BIOCHEMICAL, CYTOTOXIC AND GENOTOXIC EFFECTS OF AESCIN ON HUMAN LYMPHOCYTES AND HL-60 PROMYELOID LEUKEMIA CELL LINE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

JULY 2005

Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

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July 2005, 159 pages

Aescin is a mixture of several acidic triterpenoid saponin glycosides found in the extracts of the horse chestnut tree. Horse chestnut, *Aesculus Hipoocastanum*, is one of the 25 domestic species of Aesculus that are mostly large, ornamental shade trees. Although known to be poisonous, the nuts of the horse chestnut are used by Amerindians, after detoxification. Horse chestnuts are said to have several traditional medicinal usages including even cancer.

In this study the biochemical, genotoxic, and cytotoxic effects of aescin was studied using isolated lymphocytes, whole blood lymphocytes and HL-60 promyeloid leukemia cell lines.

Cytotoxicity of aescin was examined by trypan blue viability staining of the cells in culture treated with varying aescin concentrations. It was observed that aescin was cytotoxic at all concentrations, for all cell types studied, except whole blood lymphocytes, where it was not cytotoxic at 10^{-9} and 10^{-10} M concentrations.

Genotoxicity of aescin was examined by sister chromatid exchange and micronucleus. The genotoxic effect of Aescin was observed to be more significant over isolated lymphocytes compared to other cell lines. On the otherhand, aescin at 10^{-8} M and lower concentrations were observed to be non-genotoxic over whole blood lymphocytes whereas this concentration was considerably toxic for isolated lymphocytes and for HL-60 cell lines.

Apoptotic properties of aescin were determined by DNA fragmentation, cytochrome *c* release and negative NAPO staining. All the Aescin concentrations tested resulted in apoptosis over HL-60 cell lines, whereas necrosis was not observed. However, isolated lymphocytes showed both apoptosis and necrosis upon treatment with 10^{-6} M to 10^{-8} M aescin, exhibiting apoptosis only at 10^{-9} M and 10^{-10} M.

Biochemical effects of aescin were investigated by following GST and NAT enzyme activities. An increase in GST enzyme activity was observed over all cell lines treated with increasing aescin concentrations for 72 hours. Whereas NAT activity was decreased upon treatment with aescin in similar manner.

Key Words: Glutathione *S*-transferases, N- acetyltransferases, HL-60 promyelocytic leukemia cell line, Isolated Lymphocytes, SCE, Micronuclei, Apoptosis, DNA ladder, Western blotting.

ÖZ

ESSİNİN İNSAN LENFOSİTLERİ VE HL-60 PROMYELOİD LÖSEMİ HÜCRE HATTI ÜZERİNE BİYOKİMYASAL, SİTOTOKSİK VE GENOTOKSİK ETKİLERİ

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Temmuz 2005, 159 Sayfa

Essin, at kestanesi ağacının, *Aesculus Hipoocastanum,* tüm kısımlarında bulunan, bir takım asidik, triterpenoid saponin glikositlerin karışımı olan bir etken maddedir. At kestanesi, Aesculus cinsine ait 25 domestic türden gösterişli bir ağaçtır. Her ne kadar meyveleri zehirli olsa da Amerikan Yerlileri tarafından toksinlerinden arındırılarak kullanılmaktadır. At kestanesinin kanser de dahil olmak üzere çeşitli geleneksel tibbi kullanımları mevcuttur.

Bu çalışmada farklı essin konsantrasyonlarının, izole edilmiş limfositler, tam kan limfositleri ve HL-60 promyeloid leukemia hücre hatları üzerine biyokimyasal, sitotoksik ve genotoksik etkileri incelenmiştir.

Essinin, sitotoksik etkisi tripan mavisi hücre canlılık testiyle çalışılmıştır. Tüm hücre hatları için essinin çalışılan konsantrasyonlarının (10^{-4}-10^{-10} M) sitotoksik olduğu, ancak sadece tam kan lenfositleri için 10^{-9} ila 10^{-10} M konsantrasyonlarda sitotoksik olmadığı görülmüştür.

Essinin, genotoksik etkisi kardeş kromozom değişimi ve mikro çekirdek induksiyon tayini teknikleriyle incelenmiştir. Diğer hücre hatları ile kıyaslandığında essinin genotoksik etkisinin, izole edilmiş lenfositler üzerinde daha belirgin olduğu gözlenmiştir. Öte yandan 10^{-8} M ve daha düşük essin konsantrasyonlarının tam kan lenfositler üzerinde genotoksik olmadığı gözlenmiştir. Oysa bu konsantrasyonların izole edilmiş lenfositler ve HL-60 hücre hatları üzerinde ciddi derecede genotoksik olduğu gözlenmiştir.

Essinin apoptotik özellikleri DNA parçalanması, sitokrom *c* salınması ve negatif NAPO boyamasıyla izlenmiştir. Test edilen tüm essin konsantrasyonları HL-60 hücre hattı üzerinde apoptosise neden olmuş ancak bu hücre hattı üzerinde nekrosis görülmemiştir. İzole edilen lenfositlerde, 10^{-6} M'dan 10^{-8} M'a kadar essin konsantrasyonlarında apoptosisin yanısıra nekrosis de görülmesine rağmen, 10^{-9} M ve 10^{-10} M essin konsantrasyonlarında yalnızca apoptosis görülmüştür.

