EFFECTS OF BIOLOGICAL COMPOUND TURKISH PROPOLIS EXTRACT ON BREAST CANCER CELLS

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FOR THE DEGREE OF

MASTER OF SCIENCE

BY

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AUGUST, 2013

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

EFFECTS OF BIOLOGICAL COMPOUND TURKISH PROPOLIS EXTRACT ON BREAST CANCER CELLS

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Propolis is a resinous compound which is collected from various plants then combined with wax and bee enzymes by worker bees. There are many studies conducted on propolis or its active components aiming to find new treatment possibilities in diverse research fields such as immunology, infectious diseases, allergy, diabetes, ulcers, and oncology. Chemical analysis indicated that propolis is a multicomponent mixture of various compounds with prevalence of flavonoids and phenolic acids. Therefore it is important to investigate the propolis extract mechanisms of action in order to predict possible cytotoxic and may be therapeutic effects for cancer. The most common propolis extract is ethanol extract of propolis (EEP) whereas Turkish researchers were able to extract the propolis with *dimethyl sulfoxide* (DMSO) which can maximize the penetration of compounds from propolis to the cells as well as DMSO is a good solvent for flavonols (one of the most common compound in propolis). There are many studies conducted on propolis or its active components for treatment of cancer which reveals the potential of this biological compound in the development of novel anti-cancerous agents. However, anti-cancer activity of DMSO extract of Turkish propolis (DEP) on human breast cancer has not been investigated yet. The aim of this study was to investigate the anti-cancer effects of DMSO extract of Turkish propolis (DEP) on cancer cells. Inhibitory effects of propolis extracts collected from different regions of Turkey were analyzed on the growth of the human breast carcinoma cells. Two different

propolis extracts were used to determine their cytotoxic effects of breast carcinoma cell lines using SRB staining and IC_{50} values were determined. The results showed that propolis is cytotoxic in dose-dependent manner (IC_{50} value of diverse from 25 ug/ml to 123 ug/ml). Real time monitoring (xCELLigence system) of propolis treated cells confirmed the cytotoxic effect of propolis, since increasing concentrations of propolis decreased the cell number in a dose- and cell line- dependent way. Furthermore, propolis treatment induces apoptosis in breast carcinoma cell lines. Propolis treated cells changed their adherent morphology to round cells and detached from the surface. Hoechst 33258 staining of propolis treated cells revealed the increasing number of cells displays DNA condensation. PARP-1, a 116 kDa nuclear enzyme, is cleaved in fragments of 89 and 24 kDa during apoptosis. Western blot analysis was performed to detect the PARP-1 cleavage in propolis treated cells. Decrease in the full-length PARP-1 protein levels supports our hypothesis that propolis shows its cytotoxic effect at least partially through induction of apoptosis. The effect of propolis on cell cycle was analyzed with flow cytometer after staining the cells with Propidium iodide (PI). Increase in the G2/M cell cycle arrest was observed in propolis treated cells compare to control DMSO treated MDA-MB-231 cells. In addition to cytotoxic effects, in vitro wound healing assay revealed that propolis treated MDA-MB-231 cells shows delayed invasion of the cells to the denuded area when compared to the DMSO control cells. In conclusion, propolis showed a cytotoxic effect on breast carcinoma cell lines by inducing apoptosis, G2/M arrest as well as delaying the invasion capacity of the cells which makes it a potent anti-tumorigenic compound that may be useful in cancer chemoprevention or therapy.

Key words: Propolis, Breast Cancer, Cytotoxic, Apoptosis, xCELLigence, Scratch Assay.

ÖZET

BİYOLOJİK BİLEŞKE TÜRK PROPOLİSİNİN MEME KANSERİ HÜCRELERİNE ETKİLERİ

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Propolis, işçi arılar tarafından çeşitli bitkilerden toplanıp balmumu ve arı enzimleriyle kombine edilen, reçine tipinde bir üründür. Propolis veya aktif bileşenleriyle ilgili olarak immünoloji, enfeksiyon hastalıkları, alerji, diyabet, ülser ve onkoloji gibi çeşitli araştırma alanlarında yeni tedavi olasılıkları bulmayı hedefleyen çok sayıda çalışma vardır. Kimyasal analizler propolisin flavonoidler ve fenolik asitlerle birlikte çeşitli bileşenlerin karışımı olduğunu göstermiştir. Bu nedenle olası toksik ve terapötik etkileri öngörmek açısından propolis ekstrelerinin etki mekanizmalarını incelemek önemlidir. En sık kullanılan propolis ekstresi propolisin etanol ekstresidir (EEP). Türk araştırmacılar propolis bileşenlerinin hücrelere penetrasyonunu maksimuma çıkartabilecek şekilde propolisin *dimetil sülfoksit* (DMSO) ile ekstresini elde edebilmişlerdir. DMSO, flavonoller (propolis içinde en sık bulunan bileşenlerden) için iyi bir solventtir. Propolis veya aktif bileşenlerinin kanser tedavisinde kullanımıyla ilgili olarak yapılmış ve bu biyolojik bileşenin yeni anti-kanser ajanların geliştirilmesindeki potansiyelini gösteren çok sayıda çalışma vardır. Ancak Türk propolisinin DMSO ekstresinin (DEP) insan meme kanseri üzerindeki anti-kanser aktivitesi henüz incelenmemiştir. Bu çalışmanın amacı kanser hücreleri üzerinde Türk propolisinin DMSO ekstresinin (DEP) anti-kanser etkilerini araştırmaktır. Çalışmada Türkiye'nin çeşitli bölgelerinden toplanan propolisin insan meme karsinomu hücrelerinin büyümesi üzerindeki inhibe edici etkileri incelendi. SRB boyaması kullanılarak iki farklı propolis ekstresinin meme karsinomu hücre

hatları üzerindeki sitotoksik etkileri incelendi ve IC_{50} değerleri belirlendi. Sonuçlar propolisin doza bağımlı bir şekilde sitotoksik olduğunu gösterdi (IC_{50} değeri 25 ug/ml ile 123 ug/ml arasında değişmektedir). Propolis ile muamele edilen hücrelerin gerçek zamanlı incelenmesi (xCELLigence sistemi) propolis sitotoksik etkilerini doğruladı çünkü artan propolis konsantrasyonları hücre sayısını doza ve hücre hattına bağımlı bir şekilde azalttı. Ayrıca propolis tedavisi meme kanseri hücre hatlarında apoptozu indükledi. Muamele edilen hücrelerin adheran morfolojisi yuvarlak hücreler haline dönüştü ve Hoechst 33258 boyama yöntemi ile artan sayıda hücrede DNA kondansasyonu gösterdi. Apoptoz sırasında, 116 kDa bir nükleer enzim olan PARP-1, 89 ve 24 kDa büyüklüğündeki fragmanlara ayrılmaktadır. Propolis ile muamele edilen hücrelerde PARP-1 ayrılmasını saptamak üzere Western blot analizi yapıldı. Tam uzunlukta PARP-1 protein seviyelerinde azalma, propolisin sitotoksik etkisini en azından kısmen apoptoz indüksiyonu yoluyla gösterdiği hipotezimizi desteklemektedir. Propolisin hücre döngüsü üzerine etkisi, hücrelerin Propidium iyodür (PI) ile boyandıktan sonra bir akış sitometresi tarafından analiz edilmesiyle incelendi. Kontrol DMSO ile muamele edilmiş MDA-MB-231 hücreleriyle karşılaştırıldığında propolis ile muamele edilen hücrelerde G2/M hücre döngüsü arestinde güçlü bir artış görüldü. Sitotoksik etkilere ilaveten, in vitro yara iyileşmesi testi, propolis ile muamele edilen MDA-MB-231 hücrelerinin DMSO kontrol hücreleriyle karşılaştırıldığında soyulmuş bölgeye hücre invasyonunda gecikme olduğu saptandı. Sonuç olarak, meme kanseri hücre hatlarında apoptoz ve G2/M arestini indükleyerek ve ayrıca hücrelerin invazyon kapasitesini geciktirerek gösterdiği sitotoksik etki sayesinde propolis kanser kemoterapisi veya önlenmesinde faydalı olabilecek güçlü bir anti-tümorijenik bileşendir.

Anahtar Sözcükler: Propolis, Meme Kanseri, Sitotoksik, Apoptoz, xCELLigence, Yara İyileşmesi testi

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Abbreviations

°C	degree Celsius
APS	Ammonium persulfate
bCSCs	breast cancer stem cells
BRCA1	breast cancer type 1 susceptibility protein
BRCA2	breast cancer type 2 susceptibility protein
BSA	Bovine serum albumin
CAPE	Caffeic acid phenethyl ester
caspase-3	cysteinyl-aspartate proteases-3
CI	Cell Index
CO_2	Carbon dioxide
Conc.	Concentration
ddH_2O	Double-distilled water
DEP	DMSO extract of propolis
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEP	ethanol extract of propolis
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	estrogen receptor
etc	et cetera
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
G2/M	Gap 2/Mitosis
h	hour
HDACi	histone deacetylase inhibitor
HER2(ERBB2)	Human Epidermal Growth Factor Receptor 2
IC_{50}	The half maximal inhibitory concentration
kDa	kiloDalton
LNCaP	androgen-sensitive human prostate adenocarcinoma cells
MMP	matrix metalloproteinase
n/a	not available
NaCl	Sodium chloride

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
NP-40	nonyl phenoxypolyethoxylethanol
OD	Optical Density
p53	protein 53
PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose) Polymerase-1
PBS	Phosphate buffered saline
PI	Propidium iodide
Post-EMT	Post-Epithelial–mesenchymal transition
PR	progesterone receptor
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SRB	Sulforhodamine B
TBS for SRB	Tris Base Solution
TBS for Western	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
TNF	Tumor necrosis factors
TRAIL	TNF-related apoptosis-inducing ligand
ul	microliter
VEGF	Vascular endothelial growth factor
WEP	water extract of propolis
wt	wild type
Z	impedance

1 INTRODUCTION

1.1 Breast Cancer

Malignant tumors can invade the surrounding cells or metastasize to other parts of body. It is defined as *breast cancer* when the malignant tumor starts in the cells of breast (cancer.org; American Cancer Society). Healthy breast consists of fat, connective tissue, blood and lymph vessels (Figure 1-1).

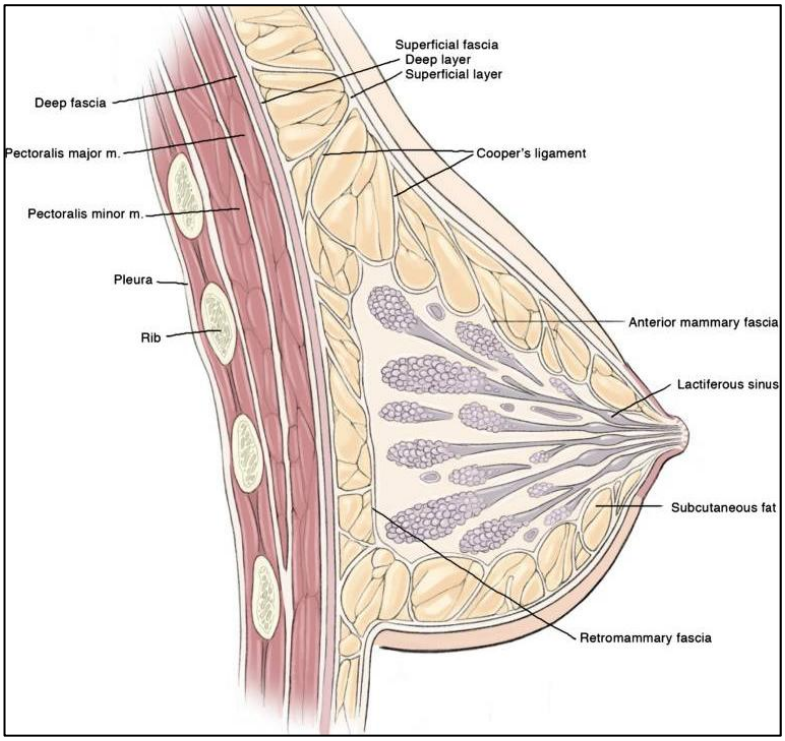


Figure 1-1 Anatomy of breast (Harness, 2011).

Breast cancer is the most common cancer type and the leading cause of cancer death among women worldwide (Table 1.1). Increase in the age is the most important risk factor and BRCA1 & BRCA2 inherited mutations increase the risk (cancer.org; American Cancer Society).

Table 1.1 The two most common types of new cancer cases and deaths by world area, 2008 (cancer.org; American Cancer Society).

	Cancer Cases				Cancer Deaths			
	Females				Females			
	First		Second		First		Second	
Eastern Africa	Cervix uteri	26.2%	Breast	14.9%	Cervix uteri	24.5%	Breast	11.3%
Middle Africa	Breast	22.1%	Cervix uteri	22.0%	Cervix uteri	20.7%	Breast	16.9%
Northern Africa	Breast	33.8%	Cervix uteri	6.4%	Breast	26.3%	Colon & rectum	5.6%
Southern Africa	Breast	23.4%	Cervix uteri	16.9%	Breast	17.5%	Cervix uteri	13.6%
Western Africa	Breast	26.4%	Cervix uteri	25.9%	Cervix uteri	24.9%	Breast	21.0%
Caribbean	Breast	24.6%	Cervix uteri	13.0%	Breast	15.8%	Lung & bronchus	12.7%
Central America	Breast	18.9%	Cervix uteri	16.9%	Cervix uteri	13.7%	Breast	11.6%
South America	Breast	26.6%	Cervix uteri	14.4%	Breast	14.6%	Cervix uteri	11.8%
North America	Breast	26.6%	Lung & bronchus	14.3%	Lung & bronchus	25.9%	Breast	14.9%
Eastern Asia	Breast	15.2%	Lung & bronchus	13.2%	Lung & bronchus	19.1%	Stomach	15.5%
South-Eastern Asia	Breast	22.4%	Cervix uteri	11.4%	Breast	15.2%	Lung & bronchus	11.5%
South-Central Asia	Cervix uteri	22.5%	Breast	22.4%	Cervix uteri	19.8%	Breast	17.1%
Western Asia	Breast	27.2%	Colon & rectum	8.2%	Breast	19.0%	Colon & rectum	8.1%
Central and Eastern Europe	Breast	23.4%	Colon & rectum	13.3%	Breast	16.8%	Colon & rectum	14.6%
Northern Europe	Breast	30.0%	Colon & rectum	12.1%	Lung & bronchus	18.6%	Breast	15.8%
Southern Europe	Breast	29.0%	Colon & rectum	14.2%	Breast	16.5%	Colon & rectum	13.1%
Western Europe	Breast	32.1%	Colon & rectum	13.8%	Breast	18.2%	Colon & rectum	13.3%
Australia/New Zealand	Breast	28.4%	Colon & rectum	14.0%	Lung & bronchus	16.4%	Breast	15.7%
Melanesia	Cervix uteri	19.5%	Breast	17.1%	Cervix uteri	18.7%	Breast	13.6%
Micronesia	Breast	35.7%	Colon & rectum	11.6%	Breast & lung	17.8%	Colon & rectum	11.2%
Polynesia	Breast	29.6%	Thyroid	12.2%	Breast	16.7%	Lung & bronchus	15.4%

1.1.1 Classification of Breast Cancer

Since breast consists of epithelial cells, almost all of the breast cancers are carcinomas. Some of them are adenocarcinomas if cancer starts from ducts or lobules of the breast which produces milk. Carcinoma *in situ* is the early stage of the cancer and this is referred to as non-invasive or pre-invasive (cancer.org; American Cancer Society). On the other hand, infiltrating carcinomas are invasive and

constitutes more than 95% of all mammary carcinomas (Yoder, 2007). Both carcinomas can be ductal or lobular.

Molecular classification is very important for breast cancer because of the heterogeneous nature of the cancer. Basal and luminal cells are the two distinct epithelial subtypes of mammary gland (Perou, 2000). Luminal subtype can be divided into Luminal A and Luminal B as their gene expression pattern (Sorlie, 2003). Also another subdivision came from Neve *et. al.* as Basal A and Basal B (Neve, 2006). Dawson *et. al.* introduced a novel categorization in recently published article. This categorization includes 10 "integrative clusters" which are generated from molecular information of genomic and transcriptomic features of breast cancer (Dawson, 2013). Categorization of Breast cancer is important for the sake of proper treatment to each subtype of breast cancer. Different drugs may affect a subtype of breast cancer which can be linked with its molecular properties.

1.1.2 Breast Carcinoma Cell Lines

BT-20 was the first breast carcinoma cell line to be established in 1958. Followed by MD Anderson series of breast carcinoma cell lines and the most famous one, MCF7 was established by Michigan Cancer Foundation in 1973. Cell lines are good models of breast cancer research (Holliday, 2011). Classification of cell lines is also important to use the right cell line as model. For example, a specific kinase inhibitor was shown that it preferentially inhibits proliferation of luminal estrogen receptor-positive human breast carcinoma cell lines (Finn, 2009). Different compounds may have selective effect on subtypes of breast cancer and this can be linked with its molecular properties of the subtype. Some of the characteristics of the breast carcinoma cell lines are summarized in the Table 1.2.

Table 1.2 Properties of Breast carcinoma cell lines. {* = amplified but not highly expressed, n/a = not available, wt = wild type, ER = estrogen receptor, PR = progesterone receptor, HER2[ERBB2] = Human Epidermal Growth Factor Receptor 2} (Neve, 2006; Kao, 2009; Finn, 2009; Holliday, 2011).

	Subtype	**ER status**	**PR status**	**HER2 status**	**p53 mutation**
MCF10A	Basal B	negative	negative	immortalized	+/- wt
MCF12A	Basal B	negative	negative	n/a	+
CAMA-1	Luminal	positive	negative	normal	+
MDA-MB 231	Basal B(Post-EMT)	negative	negative	normal	++ mutant
MDA-MB-453	Luminal	negative	negative	amplified*	- wt
MDA-MB-468	Basal A	negative	negative	normal	+
MCF7	Luminal A	positive	positive	normal	+/- wt
T47D	Luminal A	positive	positive	normal	++ mutant
MDA-MB-157	Basal B(Post-EMT)	negative	negative	normal	-
HCC-1937	Basal A (Post-EMT)	negative	negative	normal	-
BT-20	Basal A	negative	negative	normal	++ wt
MDA-MB-361	Luminal	positive	positive	amplified	- wt
BT-474	Luminal B	positive	positive	amplified	+
ZR-75-1	Luminal B	positive	negative	normal	-
hTERT-HME1	Basal B	negative	n/a	negative	n/a

1.2 Biological Compound Propolis

From ancient times to today, humankind faced to reality of breast cancer and tried different treatments varies from organic supplies to surgical operations. We are still looking for a cure for cancer in general. If we assume that nature has a remedy for all problems, we should look for the remedy for treatment of breast cancer. In this case we are looking for it in a beehive where propolis comes from.

Propolis or bee glue is a yellow-brownish resinous compound which is collected from various plants and combined with wax and bee enzymes by worker bees. This mixture is used to smooth out hive walls, to protect bees from diseases because of its antiseptic features and also to embalm the carcasses of invader insects to avoid decomposition (Sforcin, 2011). The word propolis comes from a Greek origin which pro is 'in front of' or 'at the entrance to' and polis is city -in this case, hive- therefore propolis means that a material in defense of the hive (Castaldo, 2002).

Using propolis as a medicine has a long history dating back to ancient times. For instance, Ancient Egyptians used propolis to embalm the cadavers and Ancient Greeks and Romans used propolis as an anti-inflammatory agent to heal wounds and ulcers. Also it was accepted as an official drug in London in 17th century (Salatino, 2011). Still, it is widely used among Balkan States. There are also studies that show propolis having no side effect to mice or human (Sforcin, 2007). Recently, there are many studies associated with propolis extracts aiming to find new treatment possibilities in diverse research fields such as immunology, oncology, infectious diseases, allergy, diabetes, ulcers, *etc* (Sforcin, 2011).

Chemical composition of propolis varies by the geographical status and by the different races of honeybees. Different studies are going on with local propolis extracts such as Cuban, Brazilian, Chinese, Indian *etc.* (Monzote, 2012; Sforcin, 2011; Sun, 2012; Thirugnanasampandan, 2012). Propolis is mainly composed of resins which comes from plants those honeybees collect from. Hence, propolis extracts from different geographical origin have a specific combination of chemicals that reflect the floral properties of the field (Salatino, 2011). Sibel Silici and Semiramis Kutluca showed that Turkish propolis collected form Erzurum region have a number of chemical compounds which were identified from propolis for the first time (Silici, 2005).

Diversity of propolis has both advantages and disadvantages for the research. Distinct compounds found in propolis may have novel benefits for the drug

discoveries or they may construct a novel synergistic effect with regular compounds found in propolis. Major disadvantage would be the problem of standardization of propolis as a possible drug; however there are many ways to overcome this problem. The most common solution to standardize of propolis is to categorize propolis according to its chemical composition and source of plant. Six main types of propolis are poplar propolis, birch propolis, Brazilian green propolis, red propolis, pacific propolis and Canarian propolis (Bankova, 2005). When the standardization problem is defeated, the use of propolis is "safe and less toxic than many synthetic medicines" (Castaldo, 2002).

1.2.1 Propolis Extracts

Propolis extraction is made with alcoholic solvents or water, generally. The most common solvent is absolute ethanol, methanol and water follows it. While water extraction has 7% activity, alcoholic extractions can reach up to 28% activity. Also triglyceride extraction patent is held by Japanese researchers (Ashry, 2012). Since the chemical composition of propolis is very complex (propolis consist of more than 300 components), solvent of the extraction method affects the activity of propolis. Different compounds in the mixture can solubilize in different solvents so that each extraction material gives different outcomes (Sforcin, 2007).

Turkish researchers were able to extract the propolis with *dimethyl sulfoxide* (DMSO) which can dissolve both polar and nonpolar compounds (Aliyazicioglu, 2005). DMSO maximizes the penetration of compounds to the cells as well as it is a good solvent for flavonols (one of the most common compound in propolis) (Cai, 2011). According to one of the studies, DMSO extract of propolis (DEP) is richer in polyphenols and flavonoids than water extraction of propolis (WEP). They also claim that the antioxidant potentials of those two extracts are parallel with the total phenolic compounds in each extract (Barlak, 2011).

Propolis composition is highly variable considering the plant source, bee race, geographical and seasonal diversity. In general, propolis contains flavonoids such as chrysin, acacetin, apigenin, and phenolic acids like cinnamic acid, caffeic acid, *etc* (Table 1.3). Propolis also includes some vitamins and minerals as well as fatty acids (Khalil, 2006). Some of the researchers use the whole extract of propolis whereas others prefer to use active components of it. Even though using an individual constituent of propolis is an effective way of standardization, there might be a synergic effect of components within the propolis extract. This effect can be the reason that propolis has different pharmacological activities (Banskota, 2001).

Table 1.3 Chemical Composition of Propolis (Sawicka, 2012).

Compounds	**Percentage**
Fatty and Aliphatic Acids	24-26 %
Flavonoids	18-20 %
Sugars	15-18 %
Aromatic Acids	5-10 %
Esters	2-6 %
Vitamins	2-4 %
Alcohol and Terpens	2-3.3 %
Microelements	0.5-2 %
Others	21-27 %

1.2.2 Antitumoral Activity of Propolis and Its Active Components

Propolis is a research subject for its antitumoral activity all over the world with local extracts since its composition changes with its origin. The common point of the anti-cancerous effects of propolis is the ability of propolis to trigger apoptosis in cancer cells. Some of the active components of propolis as well as alcoholic extractions or water-soluble derivatives of propolis were shown to induce apoptosis in cancer cells depending on the concentration (Sawicka, 2012). There are many studies conducted on propolis or its active components for treatment of cancer which reveals the potential of this biological compound in the development of novel anti-cancerous agents.

Numerous studies claim that polyphenols in propolis, activates TRAIL-induced apoptosis in cancer cells. Naringenin in lung cancer, Biochanin A in prostate cancer, Kaempferol in glioma and chrysin, quercetin, apigenin in various cancer cells were particularly identified as a synthesizer of TRAIL-induced apoptosis (Szliszka, 2013). Synthesizing cancer cells to TRAIL-targeted therapies with propolis or its polyphenols would increase the anticancer activity of TRAIL so that TRAIL-resistance may be overcome by propolis treatment.

Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) is one of the active components of propolis which causes significant damage to carcinoma and malignant melanoma. Intratumor injection of Artepillin C (500g, three times a week) increases the number of helper T cells in addition to suppression of tumor growth in mice (Khalil, 2006).

Caffeic acid phenethyl ester (CAPE) is a strong antioxidant, extracted from propolis and it is a well-known NF-κB specific inhibitor (Figure 1-2). It suppresses the cell proliferation of some metastatic prostate cancer cell lines as well as sensitizes cancer cells to radiation and chemotherapeutic drugs. LNCaP (androgen-sensitive human prostate adenocarcinoma cells) xenograft nude mice were orally treated with CAPE (10 mg/kg per day for six weeks) and consequently tumor volume was

reduced 50% (Liu, 2013). As a result of these findings, it is hypothesized that CAPE can be an effective adjuvant therapy for prostate cancer.

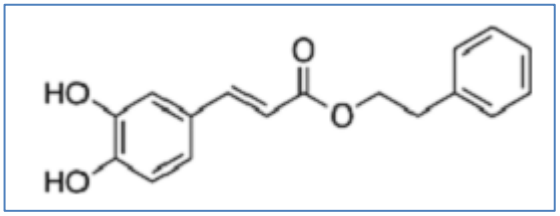


Figure 1-2 The Chemical Structure of Caffeic acid phenethyl ester (CAPE) $C_{17}H_{16}O_4$ (Akyol, 2012).

As a candidate anti-cancer agent, propolis can be a relatively inexpensive solution for cancer treatment. Administration of propolis does not lead to side effects on rats or humans along with the fact that it can reduce side effects of cancer treatment (Watanabe, 2011). A research group from Turkey also supports that usage of CAPE prevents the damages and side effects of chemotherapy and radiotherapy. They propose the usage of CAPE as a protective agent during chemotherapy in clinical trials (Akyol, 2012). A research group from Taiwan claims that CAPE has an anti-metastatic and anti-angiogenic effects on cancer cells. Even though the exact mechanism of anti-metastatic activity of CAPE is not revealed yet, they demonstrated that CAPE has effects on destruction of capillary-like tube formation, inhibition of tumor cell invasion, and elimination of VEGF level *in vitro* and *in vivo* (Liao, 2003).

Another usage of active components of propolis is as a histone deacetylase inhibitor (HDACi). with anticancer activity. Chrysin (Sun, 2012) and NMB-HD-1 (Huang, 2012) are examples of HDACis synthesized from propolis. Chrysin, a known potent anticancer compound, is a HDAC8 inhibitor as well as it can significantly inhibit tumor growth. Chrysin is also important for the standardization of Chinese propolis since it is the major index compound (Sun, 2012). NMB-HD-1 has an anti-proliferative effect and also injection of NMB-HD-1 to MDA-MB-231 breast cancer

xenograft model exhibited antitumor activity. This outcome may involve HDAC inhibition which changes chromatin core histones so that expression of cell cycle regulating genes changes. Another possibility is the suppression of PTEN/AKT pathway which inhibits cancer cell growth (Huang, 2012).

1.2.3 Synergistic Effects of Propolis with Other Chemotherapeutic Drugs

Propolis have various functional and biological properties such as antibacterial (Grange, 1990)., antimicrobial (Monzote, 2012), anti-oxidative (Thirugnanasampandan, 2012), antiviral (Viuda-Martos, 2008), hepatoprotective (Albukhari, 2009), anti-cancerious (Sawicka, 2012), anti-ulcerous (Viuda-Martos, 2008) as well as anti-inflammatory (Banskota, 2001). Especially anti-oxidative and anti-inflammatory features of propolis make it a promising candidate as an adjuvant to chemotherapy.

More than 70% of antitumor agents are natural compounds or materials derived from natural products (Watanabe, 2011). Propolis is one of the candidates for such products while paclitaxel is already one of them. Paclitaxel also known as Taxol® is an anticancer agent which was isolated from bark of *Taxus brevifolia* (Figure 1-3) (Khosroushahi, 2011).

Figure 1-3 Picture of *Taxus brevifolia* (Pacific Yew) which Paclitaxel was isolated from (McMullen, 2008).

Paclitaxel is widely used for treatment of breast cancer, non-small cell lung cancer, ovarian cancer, melanoma, head and neck cancer. Giving paclitaxel in combination with propolis, results in maximum protection from induced mammary carcinogenesis in rats. Treating breast cancer-bearing rats with 50 mg propolis per kg body weight along with 33 mg paclitaxel per kg body weight reduces the toxic side effects of paclitaxel by propolis' immunemodulatory activity. Another effect of propolis is free radical scavenging activity against alkoxyl radicals that is due to the antioxidant property of propolis. Synergistic action of propolis mixture is distinct from the action of a single component since there are diverse effects of propolis on cancer treatment (Padmavathi, 2006).

In another study, ethanolic extract of propolis was used with temozolomide to inhibit U87MG (human glioblastoma cell line) cell line growth. Researchers affirmed that propolis has cytotoxic effects as well as growth inhibiting activity in combination with temozolomide. They think there is at least partial relationship between cytotoxic properties and reduced activity of NF-κB since NF-κB is an essential survival factor for glioblastomas (Markiewicz-Żukowska, 2013).

Caffeic acid phenethyl ester (CAPE), an active component of propolis was found to change the characteristics of breast cancer stem cells (bCSCs). CAPE inhibits the self-renewal and clonal expansion in soft agar, also decrease the CD44 (cell surface markers for bCSCs) content and malignancy in bCSCs. Another effect of CAPE is that it increases the cycling state of bCSCs so that susceptibility to chemotherapeutic agents of bCSCs increases. In conclusion, CAPE can be used effectively for cancer treatment in combination with other chemotherapeutic agents (Omene, 2012).

1.3 Cell Death

Carl Vogt was the first one to describe cell death in 1842 following the establishment of cell theory. He observed the elimination of cells and replacement of them by new cells (Clarke, 2012). Stress to the cellular system causes cell death with a diverse and complex process. Apoptosis, necrosis, autophagy and mitotic cell death are the known cell death types for today. An individual cell may have a heterogeneous behavior within a population as well as cell death can also be a heterogeneous property. Both biochemical and morphological properties may cause the heterogeneity in cellular systems (Stevens, 2013). Apoptosis and autophagy are the most well-known programmed cell death mechanism however there is third one: programmed necrosis. These are the three main forms of programmed cell death and they balance survival with cell death for normal cells (Ouyang, 2012).

1.3.1 Apoptosis

In the case of cellular life, death program comes with the code which gives the life itself. In case of emergencies, cells kill themselves for the sake of population of other cells. This is also parallel with the evolutionary development since cells cannot pass their genetic information if the information is damaged.

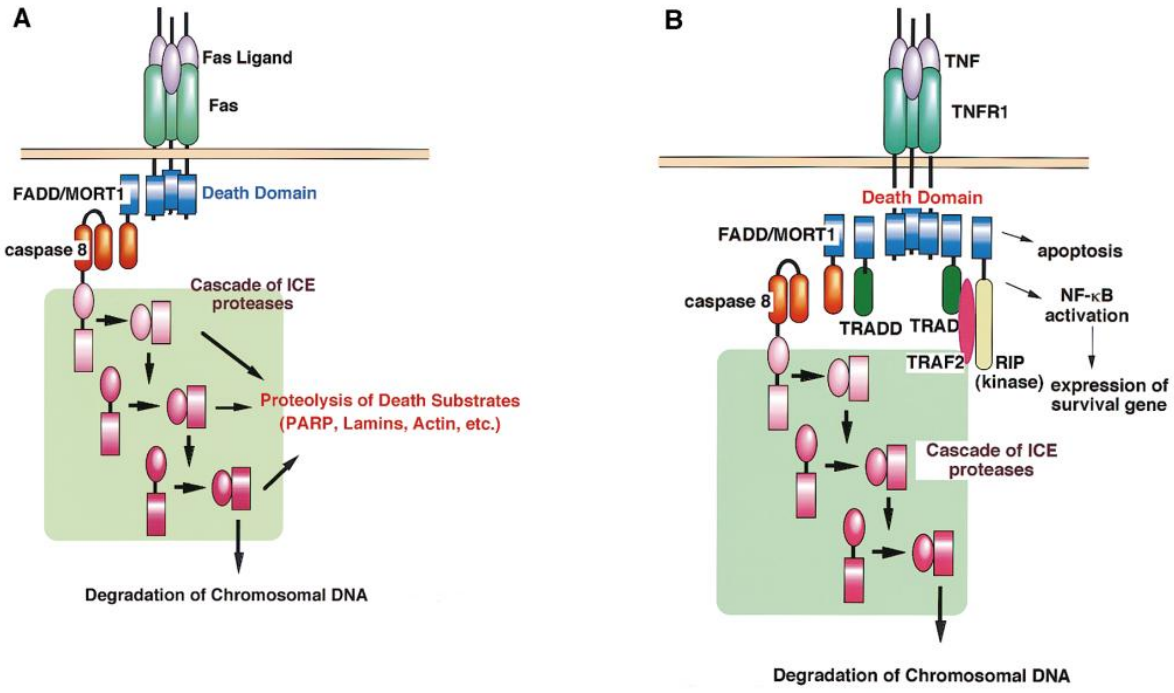


Figure 1-4 Fas-induced and TNF-induced Apoptosis Models (Nagata, 1997).

If the DNA damage is irreversible, major type of cell death is the apoptosis. Apoptosis can be triggered by two different pathways: death receptor (extrinsic) and mitochondrial (intrinsic) pathway. When plasma-membrane death receptor, Fas binds to its extracellular ligand Fas-L; the extrinsic pathway triggers. Both TNF and Fas induces extrinsic pathway of apoptosis (Figure 1-4). Mitochondrial pro-enzymes control the intrinsic pathways of apoptosis (Ouyang, 2012).

1.3.2 Poly(ADP-ribose) Polymerase-1 (PARP-1) Cleavage

Single and double stranded DNA breaks activate the nuclear protein PARP and PARP is also involved in DNA repair, cell cycle regulation, differentiation and transformation mechanisms (Whitacre, 1999). PARP binds to DNA single strand brakes and induces a structural modification to promote base excision repair. Poly(ADP-ribose) (PAR) recruits other DNA damage proteins to the close vicinity and PARP induces the synthesis of PAR. Following Poly(ADP-ribosyl)ation (PARylation), PARP is needed to be cleaved by cysteinyl-aspartate proteases-3 (caspase-3) (Nowsheen, 2012).

Caspases, calpains, cathepsins, granzymes and matrix metalloproteinases (MMPs) are some of the suicidal proteases which cleave PARP from different sites so that signature fragments appear (Figure 1-5). Each cleaved fragment has its specific molecular weight therefore fragments can be used as a biomarker for specific cell death program (Chaitanya, 2010).

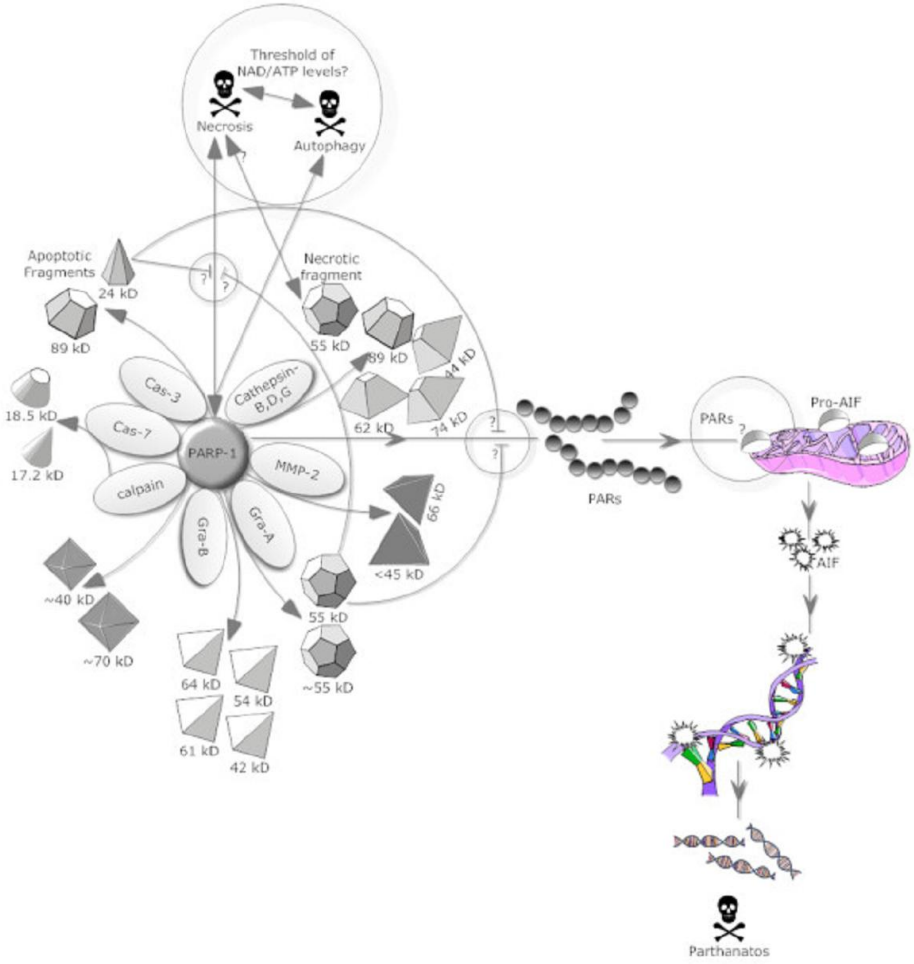


Figure 1-5 Various Fragments of PARP-1 after cleaved by specific suicidal proteases (Chaitanya, 2010).

PARP-1 cleavage by caspases is considered as a hallmark of apoptosis. There are 2 different possible fragments after the cleavage of PARP by caspases: 85 and 89 kDa fragments. These fragments are indicators of apoptosis of the cell. The full-length protein is 116 kDa and cleavage by caspase-3 results in 89 kDa fragment. However, cleavage by caspases-7 yields two specific fragments, 89 and 24 kDa (Chaitanya, 2010). Therefore detection of one of these bands shows the association of apoptosis. On the other hand, 50 kDa fragment is detected during necrosis (Buontempo, 2010).

2 Materials

2.1 Propolis Extract

Propolis extracts were kindly provided by Prof. Dr. Orhan Değer from Karadeniz Technical University, Faculty of Medicine, Department of Medical Biochemistry. Two different batches of DMSO extracts of propolis were used in this study; first batch is 25 mg/ml (Propolis-1) and second batch is 100 mg/ml (Propolis-2). Propolis was collected from different regions of Turkey by Fanus Gıda Corporation (Trabzon, Turkey) and extractions were prepared in laboratories of Karadeniz Technical University with the following method: Natural propolis was grinded and mixed until it became powder. For 25 mg/ml propolis extract, 0.5 g of propolis powder was mixed with 20 ml DMSO and vortexed. Mixture was incubated on the shaker at 150 rpm for 24 hours at 60°C for propolis to dissolve. Mixture was filtered with filter paper and the extracts were kept in dark at +4°C. 100 mg/ml propolis extract was prepared with the same procedure.

2.2 Growth Mediums

Growth mediums of cell lines are given in Table 2.1.

