleocytes exposed to 30nmol/ml 4-hydroxycyclophosphamide (HCYC) for 1hr had sufficient DNA cross-linking to decrease cell viability (Ozer et al., 1982). For this study we assumed that therapeutic serum concentrations were likely to be the highest possible concentrations acting on cells *in vivo* (Fisher et al., 2004, Skrablin et al., 2007).

1.4 Apoptosis and Necrosis

Apoptosis, also called programmed cell death, is a widespread phenomenon in the mammalian and plant cells (Morad, 2010). When self-destruction signals such as reactive oxygen species (ROS) or DNA damage are generated, cells activate the apoptosis program and commit suicide without causing inflammation (Abou-Ghali and Stiban, 2015). Apoptosis eliminates damaged cells (Castejón, 2015, Sun et al., 2009). When cells undergo apoptosis, they show typical morphological changes, including plasma membrane blebbing, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies (Ruan et al., 2012). In early apoptosis phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. This is followed by a change in mitochondrial membrane permeability, release of mitochondrial proteins, caspase-dependent activation, and DNA cleavage (Castejón, 2015, Ruan et al., 2012).

Apoptosis has two major apoptotic pathways (Figure 1.1): the extrinsic or death receptor pathway (signals coming from outside the cell) and the intrinsic or mitochondrial pathway (signals coming from inside the cell) (Abou-Ghali and Stiban, 2015, Morad, 2010, Siddik, 2014). The extrinsic pathway involves cascades of caspase activation. Then, cells execute the cleavage of different nuclear and cytoplasmic substrates. In intrinsic apoptosis, signals induced by stimuli causes the permeabilization of the outer membrane of the mitochondria, and this is mediated by members of the Bcl-2 family of proteins (Abou-Ghali and Stiban, 2015, Morad, 2010).

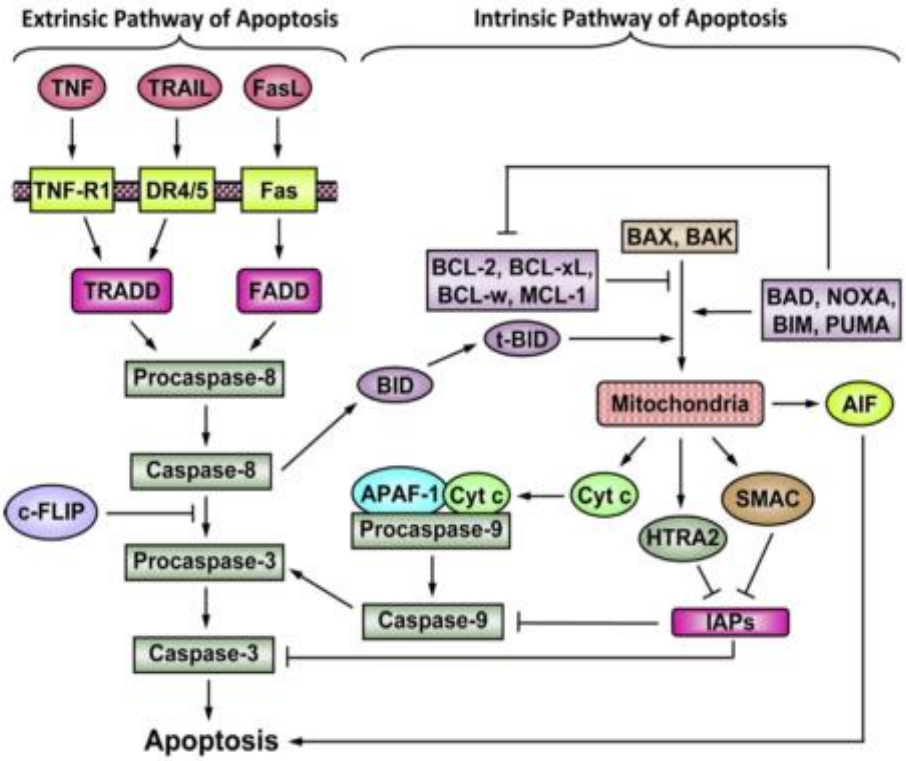


Figure 1.1 Extrinsic and intrinsic pathways of apoptosis. The two pathways are independent but connected through BID, and they share caspase-3 as the common effector protease (Siddik, 2014).

Necrosis is considered to be a toxic process in which the cell is a passive victim and follows an energy-independent mode of death which is the alternative to apoptotic cell death (Elmore, 2007, Morad, 2010). Table 1.4 shows the comparison between apoptosis and necrosis and Figure 1.2 shows the morphological characteristics of apoptosis and necrosis.

Table 1.4 Apoptosis and necrosis (Elmore, 2007, Morad, 2010)

Apoptosis	**Necrosis**
Normal of healthy cells programmed cell death through apoptosis and it is common phenomena in human bodies. It is normal and useful when healing process happens and undergoes apoptosis which can used as a defense mechanism.	External factors or illness, such as toxins or infections cause necrosis, and this process is abnormal and harmful. Sometimes necrosis is also studied as a natural process, but normally researched it as unnatural process.
In apoptosis, cell shrinks in volume and chromatin condenses, membrane budding occurs and many apoptotic bodies form. Then, it is swallowed by immune cells. It is an energy dependent process.	Membrane becomes leaky, cell swells and breaks up, and it releases many cell organelles into surroundings resulting in inflammation. It does not use ATP energy.
It does not cause inflammation so there are no obvious symptoms in this process. Signals are generated by apoptotic cell itself. It is natural process of life, and mitosis can initiate its continually cellular cycle. Normally do not need any treatment.	It can cause inflammation which reduces blood flow at affected areas and needs to remove necrotic tissues. It needs medical treatment. Untreated necrosis results in cell death or dead tissues.

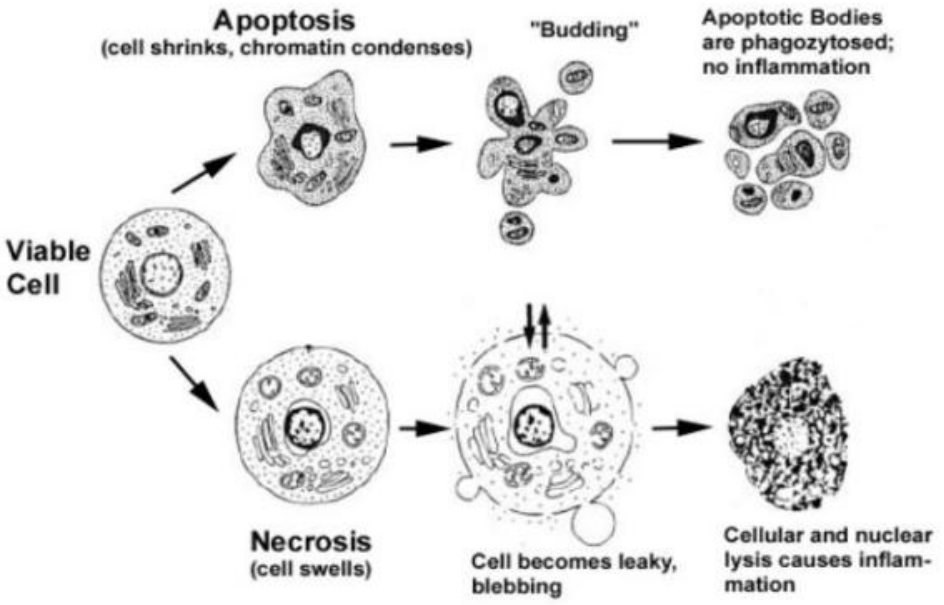


Figure 1.2 Diagram of the morphological characteristics of apoptosis and necrosis. Apoptosis causes cellular shrinks in volume, chromatin condenses and membrane budding occurs and many apoptotic bodies form without inducing inflammation. The necrotic cell swells, becomes leaky and breaks up, and releases its cell organelles into the surrounding tissues and causes inflammation (Morad, 2010).

1.5 Chemotherapeutic mechanism of action of AC

1.5.1 Mechanism of action (MOA) of doxorubicin

1.5.1.1Reactive oxygen species (ROS) generation

Doxorubicin causes ROS to be generated in many different ways and uses various biological systems to produce peroxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxide (O^-) (Chegaev et al., 2013). These free radicals attack soluble cell compounds and membranes and can reduce cell function. This eventually leads to necrosis, apoptosis or cell death (Ince et al., 2014, Madondo et al., 2016, Radomska-Lesniewska et al., 2016). Free radicals are generated when doxorubicin activates NAD(P)H oxidases (NPXs) or through a non-enzymatic mechanism involving iron-anthracycline complex (Chegaev et al., 2013, Park et al., 2012). In *vivo* DOX is transformed to doxorubicinol and this induces the release of iron from cytoplasmic aconitate which leads to ROS

generation (Chegaev et al., 2013). ROS oxidize DNA bases, which causes dozens of major DNA base changes such as thymine glycol and 5-hydroxymethyl-2'-deoxyuridine, and subsequent mismatches during DNA replication and mutagenesis. The oxidative DNA damage also can cause deletions by the base excision repair pathway (Swift and Golsteyn, 2014).

1.5.1.2 Topoisomerase II (TOP2) DNA Fragmentation

Topoisomerases regulate the overwinding or underwinding of DNA supercoiling during DNA replication and transcription (Swift and Golsteyn, 2014). There are two types of topoisomerases, type I and type II. TOP2 cuts both strands of the DNA helix simultaneously in order to manage DNA tangles and supercoils (Hurley, 2002). TOP2 catalytic inhibitors (Figure 1.3), like doxorubicin, transiently bind to the TOP2 cleavage complexes and prevent religation of the DNA strand breaks (Swift and Golsteyn, 2014). It is believed that DOX interacts with TOP2 to trap protein which is covalently bound to DNA (Hurley, 2002, Swift, 2006).

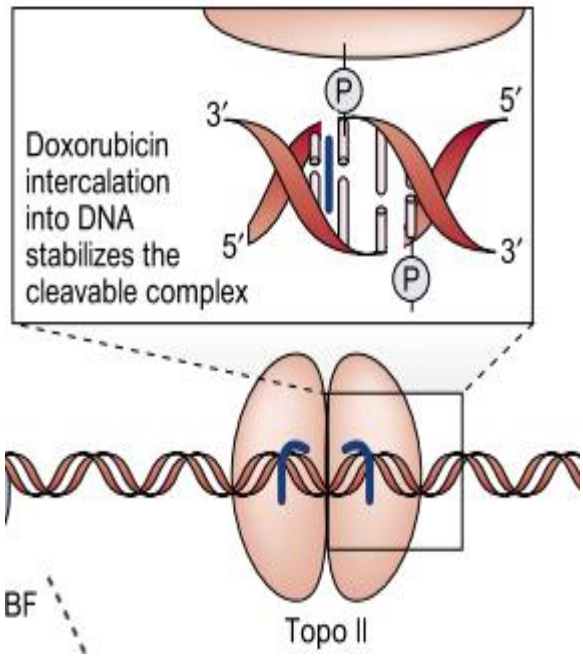


Figure 1.3 DNA-interactive drug, DOX, targets DNA transcription. DOX interacts with, and stabilizes, the TOP2–DNA complexes to produce a ‘cleavable complex’, as DNA strand breakage is associated with the protein–DNA complex

(Hurley, 2002).

1.5.1.3 Induction of apoptosis by Doxorubicin

DOX inhibits DNA replication by intercalation into the DNA strands, and this triggers apoptosis in cancer cells (Swift and Golsteyn, 2014, Swift, 2006, Zhou et al., 2012). DOX-induced apoptosis has been reported in many ways; the activation of caspases and disruption of mitochondrial membrane potential (Zhou et al., 2012); DOX leads to MAPK5-deficiency by the 26S proteasome pathway in skin, colorectal and hepatoma tumor cells induced apoptosis (Zhou et al., 2012); and various mutations of p53 and attenuation of p53 activation (Eom et al., 2005, Kugawa et al., 2004, Wang et al., 2004). When DOX activates p53, it promotes apoptosis of tumor cells (Wang et al., 2004)

1.5.2 MOA of cyclophosphamide (CYC)

CYC is one of the most efficient and commonly used chemotherapeutic drugs and is listed on the World Health Organisations List of Essential Medicines (Madondo et al., 2016). Its direct cytotoxic effect on tumors means that it has been used to treat a range of cancers including lymphomas, breast and ovarian cancer. It has also been used as an immunosuppressive drug after organ transplantations (Ince et al., 2014, Madondo et al., 2016). CYC is currently used in combination with other drugs to treat breast cancers (Fisher et al., 2004, Madondo et al., 2016). It has a number of MOAs, including ROS generation, DNA damage and T cell depletion (Ince et al., 2014, Madondo et al., 2016). CYC is hydrolysed by the hepatic cytochrome enzymes P450 to become the pharmacologically active form, 4-hydroxycyclophosphamide (HCYC) in *vivo* (Madondo et al., 2016). CYC and HCYC have been previously shown to have similar cytotoxic activity and cause comparable cross-linking of DNA at equimolar concentrations (Ozer et al., 1982).

1.5.2.1 CYC induced ROS generation

CYC can cause the generation of ROS like superoxide anion, OH^- and H_2O_2 (Ince et al., 2014). CYC-induced ROS generation causes oxidative stress, which leads to cardiotoxicity and nephrotoxicity (Ince et al., 2014, Mansour et al., 2015). The

active form of CYC, HCYC, generates ROS, such as the superoxide anion. This is thought to change cell redox balance, which causes oxidative stress, which damages cancer and healthy cells. The superoxide anion can react with other radicals such as nitric oxide, and produce peroxynitrite in the cytoplasm and mitochondria. Peroxynitrite can affect mitochondrial enzymes and cause the production of more intracellular ROS in the mitochondria (Mansour et al., 2015).

1.5.2.2 CYC induced DNA-damage

The amount of DNA damage caused by CYC depends on the dose of CYC and the time of exposure to the drug (Madondo et al., 2016). The occurrence of inter-strand cross linking has a dose - dependent relationship to the concentration of CYC up to 50 μM. When the concentrations of CYC were higher, DNA cross-linking declined but there was an onset of DNA strand breakage in *vitro* (Madondo et al., 2016). In *vivo*, DNA crosslinking is cytotoxic and can cause cell death either by apoptosis or necrosis, depending on stage of the cell cycle (Madondo et al., 2016). CYC or its metabolites, like HCYC, phosphoramide mustard and acrolein, can bind DNA and cause chromosome breaks, micronucleus formation and cell apoptosis (Ince et al., 2014).

1.5.2.3 CYC induced apoptosis

CYC has perversely been reported to trigger apoptosis in the human breast cancer cell line MCF7 (Kugawa et al., 2004). 5 and 10mM (these are very high doses) of CYC were exposed for 48hr after seeding MCF-7 cells for 48hr, and they caused dose-dependent manner on inducing apoptosis when analyzed using annexin-V-FITC and PI staining analysis (Singh et al., 2009). One of the important anti-tumor effects of CYC is to induce tumor cell apoptosis which is mediated by caspase (Yang et al., 2011), and by p38 MAPK pathway activation (Pang et al., 2011).

1.5.2.4 T cell depletion & attenuation of T cells

Several studies maintain that CYC depletes a suppressive T cell population, or

attenuates their function (Madondo et al., 2016). Regulatory T cells help tumor cells evade the immune system, so their presence is related to the progression of cancer (Madondo et al., 2016). The depletion of T cells or attenuation of T cell function can improve immune response to cancer and promote anticancer efficacy.

1.5.3 MOA for the combination of AC

There is no information about the relationship of these different MOAs to each other, nor their relative contribution to the chemotherapeutic effect. To our knowledge, there are no publications reporting on the MoA by which the combined chemotherapeutic treatment “AC” exerts anticancer effects.

1.6 Adverse side – effects of AC

DOX can cause many adverse side effects including fever, dizziness, lack of concentration, infection, cardiotoxicity and nephrotoxicity (Dhingra et al., 2014, Petrovic, 2015). CYC alone causes numerous side effects such as hemorrhagic cystitis, lung damage, cardiotoxicity and nephrotoxicity (Madondo et al., 2016, Mansour et al., 2015). When taking large doses of the combination AC chemotherapeutic agent, most of the breast cancer patients suffer all side effects (Fridrik et al., 2016) as well as nausea, fatigue, headache, menopausal problems, ovarian failure, infertility and alopecia since the non-specific distribution of AC in the body simultaneously impact both the tumor cells and the healthy cells (Banu et al., 2015). The antioxidant, α-tocopherol, reduced cardiotoxicity caused by adriamycin, but did not reduce anti-cancer efficacy which suggested that ROS caused adverse side-effects (Legha et al., 1982).

1.6.1 Effect of chemotherapy on ovary function

The ovary contains primordial, primary, secondary and antral follicles. Follicles are the basic functional units of the mammalian ovary (Forabosco and Sforza, 2007, Lindeberg et al., 2007). Primordial follicles contain 1 layer of quiescent granulosa cells around one oocyte. Primary follicles contain an activated single

layer of proliferating granulosa cells and secondary follicles contain 2 to 4 layers of proliferating granulosa cells. The mature antral follicles contain a fluid-filled antrum and can undergo ovulation (Lindeberg et al., 2007). Chemotherapeutic drugs can target rapidly dividing follicular granulosa cells (Comish et al., 2014).

Chemotherapy can cause amenorrhea, menopause or infertility depending on the patient's age and type and dose of treatment received (Gurgen et al., 2013). For example, older women are at a higher risk of infertility (Yu et al., 2010). The use of AC to treat breast cancer poses a lower risk to ovarian function than CMF; reported the survival rate as 69 % of AC compared with 34 % of CMF (Anders et al., 2008). The influence of chemotherapy on ovarian function is an important issue in the care of young breast cancer survivors.

Anti mullerian hormone (AMH) is secreted by granulosa cells of small growing follicles (secondary follicle) but not by larger developing follicles (Sedes et al., 2013). AMH suppresses the activiation of primordial follicles and regulates the primordial to growing follicle transition (Browne et al., 2011). In healthy, premenopausal females, the amount of AMH does not vary significantly at different phases of menstrual cycle (Anders et al., 2008). Hence, AMH can be a valuable marker for predicting oocyte numbers and quality in premenopausal women (Anders et al., 2008, Browne et al., 2011, Yu et al., 2010). Chemotherapy for premenopausal breast cancer patients decreases AMH levels rapidly, and it is thought this is because the proliferating granulosa cells of secondary follicles are killed directly by anticancer drugs (Hu et al., 2014). Reduced AMH levels result in the transition of primordial follicles and their entry into the growing pool. These in turn are killed by subsequent cycles of chemotherapy (Hu et al., 2014, Sedes et al., 2013).

1.6.2 ROS and infertility

Both DOX and CYC generate ROS, but ROS impair steroid hormone synthesis pathways such as these found in follicular granulosa cells (Young et al., 1995).

When ROS are at high levels, they cause oxidative stress and reduce female fertility in animal and *in vitro* models (Rudera et al., 2009).

In steroid hormone synthesis, the first step is the cleavage of the side-chain of the cholesterol molecule by cytochrome P450 side chain cleavage enzyme (P450scc). However, oxygen free radicals can cause damage such as cross-linking between P450scc and adrenodoxin (Rodgers et al., 1995, Young et al., 1995), which inhibits P450scc activity. In the cell adrenodoxin normally dissociates from the P450scc after electron transfer and reassociates with the other member of the electron transport chain, adrenodoxin reductase, to receive another electron. ROS impairment of this electron transport chain is prevented by intracellular antioxidants (superoxide dismutase and catalyase) or by other antioxidants or free radical scavengers such as vitamins A, C or E or β-carotene. The addition of more ROS generated by AC activity may overwhelm intracellular antioxidant capabilities, and shut down hormone synthesis pathways (Young et al., 1995).

1.7 Antioxidant-Vitamin E

1.7.1 Antioxidant-tocopherols and fertility

Vitamin E is the common name for the family of tocopherol molecules; α, β, γ and δ (Yang et al., 2010). These are antioxidants that can counteract oxidative stress (Nechuta et al., 2011). Bovine ovaries have high levels of the antioxidant β-carotene which prevented cross-linking between P450scc and adrenodoxin (Young et al., 1995). In addition, vitamin A and E deficiencies in many species cause a reduction in steroid hormone production with subsequent infertility (Young et al., 1995). Oxidative stress is associated with increased cell lipid peroxidation and decrease in Vitamin C and E (Kasapovic et al., 2010).

1.7.2 γ-tocopherol induced apoptosis

Gamma tocopherol (G-TOC) inhibited proliferation of breast cancer cells *in vitro* (Lee et al., 2009, Smolarek and Suh, 2011) and all anti-tumor activity in animal

model of colon and prostate cancer (Smolarek and Suh, 2011) and delayed the formation of breast cancer tumors in animal model (Smolarek et al., 2012).

Gamma tocotrienol induced apoptosis by activation of caspase-3 (Patacsil et al., 2012) and in adenocarcinoma cells (Sun et al., 2009) and in colon carcinoma HT 29 cells (Xu et al., 2009). The previous study reported G-TOC reduces the growth of human large-cell lung carcinoma cells *in vivo* and *in vitro*, whereas α tocopherol had no effect on cell viability (Yang et al., 2010). G-TOC inhibited lung tumor growth in mice by inducing caspases mediated apoptosis (Yang et al., 2010) and induced apoptosis in MCF-7 cells when assessed using phosphatidylserine (PS) externalization using Annexin V-FITC. Exposure to 40uM of G-TOC for 48 or 72hr induced 28% and 48% annexin V positive to MCF-7 cells (Yu et al., 2008).

The idea of this project is that the antioxidant can reduce AC induced ROS generation and maintain follicle hormone synthesis, whilst maintaining apoptosis and anti-cancer activity.

1.8 Cell lines

1.8.1 MCF7 cell line

The human hormone-responsive MCF7 breast cancer cell line, was derived from a pleural effusion of a patient with breast cancer in 1970 (Ray et al., 2006). The MCF7 cell line has been used to show effects of anticancer drugs (Chegaev et al., 2013, Petrovic et al., 2015, Ray et al., 2006, Roti et al., 2012). MCF7 cells have a short proliferating rate, around 33hr (Thomas et al., 1992).

1.8.2 KGN cell line

In 1984 a 63-yr-old woman was diagnosed with ovarian cancer stage III, indicative of a granulosa cell carcinoma (Ernst et al., 2016, Nishi et al., 2001). The KGN cell line can secrete pregnenolone and progesterone and the

steroidogenesis pathway is analogous to that in human granulosa cells. The KGN cells have a proliferation rate of 46.4h (Nishi et al., 2001).