Essinin biyokimyasal etkileri GST ve NAT enzim aktiviteleri ile izlenmiştir. GST aktivitesi bütün hücre hatlarında essinin artan konsantrasyonlarıyla artış göstermiştir. NAT enzim aktivitesinde ise aynı koşullarda düşüş gözlenmiştir.

Anahtar kelimeler: Glutatyon *S*-transferazler, N-acetiltransferazlar, HL-60 Pro lösemi hücre hatları, İzole Lenfositler, SCE, Mikroçekirdek, Apoptoz, DNA parçalanması, Western Blot.

To My Mother, My Husband and My Daughters

ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Dr. Mesude İşcan and Prof. Dr. Tülin Güray for their encouragement, valuable advise and continued guidance throughout this study.

I wish to express my thanks to Prof. Dr. Orhan Adalı, Prof. Dr. Zeliha Büyükbingöl, Assoc. Prof. Dr. Nursen Çoruh and Prof. Dr. Leyla Açık their valuable suggestions and corrections.

I am grateful to Prof. Dr. Mehmet Öztürk for providing the polyclonal antibodies and NAPO monoclonal antibodies.

Special thanks to Ahmet Ata Özcimen for his help in growing the HL-60 cell culture line at Hacettepe University. I am also extremely grateful to Aslı Sezer Özçelik in MTA for her support, encouragement and her great friendship. Special thanks to all of my friends, particularly Tolga Yıldırım, Evren Koban, Hava Dinç, Pembegül Uyar for their great friendship and encouragement.

Finally, I would like to express my sincere gratitude to all family members namely, my father, my husband, my brother, and my sister for their great support, patience and encouragement during the long period of my study. Particularly my lovely daughters, İdil and İpek Kolukısa were my great enthusiasm throughout my doctoral studies. I am also extremely grateful to my mother Nimet Topsoy for her valuable support.

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LIST OF ABREVIATIONS

AcCAR	:	Acetyl Carnitine
AcCoA	:	Acetyl Coenzyme A
AP	:	Alkaline phosphatase
APS	:	Ammonium persulfate
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
Bis	:	N,N′-methylene bisacrylamide
BrdUrd	:	5-bromo-2-deoxyuridine
BSA	:	Bovine serum albumin
CAT	:	Carnitine Acetyl Transferase
CDNB	:	1-Chloro-2,4-dinitrobenzene
DNA	:	Deoxyribonucleic Acid
DMAB	:	Dimethylaminobenzaldehyde
DMSO	:	Dimethyl sulfoxide
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
FPG	:	Flouresence plus giemsa
GSH	:	Reduced glutathione
GST	:	Glutathione-S-transferase
KCl	:	Potassium chloride
KCN	:	Potassium cyanide
HCSE	:	Horse chestnut seed extract
NAPO	:	Non in apoptosis
NaOH	:	Sodium Hydroxide
NBT	:	Nitro blue tetrazolium

MN	:	Micronucleus
MI	:	Mitotic Index
PABA	:	p-aminobenzoic acid
PBS	:	Phoshate buffered saline
sPBS	:	Steril Phoshate buffered saline
PHA-L	:	Phytohemaglutinin L
PVDF	:	Polyvinylidene difluoride
SCE	:	Sister-chromatid exchange
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Polyacrylamide gel electrophoresis
SMZ	:	Sulfamethazine
TBS	:	Tris-buffered saline
TE	:	Tris- Ethylenediaminetetraacetic acid
TEMED	:	N,N,N′,N′-tetramethylenediamine
Tris base	:	Tris(hydroxymethyl) aminomethane
TBS	:	Tris buffered saline
TTBS	:	Tris-buffered saline containing Tween-20

CHAPTER I

1.1 INTRODUCTION

1.1.1 The Horse Chestnut (Aesculus Hippocastanum L)

The horse chestnut *(Aesculus hippocastanum L.)* is one of the species among 25 domestic species of Aesculus, mostly large, ornamental shade, round-shaped tree growing to about 25-30 meters in height. The trunk is thick and usually short. The large leaves are divided into five or seven leaflets and have finely toothed margins. The tree is native to northern and central Asia, but is now widespread throughout the world (Martin *et al.,* 1990). Horse chestnut is also known as Rosskastanien (German), Buckeye, Chestnut, Escine, Hippocastani Cortex, Hippocastani Flos, Hippocastini folium, Hippocastani Semen, Marron European, Marron d'Inde, or Spanish chestnut. The fruit of the horse chestnut is a spiny, green sphere about 4 cm in diameter. When it ripens, the thick green husk splits to reveal one or larger, smoother, shiner brown nuts/seeds (often referred to as conkers). Whole horse chestnut is classified as an unsafe herb by the FDA and all members of this genus should be considered potentially poisonous, but after special preparation for raw horse chestnuts to remove the toxins, they are relatively safe to use.