Table 2.1 Growth mediums of cell lines

2.3 Buffers and Solutions

Table 2.2 General Solutions

10% TCA Solution	(v/v) 10% TCA in ddH_2O
1% Acetic Acid Solution	(v/v) 1% Acetic acid in ddH_2O
0.4% SRB Solution	(w/v) 0.4% SRB in 1% Acetic Acid
10 mM Tris Base Solution	10 mM Tris in ddH_2O
300 ug/ml Hoechst 33258 Stock Solution	300 ug/ml Hoechst dissolved in ddH_2O (stored in dark)
1 ug/ml Hoechst 33258 Working Solution	1 ug/ml Hoechst diluted from 300 ug/ml Hoechst stock solution dissolved in 1x PBS (kept in dark)
10x TBS	12.19 g Tris-base and 87.76 g NaCl were dissolved in 1 liter of ddH2O and the pH was adjusted to 8 to prepare 10X TBS stock solution.
0.2 % TBS-T	0.2% Tween 20 was added into 1X TBS solution.
10% APS	(w/v) 10% APS in 1% ddH_2O
5 % BSA	(w/v) 5% BSA in 0.2 % TBS-T
5 % milk	(w/v) 5% milk powder in 0.2 % TBS-T

Table 2.3 Cell Lysis Buffer

Reagent	Final concentration
Tris-HCl (pH:8.0)	50 mM
NaCl	150 mM
NP-40	1 %
SDS	0.1 %
Protease Inhibitor	1x
ddH_2O	Rest of the solution

Table 2.4 Bradford Stock Solution

Coomassie brilliant blue	17.5 mg
Ethanol	4.75 ml
Phosphoric acid	10 ml
final volume with ddH_2O=	25 ml

Table 2.5 Bradford Working Solution

Bradford stock solution	1.5 ml
95% Ethanol	0.75 ml
Phosphoric acid	1.5 ml
final volume with ddH_2O=	25 ml

Table 2.6 5x Loading Dye

Tris-HCL, pH:6.8, ,	62.5 mM
β-mercaptoethanol	5%
glycerol	15%
bromophenol blue.	0.001%
SDS	2%

Table 2.7 30% Acrylamide/Bisacrylamide Solution

Acrylamide	29 gr
Bisacrylamide	1 gr
final volume with ddH_2O=	100 ml
stored in the dark	

Table 2.8 SDS Gel Formulation to Prepare two Gels

Reagents	5% Stacking Gel	10% Resolving Gel
30% acrylamide/bisacrylamide solution	1.3 ml	6.7 ml
1.0 M Tris Solution (pH: 8.8)	-	7.5 ml
1.0 M Tris Solution (pH: 6.8)	1.0 ml	-
10% SDS solution	80 ul	200 ul
10% APS solution	80 ul	200 ul
TEMED	8 ul	8 ul
ddH_2O	5.6 ml	5.4 ml
TOTAL=	8 ml	20 ml

Table 2.9 5x Running Buffer

Tris	45 g
Glycine	216 g
SDS	15 g
final volume with ddH_2O=	3 liters

Table 2.10 Wet Transfer Buffer

Tris	6 g
Glycine	28.8g
Methanol	15%
final volume with ddH_2O=	1 liter

Table 2.11 Antibodies and Their Solutions

Primary Antibodies
PARP-1 (Cell Signaling 46D11): 1:200 in 5% BSA in 0.2 % TBS-T
β-actin (Sigma A5441): 1:5000 in 5% milk powder in 0.2% TBS-T
Secondary Antibodies
Anti-Mouse IgG (Sigma A9044): 1:5000 in 5% milk powder in 0.2% TBS-T
Anti-Rabbit IgG (Sigma A0545): 1:5000 in 5% BSA in 0.2 % TBS-T

Table 2.12 Propidium Iodide (PI) Staining Solution

Propidium Iodide (PI) stock	50 ug/ml
RNAse-A	0.1 mg/ml
Triton X	0.05%
Dissolved in PBS	

2.4 General Materials

Table 2.13 Production Information of Materials

Material	Catalog Number	Company
DMEM (Low Glucose)	SH30021.01	Hyclone
Acetic Acid	27225-2.5L-R	Sigma Aldrich
Acrylamide	BP170-500	Fisher Scientific
Adriamycin (ADRIMISIN 10 mg)	L01DB01	Saba İlaç
APS	420627	Carlo Erba
Bisacrylamide	A3636,0250	AppliChem
Bovine Pituitary Extract	13028-014	Gibco
Bromophenol blue.	B5525	Sigma Aldrich
BSA	10 735 078 001	Roche

Material	Catalog Number	Company
Coomassie brilliant blue	27816	Fluka
D-Glucose	16325	Riedel de Haen
DMEM/Ham's F12	F4815	Biochrom
DMSO	A3672,0100	AppliChem
EGF	E9644-2MG	Sigma Aldrich
Ethanol	CAS-64-17-5	AlcoMED
FBS	SV30160.03	Hyclone
Glycerol	346165	Carlo Erba
Glycine	EC200-272-2	Fisher Scientific
Hoechst 33258	861405	Sigma Aldrich
Hydrocortisone	H0888-19	Sigma Aldrich
Insulin	I1882-100MG	Sigma Aldrich
Isopropanol	1.009.952.500	MERCK
L-Glutamine	K0293	Biochrom
Methanol	24229-2.5L-R	Sigma Aldrich
Milk powder	-	Sütaş
NaCl	1,06404,1000	MERCK
Nitrocellulose Membrane	RPN3032D	Amersham
Nonessential Amino Acids	BE13-114E	Lonza
NP-40	NonidetP-40	AppliChem
Parafilm	PM.996	Pechiney
PBS	BE17-516F	Lonza
Penicillin/Streptomycin	SV30010	Hyclone
Phosphoric acid	4107	Riedel de Haen
Propidium Iodide (PI)	D4864	Sigma Aldrich
Protease Inhibitor	11873580001	Roche
RNAse-A	EN0531	Thermo Scientific
RPMI Medium	SH30096.01	Hyclone
SDS	L5750	Sigma Aldrich
Sodium Pyruvate	11360	Gibco
Sulforhodamine B (SRB)	S1402	Sigma Aldrich
Trichloroacetic Acid (TCA)	33731-100G	Sigma Aldrich
Tris	826	Amresco
Tris-Base	T1503-1KG	Sigma Aldrich
Tris-HCl	T-3253	Sigma Aldrich
Trypsin/EDTA	SH30236.01	Hyclone
Tween 20	0777-1L	Amresco
β-mercaptoethanol	M-3148	Sigma Aldrich

3 Methods

3.1 Cell Culture Techniques

3.1.1 Growth Conditions

Every cell line was grown in their appropriate growth medium which is listed in the materials section Table 2.1. Cells were passaged when their confluence was over 70%. Some of the cells were passaged 1:2 while some fast growing cell lines were passaged 1:5. When cells reached confluency the growth medium was removed and the cells were washed with 1xPBS. 1 ml pre-warmed Trypsin/EDTA was spread into 75 cm^2 flasks and incubated at 37°C for 1-2 minutes. When cells detached from the flask surface, trypsin was inactivated with FBS containing fresh culture medium. Cells were dispersed by pipetting up and down a few times. Cells were transferred to new culture flasks and incubated in a 5% air jacketed CO_2 incubator at 37^0C.

3.1.2 Cryopreservation of Cells

Cells were incubated 24 h after passaging and 75 cm^2 flask full of cells was frozen into one tube. Their growth medium was removed and washed with 1x PBS. 1 ml Trypsin/EDTA was spread into 75 cm^2 flasks and incubated at 37°C for 1-2 minutes. When cells appeared to have lost their adherence, trypsin was inactivated by adding fresh medium which contains FBS. Cells were transferred into 15 ml tubes and centrifuged at 1500 rpm for 5 minutes. Cryopreservative medium was freshly prepared by mixing 90% FBS and 10% DMSO. After centrifugation, medium was removed and cell pellets were suspended within 1 ml freezing medium. Each cell pellet was stored in separate cryotubes at -20°C for 1 hour and -80°C or in liquid nitrogen for long term storage. Each vial contains approximately 3-4 millions of cells.

3.1.3 Reculturing Frozen Cells

Frozen cells need to be thawed rapidly therefore they were melted down in a water bath at 37°C. Melted cells were mixed with 5 ml pre-warmed growth medium and centrifuged at 1500 rpm for 5 minutes. After centrifugation, DMSO containing freezing medium was removed and cell pellets were suspended within 5 ml fresh growth medium and transferred into 25 cm^2 flask. Cells were incubated at 37°C with 5% CO_2 air. Next day, the growth medium was removed and the cells were washed with 1x PBS. If the cells were confluent enough, the cells were cultured into 75 cm^2 flask as described above. Cells were incubated at 37°C with 5% CO_2 air. The cells were passaged at least once before using for further manipulations.

3.1.4 Storing Cell Pellets for Protein Isolation

The cells pellets were collected for protein extraction to use for Western blot experiments. The drug treated and un-treated cells were cultured in 6 well plates. Since apoptotic cells detach from the plate surface and remain in the growth media, growth medium in each well was collected into separate 15 ml tubes. The wells were washed with 1x PBS and PBS was added into same 15 ml tubes detached cells containing growth medium. 0.3 ml Trypsin/EDTA was spread into each well of 6 well plate to detach the adherent cells and incubated at 37°C for 1-2 minutes. When cells appeared to have lost their adherence, trypsin was inactivated by adding fresh medium which contains FBS. Cells were transferred into appropriate tubes containing the cells collected from the previous steps and centrifuged at 1500 rpm for 5 minutes at +4°C. The supernatant was removed and the cell pellets were resuspended with ice-cold 1x PBS. Tubes were centrifuged at 1500 rpm for 5 minutes at +4°C. PBS was removed and tubes were soaked in liquid nitrogen immediately to freeze them. Pellets were stored at -80°C for further experiments.

3.1.5 Cell Counting with Haemocytometer

Haemocytometer was used to determine the number of cells in the cell cultures. Certain number of cells was required for IC_{50} calculations, xCELLigence and other propolis treatment experiments. The cultured cells were trypsinized and resuspended in culture mediums as described above. Haemocytometer was cleaned with 70% ethanol and a coverslip was gently put onto the chamber area. The cell suspension was mixed gently to ensure equal distribution of the cells in the tube and then 10 ul of cell suspension was taken and placed into the edge of the chambers. Two chambers were used for counting. The cells in 16 corner squares were counted under the light microscope for both chambers of Haemocytometer.

Average of those numbers was multiplied with 10^4. This gives the approximate cell number within 1 ml of cellular solution. The certain amount of cells for each cell line was used for various experiments.

3.2 Sulforhodamine B (SRB) Assay

Different cell lines were plated (Table 3.1) into the 96 well plates and treated with Propolis-24 hours later. At a given amount of time later, cells need to be fixed to stop further cell growth. After fixation cells were stained with SRB and the color intensity was measured with ELISA reader. Resulting optical density (OD) is correlated with the amount of cells.

Table 3.1 Starting cell number for IC_{50} calculations

	Propolis-1	Propolis-2
BT-20	12,000	12,000
BT-474	20,000	10,000
CAMA-1	4,000	4,000
HCC-1937	10,000	8,000
hTERT-HME1	6,000	6,000
MCF10A	4,000	5,000
MCF12A	5,000	6,000
MCF7	3,000	3,000
MDA-MB 231	4,000	7,000
MDA-MB-157	10,000	10,000
MDA-MB-361	20,000	20,000
MDA-MB-453	4,000	8,000
MDA-MB-468	4,000	4,000
T47D	5,000	4,000
ZR-75-1	5,000	6,000

3.2.1 Trichloroacetic acid (TCA) Fixation

For propolis and DMSO control treated cells, fixation was done as following method for each well. These cells were plated and after 24 h, treated with either propolis or DMSO. Cells need to be fixed after 72 h incubation of treatment. This part was done by using multi-channel pipetting for 96 well plate.

- Remove growth medium with pipet
- Add 100-200 ul PBS at room temperature and shake gently *(Add 500 ul for 24 well plates)*
- Remove PBS with pipet
- Add 50 ul ice-cold 10% TCA solution *(Add 200 ul for 24 well plates)*
- Incubate at +4°C for 1 hour
- Wash with excess ddH_2O for 5 times
- Leave at room temperature for air dry

3.2.2 Sulforhodamine B (SRB) Staining

TCA fixed and air-dried plates were stained with SRB dye solution for further measurements as in the following method:

- 0.4% SRB prepared with 1% acetic acid solution
- Add 50 ul SRB solution to each well and make sure that solution covers all the bottom surface of each well *(Add 200 ul for 24 well plates)*
- Incubate the dye at room temperature for 10 minutes in dark.
- Wash with excess 1% acetic acid solution 5 times
- Leave at room temperature for air dry
- Add ice-cold 100 ul 10mM Tris-base solution *(Add 500 ul for 24 well plates)*
- Solubilize the dye in the Tris-base solution on the shaker for 5 minutes
- Read the results with ELISA reader at 515 nm

3.3 IC_{50} Calculations

For the IC_{50} calculations SRB assay results were used. Cells were treated with propolis and DMSO control with serial diluted concentrations of propolis for at least 5 different concentrations. Samples were collected and calculated at least in triplicates. OD results were then converted to the percent cell death values by using following formula. At a given concentration:

percent cell death = (1 – average OD propolis/average OD DMSO)*100

Percent cell death values of each concentration were calculated and drawn on an X-Y Scatter graph on Excel sheet. For this graph logarithmic trend line was added and 50% inhibition intersection was calculated with given formula on Excel:

IC_{50} value =EXP((50 – y-intersection point of trend line)/ slope of trend line)

This formula gives IC_{50} value of propolis at a given time point for particular cell line. Also coefficient of determination was checked to see the significance of calculated IC_{50} value. That was calculated by Excel by R^2 value of the trend line. For this study IC_{50} values were calculated only if the R^2 values are in between 0.75 -1.00.

3.4 Statistical Analyses

Standard Error of the Mean (SEM)

To determine the standard deviation between triplicate or quadruplicate samples, SEM analysis was performed. STDEVA function was used for the calculation of

standard deviation and the result was divided by square root of sample number. The result shows the standard error of the mean (SEM) for selected samples.

SEM = STDEVA (Sample1, sample2,…) / (√ sample number)

Student's t-test

To calculate the significance of two groups such as luminal-basal, ER positive-negative, *etc*. T-TEST function was used with Microsoft Excel. Function was used to compare the significance of two subgroups with two-tailed distribution and two-sample unequal variance. $P<0.05$ were analyzed as significant result.

3.5 Live Cell Proliferation Assay (xCELLigence)

The xCELLigence is a system that monitors dynamic cellular events in real time and gives quantitative information about biological status of the cells including cell number and viability. This system enabled to provide good sensitivity and reproducibility in monitoring an entire cell population in a culture well. The technology behind this system comes from the design of the platform. E-plate 96 is similar to 96 well plates but it contains electrode sensors integrated into the each well so that each well can be monitored separately. The electrode impedance (Z) increases as the number of cells increase on it (Figure 3-1).

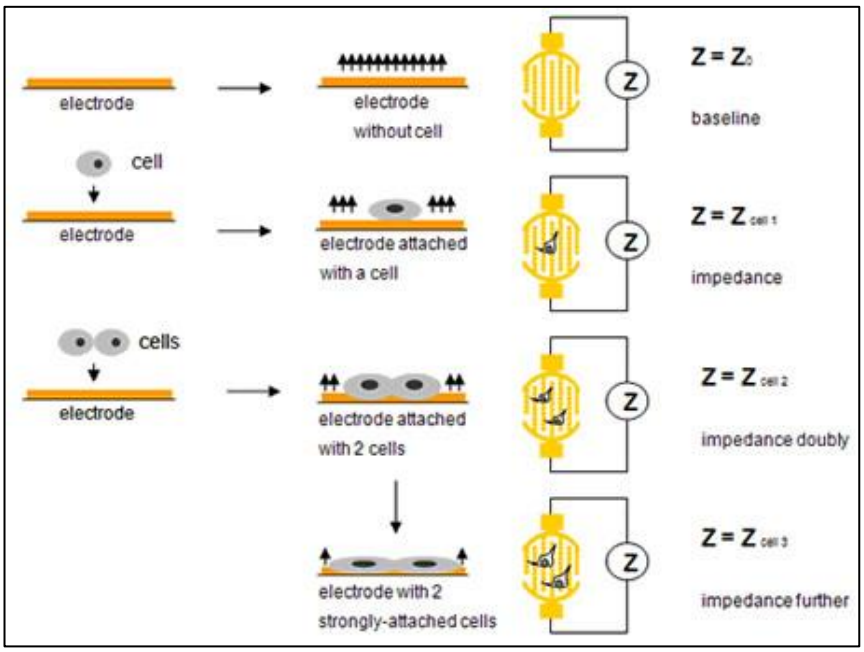


Figure 3-1 Schematic Drawing of the Working Principle of xCELLigence (ACEA Bioscience Inc.)

Before inoculating the cells to the E-plate 96, 50 ul of growth medium was put into E-plate 96 wells for each cell line to obtain background readings (takes only 1 minute). This step also allows us to determine if there are any inconvenient wells exist in that particular plate so that a problematic well can be omitted from the experiment design and replaced with another well. The certain number of cells were from cell suspensions were added on top of growth mediums with 100 ul/well volume where the total volume will be 150 ul/well in total. The xCELLigence system (RTCA SP Station) where the cells were replaced was kept at 37°C with 5% CO_2 air incubator during the experiment. Depending of the cell growth (between 24 to 72 hours), cells were treated with either DMSO or Propolis-2 with pre-determined concentrations. The treatment time point determined as the cells pass 0.75 Cell Index and before they reach to 1.25 Cell Index so that cells can be treated when they are in the log phase. At the beginning of the experiment, Cell Index values were recorded for every 30 minutes but after the drug treatment the Cell Index

values were recorded for every 10 minutes to observe the fast drug response. After the observation of fast drug response the counter changed into record Cell Index for every 30 minutes. Long-term drug response was recorded at least 72 hours after the drug treatment.

3.6 Nuclear Staining with Hoechst 33258

Cells (80,000 cells/well) were plated in cover slips in 6 well plates and after 24 h cells were treated with 0.1 % DMSO control or 100 ug/ml Propolis-2. Cells were incubated at 37°C with 5% CO_2 air. After 48 h incubation, growth medium was removed and cells were washed with 1x PBS. After removal of PBS, cells were fixed with 1 ml ice-cold 100% methanol and kept for 10 minutes at +4°C. Cells were washed with ice-cold 1x PBS. 1 ug/ml Hoechst 33258 solution was prepared with ice-cold 1x PBS. After removal of PBS, cells were stained with 400 ul Hoechst solution and incubated at room temperature for 5 minutes in the dark. Then the cells were destained with ddH_2O for 10 minutes at room temperature in the dark. Water was removed and cover slips were mounted on glass slides with glycerol. Cover slips were fixed onto the slides by wiping transparent nail polish to the edges. The stained cells' nuclear morphology was examined under fluorescent microscope.

3.7 Western Blot

3.7.1 Cell Lysis

Cell pellets were taken from -80°C freezer and thawed on ice. Each pellet was resuspended with 50 ul (depending on to cell pellet size) cell lysis buffer, vortexed 3

times and left on ice for 30 minutes with occasional vortexing. Samples were centrifuged at 13000 rpm at +4°C for 15 minutes. The supernatant was taken into the fresh tubes. Protein samples were always stored in -80°C and kept on ice during the experiments.

3.7.2 Bradford Assay for Protein Quantitation and Sample Preparation

Bradford assay was used to determine the amount of protein in each cell lysate. BSA Standard Curve was prepared before using Bradford working solution. BSA samples were prepared in Cuvettes as in Table 3.2. Samples were measured with spectrophotometer at 515 nm wavelength and absorbance results were used to draw a BSA Standard Curve (Figure 3-2). Trendline of this curve was used for protein quantitation of samples.

Table 3.2 Sample Preparation for BSA Standard Curve

Vial #	Blank	1	2	3	4	5	6
BSA (ug/ml)	-	1	2	4	8	16	32
ddH2O (ul)	100	99	98	96	92	84	68
Bradford (ul)	900	900	900	900	900	900	900
TOTAL (ul)	1000	1000	1000	1000	1000	1000	1000

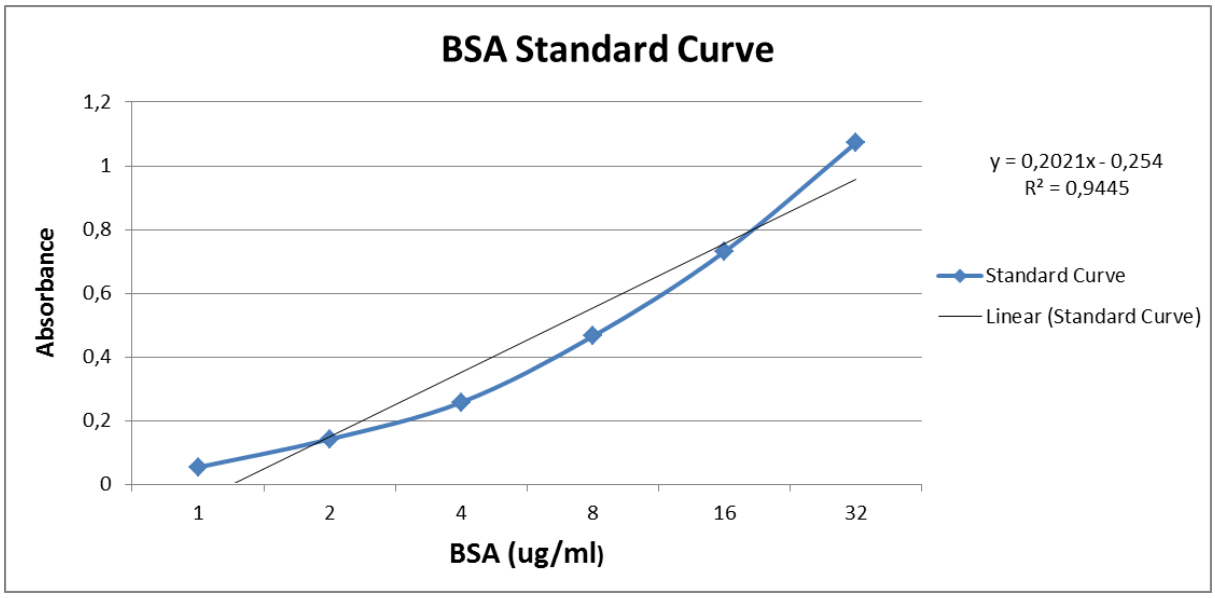


Figure 3-2 BSA Standard Curve

After generating the BSA Standard Curve, protein samples were prepared as in Table 3.3 to measure with spectrophotometer at 515 nm wave length. The measurements were taken and the results were calculated with the trendline equation of BSA Standard Curve (y=0.2021x-0.2554). Since 2 ul of samples were loaded to the cuvettes, the results were divided by 2. Absorbance of samples and their protein concentrations were calculated and given in Table 3.4.

Table 3.3 Sample Preparation for Protein Quantitation with Spectrophotometer

Vial #	Blank	Samples
Cell Lysis Buffer (ul)	2	-
Protein Sample (ul)	-	2
ddH2O (ul)	98	98
Bradford (ul)	900	900
TOTAL (ul)	1000	1000

Table 3.4 Absorbance and Protein Concentrations of Samples for Western Blot Analysis.

Sample Name		Absorbance	Protein Conc. (ug/ul)	for 50 ug (ul)	ddH2O (ul)	5X Loading dye (ul)
MCF10A	DMSO(%0.1)	0,7606	22,99	2,18	9,82	3
	Propolis(100ug/ml)	0,4599	16,18	3,09	8,91	3
MDA-MB-231	DMSO(%0.1)	0,5867	19,05	2,62	9,38	3
	Propolis(100ug/ml)	0,4558	16,09	3,11	8,89	3
T47D	DMSO(%0.1)	0,4038	14,91	3,35	8,65	3
	Propolis(100ug/ml)	0,2771	12,05	4,15	7,85	3
CAMA-1	DMSO(%0.1)	0,4285	15,47	3,23	8,77	3
	Propolis(100ug/ml)	0,1991	10,28	4,86	7,14	3
BT-20	DMSO(%0.1)	0,8366	24,71	2,02	9,98	3
	Propolis(100ug/ml)	0,353	13,76	3,63	8,37	3
BT-474	DMSO(%0.1)	0,3074	12,73	3,93	8,07	3
	Propolis(100ug/ml)	0,0323	6,51	7,68	4,32	3
MDA-MB-231	DMSO(%0.1)	0,5058	17,22	2,90	9,10	3
	IC50(74 ug/ml)	0,0082	5,96	8,38	3,62	3
	Propolis(100 ug/ml)	0,2985	12,53	3,99	8,01	3
	IC100(148 ug/ml)	-0,0519	4,60	10,86	1,14	3
MDA-MB-231 (Positive Control)	Adriamycin 500 ng/ml	0,2178	10,71	4,67	7,33	3
	Adriamycin 750 ng/ml	0,3035	12,64	3,95	8,05	3
	Adriamycin 1000 ng/ml	0,1351	8,83	5,66	6,34	3
MCF10A	Untreated Cell Lines	0,1726	9,68	5,16	6,84	3
T47D		0,0819	7,63	6,55	5,45	3
CAMA-1		0,038	6,64	7,53	4,47	3
BT-20		-0,0734	4,12	12,14	-0,14	3
BT-474		0,0431	6,75	7,40	4,60	3
MDA-MB-231		0,2143	10,63	4,71	7,29	3
BSA curve is y=0.2021x-0.2554			TOTAL	15 ul		

Western blot analysis was performed with these protein extracts. 50 mg of protein was mixed with 1x loading dye and added up to final volume of 15 ul with ddH_2O

(Table 3.4). Samples were incubated on boiling water for 5 minutes. After a quick spin down, samples were ready to load into SDS gel. The samples were always kept on ice during the experiments.

3.7.3 Preparation of SDS gel and Its Transfer to Nitrocellulose Membrane

The material used for gel preparation was pre-washed with soap, rinsed with tap water and then with distilled water to get rid of the debris on the material. The apparatus was set and 10% resolving gel was prepared and poured in between the glasses and then filled with isopropanol. After polymerization of resolving gel, isopropanol was removed and the empty area was washed with distilled water to remove remaining alcohol. 5% stacking gel was prepared, poured on top of resolving gel and combs were placed in between glasses. After polymerization of gels, glasses with gels were placed in the tank that was filled with 1x running buffer. Combs were carefully removed and the wells were cleared with a syringe. The first well was loaded with 5 ul of PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and samples were loaded into the wells. Power supply was adjusted to 90 Volts until proteins passed through the stacking gel and then increased to 120 Volts. The gels were transferred to nitrocellulose membrane with wet transfer method.

Wet transfer buffer was prepared with 15% methanol concentration. Whatman papers, sponges and membranes were soaked in wet transfer buffer for a few minutes. From negative plate towards positive plate; sponge, two Whatman papers, gel, nitrocellulose membrane, two Whatman papers, and sponge were aligned one top of each other. Before putting the last sponge, air bubbles were destroyed by rolling a tube on the layers. Transfer cassettes were placed in tank and filled with wet transfer buffer. Power supply was adjusted to 80 Volts and the gels were run for approximately 2 hours.

3.7.4 Blocking & Antibody Incubations

Blocking solution was prepared with 5% BSA in 0.2 % TBS-T. Each membrane was placed in an appropriate container and the container was filled with blocking solution. Membranes were incubated for 1 hour on shaker (very slow) at room temperature. At the end, membranes were prepared for antibody incubation: Each membrane was cut from the 55 kDa and 35 kDa marker band for different primary antibodies. (Figure 3-3: The protein loading marker and the gel configuration where the dashed lines show the cutting sites of the gels.)

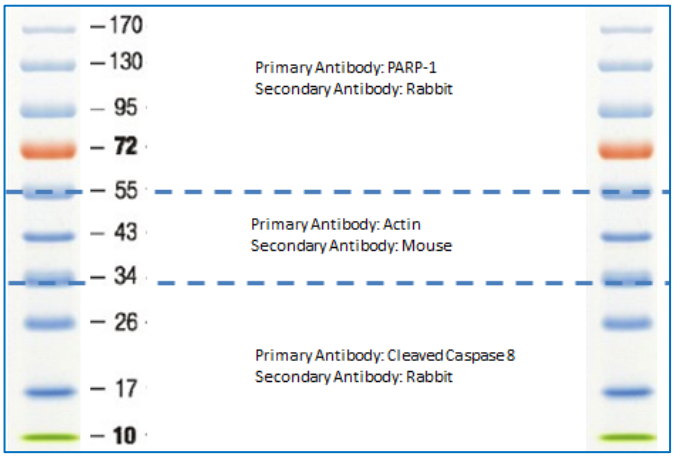


Figure 3-3: The protein loading marker and the gel configuration where the dashed lines show the cutting sites of the gels.

After blocking, membranes were placed in an appropriate container and containers were filled with its primary antibody (Table 2.11 and Figure 3-3). They were incubated with constant shaking (very slow) at +4°C over-night. After incubation, membranes were washed with 0.2 % TBS-T for 10 minutes on shaker (fast) at room-temperature, 3 times. Then, secondary antibody (Table 2.11 and Figure 3-3) incubation was done, on shaker (very slow) at room temperature for 2 hours. After

incubation, membranes were washed three times with 0.2 % TBS-T for 10 minutes on shaker (fast) at room-temperature.

Clarity™ Western ECL Substrate (Bio-Rad) material was used for detecting the signal of secondary antibodies. The membranes were placed on a 10x10 cm parafilm on the bench. Each membrane was coved with 250-350 ul (according to area of the membrane) 1:1 mixed ECL solution drop by drop. Membranes were incubated for 5 minutes at room-temperature and plastic covers were put on top of every membrane so that solution was not exposed to light. After incubation membranes were placed on glass plate. The x-ray film was used to capture image of the membranes in the dark room. Membranes were exposed to film between 1-5 seconds depending of the intensity of the bands.

3.7.5 ImageJ Analysis

Resulting bands on film were analyzed with ImageJ software (National Institutes of Health (NIH)) which is a public domain Java image processing program. The resulted band intensities need to be quantified to be able to compare the western blot results. Films were scanned and saved as jpeg photo. These photos were used for ImageJ analysis. The bands were selected with a rectangular shape option of the program and all the lanes were repeated with the same rectangular selection. At the end rectangular area was plotted by the program and the resulting band intensity was quantified. The comparable data was calculated by dividing PARP-1 protein intensity to its β-actin loading control. The resulting values were displayed in bar graphs.

3.8 FACS

3.8.1 Cell Fixation

Propolis-treated and un-treated cells were used for staining with Propidium iodide (PI) to analyze the cell cycle stages with FACS method. Six well plates were used for culturing the cells and for treatment. Since apoptotic cells detach from the plate surface and remain in the growth media, each well was transferred into separate 15 ml tubes and labeled. Then, each well was washed with 1x PBS and PBS was collected into same tubes containing the detached cells. 0.3 ml Trypsin/EDTA was put into each well of 6 well plate and incubated at 37°C for 1-2 minutes to detach the adherent cells. When the cells appeared to have lost their adherence, trypsin was inactivated by adding fresh medium which contains FBS. Cells were transferred into appropriate tubes containing the cells collected in previous steps and centrifuged at 1500 rpm for 5 minutes at +4°C. Supernatant was removed and cell pellets were resuspended by gentle pipetting with 1 ml 1x PBS. Cell suspensions were vortexed vigorously and 2.5 ml of ice-cold 100% absolute ethanol was added drop wise to prevent cell clumps during fixation. Cell suspensions were incubated for 30 minutes on ice with occasional vortexing. Fixed cell suspensions were stored at +4°C overnight before the PI staining procedure.

3.8.2 Cell Staining with Propidium iodide (PI)

Fixed cell suspensions were centrifuged at 1500 rpm for 5 minutes at +4°C. The supernatant was aspirated. Cells were stained with 500 ul PI Staining Solution and suspensions were pipetted gently to break cell clumps. Since PI is light-sensitive,

tubes need to be kept in dark after this step. Cells were incubated at 37°C for 40 minutes with occasional vortexing. After incubation, 3 ml 1x PBS was added onto each tube and tubes were centrifuged at 1500 rpm for 5 minutes at +4°C. The supernatant was removed and cell pellets were resuspended in 200-500 ul 1x PBS (according to size of the pellet). Cell suspensions were transferred into special tubes to analyze them in Flow Cytometer.

3.9 Scratch Assay

The in vitro scratch assay (wound healing assay) is an easy, low-cost and well-developed method to measure cell invasion *in vitro*. A straight line of cells are scraped from the plate and the invasion of the cells through this scraped line is observed via light microscope.

FBS concentration in the growth medium was dropped from 10 % to 0.1 % to prevent cells growing through the scratch. By this way, wound healing assay can display the invasion properties of cells but not the proliferation. There were 6 conditions for this set up to observe the invasion rate:

- 10 % FBS containing growth medium treated cells (untreated, mock cells) to observe the effect of proliferation and compare it with 0.1 % FBS medium treated cells,
- 0.1 % FBS containing growth medium treated cells to observe the effect of decreased FBS and compare it with DMSO control cells,
- 0.1 % FBS + 0.1 % DMSO containing growth medium treated cells as a control for propolis treated cells,

- 0.1 % FBS + 50, 75 (IC50 value propolis for MDA-MB-231 cells) and 100 ug/ml Propolis-2 containing growth medium treated cells to see the effect of gradually increasing concentrations of propolis on cell invasion ability.

Cells (500,000 cells/well) were seeded in 6 well plate to obtain high confluency and 24 hours later, scratches were made with a 200 ul micropipette tip. Cells were washed with 1X PBS twice to get rid of cell debris in the medium and then cells were treated with the growth mediums listed above. Right after the scratches were performed, time zero photos were taken to observe the scratches borders clearly. Then, the photos were taken at 6, 12, 24, 36 and 48 h intervals under the light microscope with 10X magnification.

4 RESULTS

4.1 Analysis of Propolis' Effects in Cancer Cells

Two different propolis extracts were used in this study to analyze its cytotoxic effects on cancer cells by using SRB staining and ELISA reading. First propolis extract received from Karadeniz Technical University (Propolis-1) was prepared in DMSO and the stock at a concentration of 25 mg/ml. The initial studies were performed with increasing concentrations (starting from 100 ng/ml up to 2000 ng/ml) of propolis by using FOCUS cell line at two different time points (48 and 72 h). The ELISA reading as a result of SRB staining was given at Table 4.1 and results of these experiments were analyzed in Figure 4-1.

Table 4.1: The ELISA reading results that show the cytotoxic effects of Propolis-1 and DMSO on FOCUS cells (A, 48h; B, 72h incubations).

A)

FOCUS 48h 3000 cells/well	Concentration (ng/ml)	Set 1	Set 2	Set 3	Average	SEM
DMSO	100	0.011	0.026	0.012	0.016	0.005
	250	0.005	0.023	0.046	0.025	0.012
	500	0.004	0.01	0.012	0.009	0.002
	750	0.033	0.03	0.029	0.031	0.001
	1000	0.005	0.007	0.013	0.008	0.002
	2000	-0.003	0	-0.003	-0.002	0.001
Propolis	100	0.046	0.051	0.027	0.041	0.007
	250	0.018	0.027	0.011	0.019	0.005
	500	0.013	0.019	0.015	0.016	0.002
	750	0.019	0.015	0.003	0.012	0.005
	1000	0.011	-0.007	0.001	0.002	0.005
	2000	0.008	-0.001	0.001	0.003	0.003

B)

FOCUS 72h 3000 cells/well	Concentration (ng/ml)	Set 1	Set 2	Set 3	Average	SEM
DMSO	100	0.037	0.032	0.157	0.075	0.041
	250	0.136	0.178	0.091	0.135	0.025
	500	0.012	0.029	0.026	0.022	0.005
	750	0.048	0.052	0.033	0.044	0.006
	1000	0.024	0.057	0.036	0.039	0.01
	2000	0.017	0.015	0.023	0.018	0.002
Propolis	100	0.108	0.126	0.084	0.106	0.012
	250	0.038	0.02	0.078	0.045	0.017
	500	0.023	0.01	0.025	0.019	0.005
	750	0.018	0.008	0.09	0.039	0.026
	1000	0.029	0.019	0.021	0.023	0.003
	2000	0.031	0.001	0.037	0.023	0.011

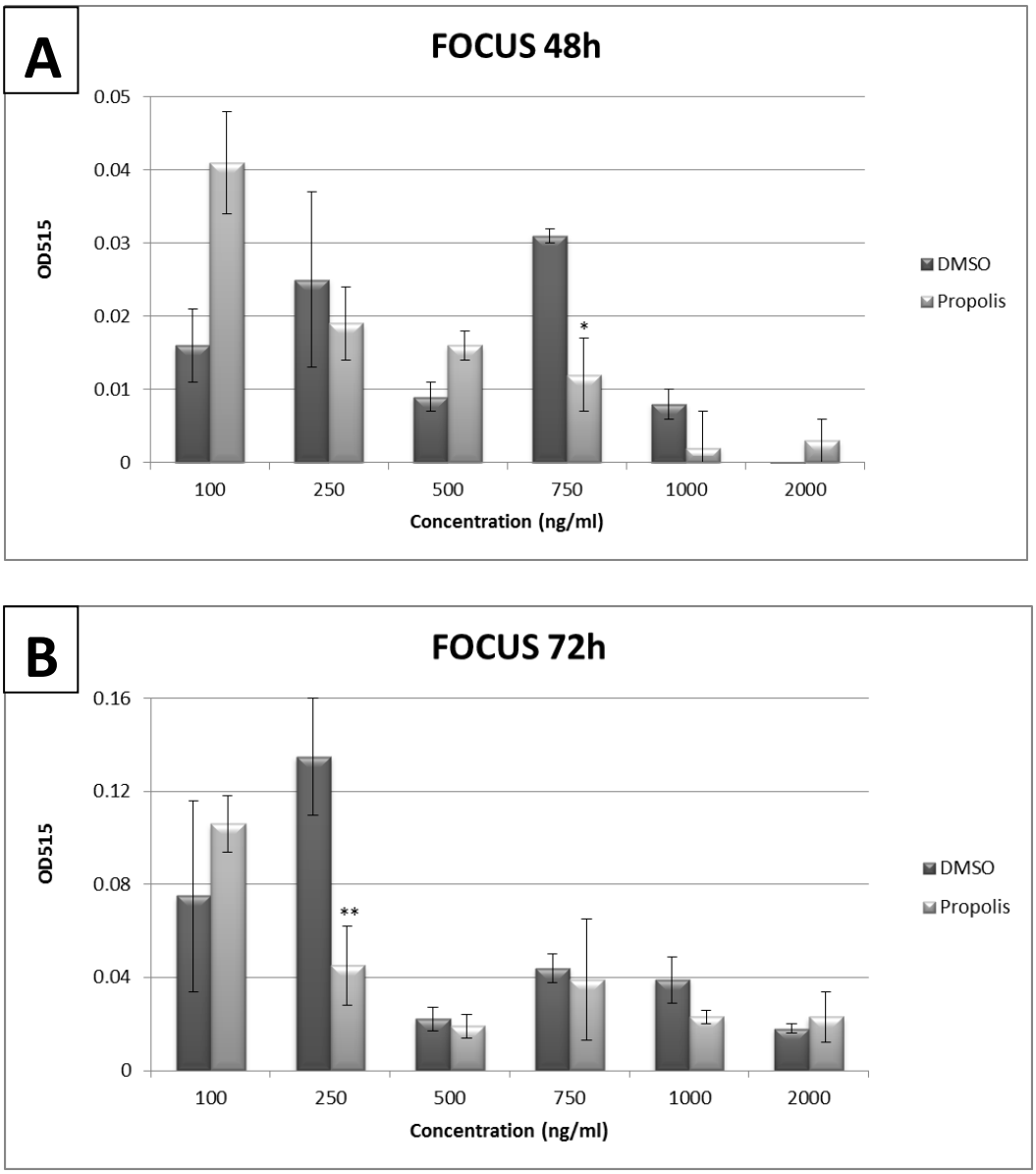


Figure 4-1: The cell proliferation rate of FOCUS cells after propolis treatment. The cells were treated with Propolis-1 and DMSO at 48 h (A) and 72 h (B). Standard error of the mean (SEM) for each calculation is given at Table 4.1. (*, $p<0.1$; **, $p<0.05$)

These analyses showed that the Propolis-1 at low concentrations (100 ng/ml up to 2000 ng/ml) did not result with a significant cytotoxic effect. Therefore, the concentration of Propolis-1 was increased to 50 ug/ml. The cell proliferation rate

was measured at different time points (24, 48 and 72 h) by using FOCUS and Huh7 hepatocellular carcinoma cell lines. The ELISA reading as a result of SRB staining was given at Table 4.2 and the results of these experiments were analyzed in Figure 4-2.

Table 4.2: The ELISA reading results that show the cytotoxic effects of Propolis-1 and DMSO on FOCUS (A) and Huh7 (B) cells at different time points (24 h, 48 h, and 72 h). The starting cell number was 20,000 cells/well into 24 well plates.