1.9 Measures of cell viability

1.9.1 Crystal violet assay

The crystal violet (CV) assay is a colorimetric method that uses crystal violet (Tris (4-(dimethylamino) phenyl) methylium chloride) to stain DNA-associated proteins in cell nuclei and then applies acetic acid to solubilize the cells and dye. Subsequent optical density measurements provide a measure of the relative number of viable cells (Reid et al., 2015, Vandersickel et al., 2011). Crystal violet assays have been used to detect cell viability or evaluate cytotoxicity after exposure to toxins of different tumor cell lines (Kitagaki et al., 2006, Lison et al., 1989, Reid et al., 2015, Vandersickel et al., 2011). Crystal violet assays show a good linear correlation between cell number and optical density, and are rapid, sensitive, objective and easy to perform (Reid et al., 2015, Lison et al., 1989, Vandersickel et al., 2011).

1.9.2 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been used to measure cell viability or drug cytotoxicity for around 30yrs, and is based on the abundance and activity of the mitochondrial enzyme succinate dehydrogenase in viable cells. This enzyme converts water-soluble yellow MTT into purple formazan crystals (Figure 1.4) that are not soluble in the aqueous solutions typical of *in vitro* cell culture system (Angius and Floris, 2015, Boncler et al., 2014, Reid et al., 2015, Soman et al., 2009, Stepanenko and Dmitrenko, 2015, Stockert et al., 2012).

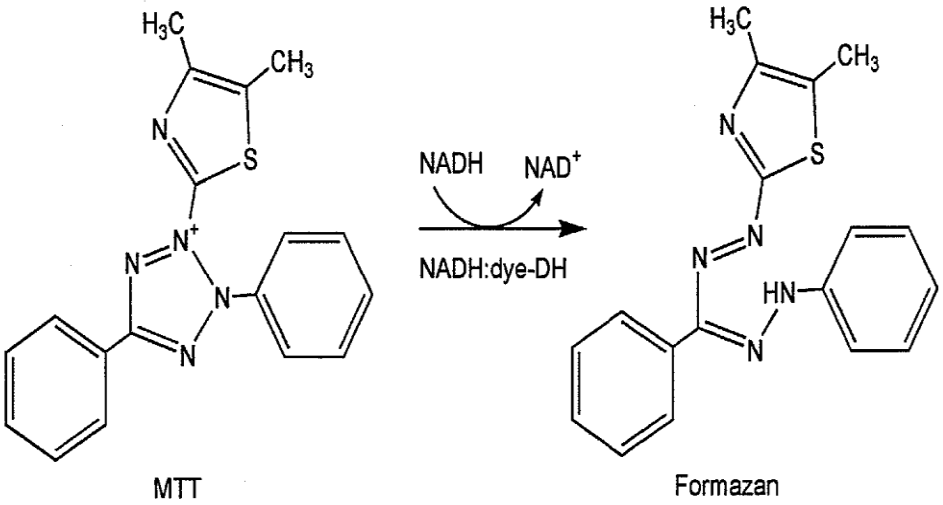


Figure 1.4 Reduction of yellow MTT into purple formazan by the mitochondrial enzyme succinate dehydrogenase (Stockert et al., 2012)

It is therefore necessary to dissolve the dark purple formazan crystals in an organic solvent; 20% SDS in 0.02M HCL (Soman et al., 2009, Stepanenko and Dmitrenko, 2015, Young et al., 2005). MTT-formazan is measured at 570 nm absorbance with 630 nm as the wavelength reference (Angius and Floris, 2015).

1.10 Flow Cytometry

Flow cytometry is a laser-based technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, which are around 0.2–150μm in size (Castejón, 2015). Particles or suspended cells flow into a fluid stream through a beam of light which scatters in both forward and sideways (90°) directions as cells pass through the interrogation point (Yurkin et al., 2005). The signals (amount of light scattered) are related to the size and internal complexity and granularity of each of the cells analysed (Mandy and Gratama, 2009). These light scattering signals are assembled by certain detectors, transformed to digital signals and finally presented as dot plots for analysis (Mandy and Gratama, 2009).

- **Annexin V and propidium iodide (PI)**

The evaluation of cellular viability to determinate the type of cell death can be achieved by a double stain with annexin V-FITC (AnV-FITC) and propidium iodide (PI) (Elmore, 2007). Annexin V and propidium iodide (PI) staining enhanced with flow cytometry can be used to assess apoptosis. During the early stages of apoptosis, phosphatidylserine (PS) moves from the inner layer to the outer layer of the cell membrane and thus PS is exposed to the external environment (Castejón, 2015, Elmore, 2007). FITC-labelled Annexin V (green fluorescence) can strongly interact with the PS and therefore early apoptosis can be detected (figure1.5). However, PS exposure at the outer cell surface also takes place during the later stages of apoptosis as well as during necrosis, thus requiring additional staining to differentiate between apoptosis or necrosis (Rieger et al., 2011, Ruan et al., 2012).

PI can enter a cell when the permeability of the cell membrane is disrupted during necrosis, but PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane. However, in late apoptosis and necrosis, PI can pass through the membranes because the integrity of the plasma and nuclear membranes decreases (Rieger et al., 2011). Then, PI intercalates into nucleic acids, and displays red fluorescence (Ruan et al., 2012). In this assay, viable cells are Annexin V and PI negative, cells going through the early stages of apoptosis are Annexin V positive, but PI negative, and cells that are already dead or in the later stages of apoptosis are Annexin V and PI positive (Castejón, 2015, Elmore, 2007).

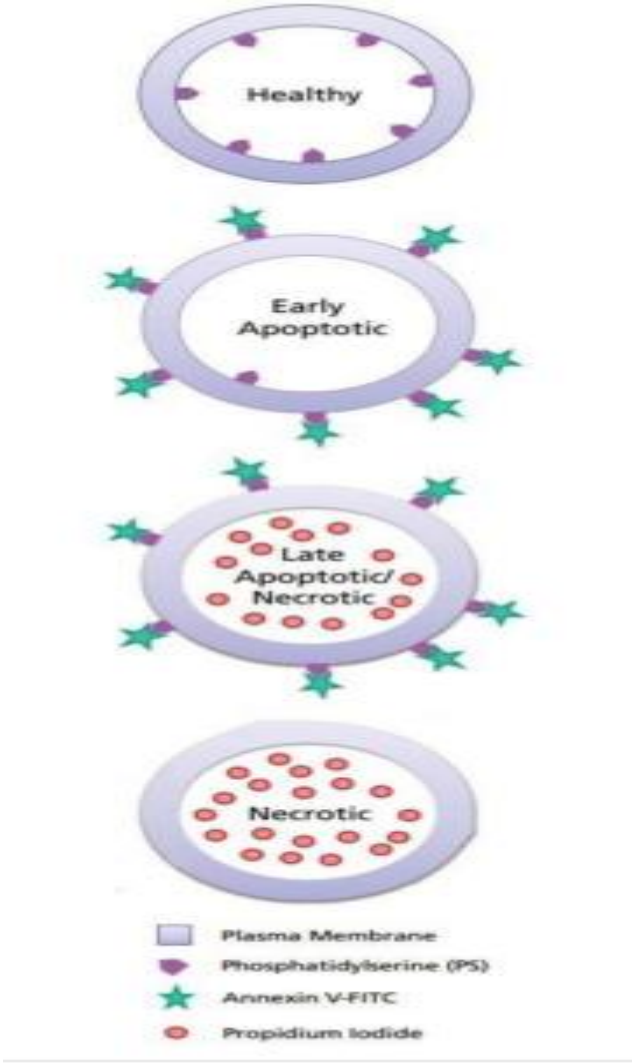


Figure 1.5: Healthy and apoptotic cells with markers for detection of apoptosis (Castejón, 2015).

1.11 Hypotheses and Aims

1. DOX or CYC alone will cause dose-dependent cytotoxicity in MCF-7 breast cancer cell and KGN granulosa cell.
2. The combination of DOX and CYC will be more cytotoxic than either chemotherapeutic alone in MCF-7 and KGN cells.
   - The combination of EC25 value of DOX and EC25 value of CYC will be cytotoxic more than 50% of MCF-7 cells because there will be synergism.
3. Alpha tocopherol (A-TOC) will not affect MCF-7 or KGN granulosa cell viability.

4. Gamma tocopherol (G-TOC) will cause dose dependent cytotoxicity to MCF7 because it previously induced apoptosis in cancer cells (Yang et al., 2010).
5. A-TOC will reduce the cytotoxicity of AC because its antioxidant activity will reduce ROS generation in MCF-7 and KGN cells.
   - The combination of EC25 value of DOX, EC25 value of CYC and 100uM A-TOC will reduce cytotoxicity in MCF-7 cells as AC alone.
6. G-TOC will increase the cytotoxicity of AC in MCF-7 and KGN cells.
   - The combination of EC25 value of DOX, EC25 value of CYC and EC25 value of G-TOC will be cytotoxic in more than 75% of MCF-7 cells because there will be synergism.

1.12 Academic and Biotechnology Significance

The most common cancer in women is breast cancer (Ghoncheh et al., 2015). Doxorubicin (Adriamycin) and Cyclophosphamide (AC) treatment is the most common chemotherapeutic treatment, which nearly all breast cancer patients are treated with AC (Fisher et al., 2004, Senchukova et al., 2015). ROS generation, DNA fragmentation and apoptosis are some of the major MOAs of DOX and CYC alone (Chegaev et al., 2013, Madondo et al., 2016, Mansour et al., 2015, Swift, 2006, Zhou et al., 2012). However, there is no information about the relationship of these different MOAs to each other, or their relative contribution to the chemotherapeutic effect.

AC treatment causes adverse side effects in breast cancer patients, and CYC (Ezoe et al., 2014, Gurgen et al., 2013) and DOX cause infertility (Roti et al., 2012). Antioxidants, like α, β, γ and δ tocopherol which are vitamin E (Yang et al., 2010), can counteract oxidative stress (Nechuta et al., 2011). Therefore, studying the cytotoxic effect of DOX, CYC, A-TOC and G-TOC alone and in combination on MCF7 and KGN cells is required. From this study, we expect A-TOC and G-TOC will reduce the damage caused by ROS, but maintain or increase apoptosis of in cancer cells *in vitro*.

It is possible that treatment which antioxidants could become an adjunct to current chemotherapeutic treatment methods or a new way to treat breast cancer patients. In addition, our understanding of the mechanism of action of the AC combination will be increased from this study. Future experiments can be conducted to determine if vitamin E reduces ROS generation and maintain in the granulosa cell hormone production in the presence of AC.

CHAPTER 2: Materials and Methods

2. Materials and Methods

All chemicals and reagents were obtained from Sigma-Aldrich (Castle Hill, Australia) unless otherwise noted. MCF-7 and KGN cell lines were obtained from American Type Culture Collection (ATCC; The Global Bio resource center).

2.1 Overview of Experimental Design

In this study, DOX, HCYC, A-TOC, G-TOC and combinations of these were examined for their cytotoxicity towards the MCF-7 and KGN cell lines (Figure 2.1). Human *in vivo* clinical serum concentrations of DOX and CYC after chemotherapeutic administration were reported as being in the ratio of 100:1 (Network, 2015, Skrablin et al., 2007), but a preliminary *in vitro* dose response study found that doxorubicin was more cytotoxic *in vitro*, therefore in this study the combination of DOX and CYC was applied in a ratio of 10:1.

The crystal violet assay also used to determine the EC25, EC50 and EC75 values of DOX, HCYC, A-TOC and G-TOC which used on apoptosis assay to MCF-7 cells (Figure 2.2).

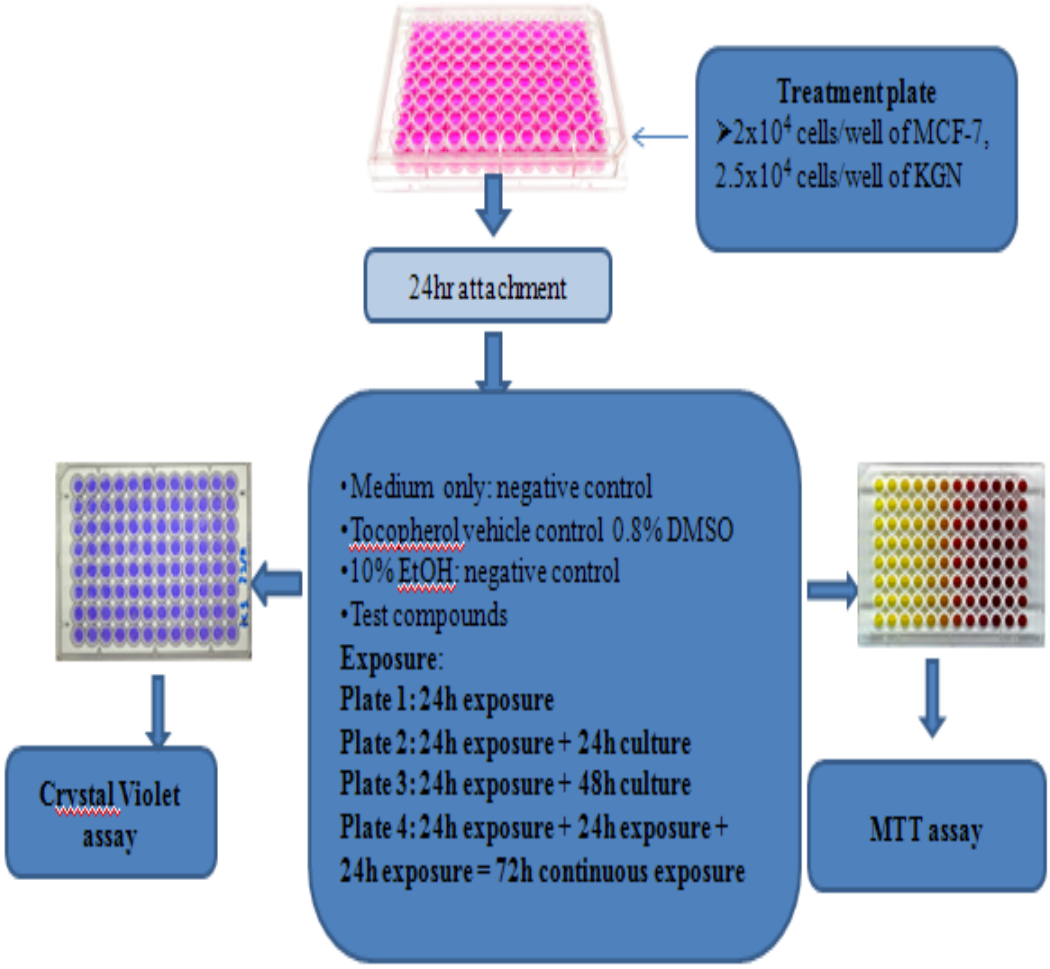


Figure 2.1 Overview of the cytotoxicity experimental design. Repeated on four separate occasions (n=4)

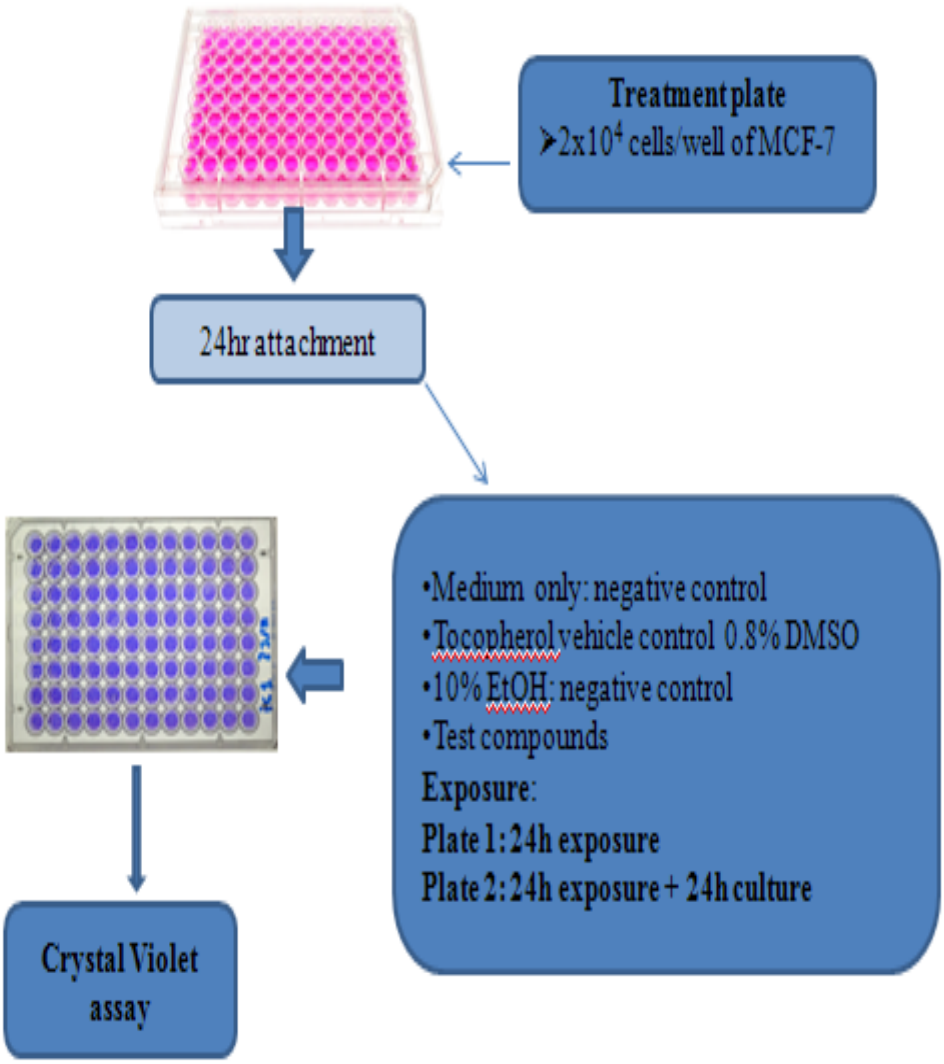


Figure 2.2 Overview of the EC50 experimental design. Repeated on four separate occasion (n=4)

Annexin V FITC Apoptosis Detection Kit I (BP Pharmingen) was used to examine the effect of DOX, HCYC, A-TOC, G-TOC and their different combinations on MCF-7 cell lines. Cells were exposed to the EC25, EC50 and EC75 values of DOX, HCYC and G-TOC, and A-TOC was used at 100uM because the highest concentration tested was not cytotoxic. The IC25 values were used to examine combinations. The controls were medium alone (negative control), or untreated

cells with Propidium Iodide (PI) or annexin added at the end of culture (negative controls for flow cytometry), 10% Ethanol (EtOH) (cytotoxic and possibly necrosis positive control), 5uM staurosporine (apoptosis positive control), 0.8% DMSO and 0.25% DMSO (tocopherol vehicle controls). Cells were attached to 6-well plates for 24hr before a 24hr exposure. The apoptosis assay reagents were added according to the manufactures instructions, and the cells were examined using Accuri C6 flow cytometer (Figure 2.3)

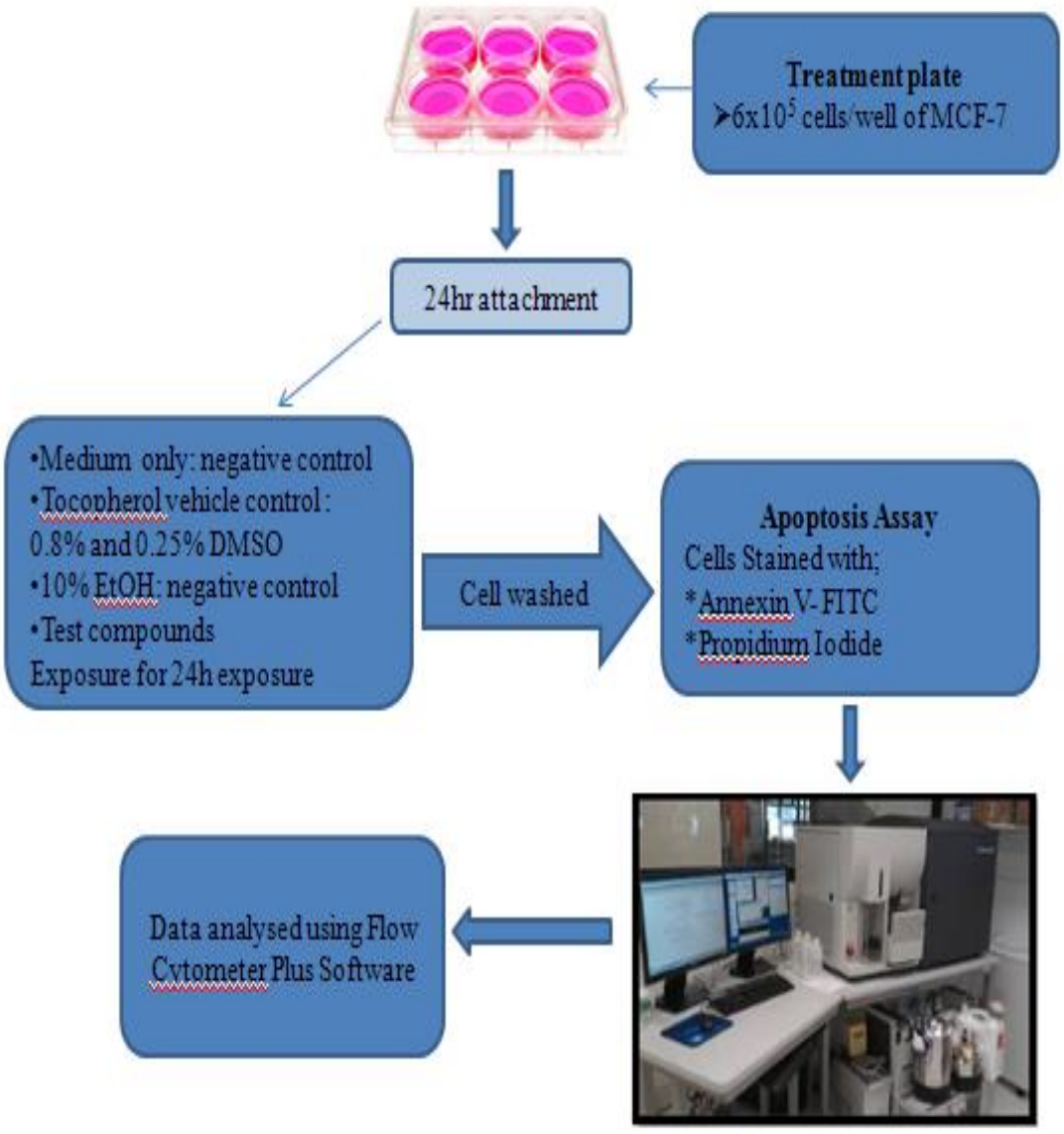


Figure 2.3 Overview of the Apoptosis assay. Experiment conducted once (n=1).