1.1.2 Medical Usage of Plants

Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago (Solecki *et al.,* 1975). From that point the development of traditional medical systems incorporating plants as a means of therapy can be traced back only as far as recorded documents of their likeness. However, the value of these systems is much more than a significant anthropologic or archeological fact. Their value is as a methodology of medicinal agents, which, according to the World Health Organization (WHO), almost 65% of the world's population has incorporated into their primary modality of health care (Farnsworth *et al.,* 1985). The goals of using plants as sources of therapeutic agents are a) to isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine; b) to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, weraparnil, and amiodarone, which are based, respectively, on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol c) to use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline; and d) to use the whole plant or part of it as a herbal remedy, e.g., cranberry, Echinacea, feverfew, garlic, ginkgo biloba, horse chestnut, St. John's wort.

The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated at 250.000 (Ayensu *et al.,* 1978). Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Verpoorte, 2000). With high throughput screening methods becoming more advanced and available, these numbers will change, but the primary discriminator in evaluating one plant species versus another is the matter of approach to finding leads. There are some broad starting points to selecting and obtaining plant material of potential therapeutic interest.

Plants have an advantage in this area based on their long-term use by humans (often hundreds of thousands of years) (Daniel *et al.,* 2001).

1.1.2.1 Traditional Usage of Horse Chestnut (Aesculus Hippocastanum L) for Treatment of some Diseases

Horse chestnuts have been used for centuries in traditional medicine and other purposes. Although known to be poisonous, the nuts of the horse chestnut are roasted by Amerindians, then peeled, mashed and leached in lime water to remove the toxins before used.

Traditionally, people have taken horse chestnut seeds for the treatment of varicose veins, hemorrhoids, vein inflammation (phlebitis), diarrhea, fever, and enlarged prostate (Martin *et al.,* 1990; Diehm *et al.,* 1996). Horse chestnut bark has traditionally been used for the treatment of malaria and dysentery (Martin *et al.,* 1990; Diehm *et al.,* 1996). Horse chestnut leaf has traditionally been used to treat eczema, menstrual pain, soft tissue swelling, cough, arthritis and rheumatism (Martin *et al.,* 1990; Diehm *et al.,* 1996). As a topical application, horse chestnut bark is sometimes used to treat lupus and skin ulcers (Diehm *et al.,* 1996).

1.1.2.2 Medical Usage of Horse Chestnut (Aesculus Hippocastanum L) for Treatment of some Diseases

Serious German research of this herb began in the 1960s and ultimately led to the approval of a horse chestnut extract for vein diseases of the legs. Horse chestnut is the third most common single herb product sold in Germany, after ginkgo and St. John's wort (Dustmann *et al.,* 1984). A daily dose of 600 mg of standardized HCSE will contain between 100-150 mg of aescin (Erdlen, 1989). In Japan, an

injectable form of horse chestnut is widely used to reduce inflammation after surgery or injury; however, it is not available in the United States, and it may present safety risks (Erdlen, 1989).

Horse chestnut seed extract is administered orally for the treatment of chronic venous insufficiency (CVI) and a variety of other conditions, or as a topical preparation for the treatment of lupus and skin ulcers (Bombardelli *et al.,* 1996).

CVI-affected legs have an accumulation of white blood cells (leucocytes). The presence of these leucocytes results in the activation and release of certain enzymes (elastase and hyaluronidase). These enzymes are involved in breakdown of protein within the capillary (very small veins) walls (Martinez *et al.,* 2003), which in turn leads to swelling and the skin changes associated with CVI. Cellular studies have shown that one of the components of HCSE; aescin can stop the activity of the elastase and hyaluronidase enzymes (Bisler *et al.,* 1986; Diehm *et al.,* 1992). Aescin also has a diuretic effect (promotes the passing of urine) (Diehm *et al.,* 1992; Cloarec, 1992). Aescin appears to reduce swelling and inflammation (Newall *et al.,* 1996; Sirtori, 2001). It's not exactly clear how aescin might work, but theories include "sealing" leaking capillaries, improving the elastic strength of veins, preventing the release of enzymes (known as glycosaminoglycan hydrolases) that break down collagen and open holes in capillary walls, decreasing inflammation, and blocking other various physiological events that lead to vein damage (Kreysel *et al.,* 1983; Sirtori, 2001).

Individuals with severe kidney problems should avoid horse chestnut (Grasso *et al.,* 1976; Rothkopf *et al.,* 1977; Reynolds,-1989). In addition, inject able forms of horse chestnut can be toxic to the liver (Takegoshi *et al.,* 1986). The safety of horse chestnut in young children and pregnant or nursing women has not been established. However, 13 pregnant women were given horse chestnut in a controlled study without noticeable harm (Schulz *et al.,* 1998). Furthermore, studies in pregnant rats and rabbits found no injury to embryos at doses up to 10 times the human dose (25-100mg/day), and changes of questionable significance at 30 times the dose

(Hitzenberger, 1989). Horse chestnut should not be combined with anticoagulant, or blood-thinning, drugs, as it may amplify their effect (Brooks, 1995; Brinker, 1998).

Horse chestnut seed extract can be obtained as an "over the counter" product from health food shops, supermarkets, pharmacies, etc. A medical herbalist may also prescribe horse chestnut seed extract. The practice of herbal medicine is not currently regulated by legislation in New Zealand, however many herbal medicine practitioners are affiliated with a self-regulated professional body (such as the New Zealand Association of Medical Herbalists).