A)

FOCUS	Time	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Average	SEM
Mock	24h	0.094	0.097	0.085	0.105	0.098	0.108	0.098	0.003
	48h	0.216	0.221	0.212	0.24	0.217	0.214	0.22	0.004
	72h	0.338	0.43	0.383	0.392	0.382	0.31	0.373	0.017
DMSO	24h	0.084	0.088	0.094	0.096	0.091	0.09	0.091	0.002
	48h	0.214	0.243	0.229	0.224	0.266	0.248	0.237	0.008
	72h	0.125	0.116	0.147	0.102	0.104	0.093	0.115	0.008
Propolis-1 50 ug/ml	24h	0.069	0.067	0.068	0.078	0.062	0.064	0.068	0.002
	48h	0.109	0.096	0.078	0.091	0.097	0.082	0.092	0.005
	72h	0.038	0.03	0.026	0.016	0.018	0.016	0.024	0.004

B)

Huh7	Time	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Average	SEM
Mock	24h	0.064	0.062	0.069	0.073	0.07	0.064	0.067	0.002
	48h	0.136	0.138	0.134	0.139	0.154	0.148	0.142	0.003
	72h	0.289	0.295	0.3	0.306	0.309	0.264	0.294	0.007
DMSO	24h	0.065	0.057	0.057	0.053	0.059	0.056	0.058	0.002
	48h	0.118	0.13	0.128	0.143	0.136	0.143	0.133	0.004
	72h	0.324	0.3	0.266	0.293	0.32	0.307	0.302	0.009
Propolis-1 50 ug/ml	24h	0.043	0.043	0.038	0.042	0.039	0.043	0.041	0.001
	48h	0.067	0.075	0.063	0.064	0.076	0.078	0.071	0.003
	72h	0.083	0.063	0.087	0.083	0.084	0.084	0.081	0.004

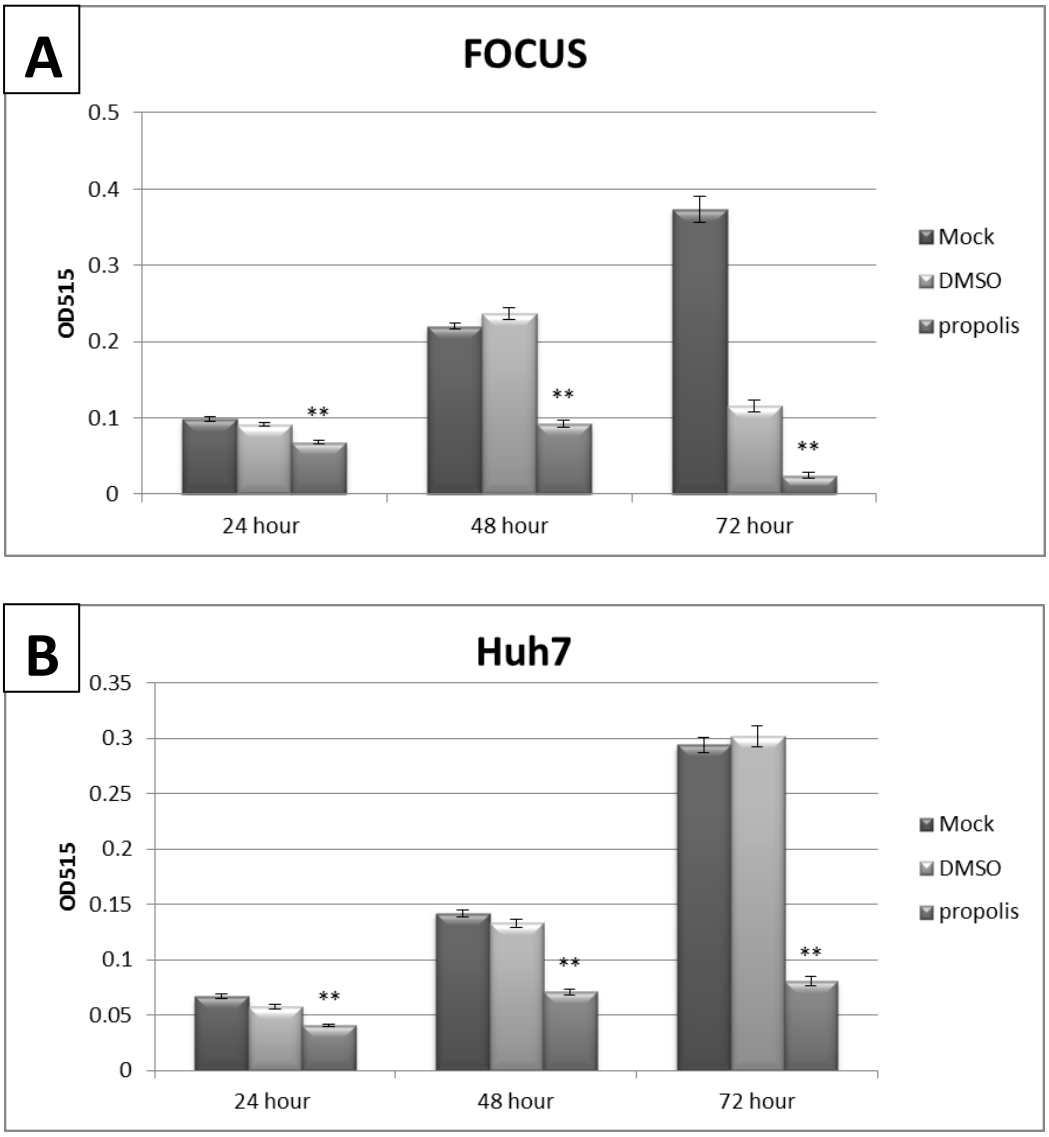


Figure 4-2: The cell proliferation rate of FOCUS (A) and Huh7 (B) cells after treatment with Propolis-1 and DMSO at 24 h, 48 h, and 72 h time points. Standard error of the mean (SEM) for each calculations were given at Table 4.2. (**, $p<0.05$)

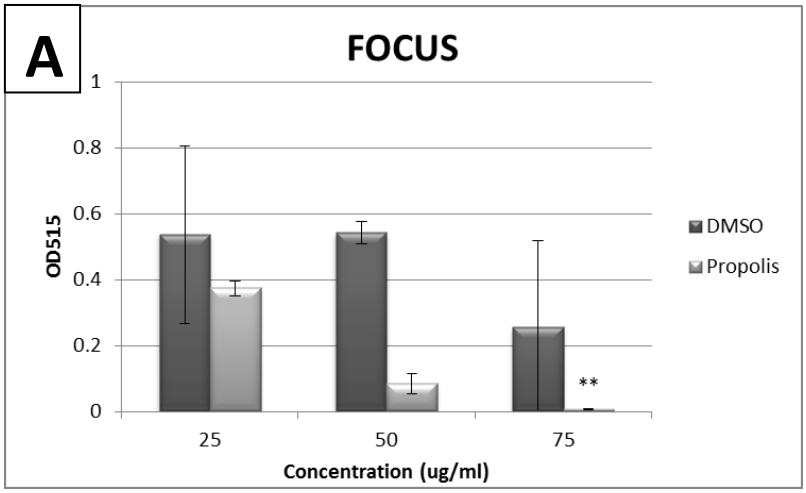
As a result of the above analysis, the most significant time point on HCC cell lines showing significant cytotoxic effect was observed at 72 h with 50 ug/ml of Propolis-1. Then the following experiments were designed to analyze the time points and

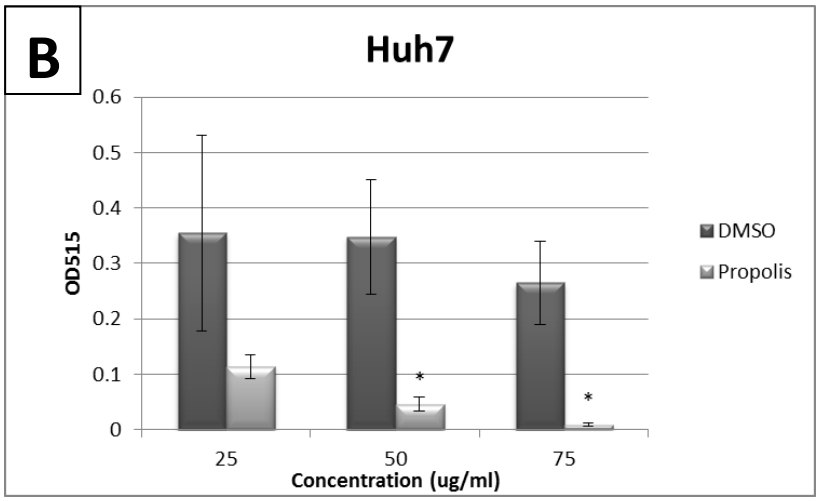
different concentration of Propolis-1 (25, 50 and 75 ug/ml) on five different cell lines (FOCUS and Huh7 are hepatocellular carcinoma cells; SK-LC is a lung cancer cell line; MDA-MB-231 is breast carcinoma cell line) and non-tumorigenic breast cell line (MCF12A). For each cell line 20000 cells/well were plated on 24 well plates and experiments were designed as triplicates. The cells were treated with indicated concentrations of Propolis-1 and DMSO after 24 h incubation. The cells were fixed and cell numbers were counted with SRB staining method at 72 h time point after 24 h treatment. The ELISA reading as a result of SRB staining was given at Table 4.3 and the results of these experiments were analyzed in Figure 4-3.

Table 4.3: The ELISA reading results of Propolis-1 and DMSO on FOCUS, Huh7, SK-LC, MDA-MB-231 and MCF12A cell lines.

FOCUS (20000 cells/well)	**Set 1**	**Set 2**	**Set 3**	**Average**	**SEM**
25ug/ml DMSO	0.857	0.752	0.003	0.537	0.269
25ug/ml Propolis	0.306	0.402	0.415	0.374	0.034
50ug/ml DMSO	0.642	0.942	0.043	0.542	0.264
50ug/ml Propolis	0.042	0.095	0.119	0.085	0.023
75ug/ml DMSO	0.246	0.312	0.211	0.256	0.03
75ug/ml Propolis	0.005	0.007	0.008	0.007	0.001
Huh7 (20000 cells/well)	**Set 1**	**Set 2**	**Set 3**	**Average**	**SEM**
25ug/ml DMSO	0.431	0.616	0.019	0.355	0.176
25ug/ml Propolis	0.072	0.141	0.128	0.114	0.021
50ug/ml DMSO	0.331	0.535	0.177	0.348	0.104
50ug/ml Propolis	0.025	0.043	0.07	0.046	0.013
75ug/ml DMSO	0.279	0.387	0.13	0.265	0.075
75ug/ml Propolis	0.004	0.013	0.011	0.009	0.003
SK-LC (20000 cells/well)	**Set 1**	**Set 2**	**Set 3**	**Average**	**SEM**
25ug/ml DMSO	0.219	0.287	0.002	0.169	0.086
25ug/ml Propolis	0.035	0.042	0.051	0.043	0.005
50ug/ml DMSO	0.144	0.247	0.025	0.139	0.064
50ug/ml Propolis	0.038	0.035	0.034	0.036	0.001
75ug/ml DMSO	0.108	0.162	0.111	0.127	0.018
75ug/ml Propolis	0.002	0.004	0.002	0.003	0.001

MDA-MB-231 (20000 cells/well)	**Set 1**	**Set 2**	**Set 3**	**Average**	**SEM**
25ug/ml DMSO	0.122	0.101	0.093	0.105	0.009
25ug/ml Propolis	0.057	0.064	0.061	0.061	0.002
50ug/ml DMSO	0.126	0.09	0.097	0.104	0.011
50ug/ml Propolis	0.049	0.043	0.043	0.045	0.002
75ug/ml DMSO	0.074	0.118	0.116	0.103	0.014
75ug/ml Propolis	0.012	0.01	0.007	0.01	0.001
MCF12A (20000 cells/well)	**Set 1**	**Set 2**	**Set 3**	**Average**	**SEM**
25ug/ml DMSO	0.052	0.075	0.052	0.06	0.008
25ug/ml Propolis	0.043	0.038	0.044	0.042	0.002
50ug/ml DMSO	0.041	0.066	0.088	0.065	0.014
50ug/ml Propolis	0.031	0.033	0.037	0.034	0.002
75ug/ml DMSO	0.041	0.057	0.013	0.037	0.013
75ug/ml Propolis	0.003	0.005	0.004	0.004	0.001

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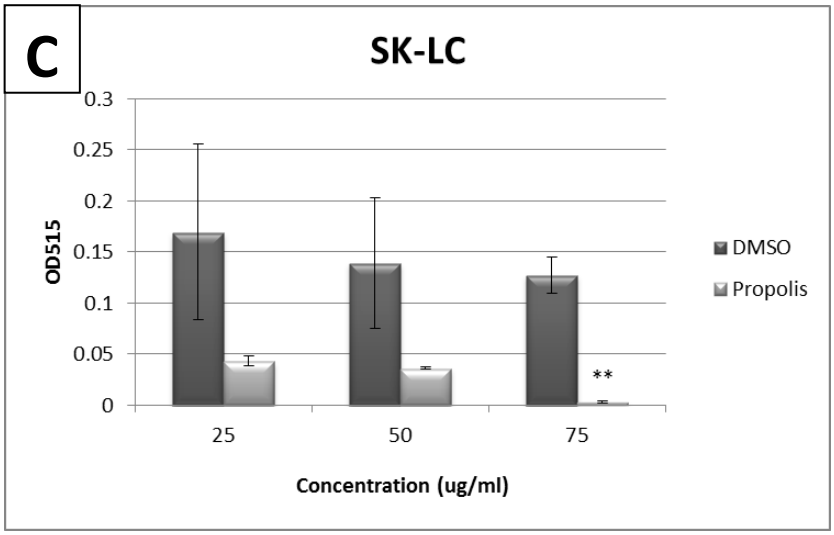
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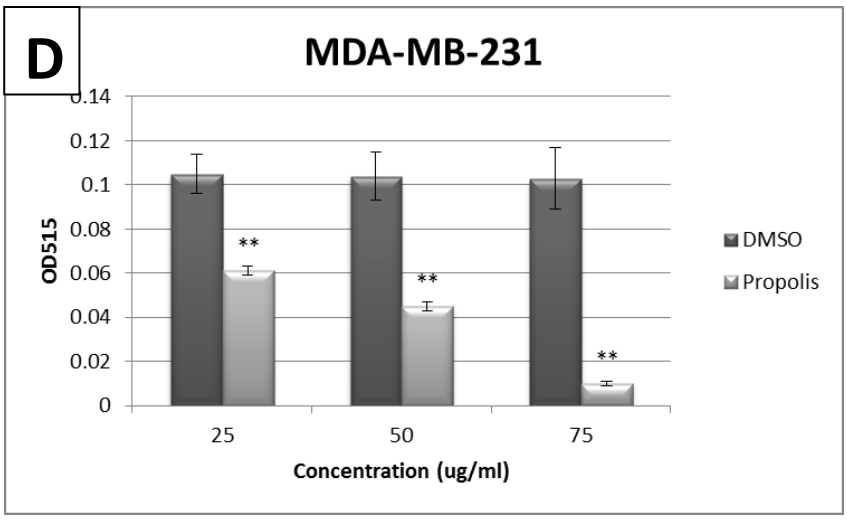
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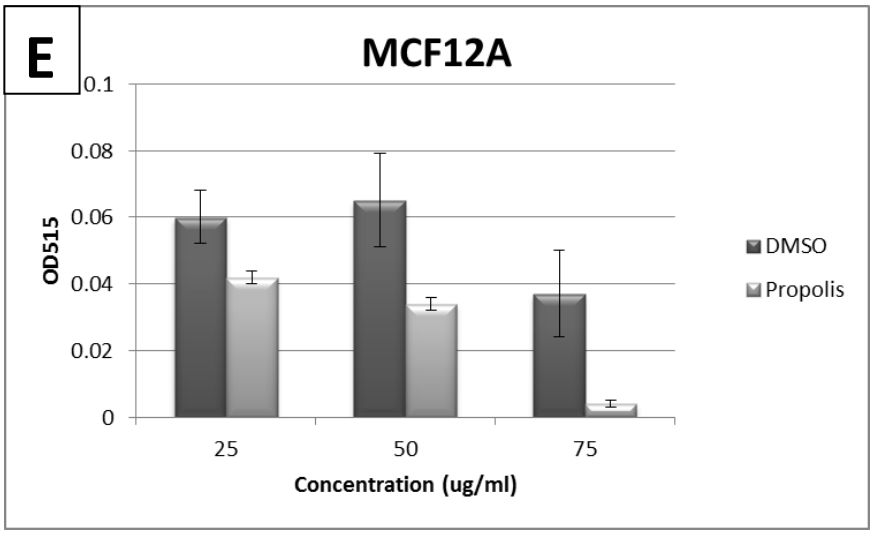


Figure 4-3: Cell proliferation of FOCUS (A), Huh7 (B), SK-LC (C), MDA-MB-231 (D) and MCF12A (E) cell lines with increasing concentrations of Propolis-1 and DMSO. Values are the average of at least three independent data sets. Error bars represent standard error of the mean of triplicates given at Table 4.3. (*, $p<0.1$; **, $p<0.05$)

According to above analysis, higher concentrations of propolis have a greater effect on cell number decrease in each cell line. When the ratio of DMSO over propolis was calculated (Table 4.4), the effect of propolis was revealed more clearly (Figure 4-4). As the concentration of propolis increases, decrease in the cell number became up to 10 fold more than low concentrations of propolis.

Table 4.4: Comparison of control DMSO and propolis treated cells proliferation ratio. Each cell lines fold decrease ratio was calculated by dividing control DMSO cell number to propolis value (data was taken from Figure 4-3).

Fold Decrease	FOCUS	Huh7	SK-LC	MDA-MB-231	MCF12A
25 ug/ml	1,4	3,1	3,9	1,7	1,4
50 ug/ml	6,4	7,6	3,9	2,3	1,9
75 ug/ml	36,6	29,4	42,3	10,3	9,3

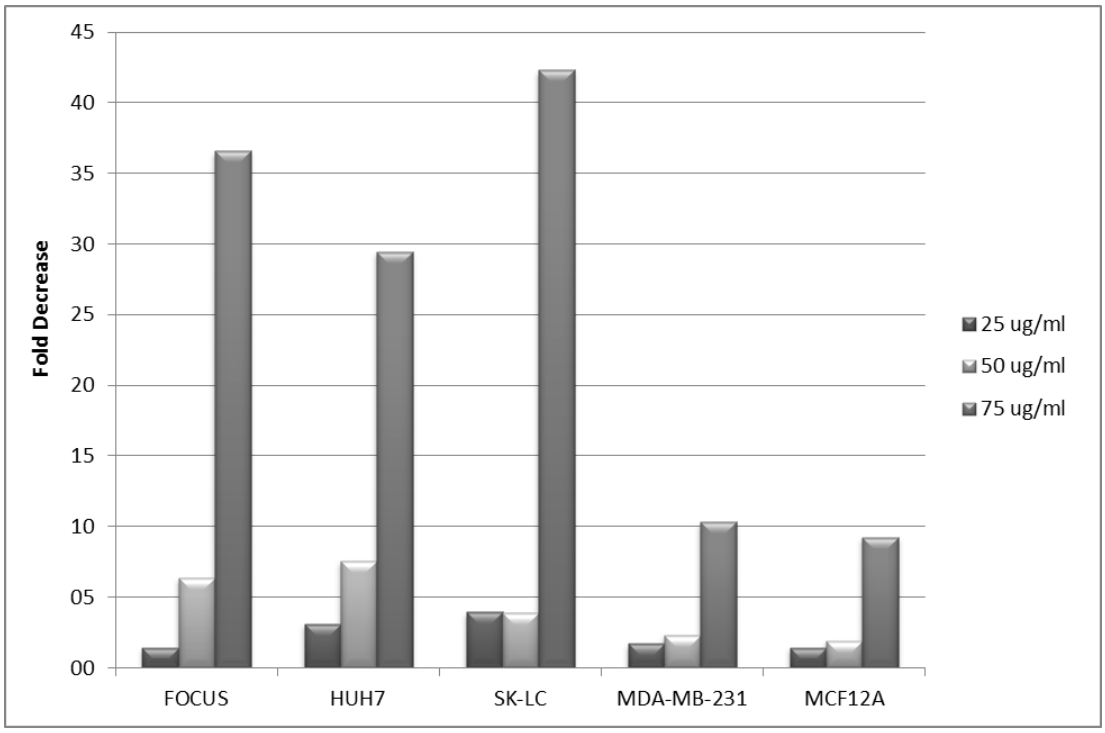


Figure 4-4: The relative fold change of each propolis treated cell line at different concentrations of Propolis-1. Each cell lines fold decrease ratio was calculated by dividing control DMSO value to propolis value (data was taken from **Table 4.3**).

4.2 IC_{50} Values for Propolis extracts for Breast Carcinoma Cell Lines

IC_{50} value determination was started with inoculation of breast carcinoma cells into 96 well plates. The numbers of cells were different for each cell line and indicated in the related sections. 24 hours later, cells were treated with serial dilutions of propolis and control DMSO. Propolis extract dissolved in DMSO, therefore the same volume of DMSO was used in serial dilutions as controls. Cells were fixed with TCA to 72 hours after the treatment. Cells were stained with SRB and the color intensity was measured with ELISA reader. Resulting optical density (OD_{515}) is correlated with

the amount of cells. Samples were collected and calculated at least in triplicates and standard error of mean (SEM) was calculated according to sample number. IC_{50} values were analyzed with at least 5 different concentrations of propolis. All the IC_{50} value calculations were analyzed the same way. Two different batches of DMSO extracts of propolis were used in this study; first batch is 25 mg/ml (Propolis-1) and second batch is 100 mg/ml (Propolis-2). Propolis was collected from different regions of Turkey by Fanus Gıda Corporation (Trabzon, Turkey) and extractions were prepared in laboratories of Karadeniz Technical University, Medical biochemistry laboratories, Trabzon.

IC_{50} Values of Propolis Extracts for Non-Tumorigenic Cell Lines (MCF10A and MCF12A)

Analysis of IC_{50} value of propolis extracts on MCF10A was performed with following conditions:

Propolis-1: 100-50-25-12.5-6 ug/ml, 4000 cells were used in each well. MCF10A cell line's IC_{50} value was calculated as 45 ug/ml (Figure 4-5, A). The experiment was performed in triplicate.

Propolis-2: 200-150-100-75-37.5 ug/ml, 5000 cells were used in each well. MCF10A cell line's IC_{50} value was calculated as 72 ug/ml (Figure 4-5, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.1 for Propolis-1 and Appendix Table 8.2 for Propolis-2.

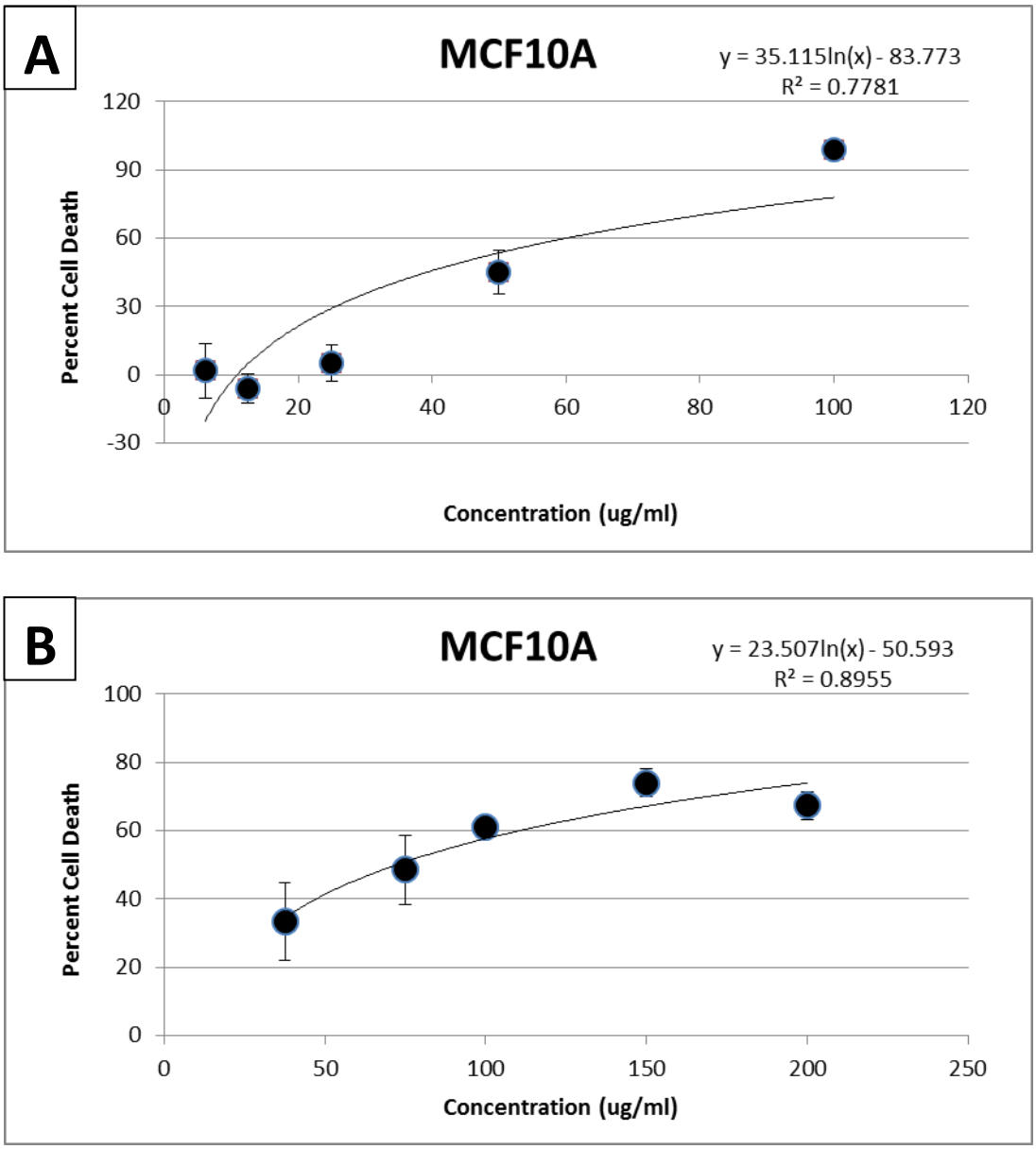


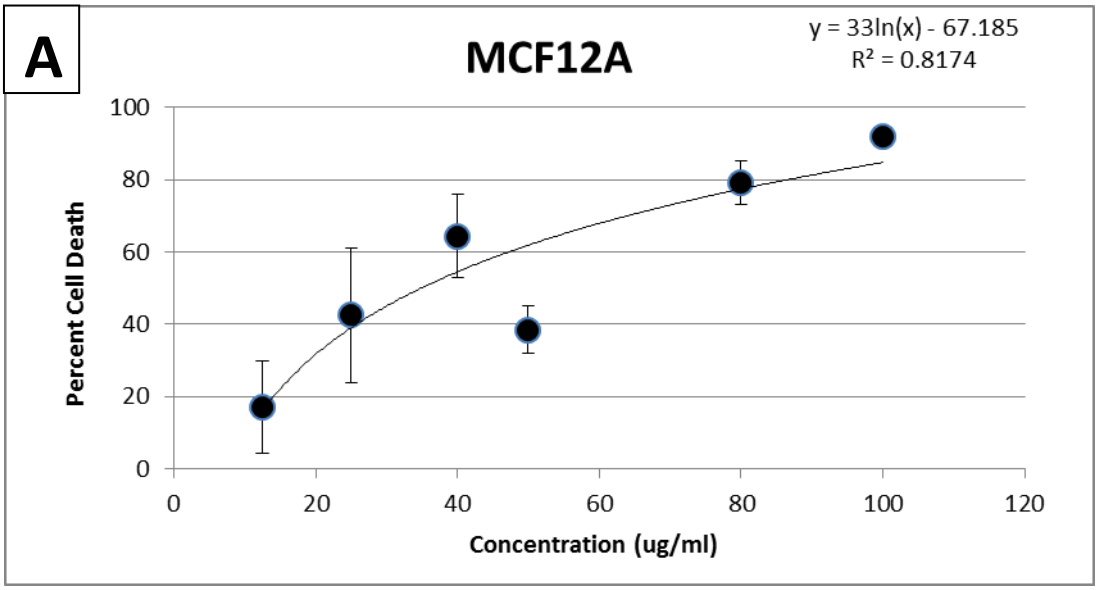
Figure 4-5: **Calculation of IC_{50} values for propolis extracts in MCF10A cells.** Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC_{50} value for Propolis-1 is 45 ug/ml and R^2 value is R^2=0.7781. B, The IC_{50} value for Propolis-2 is 72 ug/ml and R^2 value is R^2=0.8955. Error bars represent standard error of the mean (SEM).

Analysis of IC_{50} value of propolis extracts on MCF12A was performed with following conditions:

Propolis-1: 100-80-50-40-25-12.5 ug/ml, 5000 cells were used in each well. MCF12A cell line's IC_{50} value was calculated as 35 ug/ml (**Figure 4-6**, A). The experiment was performed in triplicate.

Propolis-2: 200-150-100-75-50-37.5 ug/ml, 6000 cells were used in each well. MCF12A cell line's IC_{50} value was calculated as 45 ug/ml (Figure 4-6, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.3 for Propolis-1 and Appendix Table 8.4 for Propolis-2.



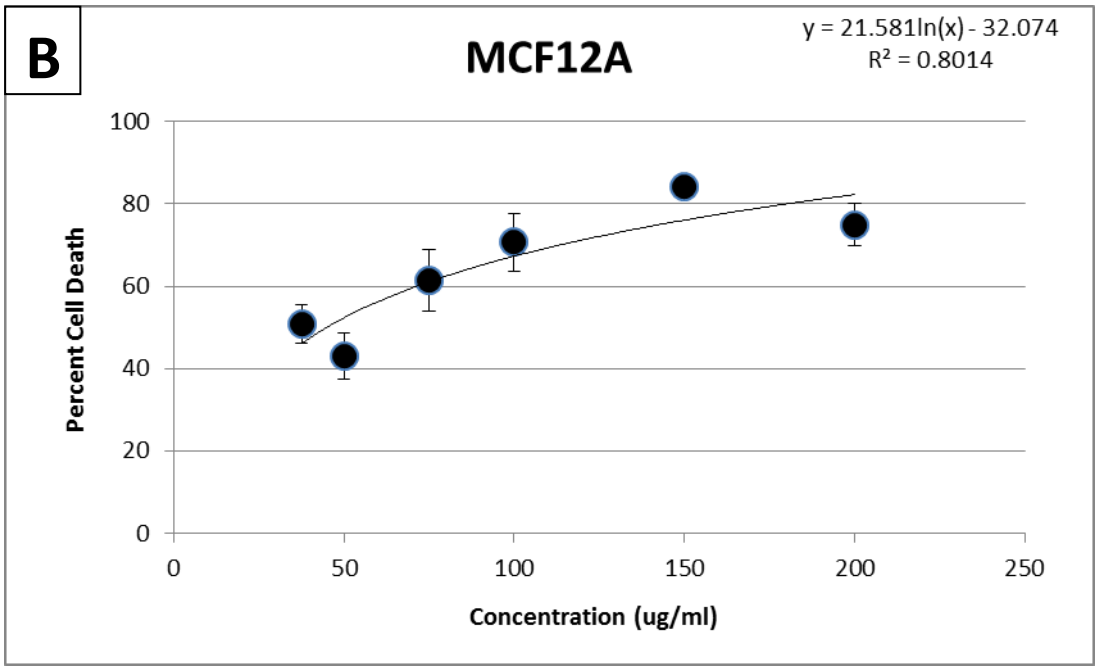


Figure 4-6: Calculation of IC_{50} values for propolis extracts in MCF12A cells. Each point shows the percent cell death at different concentrations of Propolis extracts. **A**, The IC_{50} value for Propolis-1 is 35 ug/ml and R^2 value is R^2=0.8174. **B**, The IC_{50} value for Propolis-2 is 45 ug/ml and R^2 value is R^2=0.8014. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for MDA-MB-231 Cell line

Analysis of IC_{50} value of propolis extracts on MDA-MB-231 was performed with following conditions:

Propolis-1: 100-75-50-25-12.5-6 ug/ml, 4000 cells were used in each well. MDA-MB-231 cell line's IC_{50} value was calculated as 26 ug/ml (**Figure 4-6**, A). The experiment was performed in quadruplicate.

Propolis-2: 150-120-75-60-37.5-30 ug/ml, 7000 cells were used in each well. MDA-MB-231 cell line's IC_{50} value was calculated as 74 ug/ml (Figure 4-6, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.5 for Propolis-1 and Appendix Table 8.6 for Propolis-2.

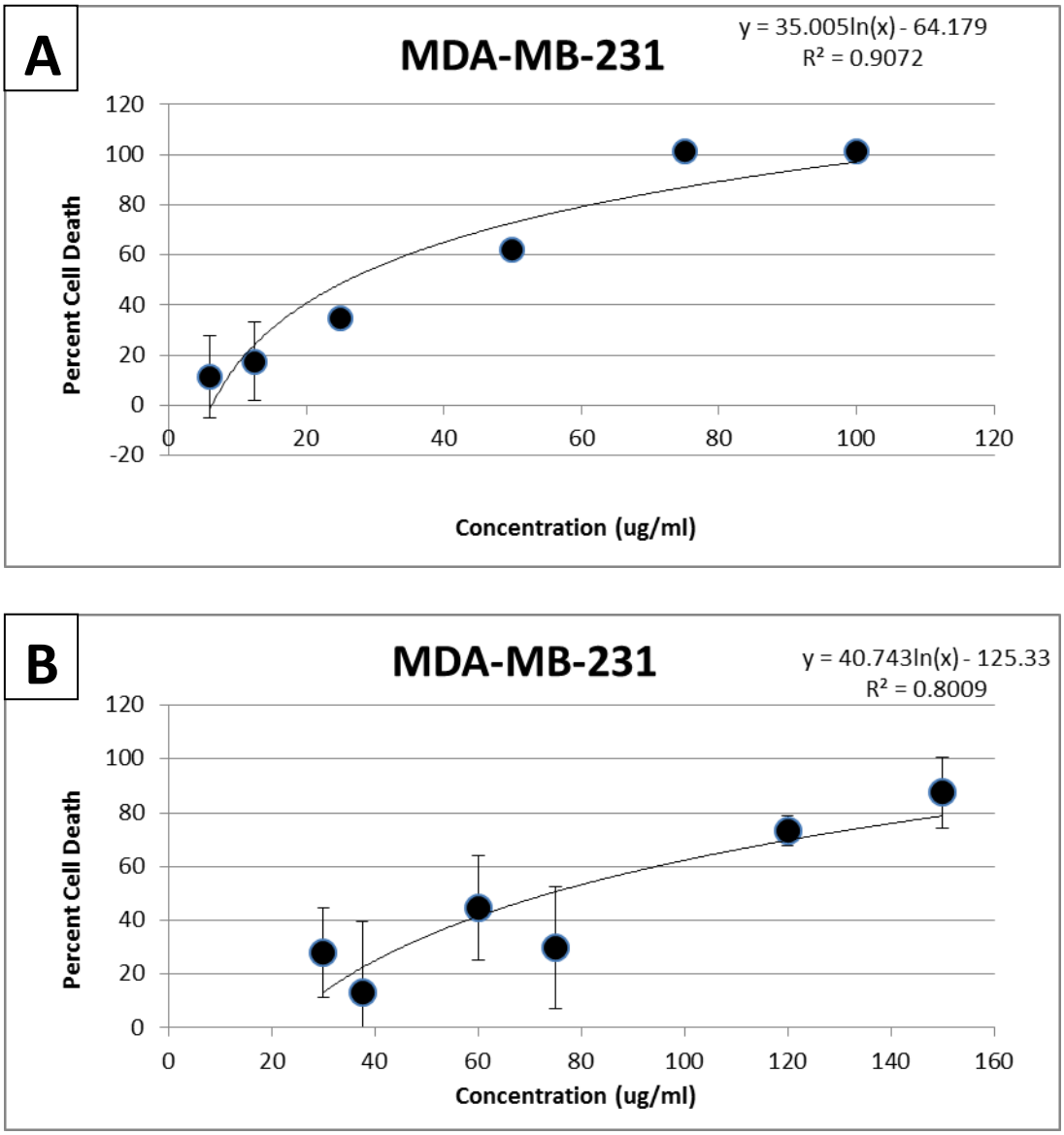


Figure 4-7 Calculation of IC_{50} values for propolis extracts in MDA-MB-231 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC_{50} value for Propolis-1 is 26 ug/ml and R^2 value is R^2 =0.9072. B, The IC_{50} value for Propolis-2 is 74 ug/ml and R^2 value is R^2 =0.8009. Error bars represent standard error of the mean (SEM).

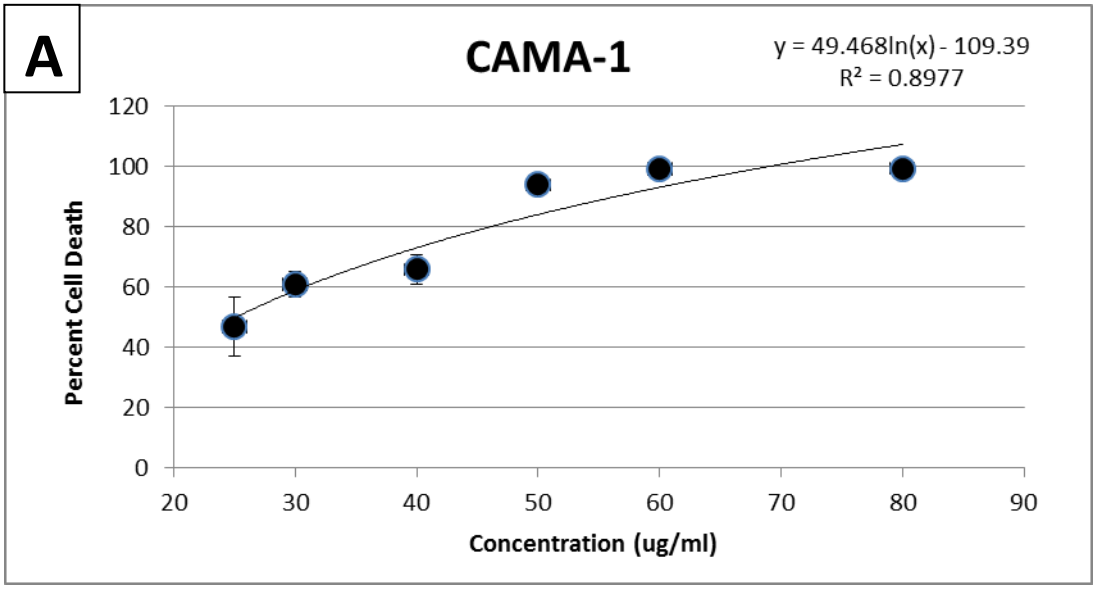
IC_{50} Values of Propolis extracts for CAMA-1 Cell line

Analysis of IC_{50} value of propolis extracts on CAMA-1 was performed with following conditions:

Propolis-1: 80-60-50-40-30-25 ug/ml, 4000 cells were used in each well. CAMA-1 cell line's IC_{50} value was calculated as 25 ug/ml (Figure 4-8, A). The experiment was performed in quadruplicate.

Propolis-2: 100-80-50-40-25-20 ug/ml, 4000 cells were used in each well. CAMA-1 cell line's IC_{50} value was calculated as 35 ug/ml (Figure 4-8, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.7 for Propolis-1 and Appendix Table 8.8 for Propolis-2.



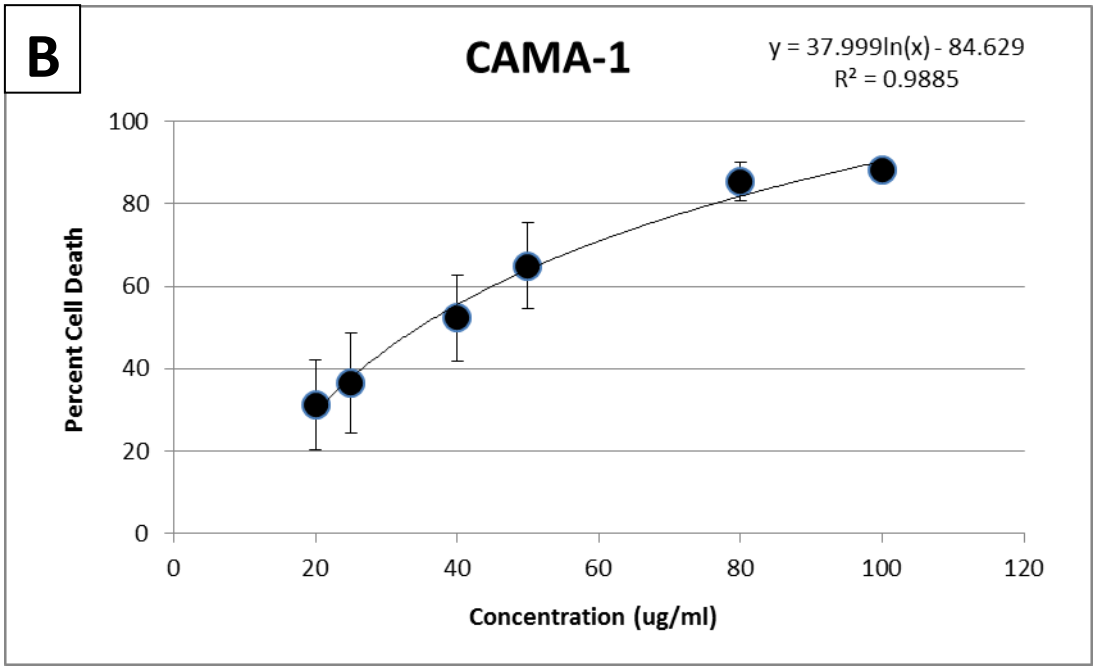


Figure 4-8: Calculation of IC50 values for propolis extracts in CAMA-1 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 25 ug/ml and R^2 value is R^2=0. 8977. B, The IC50 value for Propolis-2 is 35 ug/ml and R^2 value is R^2=0. 9885. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for MDA-MB-453 Cell line

Analysis of IC_{50} value of propolis extracts on MDA-MB-453 was performed with following conditions:

Propolis-1: 80-60-40-30-20 ug/ml, 4000 cells were used in each well. MDA-MB-453 cell line's IC_{50} value was calculated as 47 ug/ml (Figure 4-9, A). The experiment was performed in triplicate.