2.2 Cell Culture

2.2.1 Cell lines

MCF-7 breast cancer cells were grown in vented T75 cm^2 cell culture flasks in RPMI 1640 medium (appendix 1.1) supplemented with 10% (v/v) of heat-inactivated foetal bovine serum (FBS), and 1% (v/v) antibiotics of 100U/ml penicillin and 100 μg/ml streptomycin.

KGN granulosa cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM/F12) medium in vented T75 cm^2 cell culture flasks (appendix 1.2) supplemented with 10% (v/v) of FBS, 1% (v/v) of antibiotics of 100U/ml penicillin and 100 μg/ml streptomycin, and 1% (v/v) of Insulin-Transferrin-Selenium (100 x ITS).

All the cells were grown in a humidified atmosphere at 37 ℃ and 5% CO_2 in a cell culture incubator.

2.2.2 Cell line passaging

Passaging of cells was performed twice a week for MCF-7 cells and once a week for KGN cells. The cells were sub-cultured when they reached about 85% confluence. The cells were washed twice with 5ml of 1x PBS (appendix 2.2). To detach cells, 2.5 ml trypsin EDTA (appendix 2.3) were added for 5 mins at 37℃. After checking cell detachment, 4ml fresh media were added and cells were transfered into new 15ml tubes to centrifuge at 1200G for 5mins. The cell pellets were resuspended in fresh media and used to seed new flasks or for experiments. The passage number of MCF-7 and KGN cells were used range from 6 to 20.

2.2.3 Freezing and Storage of MCF-7 and KGN cells

MCF-7 and KGN cells were frozen at 2 x 10^6 cells/ml in growth medium with 5% (v/v) Dimethyl sulfoxide (DMSO). The cells were distributed into 1ml cryovials and kept on ice for 10 minutes, then placed in a freezer at -20℃ for 1 hour. The

cells were placed in a freezer at - 80℃ for 24 hours, before storage in liquid nitrogen.

2.2.4 Thawing of MCF-7 and KGN cells

The MCF-7 and KGN cells were thawed by placing 9ml of RPMI media or DMEM/F12 media respectively, into sterile 10ml centrifuge tubes. Each of the frozen vials was thawed in a 37℃ water bath. 1ml of warm RPMI medium or DMEM/F12 medium was added to each vial, before centrifugation at 1000 g for 5mins. The supernatant was discarded and 5ml of media were added to each pellet. The resuspended cells were added to T25cm^2 sterile culture flasks and incubated at 37℃ with 5% CO_2 until the cells achieved ~85% confluence, after which, the cells were passaged into T75cm^2 flasks.

2.3 Trypan Blue Exclusion Assay

After resuspending a cell pellet in fresh media, two 50µl aliquots of cell suspension were transferred to a 96-well plate and diluted 1:1 with 50µl Trypan blue (appendix 2.4). The haemocytometer (Neubauer Improved) was loaded with 10µl stained cell suspension, and the number of viable (golden) and non-viable (blue) cells were counted (Figure 2.4).

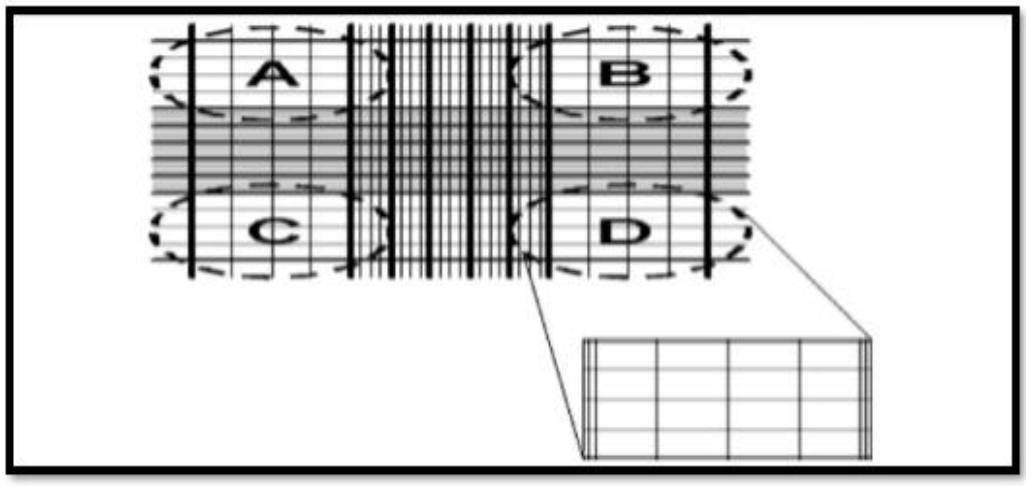


Figure 2.4 Haemocytometer used in a trypan blue exclusion assay.

Calculation of viable cell concentration:

Average viable cell count = (viable cells) / (large squares counted)

Viable cell concentration = (average viable cells) x 10^4 (correction factor) x 2 (dilution factor) cells/ml

2.4 Cell Viability Assays

2.4.1 Standard curves

Standard curves for the crystal violet (CV) and MTT assays were prepared using 1:2 serial dilutions of cells in flat bottomed sterile 96-well plates (Figure 2.5A). The highest cell density was 8 x 10^4 cells/well, and the lowest cell density was 313 cells/well. Each cell density was examined in six replicate wells, as well as a medium control that did not have any cells. Each well contained 100μl of medium. Cells were cultured for 24hr before determination of cell number using the CV or MTT assays.

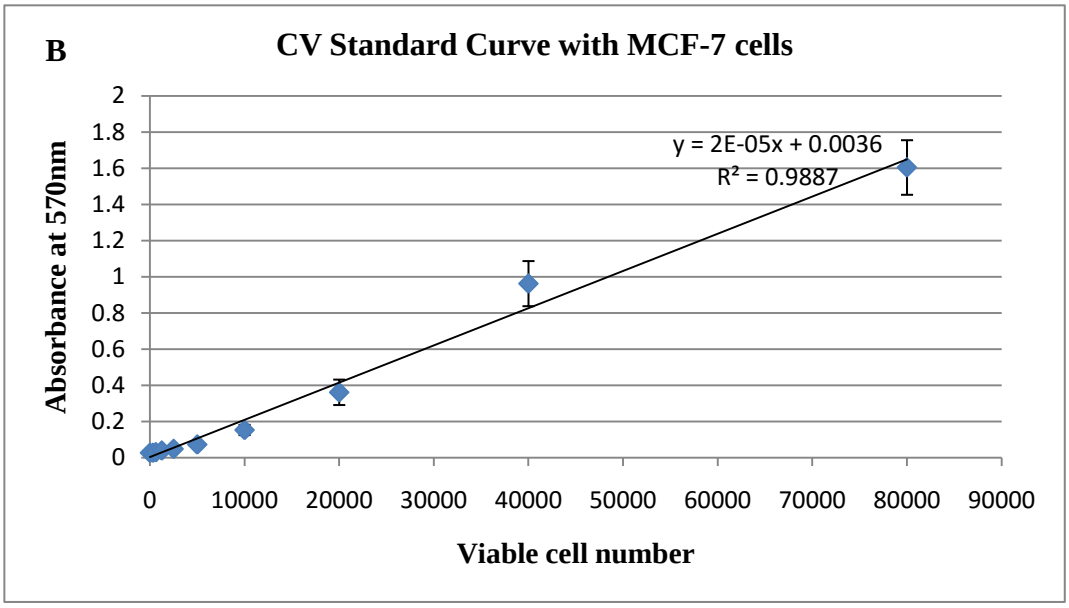
2.4.2 Crystal Violet (CV) Assay Procedure

To count the number of viable cells, 50μl of 0.5% crystal violet in 50% methanol was added to each well and cells were incubated for 10mins at room temperature. The cells were washed by demineralised water and air dried. On the following day, the cells were destained with acetic acid (33% v/v) for 10mins. The optical density (OD) was measured at 570nm with 630nm as a reference wavelength on an automatic spectrophotometer with KC Junior software.

OD values in experimental test wells were converted to number of cells/well using the equation that provided the linear regression between cell density and OD (Figure 2.5B) in the same experimental replicate.

Figure 2.5A 96-well plate layout for cell viability standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A		Standard Curve Plate										
B		Media only (zero cells per well)	312.5 cells per well	625 cells per well	1250 cells per well	2500 cells per well	5000 cells per well	10000 cells per well	20000 cells per well	40000 cells per well	80000 cells per well	
C												
D												
E												
F												
G												
H												



C. The equation of the straight line in the form y = m(x) + b

m is the slope

b is the y-intercept

The treatment wells will each have an OD value, equivalent to 'y', for each well.

Using the equation to solve for 'x' - the cell number:

y = 2E-05x + 0.0036

x = (y-b)/m = [(OD value - 0.0036)/0.00002]cells/well

Figure 2.5 Standard Curves for cell viability assays. Prior to viability assay, cells were seeded into a 96-well flat bottom sterile plate in six replicate wells for

each cell density (A) then incubated for at 37℃ with 5% CO_2 for 24 hours. After completing the MTT or Crystal Violet (B) assay, the optical density corresponds to the number of viable cells in the well. Data shown as means ± SD of six replicate wells. A liner regression was conducted (C), and this was used to derive viable cell numbers in experimental test wells.

2.4.3 MTT Assay Procedure

To count the number of viable cell in the standard curve or experimental test plates, standard curves for the MTT assay were repeated using 1:2 serial dilutions of cells as before (Figure 2.5A+B) 200µl of 0.5 mg/mL MTT in media were added to each well for 24hr incubation at 37℃ in 5% CO_2. Then 80µl of 20% (w/v) sodium dodecyl sulphate in 0.02M HCl were added into each well. The cells were incubated overnight in the dark before measuring the optical density (OD) at 570nm with 630nm as a reference wavelength using an automatic spectrophotometer with KC Junior software.

Optical density (OD) values were converted to number of cells/well using the equation that provided the linear regression between cell density and OD (Figure 2.5C).

2.5 Cytotoxicity Experiment

After adding $2x10^4$ cells/well of MCF-7 or $2.5x10^4$ cells/well of KGN into 96-well plates and incubating at 37℃ with 5% CO_2 for 24 hours, the cells were exposed to control treatment, DOX, HCYC, A-TOC, G-TOC and their different combinations (Table 2.1). Cells were exposed to medium only (negative control), 0.25% and 0.8% DMSO (tocopherol vehicle controls), 10% EtOH (positive control), doxorubicin (5, 10 and 25uM), 4 hydroxy cyclophosphamide (0.5, 1 and 2.5uM), alpha tocopherol (50, 75 and 100uM), gamma tocopherol (50, 75 and 100uM), the low dose combination AC (10uM of doxorubicin + 1uM of 4 hydroxy cyclophosphamide), the high dose combination of AC (25uM of doxorubicin +

2.5uM of 4 hydroxy cyclophosphamide), the low dose combination of AC with the middle dose of tocopherols (10uM of doxorubicin + 1uM of 4 hydroxy cyclophosphamide + 75uM of alpha tocopherol, 10uM of doxorubicin + 1uM of 4 hydroxy cyclophosphamide + 75uM of gamma tocopherol) and the high dose combination of AC with the middle dose tocopherols (25uM of doxorubicin + 2.5uM of 4 hydroxy cyclophosphamide + 75uM of alpha tocopherol, or 25uM of doxorubicin + 2.5uM of 4 hydroxy cyclophosphamide + 75uM of gamma tocopherol). Four different exposures were set up:

- **Plate 1: 24h exposure (24hr culture)**
- **Plate 2: 24h exposure + 24h culture (48hr culture)**
- **Plate 3: 24h exposure + 48h culture (72hr culture)**
- **Plate 4: 24h exposure + 24h exposure + 24h exposure = 72h continuous exposure**

The cytotoxicity experiment was repeated on four separate occasions (n=4), except the MTT assay on KGN (n=3).

Table 2.1 96-well plate layout for cytotoxicity experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Complete Media	Dox 5	Dox 10	Dox 25	HCyc 0.5	HCyc 1	HCyc 2.5	Dox 10+HCyc 1	Dox 25 + HCyc 2.5	0.25% DMSO in Media	Complete Media	10% EtOH
C												
D												
E	0.8% DMSO in Media	a Toc 50	a Toc 75	aToc 100	gToc 50	GToc 75	gToc 100	Dox 10 + HCyc 1+aToc 75	Dox 10 + HCyc 1+gToc 75	Dox 25+HCyc 2.5+ aToc 75	Dox 25+HCyc 2.5+ gToc 75	Complete Media
F												
G												
H												

2.5.1 Modified MCF-7 Cytotoxicity Experiment using a lower seeding density

A 72hr culture supported MCF-7 proliferation that resulted in cell numbers that were higher than 80,000 cells per well, the upper limit of the viability standard

curve. Therefore, the experiment was repeated with a lower cell seeding density; 1 x 10^4 cells/well of MCF-7 cells and an MTT viability assay. Time limitation prevented repetition of the entire experiment with lower seeding densities. The cytotoxicity experiment with the lower seeding density of MCF-7 cells and the MTT assay was repeated on three separate occasions (n=3).

2.6 Determination of EC25, EC50 & EC75 Values

2 x 10^4 cells/well of MCF-7 were adhered to 96-well plates for 24 hours; then they were exposed to different concentrations of DOX, HCYC, A-TOC, G-TOC, vehicle control (0.25% or 0.8% DMSO) and positive control (10% EtOH) for 24hr or 24hr exposure followed by 24hr culture (Table 2.2). Cells were exposed to medium only (negative control), 0.25% and 0.8% DMSO (tocopherol vehicle controls), 10% EtOH (positive control), doxorubicin (2.5, 5, 10 and 25uM), 4 hydroxy cyclophosphamide (1, 2.5, 5, 10, 20 and 40uM), alpha tocopherol (50, 75, 100, 200 and 500uM) and gamma tocopherol (50, 75, 100 and 200uM).

Table 2.2 96-well plate layout for calculating EC50

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Complete Media	Dox 2.5	Dox 5	Dox 10	Dox 25	ATOC 50	ATOC 75	ATOC 100	ATOC 200	ATOC 500	0.25% DMSO in Media	10% EtOH
C												
D												
E	0.8% DMSO in Media	HCYC 1	HCYC2.5	HCYC 5	HCYC 10	HCYC20	HCYC 40	GTOC 50	GTOC 75	GTOC 100	GTOC 200	Complete Media
F												
G												
H												

The numbers of viable cell numbers were determined in a crystal violet assay. The whole experiment was repeated on four separate occasions (n=4). Then, EC50 was calculated through a non-linear regression analysis using GraphPad Prism 5.1 based on the dose response data and EC25 and EC75 values were calculated using

the calculation template software (http://www.graphpad.com/quickcalcs/Ecanything1.cfm).

2.7 Apoptosis Assay

20,000 cells/well were seeded in a 96-well plate, and the area of each well was 0.32cm^2. In 6-well plate, the area of each well was 9.5cm^2. The 6-well plates were therefore seeded with 6 x 10^5 MCF-7 cells/well (9.5 x 20,000)/0.32) and cultured for 24hr to allow cells to attach to the bottom of wells. The cells were then exposed to the control treatments, EC25, EC50 and EC75 values of DOX, HCYC and G-TOC and their combinations for 24hr (Table 2.4)

Table 2.3 List of the concentration of test reagents for apoptosis assay

Unstained Medium
Medium stained with Annexin
Medium stained with PI
0.25%DMSO
0.8%DMSO
5uM Staurosporine
10% EtOH
A-TOC 100uM
DOX EC25 (1.21uM)
DOX EC50 (3.63uM)
DOX EC75 (10.89uM)

HCYC EC25 (21.23uM)
HCYC EC50 (63.69uM)
HCYC EC75 (191.07uM)
G-TOC EC25 (35.1uM)
G-TOC EC50 (105.3uM)
G-TOC EC75 (315.9uM)
DOX EC25 (1.21uM) + HCYC EC25 (21.23uM)
DOX EC25 (1.21uM) + HCYC EC25 (21.23uM) + G-TOC EC25
DOX EC25 (1.21uM) + HCYC EC25 (21.23uM) + A-TOC 100uM

The controls were medium only, cells culture in medium but with PI or annexin added at the end of culture, 10% EtOH, 5uM staurosporine, 0.8% DMSO and 0.25% DMSO. After exposure the media supernatants containing the unattached dead cells and Trypsin-EDTA cells collected from wells were added separately into 5 ml tubes and centrifuged for 5min at 1000 g. After washing cells twice, the pellets were pooled. The cells were washed twice with 1ml cold 0.1% sodium azide in PBS (appendix 2.7), then resuspended in 1 x binding buffer (appendix 2.8). 1µl of Annexin V-FITC and 1µl of PI were added to the cells, which were gently resuspended and incubated for 15min at RT in the dark. 1 x binding buffer was added and cells were analyzed by flow cytometry within one hour. The flow cytometer (Accuri C6) used the wave lengths of 525nm for AnV-FITC and 640nm for PI. The whole experiment was repeated only once (n=1) because the staurosporine positive control did not induce apoptosis.

Data was obtained using Accuri C6 Flow Cytometer and C Flow Plus software, from which a work list was created such that the samples were collected

automatically and the acquisition criteria were defined at 50,000 events for each sample.

The report generated by the software included the plots and gates for treated and untreated samples:

a. Annexin V–/PI–: viable cells or no measurable cell death

b. Annexin V+/PI–: cells in early apoptosis with intact membranes

c. Annexin V+/PI+: cells in late apoptosis or necrosis

d. Annexin V–/PI+: cells in necrosis

2.8 Statistical Analysis

Each cytotoxicity assay had three or four independent experimental replicates, and data were presented as mean ± SD. Statistical analysis was performed using GraphPad Prism 5. The cytotoxicity of DOX, HCYC, A-TOC, G-TOC and their different combinations was analysed using a 2-way ANOVA. Statistical significance was assigned at $p < 0.05$, $p < 0.01$, and $p < 0.001$. The EC50 was automatically calculated through a non-linear regression analysis using GraphPad Prism 5.1 and EC25 and EC75 values were calculated using the calculation template software (http://www.graphpad.com/quickcalcs/Ecanything1.cfm).

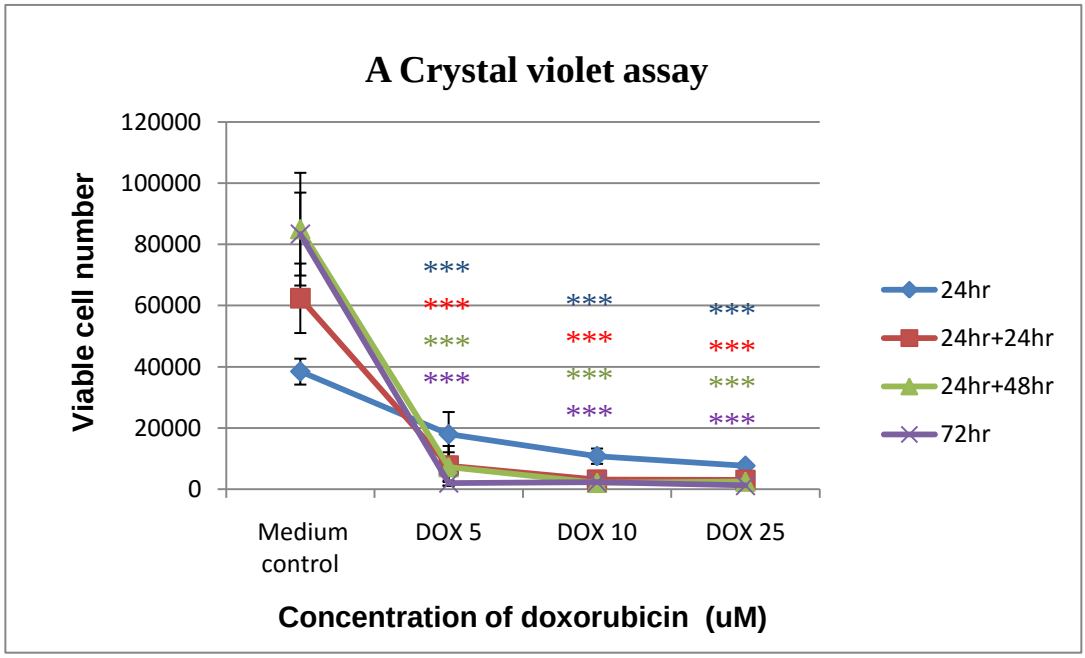
CHAPTER 3: Result

3. Result

3.1 Effect of chemotherapeutic drugs and tocopherols on MCF7 breast cancer cells

All concentrations and exposures of DOX caused significantly cytotoxicity compared to the medium control for the same exposure in the CV assay ($p<0.001$, Figure 3.1.1A). There was a dose-dependent effect of DOX after 24hr exposure, and the 72hr continuous exposure of DOX 5 caused significantly more cytotoxicity than 24hr exposure, suggesting a time-dependent increase in cytotoxocity (Figure 3.1.1A).

All concentrations and exposures of DOX caused significantly cytotoxicity to the medium control in the MTT assay ($p<0.001$, Figure 3.1.1B). There was a dose-dependent effect of DOX after 24hr exposure, and a time-dependent increase in cytotoxicity at all concentrations (Figure 3.1.1B).