Horse chestnut extracts are said to have alternative analgesic, antipyretic, astringent, expectonant, narcotic, tonic, sternutatory, and vasoconstricting properties. They have been used as cataplasm for mammary indurations and other types of topical ulcer and even cancer. There is limited result about action of aescin on cancer treatment although aescin structure contains terpenoid. In experimental studies, terpenoids have prevented the occurrence of cancer in many tissues including lung, prostate, breast, colon, stomach, pancreas, liver and skin (Kawari *et al.,* 1996; So *et al.,* 1996; Reddy *et al.,* 1997). The presence of terpenoids and flavonoids having antioxidant activity in coriander and of related polyphenols in the other extracts might be responsible for their observed antioxidant activity (Kinsella *et al.,* 1993; Takuro *et al.,* 1999).

Cancer is one of stress related disorder. In order to evaluate effect of aescin on treatment and/or prevention of cancer, further studies are needed to establish.

1.1.3 The Components of Horse Chestnut Extract

The active chemical constituents aescin (aka escin); a complex mixture of triterpenoid saponin glycosides, coumarin derivatives; aesculin, fraxin, scopolin, flavonoids; quercetin, kaempferol, astragalin, isoquercetrin, rutin, leucocyanidine,

essential oils; oleic acid, linoleic acid, other; amino acids (adenosine, adenine, guanine), allantoin, argyrin, carotin, choline, citric acid, epicatechin, leucodelphinindin, phyosterol, resin, scopoletin, tannins, and uric acid (Duke, 1985; Newall *et al.,* 1996; Robbers, 1999; Bedard, 2002; Martinez *et al.,* 2003; Pittler *et al.,* 2004). Aescin can be fractionated into beta-aescin, an easily crystallizable mixture, and alpha-aescin, which is water-soluble (Neumann-Mangoldt, 1979). Saponins are steroid or triterpene glycosides widely distributed in the plant and marine animal kingdoms (Lacaille-Dubois, and Wagner, 1996). Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating cholesterol. When saponins are near cell membranes, hey create pore-like structures in the membrane which cause the membrane to burst. Haemolysis of erythrocytes is an example of this phenomenon, which is a property of certain, but not all, saponins. The terpenoids are oxygen containing analogous of terpenes which are hydrocarbons containing one or more C=C bonds.

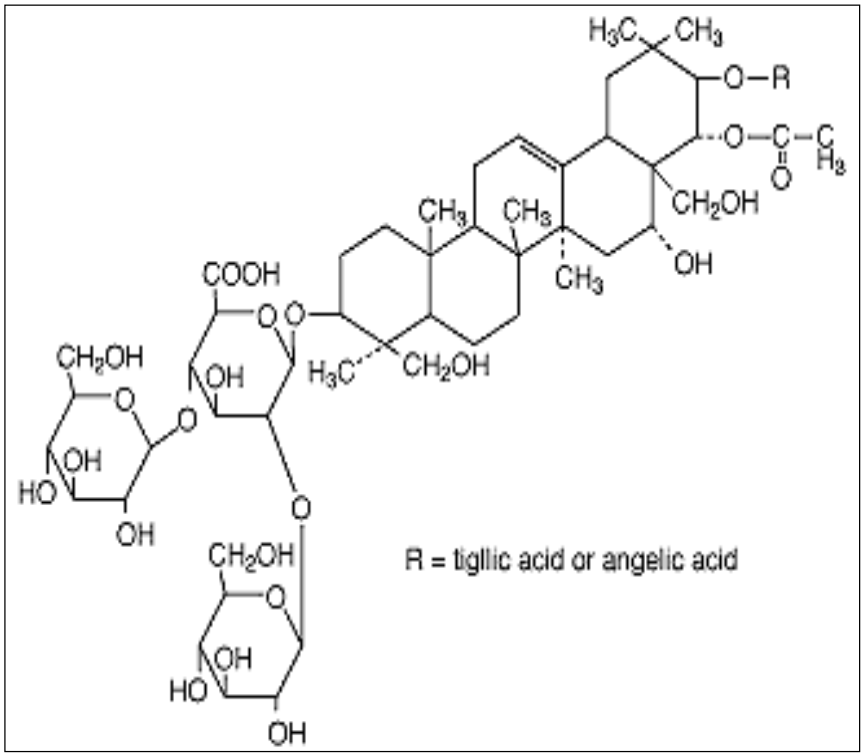
1.1.3.1 Aescin (β-Aescin)

The principal extract and medicinal constituent of horse chestnut seed is aescin, a mixture of triterpenoid saponin glycosides. Aescin [aescigenin-(2-methyl-3-acetoxy-butyrate)-(2-xyloside-4-glucosidoglucuronoside)] is a mixture of several acidic triterpenoid saponin glycosides found in the extracts of the horse chestnut tree, Aesculus Hippocastanum.

The molecular structure of aescin containing aescigenin, 2 glucose, 1 glucuronic acid and 1 triglic acid, is pentacyclic triterpenoid saponin is presented in Figure 1.