Propolis-2: 120-100-60-50-30 ug/ml, 8000 cells were used in each well. MDA-MB-453 cell line's IC_{50} value was calculated as 53 ug/ml (Figure 4-9, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.9 for Propolis-1 and Appendix Table 8.10 for Propolis-2.

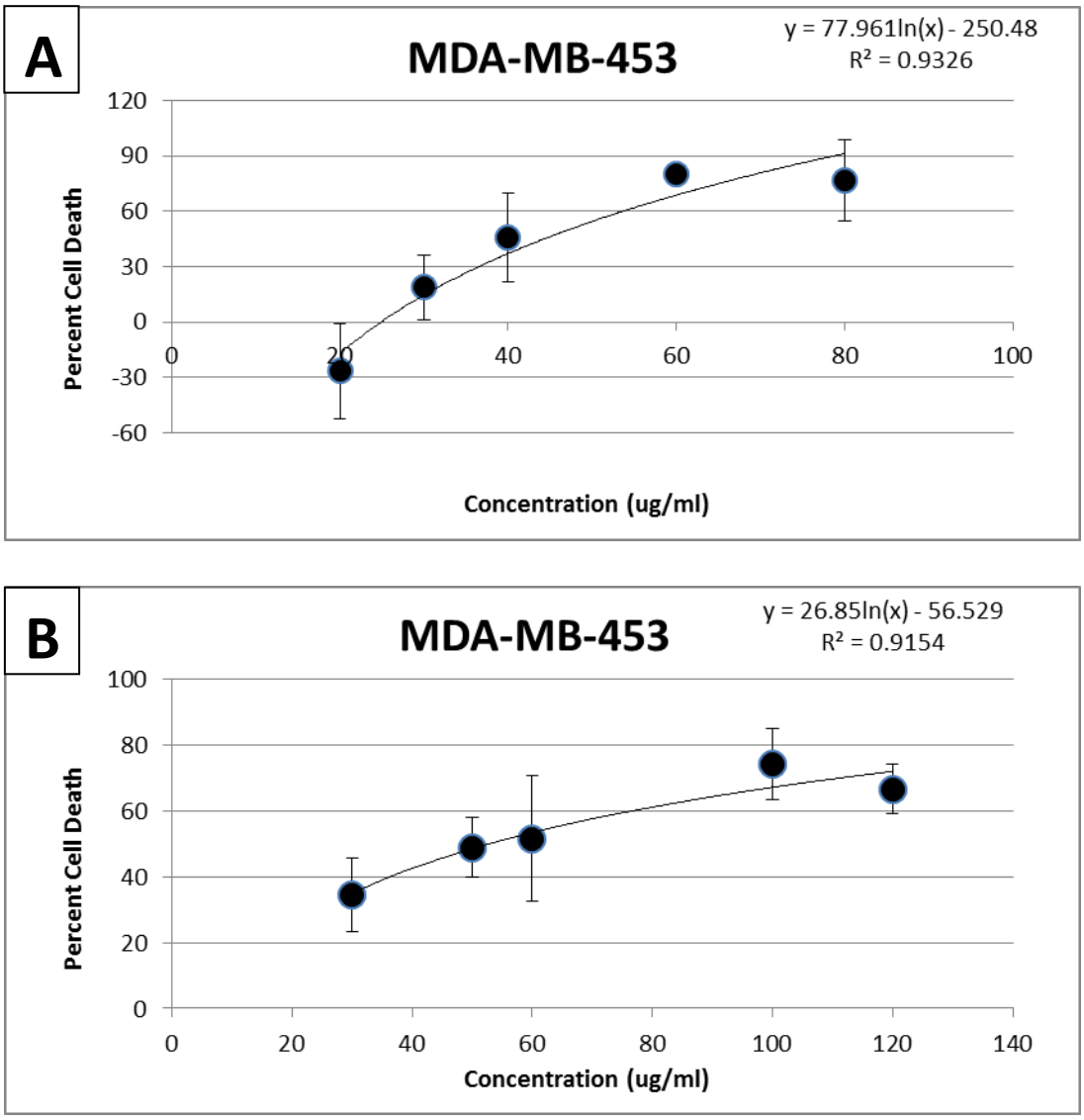


Figure 4-9: Calculation of IC50 values for propolis extracts in MDA-MB-453 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 47 ug/ml and R^2 value is R^2=0. 9326. B, The IC50 value for Propolis-2 is 53 ug/ml and R^2 value is R^2=0. 9154. Error bars represent standard error of the mean (SEM).

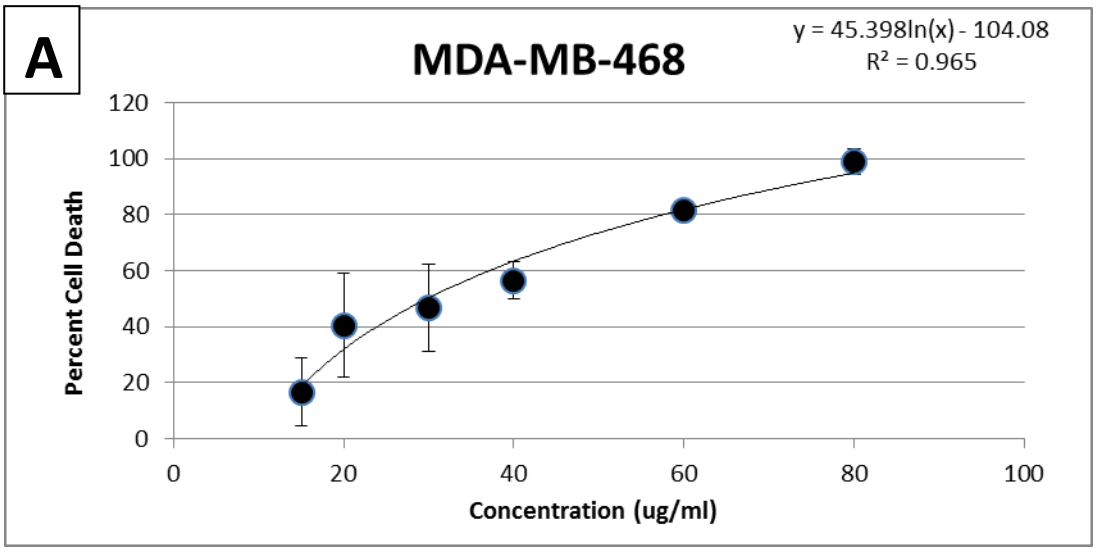
IC_{50} Values of Propolis extracts for MDA-MB-468 Cell line

Analysis of IC_{50} value of propolis extracts on MDA-MB-468 was performed with following conditions:

Propolis-1: 80-60-40-30-20-15 ug/ml, 4000 cells were used in each well. MDA-MB-468 cell line's IC_{50} value was calculated as 30 ug/ml (Figure 4-10, A). The experiment was performed in quadruplicate.

Propolis-2: 120-100-60-50-30 -25 ug/ml, 4000 cells were used in each well. MDA-MB-468 cell line's IC_{50} value was calculated as 26 ug/ml (Figure 4-10, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.11 for Propolis-1 and Appendix Table 8.12for Propolis-2.



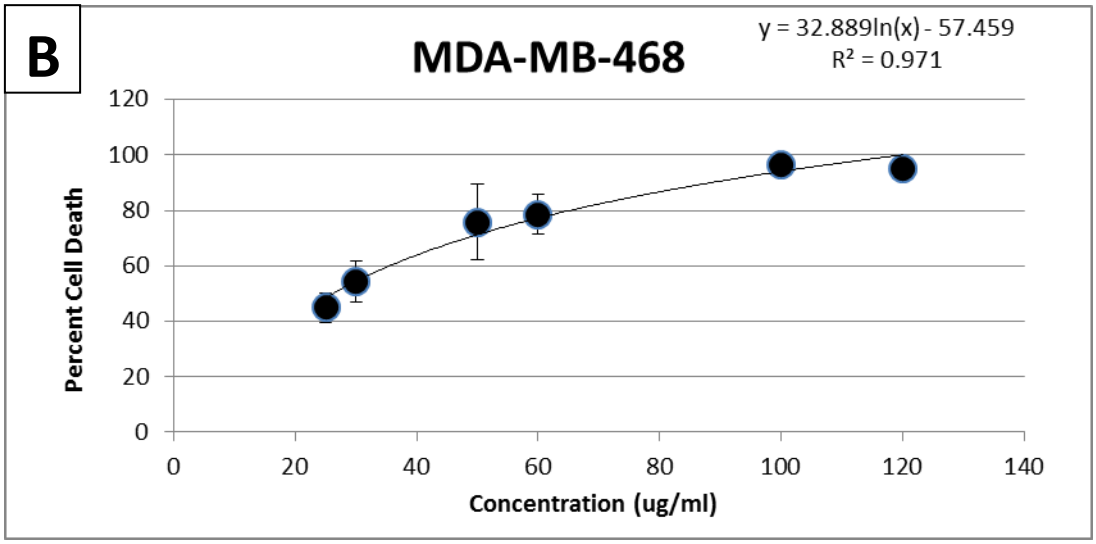


Figure 4-10: Calculation of IC50 values for propolis extracts in MDA-MB-468 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 30 ug/ml and R^2 value is R^2=0. 965. B, The IC50 value for Propolis-2 is 26 ug/ml and R^2 value is R^2=0. 971. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for T47D Cell line

Analysis of IC_{50} value of propolis extracts on T47D was performed with following conditions:

Propolis-1: 80-60-50-40-30-25 ug/ml, 5000 cells were used in each well. T47D cell line's IC_{50} value was calculated as 36 ug/ml (Figure 4-11, A). The experiment was performed in quadruplicate.

Propolis-2: 100-80-50-40-25-20 ug/ml, 4000 cells were used in each well. T47D cell line's IC_{50} value was calculated as 43 ug/ml (Figure 4-11, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.13 for Propolis-1 and Appendix Table 8.14 for Propolis-2.

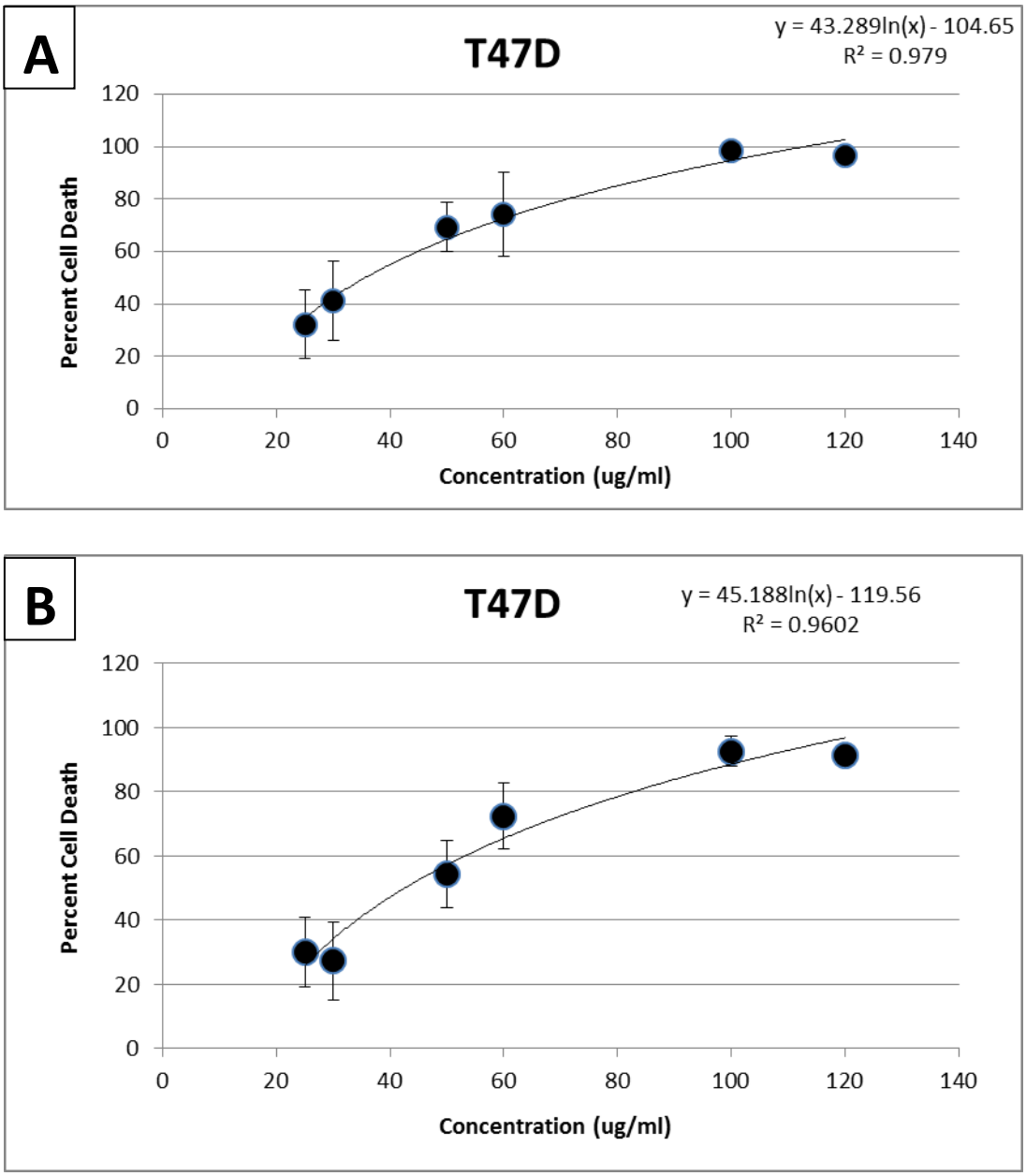


Figure 4-11: Calculation of IC50 values for propolis extracts in T47D cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 36 ug/ml and R^2 value is R^2=0. 979. B, The IC50 value for Propolis-2 is 43 ug/ml and R^2 value is R^2=0. 9602. Error bars represent standard error of the mean (SEM).

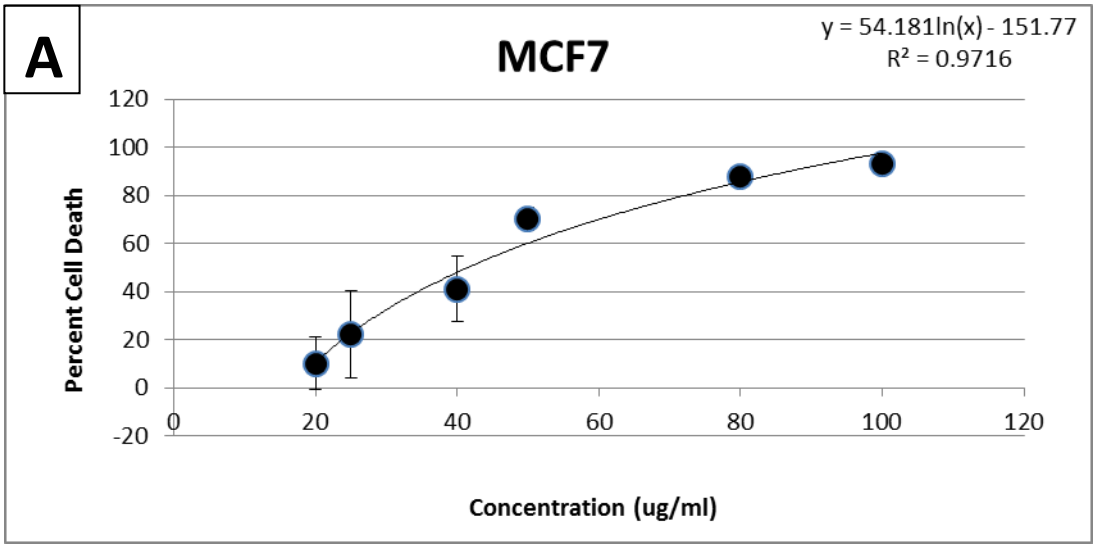
IC_{50} Values of Propolis extracts for MCF7 Cell line

Analysis of IC_{50} value of propolis extracts on MCF7 was performed with following conditions:

Propolis-1: 100-80-50-40-25-20 ug/ml, 3000 cells were used in each well. MCF7 cell line's IC_{50} value was calculated as 41 ug/ml (Figure 4-12, A). The experiment was performed in quadruplicate.

Propolis-2: 120-100-60-50-30 -25 ug/ml, 3000 cells were used in each well. MCF7 cell line's IC_{50} value was calculated as 61 ug/ml (Figure 4-12, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.15 for Propolis-1 and Appendix Table 8.16 for Propolis-2.



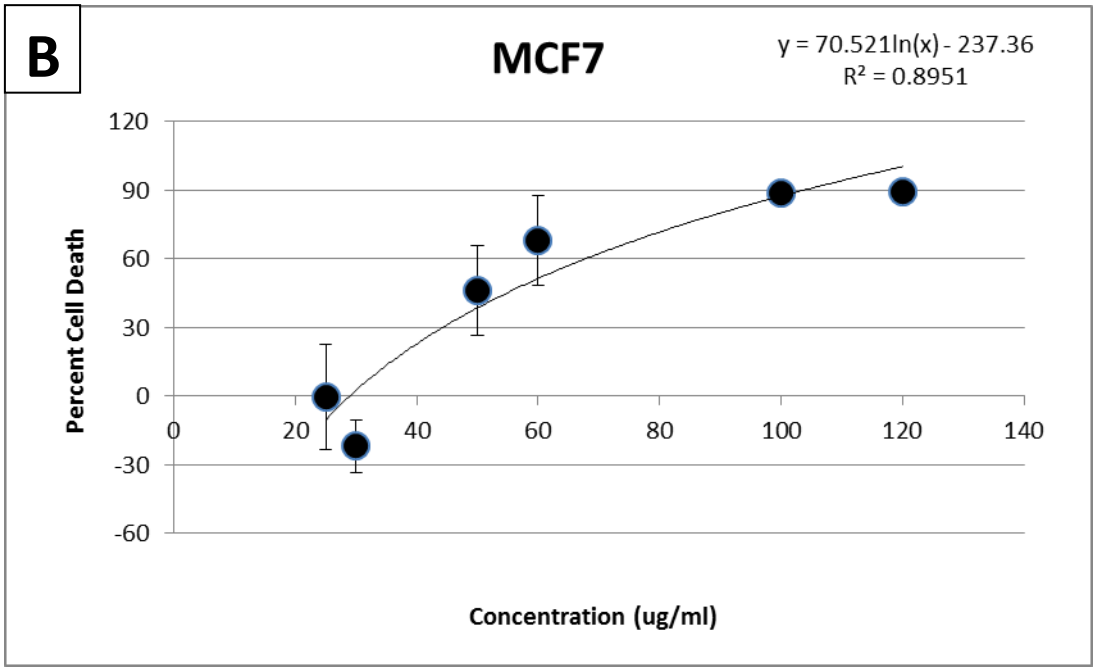


Figure 4-12: Calculation of IC50 values for propolis extracts in MCF7 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 41 ug/ml and R^2 value is R^2=0. 9716. B, The IC50 value for Propolis-2 is 61 ug/ml and R^2 value is R^2=0. 8951. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for HCC-1937 Cell line

Analysis of IC_{50} value of propolis extracts on HCC-1937 was performed with following conditions:

Propolis-1: 150-120-75-60-37.5-30 ug/ml, 10000 cells were used in each well. HCC-1937 cell line's IC_{50} value was calculated as 123 ug/ml (Figure 4-13, A). The experiment was performed in quadruplicate.

Propolis-2: 150-120-75-60-30 ug/ml, 8000 cells were used in each well. HCC-1937 cell line's IC_{50} value was calculated as 119 ug/ml (Figure 4-13, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.17 for Propolis-1 and Appendix Table 8.18 for Propolis-2.

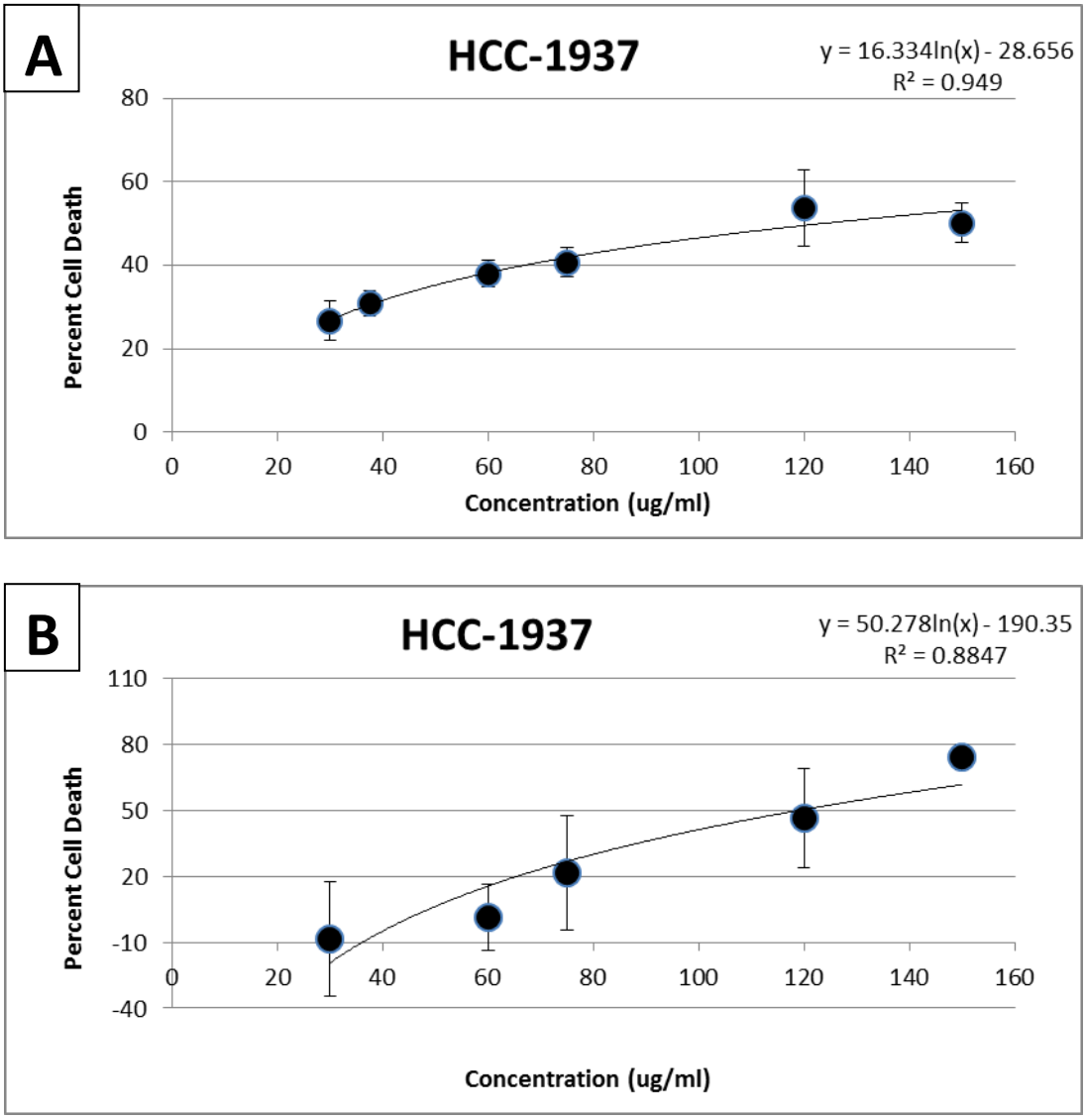


Figure 4-13: Calculation of IC50 values for propolis extracts in HCC-1937 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 123 ug/ml and R^2 value is R^2=0. 949. B, The IC50 value for Propolis-2 is 119 ug/ml and R^2 value is R^2=0. 8847. Error bars represent standard error of the mean (SEM).

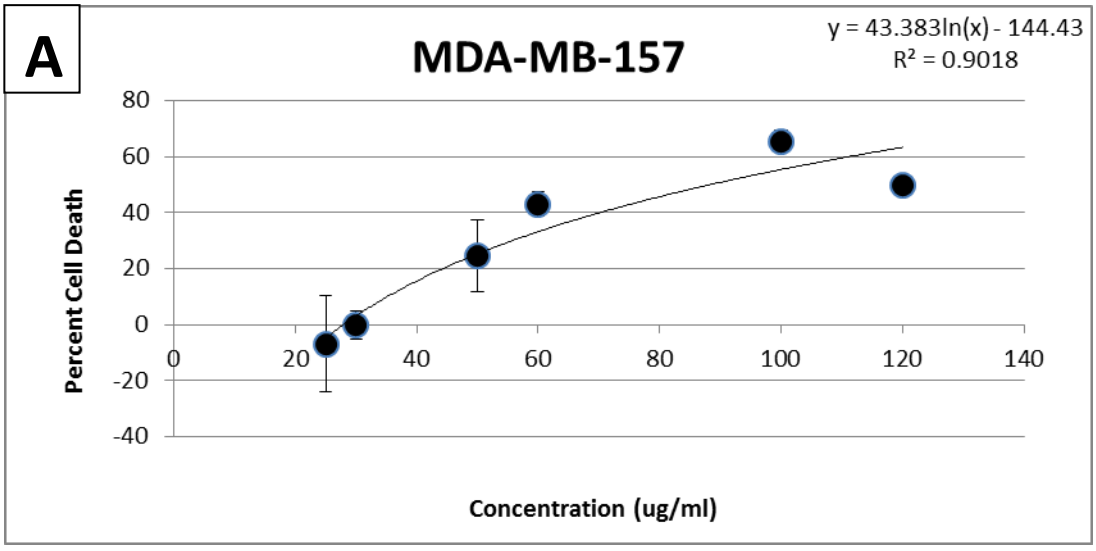
IC_{50} Values of Propolis extracts for MDA-MB-157 Cell line

Analysis of IC_{50} value of propolis extracts on MDA-MB-157 was performed with following conditions:

Propolis-1: 120-100-60-50-30 -25 ug/ml, 10000 cells were used in each well. MDA-MB-157cell line's IC_{50} value was calculated as 88 ug/ml (Figure 4-14, A). The experiment was performed in quadruplicate.

Propolis-2: 150-120-75-60-37.5-30 ug/ml, 10000 cells were used in each well. MDA-MB-157cell line's IC_{50} value was calculated as 61 ug/ml (Figure 4-14, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.19 for Propolis-1 and Appendix Table 8.20 for Propolis-2.



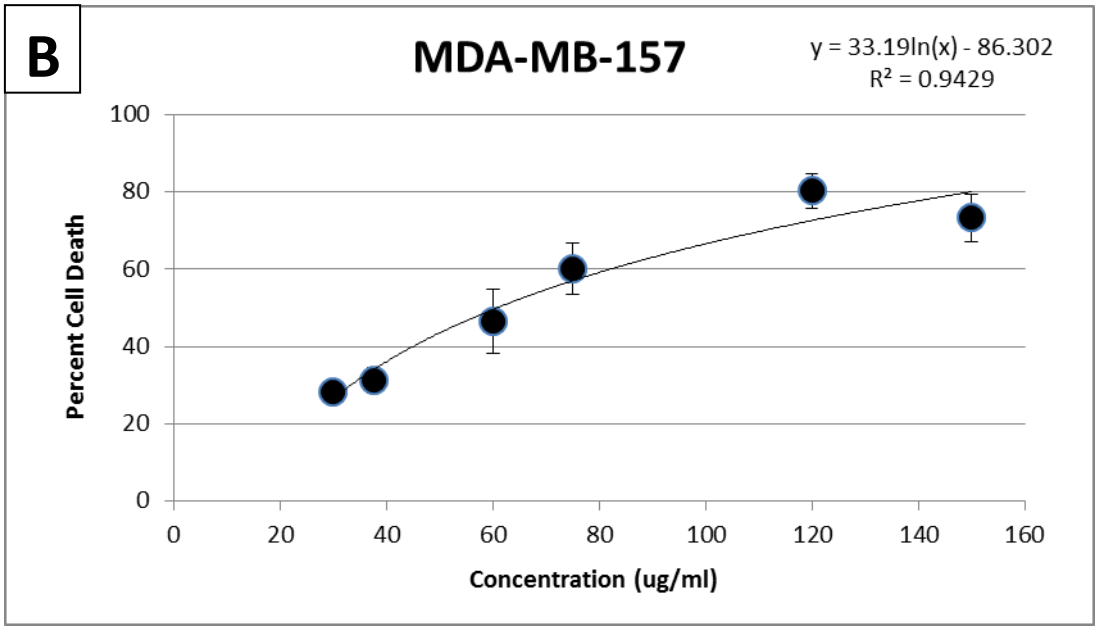


Figure 4-14: Calculation of IC50 values for propolis extracts in MDA-MB-157cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 88 ug/ml and R^2 value is R^2=0. 9018. B, The IC50 value for Propolis-2 is 61 ug/ml and R^2 value is R^2=0. 9429. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for BT-20 Cell line

Analysis of IC_{50} value of propolis extracts on BT-20 was performed with following conditions:

Propolis-1: 150-120-75-60-37.5-30 ug/ml, 12000 cells were used in each well. BT-20 cell line's IC_{50} value was calculated as 73 ug/ml (Figure 4-15, A). The experiment was performed in quadruplicate.

Propolis-2: 120-100-60-50-30 ug/ml, 12000 cells were used in each well. BT-20 cell line's IC_{50} value was calculated as 117 ug/ml (Figure 4-15, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.21 for Propolis-1 and Appendix Table 8.22 for Propolis-2.

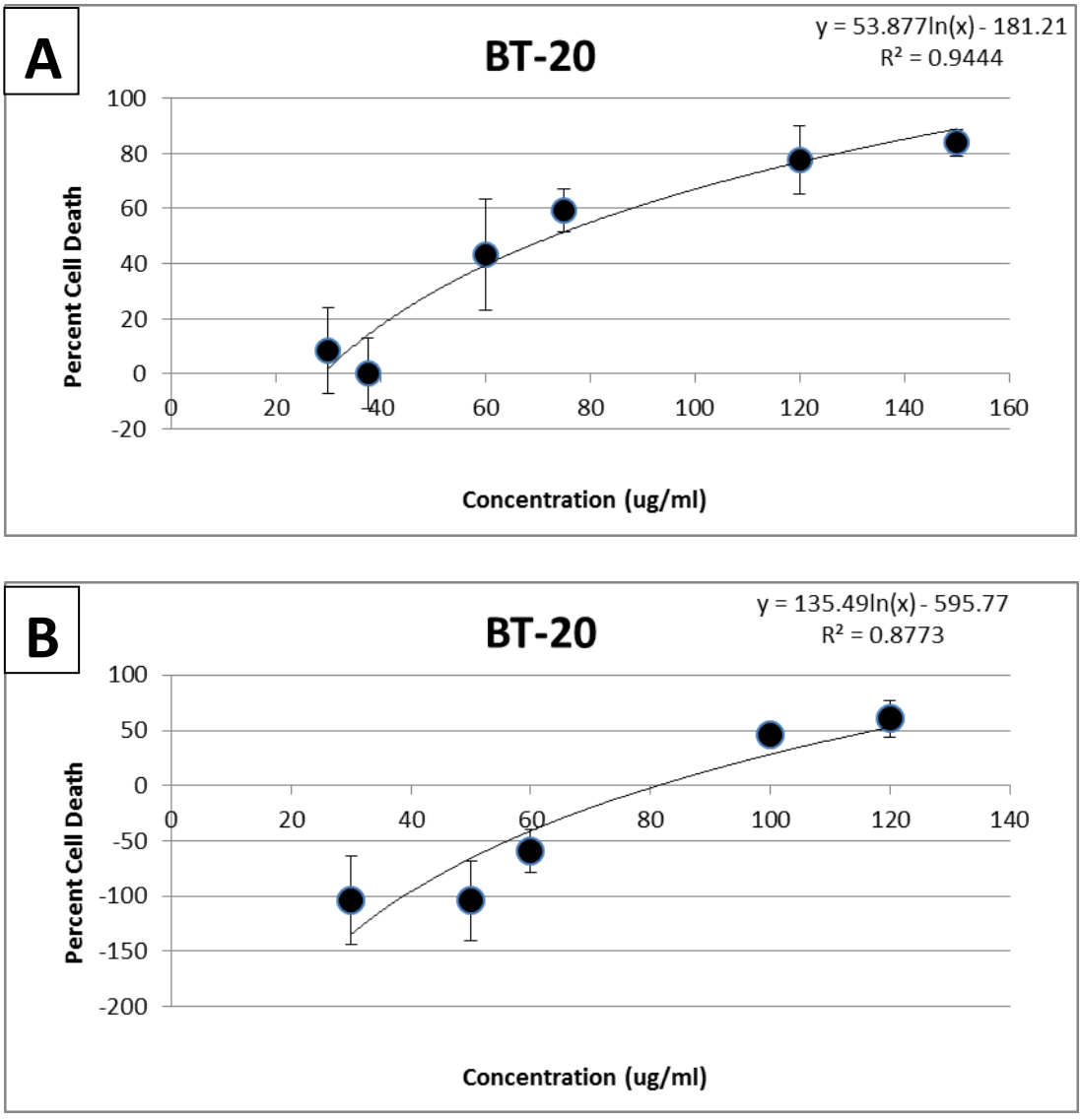


Figure 4-15: Calculation of IC50 values for propolis extracts in BT-20 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 73 ug/ml and R^2 value is R^2=0. 9444. B, The IC50 value for Propolis-2 is 117 ug/ml and R^2 value is R^2=0. 8773. Error bars represent standard error of the mean (SEM).

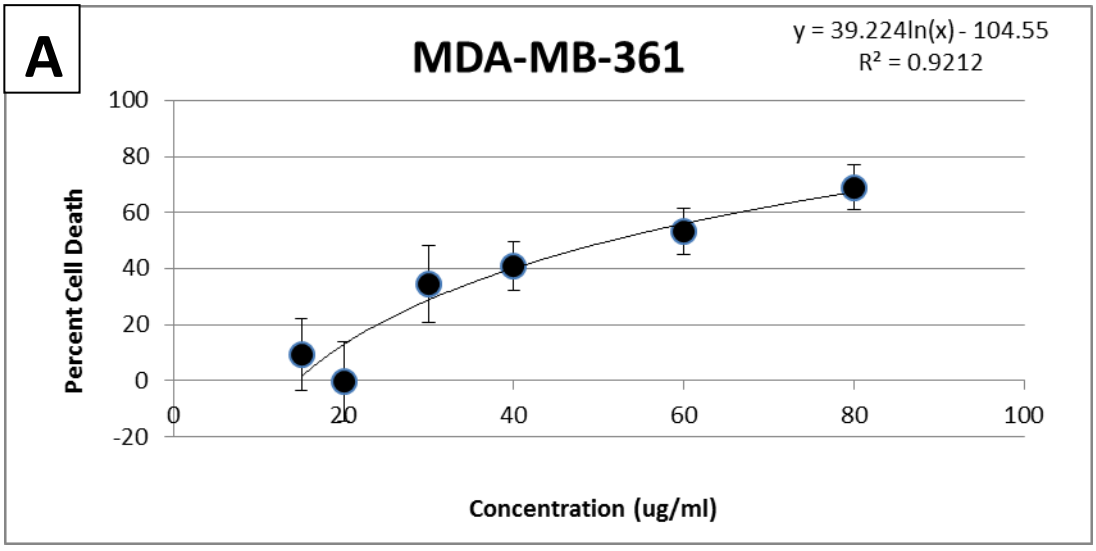
IC_{50} Values of Propolis extracts for MDA-MB-361 Cell line

Analysis of IC_{50} value of propolis extracts on MDA-MB-361 was performed with following conditions:

Propolis-1: 80-60-40-30-20-15 ug/ml, 20000 cells were used in each well. MDA-MB-361 cell line's IC_{50} value was calculated as 51 ug/ml (Figure 4-16, A). The experiment was performed in quadruplicate.

Propolis-2: 150-120-75-60-37.5-30 ug/ml, 20000 cells were used in each well. MDA-MB-361 cell line's IC_{50} value was calculated as 57 ug/ml (Figure 4-16, B). The experiment was performed in quadruplicate.

The original data for ELISA reading results are given in Appendix Table 8.23 for Propolis-1 and Appendix Table 8.24 for Propolis-2.



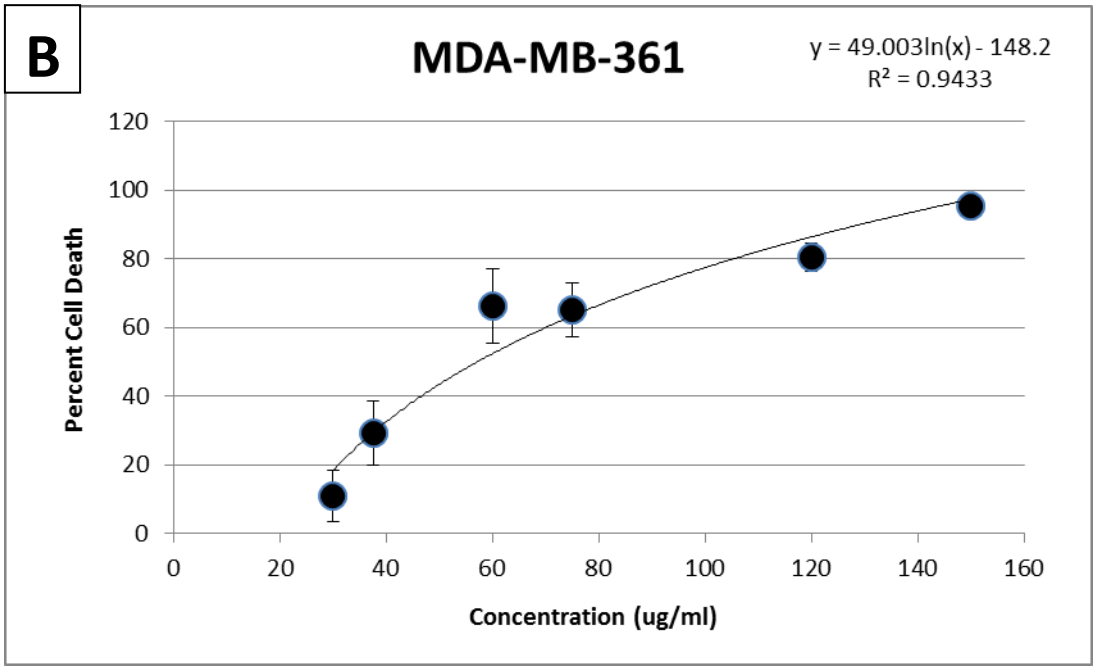


Figure 4-16: Calculation of IC50 values for propolis extracts in MDA-MB-361 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 51 ug/ml and R^2 value is R^2=0. 9212. B, The IC50 value for Propolis-2 is 57 ug/ml and R^2 value is R^2=0. 9433. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for BT-474 Cell line

Analysis of IC_{50} value of propolis extracts on BT-474 was performed with following conditions:

Propolis-1: 150-120-75-60-37.5-30 ug/ml, 20000 cells were used in each well. BT-474 cell line's IC_{50} value was calculated as 50 ug/ml (Figure 4-17, A). The experiment was performed in triplicate.

Propolis-2: 150-120-75-60-37.5-30 ug/ml, 10000 cells were used in each well. BT-474 cell line's IC_{50} value was calculated as 92 ug/ml (Figure 4-17, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.25 for Propolis-1 and Appendix Table 8.26 for Propolis-2.