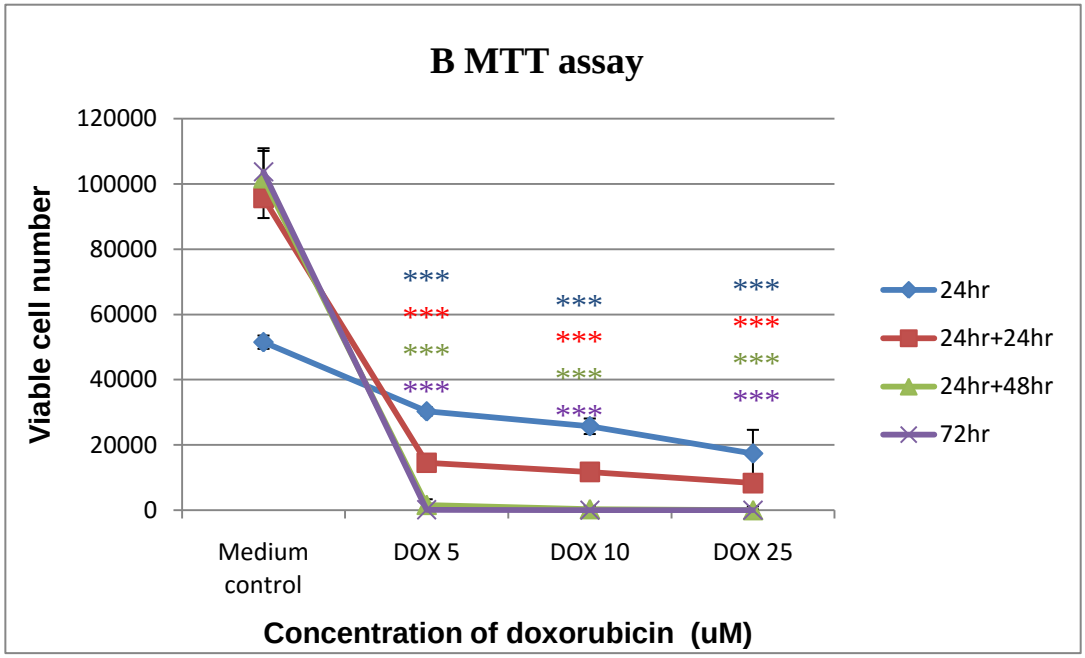


Figure 3.1.1 Effect of DOX on breast cancer cell viability. A) represents CV assay and B) represents MTT assay. MCF-7 cells were seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to 5uM, 10uM and 25uM of DOX for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed before determination of the viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

In the CV assay, almost all concentrations of HCYC had no effect on cell viability compared to medium control at same exposure period except 72 exposure (Figure

3.1.2A). 2.5uM of HCYC was significantly more cytotoxicity than medium control at same exposure (72hr p< 0.001, Figure 3.1.2A). There was a time-dependent increase in viable cell numbers in the medium control.

In the MTT assay, all concentrations of HCYC had no effect on cell viability compared to medium control at same exposure period (Figure 3.1.2B).

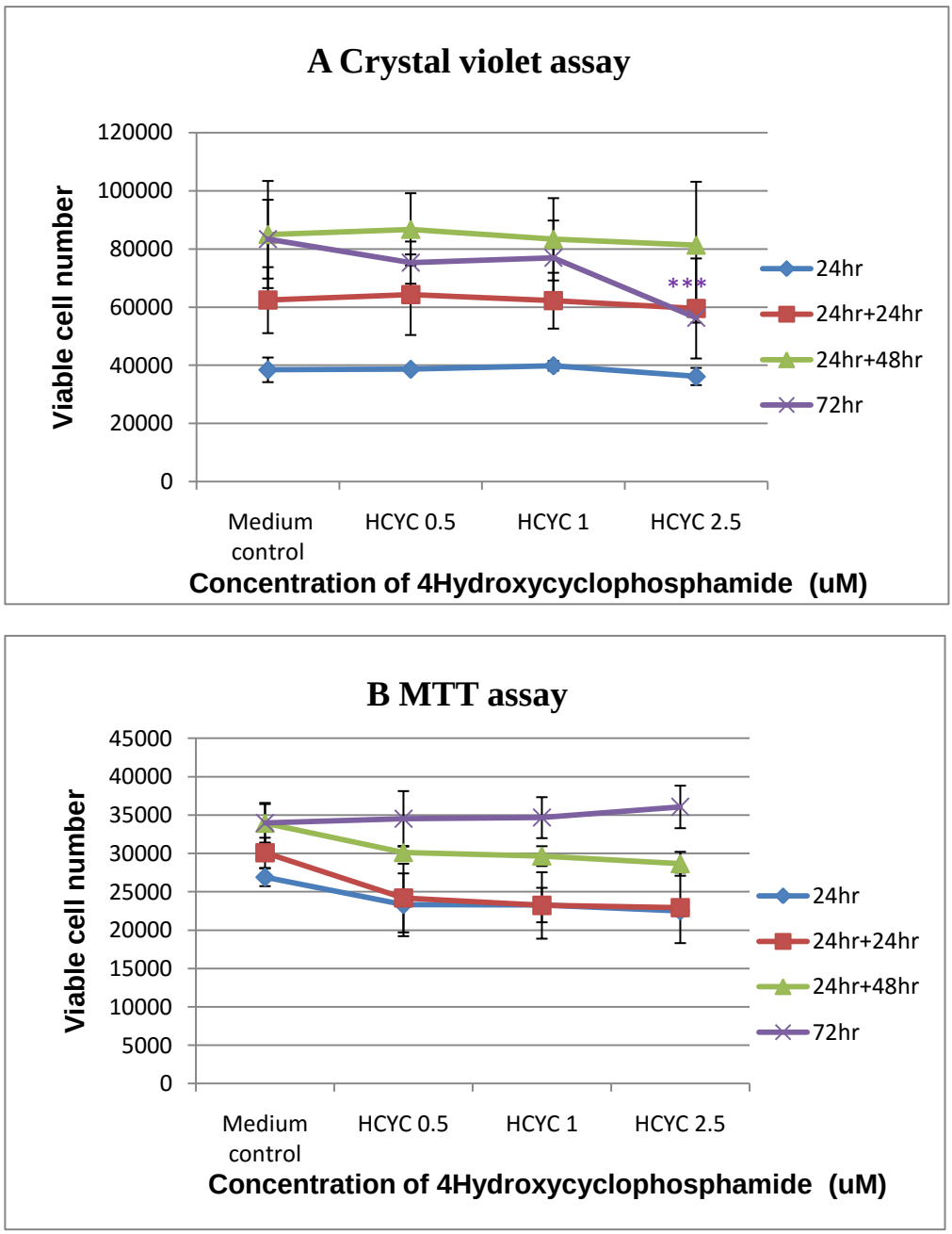


Figure 3.1.2 Effect of HCYC on breast cancer cells. A) represents CV assay and B) represents MTT assay. MCF-7 cells were seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to 0.5uM, 1uM and

2.5uM of HCYC for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed before determination of viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.05$ (*), $p < 0.01$(**), and $p < 0.001$ (***).

The 0.8% DMSO vehicle control for the tocopherols did not affect cell viability (Figure 3.1.3A), because the cell numbers were similar to those in control medium (Figure 3.1.2A). In CV assay, A-TOC did not have a dose-dependent effect on MCF-7 cell viability except after the 72hr continuous exposure, which caused a significant decrease in cell viability compared to the 0.8% DMSO control ($p<0.01$, Figure 3.1.3A). In MTT assay, A-TOC did not have a dose-dependent effect on MCF-7 cell viability (Figure 3.1.3B).

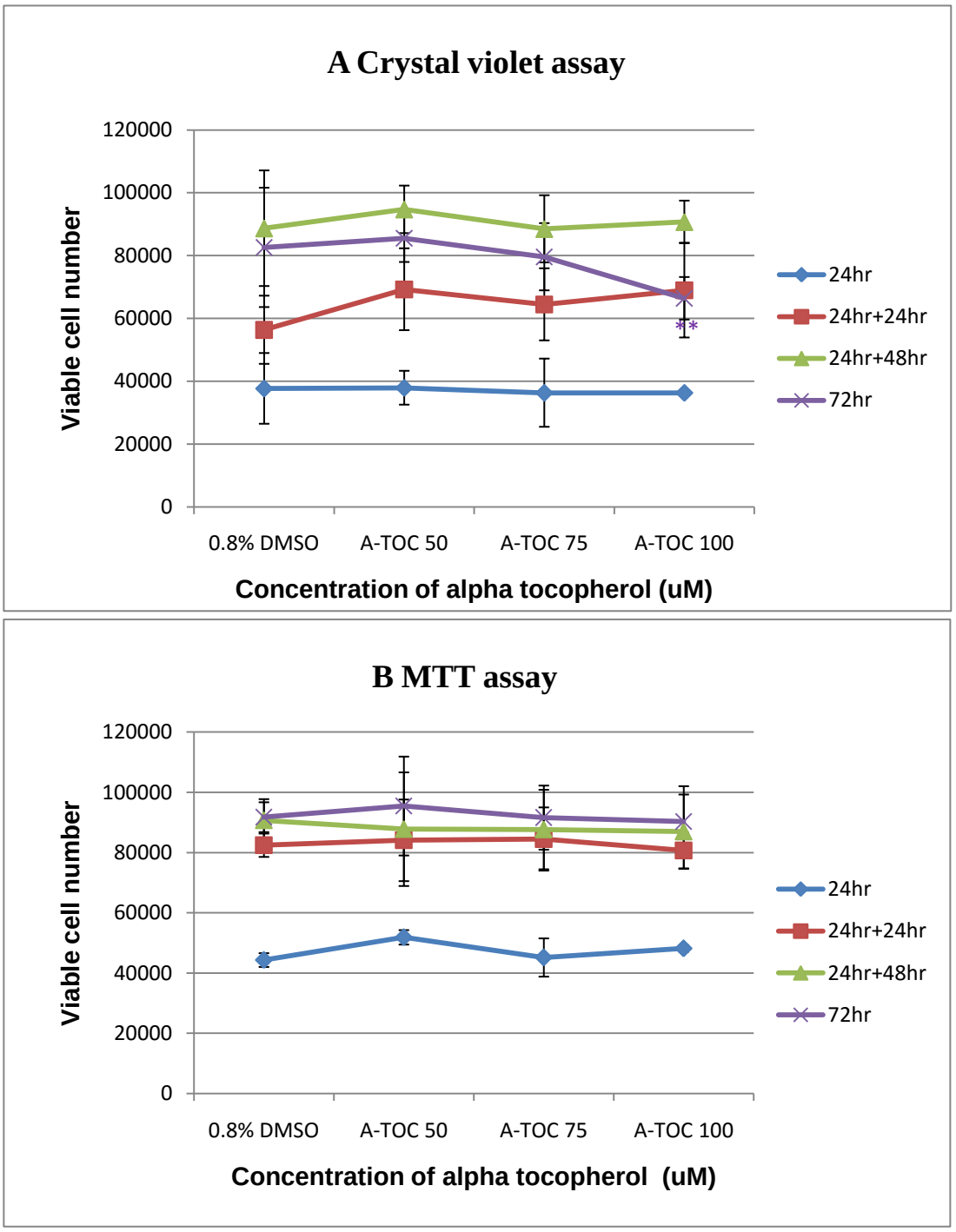
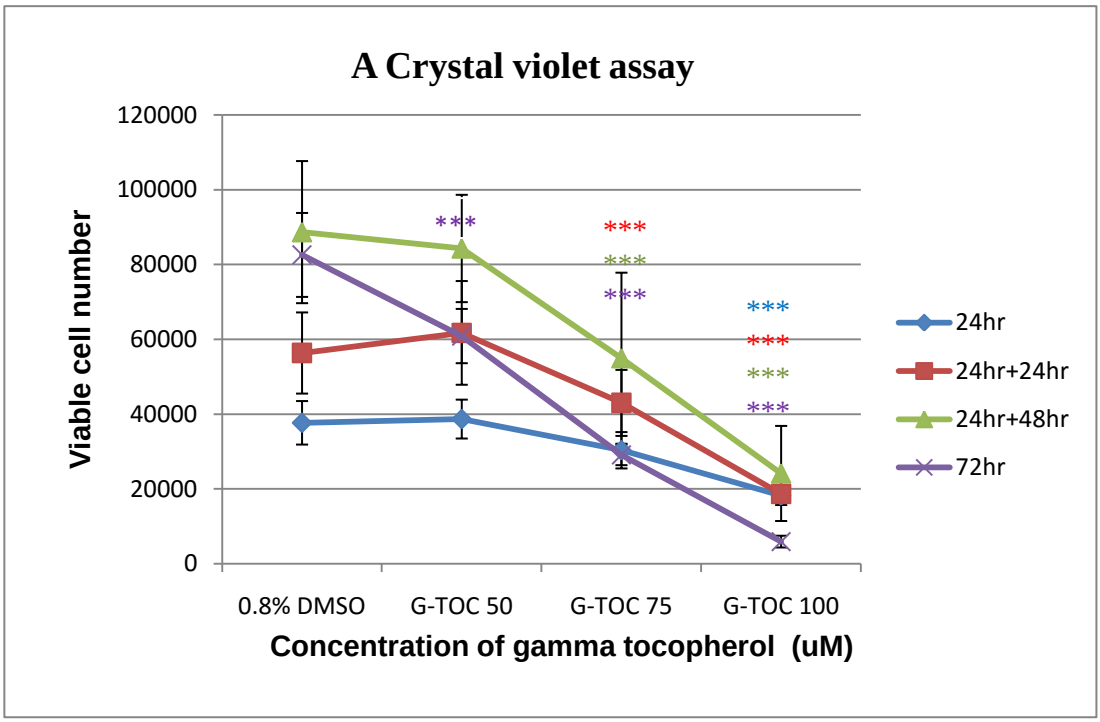


Figure 3.1.3 Effect of A-TOC on breast cancer cells. A represents CV assay and B represents MTT assay. MCF-7 cells were seeded at 20,000 cells/well in 96-well plate for 24hr. Then MCF-7 cells were exposed to 50uM, 75uM and 100uM of A-TOC for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed before determination of viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.01$(**).

The 0.8% DMSO vehicle control for the tocopherols did not affect cell viability (Figure 3.1.4A), because the cell numbers were similar to these in control medium (Figure 3.1.2A). All exposures of 75 and 100uM of G-TOC caused significant cytotoxicity compared to the 0.8% DMSO control in the CV assay except 75uM of G-TOC at 24hr culture ($p<0.001$, Figure 3.1.4A). There was a dose-dependent effect of G-TOC after 72hr continuous exposure (Figure 3.1.4A).

All concentrations and exposures of G-TOC caused significant cytotoxicity compared to the 0.8% DMSO control in the MTT assay except 24hr culture at any concentration ($p<0.001$, Figure 3.1.4B). There was a dose-dependent effect of G-TOC after any exposure except 24hr exposure (Figure 3.1.4B).



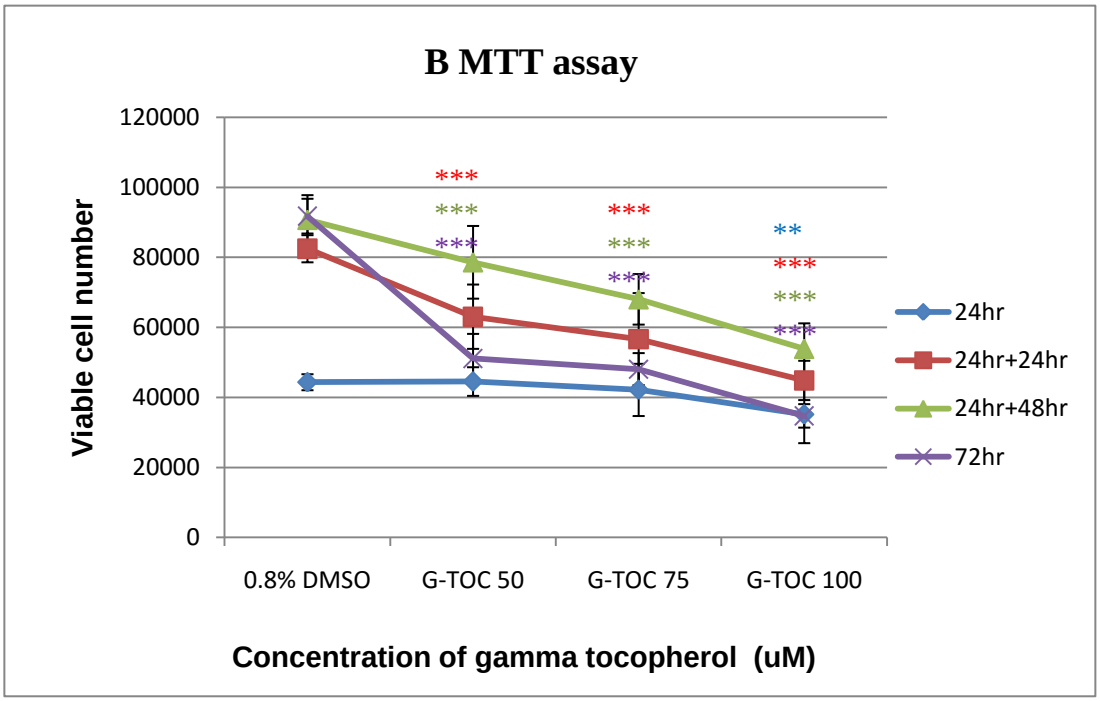
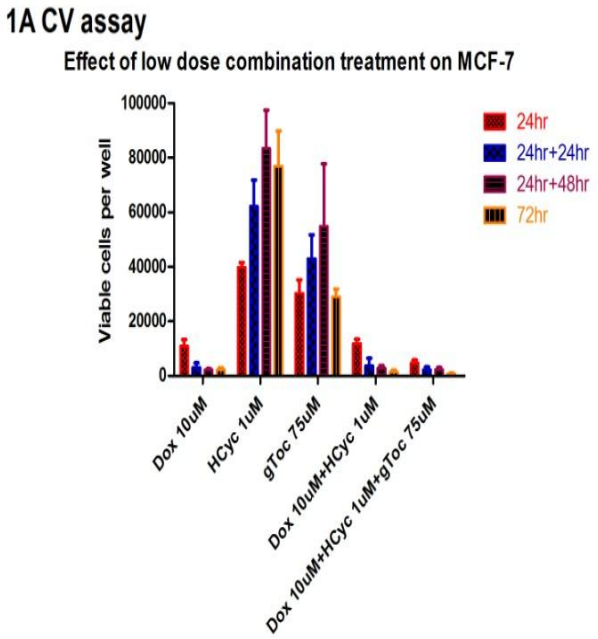
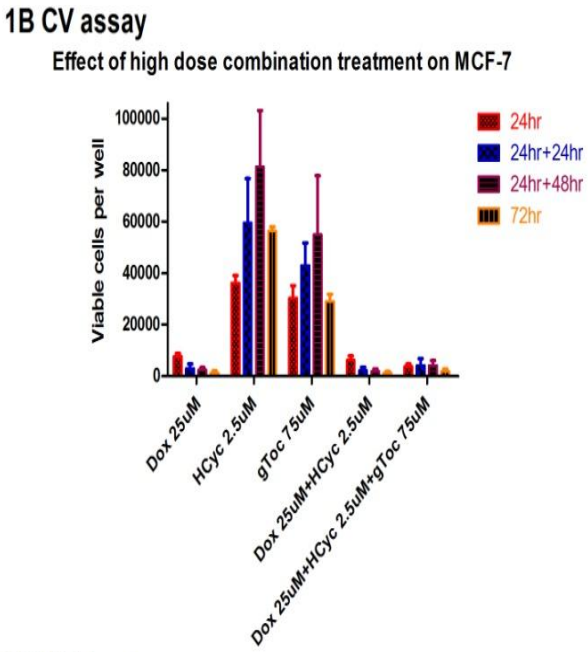
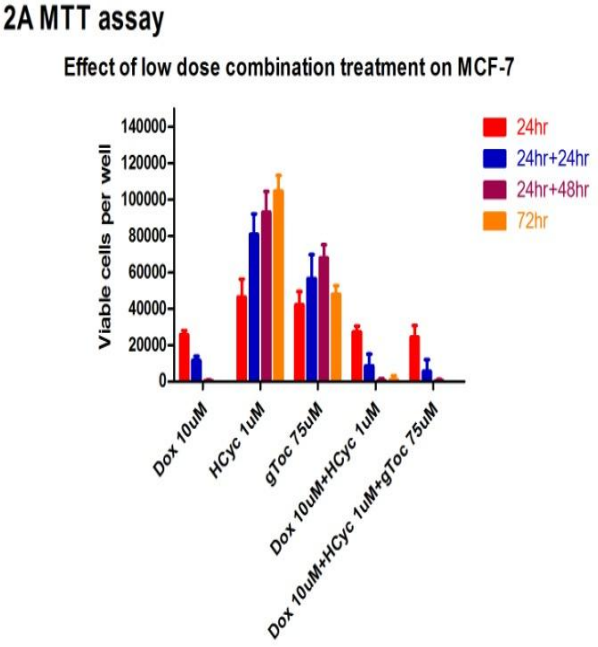


Figure 3.1.4 Effect of G-TOC on breast cancer cells. A) represents CV assay and B) represents MTT assay. MCF-7 cells were seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to 50uM, 75uM and 100uM of G-TOC for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed before determination of viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.01$(**), and $p < 0.001$ (***).

Both low dose and high dose combinations of DOX + HCYC (AC) caused the same cytotxicity as DOX alone either in CV assay or MTT assay (Figure 3.1.5). The addition of G-TOC to the AC combination had no significant effect on cell viability. All treatment containing DOX caused significantly more cytotoxicity than exposure to either HCYC or G-TOC alone at any exposure. There was no significant difference between cytotoxicity of low dose and high dose DOX alone, AC or AC with G-TOC (Figure 3.1.5).







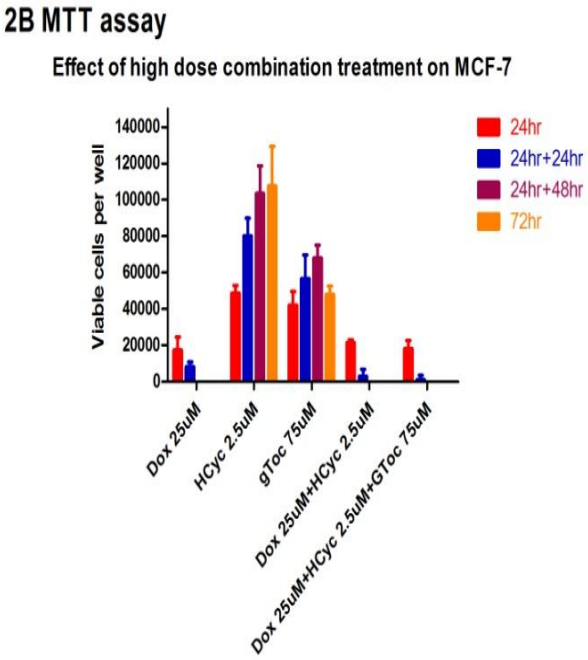


Figure 3.1.5 Effect of the combination of chemotherapeutic drugs with G-TOC on breast cancer cells. 1A and 1B represent CV assay and 2A and 2B represent MTT assay. Figure A represents the low dose combinations of DOX, HCYC and middle dose of G-TOC compared with each drug alone. Figure B represents high dose combinations of DOX, HCYC and middle dose of G-TOC compared with each drug alone. MCF-7 cells were seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to different doses of treatment for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure Treatment media were disposed before determination of viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and no significance showed.