Aescin decreases the transcapillary filtration of water and proteins (Neumann-Mangoldt, 1979; Pittler *et al.,* 1998). It has been used to treat a wide variety of inflammatory and edematous conditions, to reduce swelling associated with bruises,

fractures, brain trauma, post-operative and post-traumatic soft tissue swelling, and acute thrombophlebities (Pittler *et al.,* 1998; Diehm *et al.,* 1992). It is not exactly clear how aescin might work, but theories include "sealing" leaking capillaries, improving the elastic strength of veins, preventing the release of enzymes (known as glycosaminoglycan hydrolases) that break down collagen and open holes in capillary walls, decreasing inflammation, blocking other various physiological events that lead to vein damage (Kreysel *et al.,* 1983; Bougelet *et al.,* 1998; Sirtori, 2001). Aescin reduces lysosomal enzyme activity by stabilizing lysosomal membranes and limiting enzyme release (Cloarec, 1992; Newall *et al.,* 1996).



Formula: $C_{54}H_{84}O_{23}$ **Molecular Weight:** 1101.

Figure 1 The molecular structure of Aescin.

In clinical use, aescin is administered orally and intravenously, and gel formulations are widely used in percutaneous therapy.

Orally administered aescin is either sparingly absorbed by the gastrointestinal tract or undergoes a substantial first-pass effect. It has an absorption half-life of about one hour, peak serum levels in two to three hours, peak response in terms of vascular protection in 16 to 20 hours, and an elimination half-life of about 20 hours. Aescin is filtered through the glomerula and is neither reabsorbed nor excreted in tubules. It binds to plasma proteins, preventing nephrotoxicity. The amount of free aescin excreted through the glomerula is small, and the concentration too low to have cytotoxic effects on the tubular epithelium (Kreysel *et al.,* 1983). Aescin is fully dialyzable (Bougelet *et al.,* 1998). The coumarin aesculine has a moderate diuretic effect. Aesculin is reported to possess microvasculokinetic activity, and is used in the treatment of cellulites and hair loss (Diehm *et al.,* 1996).

1.1.4 Toxicity of Horse Chestnut Extract (Aescin)

Unprocessed horse chestnut seeds containing esculin are extremely poisonous and can cause severe gastrointestinal (vomiting, diarrhea) reactions and neurotoxic reactions (muscle twitching, weakness, loss of coordination, dilated pupils, paralysis and stupor) (Neumann-Mangoldt, 1979) and may increase the risk of bleeding due to its ability to stop blood clots forming (antithrombotic) (Bedard, 2002), if they are eaten. The seeds of the horse chestnut should not be confused with the edible seeds of the sweet chestnut; Castanea sativa L., (Bombardelli *et al.,* 1996).

Horse chestnut seeds are therefore processed to remove the toxic component, resulting in purified horse chestnut seed extract (HCSE). Trade names for HCSE include Aescorin, Catarrh cream, Noricaven, Hoevenol, Reparil, Tecura, Venastat, Venosin, Venoplant, Venostat, Venostasin, Venalot, Veinotonyl 75 (Martin *et al.,* 1990, Pittler *et al.,* 1998).

The most common dosage of horse chestnut extract is 300 mg twice daily, standardized to contain 50 mg aescin per dose, for a total daily dose of 100 mg aescin. After good results have been achieved, the dosage can be reduced by about half for maintenance.

In animal studies, horse chestnut and its principal ingredient aescin have shown a low degree of toxicity, producing no measurable effects when taken at dosages seven times higher than normal (Hitzenberger, 1989; Schulz, 1998). Dogs and rats have been treated for 34 weeks with this herb without harmful effects.

Although aescin is known as safety component of horse chestnut, the dosage is important for all compounds before usage. Beside this knowledge, to follow the cancer treatment and/or to prevent ability of aescin, biochemical, cytotoxic and genototoxic affect of aescin on both lymphocytes used for the type of normal cells and HL-60 cells used for the type of cancer cells were tested in this study.

1.2 SCREENING OF AESCIN ACTION

1.2.1 In vitro Screening of Aescin on cancer

1.2.1.1 Cancer

Cancer is a group of many related diseases that originate in genome. Although there are many kinds of cancers, their common feature is loss of cell division control. Oncogenes and loss or mutation in tumor suppressor genes play major role in that abnormal cell growth. Oncogenes are normal regulatory genes whose activity increased as a consequence of genetic alteration. Only one allele of an oncogene needs to be change. The effect is dominant. Beside oncogenes the other players are suppressor genes that are coding for cell division inhibitory proteins whose function is lost in cancer. Oncogenes and repressors cooperate in

carcinogenesis. As a consequence of extra cells a mass of tissue called tumor forms (King, 2000).

The life history of a cancer can be divided into stages. Carcinogenesis, the process of cancer development from normal cells, is divided into initiation and promotion stages. Progression describes the additional changes occurring after initial cancer cells have been formed. Risk factors associated with cancer are tobacco use, diet, ultraviolet radiation, alcohol, ionizing radiation, chemicals and hormone replacement therapy as well as life style.

According to the 2005 estimates of American Cancer Society, cancer is the second leading cause of death among the other diseases. Prostate cancers among men and breast cancer among women are the two primary cancers according to the incidence rate. Although leukemia ranks at the ninth position for incidence rate (%3), it rises up to sixth position when death rates are considered.

1.2.1.2 Leukemia

Leukemia is a cancer of blood-forming cells. It occurs when immature or mature cells multiply in an uncontrolled manner in the bone marrow. It is classified as lymphocytic or myeloid, according to the type of cell that is multiplying abnormally, and either acute, signifying rapidly progressing disease with a predominance of highly immature (blastic) cells, or chronic, which denotes slowly progressing disease with greater numbers of more mature cells (Cook, 1996).