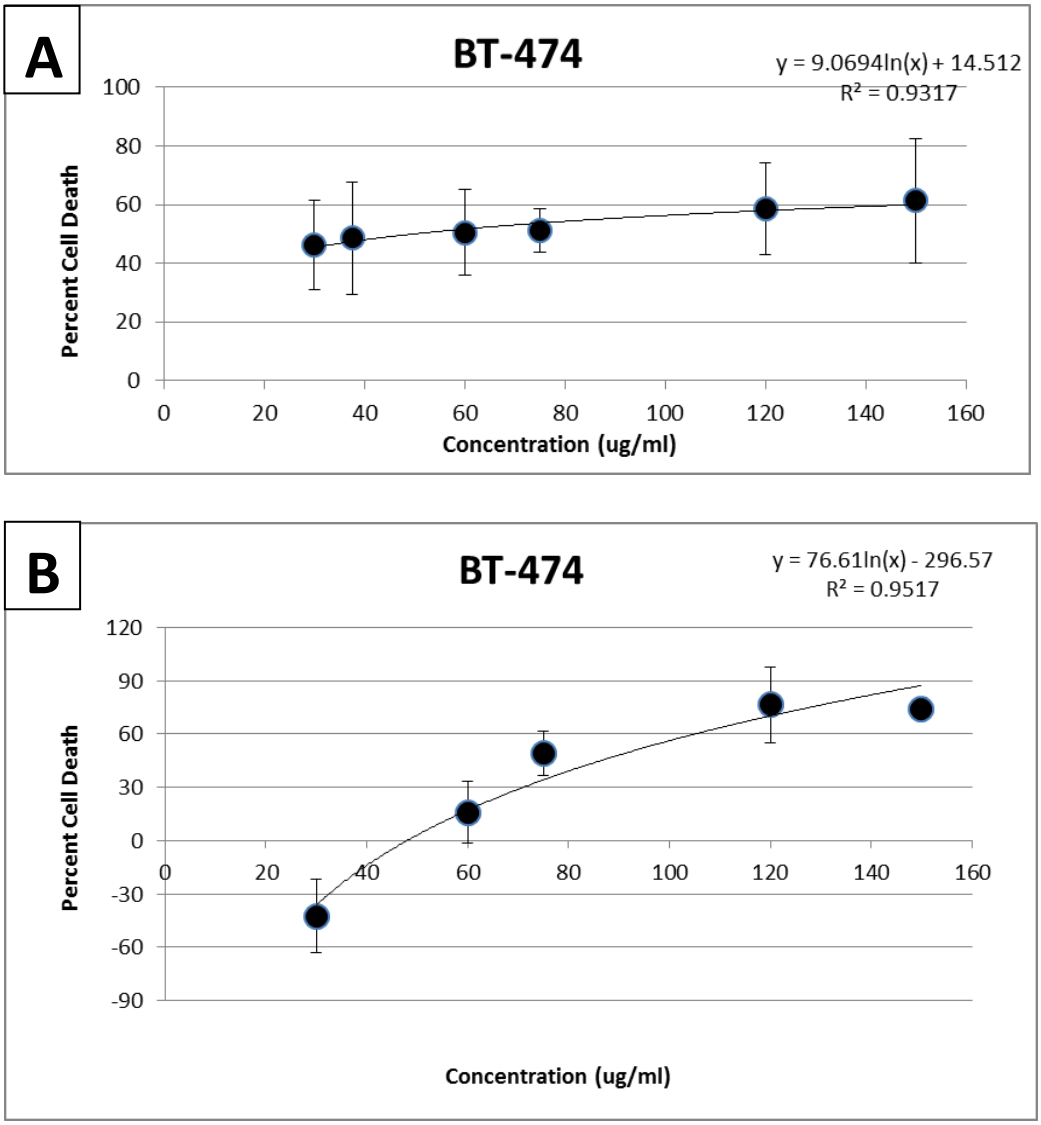


Figure 4-17: Calculation of IC50 values for propolis extracts in BT-474 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 50 ug/ml and R^2 value is R^2=0. 9317. B, The IC50 value for Propolis-2 is 92 ug/ml and R^2 value is R^2=0. 9517. Error bars represent standard error of the mean (SEM).

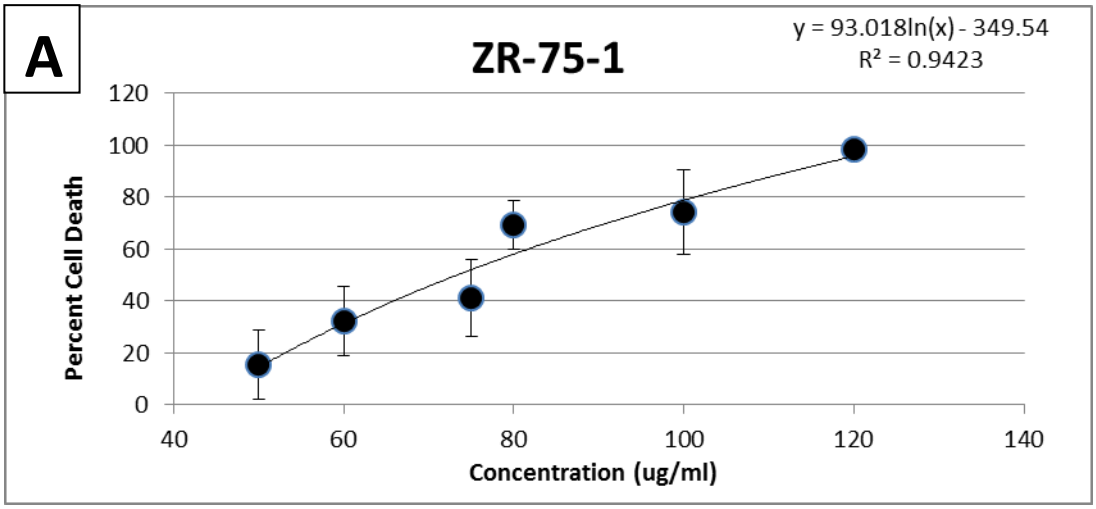
IC_{50} Values of Propolis extracts for ZR-75-1 Cell line

Analysis of IC_{50} value of propolis extracts on ZR-75-1 was performed with following conditions:

Propolis-1: 120-100-80-75-60-50 ug/ml, 5000 cells were used in each well. ZR-75-1 cell line's IC_{50} value was calculated as 73 ug/ml (Figure 4-18, A). The experiment was performed in quadruplicate.

Propolis-2: 120-100-60-50-30 ug/ml, 6000 cells were used in each well. ZR-75-1 cell line's IC_{50} value was calculated as 76 ug/ml (Figure 4-18, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.27 for Propolis-1 and Appendix Table 8.28 for Propolis-2.



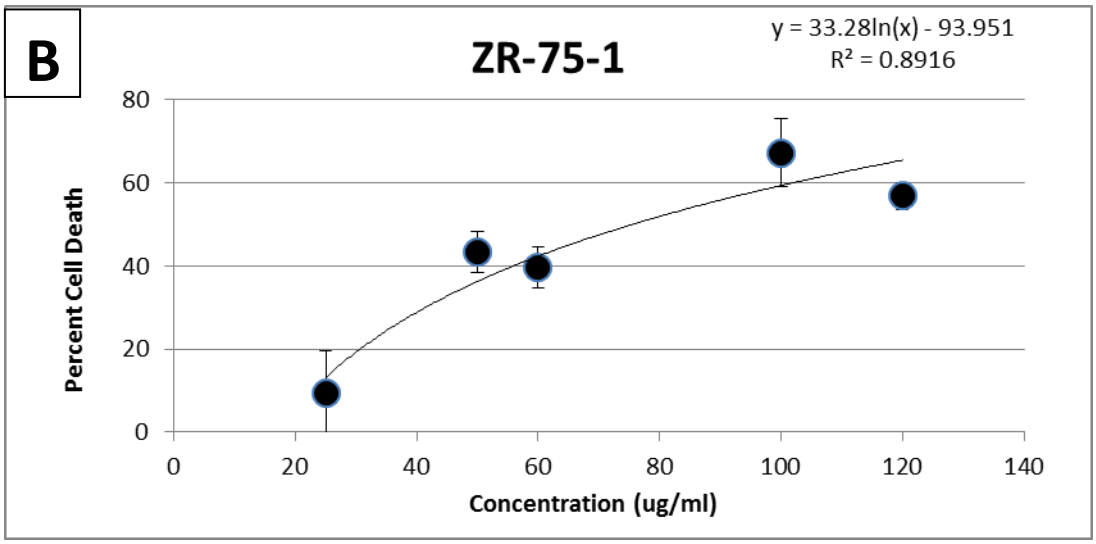


Figure 4-18: Calculation of IC50 values for propolis extracts in ZR-75-1 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 73 ug/ml and R^2 value is R^2=0. 9423. B, The IC50 value for Propolis-2 is 76 ug/ml and R^2 value is R^2=0. 8916. Error bars represent standard error of the mean (SEM).

IC_{50} Values of Propolis extracts for hTERT-HME-1 Cell line

Analysis of IC_{50} value of propolis extracts on hTERT-HME-1 was performed with following conditions:

Propolis-1: 120-100-60-50-30 ug/ml, 6000 cells were used in each well. hTERT-HME-1 cell line's IC_{50} value was calculated as 41 ug/ml (Figure 4-19, A). The experiment was performed in quadruplicate.

Propolis-2: 150-120-75-60-37.5 ug/ml, 6000 cells were used in each well. hTERT-HME-1 cell line's IC_{50} value was calculated as 114 ug/ml (Figure 4-19, B). The experiment was performed in triplicate.

The original data for ELISA reading results are given in Appendix Table 8.29 for Propolis-1 and Appendix Table 8.30 for Propolis-2.

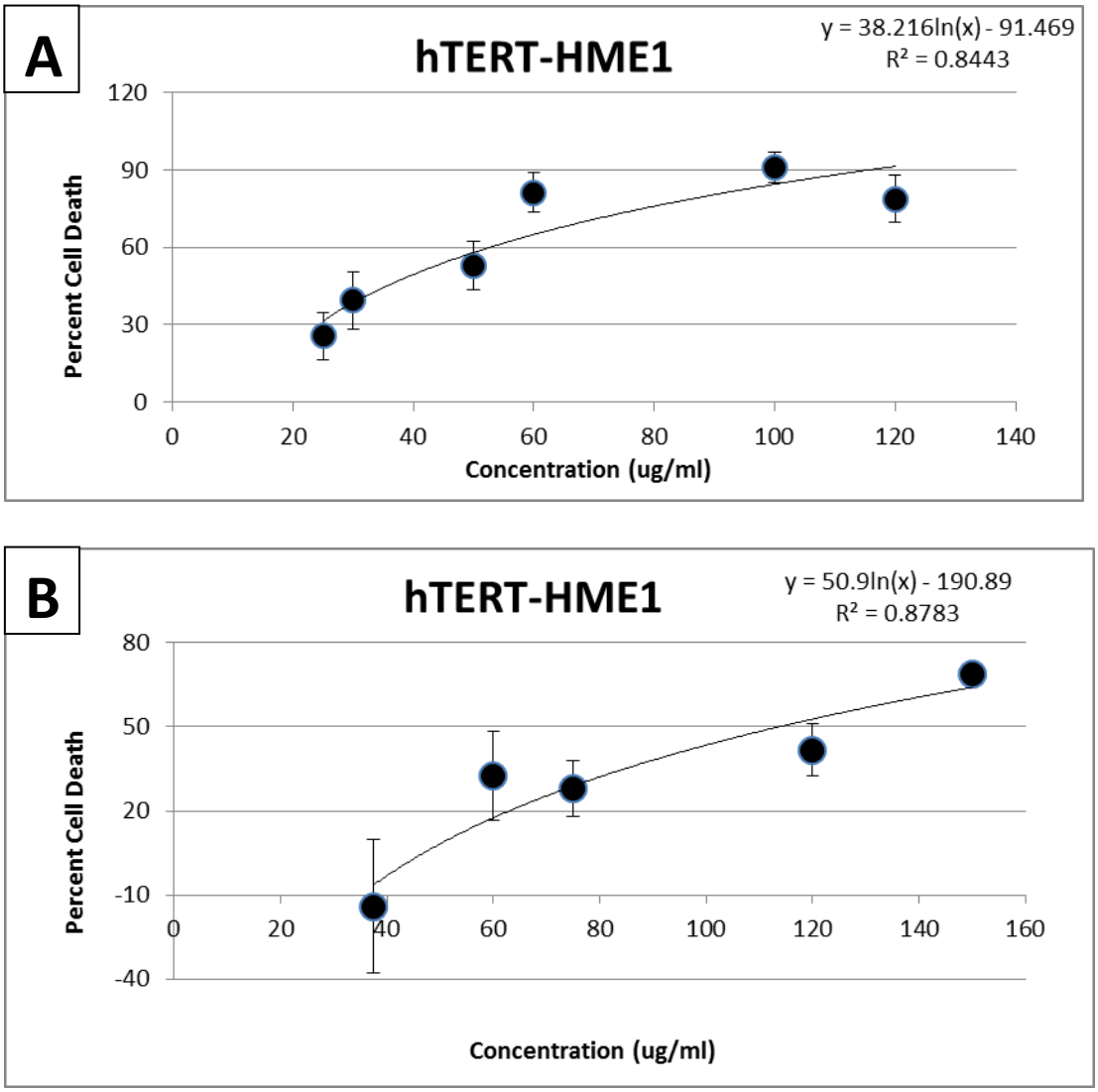


Figure 4-19: Calculation of IC50 values for propolis extracts in hTERT-HME-1 cells. Each point shows the percent cell death at different concentrations of Propolis extracts. A, The IC50 value for Propolis-1 is 41 ug/ml and R^2 value is R^2=0. 8443. B, The IC50 value for Propolis-2 is 114 ug/ml and R^2 value is R^2=0. 8783. Error bars represent standard error of the mean (SEM).

4.3 Association of IC_{50} Values of Propolis extracts with Properties of Breast Carcinoma Cell Lines

Previously calculated IC_{50} values of propolis extracts for breast carcinoma cell lines were classified according to cell lines' molecular properties (Table 4.5). Statistical analysis was performed to analyze whether there is any correlation between IC_{50} values of propolis and the molecular status of cell line with Microsoft Excel program by using T.TEST function. Student's t-test was calculated with two-tailed distribution and two-sample unequal variance. Fold change of propolis extracts was calculated by dividing IC_{50} values of Propolis-2 extract to IC_{50} values of Propolis-1 extract(Figure 4-20 and Figure 4-21).

Table 4.5 Classification of Breast carcinoma cell lines According to Their Molecular Status with IC_{50} Values of Propolis Extracts (Neve, 2006; Kao, 2009; Finn, 2009; Holliday, 2011).

Cell Lines	IC_{50} (ug/ml)		Molecular Status of Cell Lines			
	Propolis-1	Propolis-2	Subtype	Estrogen Receptor (ER)	Progesterone Receptor (PR)	HER2
BT-20	73	117	basal A	negative	negative	normal
BT-474	50	92	luminal B	positive	positive	amplified
CAMA-1	25	35	luminal	positive	negative	normal
HCC-1937	123	119	basal A	negative	negative	normal
hTERT-HME1	41	114	basal B	negative	n/a	negative
MCF10A	45	72	basal B	negative	negative	immortalized
MCF12A	35	45	basal B	negative	negative	n/a
MCF7	41	61	luminal A	positive	positive	normal
MDA-MB 231	26	74	basal B	negative	negative	normal
MDA-MB-157	88	61	basal B	negative	negative	normal
MDA-MB-361	51	57	luminal	positive	positive	amplified
MDA-MB-453	47	53	luminal	negative	negative	amplified
MDA-MB-468	30	26	basal A	negative	negative	normal
T47D	36	43	luminal A	positive	positive	normal
ZR-75-1	73	76	luminal B	positive	negative	normal

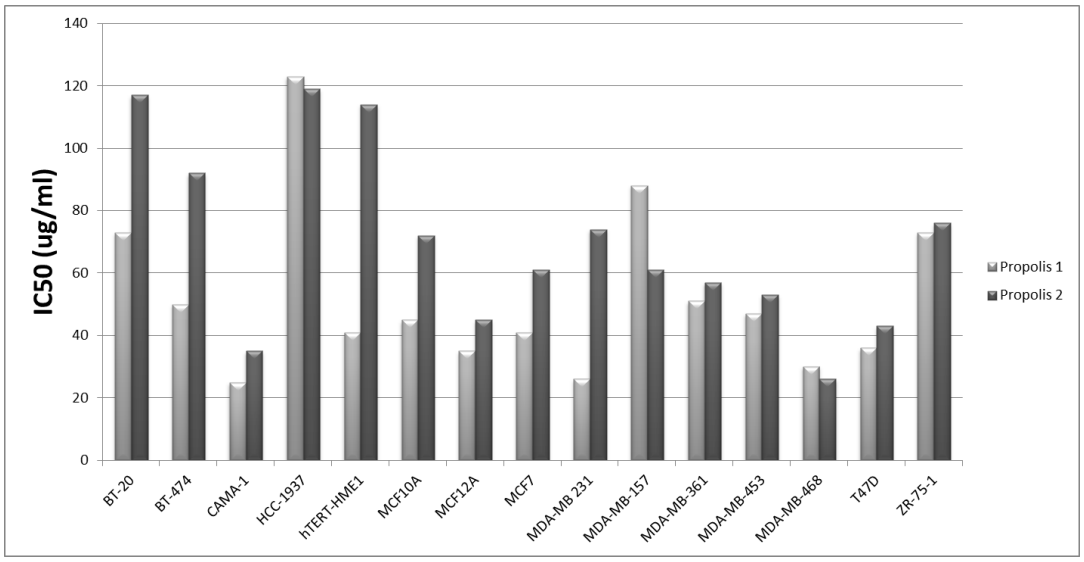


Figure 4-20 Comparison of IC_{50} Values of Two Different Propolis Extracts on Breast carcinoma cell lines

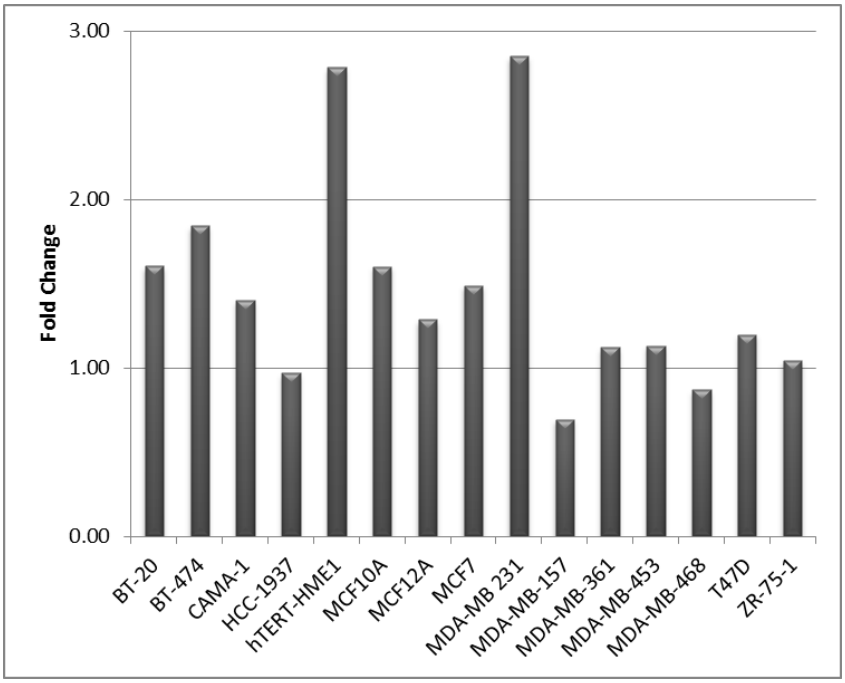


Figure 4-21 Fold change of IC_{50} values of two different propolis extracts on breast carcinoma cell lines (p= 0,0016, IC_{50} values of Propolis-2 are significantly higher than IC_{50} values of Propolis-1).

The result showed that IC_{50} values of Propolis-2 extract were significantly higher than IC_{50} values of Propolis-1 extract among all cell lines. Pearson correlation was also calculated and the resulted correlation was found as significant at the 0.05 level (2-tailed).

The calculated IC_{50} for each cell line was analyzed for the cell lines molecular classification groups. Basal cell line group was compared to luminal cell lines to analyze if there is any significant difference between the subgroups responding to propolis extracts. The results showed that there is no significant correlation between these two groups for any of the two extracts (Propolis-1, p=0.4 and Propolis-2, p=0.2).

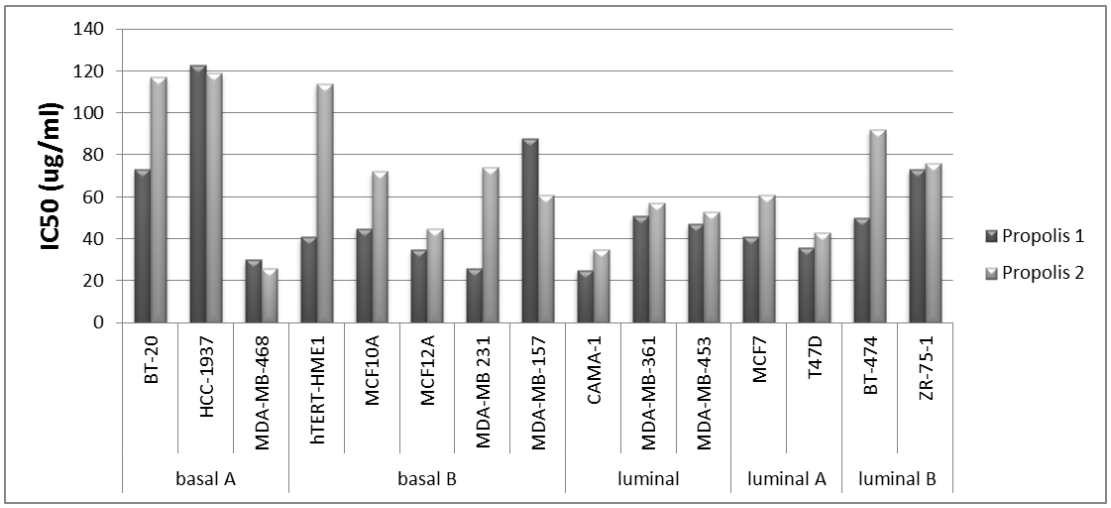


Figure 4-22 IC_{50} values of propolis extracts were used to analyze according to the molecular subtypes of breast carcinoma cell lines. Basal cell line group was compared to luminal cell line group for both Propolis-1 and Propolis-2 (Propolis-1, p=0.4 and Propolis-2, p=0.2).

The calculated IC_{50} for each cell line was analyzed for ER status (positive/negative) of the breast carcinoma cell lines. The results showed that there is no significant correlation between these two groups for any of the two extracts (Propolis-1, p=0.4 and Propolis-2, p=0.3).

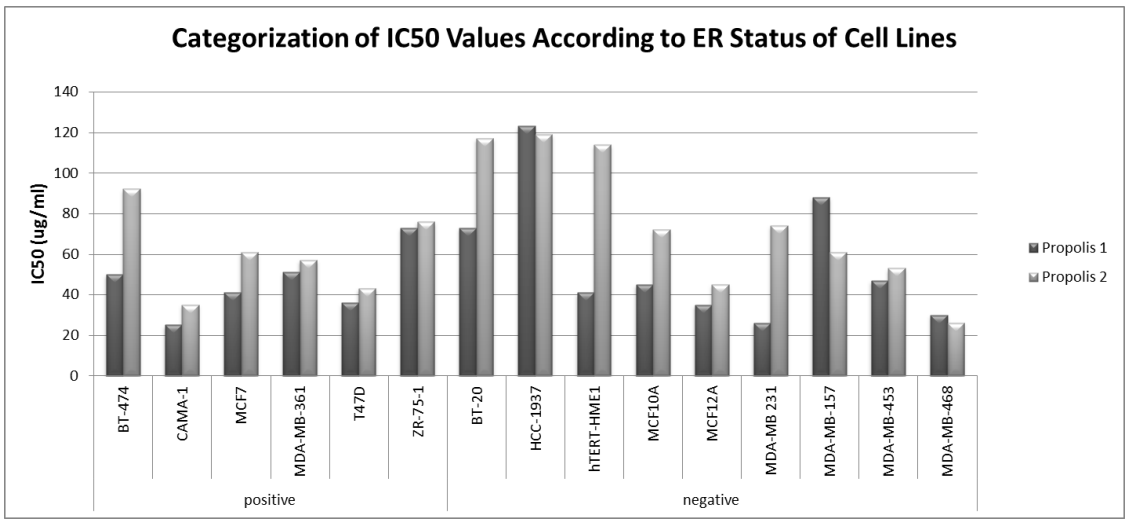


Figure 4-23 IC_{50} values of propolis extracts were used to analyzed according to the ER status of breast carcinoma cell lines. ER positive cell line group was compared to ER negative cell line group for both Propolis-1 and Propolis-2 (Propolis-1, p=0.4 and Propolis-2, p=0.3).

The calculated IC_{50} for each cell line was analyzed for PR status (positive/negative) of the cell lines. The results showed that there is no significant correlation between these two groups for any of the two extracts (Propolis-1, p=0.3 and Propolis-2, p=0.8).

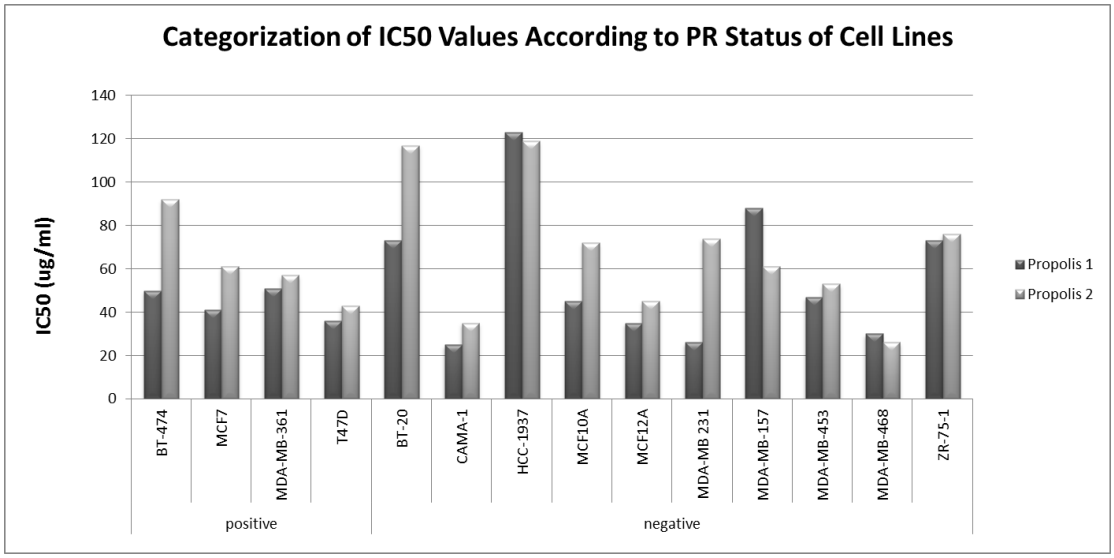


Figure 4-24 IC_{50} values of propolis extracts were used to analyzed according to the PR status of breast carcinoma cell lines. PR positive cell line group was compared to PR negative cell line group for both Propolis-1 and Propolis-2 (Propolis-1, p=0.3 and Propolis-2, p=0.8).

The calculated IC_{50} for each cell line was analyzed for HER2 status (normal/amplified) of the cell lines. The results showed that there is no significant correlation between these two groups for any of the two extracts (Propolis-1, p=0.5 and Propolis-2, p=1.0).

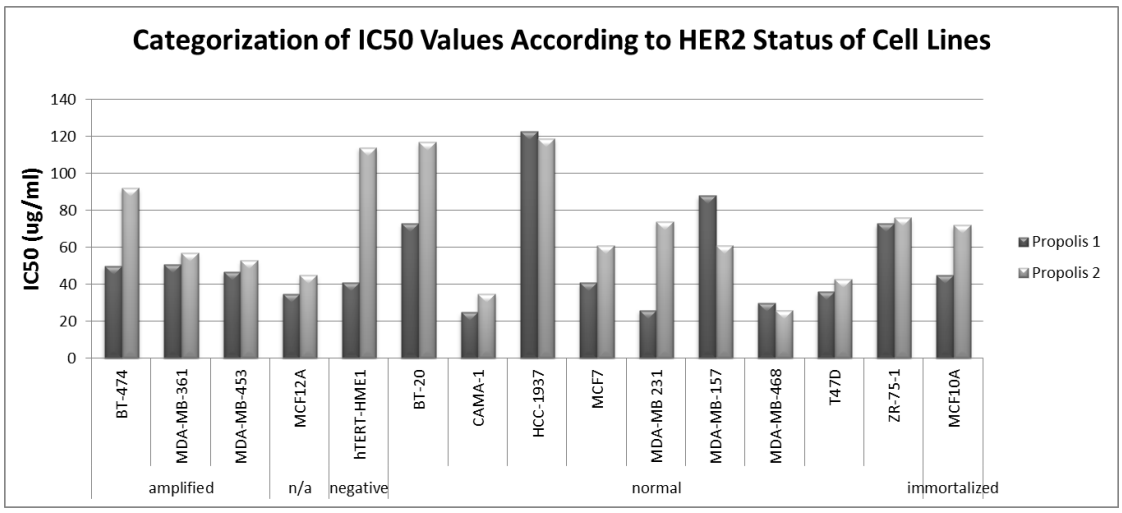


Figure 4-25 IC_{50} values of propolis extracts were used to analyzed according to the HER2 status of breast carcinoma cell lines. HER2 normal cell line group was compared to HER2 amplified cell line group for both Propolis-1 and Propolis-2 (Propolis-1, p=0.5 and Propolis-2, p=1.0).

4.4 Dynamic Cell Proliferation of Propolis Treated Cells with xCELLigence

The xCELLigence Systems allow for label-free and real-time monitoring of cellular processes such as cell proliferation. The cell growth curves were automatically recorded on the xCELLigence System in real time. IC_{50} value calculations were restricted to the one time point whereas response to a treatment is dynamic phenomena. Therefore observing the effect of propolis on growing cells (real-time) is an important analysis. xCELLigence (Roche) system was used to monitor the growing cells in real time. Dynamic monitoring of the logarithmic growth of the cells was correlated with cell index (CI). Cells were inoculated to E plate 96 with indicated cell numbers and experiments were performed in triplicate (Table 4.6). Cells were monitored and measurements were collected every 10 minutes. When the cell index reaches to 0,75 before it exceeds the cell index 1,20, cells were treated with three different Propolis extract-2 concentrations (IC_{50} values, half and double of IC_{50} values of each cell line) and control DMSO. Then the measurements were collected for every 30 min for minimum of 72 h. DMSO amount was adjusted in each propolis-2 concentration to the same dilution so that only one control DMSO dilution was used. The logarithmic growth of each cell line was given from Figure 4-26 to **Figure 4-31**.

Table 4.6 Starting Cell Numbers of Cell Lines for xCELLigence Monitoring

Cell Line	Starting Cell Numbers
MCF10A	2000 cells/well
BT-474	4000 cells/well
T47D	2000 cells/well
BT-20	2000 cells/well
CAMA-1	4000 cells/well
MDA-MB-231	3000 cells/well

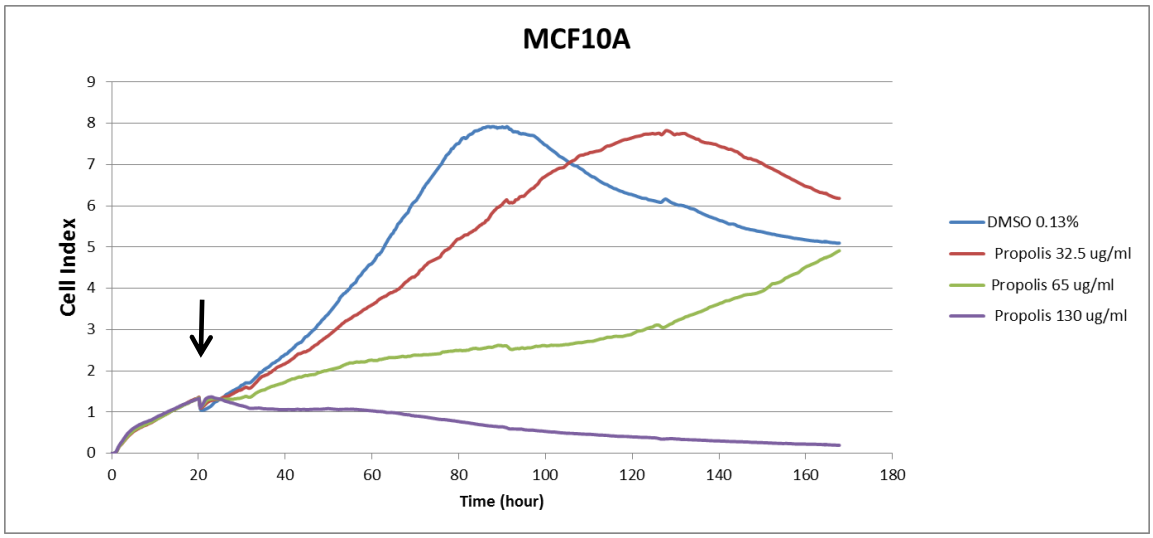


Figure 4-26 Dynamic monitoring of cell proliferation using the xCELLigence system in MCF10A. MCF10A (IC_{50}=72 ug/ml) cells treated with propolis-2 at 32.5 ug/ml, 65 ug/ml, and 130ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

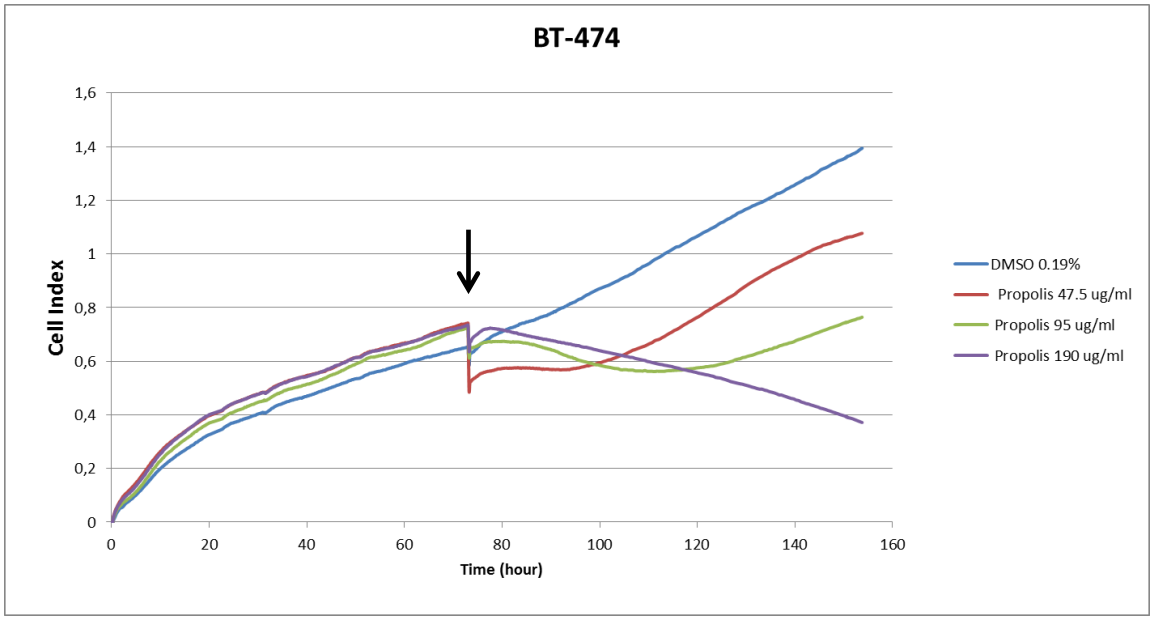


Figure 4-27 Dynamic monitoring of cell proliferation using the xCELLigence system in BT-474. BT-474 (IC_{50}= 92 ug/ml) cells treated with propolis-2 at 47.5 ug/ml, 95ug/ml and 190ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

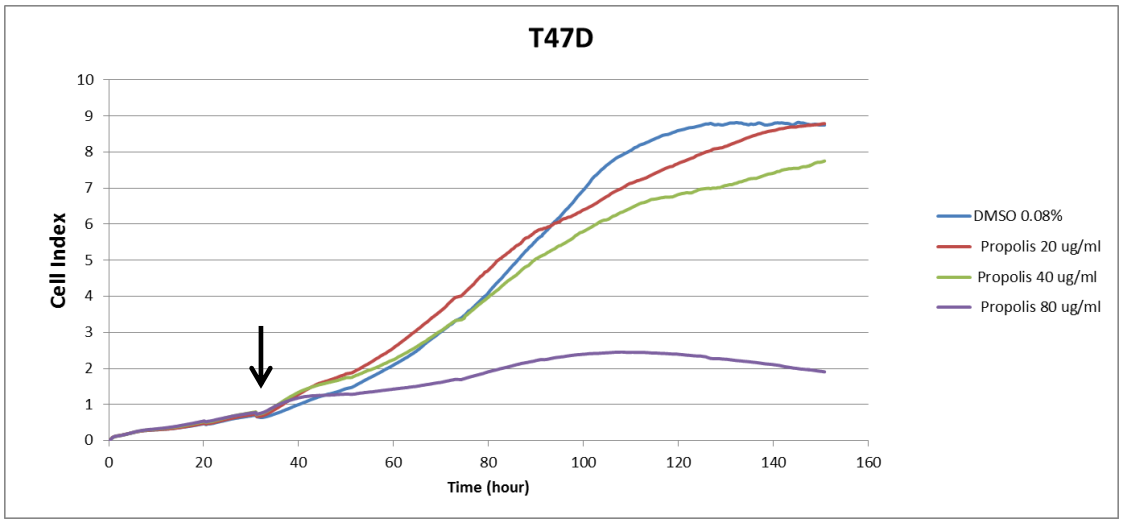


Figure 4-28 **Dynamic monitoring of cell proliferation using the xCELLigence system in T47D.** T47D (IC_{50}= 43 ug/ml) cells treated with propolis-2 at 20 ug/ml, 40ug/ml and 80ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

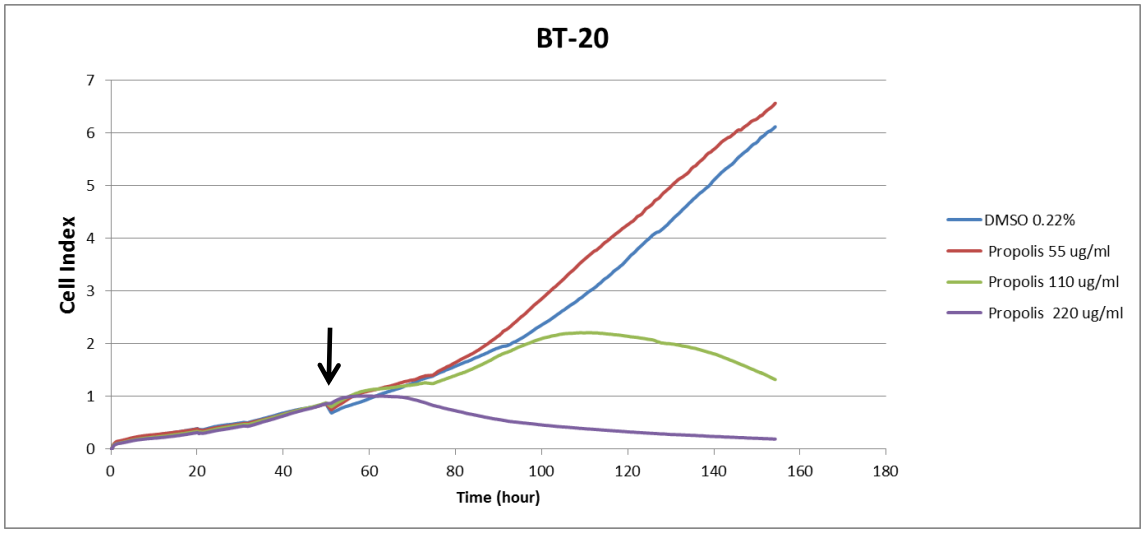


Figure 4-29 Dynamic monitoring of cell proliferation using the xCELLigence system in BT-20. BT-20 (IC_{50}= 117 ug/ml) cells treated with propolis-2 at 55 ug/ml, 110 ug/ml and 220ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

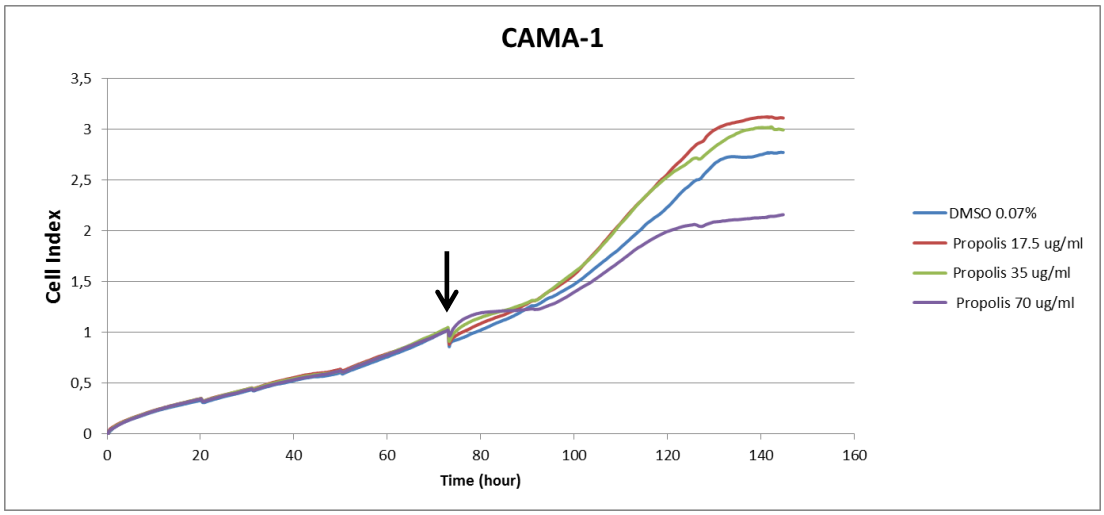


Figure 4-30 Dynamic monitoring of cell proliferation using the xCELLigence system in CAMA-1. CAMA-1 (IC_{50}= 35 ug/ml) cells treated with propolis-2 at 17.5 ug/ml, 35ug/ml and 70ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

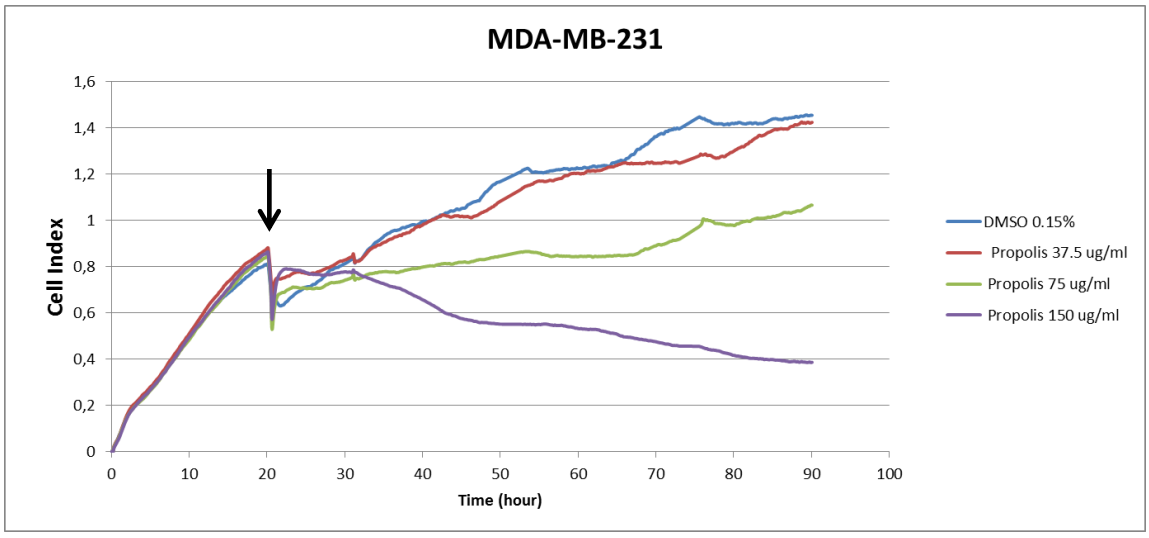


Figure 4-31 Dynamic monitoring of cell proliferation using the xCELLigence system in MDA-MB-231. MDA-MB-231 (IC_{50}= 74 ug/ml) cells treated with propolis-2 at 37.5 ug/ml, 75ug/ml and 150ug/ml concentrations and control DMSO. The arrow shows the time point where the propolis-2 and DMSO were added to the cells.