The addition of A-TOC to the AC combination had no significant effect on cell viability. All treatment containing DOX caused significantly more cytotoxicity than exposure to either HCYC or A-TOC alone at any exposure. There was no significant difference between cytotoxicity of low dose and high dose DOX alone, AC or AC with A-TOC (Figure 3.1.6).

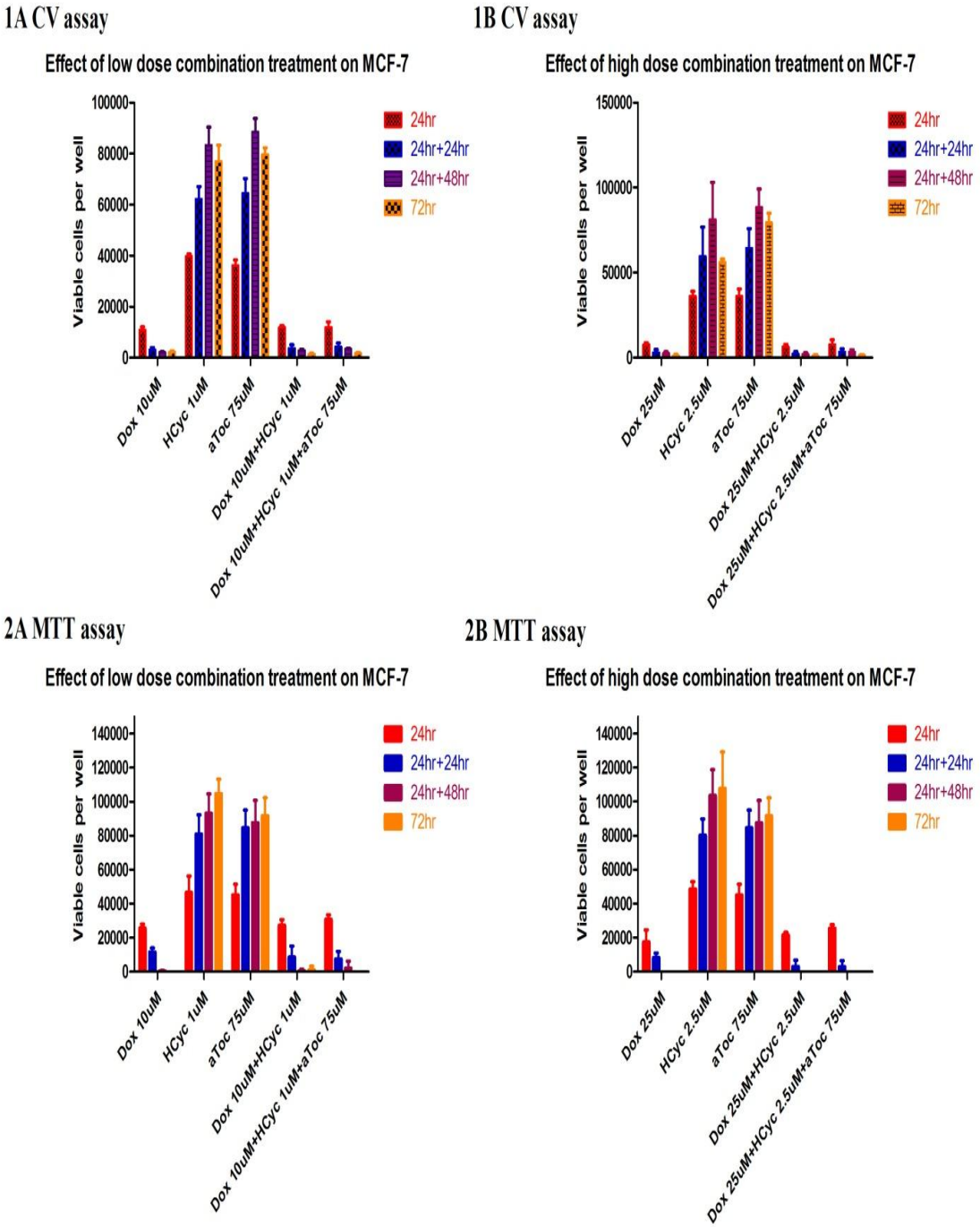


Figure 3.1.6 Effect of the combination of chemotherapeutic drugs with A-TOC on breast cancer cells. 1A and 1B represent CV assay and 2A and 2B represent MTT assay. Figure A represents the low dose combinations of DOX, HCYC and middle dose of A-TOC compared with each drug alone. Figure B represents high dose combinations of DOX, HCYC and middle dose of A-TOC compared with each drug alone. MCF-7 cell was seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to different doses of treatment for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed

before determination of viable cell number. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown. Data was analyzed using a two-way ANOVA and no significance showed.

3.2 Effect of chemotherapeutic drugs and tocopherols on lower seeding density of MCF-7 cells (10,000 cells/well)

All concentrations and exposures of DOX were significantly cytotoxicity compared to the medium control in the MTT assay ($p<0.001$, Figure 3.2.1). DOX did not have a dose-dependent effect at any exposure period, 24hr exposure was significantly less cytotoxic than any other exposure and culture regime (Figure 3.2.1).

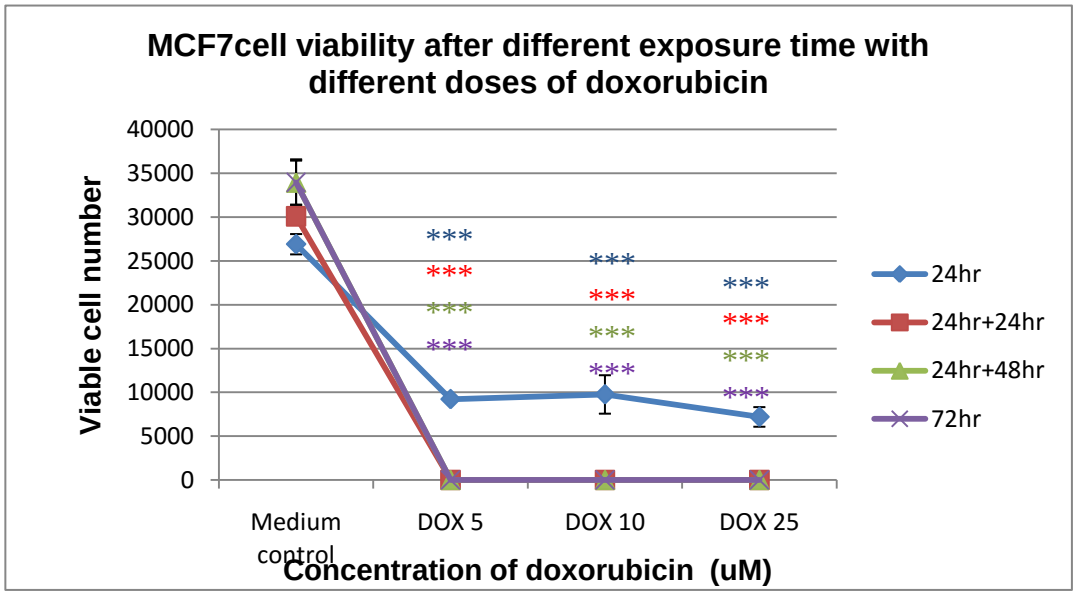


Figure 3.2.1 Effect of DOX on breast cancer cell viability by MTT assay. MCF-7 cells were seeded at 10,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to 5uM, 10uM and 25uM of DOX for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. Treatment media were disposed before determination of viable cell number. The experiment (including standard curves) was replicated on three separate occasions (n=3) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

Almost all concentrations of HCYC and A-TOC had no significant cytotoxic effects, and results were similar to those obtained with a seeding cell density of

20,000 cells/well. G-TOC had cytotoxic activity to MCF-7 cells which was also similar to the results obtained using higher seeding cell density in the MTT assay.

When MCF-7 cells were seeded at lower 10,000 cells/well density, however, the addition of G-TOC to the AC combination had a significant effect on cell viability after a 24hr exposure compared with DOX alone or the combination of AC ($p<0.001$, Figure 3.2.2.1). The addition of A-TOC to the AC combination had no significant effect on cell viability (Figure 3.2.2). All treatment containing DOX caused significantly more cytotoxicity than exposure to either HCYC or tocopherols alone at any exposure. There was no significant difference between cytotoxicity of low dose and high dose DOX alone or AC (Figure 3.2.2).

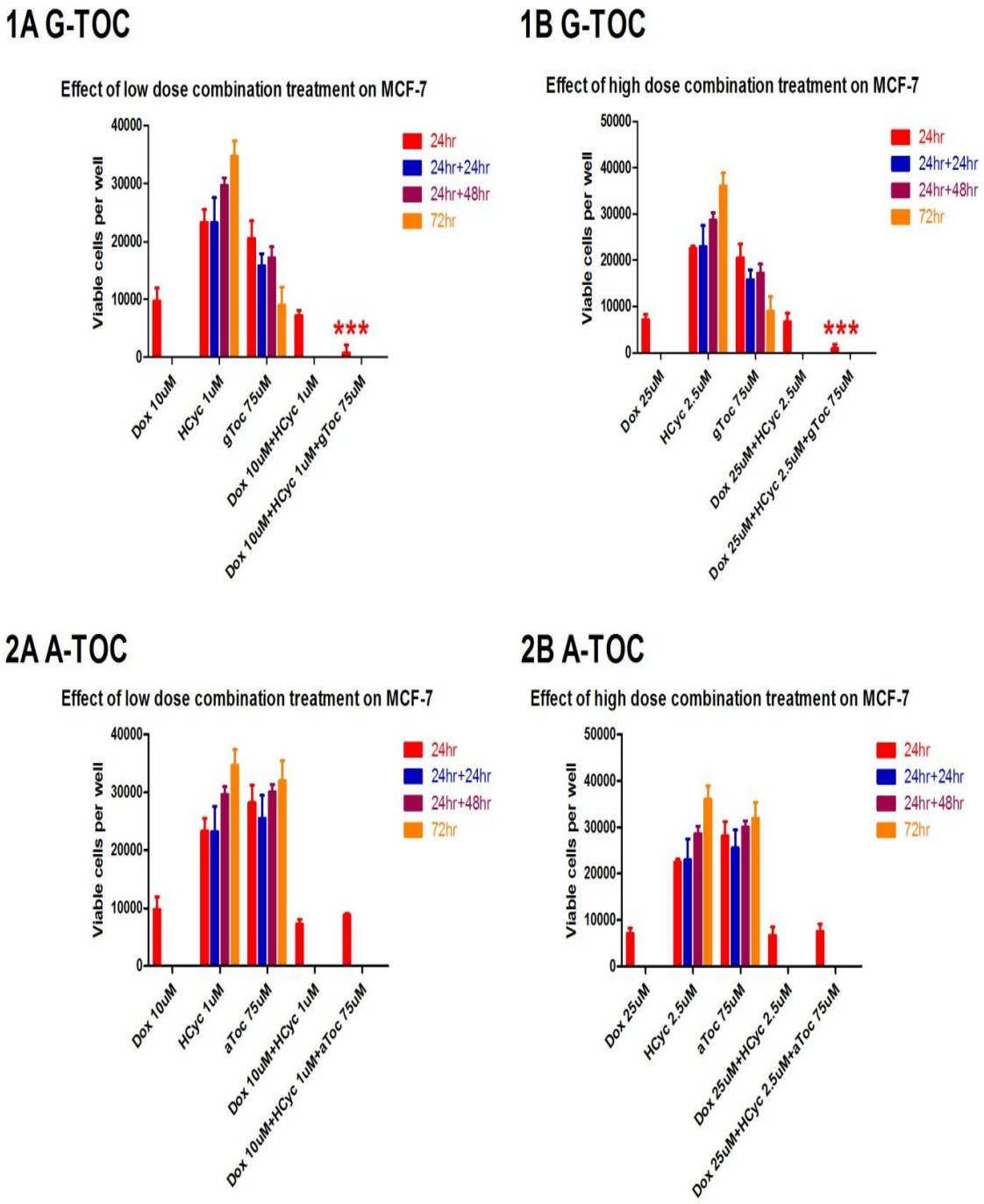


Figure 3.2.2 Effect of the combination of chemotherapeutic drugs with tocopherols on lower density breast cancer cells. Figure 1 represents the low and high doses combinations of DOX, HCYC and middle dose of G-TOC compared with their each drug alone. Figure 2 represents the low and high doses combinations of DOX, HCYC and middle dose of A-TOC compared with each drug alone. MCF-7 cell was seeded at 10,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposed to different doses of treatment for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure Treatment media were disposed before determination of

viable cell number. The experiment (including standard curves) was replicated on three separate occasions (n=3) and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

3.3 Effect of chemotherapeutic drugs and tocopherols on KGN granulosa cells

All concentrations and exposures of DOX caused significant cytotoxicity compared to the medium control in the CV assay ($p<0.001$, Figure 3.3.1A). The 72hr continuous exposure to DOX 5 caused significantly more cytotoxicity than the 24hr exposure, suggesting a time-dependent increase in cytotoxocity (Figure 3.3.1A). All concentrations and exposures of DOX caused significant cytotoxicity compared to the medium control in the MTT assay ($p<0.001$, Figure 3.3.1B). There was a dose-dependent effect of DOX after 72hr continuous exposure (Figure 3.3.1B).

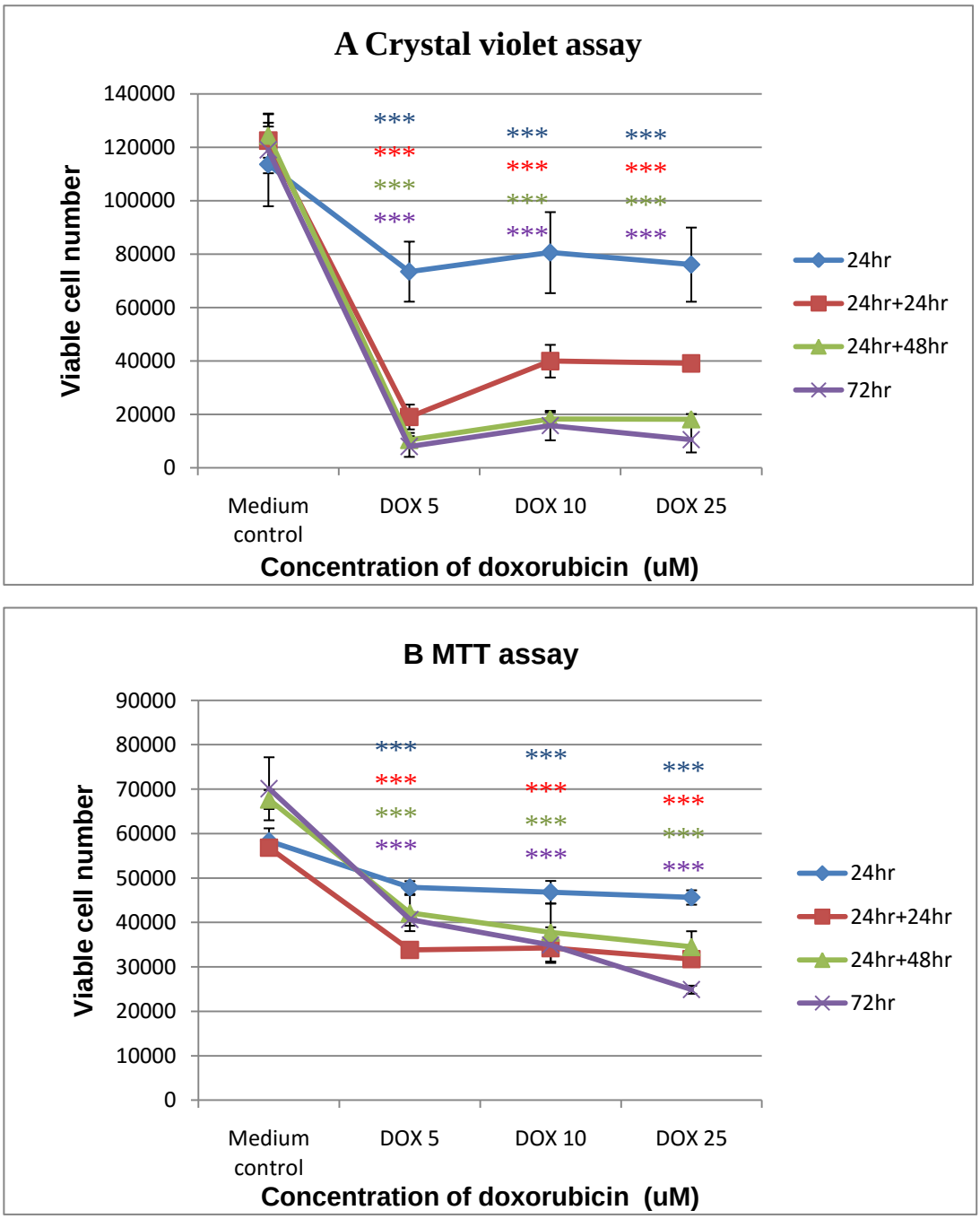


Figure 3.3.1 Effect of DOX on ovarian cancer cell viability. A) represents CV assay and B) represents MTT assay. KGN cells were seeded at 25,000 cells/well in 96-well plates for 24hr. Then KGN cells were exposed to 5uM, 10uM and 25uM of DOX for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. The experiment (including standard curves) was replicated on four separate occasions (n=4) for the CV assay and three separate occasions (n=3) for the MTT assay and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

The 0.8% DMSO vehicle control for the tocopherols did not affect cell viability

(Figure 3.3.2A), because the cell numbers were similar to these in control medium (Figure 3.3.1A). All concentrations and exposures of G-TOC had no significant effect on cell viability in the CV nor the MTT assay except 100uM of G-TOC after 72hr continuous exposure in the MTT assay ($p<0.05$, Figure 3.3.2B). There was dose-dependent cytotoxicity after 72hr continuous exposure to G-TOC (Figure 3.1.2B).

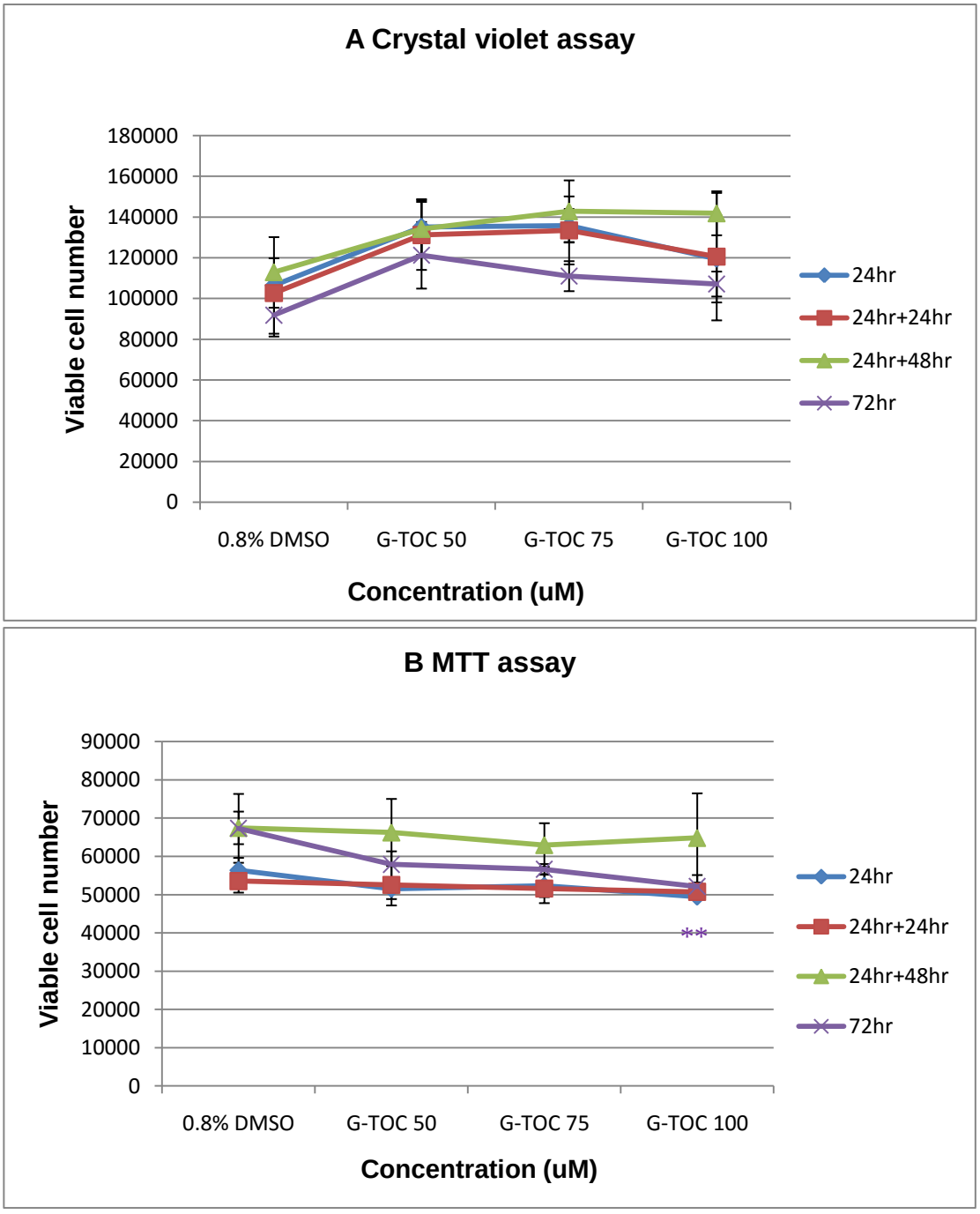


Figure 3.3.2 Effect of G-TOC on ovarian cancer cells. A) represents CV assay and B) represents MTT assay. KGN cells were seeded at 25,000 cells/well in

96-well plates for 24hr. Then KGN cells were exposed to 50uM, 75uM and 100uM of G-TOC for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. The experiment (including standard curves) was replicated on four separate occasions (n=4) for the CV assay and three separate occasions (n=3) for the MTT assay and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.01$(**).

Both low and high dose combinations of DOX + HCYC (AC) caused the same cytotoxicity as DOX alone in the CV assay, and the addition of G-TOC had no significant effect on KGN cell viability (Figure 3.3.3A). All treatments containing DOX caused significantly more cytotoxicity than exposure to either HCYC or G-TOC alone at any exposure in the CV and the MTT assay. In the MTT assay, the low dose combination of AC caused the same cytotoxicity as DOX alone, but the high dose combination of AC was significantly more cytotoxic than DOX alone after 72hr continuous exposure ($p<0.001$, Figure 3.3.3.2B). The addition of G-TOC had a significant effect on cell viability after 24hr exposure plus 24hr culture or 72hr continuous exposure compare to DOX alone, or the combination of AC ($p<0.001$, Figure 3.3.3.2B).