When incidence of leukemia is considered they cover only 2-3 % of all cancer types but the death rates increase to 4% according to 2005 estimates of American Cancer Society. That interesting data makes the people study on the factors causing leukemia and the treatment strategies for the disease. Although the causes of leukemia are not fully understood, certain factors are known to increase the risk of

developing the disease. Among these are exposure to radiation, genetic and congenital factors, exposure to mutagenic chemicals and infection with certain viruses (Lin *et al.,* 1999).

Chronic leukemia is a cancer in which too many mature white blood cells are produced and build up in the body. Chronic leukemia develops slowly, often with no symptoms, and remains undetected for a long while. It is classified into two subgroups, according to the cell type that is affected, Chronic Lymphocytic and Chronic Myeloid Leukemia (Lin *et al.,* 1999).

Acute leukemias seem to arise when the normal process of cell maturation (also called differentiation) is interrupted at a very early (blast) stage of cell's life. The result is an accumulation of immature, nonfunctional, leukemic blast cells in the bone marrow and to a lesser extends in the blood. Acute leukemias are fatal if not treated. The distinction between the two subtypes acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML) is that blast cells associated with ALL are those that would mature into lymphoid cells, whereas blasts that would mature into myeloid cells are associated with AML.

Acute lymphocytic leukemia (ALL) is a clonal malignant disorder characterized by the excessive proliferation and arrest of differentiation of cells and the loss of programmed cell death (apoptosis). It is the most common pediatric malignancy. Nearly 80% of all pediatric leukemias are ALL. Chromosomal abnormalities (Downs' Syndrome, Klinifelter's Syndrome), DNA repair disorders, and environmental exposures are the main causes of ALL.

According to French-American-British (FAB) Classification, ALL has been divided into subgroups L1 to L3 as described in Table 1.

Acute Myeloid Leukemia (AML) is the second subtypes of acute leukemia. Myeloid cells are affected in AML. The clinical features of AML result from the lack of the normal products of hemopoiesis (production of blood cells) as a consequence

of the proliferation of immature leukemic cells. Even one mutation in DNA is enough for the formation of AML. It covers %90 of all acute leukemias. AML has been divided into subgroups M0 to M7 according to FAB Classification as described in Table 2.

Table 1 French-American-British Classification of Acute Lymphocytic Leukemia

L1	The lymphocytes tend to be small, with little cytoplasm and regularly shaped nuclei. More mature in appearance than other types.
L2	The lymphoblasts appear more immature, varying in size and nuclear shape.
L3	The lymphoblasts tend to be large, with abundant cytoplasm and similarly shaped nuclei.

Table 2 French-American-British Classification of Acute Myeloid Leukemia

Designation	Description
MO	Undifferentiated Leukemia
M1	AML with immature cells
M2	AML with some mature cells
M3	Acute Promyelocytic Leukemia
M4	Acute Myelomonocytic Leukemia
M5	Acute Monocytic Leukemia
M6	Erythroleukemia
M7	Acute Megakaryotic Leukemia

1.2.2 Cell Line Usage in vitro Studies

Cell lines that biologically resemble AML have been available since the 1980s and allowed careful study of the growth of leukemia cells under controlled conditions and of the effects of antileukemic agents (Devita *et al.,* 2001).

1.2.2.1 HL-60 (Acute Promyelocytic Leukemia)

One of the cell lines resembling AML is HL-60. The HL-60 cell line is a promyelocytic cell line derived from peripheral blood cells from a 36-year-old Caucasian female with acute promyelocytic leukemia. Because of its morphological and genetic characteristics it is classified as FAB-M3 (Table 3) (Gallagher *et al.,* 1979). Typical characteristic of promyelocytic leukemia is owned by HL-60 cells, which is translocation between chromosomes 15 and 17 (t (15; 17)) (Dalton *et al.,* 1988). The HL-60 cell line, derived from a single patient with acute promyelocytic leukemia, provides a unique in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage (Collins, 1987). HL-60 cells grow in suspension culture. The predominant cell population consists of neutrophilic promyelocytes.

1.2.2.2 Lymphocytes

There are several kinds of lymphocytes (although they all look alike under the microscope), each with different functions to perform. The most common types of lymphocytes are B-Lymphocytes ("B cells") which are responsible for making antibodies; T lymphocytes ("T cells"). There are several subsets of T lymphocytes. They are inflammatory T cells that recruit macrophages and neutrophils to the site of infection or other tissue damage; cytotoxic T lymphocytes (CTLs) that kill virus-infected and, perhaps, tumor cells; helper T cells that enhance the production of antibodies by B cells.

Although bone marrow is the ultimate source of lymphocytes, the lymphocytes that will become T cells migrate from the bone marrow to the thymus where they mature. Both B cells and T cells also take up residence in lymph nodes, the spleen and other tissues where they encounter antigens; continue to divide by mitosis; mature into fully functional cells.

1.2.3 Activation of Detoxification Systems

Detoxification enzyme activity is the most reliable way to follow the toxicity effect of compounds, biochemically.