Real Time Growth Curves of breast carcinoma cell lines with different concentrations of propolis-2 were monitored by xCelligence system. The arrow shows the time point where the propolis-2 and control DMSO were added to the cells.

All the cell lines , except CAMA1, showed decrease in growth rate compare to only DMSO treated cells. Increasing concentrations of propolis-2 was decreases the cell number in a dose- and cell line- dependent way.

4.5 Propolis Induces Apoptosis on Breast Carcinoma Cell Lines

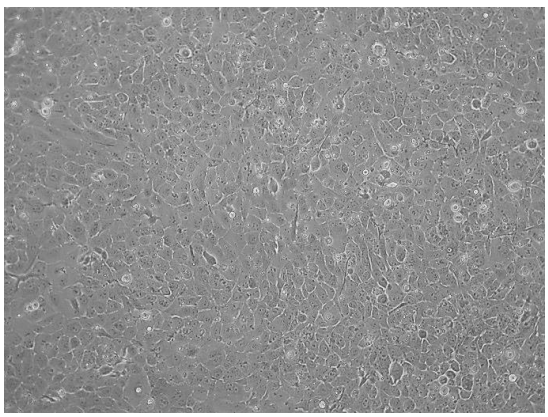
The observation of propolis anti-proliferative effect led us to evaluate its cellular mechanism. To evaluate the anti-proliferative effects of propolis , the cell lines were analyzed for cell death.

4.5.1 Morphology of the Cells Changes with Propolis Treatment

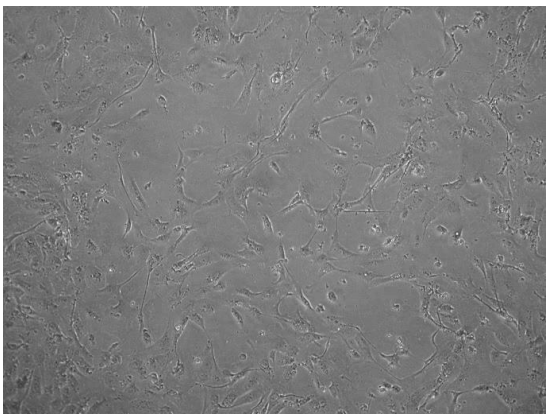
The cells were treated with propolis and their morphology was observed under the light microscope. Morphological changes indicate apoptotic cell death. The effected cells were changed their adherent morphology to round cells and after a few hours later, those round cells were detached from the surface of the plate.

Breast carcinoma cell lines were cultured on 6 well plates as 80,000 cells/well. The cells were treated with either 0.1 % DMSO or 100 ug/ml Propolis-2 after 24 hours. The cells were observed under light microscope and photos were taken with 10X magnification, 48 hours after the treatment (Figure 4-32).

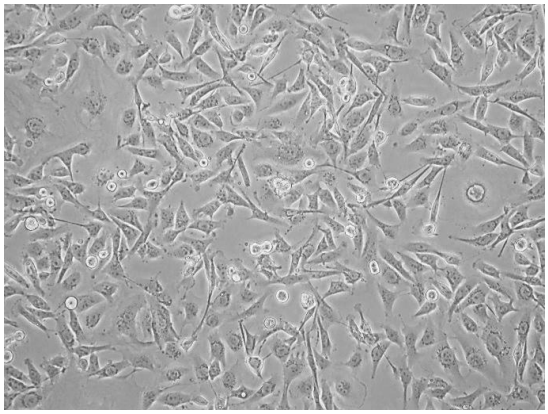
A-1) MCF10A, DMSO



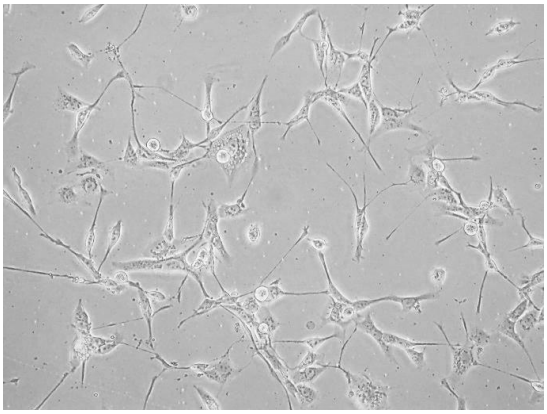
A-2) MCF10A, Propolis



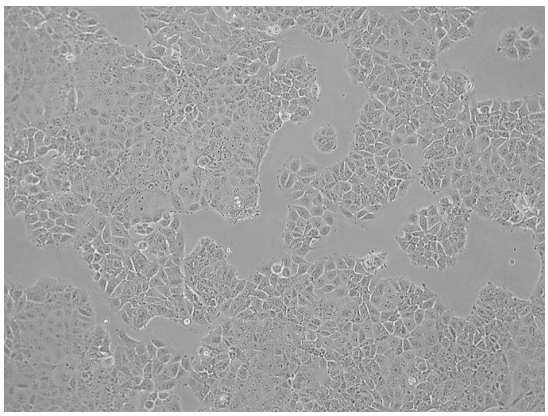
B-1) MDA-MB-231, DMSO



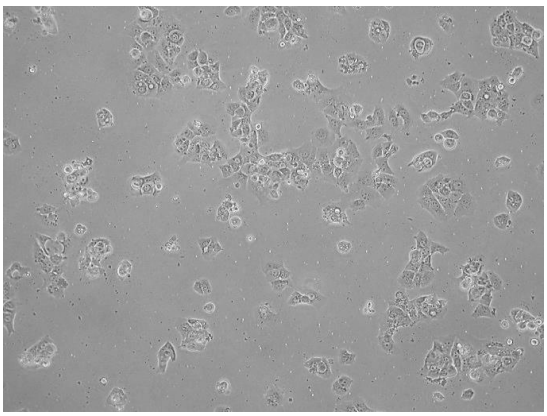
B-2) MDA-MB-231, Propolis



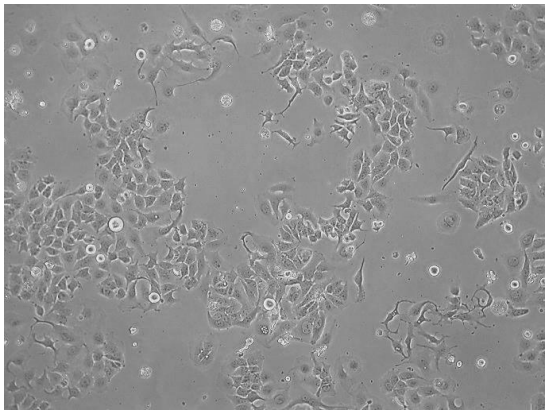
C-1) T47D, DMSO



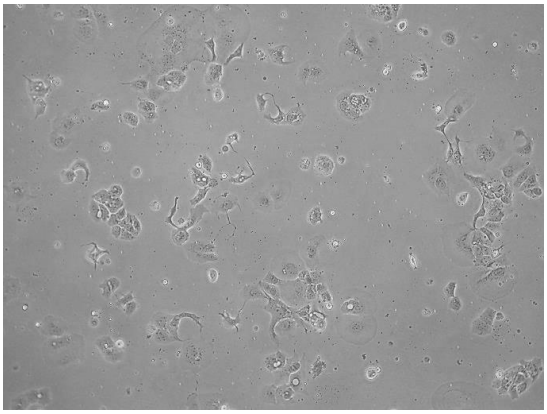
C-2) T47D, Propolis



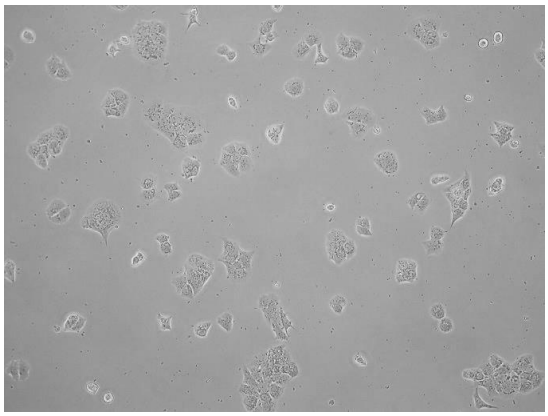
D-1) BT-20, DMSO



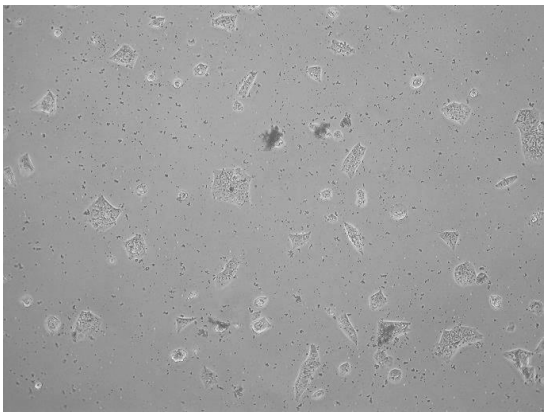
D-2) BT-20, Propolis



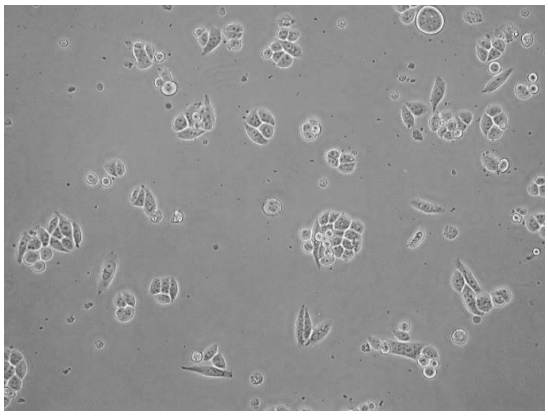
E-1) BT-474, DMSO



E-2) BT-474, Propolis



F-1) CAMA-1, DMSO, 10X



F-2) CAMA-1, Propolis, 10X

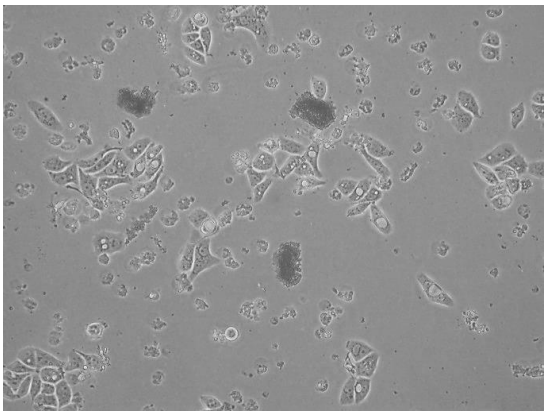


Figure 4-32 The morphological appearance of treated cell lines under the light microscope. The cells were treated with 100 ug/ml Propolis and 0.1% DMSO control

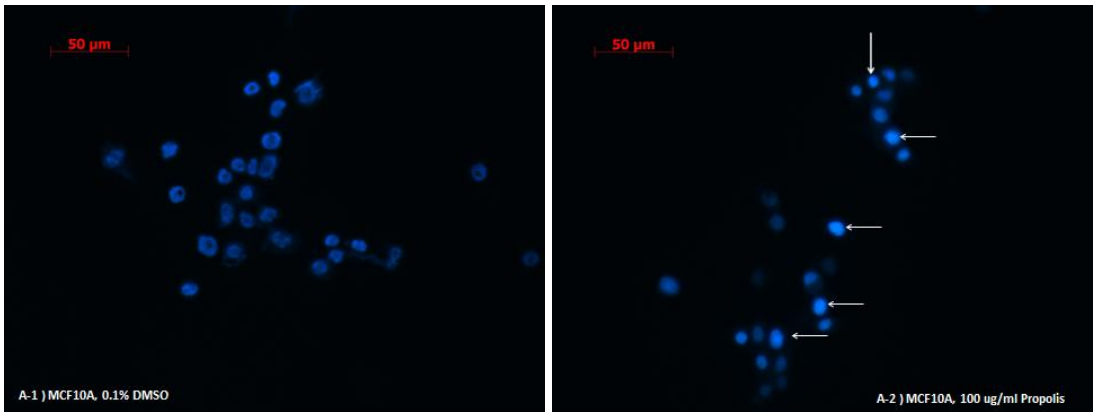
and the photos were taken after 48 h of treatment. MCF10A (A), MDA-MB-231 (B), T47D (C), BT-20 (D), BT-474 (E), CAMA-1 (F), (10X magnification).

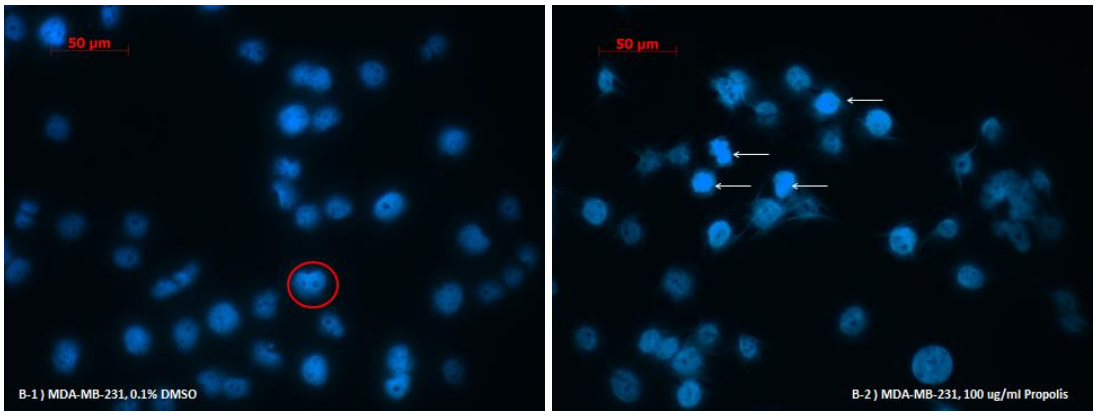
Cell death effect of propolis in cell lines were evaluated by treating cells with propolis and the morphology of the cells were observed under light microscope. The cell morphology changed after treatment. It was observed that the cells become more round and the cell edges were sharper than control cells. The increased number of round and detached cells were observed which made the apoptosis as a suspected cell death mechanism.

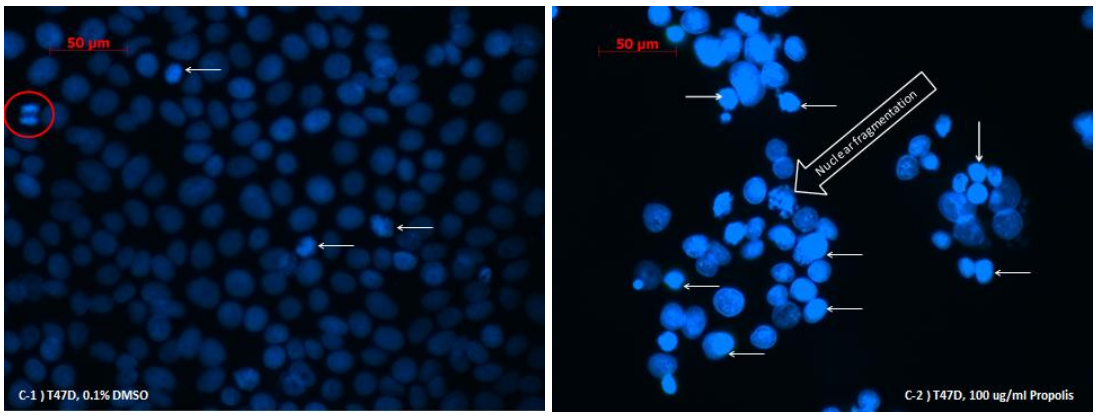
4.5.2 Chromatin Condensation and Nuclear Fragmentation

To analyze the apoptosis the Propolis and DMSO treated cells were stained with well-known nuclear dye Hoechst 33258 and common properties of apoptotic cells, chromatin condensation and nuclear fragmentation, were observed.

The cells were seeded onto glass coverslips in 6 well plates as 80,000 cells/well and 24 hours later, cells were treated with either 0.1 % DMSO or 100 ug/ml Propolis-2. Cells were stained with Hoechst 33258, 48 hours after the treatment. Photos were taken under fluorescent microscope with 40X magnification (Figure 4-33). Condensed chromatins were identified by their intense staining while normal cells can be depicted with clear and uniformly dispersed nuclei. In DMSO control treated cells, the nucleolus could be observed (black dots in the cells) . White arrows in the pictures indicate apoptotic cells with condensed chromatin and red circles indicate the dividing cells. The bold white arrow in Figure 4-33, C-2 shows the nuclear fragmented apoptotic cell.


50 µm
A-1) MCF10A, 0.1% DMSO
50 µm
A-2) MCF10A, 100 ug/ml Propolis


50 µm
B-1) MDA-MB-231, 0.1% DMSO
50 µm
B-2) MDA-MB-231, 100 ug/ml Propolis


50 µm
C-1) T47D, 0.1% DMSO
50 µm
Nuclear fragmentation
C-2) T47D, 100 ug/ml Propolis

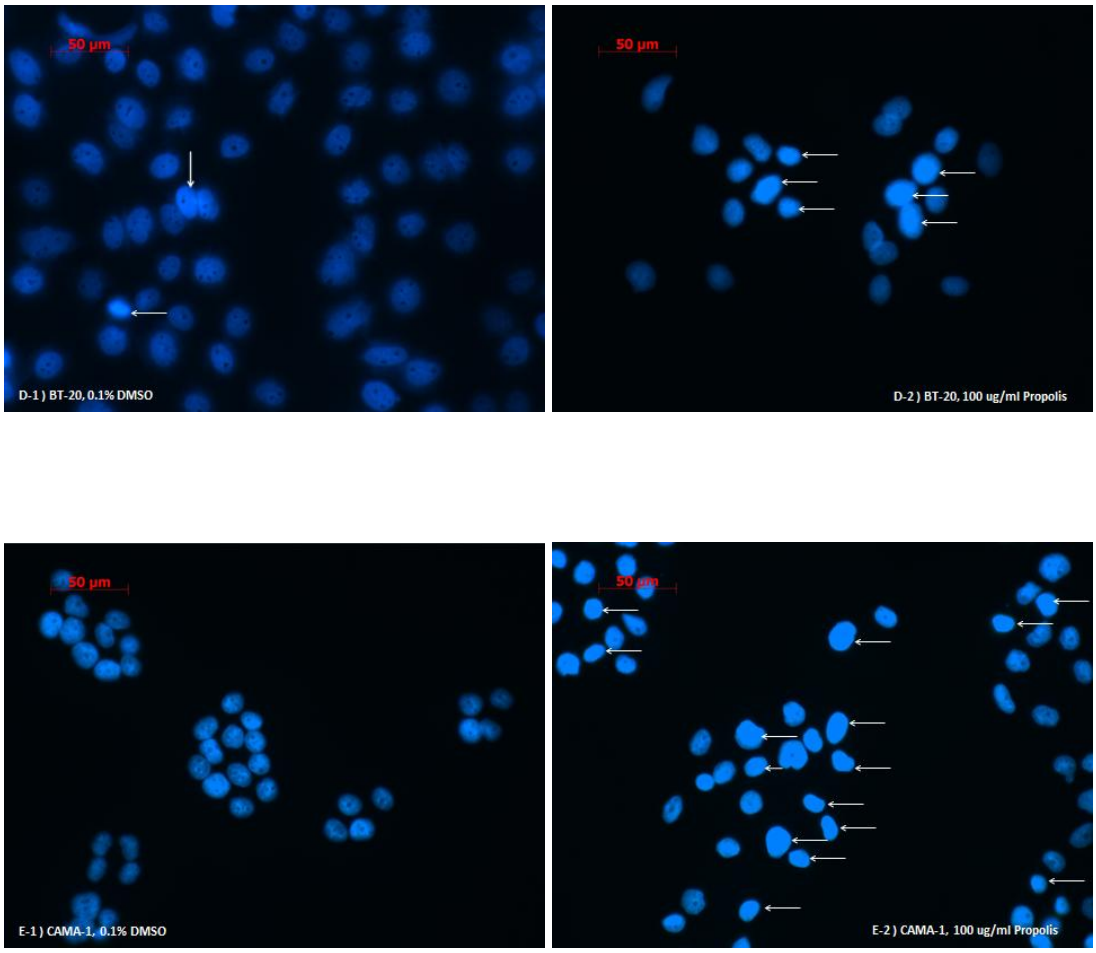


Figure 4-33 Hoechst 33258 staining and morphological appearance of cell nucleus after treatment. The MCF10A (A), MDA-MB-231 (B), T47D (C), BT-20 (D), CAMA-1 (E) cells were incubated with Propolis-2 with either 0.1% DMSO (1) or 100 ug/ml Propolis-2 (2) for 48 h. Then the cells were stained with Hoechst 33258. Photos were taken under the flourescent microscope with 40X magnification. Chromatin condensations were indicated with arrows, red circles indicate dividing cells, and big white arrow in C-2 shows the nuclear fragmented apoptotic cells.

The result showed that there is an increase in the number of apoptotic cells when they were treated with propolis.

4.5.3 Western Blot Analysis of PARP-1 cleavage

The Poly (ADP-Ribose) Polymerase 1 (PARP-1) is an ADP-ribosylating enzyme essential for initiating various forms of DNA repair. PARP-1, a 116 kDa nuclear enzyme, is cleaved in fragments of 89 and 24 kDa during apoptosis. This cleavage has become a useful hallmark of apoptosis. Western blot analysis was performed to detect the PARP-1 cleavage in propolis treated cells. Cells were seeded on 6 well plates as 80,000 cells/well and cells were treated with either 0.1 % DMSO or 100 ug/ml Propolis-2 after 24 hours. Cell pellets were collected after 48 hours of treatment. Protein extraction was performed and 50 ug proteins were run in SDS-PAGE. PARP-1 protein was detected in the membranes and β-actin was used as equal loading control (Figure 4-37). The PARP-1 antibody can both target the full length PARP-1 (116 kDa) as well as its cleaved fragment (89 kDa). The image photos were analyzed with ImageJ software to obtain quantified, comparable data. Each band's intensity was quantified by using ImageJ to obtain numerical results of its intensity. Each sample's normalization was calculated by dividing its PARP-1 protein band intensity to its β-actin protein band intensity (from Figure 4-38 to Figure 4-36).

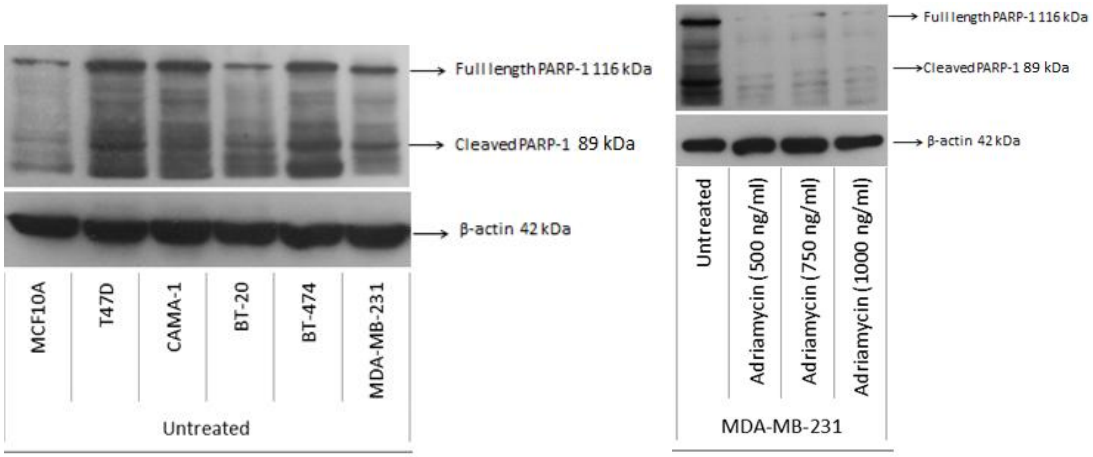


Figure 4-34 Western blot analysis of PARP-1 cleavage in untreated and Adriamycin treated breast carcinoma cell lines. Untreated cell lines (left), Adriamycin treated (as a positive control) cells (right) were analysed for their PARP-1 protein cleavage.

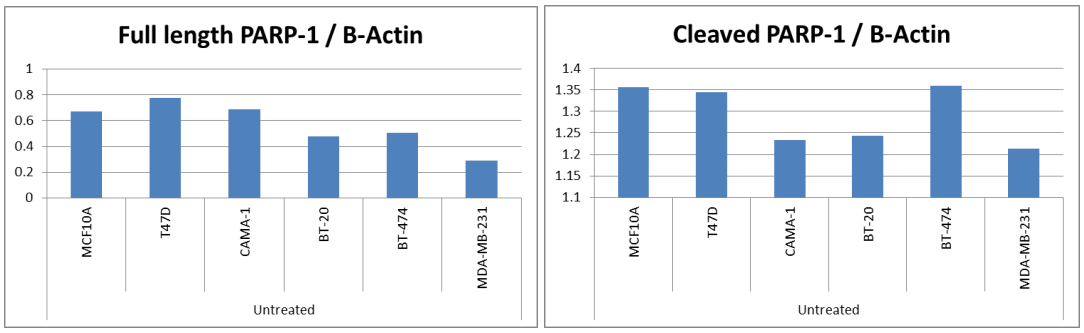


Figure 4-35 Graphical representation of normalized data of PARP-1 band intensities in untreated breast carcinoma cell lines. Each sample's normalization was calculated by dividing its full length PARP-1 protein (116 kDa) band intensity to its β-actin protein (42 kDa) band intensity (left) and cleaved PARP-1 protein (89 kDa) band intensity to its β-actin protein (42 kDa) band intensity (right)(images from Figure 4-34).

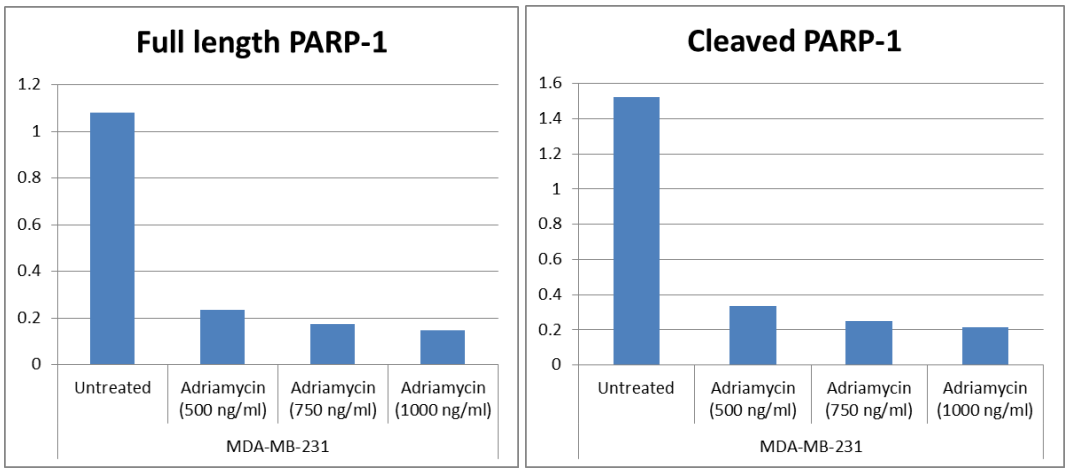
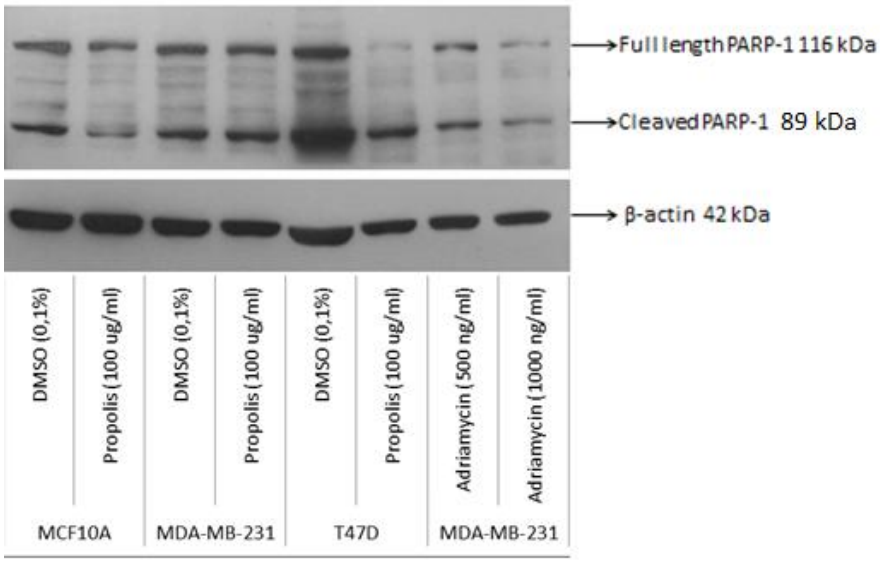


Figure 4-36 Graphical representation of normalized data of PARP-1 band intensities in untreated and Adriamycin treated MDA-MB-231 cells. Each sample's normalization was calculated by dividing its full length PARP-1 protein (116 kDa) band intensity to its β-actin protein (42 kDa) band intensity (left) and cleaved PARP-1 protein (89 kDa) band intensity to its β-actin protein (42 kDa) band intensity (right)(images from Figure 4-34).

Adriamycin is a well know DNA damaging drug that directly intercalates into double-stranded DNA. It prevents DNA replication and induces apoptosis therefore Adriamycin was used as a positive control of apoptosis. In the increasing amount of Adriamycin treatment, PARP-1 full length as well as cleaved fragment of PARP-1 were decreased.



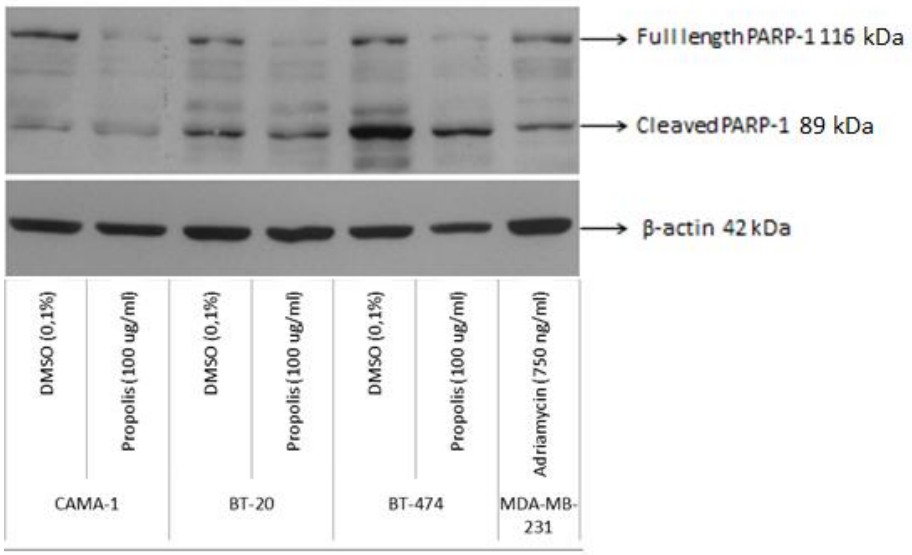


Figure 4-37 Western blot analysis of PARP-1 cleavage in DMSO and Propolis-2 treated breast carcinoma cell lines. Adriamycin treated cells were analysed for their PARP-1 protein cleavage as a positive control.

The propolis treated sample intensity was compared to DMSO control sample intensity for PARP-1 changes after β-actin normalization.

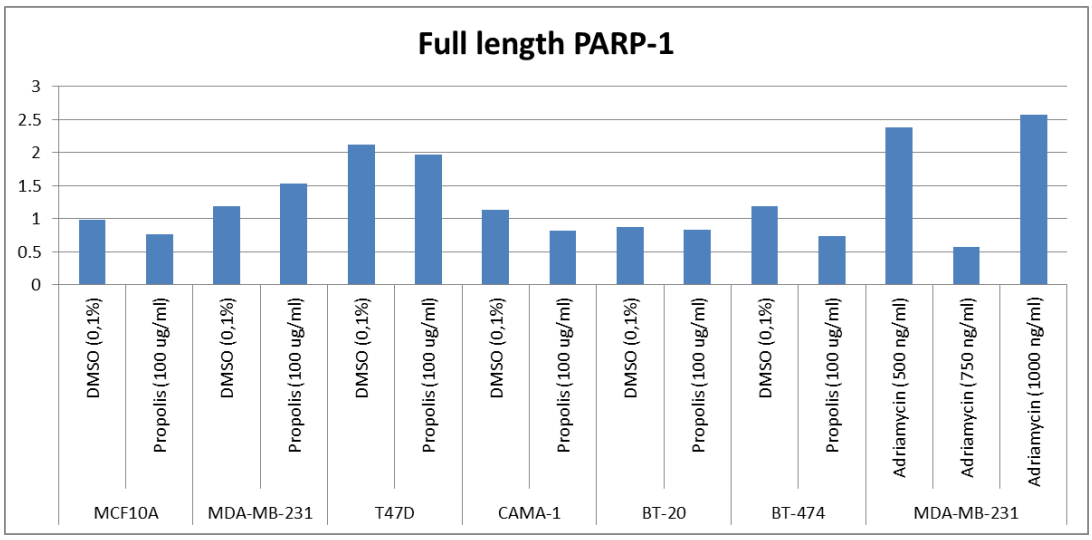


Figure 4-38 Graphical representation of normalized data of full length PARP-1 band intensity in breast carcinoma cell lines. Each sample's normalization was calculated by dividing its full length PARP-1 protein (116 kDa) band intensity to its β-actin protein (42 kDa) band intensity (images from Figure 4-37).

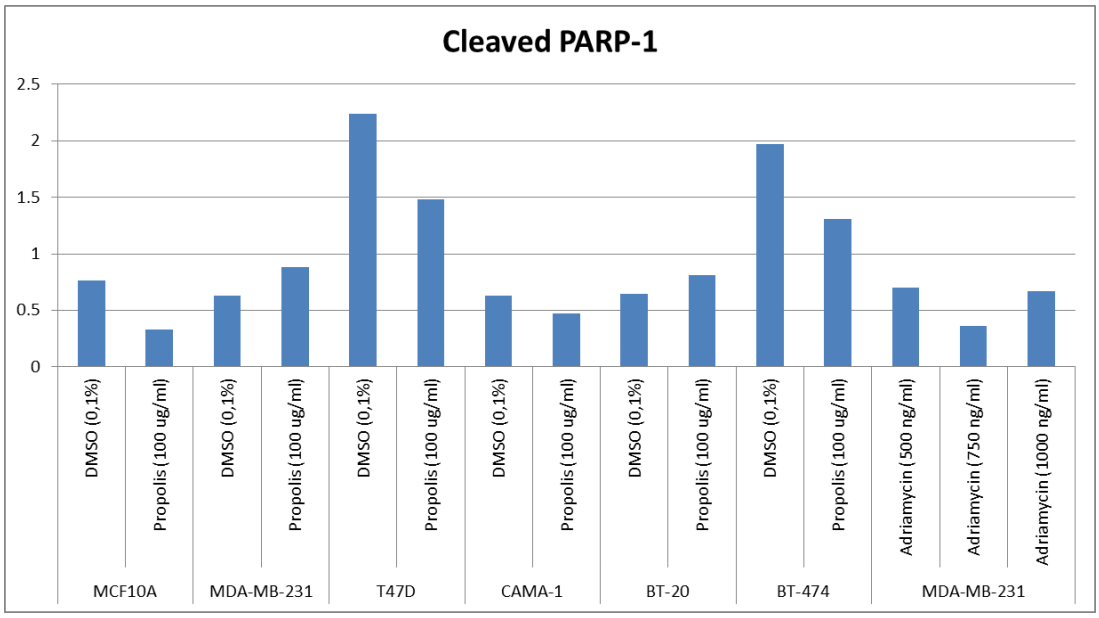


Figure 4-39 Graphical representation of normalized data of cleaved PARP-1 band intensity in breast carcinoma cell lines. Each sample's normalization was calculated by dividing its cleaved fragment of PARP-1 protein (89 kDa) band intensity to its β-actin protein (42 kDa) band intensity (images from Figure 4-37).

Decrease in the full length PARP-1 protein levels (except MDA-MB-231 cell line) supports our hypothesis that propolis treated cells enter to apoptotic state. Increase in the cleaved PARP-1 was observed only in MDA-MB-231 and BT-20 cell lines. To analyze the relative effect of full length and cleaved PARP-1 protein levels, another analysis was performed by calculating the intensity ratio of full length PARP-1 and cleaved PARP-1 proteins. Since the analysis gave an expected result with positive control samples (Figure 4-40), the same analysis was performed with propolis and control DMSO treated cells ().

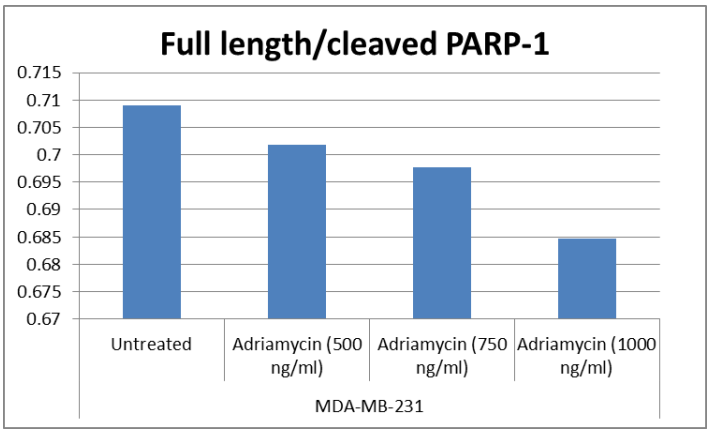


Figure 4-40 Graphical representation of normalized data of full length PAPR-1 and cleaved PARP-1 band intensity ratio in untreated and Adriamycin treated MDA-MB-231 cells. Each sample's ratio was calculated by dividing its full length PARP-1 protein (116 kDa) band intensity to cleaved PARP-1 protein (89 kDa) band intensity (images from Figure 4-34).