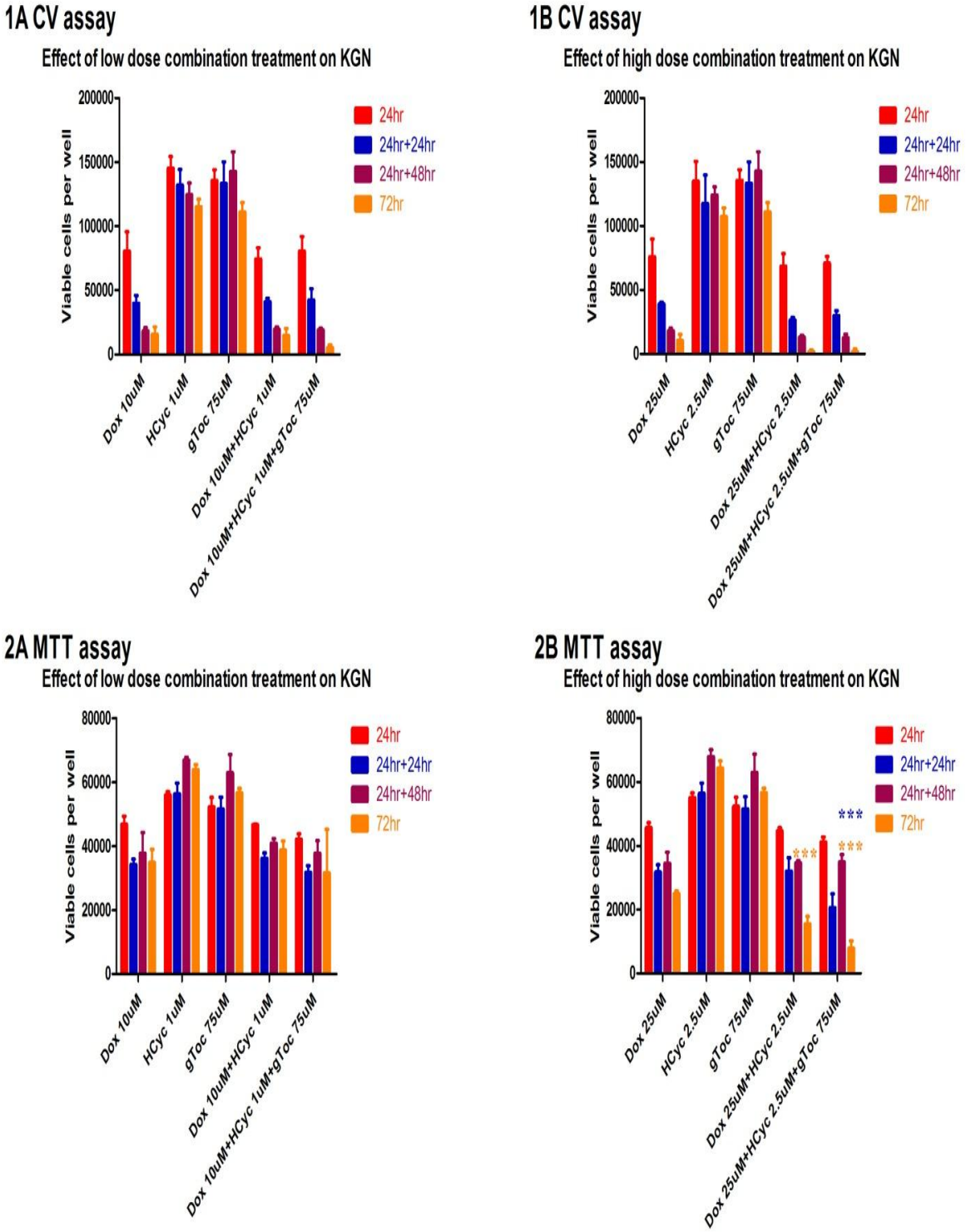


Figure 3.3.3 Effect of the combination of chemotherapeutic drugs with G-TOC on ovarian cancer cells. 1A and 1B represent CV assay and 2A and 2B represent MTT assay. Figure A represents the low dose combinations of DOX, HCYC and middle dose of G-TOC compared with each drug alone. Figure B represents high dose combinations of DOX, HCYC and middle dose of G-TOC compared with each drug alone. KGN cell was seeded at 25,000 cells/well in

96-well plates for 24hr. Then KGN cells were exposed to different doses of treatment for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. The experiment (including standard curves) was replicated on four separate occasions (n=4) for the CV assay and three separate occasions (n=3) for the MTT assay and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

In the CV and MTT assay, almost all the concentrations of HCYC and A-TOC were not significantly cytotoxic towards the KGN cells (Figure 3.3.4).

In the CV assay, the addition of A-TOC to both the low and high dose combinations of DOX + HCYC (AC) had no significant effect on cell viability (Figure 3.3.4A). All treatments containing DOX caused significantly more cytotoxicity than exposure to either HCYC or A-TOC alone at any exposure. There was no significant difference between cytotoxicity of low dose and high dose DOX alone, AC and AC with A-TOC (Figure 3.3.4). In the MTT assay, the low dose combination of AC caused the same cytotoxicity as DOX alone, but the high dose combination of AC had a significantly cytotoxicity compared to DOX alone after 72hr continuous exposure ($p<0.001$, Figure 3.3.4.2B). The addition of A-TOC also had a significant effect on cell viability at 72hr continuous exposure compared to DOX alone or the combination of AC ($p<0.001$, Figure 3.3.4.2B) but was the same as the high dose combination of AC.

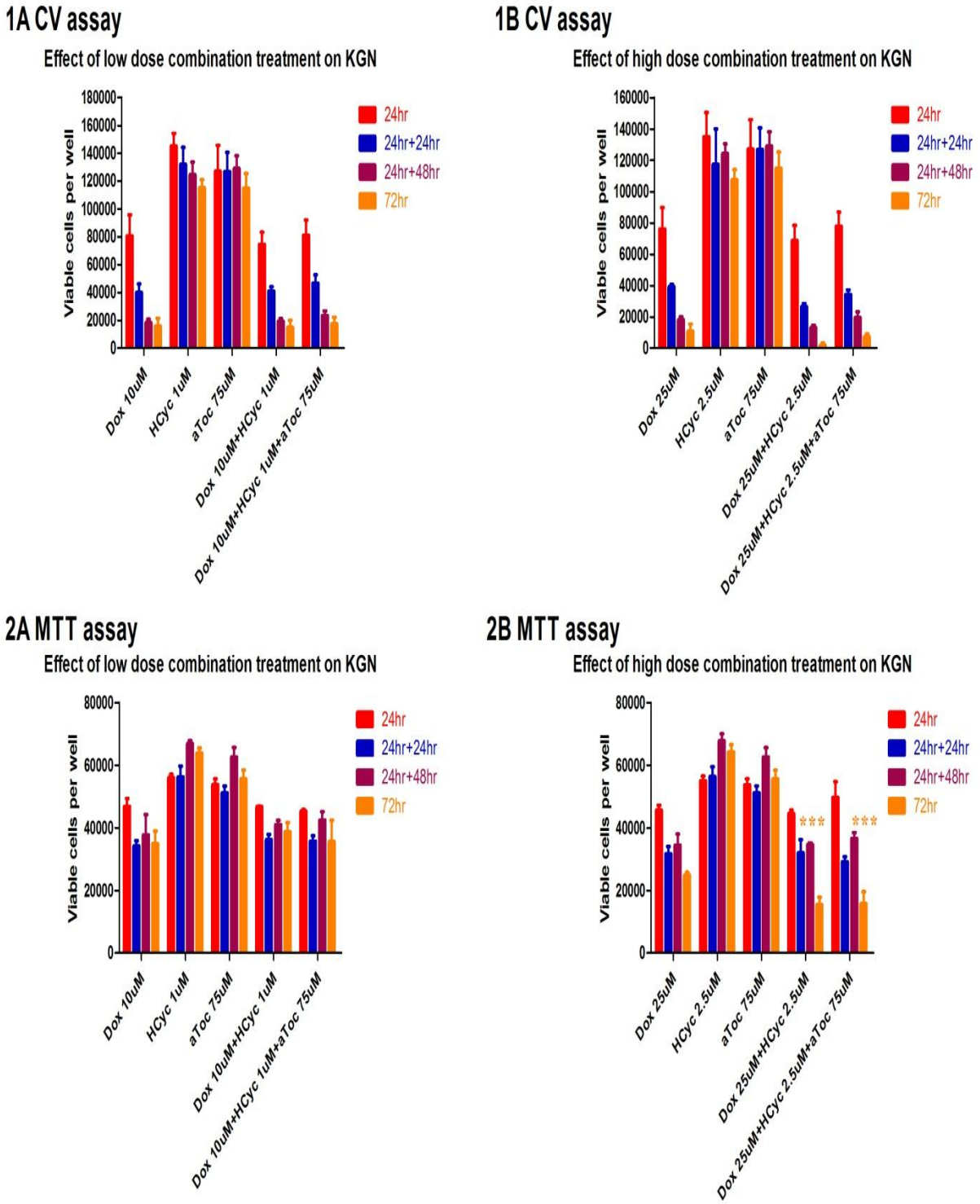


Figure 3.3.4 Effect of the combination of chemotherapeutic drugs with A-TOC on ovarian cancer cells. 1A and 1B represent CV assay and 2A and 2B represent MTT assay. Figure A represents the low dose combinations of DOX, HCYC and middle dose of A-TOC compared with each drug alone. Figure B represents high dose combinations of DOX, HCYC and middle dose of A-TOC compared with each drug alone. KGN cell was seeded at 25,000 cells/well in

96-well plates for 24hr. Then KGN cells were exposed to different doses of treatment for 24hr exposure, 24hr exposure plus 24hr culture, 24hr exposure plus 48hr culture and 72hr continuous exposure. The experiment (including standard curves) was replicated on four separate occasions (n=4) for the CV assay and three separate occasions (n=3) for the MTT assay and the means ± SD shown. Data was analyzed using a two-way ANOVA and significance was assigned at $p < 0.001$ (***).

3.4 Determination of EC25, EC50 & EC75 of DOX, HCYC and tocopherols on MCF7 by CV assay

3.4.1 EC50 Calculation

The term half maximal effective concentration (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time

The cytotoxicity EC50 value of DOX was automatically calculated by generating the EC50 curve in GraphPad Prism 5.1. A non-linear regression analysis was used to calculate the EC50 values for DOX, which were 3.63uM after 24hr exposure (Figure 3.4.1A) and 3.24uM for 48hr culture (Figure 3.4.1B). The EC50 values for HCYC were 63.69uM after 24hr exposure (Figure 3.4.1C) and 54.65uM after 24hr exposure with 24hr culture (Figure 3.4.1D). The EC50 values for G-TOC were 105.3uM after 24hr exposure (Figure 3.4.1E) and 78.13uM after 24hr exposure with 24hr culture (Figure 3.4.1F).

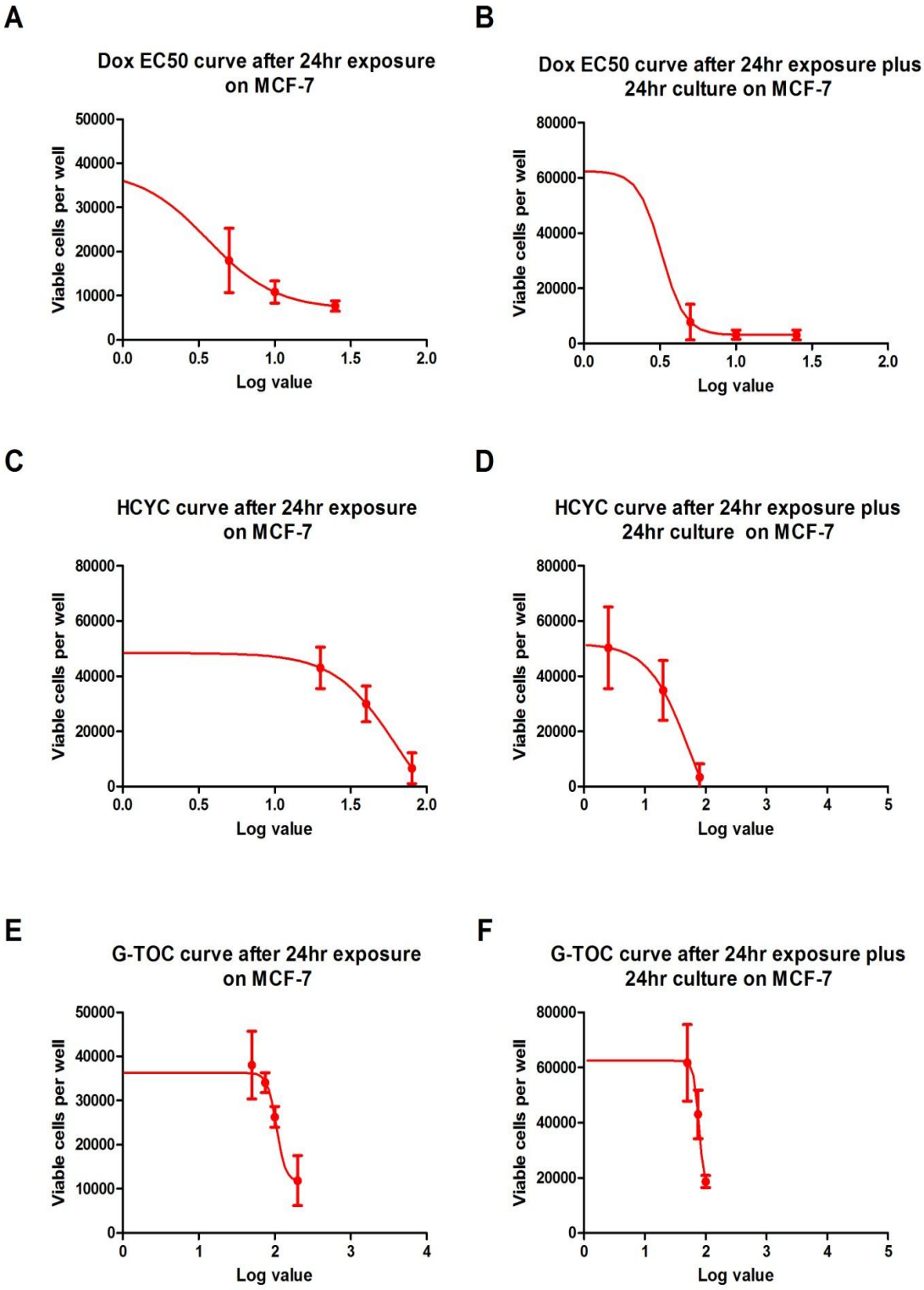


Figure 3.4.1 Effect of chemotherapeutic drugs and gamma tocopherol on breast cancer cells by CV assay. A and B show DOX after 24hr exposure and 24hr exposure plus 24hr culture. C and D show HCYC after 24hr exposure and 24hr exposure plus 24hr culture. E and F show G-TOC after 24hr exposure and 24hr exposure plus 24hr culture. The X axis represents log value of concentration

of test reagents, and the Y axis represents viable cell number after exposure to test reagents. MCF-7 cells were seeded at 20,000 cells/well in 96-well plates for 24hr. Then MCF-7 cells were exposured to different doses of the test reagents for 24hr exposure or 24hr exposure plus 24hr culture. The viable cell number was determined by CV assay. The experiment (including standard curves) was replicated on four separate occasions (n=4) and the means ± SD shown.

3.4.2 EC25 and EC75 Calculation

EC25 and EC75 values (Table 3.1) were calculated by using a calculation template GraphPad software (http://www.graphpad.com/quickcalcs/Ecanything1.cfm)

Table 3.1 EC25, EC50 and EC75 values for DOX, HCYC and G-TOC on MCF-7 cells

		EC25	EC50	EC75
DOX	24hr	1.21uM	3.63uM	10.89uM
	24hr+24hr culture	1.08uM	3.24uM	9.7uM
HCYC	24hr	21.23uM	63.69uM	191.07uM
	24hr+24hr culture	18.22uM	54.65uM	163.95uM
G-TOC	24hr	35.1uM	105.3uM	315.9uM
	24hr+24hr culture	26.04uM	78.13uM	234.39uM

3.5 Effect of chemotherapeutics and tocopherols on MCF-7 apoptosis

The following reports preliminary results, because there was only one experimental replicate. Need to repeat on at least 3 separate occasions and determine mean ± SD for each treatment.

MCF-7 cells were cultured in medium in three sets of duplicate wells. After 24hr attachment and 24hr culture, PI and annexin V were added to two of the sets of duplicate wells for examination in flow cytometry apoptosis assay, but the cells in the three "treatment" were replicates. They were collated (Table 3.5.1) to show the variation in the viable cell numbers.

Table 3.5.1 MCF-7 control cell viability, late apoptosis / necrosis and early apoptosis. The number of table shows the percentage of MCF-7 cells.

	Viable %	Late apoptotic/ necrotic %	Early Apoptotic %
Medium	99.5	0.4	0
Medium + PI	92.5	7.6	0
Medium+annexinV	90.7	0.3	9

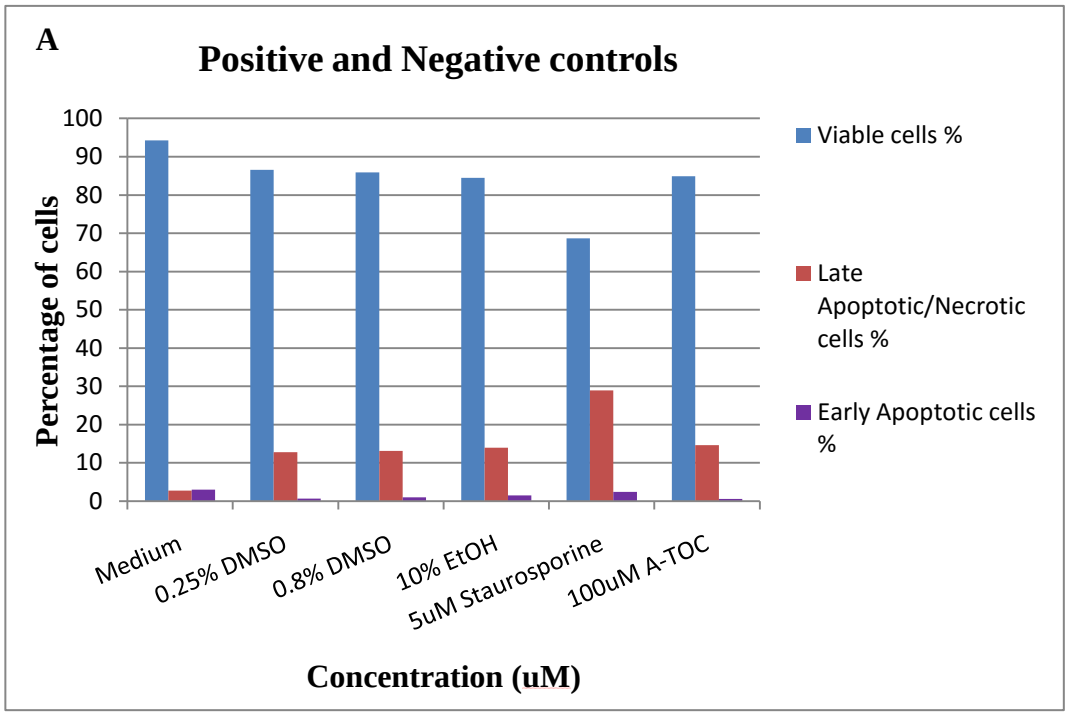
In this single replicate, there were no obvious differences between the controls cells cultured in the culture medium, DMSO vehicle negative controls, 10% EtOH, 5uM staurosporine, or 10uM A-TOC (Figure 3.5.1A) except the percentage of necrotic cell of DMSO, 10% EtOH, 5uM staurosporine and 10uM A-TOC appeared higher than medium only (Table 3.5.1).

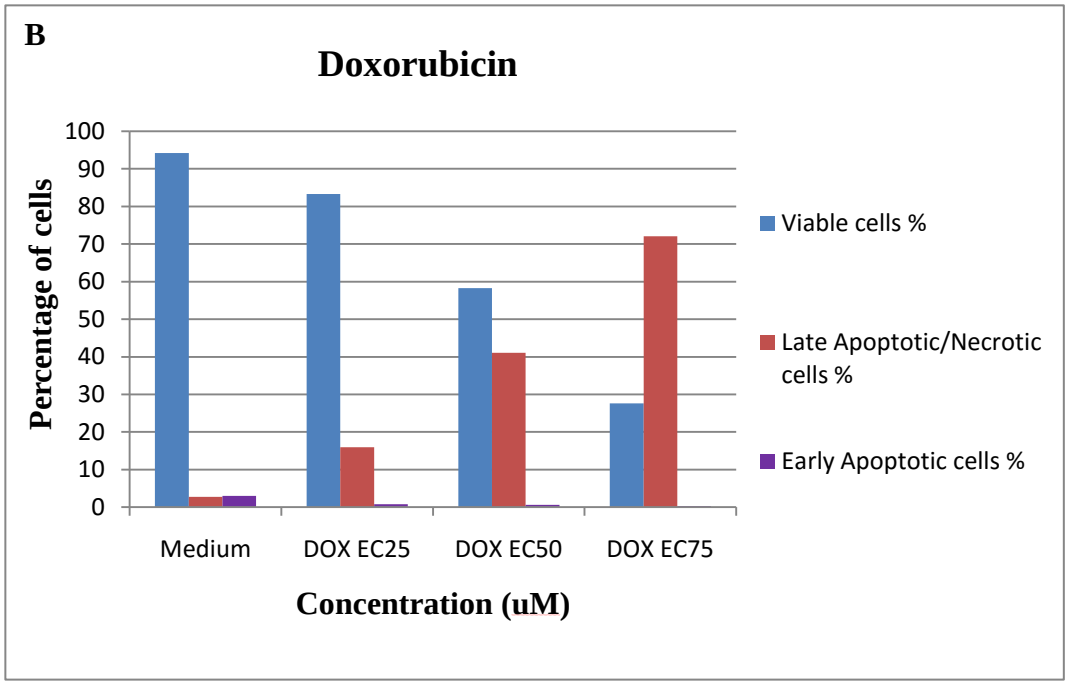
5uM staurosporine caused more necrosis compared to medium control. It was supposed to induce apoptosis, but only 2.4% cells were annexin V positive. This dot plot (appendix Figure 3.1.1E) shows lower cell densities than other treatments, suggesting fewer cells were examined in the flow cytometry. This may have been because the staurosporine dose was too high, it caused rapid cell death and resulting cell debris was not detected in the flow cytometer assay.