Every individual is unique. This includes not only personal appearance and preferences but, most importantly, responses to the environment and susceptibility to diseases. Genetic variability gives a species the ability to adapt to the environment over time and at this point in human evolution, has resulted in various environmental response phenotypes. For example, some individuals may not respond to pain medications while others fall into a deep sleep at a similar dose. Some people may develop cancer after exposure to a hazardous chemical while co-workers with similar exposures never develop tumors. These susceptibilities and responses can often be linked to an individual's ability to metabolize toxicants or repair damage from toxicants encountered in the environment. An individual's response to environmental chemicals will vary depending on the presence of DNA sequence variations (polymorphisms) within critical genes in that individual. These genetic polymorphisms may affect the level of expression, the structure, or the catalytic activity of metabolic or DNA repair enzymes, thereby influencing toxicant susceptibility. Some specific examples include the following: (1) the relationship between enzymes that activate (e.g. CYP, N-acetyltransferases) or detoxification of (e.g. glutathione S-transferases) carcinogens; (2) the susceptibility of individuals with base excision and nucleotide excision repair genes (XRCC1 or XPD) polymorphisms to various types of genotoxic damage (Miller III *et al.,* 2001).

Foreign compounds even drugs are metabolized through the enzyme systems which may be divided into Phase I and Phase II enzyme systems, involved in metabolic transformation and conjugation reactions, respectively.

1.2.3.1 Glutathione S-Transferases (GSTs)

Glutathione S-transferases (GSTs) (EC 2.5.1.18) are one of the important members of the phase II enzyme system, which are involved in the detoxification of a broad spectrum of electrophilic, hydrophobic compounds both from endogenous and exogenous origin, by catalyzing the nucleophilic attack of the sulfur atom of GSH on electrophilic groups in a second substrate. Accordingly, GSTs represent one of the body's most important defense mechanisms against exogenously and endogenously generated electrophilic intermediates by preventing these metabolites from possible interaction with macromolecules. There are 4 types of Glutathione S-transferase isozyme; alpha, mu, pi, theta. GST isoenzymes are thought to function as antioxidants by conjugating GSH to endogenously produced products of lipid peroxidation such as alkenes, aldehydes and epoxides (Pickett and Lu, 1989).

The central role of glutathione-dependent enzymes in the detoxification of toxic electrophiles implies that their expression with tumors will be an important aspect of tumor sensitivity to cytotoxic drugs (Chabner, 1982). In addition to their constitutive expression in tumors, there is a growing body of evidence which indicates that these enzymes become over expressed in tumor cells following exposure to cytototic chemicals (Batist *et al.,* 1986). Recently, active oxygen metabolites have been increasingly thought to have important biological significance, especially in relation to carcinogenesis (Cerutti, 1985).

The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is less reactive than the parental compound (Chasseaund, 1979), and therefore the action of GST generally results in

detoxification. GSH conjugation is thought to be of value not only because it increases the solubility of hydrophobic xenobiotics and, by preventing their partitioning into membrane lipid, decreases their half-life in the body.

The conjugation reaction between GSH and xenobiotics represents the first step in the synthesis of mercapturic acids, an important group of excretion products that were first identified more than 100 years ago in the urine of animals treated with bromobenzene. As shown in Figure 2, following conjugation with GSH, the subsequent steps include the conversion of the glutathione conjugate to the corresponding cysteine conjugate following sequential removal of glutamate and glycine. Cysteine conjugate is either metabolized to a mercapturate by acetylation or cleaved to a mercaptan by β-lyase (C-S lyase). In addition to the mercapturic acid pathway, methylation of the thiol to form the methylthio-containing metabolite and the glucuronydation of the mercaptan to form the thioglucuronide represent important metabolic steps for the biotransformation of the cysteine conjugate (Boyland and Chasseaud, 1969 and Pickett and Lu, 1989).

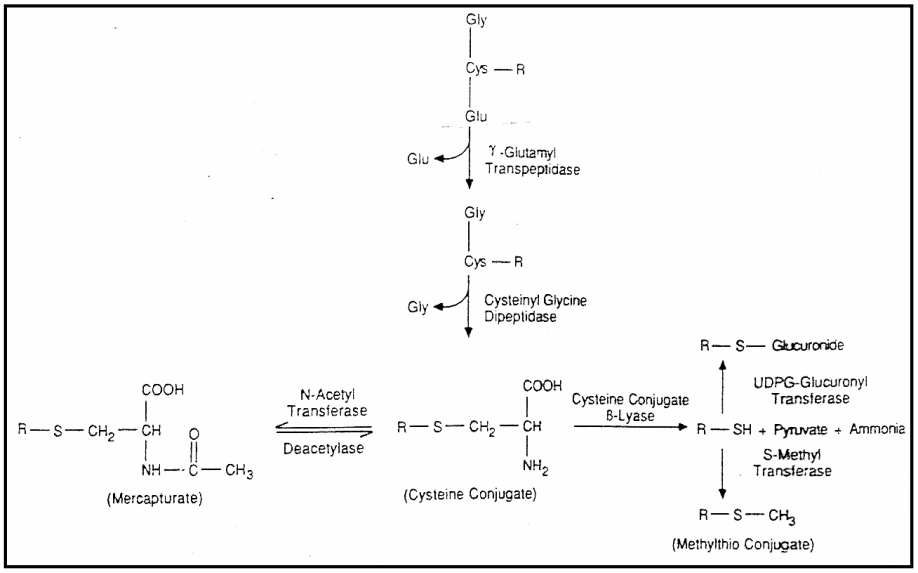


Figure 2 Metabolism of glutathione conjugates (Pickett and Lu, 1989).