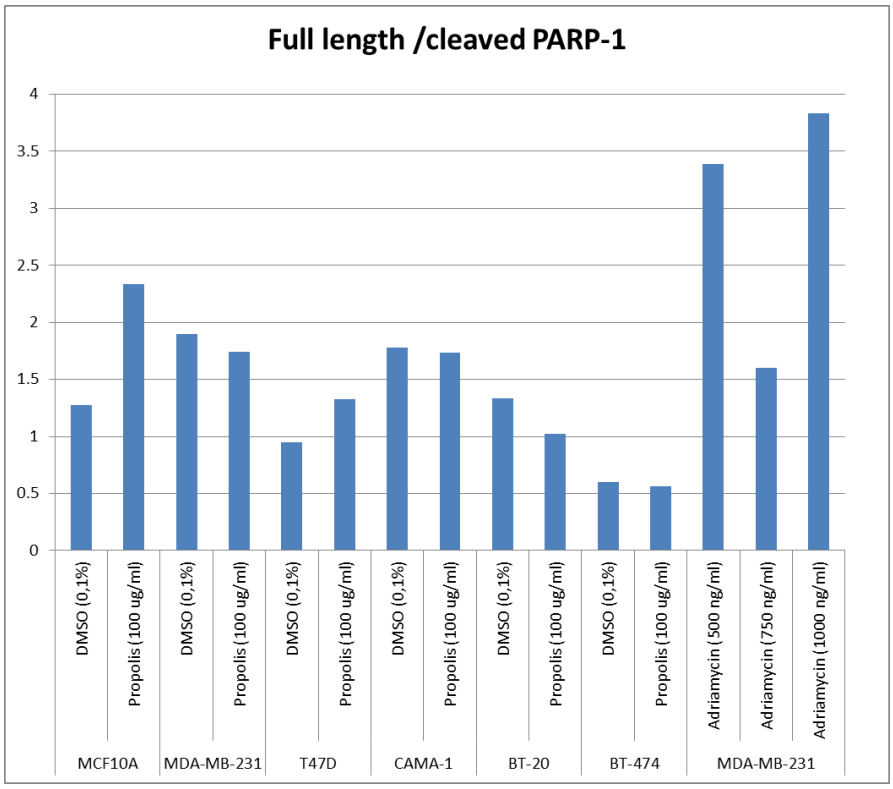


Figure 4-41 Graphical representation of normalized data of full length PAPR-1 and cleaved PARP-1 band intensity ratio in breast carcinoma cell lines. Each sample's

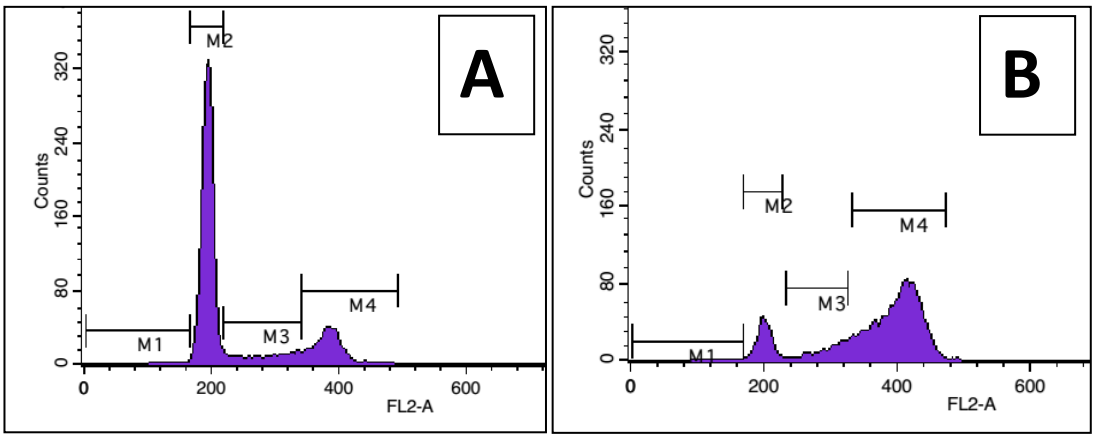
ratio was calculated by dividing its full length PARP-1 protein (116 kDa) band intensity to cleaved PARP-1 protein (89 kDa) band intensity (images from Figure 4-37).

MDA-MB-231, CAMA-1, BT-20 and BT-474 cell lines were showed a decrease in the ratio of full length PAPR-1 over cleaved PAPR-1 protein band intensity which supports the apoptosis possibility of cell death mechanism.

4.6 Propolis Slightly Increases G2/M arrest of MDA-MB-231 Cells

The effect of propolis on cell cycle was analyzed with flow cytometer. MDA-MB-231 cells were seeded on 6 well plates (80,000 cells/well) and 24 hours later, cells were treated with DMSO, 75 ug/ml Propolis-2 (IC_{50} value for this cell line), 250 ng/ ml Adriamycin (as a positive control for G2/M arrest) or untreated (control for Adriamycin treated cells) for 72 hours. Cell pellets were maintained and stained with Propidium Iodide (PI) and stained cells were counted with flow cytometer.

Initially, the Adriamycin treated MDA-MB-231 cells were analyzed with flow cytometer as a control experiment to observe cell cycle changes. The cells were treated with Adriamycin for 72 hours (Figure 4-42).



Histogram Statistics

File: mda mb 231 72h mock
Gate: G1
Total Events: 10000
Acquisition Date: 08-Feb-13
Gated Events: 9340
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Peak Ch
All	0, 1023	9340	100.00	93.40	197
M1	3, 165	61	0.65	0.61	127
M2	165, 217	6432	68.87	64.32	197
M3	217, 340	1010	10.81	10.10	325
M4	340, 490	1867	19.99	18.67	381

Histogram Statistics

File: mda mb 231 72h adriamycin
Gate: G4
Total Events: 10000
Acquisition Date: 08-Feb-13
Gated Events: 8090
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Peak Ch
All	0, 1023	8090	100.00	80.90	412
M1	3, 168	110	1.36	1.10	113
M2	168, 227	1057	13.07	10.57	197
M3	233, 325	903	11.16	9.03	324
M4	331, 472	5857	72.40	58.57	412

Figure 4-42 Cell cycle analysis of untreated and Adriamycin treated MDA-MB-231 cell line with flow cytometer. Untreated cells (A) and Adriamycin treated cells (B). The cell cycle phases were represented in the histogram as M1: Sub-G1; M2: G1; M3: S; M4: G2/M.

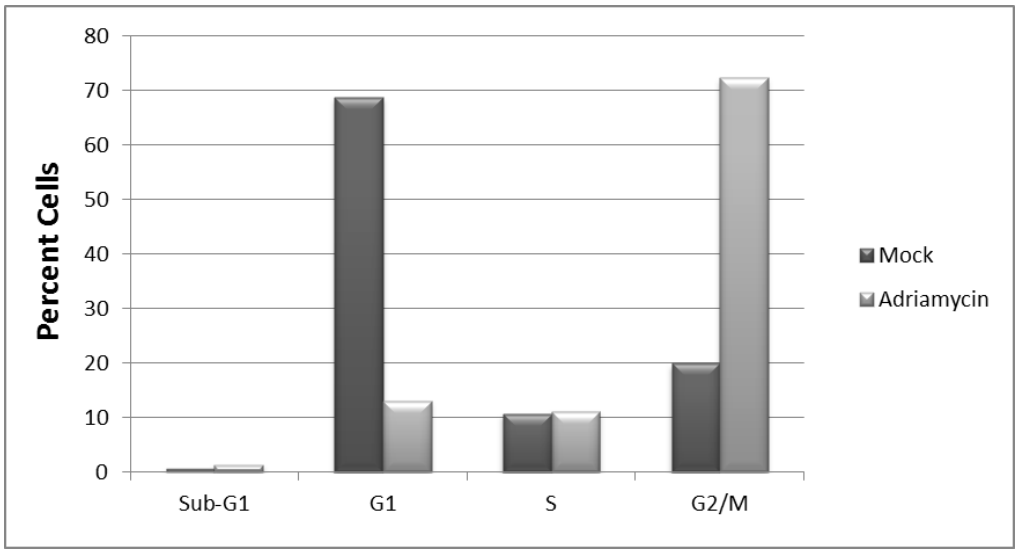
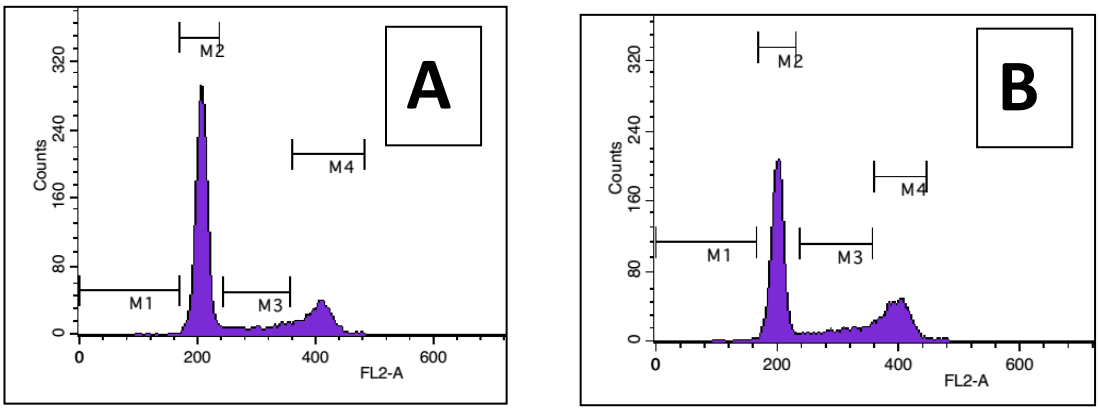


Figure 4-43 Graphical representation of cell cycle analysis of untreated and Adriamycin treated MDA-MB-231 cell line. Data in Figure 4-42. was used to show the cell cycle phase differences between untreated and Adriamycin treated cells.

It was observed that the Adriamycin treated cells were entered G2/M cell cycle arrest. After this control experiment, the same analysis was performed with DMSO and propolis treated MDA-MB-231 cells.



Histogram Statistics

File: mda mb 231 72h DMSO
Gate: G2
Total Events: 10000
Acquisition Date: 08-Feb-13
Gated Events: 9108
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Peak Ch
All	0, 1023	9108	100.00	91.08	207
M1	0, 168	45	0.49	0.45	158
M2	168, 236	6162	67.65	61.62	207
M3	242, 355	909	9.98	9.09	354
M4	358, 481	1938	21.28	19.38	403

Histogram Statistics

File: mda mb 231 72h propolis
Gate: G3
Total Events: 10000
Acquisition Date: 08-Feb-13
Gated Events: 8624
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Peak Ch
All	0, 1023	8624	100.00	86.24	203
M1	0, 165	102	1.18	1.02	134
M2	168, 230	4635	53.75	46.35	203
M3	236, 355	1348	15.63	13.48	307
M4	358, 444	2371	27.49	23.71	405

Figure 4-44 Cell cycle analysis of DMSO and propolis treated MDA-MB-231 cell line with flow cytometer. DMSO treated cells (A) and Propolis-2 treated cells (B). The cell cycle phases were represented in the histogram as M1: Sub-G1; M2: G1; M3: S; M4: G2/M.

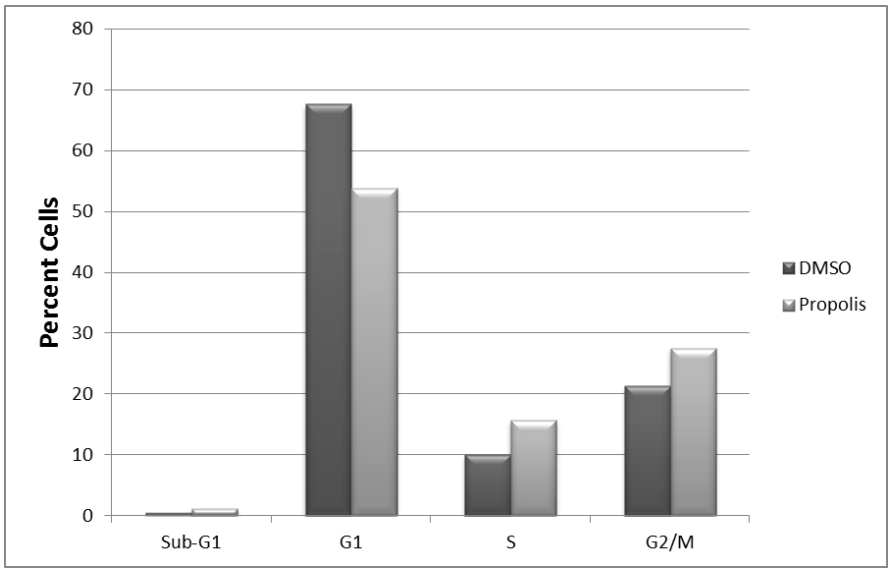


Figure 4-45 Graphical representation of cell cycle analysis DMSO and propolis treated MDA-MB-231 cell line. Data in Figure 4-44. was used to show the cell cycle phase differences between DMSO and propolis treated cells.

Table 4.7 Percent gate comparison of untreated, DMSO, propolis and Adriamycin treated MDA-MB-231 cell line for cell cycle analysis. Data was taken from Figure 4-42 an Figure 4-44.

% Gate	Untreated	DMSO	Propolis	Adriamycin
Sub-G1	0.65	0.49	1.18	1.36
G1	68.87	67.65	53.75	13.07
S	10.81	9.98	15.63	11.16
G2/M	19.99	21.28	27.49	72.4

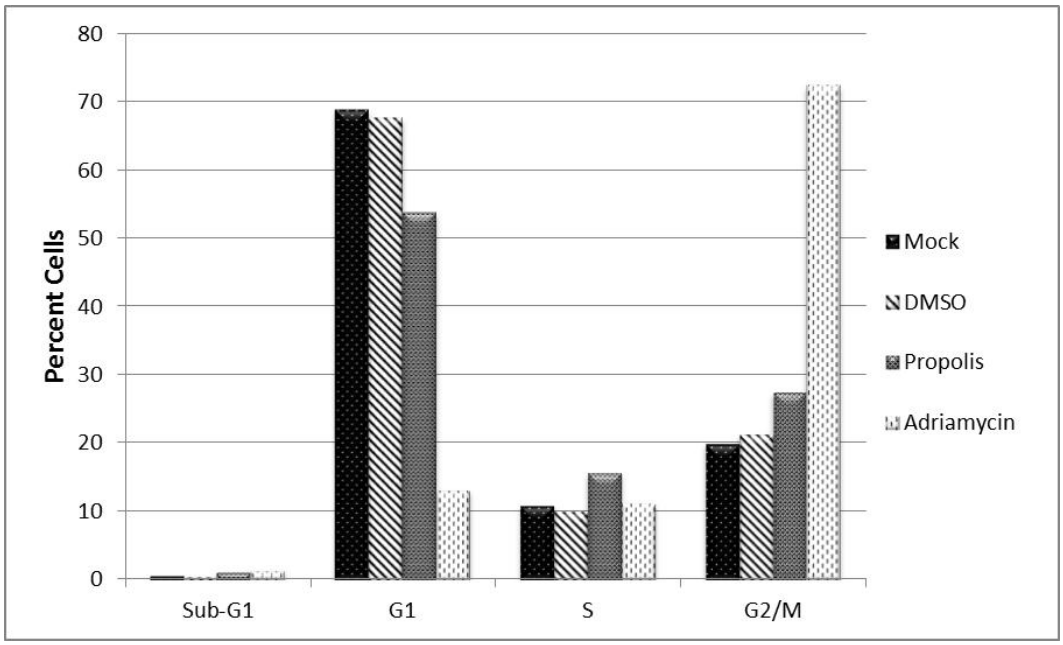


Figure 4-46 Graphical representation of cell cycle analysis of untreated, DMSO, propolis and Adriamycin treated MDA-MB-231 cell line. Data was taken from Figure 4-42 an Figure 4-44.

Above analyses demonstrate that propolis treatment induces G2/M arrest of MDA-MB-231 cell line.

4.7 Propolis Blocks Invasion of MDA-MB-231 Cells

The in vitro scratch assay (wound healing assay) is an easy, low-cost and well-developed method to measure cell invasion *in vitro*. A straight line of cells are scraped from the plate and the invasion of the cells through this scraped line is observed via light microscope.

FBS concentration in the growth medium was dropped from 10 % to 0.1 % to prevent cells growing through the scratch. By this way, wound healing assay can

display the invasion properties of cells but not the proliferation. There were 6 conditions for this set up to observe the invasion rate:

- 10 % FBS containing growth medium treated cells (untreated, mock cells) to observe the effect of proliferation and compare it with 0.1 % FBS medium treated cells,
- 0.1 % FBS containing growth medium treated cells to observe the effect of decreased FBS and compare it with DMSO control cells,
- 0.1 % FBS + 0.1 % DMSO containing growth medium treated cells as a control for propolis treated cells,
- 0.1 % FBS + 50, 75 (IC_{50} value propolis for MDA-MB-231 cells) and 100 ug/ml Propolis-2 containing growth medium treated cells to see the effect of gradually increasing concentrations of propolis on cell invasion ability.

Cells (500,000 cells/well) were seeded in 6 well plate to obtain high confluency and 24 hours later, scratches were made with a 200 ul micropipette tip. Cells were washed with 1X PBS twice to get rid of cell debris in the medium and then cells were treated with the growth mediums listed above. Right after the scratches were performed, time zero photos were taken to observe the scratches borders clearly. Then, the photos were taken at 6, 12, 24, 36 and 48 h intervals under the light microscope with 10X magnification.

As shown in Figure 4-47, cells treated with propolis shows decreased invasion rate of the cells to the denuded area when compared to the DMSO control cells.

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Figure 4-47 Light microscope image to evaluate wound healing *in vitro* in the scratch assay using a confluent monolayer of MDA-MB-231 Cells. Cell migration into the wound was observed in response to an artificial injury of the cells.

5 Discussion

The aim of this study was to evaluate the organic compound propolis effect on cancer cells. We are interested in the effects of propolis on cell growth in human cancer cells, as predictors of novel agents that may be useful in cancer chemoprevention or therapy. In recent years, it has been shown that propolis have antiviral, antimicrobial and antifungal and anti-carcinogenic activity (Sun, 2012). Propolis is a resinous material gathered by honeybees from the buds, leaf and bark of certain trees and plants. It is claimed to improve human health and prevent diseases, such as diabetes, inflammation and cancer (Viuda-Martos, 2008). There are many studies conducted on propolis or its active components for treatment of cancer which reveals the potential of this biological compound in the development of novel anti-cancerous agents (Markiewicz-Zukowska, 2013). Chemical analysis indicated that propolis is a multicomponent mixture of various compounds with prevalence of flavonoids and phenolic acids. Therefore it is important to investigate the propolis extract mechanisms of action in order to predict possible toxic and may be therapeutic effects. The information may help us to develop new drugs that are even more effective for the prevention and treatment of cancer.

In this study, the inhibitory effects of propolis collected from different regions of Turkey were analyzed on the growth of the human breast carcinoma cells. Two different extracts of propolis used in this study were provided by Prof. Dr. Orhan Deger at Karadeniz Technical University, Medical Biology Department. The propolis was extracted and diluted in DMSO and added to the cultures.

Initial experiments to evaluate the effect of propolis on cancer cells was analyzed with low concentrations (100-2000 ng/ml). The low concentrations did not show

any significant effect on cancer cells. One of the reasons can be the very low cell number of the living cells at the end of incubation period. However, the studies conducted so far show that propolis from different regions of the world are cytotoxic to cancer cells for different specific isolates of the extract with differing concentrations. Therefore, the analysis was repeated with higher concentrations of propolis and the result was promising. After the most effective incubation period was determined as 72 hours, the following experiments were conducted with 72 hour incubation. The starting analysis was done with hepatocellular carcinoma cells, lung cancer cells, breast cancer cells as well as normal breast cell line and higher concentrations (25-50-75 ug/ml) of propolis were found to be significantly increasing the cell death among cancer cell lines. However, we decided to focus on one cancer type, breast cancer, to carry on further analysis.

The first step of screening was performed with breast carcinoma cell line panel by calculating the IC_{50} values of propolis on each breast carcinoma cell line. The resulting IC_{50} values of 15 breast carcinoma cell lines for Propolis-1 extract were found to be ranging from 25 ug/ml to 123 ug/ml. Since propolis is a biological compound, a new propolis extract (Propolis-2) was also screened with the same breast carcinoma cell line panel by calculating the IC_{50} values to confirm that the effect of propolis. In this case, IC_{50} values of those breast carcinoma cell lines for Propolis-2 extract were found to be ranging from 26 ug/ml to 119 ug/ml. We concluded that propolis is cytotoxic to breast cancer cells with dose-dependent manner.

Student's t-test and Pearson correlation analyses show that there is significant increase in the IC_{50} values of Propolis-2 extract on breast carcinoma cell lines when compared with the IC_{50} values of Propolis-1 extract. This result may stem from the nature of a biological compound which can display fluctuating results according to the different propolis collections. Since propolis is a mixture of many compounds, plants that bees collect the propolis from may influence the effects on composition of propolis. Even the season that propolis collected in has an effect on active

components of propolis (Barlak, 2011). Because of these reasons, having different results from different extracts of a biological compound is inevitable.

Another statistical analysis was processed to assess the possible correlation between molecular properties of different breast carcinoma cell lines and their IC_{50} values for propolis extracts. Student's t-test results show that there is no significant correlation between subtypes of breast carcinoma cell lines and IC_{50} values of propolis extracts. ER status as well as PR status and HER2 status of breast carcinoma cell lines do not result in a significant correlation with IC_{50} values of propolis extracts. We concluded that there is not a significant correlation between the cytotoxic effect of propolis extracts with different molecular properties of breast carcinoma cell lines.

xCELLigence analysis is a powerful method to show the effect of drug on living cells. In this study, real time monitoring was a confirmative assay to show the effect of propolis on breast carcinoma cell lines. xCELLigence analysis shows the effect of propolis on cells in a short time intervals whereas SRB experiments can be performed only one particular time point. Results support the cytotoxicity of propolis on breast carcinoma cells. We could see the gradual decrease in the cell number as the concentration of propolis increases. Lower concentration of propolis behaves similar to control treatments however high concentrations of propolis almost kill all the cells after 72-100 hours. Also SRB measurements were correlated with this experiment. Other than T47D and CAMA-1 cell lines, the result showed that IC_{50} calculations had a similar effect on both SRB staining and xCELLigence experiments. This data reveals that propolis has cytotoxic effects on breast carcinoma cells and this effect is dose- and time- dependent.

Induction of apoptosis in cancer cells is an important mechanism to eliminate the cancer cells. Since propolis extracts were toxic to the carcinoma cells, we evaluated the apoptosis effect of propolis in cell lines. To reveal if propolis effects apoptosis the cells were treated with the compound and the morphology of the cells were

first evaluated under light microscope and then stained with Hoechst, evaluated under fluorescence microscope. The cell morphology changed after treatment. It was observed that the cells become more round and the cell edges were sharper than control cells. The increased number of round and detached cells were observed. The Hoechst staining was performed to evaluate this observation. The staining results showed chromatin condensation and partial nuclear fragmentation in some cells. These data strengthen the hypothesis that Turkish propolis cytotoxic effect resulted with cell death through apoptosis.

To further support this hypothesis, PARP-1 protein cleavage was investigated with western blot analysis. PAPR-1 is a well-known marker protein for apoptosis. The cleavage of PARP-1 into 89 kDa and 24 kDa fragments is another reliable marker of apoptotic cells (Chaitanya, 2010). Adriamycin was used as a positive control of apoptosis (Bilim, 1997). The Western blot results reveal that majority of the breast carcinoma cell lines had less amount of full length PARP-1 protein when treated with propolis than DMSO. The decrease in full length PARP-1 protein amount in propolis treated cells indicates the apoptosis was taken place in the cells. However we could not see a significant increase in the apoptotic 89 kDa PARP-1 fragment when compared with control cells. Therefore further analysis are required to confirm the cell western blot analysis. Caspase-3 or 7 activities can be detected or other apoptotic markers can be analyzed. Also to eliminate the necrosis as a cell death another PARP-1 western blot analysis can be done with a different antibody which can recognize the 50 kDa fragment of PARP-1 protein which is associated with necrosis (Buontempo, 2010). Although increase in the amount 89 kDa cleaved protein was not clearly observed in propolis treated cells, observation of decrease in the full length PARP-1 protein after treatment shows that apoptosis takes place in these cell lines. Another analysis which compares the ratio of full length PAPR-1 over cleaved PARP-1 protein band intensity showed a better result for supporting the apoptosis. MDA-MB-231, CAMA-1, BT-20 and BT-474 cell lines were found to have decreased ratio of full length PAPR-1 over cleaved PARP-1 protein band

intensity which combines the expected result of decrease in the full length PAPR-1 band intensity while increase in the cleaved PAPR-1 band intensity.

The effect of propolis on cell cycle was also analyzed by flow cytometer. In this assay Adriamycin treated cells were used as a positive control for G2/M arrest (Bilim, 2000). The cell cycle analysis with Propidium iodide (PI) staining of propolis treated cells showed that the cells enter cell cycle arrest in G2/M phase. When the results were compared, increase in the propolis treated cells' G2/M arrest was not as dramatic as Adriamycin effect. Therefore we can conclude that propolis slightly increases the number of cells stuck in the G2/M phase.

Previously CAPE was showed as an inhibitor of invasion in hepatocellular carcinoma (Jin, 2005). We used scratch assay to see the effect of propolis on breast cancer cells. Scratch assay was an easy but informative assay to analyze the effect of propolis on invasion property of breast cancer cells. For this experiment one of the most invasive breast cancer cell, MDA-MB-231 was chosen. When treated with higher concentrations of propolis, cells could not invade the scratched area while DMSO treated cells could fill up the scratched area within 2 days. Since we dropped the serum concentration in the growth media, we can say that invasion of the control samples are not due to the reproduction of existing cells but the invasion of the neighboring cells. This assay is a strong evidence to show the ability of propolis to block the invasion on breast carcinoma cell lines.

In conclusion, propolis showed a cytotoxic effect on breast carcinoma cell lines by inducing apoptosis, G2/M arrest as well as delaying the invasion capacity of the cells which makes it a potent anti-tumorigenic compound that may be useful in cancer chemoprevention or therapy.

6 Future Perspectives

We are interested in the effects of propolis on cell growth in human cancer cells, as predictors of novel agents that may be useful in cancer chemoprevention or therapy. Anti-proliferative activity of DMSO and water extracts of Turkish propolis was previously demonstrated with prostate cancer cell lines (Barlak, 2011). In this study, we showed that DMSO extracts of Turkish propolis has an anti-proliferative activity on breast carcinoma cell lines. Effects of Turkish propolis can be investigated in other cancer types such as hepatocellular carcinoma and lung carcinoma which we have preliminary data that shows cytotoxic effect.

Propolis and its polyphenols target TRAIL-induced apoptosis signaling pathway in tumor cells and sensitizes the TRAIL-resistant cancer cells (Szliszka, 2013). Therefore, more comprehensive study on apoptosis pathway that propolis induce can be designed to reveal the molecular mechanism behind the cytotoxic effect of propolis on carcinoma cells. We showed that DMSO extracts of Turkish propolis increase the apoptosis rate in breast carcinoma cells however the responsible apoptosis pathway is yet to be unknown. The activity of caspases (caspase3 and 7) and other apoptotic molecules on propolis treated carcinoma cells is required to be analyzed.

To eliminate the necrosis as a cell death another PARP-1 western blot analysis can be done with a different antibody which can recognize the 50 kDa fragment of PARP-1 protein which is associated with necrosis (Buontempo, 2010). To eliminate the senescence as a cell death, senescence β-Galactosidase staining can be performed to propolis treated cells.

Due to the technical problems, we performed the cell cycle analysis of only one cell line (MDA-MB-231). The following experiments are required to analyze the effects of propolis on cell cycle with remaining breast carcinoma cell lines. Flow cytometer can be used for both PI staining which is used for cell cycle analysis and Annexin-V

staining for apoptosis detection. Therefore, Annexin-V staining can be performed to confirm the apoptotic cell death of propolis treated cell lines.

To confirm the scratch assay results, matrigel assay can be performed so that prevention of invasion can be proved. In addition, epithelial to mesenchymal transition (EMT) markers (vimentin as mesenchymal and E-cadherin as an epithelial marker) can be detected with immunofluorescence method to analyze the effect of propolis on EMT.

Anti-proliferative activities of CAPE and Chrysin are not always based on similar mechanisms as whole propolis extract was revealed by Sawicka *et. al*. in 2012. Therefore, chemical analysis of propolis compound can be performed and the active components of the propolis extract can be studied separately or in combinations to see the individual effects of chemicals in propolis. We also believe in the synergistic effects of individual compounds, depending on their concentrations. For example, CAPE is an effective adjuvant by targeting Akt signaling in advanced prostate cancer. CAPE treatment reduces the dosage of chemotherapeutic agents required therefore it can be used as a potential adjuvant therapy since it is a safe, natural product (Liu, 2013). The resulting active components of Turkish propolis can be investigated in synergistic effects with existing chemotherapeutic agents.

Protective role of flavonoids in propolis were demonstrated on rats to reduce the toxicity as an adjuvant to chemotherapeutic agents (Padmavathi, 2006). According to this study, there are strong evidences for propolis being a cytotoxic material so that animal cancer models can be treated with Turkish propolis to see the effects on healing. Clinical studies to substantiate these results can help to show the beneficial effects of Turkish propolis since little information is available concerning propolis efficiency clinically. Pharmacological variability of preparations is expected, but biological properties of propolis could be linked to its chemical composition and to its botanical sources (Sforcin, 2011). If Turkish propolis is standardized based on most important active constituents, it can be subjected to clinical trials.

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8 Appendix

8.1 IC_{50} Analysis of Propolis

Table 8.1: The ELISA reading results of MCF10A cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF10A	Concentration (ug/ml)				
Propolis-1	**100**	**50**	**25**	**12.5**	**6.125**
Set 1	0.003	0.448	1.161	1.692	1.415
Set 2	-0.002	1.018	1.859	2.192	1.729
Set 3	0.019	0.751	1.687	2.302	2.282
AVERAGE	0.007	0.739	1.569	2.062	1.809
SEM	0.006	0.165	0.21	0.188	0.253

MCF10A	Concentration (ug/ml)				
DMSO	**100**	**50**	**25**	**12.5**	**6.125**
Set 1	0.858	1.288	1.505	1.824	1.812
Set 2	0.904	1.367	1.672	1.829	1.925
Set 3	0.757	1.388	1.789	2.184	1.801
AVERAGE	0.84	1.348	1.655	1.946	1.846
SEM	0.043	0.03	0.082	0.119	0.04

Percent Cell Death	Concentration (ug/ml)				
	100	**50**	**25**	**12.5**	**6.125**
Set 1	99.65	65.217	22.857	7.237	21.909
Set 2	100.221	25.53	-11.184	-19.847	10.182
Set 3	97.49	45.893	5.702	-5.403	-26.707
AVERAGE	99.167	45.178	5.196	-5.961	2.004
SEM	0.679	9.356	8.025	6.388	11.96

y-int	-83.773			
slope	35.115	**IC50**	45	ug/ml
R2	0.78			

Table 8.2: The ELISA reading results of MCF10A cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF10A	Concentration (ug/ml)				
Propolis-2	**200**	**150**	**100**	**75**	**37.5**
Set 1	0.103	0.109	0.246	0.327	0.406
Set 2	0.147	0.197	0.492	0.687	0.747
Set 3	0.101	0.169	0.376	0.418	0.653
AVERAGE	0.117	0.158	0.371	0.477	0.602
SEM	0.015	0.026	0.071	0.108	0.102

MCF10A	Concentration (ug/ml)				
DMSO	**200**	**150**	**100**	**75**	**37.5**
Set 1	0.329	0.584	0.786	0.959	0.923
Set 2	0.349	0.766	1.16	0.913	1.075
Set 3	0.397	0.47	0.913	0.91	0.713
AVERAGE	0.358	0.607	0.953	0.927	0.904
SEM	0.02	0.086	0.11	0.016	0.105

Percent Cell Death	Concentration (ug/ml)				
	200	**150**	**100**	**75**	**37.5**
Set 1	68.693	81.336	68.702	65.902	56.013
Set 2	57.88	74.282	57.586	24.754	30.512
Set 3	74.559	64.043	58.817	54.066	8.415
AVERAGE	67.318	73.97	61.07	48.544	33.407
SEM	3.989	4.105	2.878	9.986	11.24

y-int	-50.593			
slope	23.507	**IC50**	72	ug/ml
R2	0.9			

Table 8.3: The ELISA reading results of MCF12A cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF12A	Concentration (ug/ml)					
Propolis-1	**100**	**80**	**50**	**40**	**25**	**12.5**
Set 1	0.006	0.034	0.203	0.022	0.073	0.529
Set 2	0.096	0.19	0.526	0.298	0.373	0.641
Set 3	0.021	0.189	0.332	0.466	0.615	0.707
Set 4	0.012	0.149	0.478	0.308	0.743	0.707
AVERAGE	0.034	0.141	0.385	0.274	0.451	0.646
SEM	0.021	0.037	0.073	0.092	0.148	0.042

MCF12A	Concentration (ug/ml)					
DMSO	**100**	**80**	**50**	**40**	**25**	**12.5**
Set 1	0.404	0.807	0.326	0.922	0.725	0.394
Set 2	0.661	0.851	0.64	0.836	0.927	0.88
Set 3	0.313	0.67	0.724	0.741	0.806	0.905
Set 4	0.322	0.4	0.815	0.58	0.682	0.935
AVERAGE	0.425	0.682	0.626	0.77	0.785	0.779
SEM	0.081	0.102	0.106	0.073	0.054	0.129

Percent Cell Death	Concentration (ug/ml)					
	100	**80**	**50**	**40**	**25**	**12.5**
Set 1	98.515	95.787	37.73	97.614	89.931	-34.264
Set 2	85.477	77.673	17.813	64.354	59.763	27.159
Set 3	93.291	71.791	54.144	37.112	23.697	21.878
Set 4	96.273	62.75	41.35	46.897	-8.944	24.385
AVERAGE	92	79.326	38.498	64.416	42.548	17.073
SEM	2.484	6.062	6.517	11.53	18.611	12.855

y-int	-67.185			
slope	33	**IC50**	35	ug/ml
R2	0.82			

Table 8.4: The ELISA reading results of MCF12A cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF12A	Concentration (ug/ml)					
Propolis-2	**37.5**	**50**	**75**	**100**	**150**	**200**
Set 1	0.06	0.068	0.05	0.058	0.027	0.007
Set 2	0.174	0.235	0.144	0.107	0.044	0.038
Set 3	0.265	0.288	0.263	0.183	0.069	0.049
Set 4	0.285	0.304	0.216	0.155	0.089	0.049
AVERAGE	0.196	0.224	0.168	0.126	0.057	0.036
SEM	0.051	0.054	0.046	0.027	0.014	0.01

MCF12A	Concentration (ug/ml)					
DMSO	**37.5**	**50**	**75**	**100**	**150**	**200**
Set 1	0.173	0.169	0.193	0.193	0.143	0.113
Set 2	0.413	0.338	0.367	0.268	0.319	0.115
Set 3	0.45	0.466	0.412	0.339	0.487	0.168
Set 4	0.554	0.601	0.771	0.921	0.489	0.179
AVERAGE	0.398	0.394	0.436	0.43	0.36	0.144
SEM	0.081	0.092	0.121	0.166	0.082	0.017

Percent Cell Death	Concentration (ug/ml)					
	37.5	**50**	**75**	**100**	**150**	**200**
Set 1	65.318	59.763	74.093	69.948	81.119	93.805
Set 2	57.869	30.473	60.763	60.075	86.207	66.957
Set 3	41.111	38.197	36.165	46.018	85.832	70.833
Set 4	48.556	49.418	71.984	83.17	81.8	72.626
AVERAGE	50.754	43.147	61.468	70.698	84.167	75
SEM	4.618	5.562	7.538	6.923	1.152	5.231

y-int	-32.074			
slope	21.581	**IC50**	45	ug/ml
R2	0.8			

Table 8.5: The ELISA reading results of MDA-MB-231 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-231	Concentration (ug/ml)					
Propolis-1	**100**	**75**	**50**	**25**	**12.5**	**6**
Set 1	-0.006	-0.006	0.267	0.358	0.465	0.61
Set 2	-0.004	-0.013	0.222	0.41	0.559	0.449
Set 3	-0.005	-0.006	0.216	0.424	0.692	0.764
Set 4	-0.014	-0.001	0.283	0.409	0.651	0.692
AVERAGE	-0.007	-0.007	0.247	0.4	0.592	0.629
SEM	0.002	0.002	0.017	0.014	0.051	0.068

MDA-MB-231	Concentration (ug/ml)					
DMSO	**100**	**75**	**50**	**25**	**12.5**	**6**
Set 1	0.616	0.527	0.714	0.663	0.966	0.801
Set 2	0.694	0.68	0.703	0.637	0.746	0.888
Set 3	0.574	0.499	0.625	0.608	0.664	0.61
Set 4	0.481	0.565	0.549	0.539	0.495	0.544
AVERAGE	0.591	0.568	0.648	0.612	0.718	0.711
SEM	0.044	0.04	0.038	0.027	0.098	0.08

Percent Cell Death	Concentration (ug/ml)					
	100	**75**	**50**	**25**	**12.5**	**6**
Set 1	100.974	101.139	62.605	46.003	51.863	23.845
Set 2	100.576	101.912	68.421	35.636	25.067	49.437
Set 3	100.871	101.202	65.44	30.263	-4.217	-25.246
Set 4	102.911	100.177	48.452	24.119	-31.515	-27.206
AVERAGE	101.184	101.232	61.883	34.641	17.549	11.533
SEM	0.463	0.31	3.832	4.021	15.706	16.42

y-int	-64.179			
slope	35.005	**IC50**	26	ug/ml
R2	0.91			

Table 8.6: The ELISA reading results of MDA-MB-231 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-231	Concentration (ug/ml)					
Propolis-2	**150**	**120**	**75**	**60**	**37.5**	**30**
Set 1	0.001	0.012	0.018	0.036	0.054	0.052
Set 2	0.017	0.039	0.144	0.073	0.139	0.149
Set 3	0.007	0.028	0.152	0.11	0.125	0.134
Set 4	0	0.052	0.127	0.135	0.108	0.122
AVERAGE	0.006	0.033	0.11	0.089	0.107	0.114
SEM	0.004	0.008	0.031	0.022	0.019	0.021

MDA-MB-231	Concentration (ug/ml)					
DMSO	**150**	**120**	**75**	**60**	**37.5**	**30**
Set 1	0.014	0.118	0.201	0.211	0.193	0.223
Set 2	0.026	0.142	0.187	0.187	0.138	0.147
Set 3	0.083	0.107	0.142	0.128	0.073	0.141
Set 4	0.07	0.125	0.099	0.117	0.086	0.12
AVERAGE	0.048	0.123	0.157	0.161	0.123	0.158
SEM	0.017	0.007	0.023	0.023	0.027	0.023

Percent Cell Death	Concentration (ug/ml)					
	150	**120**	**75**	**60**	**37.5**	**30**
Set 1	92.857	89.831	91.045	82.938	72.021	76.682
Set 2	34.615	72.535	22.995	60.963	-0.725	-1.361
Set 3	91.566	73.832	-7.042	14.063	-71.233	4.965
Set 4	100	58.4	-28.283	-15.385	-25.581	-1.667
AVERAGE	87.5	73.171	29.936	44.72	13.008	27.848
SEM	13.244	5.567	22.642	19.386	26.284	16.617

y-int	-125.33				
slope	40.743	**IC50**	74	ug/ml	
R2	0.8				

Table 8.7: The ELISA reading results of CAMA-1 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

CAMA-1	Concentration (ug/ml)					
Propolis-1	**80**	**60**	**50**	**40**	**30**	**25**
Set 1	0	0	0	0.034	0.058	0.063
Set 2	0	0	0.019	0.067	0.069	0.083
Set 3	0.007	0.001	0.02	0.073	0.091	0.113
Set 4	-0.002	0.001	0.007	0.071	0.091	0.114
AVERAGE	0.001	0.001	0.012	0.061	0.077	0.093
SEM	0.002	0	0.005	0.009	0.008	0.012