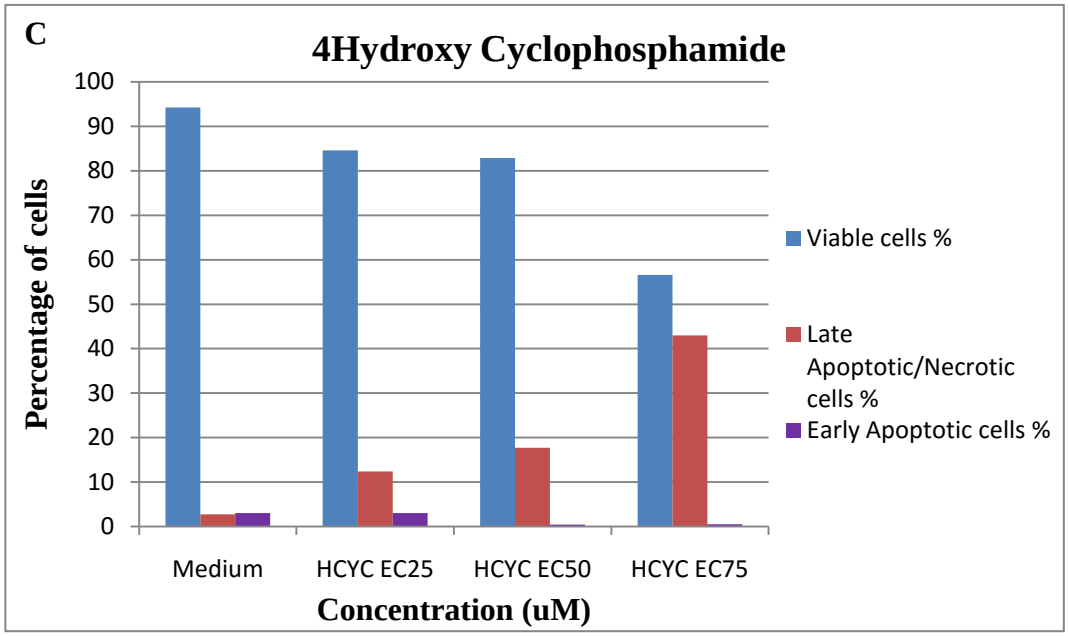
The EC25, EC50 and EC75 values of DOX did not cause a dose-dependent increase in apoptosis, but they caused a dose-dependent decrease in cell viability (Figure 3.5.1B). The EC25, EC50 and EC75 values of HCYC also did not cause a dose-dependent increase in apoptosis but did cause a dose-dependent decrease in cell viability (Figure 3.5.1C). The EC25, EC50 and EC75 values of G-TOC neither caused a dose-dependent increase in apoptosis, nor a dose-dependent decrease in cell viability. The percentage of viable cells after exposure to the

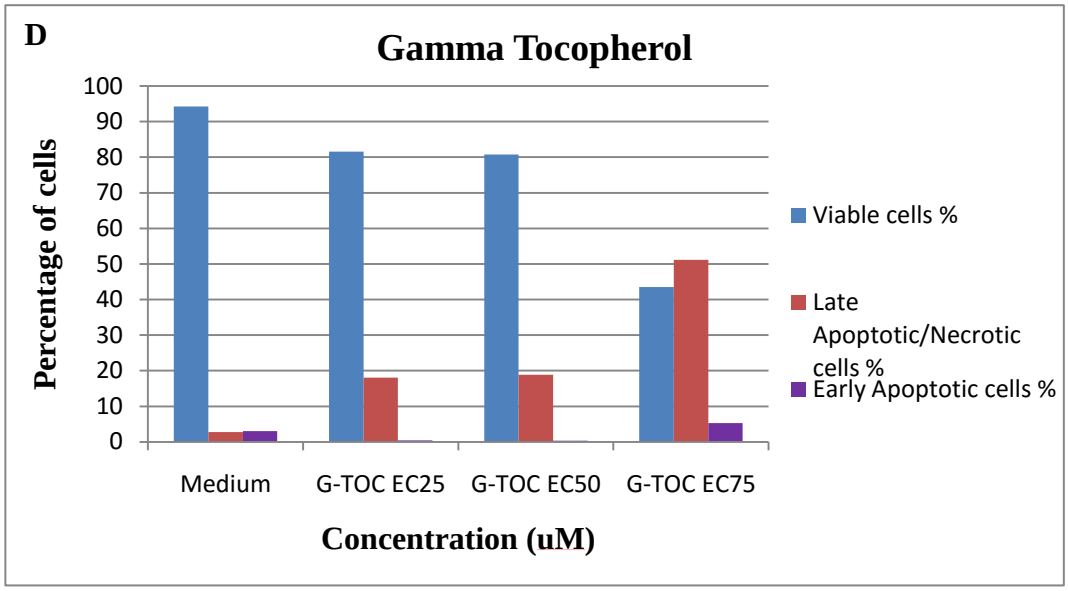
EC25 and EC50 values of G-TOC were similar, but the EC75 values of G-TOC had similar percentages of viable and necrotic cells and induced same apoptosis (Figure 3.5.1E).

The combination of AC with G-TOC and AC with A-TOC caused similar amount of necrosis but no apoptosis (Figure 3.5.1E).






C
4Hydroxy Cyclophosphamide
Percentage of cells
100
90
80
70
60
50
40
30
20
10
0
Medium
HCYC EC25
HCYC EC50
HCYC EC75
Concentration (uM)
Viable cells %
Late Apoptotic/Necrotic cells %
Early Apoptotic cells %


D
Gamma Tocopherol
Percentage of cells
100
90
80
70
60
50
40
30
20
10
0
Medium
G-TOC EC25
G-TOC EC50
G-TOC EC75
Concentration (uM)
Viable cells %
Late Apoptotic/Necrotic cells %
Early Apoptotic cells %

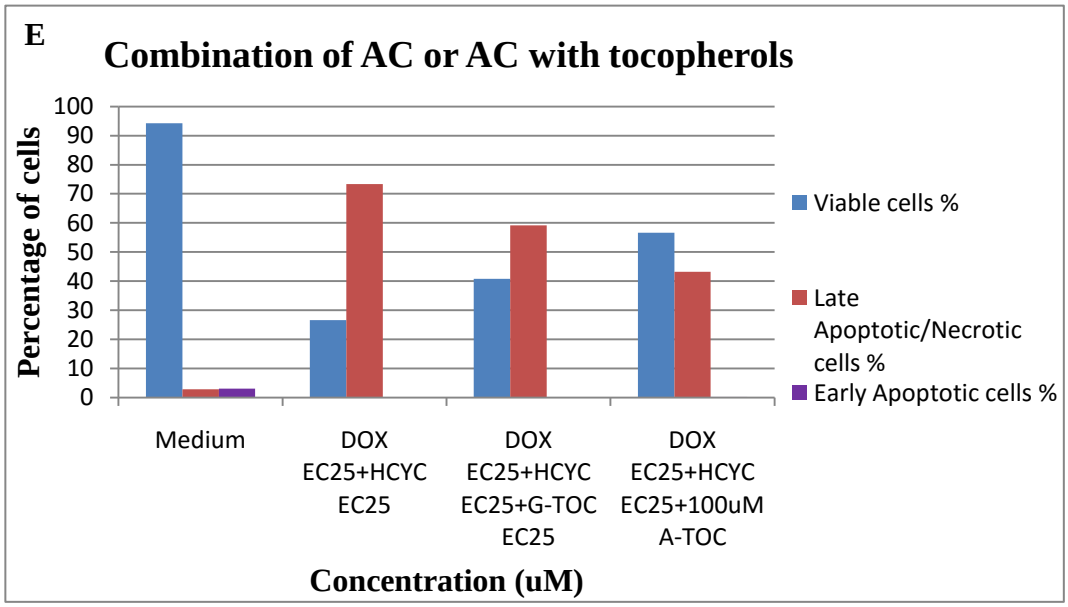

E
Combination of AC or AC with tocopherols
Percentage of cells
100
90
80
70
60
50
40
30
20
10
0
Medium
DOX EC25+HCYC EC25
DOX EC25+HCYC EC25+G-TOC EC25
DOX EC25+HCYC EC25+100uM A-TOC
Concentration (uM)
Viable cells %
Late Apoptotic/Necrotic cells %
Early Apoptotic cells %

Figure 3.5.1 Induction of cell death in the MCF-7 cells line by AC and tocopherols. A) represents the controls and 100uM A-TOC, B) represents DOX, C) represents HCYC, D) represents G-TOC and E) represents the. The MCF-7 cells ($6x10^5$ cells/well) were added to 6-well plates, pre-cultured and exposed to different concentrations of drugs for 24 hours. MCF-7 cells were collected, centrifuged and washed before staining with Annexin V-FITC and propidium iodide. The stained cells were examined by using an Accuri C6 Flow Cytometer and the data analysed using CFlow Plus. The CFlow Plus software calculated the number of cells. The whole experiment was repeated once (n=1).

CHAPTER 4: Discussions & Conclusions

4 Discussions and Conclusions

4.1 MCF-7 cytotoxicity

4.1.1 MCF-7 cytotoxicity and single reagents

In the present study, DOX and G-TOC were cytotoxic and killed MCF-7 cells, and all concentrations of DOX were significantly cytotoxic even the lowest concentration, 5uM. However, HCYC and A-TOC were not cytotoxic. The DOX cytotoxicity was similar to previous studies in that it had the strongest cytotoxicity, even though it was used at the lowest concentration *in vitro* (Kugawa et al., 2004, Park et al., 2012).

In previous studies, G-TOC reduced the growth of human lung tumor cells *in vivo* and *in vitro* and reduce the proliferation of lung cancer HT-29 cells whereas α tocopherol had no effect on cell viability (Yang et al., 2010, Xu et al., 2009). H1299 human lung cancer cells were exposure to A-TOC or G-TOC for 48 or 72hr, A-TOC (0-100uM) had no effect on cell viability, but G-TOC (0-100uM) caused a dose-dependent decrease to 70% (48hr) or 40% (72hr) of control (Lu et al., 2010). The present study showed a similar pattern on the cytotoxic effects of tocopherols towards MCF-7 cells. MCF-7 human breast cancer cells were exposure to A-TOC or G-TOC for 24hr, further 24hr or 48hr culture, and 72hr continuous exposure, A-TOC (0-100uM) had no effect on cell viability, but G-TOC (0-100uM) caused a dose-dependent decrease after any exposure except 24hr exposure, about 37% (24hrexposure + 24hr culture), 33% (24hrexposure + 48hr culture) or 17% (72hr continuous exposure) of control.

It was interesting that the low concentrations of HCYC were not cytotoxic in either the CV or the MTT assay because we expected that 0.5uM of HCYC would kill MCF-7 cells after 24hr exposure in accordance with previous *in vivo* findings (Struck et al., 1987). The present study used 4-hydroxycyclophasphamide (HCYC)

to represent cyclophosphamide (CYC) after activation by hepatic cells *in vivo* (Ozer et al., 1982, Struck et al., 1987). The HCYC compound was artificially synthesized whereas *in vivo* activation is complicated and dependent upon many types of enzyme (Madondo et al., 2016). The cytotoxicity effects of HCYC *in vitro* were not equal to the effects of CYC *in vivo,* and this may have been because the HCYC was generated through activating CYC by the hepatic cytochrome P-450 enzyme system *in vitro*, and because CYC undergoes absorption, distribution, metabolism and excretion by digestive, circulatory, endocrine, urinary and immune system *in vivo* (Nishikawa et al., 2015). MCF-7 breast cancer cells *in vitro* are not equal to breast tumor cells *in vivo*, because in breast carcinoma, there are many types of breast cancer cells surrounded by blood vessels, multi-enzymes and immune cells such as macrophages and T cells. The breast tumor cells also have abundant and sufficient nutrition and suitable environment to live, but the breast cancer cell line, MCF-7, only has artificial media containing basic nutrition, a major difference between *in vivo* and *in vitro* study. Therefore, these could be the reasons that why HCYC was not cytotoxic to MCF-7 cells.

4.1.2 Cytotoxicity and combinations of reagents

The combination of DOX and HCYC (AC) and the combination of AC with gamma tocopherol did not show synergistic cytotoxicity as we expected. In a previous study, there was a synergistic anti-tumor activity of the combination of AC in treating tumor-specific T cell responses in tumor-bearing hosts *in vivo* (Tongu et al., 2010), and HCYC in combination with DOX also shown synergistic antitumor activity in a murine lung metastatic B16BL6 melanoma model (Ishida et al., 2009). Perhaps the synergistic anti-tumor activity of the combination of AC was different due to the complicated environment *in vivo* as opposed to the simple environment *in vitro*.

4.2 MCF-7 cytotoxicity at lower seeding density (10,000 cells/well)

In the MTT cytotoxicity assays for MCF-7 cells, the highest viable cells number, around 100,000 cells/well, in medium control exceeded the maximum viable cells number, 80,000 cells/well, in the standard curve. Hence, the MTT assay results were unreliable compared with the CV assay. The MTT assay has a further 24hr culture in incubator after staining cells with MTT. When the cell number of each well exceeded 110,000, there was likely to be nutrition depletion which would impair the result. Therefore, the MCF-7 seeding cell density for the MTT assay was reduced to 10,000 cells/well.

Most of reagents had similar pattern of cytotoxicity which was that DOX and G-TOC were cytotoxic and HCYC and A-TOC were not. However, all concentrations of DOX and all the combinations killed MCF-7 cells. The lowest dose of DOX (5uM) killed more than 75,000 cells/well after 24hr exposure plus 24hr culture, so it was made sense that all the cells were killed in lower seeding cell density experiment. There was synergistic cytotoxicity after exposure to the low and high dose combinations of G-TOC with AC and lower seeding cell densities. The addition of G-TOC to AC was more cytotoxic than DOX alone or the combination of AC. The cytotoxicity assay results from the experiment with higher seeding densities of 20,000 cells/well was no significantly different.

4.3 KGN cytotoxicity

In the present study, DOX was significantly cytotoxic to KGN cells, and all concentrations of DOX were significantly cytotoxic. However, HCYC, G-TOC and A-TOC were not cytotoxic.

In previous studies, G-TOC was cytotoxic to HT-29 lung cancer cells, breast cancer cells and prostate cancer cells whereas α tocopherol had no effect on cell viability (Carolin et al., 2002, Nechuta et al., 2011, Xu et al., 2009). G-TOC only had anti-cancer activity in tumor cells but did not affect proliferation of human normal cells (Xu et al., 2009). The present study showed the similar cytotoxic

effects. G-TOC was significantly cytotoxic to MCF-7 breast cancer cells but not to KGN cells even though the KGN cells were derived from a human ovarian carcinoma. It could because cytotoxic mechanism of action of G-TOC in MCF-7 cells was different from KGN cells. In previous study, G-TOC induced MCF-7 cell apoptosis by activation of caspases (Patacsil et al., 2012, Sun et al., 2009, Xu et al., 2009), but maybe KGN cells have different apoptosis induction pathways.

4.4 EC value Chemptherapeutic drugs and Tocopherols on MCF-7 cells

The EC50 value of HCYC, 63.69uM, was much higher than we expected compared with the human serum concentration, 0.02uM, in the 24hr period after administration (Struck et al., 1987, Swenson et al., 2003). It could be the difference between HCYC and CYC *in vitro* and *in vivo* study. A-TOC did not have EC50 values because it was not cytotoxic even on higher concentration, 500uM. In previous studies, 400 cells/well MCF-7 cells were seeded into 12-well plates and exposed to G-toc for 12 days and the EC50 value was 35uM (Yu et al., 2008). However, the EC50 value of G-TOC of this study was 105.3uM after 24hr exposure and 78.13uM after 24hr exposure with 24hr culture. This could be the exposure time of present study was too short and the cell seeding densities was too high compared to previous study.

4.5 Apoptosis assay on MCF-7 cells

In this study, due to time limitations only one replicate apoptosis experiment was performed. The preliminary data, suggested that all tested reagents did not cause cell apoptosis but caused more cell necrosis. The result was different compared to previous studies in which DOX, HCYC and G-TOC had caused cells apoptosis in tumor cells (Anderson et al., 2004, Pang et al., 2011, Singh et al., 2009, Zhou et al., 2012). In the previous studies 40uM of G-TOC were exposed to MCF-7 cells

for 48hr and induced death receptor (DR5) mediated apoptosis (Yu et al., 2008). The EC25 (35.1uM) should have caused apoptosis detectable by the Annexin V FITC flow cytometry assay, but no apoptosis was detectable. Possibly because neither of the EC25 (35.1uM) or EC50 (105.3uM) concentrations were optimal for inducing apoptosis, and the 24hr exposure was too long. The apoptosis positive control, 5uM staurosporine, did not work in the apoptosis assay. This could be the concentrations of tested reagents were too high and they caused rapid cell apoptosis and secondary necrosis which was identified in the assay. The dot plot showed lower cell densities than other treatments, suggesting fewer cells were examined in the flow cytotomer. That could be the concentration of staurosporine was too high and it killed most of cells and the cell debris was washed out away.

4.6 Future directions

Firstly, the apoptosis assay of this study in MCF-7 cells was limited because only one experiment replicate was conducted. For the future development of apoptosis assay, the concentration of staurosporine (apoptosis positive control) needs to be diluted to a lower concentration because 5uM staurosporine caused too much cytotoxicity. Before conducting the apoptosis assay in MCF-7 cells, a time cause for apoptosis should be examined. Secondly, the EC25, EC50 and EC75 values of the reagents should be determined for the KGN cells.

Future studies could examine ROS generation by chemotherapeutics and tocopherols in MCF-7 and KGN cells and to see if the addition of tocopherols can maintain anticancer activities and reduce ROS generation in KGN cells and maintain hormone synthesis (Rudera et al., 2009).

4.7 Conclusions

This study analyzed the cytotoxic effects of DOX, HCYC, G-TOC, A-TOC and their combinations to MCF-7 breast cancer and KGN granulosa cell lines by the crystal violet and MTT assay. DOX and their combinations were significantly cytotoxic to both cell lines, but G-TOC was only cytotoxic to the MCF-7 breast cancer cell line. HCYC and A-TOC were not significantly cytotoxic to either cell.

Almost all exposure periods of the combination DOX and HCYC (AC), the combination of G-TOC and AC and the combination of A-TOC and AC failed to show synergistic cytotoxicity. However, the low and high dose combination of G-TOC and AC after 24hr exposure to a lower seeding cell density was synergistic in MCF-7 cells; the high dose combination of AC at 72hr continuous exposure in MTT assay also had a significant synergistic effect towards KGN cells and the combination of G-TOC and AC at 48hr culture and 72hr continuous exposure and the combination of A-TOC and AC at 72hr continuous exposure in MTT assay had a significantly synergism effect in KGN cells.

The concentrations of HCYC used in the cytotoxicity assay were not cytotoxic, but its EC50 value for 24hr exposure, 63.69uM, was much higher than we expected and much higher than EC50 values of DOX for 24hr exposure, 3.63uM.

The limited apoptosis assay showed that all the chemotherapeutics, drugs and their combinations did not cause apoptosis, but they all caused cell necrosis which corresponded to their cytotoxic effects.

Antioxidants, such as vitamin E, have been proposed as an additional treatment to treating many types of tumour cells (Anderson et al., 2004, Nechuta et al., 2011, Sun et al., 2009, Wong et al., 2012, Xu et al., 2009). Thus, it is worth studying them further to determine their mechanisms of action and anti-cancer activity.

MCF-7 cytotoxicity

DOX was significantly cytotoxicity to the MCF-7 cells, even the lowest concentration: 5 μM DOX was significantly cytotoxic after 24hr exposure and the

left amount viable cell numbers takes up 46.7% of medium control and its EC50 value was 3.63 μM after 24hr exposure.

There was no synergism between the combination of AC or AC with tocopherols compared to DOX alone

KGN cytotoxicity

DOX was also significantly cytotoxicity to the KGN cells, and on the tested lowest concentration: 5 μM DOX was significantly cytotoxic after 24hr exposure and the left amount viable cell numbers takes up 64.68% of medium control. Most importantly, there was a synergism that the addition of G-TOC to the combination of AC was more cytotoxic than DOX alone or the combination of AC.

Therefore, DOX was more cytotoxic to the MCF-7 cells than to the KGN cells, G-TOC was cytotoxic to the MCF-7 cells but not to the KGN cells, and G-TOC increased the chemotherapeutic effect of AC with KGN cells but not to the MCF-7 cells which could be AC damaged KGN first and made them susceptible to G-TOC.

CHAPTER 5: Reference

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CHAPTER 6: Appendix

Appendix 1. Cell Culture Media

1.1 Preparation of RPMI-1640 for MCF-7 cells

a. Bottle of 500mL RPMI-1640 medium (containing PH indicator phenol red) (Cat#R8758).

b. Measure 50ml foetal bovine serum (FBS) (HyClone; ISO-010-02) and filter it with 0.45μm Ministart syringe filter (Sartorius) into the RPMI medium.

c. Add 5ml penicillin-streptomycin (Cat#P4333) and filter it with 0.22μm Ministart syringe filter (artorius) into the RPMI medium.

d. Mix them very well and remove 5ml into T25 flask. Then, incubate it at 37℃ incubator for few days and check it under microscope to make sure it is not contaminated.

e. Store the bottle of mixed RPMI 1640 medium at 4^{o}C for a maximum of 2 months.

f. Before using media, warm it to 37℃.

1.2 Preparation of DMEM /F12 for KGN cells

a. Bottle of 500mL DMEM /F12 medium (Cat#D8437).

b. Measure 50mL FBS (HyClone; ISO-010-02) and filter it with 0.45μm Ministart syringe filter (Sartorius) into the DMEM /F12 medium.

c. Repeat (1.1 c) above.

d. Add 1% (v/v) of Insulin-Transferrin-Selenium (ITS) and filter it with 0.22μm Ministart syringe filter (Sartorius) into the media bottle.

g. Mix them very well and remove 5ml into T25 flask. Then, incubate it into 37℃ incubator for few days and check it under microscope to make sure it is not contaminated.

e. Store the bottle of mixed DMEM /F12 medium at 4^{o}C for a maximum of 2 months.

f. Before using media, warm it at 37℃.

Appendix 2. Experiment & reagents

Table 2.1 List of equipment

Equipment	Supplier/Brand
Centrifuge 5804	Eppendorf
CO2 Incubator	Sanyo, CO2 Incubator
Culture Flasks (T25 & T75)	Corning, USA
Flat Bottom 6-well Plate	Costar 3596, USA
Flat Bottom 96-well Plate	Costar 3596, USA
Haemocytometer	Neubauer Imprived
Microscope	HWS series (HWS-120-BHA-120)
Spectrophotometer	Bio Tek Instruments. INC
10 and 50ml centrifuge tubes	Corning, USA
0.22 and 0.45μm filter	Ministart

Table 2.2 List of reagents

Chemicals	Supplier/Brand
Acetic Acid	Univar, USA
Alpha tocopherol (A-TOC)	Sigma-Aldrich, MO, USA
Crystal Violet	Sigma-Aldrich, MO, USA
DMEM /F12 Medium	Sigma-Aldrich, MO, USA
Dimethyl sulfoxide (100% DMSO)	Sigma-Aldrich, MO, USA
Doxorubicin (DOX)	Sigma-Aldrich, MO, USA
EtOH (100%)	Merck, Kilsyth, Victoria
Fetal bovine serum (FBS)	HyClone
Gamma tocopherol (G-TOC)	Sigma-Aldrich, MO, USA
Hydrochloric Acid (HCl)	BDH chemicals, Kilsyth, Victoria
4 Hydroxycyclophosphamide (HCYC)	Sigma-Aldrich, MO, USA
Insulin-Transferrin-Selenium (ITS)	Sigma-Aldrich, MO, USA
Methanol	Merck, Kilsyth, Victoria
Milli-Q Water	Milli-Q water purification system
Penicillin/Streptomycin	Sigma-Aldrich, MO, USA
RPMI 1640 Medium	Sigma-Aldrich, MO, USA
Trypan Blue	Sigma-Aldrich, MO, USA
Trypsin EDTA	Sigma-Aldrich, MO, USA
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, MO, USA

2.1 Preparation of 20 x Phosphate Buffered Saline (PBS)

To make 1L of 20 x PBS:

1. Weigh out the following and add to 1L volumetric flask:

   NaCl – 160g

   KCl – 4g

   Na_2HPO_4 – 28.8g

   KH_2PO_4 – 4.8g

2. Add 900mL RO water. Shake well to mix.

3. Make up to 1L using RO water. Then, autoclave it.

4. Store at room temperature.

2.2 Make 1L of 1 x PBS solution from 20x stock solution

1. Measure 50mL of 20 x PBS stock solution in a 100mL measuring cylinder.

2. Measure 950mL RO water in a 1 L measuring cylinder.

3. Add the 50mL of 20 x PBS stock solution to the 950mL RO water.

4. Pour the 1 x PBS solution into Schott bottle and mix well.

5. Adjust to pH7.2-7.4.

6. Store at room temperature.

2.3 Preparation of 1 x Trypsin EDTA

To make 100ml:

1. Filter 5ml of sterile trypsin EDTA (Sigma Aldrich; Cat#T4147;

Lot#SLBH0775) and filter it with 0.22µm syringe filter into 50ml centrifuge tube.

2. Filter 45ml sterile 1x PBS solution with 0.45µm Ministart syringe filter (Sartorius) into same 50ml centrigfuge tube; then mix them very well.

3. Store aliquots at -20^{o}C until required.

2.4 Trypan Blue Staining Solution Preparation

To make 100ml:

1. Measure 100ml of MQ H_2O with volumetric flask and add to 100ml Schott bottle

2. Weigh out 0.9g of NaCl and add to Schott bottle

3. Shake to mix

4. Add 0.2g Trypan Blue to solution

5. Shake to mix

6. Filter solution with 0.22µm syringe filter into 1.5ml microfuge tubes

2.5 Crystal Violet Preparation

1. Prepare 50% methanol (make 100ml) in fume hood. If methanol is in a large 2L bottle, use a glass 25ml pipette to transfer into the volumetric flask.

    a. Measure 50mls reverse osmosis water using volumetric flask add to 100ml volumetric flask.

    b. Measure 50ml methanol in 50ml volumetric flask and then add to 50ml RO water.

c. Mix well and store in 100ml Schott bottle.

2. Weigh out crystal violet stain - 0.5g per 100ml volume (0.5%).

3. Add crystal violet to 50% methanol solution.

2.6 Acetic Acid Preparation

Prepare 33% acetic acid (Univar, USA) de-stain solution for the crystal violet assay. To make 100ml measure 67ml of RO water and add to 100ml Schott bottle.

1. Measure out 33mls acetic acid and add to RO water in Schott bottle.

2.7 Preparation of 5mg/ml MTT

a. Prepare a stock solution of 5mg/ml and store in aliquots (to avoid repeat thaw/freeze).

e.g. To make 50mls at 5mg/ml

$$\frac{5\text{mg} \times 50\text{ml}}{1\text{ml}} = 250\text{mg}$$

b. Wear mask and gloves and weigh out required amount of MTT. Make up to required volume with 1 x PBS and cover the beaker with foil. Mix well using magnetic stirrer.

c. Filter MTT solution through a 0.22μm sterile Ministart syringe filter (Sartorius) into sterile tubes.

d. Store aliquots at -20^{o}C until required.

2.8 0.02M HCl Preparation

a. To Make a stock solution of 0.02M HCl for the MTT assay

To prepare 500mls of solution of 0.02M HCl from 12M HCl stock will require:

$$\text{e.g. } \frac{0.02\text{mol.l}^{-1} \quad x \quad 0.5\text{L}}{12\text{mol.l}^{-1}} = 8.3\text{x}10^{-4}\text{L or } 0.8333\text{ml}$$

b. Add ~400ml of reverse osmosis (RO) water to a 500ml volumetric flask.

c. In fume hood add 0.8333ml of 12M HCl to flask and bring volume up to 500ml mark with RO water.

d. Store in 500ml glass Schott bottle with a clear chemical label for HCl in fume hood.

2.9 20% SDS in 0.02M HCl Preparation

a) Use a face mask when weighing out SDS powder.

    a. To make 200ml, weigh out 40g SDS powder (20%) and add to a 500ml glass beaker.

b) In a fume hood, add ~100ml of 0.02M HCl to beaker. Cover beaker with alfoil or gladwrap to stop fumes escaping. Stir in fume hood with magnetic flea under low heat until dissolved.

c) Decant SDS 0.02M HCl solution into a volumetric flask and increase volume to 200ml with 0.02M HCl solution.

d) Store in labelled 500ml container in fume hood until required.

2.10 0.1% Sodium Azide in PBS

a. To make 500ml, weigh out 0.5g sodium azide powder (0.1%) and add to a 500ml glass beaker by wearing mask and gloves

b. Make up to required volume with 1 x PBS and cover the beaker with foil. Mix well using magnetic stirrer.

c. Store aliquots at -4^{o}C until required.

2.11 1 x Binding Buffer Preparation

10 X Annexin V Binding Buffer from FITC Annexin V Apoptosis Detection Kit I. The solution was 0.22µm sterile filtered. For a working solution (1X), dilute 1 part binding buffer to 9 parts distilled H_2O.

2.12 Test reagents Preparation

Table 2.3 Preparation of test reagents in RPMI or DMEM /F12 medium

	Test Reagents	Final Concentration 2	Final Volume 2 (µl)	Concentra-tion 1	Volume 1 (µl)	Volume of Media (µl)
Working Solution	DOX	100uM	9000	5mg/ml	53.82	8946.18
	HCYC	1000uM	500	5mg/ml	29.308	470.692
		10uM	4000	1000uM	40	3960
	A-TOC	100uM	4000	10mg/ml	189.1	3810.9
		40uM	4000	1000uM	1600	2400
	G-TOC	1000uM	4000	5mg/ml	333.344	3666.66
		400uM	4000	1000uM	1600	2400
Concentr−ation Needed	DMSO	1.6%	10000	100%	160	9840
		0.5%	10000	100%	50	9950
	EtOH	20%	4000	100%	800	3200
	DOX	50uM	4800	100uM	2400	2400
		20uM	4500	50uM	1800	2700
		10uM	3000	20uM	1500	1500
	HCYC	5uM	4800	10uM	2400	2400
		2uM	4500	5uM	1800	2700
		1uM	3000	2uM	1500	1500
	A-TOC	200uM	3000	400uM	1500	1500
		150uM	3000	400uM	1125	1875
		100uM	3000	400uM	750	2250
	G-TOC	200uM	3000	400uM	1500	1500
		150uM	3000	400uM	1125	1875
		100uM	3000	400uM	750	2250
	DOX +	50uM	3000	100uM	1500	1485

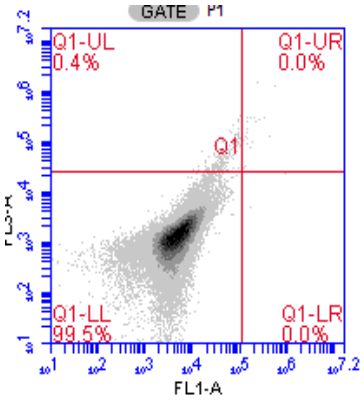
	HCYC	5uM		1000uM	15	
	DOX + HCYC	20uM	3000	100uM	600	1200
		2uM		5uM	1200	
	DOX + HCYC + A-TOC	50uM	3000	100uM	1500	1035
		5uM		1000uM	15	
		150uM		1000uM	450	
	DOX + HCYC + A-TOC	20uM	3000	100uM	600	1944
		2uM		1000uM	6	
		150uM		1000uM	450	
	DOX + HCYC + G-TOC	50uM	3000	100uM	1500	1035
		5uM		1000uM	15	
		150uM		1000uM	450	
	DOX + HCYC + G-TOC	20uM	3000	100uM	600	1944
		2uM		1000uM	6	
		150uM		1000uM	450	
	Staurospo -rine	1000uM	100	1g/ml	46.653	53.347
		5uM	4000	1000uM	20	3980

DOX represents Doxorubicin (Sigma Cat#44583), CYC represents 4-hydroxycyclophosphamide (Santa Cruz, CAS 39800-16-3), A-TOC represents alpha tocopherol (Cat#T3001-10G), G-TOC represents gamma tocopherol (Cat#T1782), EtOH represents ethanol and DMSO represents Dimethyl sulfoxide (Sigma). The method of preparing test reagents was measuring and removing volume 1 into the RPMI or DMEM /F12 medium based on the cell line type, and the volume of media was shown as table 2.1.

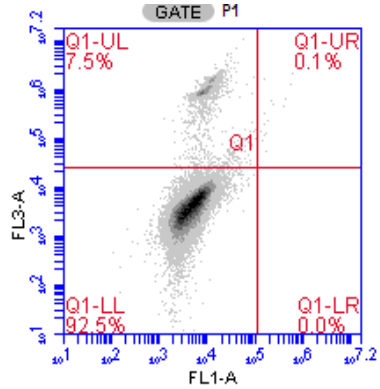
Appendix 3 Result

3.1 Examples of Flow Cytometry dot plot for apoptosis analysis (Annexin V-FITC and PI)

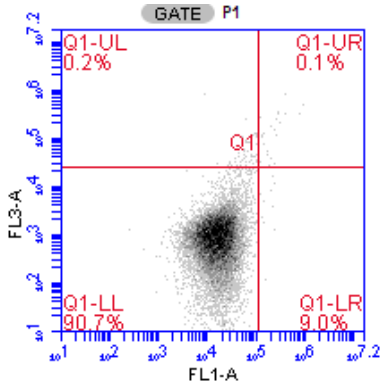
A. Medium alone without PI or annexin.



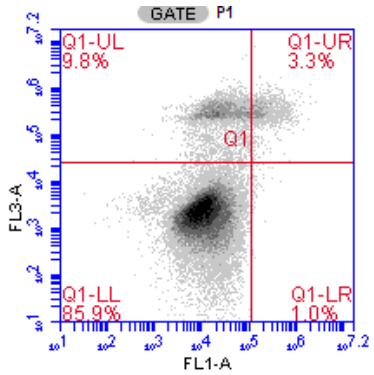
B. Medium with PI



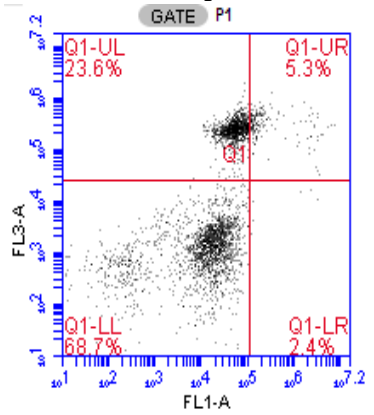
C. Medium with annexin V



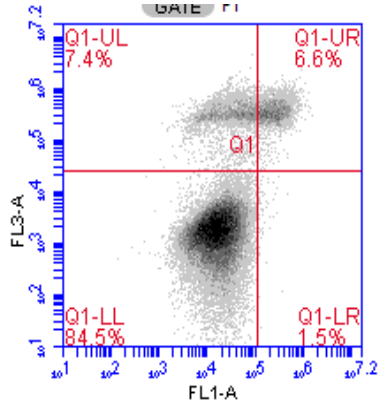
D. 0.8% DMSO



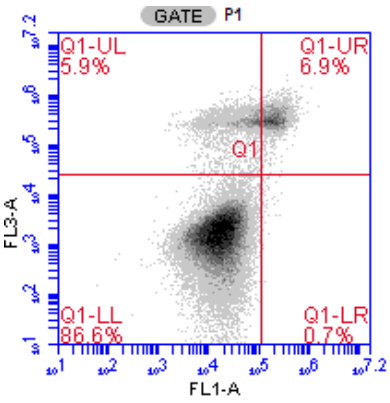
E. 5uM Staurosporine



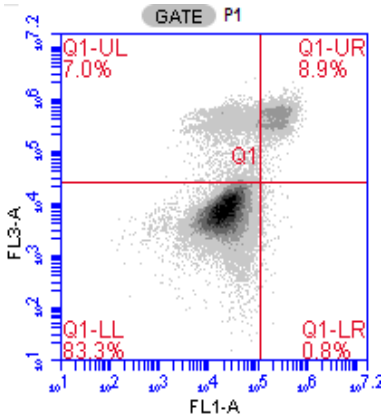
F. 10% EtOH



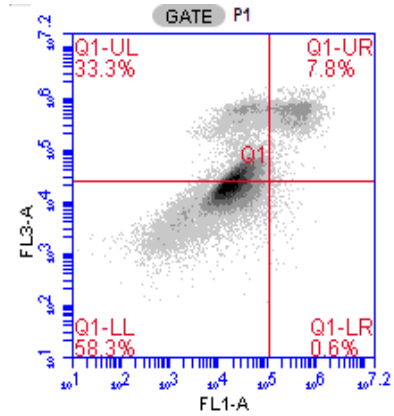
G. 0.25% DMSO



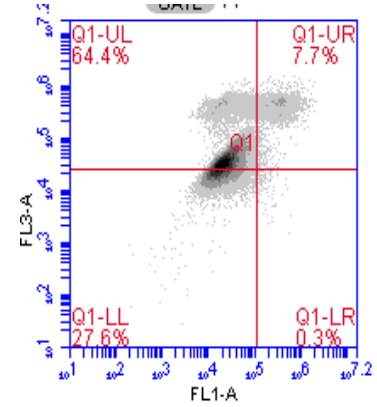
H. DOX EC25



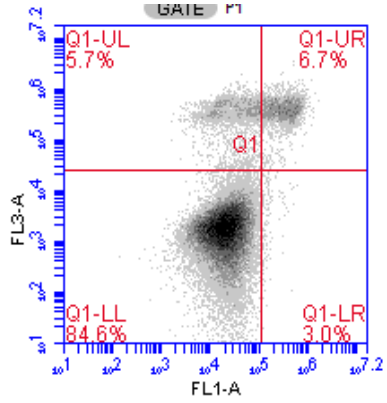
I. DOX EC50



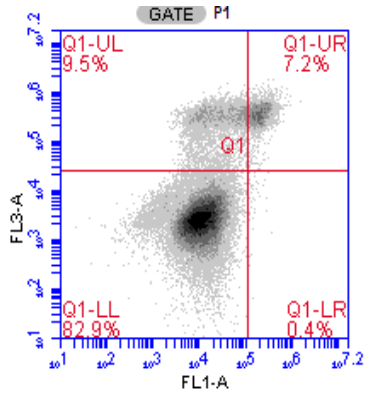
J. DOX EC75



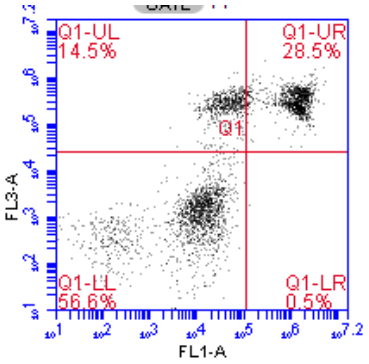
K. HCYC EC25.



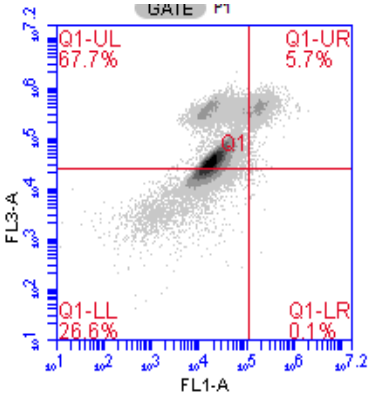
L. HCYC EC50



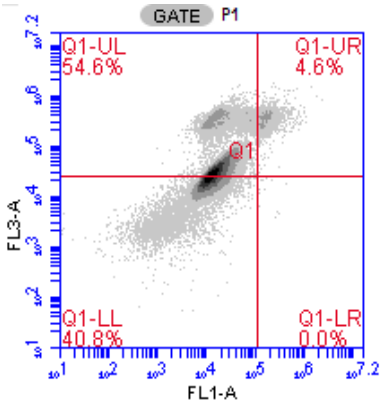
M. HCYC EC75



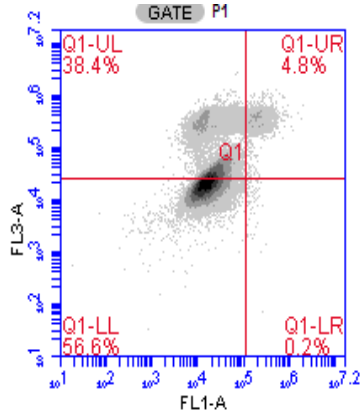
N. DOX EC25+HCYC EC25



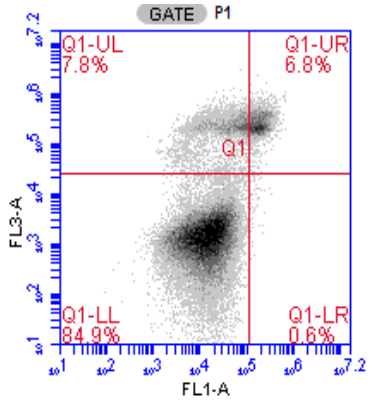
O. DOX EC25+HCYC EC25+G-TOC EC25



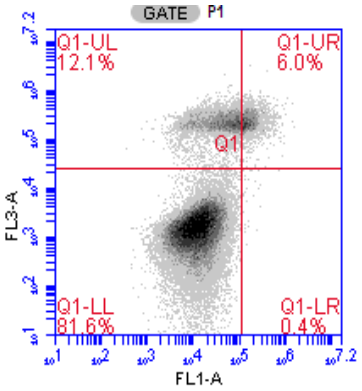
P. DOX EC25+HCYC EC25+A-TOC 100uM:



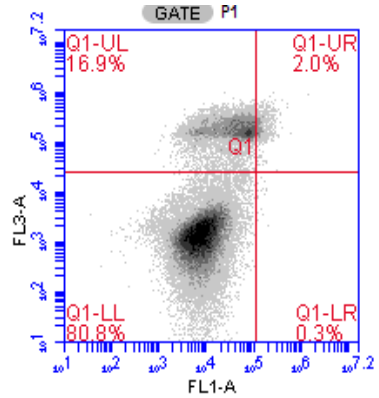
Q. A-TOC 100uM



R. G-TOC EC25



S. G-TOC EC50



T. G-TOC EC75

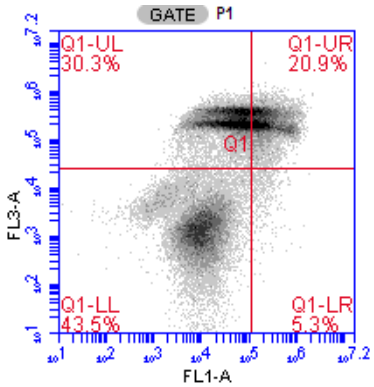


Figure 3.1.1 Induction of MCF-7 apoptosis by AC and tocopherols. The induction of apoptosis on MCF-7 cells by different doses of DOX, CYC, A-TOC, G-TOC and their combinations after 24 hours treatment was detected using Flow Cytometry with the aid of FITC Annexin V/PI staining. A represents an example of Medium without PI or FITC Annexin V (negative control), B represents an example of Medium with PI (control), C represents an example of Medium with FITC Annexin V (control), D represents an example of 0.8% DMSO (vehicle control), E represents an example of 5uM Staurosporine (positive control), F represents an example of 10% EtOH (positive control), G represents an example of 0.25% DMSO (vehicle control), H represents an example of EC25 value of DOX, I represents an example of EC50 value of DOX, J represents an example of EC75 value of DOX, K represents an example of EC25 value of HCYC, L represents an example of EC50 value of HCYC, M represents an example of EC75 value of HCYC, N represents an example of EC25 value of DOX plus EC25 value of HCYC, O represents an example of EC25 value of DOX plus EC25 value of HCYC with EC25 value of G-TOC, P represents an example of EC25 value of DOX plus EC25 value of HCYC with 100uM of A-TOC, Q represents an example of 100uM of A-TOC, R represents an example of EC25 value of G-TOC, S represents an example of EC50 value of G-TOC, T represents an example of EC75 value of G-TOC. 6 x 10^5 MCF-7 cells/well were added to 6-well plates, pre-cultured and exposed to compound for 24 hours. The compounds were diluted in RPMI media. MCF-7 cells were collected, centrifuged and washed before being stained with Annexin V-FITC and Propidium iodide (PI). Data was then collected using an Accuri C6 Flow Cytometer and later analysed using CFlow Plus software. The whole experiment was repeated once (n=1). The lower left corner of each plot represents the live cells (negative for both PI and Annexin-V). The lower right corner represents the early apoptotic cells (negative for PI and positive for Annexin-V). The upper right corner represents the late apoptotic or necrotic cells (positive for both PI and Annexin-V). The figures show the percentage of cells in each population.