CAMA-1	Concentration (ug/ml)					
DMSO	**80**	**60**	**50**	**40**	**30**	**25**
Set 1	0.087	0.157	0.196	0.191	0.201	0.202
Set 2	0.202	0.187	0.198	0.18	0.207	0.184
Set 3	0.22	0.158	0.21	0.182	0.183	0.177
Set 4	0.158	0.129	0.19	0.164	0.198	0.138
AVERAGE	0.167	0.158	0.199	0.179	0.197	0.175
SEM	0.03	0.012	0.004	0.006	0.005	0.013

Percent Cell Death	Concentration (ug/ml)					
	80	**60**	**50**	**40**	**30**	**25**
Set 1	100	100	100	82.199	71.144	68.812
Set 2	100	100	90.404	62.778	66.667	54.891
Set 3	96.818	99.367	90.476	59.89	50.273	36.158
Set 4	101.266	99.225	96.316	56.707	54.04	17.391
AVERAGE	99.401	99.367	93.97	65.922	60.914	46.857
SEM	0.822	0.189	2.038	4.97	4.314	9.71

y-int	-109.394			
slope	49.468	**IC50**	25	ug/ml
R2	0.9			

Table 8.8: The ELISA reading results of CAMA-1cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

CAMA-1	Concentration (ug/ml)					
Propolis-2	**100**	**80**	**50**	**40**	**25**	**20**
Set 1	0.022	0.025	0.072	0.078	0.101	0.109
Set 2	0.013	0.028	0.056	0.063	0.097	0.054
Set 3	0.016	0.013	0.054	0.101	0.039	0.054
Set 4	0.017	0.032	0.15	0.123	0.139	0.12
AVERAGE	0.017	0.025	0.083	0.091	0.094	0.084
SEM	0.002	0.004	0.023	0.013	0.021	0.018

CAMA-1	Concentration (ug/ml)					
DMSO	**100**	**80**	**50**	**40**	**25**	**20**
Set 1	0.173	0.077	0.292	0.177	0.19	0.108
Set 2	0.156	0.202	0.294	0.249	0.116	0.1
Set 3	0.122	0.203	0.155	0.189	0.131	0.122
Set 4	0.127	0.207	0.208	0.148	0.154	0.158
AVERAGE	0.145	0.172	0.237	0.191	0.148	0.122
SEM	0.012	0.032	0.034	0.021	0.016	0.013

Percent Cell Death	Concentration (ug/ml)					
	100	**80**	**50**	**40**	**25**	**20**
Set 1	87.283	67.532	75.342	55.932	46.842	-0.926
Set 2	91.667	86.139	80.952	74.699	16.379	46
Set 3	86.885	93.596	65.161	46.561	70.229	55.738
Set 4	86.614	84.541	27.885	16.892	9.74	24.051
AVERAGE	88.276	85.465	64.979	52.356	36.486	31.148
SEM	1.034	4.801	10.357	10.477	12.155	10.91

y-int	-84.629			
slope	37.999	**IC50**	35	ug/ml
R2	0.99			

Table 8.9: The ELISA reading results of MDA-MB-453 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-453	Concentration (ug/ml)				
Propolis-1	**80**	**60**	**40**	**30**	**20**
Set 1	0.001	0.046	0.041	0.043	0.034
Set 2	0.003	0.027	0.073	0.085	0.197
Set 3	0.052	0.024	0.077	0.086	0.129
Set 4	0.036	0.016	0.045	0.185	0.134
AVERAGE	0.023	0.028	0.059	0.1	0.124
SEM	0.013	0.006	0.009	0.03	0.034

MDA-MB-453	Concentration (ug/ml)				
DMSO	**80**	**60**	**40**	**30**	**20**
Set 1	0.119	0.093	0.168	0.078	0.144
Set 2	0.115	0.13	0.106	0.141	0.086
Set 3	0.059	0.199	0.052	0.151	0.065
AVERAGE	0.098	0.141	0.109	0.123	0.098
SEM	0.019	0.031	0.034	0.023	0.024

Percent Cell Death	Concentration (ug/ml)				
	80	**60**	**40**	**30**	**20**
Set 1	97.391	79.231	31.132	39.716	-129.07
Set 2	11.864	87.94	-48.077	43.046	-98.462
Set 3	38.983	91.96	13.462	-22.517	-106.154
AVERAGE	76.531	80.142	45.872	18.699	-26.531
SEM	22.041	3.556	23.844	17.413	25.576

y-int	-250.482			
slope	77.961	**IC50**	47	ug/ml
R2	0.93			

Table 8.10: The ELISA reading results of MDA-MB-453 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-453	Concentration (ug/ml)				
Propolis-2	**30**	**50**	**60**	**100**	**120**
Set 1	0.045	0.042	0.045	0.018	0.014
Set 2	0.108	0.093	0.095	0.06	0.027
Set 3	0.118	0.079	0.073	0.053	0.027
Set 4	0.093	0.073	0.101	0.058	0.028
AVERAGE	0.091	0.072	0.079	0.047	0.024
SEM	0.016	0.011	0.013	0.01	0.003

MDA-MB-453	Concentration (ug/ml)				
DMSO	**30**	**50**	**60**	**100**	**120**
Set 1	0.128	0.052	0.179	0.222	0.071
Set 2	0.186	0.134	0.213	0.205	0.092
Set 3	0.125	0.185	0.176	0.214	0.077
Set 4	0.115	0.191	0.082	0.086	0.047
AVERAGE	0.139	0.141	0.163	0.182	0.072
SEM	0.016	0.032	0.028	0.032	0.009

Percent Cell Death	Concentration (ug/ml)				
	30	**50**	**60**	**100**	**120**
Set 1	64.844	19.231	74.86	91.892	80.282
Set 2	41.935	30.597	55.399	70.732	70.652
Set 3	5.6	57.297	58.523	75.234	64.935
Set 4	19.13	61.78	-23.171	32.558	40.426
AVERAGE	34.532	48.936	51.534	74.176	66.667
SEM	11.289	9.047	19.138	10.956	7.38

y-int	-56.529			
slope	26.85	**IC50**	53	ug/ml
R2	0.92			

Table 8.11: The ELISA reading results of MDA-MB-468 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-468	Concentration (ug/ml)					
Propolis-1	**15**	**20**	**30**	**40**	**60**	**80**
Set 1	0.268	0.199	0.048	0.033	0.017	0.004
Set 2	0.331	0.053	0.233	0.172	0.032	-0.003
Set 3	0.335	0.292	0.301	0.204	0.076	-0.004
Set 4	0.325	0.293	0.233	0.244	0.142	0.009
AVERAGE	0.315	0.209	0.204	0.163	0.067	0.002
SEM	0.016	0.057	0.054	0.046	0.028	0.003

MDA-MB-468	Concentration (ug/ml)					
DMSO	**15**	**20**	**30**	**40**	**60**	**80**
Set 1	0.492	0.433	0.466	0.186	0.146	0.264
Set 2	0.373	0.362	0.423	0.372	0.342	0.212
Set 3	0.272	0.344	0.395	0.388	0.372	0.196
Set 4	0.375	0.263	0.25	0.555	0.606	0.045
AVERAGE	0.378	0.351	0.384	0.375	0.367	0.179
SEM	0.045	0.035	0.047	0.075	0.094	0.047

Percent Cell Death	Concentration (ug/ml)					
	15	**20**	**30**	**40**	**60**	**80**
Set 1	45.528	54.042	89.7	82.258	88.356	98.485
Set 2	11.26	85.359	44.917	53.763	90.643	101.415
Set 3	-23.162	15.116	23.797	47.423	79.57	102.041
Set 4	13.333	-11.407	6.8	56.036	76.568	80
AVERAGE	16.667	40.456	46.875	56.533	81.744	98.883
SEM	12.201	18.478	15.567	6.694	2.97	4.584

y-int	-104.079			
slope	45.398	**IC50**	30	ug/ml
R2	0.97			

Table 8.12: The ELISA reading results of MDA-MB-468 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-468	Concentration (ug/ml)					
Propolis-2	**25**	**30**	**50**	**60**	**100**	**120**
Set 1	0.119	0.075	0.036	0.06	0.01	0.014
Set 2	0.546	0.435	0.195	0.234	0.008	0.014
Set 3	0.447	0.35	0.188	0.236	0.048	0.004
Set 4	0.457	0.379	0.19	0.201	0.035	0.02
AVERAGE	0.392	0.31	0.152	0.183	0.025	0.013
SEM	0.094	0.08	0.039	0.042	0.01	0.003

MDA-MB-468	Concentration (ug/ml)					
DMSO	**25**	**30**	**50**	**60**	**100**	**120**
Set 1	0.301	0.234	0.891	0.873	0.931	0.09
Set 2	0.793	0.619	0.773	1.123	0.798	0.303
Set 3	0.83	0.859	0.606	0.985	0.606	0.425
Set 4	0.927	1.004	0.247	0.428	0.403	0.253
AVERAGE	0.713	0.679	0.629	0.852	0.685	0.268
SEM	0.14	0.168	0.14	0.15	0.115	0.069

Percent Cell Death	Concentration (ug/ml)					
	25	**30**	**50**	**60**	**100**	**120**
Set 1	60.465	67.949	95.96	93.127	98.926	84.444
Set 2	31.148	29.725	74.774	79.163	98.997	95.38
Set 3	46.145	59.255	68.977	76.041	92.079	99.059
Set 4	50.701	62.251	23.077	53.037	91.315	92.095
AVERAGE	45.021	54.345	75.835	78.521	96.35	95.149
SEM	5.306	7.404	13.481	7.233	1.835	2.747

y-int	-57.459				
slope	32.889	**IC50**	26	ug/ml	
R2	0.97				

Table 8.13: The ELISA reading results of T47D cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

T47D	Concentration (ug/ml)					
Propolis-1	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.045	0.064	0.039	0.097	0.365	0.563
Set 2	0.032	0.006	0.39	0.749	1.636	1.658
Set 3	0.035	0.025	0.758	0.946	1.697	2.067
Set 4	0.031	0.031	0.99	0.931	1.955	2.009
AVERAGE	0.036	0.032	0.544	0.681	1.413	1.574
SEM	0.003	0.012	0.209	0.2	0.356	0.349

T47D	Concentration (ug/ml)					
DMSO	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	1.152	2.009	2.511	2.554	2.452	2.159
Set 2	1.461	2.105	2.543	2.458	2.518	2.548
Set 3	1.466	1.679	2.233	2.137	2.668	2.483
Set 4	0.263	1.498	1.15	1.728	1.975	2.099
AVERAGE	1.086	1.823	2.109	2.219	2.403	2.322
SEM	0.284	0.142	0.327	0.186	0.15	0.113

Percent Cell Death	Concentration (ug/ml)					
	120	**100**	**60**	**50**	**30**	**25**
Set 1	96.094	96.814	98.447	96.202	85.114	73.923
Set 2	97.81	99.715	84.664	69.528	35.028	34.929
Set 3	97.613	98.511	66.055	55.732	36.394	16.754
Set 4	88.213	97.931	13.913	46.123	1.013	4.288
AVERAGE	96.685	98.245	74.206	69.311	41.199	32.214
SEM	2.007	0.523	16.146	9.443	14.988	13.147

y-int	-104.651			
slope	43.289	**IC50**	36	ug/ml
R2	0.98			

Table 8.14: The ELISA reading results of T47D cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

T47D	Concentration (ug/ml)					
Propolis-2	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.12	0.134	0.203	0.283	1.062	0.64
Set 2	0.06	0.252	0.391	1.502	1.724	1.616
Set 3	0.126	0.07	1.041	1.547	1.847	1.715
Set 4	0.085	0.069	0.585	1.05	1.935	2.164
AVERAGE	0.098	0.131	0.555	1.096	1.642	1.534
SEM	0.015	0.043	0.18	0.293	0.198	0.321

T47D	Concentration (ug/ml)					
DMSO	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.737	1.419	2.105	2.503	2.482	2.273
Set 2	1.022	2.07	1.999	2.324	2.352	2.298
Set 3	1.424	2.336	1.978	2.582	2.374	1.981
Set 4	1.357	1.269	1.967	2.186	1.823	2.216
AVERAGE	1.135	1.774	2.012	2.399	2.258	2.192
SEM	0.159	0.256	0.032	0.089	0.148	0.072

Percent Cell Death	Concentration (ug/ml)					
	120	**100**	**60**	**50**	**30**	**25**
Set 1	83.718	90.557	90.356	88.694	57.212	71.843
Set 2	94.129	87.826	80.44	35.37	26.701	29.678
Set 3	91.152	97.003	47.371	40.085	22.199	13.428
Set 4	93.736	94.563	70.259	51.967	-6.144	2.347
AVERAGE	91.366	92.616	72.416	54.314	27.281	30.018
SEM	2.096	1.771	7.976	10.454	11.243	13.202

y-int	-119.562			
slope	45.188	**IC50**	43	ug/ml
R2	0.96			

Table 8.15: The ELISA reading results of MCF7 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF7	Concentration (ug/ml)					
Propolis-1	**100**	**80**	**50**	**40**	**25**	**20**
Set 1	0.025	0.11	0.259	0.335	0.298	0.975
Set 2	0.056	0.154	0.533	1.137	1.659	1.637
Set 3	0.077	0.253	0.619	1.166	1.525	1.223
Set 4	0.063	0.166	0.502	1.044	1.334	1.338
AVERAGE	0.055	0.171	0.478	0.921	1.204	1.293
SEM	0.011	0.03	0.077	0.197	0.309	0.137

MCF7	Concentration (ug/ml)					
DMSO	**100**	**80**	**50**	**40**	**25**	**20**
Set 1	0.498	1.404	1.708	1.753	1.697	1.517
Set 2	0.634	1.742	1.757	1.767	1.719	1.562
Set 3	1.067	1.543	1.709	1.61	1.533	1.573
Set 4	1.078	0.901	1.225	1.132	1.248	1.106
AVERAGE	0.819	1.398	1.6	1.566	1.549	1.44
SEM	0.149	0.179	0.125	0.149	0.109	0.112

Percent Cell Death	Concentration (ug/ml)					
	100	**80**	**50**	**40**	**25**	**20**
Set 1	94.98	92.165	84.836	80.89	82.44	35.728
Set 2	91.167	91.16	69.664	35.654	3.49	-4.802
Set 3	92.784	83.603	63.78	27.578	0.522	22.25
Set 4	94.156	81.576	59.02	7.774	-6.891	-20.976
AVERAGE	93.284	87.768	70.125	41.188	22.272	10.208
SEM	0.723	2.308	4.862	13.406	18.163	11.122

y-int	-151.771				
slope	54.181	**IC50**	41	ug/ml	
R2	0.97				

Table 8.16: The ELISA reading results of MCF7 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MCF7	Concentration (ug/ml)					
Propolis-2	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.116	0.123	0.332	0.259	1.878	0.724
Set 2	0.053	0.099	0.351	1.036	1.735	2.044
Set 3	0.087	0.178	0.624	1.355	0.602	2.06
Set 4	0.099	0.15	0.71	1.358	0.7	0.397
AVERAGE	0.089	0.138	0.504	1.002	1.807	1.609
SEM	0.013	0.017	0.096	0.259	0.051	0.383

MCF7	Concentration (ug/ml)					
DMSO	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.907	1.16	2.113	2.175	2.09	2.049
Set 2	0.615	1.312	1.887	2.293	1.917	2.039
Set 3	0.741	0.952	1.613	1.853	0.996	1.41
Set 4	1.071	1.488	0.637	1.144	0.922	0.909
AVERAGE	0.834	1.228	1.563	1.866	1.481	1.602
SEM	0.099	0.114	0.325	0.258	0.304	0.275

Percent Cell Death	Concentration (ug/ml)					
	120	**100**	**60**	**50**	**30**	**25**
Set 1	87.211	89.397	84.288	88.092	10.144	64.666
Set 2	91.382	92.454	81.399	54.819	9.494	-0.245
Set 3	88.259	81.303	61.314	26.875	39.558	-46.099
Set 4	90.756	89.919	-11.46	-18.706	24.078	56.326
AVERAGE	89.329	88.762	67.754	46.302	-22.012	-0.437
SEM	0.861	2.095	19.622	19.668	11.379	22.883

y-int	-270.841				
slope	78.093	**IC50**	61	ug/ml	
R2	0.89				

Table 8.17: The ELISA reading results of HCC-1937 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

HCC-1937	Concentration (ug/ml)					
Propolis-1	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.63	0.69	0.552	0.555	0.433	0.443
Set 2	0.631	0.559	0.516	0.437	0.417	0.332
Set 3	0.427	0.52	0.387	0.463	0.099	0.291
Set 4	0.629	0.504	0.492	0.397	0.403	0.276
AVERAGE	0.579	0.568	0.487	0.463	0.338	0.336
SEM	0.051	0.042	0.035	0.034	0.08	0.038

HCC-1937	Concentration (ug/ml)					
DMSO	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.807	0.864	0.827	0.806	0.775	0.68
Set 2	0.775	0.86	0.81	0.81	0.732	0.704
Set 3	0.746	0.788	0.758	0.738	0.707	0.627
Set 4	0.833	0.772	0.745	0.771	0.713	0.689
AVERAGE	0.79	0.821	0.785	0.781	0.732	0.675
SEM	0.019	0.024	0.02	0.017	0.015	0.017

Percent Cell Death	Concentration (ug/ml)					
	30	**37.5**	**60**	**75**	**120**	**150**
Set 1	21.933	20.139	33.253	31.141	44.129	34.853
Set 2	18.581	35	36.296	46.049	43.033	52.841
Set 3	42.761	34.01	48.945	37.263	85.997	53.589
Set 4	24.49	34.715	33.96	48.508	43.478	59.942
AVERAGE	26.709	30.816	37.962	40.717	53.825	50.222
SEM	4.686	3.131	3.177	3.471	9.193	4.669

y-int	-28.656			
slope	16.334	**IC50**	123	ug/ml
R2	0.95			

Table 8.18: The ELISA reading results of HCC-1937 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

HCC-1937	Concentration (ug/ml)				
Propolis-2	**150**	**120**	**75**	**60**	**30**
Set 1	0.01	0.06	0.104	0.096	0.172
Set 2	0.022	0.169	0.324	0.445	0.781
Set 3	0.037	0.204	0.437	0.482	0.778
Set 4	0.052	0.245	0.319	0.563	0.588
AVERAGE	0.03	0.17	0.296	0.397	0.58
SEM	0.009	0.04	0.07	0.103	0.143

HCC-1937	Concentration (ug/ml)				
DMSO	**150**	**120**	**75**	**60**	**30**
Set 1	0.09	0.419	0.457	0.238	0.626
Set 2	0.129	0.43	0.515	0.39	0.601
Set 3	0.098	0.231	0.313	0.475	0.55
Set 4	0.147	0.187	0.223	0.505	0.361
AVERAGE	0.116	0.317	0.377	0.402	0.535
SEM	0.013	0.063	0.067	0.06	0.06

Percent Cell Death	Concentration (ug/ml)				
	150	**120**	**75**	**60**	**30**
Set 1	88.889	85.68	77.243	59.664	72.524
Set 2	82.946	60.698	37.087	-14.103	-29.95
Set 3	62.245	11.688	-39.617	-1.474	-41.455
Set 4	64.626	-31.016	-43.049	-11.485	-62.881
AVERAGE	74.138	46.372	21.485	1.244	-8.411
SEM	5.735	22.72	25.813	15.135	26.119

y-int	-190.347			
slope	50.278	**IC50**	119	ug/ml
R2	0.88			

Table 8.19: The ELISA reading results of MDA-MB-157 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-157	Concentration (ug/ml)					
Propolis-1	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.179	0.105	0.267	0.307	0.376	0.386
Set 2	0.232	0.161	0.215	0.497	0.474	0.689
Set 3	0.211	0.195	0.263	0.446	0.48	0.585
Set 4	0.233	0.158	0.299	0.175	0.484	0.369
AVERAGE	0.214	0.155	0.261	0.356	0.454	0.507
SEM	0.013	0.019	0.017	0.073	0.026	0.078

MDA-MB-157	Concentration (ug/ml)					
DMSO	**120**	**100**	**60**	**50**	**30**	**25**
Set 1	0.421	0.478	0.477	0.483	0.41	0.536
Set 2	0.459	0.46	0.454	0.465	0.484	0.426
Set 3	0.403	0.445	0.478	0.5	0.503	0.506
Set 4	0.413	0.413	0.421	0.439	0.413	0.427
AVERAGE	0.424	0.449	0.458	0.472	0.453	0.474
SEM	0.012	0.014	0.013	0.013	0.024	0.028

Percent Cell Death	Concentration (ug/ml)					
	120	**100**	**60**	**50**	**30**	**25**
Set 1	57.482	78.033	44.025	36.439	8.293	27.985
Set 2	49.455	65	52.643	-6.882	2.066	-61.737
Set 3	47.643	56.18	44.979	10.8	4.573	-15.613
Set 4	43.584	61.743	28.979	60.137	-17.191	13.583
AVERAGE	49.528	65.479	43.013	24.576	-0.221	-6.962
SEM	2.527	4.016	4.288	12.708	4.926	17.15

y-int	-144.434			
slope	43.383	**IC50**	88	ug/ml
R2	0.9			

Table 8.20: The ELISA reading results of MDA-MB-157 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-157	Concentration (ug/ml)					
Propolis-2	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.983	0.782	0.684	0.371	0.138	0.181
Set 2	0.88	0.905	0.953	0.742	0.341	0.287
Set 3	0.828	1.086	1.097	0.841	0.445	0.478
AVERAGE	0.897	0.924	0.911	0.651	0.308	0.315
SEM	0.046	0.088	0.121	0.143	0.09	0.087

MDA-MB-157	Concentration (ug/ml)					
DMSO	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	1.301	1.007	1.896	1.553	1.534	1.045
Set 2	1.303	1.34	1.654	1.711	1.487	1.353
Set 3	1.145	1.689	1.56	1.634	1.661	1.141
AVERAGE	1.25	1.345	1.703	1.633	1.561	1.18
SEM	0.052	0.197	0.1	0.046	0.052	0.091

Percent Cell Death	Concentration (ug/ml)					
	30	**37.5**	**60**	**75**	**120**	**150**
Set 1	24.443	22.344	63.924	76.111	91.004	82.679
Set 2	32.464	32.463	42.382	56.634	77.068	78.788
Set 3	27.686	35.702	29.679	48.531	73.209	58.107
AVERAGE	28.24	31.301	46.506	60.135	80.269	73.305
SEM	1.902	3.301	8.168	6.683	4.413	6.226

y-int	-86.302			
slope	33.19	**IC50**	61	ug/ml
R2	0.94			

Table 8.21: The ELISA reading results of BT-20 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

BT-20	Concentration (ug/ml)					
Propolis-1	**150**	**120**	**75**	**60**	**37.5**	**30**
Set 1	0.02	0.089	0.101	0.095	0.168	0.202
Set 2	0.004	0.082	0.189	0.743	0.683	0.731
Set 3	0.025	-0.011	0.566	0.4	0.91	0.642
Set 4	0.028	0.303	0.51	0.759	0.87	1.013
AVERAGE	0.019	0.116	0.342	0.499	0.658	0.647
SEM	0.005	0.066	0.115	0.158	0.171	0.168

BT-20	Concentration (ug/ml)					
DMSO	**150**	**120**	**75**	**60**	**37.5**	**30**
Set 1	0.146	0.139	0.362	0.962	0.335	0.359
Set 2	0.103	0.412	0.915	0.976	0.663	0.542
Set 3	0.126	0.738	0.921	0.945	0.777	0.948
Set 4	0.092	0.778	1.16	0.641	0.858	0.974
AVERAGE	0.117	0.517	0.84	0.881	0.658	0.706
SEM	0.012	0.15	0.169	0.08	0.115	0.152

Percent Cell Death	Concentration (ug/ml)					
	150	**120**	**75**	**60**	**37.5**	**30**
Set 1	86.301	35.971	72.099	90.125	49.851	43.733
Set 2	96.117	80.097	79.344	23.873	-3.017	-34.871
Set 3	80.159	101.491	38.545	57.672	-17.117	32.278
Set 4	69.565	61.054	56.034	-18.409	-1.399	-4.004
AVERAGE	83.761	77.563	59.286	43.36	0	8.357
SEM	4.821	12.199	7.872	20.164	12.818	15.497

y-int	-181.212			
slope	53.877	**IC50**	73	ug/ml
R2	0.94			

Table 8.22: The ELISA reading results of BT-20 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

BT-20	Concentration (ug/ml)				
Propolis-2	**120**	**100**	**60**	**50**	**30**
Set 1	0.084	0.45	0.933	1.06	1.188
Set 2	0.115	0.488	1.194	1.352	1.41
Set 3	0.056	0.625	1.219	1.186	1.184
AVERAGE	0.085	0.521	1.115	1.199	1.261
SEM	0.017	0.053	0.091	0.085	0.075

BT-20	Concentration (ug/ml)				
DMSO	**120**	**100**	**60**	**50**	**30**
Set 1	0.161	1.154	0.961	0.991	1.168
Set 2	0.139	1.537	0.817	0.525	0.546
Set 3	0.416	0.876	0.694	0.596	0.509
AVERAGE	0.21675	0.96525	0.6995	0.58725	0.61875
SEM	0.089	0.192	0.077	0.145	0.214

Percent Cell Death	Concentration (ug/ml)				
	120	**100**	**60**	**50**	**30**
Set 1	47.826	61.005	2.914	-6.963	-1.712
Set 2	17.266	68.25	-46.144	-157.524	-158.242
Set 3	86.538	28.653	-75.648	-98.993	-132.613
AVERAGE	60.784	46.024	-59.4	-104.172	-103.798
SEM	16.63	10.12	19.559	36.089	39.62

y-int	-595.77			
slope	135.493	**IC50**	117	ug/ml
R2	0.88			

Table 8.23: The ELISA reading results of MDA-MB-361 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-361	Concentration (ug/ml)					
Propolis-1	**15**	**20**	**30**	**40**	**60**	**80**
Set 1	1.109	1.177	0.827	0.918	0.692	0.549
Set 2	1.923	1.847	1.143	0.857	0.954	0.981
Set 3	2.292	1.497	1.469	0.842	1.11	0.084
Set 4	2.292	1.711	0.969	1.161	1.138	0.779
AVERAGE	1.904	1.558	1.102	0.945	0.974	0.598
SEM	0.279	0.146	0.138	0.074	0.102	0.193

MDA-MB-361	Concentration (ug/ml)					
DMSO	**15**	**20**	**30**	**40**	**60**	**80**
Set 1	2.158	0.795	1.783	1.835	2.389	2.032
Set 2	2.048	1.515	2.072	1.403	2.334	2.264
Set 3	1.872	1.807	2.045	1.886	1.514	1.678
Set 4	2.313	2.109	0.824	1.297	2.103	1.735
AVERAGE	2.098	1.557	1.681	1.605	2.085	1.927
SEM	0.093	0.281	0.293	0.149	0.2	0.136

Percent Cell Death	Concentration (ug/ml)					
	15	**20**	**30**	**40**	**60**	**80**
Set 1	48.61	-48.05	53.617	49.973	71.034	72.982
Set 2	6.104	-21.914	44.836	38.917	59.126	56.67
Set 3	-22.436	17.156	28.166	55.355	26.684	94.994
Set 4	0.908	18.872	-17.597	10.486	45.887	55.101
AVERAGE	9.247	-0.064	34.444	41.121	53.285	68.967
SEM	12.82	14.16	13.825	8.679	8.253	8.039

y-int	-104.55			
slope	39.224	**IC50**	51	ug/ml
R2	0.92			

Table 8.24: The ELISA reading results of MDA-MB-361 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

MDA-MB-361	Concentration (ug/ml)					
Propolis-2	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.455	0.317	0.219	0.401	0.127	0.03
Set 2	0.779	0.797	0.489	0.692	0.432	0.053
Set 3	1.757	1.289	0.724	0.471	0.39	0.059
Set 4	1.748	1.353	0.622	0.489	0.269	0.099
AVERAGE	1.185	0.939	0.514	0.513	0.305	0.06
SEM	0.334	0.242	0.109	0.063	0.069	0.014

MDA-MB-361	Concentration (ug/ml)					
DMSO	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.74	0.825	1.908	1.797	1.631	1.382
Set 2	0.927	0.885	1.713	1.684	1.557	1.54
Set 3	1.728	1.737	1.586	1.614	1.442	1.594
Set 4	1.924	1.857	0.88	0.773	1.628	0.878
AVERAGE	1.33	1.326	1.522	1.467	1.565	1.349
SEM	0.292	0.273	0.224	0.234	0.044	0.163

Percent Cell Death	Concentration (ug/ml)					
	30	**37.5**	**60**	**75**	**120**	**150**
Set 1	38.514	61.576	88.522	77.685	92.213	97.829
Set 2	15.965	9.944	71.454	58.907	72.254	96.558
Set 3	-1.678	25.792	54.351	70.818	72.954	96.299
Set 4	9.148	27.141	29.318	36.74	83.477	88.724
AVERAGE	10.902	29.186	66.229	65.031	80.511	95.552
SEM	7.425	9.431	11.004	7.828	4.114	1.799

y-int	-148.202			
slope	49.003	**IC50**	57	ug/ml
R2	0.94			

Table 8.25: The ELISA reading results of BT-474 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

BT-474	Concentration (ug/ml)					
Propolis-1	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	1.454	1.752	0.715	0.779	1.04	1.433
Set 2	1.878	0.402	0.119	0.51	1.517	0.682
Set 3	0.547	1.032	1.333	0.702	0.138	-0.012
AVERAGE	1.293	1.062	0.722	0.664	0.898	0.701
SEM	0.393	0.39	0.35	0.08	0.404	0.417

BT-474	Concentration (ug/ml)					
DMSO	**30**	**37.5**	**60**	**75**	**120**	**150**
Set 1	2.128	1.787	1.21	1.612	1.697	1.734
Set 2	2.317	2.219	1.176	0.728	2.337	1.015
Set 3	2.744	2.19	1.989	1.73	2.464	2.679
AVERAGE	2.396	2.065	1.458	1.357	2.166	1.809
SEM	0.182	0.139	0.266	0.316	0.237	0.482

Percent Cell Death	Concentration (ug/ml)					
	30	**37.5**	**60**	**75**	**120**	**150**
Set 1	31.673	1.959	40.909	51.675	38.715	17.359
Set 2	18.947	81.884	89.881	29.945	35.088	32.808
Set 3	80.066	52.877	32.981	59.422	94.399	100.448
AVERAGE	46.035	48.571	50.48	51.069	58.541	61.249
SEM	15.218	19.093	14.576	7.298	15.688	21.075

y-int	14.512			
slope	9.069	**IC50**	50	ug/ml
R2	0.93			

Table 8.26: The ELISA reading results of BT-474 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

BT-474	Concentration (ug/ml)				
Propolis-2	**30**	**60**	**75**	**120**	**150**
Set 1	0.513	0.418	0.45	0.172	0.064
Set 2	0.508	0.91	0.911	0.384	0.157
Set 3	0.346	1.081	0.835	0.134	0.088
AVERAGE	0.456	0.803	0.732	0.23	0.103
SEM	0.055	0.199	0.143	0.078	0.028

BT-474	Concentration (ug/ml)				
DMSO	**30**	**60**	**75**	**120**	**150**
Set 1	0.283	0.455	1.81	1.667	0.468
Set 2	0.319	0.676	1.276	0.428	0.503
Set 3	0.359	1.73	1.22	0.823	0.224
AVERAGE	0.32	0.954	1.435	0.973	0.398
SEM	0.022	0.393	0.188	0.365	0.088

Percent Cell Death	Concentration (ug/ml)				
	30	**60**	**75**	**120**	**150**
Set 1	-81.272	8.132	75.138	89.682	86.325
Set 2	-59.248	-34.615	28.605	10.28	68.787
Set 3	3.621	37.514	31.557	83.718	60.714
AVERAGE	-42.5	15.828	48.99	76.362	74.121
SEM	20.787	17.454	12.334	21.299	6.204

y-int	-296.566			
slope	76.61	**IC50**	92	ug/ml
R2	0.95			

Table 8.27: The ELISA reading results of ZR-75-1 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

ZR-75-1	Concentration (ug/ml)					
Propolis-1	**120**	**100**	**80**	**75**	**60**	**50**
Set 1	0.064	0.039	0.097	0.365	0.563	0.914
Set 2	0.006	0.39	0.749	1.636	1.658	2.378
Set 3	0.025	0.758	0.946	1.697	2.067	2.441
Set 4	0.031	0.99	0.931	1.955	2.009	2.378
AVERAGE	0.032	0.544	0.681	1.413	1.574	2.028
SEM	0.012	0.209	0.2	0.356	0.349	0.372

ZR-75-1	Concentration (ug/ml)					
DMSO	**120**	**100**	**80**	**75**	**60**	**50**
Set 1	2.009	2.511	2.554	2.452	2.159	2.369
Set 2	2.105	2.543	2.458	2.518	2.548	2.307
Set 3	1.679	2.233	2.137	2.668	2.483	2.505
Set 4	1.498	1.15	1.728	1.975	2.099	2.386
AVERAGE	1.823	2.109	2.219	2.403	2.322	2.392
SEM	0.142	0.327	0.186	0.15	0.113	0.041

Percent Cell Death	Concentration (ug/ml)					
	120	**100**	**80**	**75**	**60**	**50**
Set 1	96.814	98.447	96.202	85.114	73.923	61.418
Set 2	99.715	84.664	69.528	35.028	34.929	-3.078
Set 3	98.511	66.055	55.732	36.394	16.754	2.555
Set 4	97.931	13.913	46.123	1.013	4.288	0.335
AVERAGE	98.245	74.206	69.311	41.199	32.214	15.217
SEM	0.523	16.146	9.443	14.988	13.147	13.349

y-int	-349.542				
slope	93.018	**IC50**	73	ug/ml	
R2	0.94				

Table 8.28: The ELISA reading results of ZR-75-1 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

ZR-75-1	Concentration (ug/ml)				
Propolis-2	**120**	**100**	**60**	**50**	**25**
Set 1	0.103	0.049	0.589	0.526	0.701
Set 2	0.125	0.08	0.635	0.684	0.755
Set 3	0.078	0.271	0.681	0.785	0.838
AVERAGE	0.102	0.133	0.635	0.665	0.765
SEM	0.014	0.069	0.027	0.075	0.04

ZR-75-1	Concentration (ug/ml)				
DMSO	**120**	**100**	**60**	**50**	**25**
Set 1	0.198	0.091	0.94	1.066	0.93
Set 2	0.32	0.409	1.258	1.318	0.886
Set 3	0.191	0.716	0.958	1.139	0.72
AVERAGE	0.236	0.405	1.052	1.174	0.845
SEM	0.042	0.18	0.103	0.075	0.064

Percent Cell Death	Concentration (ug/ml)				
	120	**100**	**60**	**50**	**25**
Set 1	47.98	46.154	37.34	50.657	24.624
Set 2	60.938	80.44	49.523	48.103	14.786
Set 3	59.162	62.151	28.914	31.08	-16.389
AVERAGE	56.78	67.16	39.639	43.356	9.467
SEM	3.319	8.18	4.894	5.017	10.107

y-int	-93.951			
slope	33.28	**IC50**	76	ug/ml
R2	0.89			

Table 8.29: The ELISA reading results of hTERT-HME1 cell numbers when treated with Propolis-1 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

hTERT-HME1	Concentration (ug/ml)					
Propolis-1	**25**	**30**	**50**	**60**	**100**	**120**
Set 1	0.118	0.05	0.05	0.039	0.036	0.045
Set 2	0.548	0.557	0.624	0.093	0.041	0.092
Set 3	0.893	0.719	0.538	0.387	0.153	0.087
Set 4	0.723	0.713	0.371	0.155	0.082	0.087
AVERAGE	0.571	0.51	0.396	0.169	0.078	0.078
SEM	0.166	0.158	0.127	0.077	0.027	0.011

hTERT-HME1	Concentration (ug/ml)					
DMSO	**25**	**30**	**50**	**60**	**100**	**120**
Set 1	0.237	0.304	0.272	0.922	1.02	0.399
Set 2	0.85	0.78	0.909	1.161	1.048	0.38
Set 3	0.889	1.065	1.091	1.15	1.122	0.542
Set 4	1.093	1.219	1.099	0.389	0.251	0.152
AVERAGE	0.767	0.842	0.843	0.906	0.86	0.368
SEM	0.185	0.201	0.195	0.181	0.204	0.081

Percent Cell Death	Concentration (ug/ml)					
	25	**30**	**50**	**60**	**100**	**120**
Set 1	50.211	83.553	81.618	95.77	96.471	88.722
Set 2	35.529	28.59	31.353	91.99	96.088	75.789
Set 3	-0.45	32.488	50.687	66.348	86.364	83.948
Set 4	33.852	41.509	66.242	60.154	67.331	42.763
AVERAGE	25.554	39.43	53.025	81.347	90.93	78.804
SEM	9.338	11.055	9.368	7.789	5.99	9.075

y-int	-91.469			
slope	38.216	**IC50**	41	ug/ml
R2	0.84			

Table 8.30: The ELISA reading results of hTERT-HME1 cell numbers when treated with Propolis-2 and DMSO. Percent cell death and IC_{50} value were calculated according to this data.

hTERT-HME1	Concentration (ug/ml)				
Propolis-2	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.684	0.638	0.516	0.216	0.093
Set 2	0.679	0.636	0.561	0.314	0.097
Set 3	0.379	0.581	0.585	0.434	0.066
AVERAGE	0.581	0.618	0.554	0.321	0.085
SEM	0.101	0.019	0.02	0.063	0.01

hTERT-HME1	Concentration (ug/ml)				
DMSO	**37.5**	**60**	**75**	**120**	**150**
Set 1	0.421	0.565	0.839	0.252	0.255
Set 2	0.516	1.059	0.874	0.576	0.275
Set 3	0.59	1.13	0.591	0.524	0.288
AVERAGE	0.509	0.918	0.768	0.55	0.273
SEM	0.049	0.178	0.089	0.1	0.01

Percent Cell Death	Concentration (ug/ml)				
	37.5	**60**	**75**	**120**	**150**
Set 1	-62.47	-12.92	38.498	14.286	63.529
Set 2	-31.589	39.943	35.812	45.486	64.727
Set 3	35.763	48.584	1.015	17.176	77.083
AVERAGE	-14.145	32.68	27.865	41.636	68.864
SEM	23.729	15.844	9.888	9.346	3.539

y-int	-190.891			
slope	50.9	**IC50**	114	ug/ml
R2	0.88			

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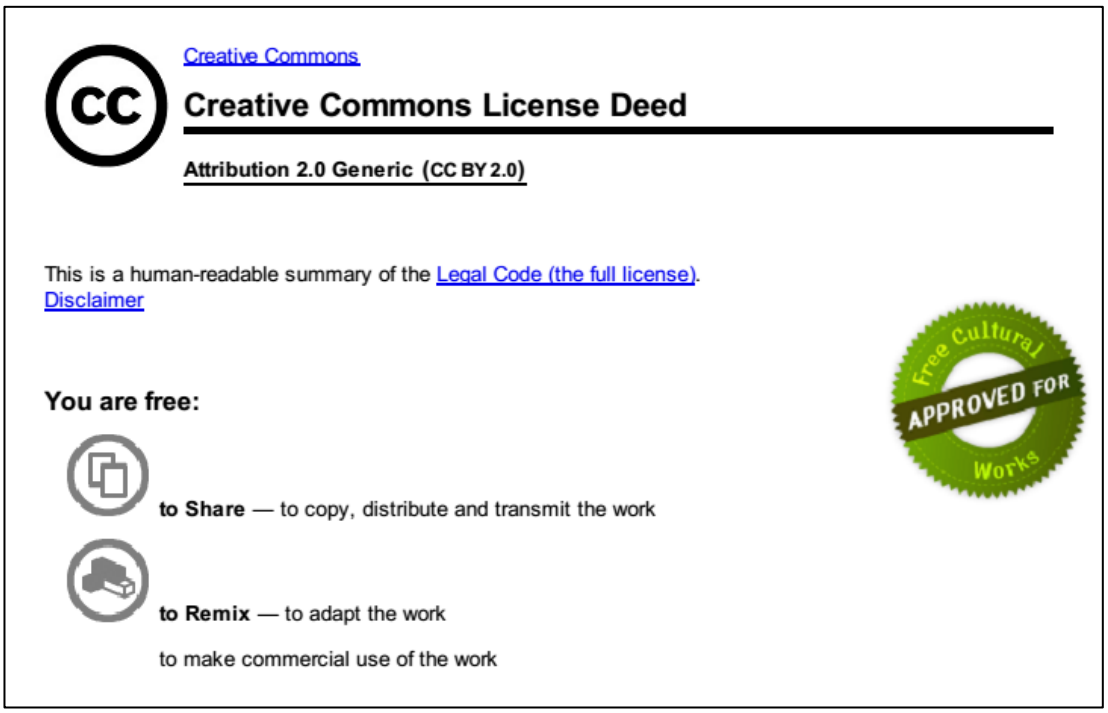

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