1.2.3.2 N-acetyltransferases (NATs)

N-acetyltransferases, which belong to Phase II of xenoboitic transformation, are involved in reactions of acetylation of arylamines, components of both carcinogenic compounds and various drugs. In human two isoforms, NAT1 and NAT2 with different acetylation phenotypes (rapid or slow), characterized by different response to certain drugs are observed. Thus N-acetlylation polymorphism determines individual variability in biotransformation of aromatic amines (4-aminobiphenyl), hydrazines, including acetoxy esters, arylamines, and heterocyclic amines (carcinogen responsible for the urinary bladder cancer) present in tobacco smoke and supplied with the diet. In the human genome, three genetic loci have been found, strictly connected with NAT expression. One of them, NATP, seems to be a pseudogene coding a non-functional protein. The nucleotide sequence of the other two expressed genes NAT1 and NAT2 is identical in 87%. Different nucleotide changes occur in NAT sequences: nucleotide substitutions (all NAT2, some NAT1 alleles), insertions or deletions (NAT1). So far, 24 different NAT1 alleles and 26 different NAT2 alleles have been found (Reszka, 2002).

NATs catalyze the metabolism of xenobiotics and carcinogens by transferring an acetyl group to the compounds in question. As compared to the CYPs and GSTs the variety of molecules the NATs metabolize is limited, consisting mainly of aromatic and heterocyclic amines and hydrazines. However, several of these are strong carcinogens, and since some of them are used in industrial processes, e.g. in the rubber and dye industries, while some may be formed during cooking or cigarette smoking, they may be highly relevant to human cancer. The NATs comprise one of the major enzyme systems catalyzing their metabolism, and it is therefore reasonable to assume that the NATs play a role in human carcinogenesis (Smith *et al.,* 1995)

Much of the evidence on the role human NATs in carcinogenesis has been obtained in studies on bladder cancer. Epidemiological studies in which bladder cancer patients and controls with high exposure to aromatic amines were analyzed

for their NAT phenotype have shown that the slow acetylator phenotype is associated with an increased risk of bladder cancer (Cartwright *et al.,* 1982). This suggests that acetylation has a protective role in bladder carcinogenesis. This is also consistent with the facts that (i) for smokers the level of 4-aminophenyl hemoglobin adducts is higher among slow acetylators than among fast acetylators (Vineis *et al.,* 1990) and (ii) for smokers or workers exposed to aromatic amines the urine of slow acetylators is more mutagenic than that of fast acetylators. This indirect evidence suggests that acetylation reactions inactivate carcinogens, at least in the case of bladder cancer.

In spite of this evidence the role of NATs in human carcinogenesis is not straightforward. First, the fast acetylation phenotype does not appear to protect against colon cancer; indeed, the contrary appears true (Smith *et al.,* 1995). Second, at least in some in vitro systems, NAT seems to activate carcinogens (Grant *et al.,* 1992). In this connection it has been found that, with regard to some carcinogens, NAT may actually catalyze 0-acetylation, which appears to be an activating step, instead of the normal N-acetylation (Hein, 1988).

1.2.4 Cytotoxicity

Cytotoxicity is the quality of being poisonous to cells. This can be a chemical substance or an immune cell.

Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death, whereas survival assays measure the end result of such metabolic perturbations which may be either cell recovery or cell death. In order to follow cytotoxic effect of compounds on cell viability, there are many techniques are used such as MTT assay, dye exclusion technique.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction is one of the most frequently used methods for measuring cell proliferation and neural cytotoxicity.

1.2.4.1 Cell Viability

Dye exclusion technique is the other type of test to follow cell viability. In this technique, Tryphan blue is a specific dye used. The basis of Tryphan blue staining is the exclusion of dyes by living cells, so that under microscope only the dead cells appear as blue, whereas the living cells remain colorless because of their dye exclusion ability.

In addition, cytotoxicity of any compound is also determined by apoptosis or/and necrosis, briefly cell death. The cytotoxic effect of most immuno- and chemotherapeutic agents in vitro and in vivo depends on induction of apoptosis in susceptible tumor cells.

1.2.4.2 Apoptosis and Necrosis

One of the most widely used principles in cancer treatment is causing the cancer cells die by apoptosis. Apoptosis (also called programmed cell death) is the term used to describe how cells die under a variety of physiological and pathological conditions (Arends *et al,* 1991).

Apoptosis is defined as genetically programmed mechanism of the cell death resulting in the morphological changes, which are characterized by blebbing of the plasma membrane, shrinkage of the cell, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies. Also, cleavage of

the DNA between histone octamers to generate the so-called nucleosomal ladder of fragments occurs during programmed cell death.

A second mode of cell death, necrosis, is the classically defined form of cell death. Apoptosis and necrosis completely differ in many respects; apoptosis is a genetically regulated event whereas necrosis occurs when the cells were subjected to harsh conditions and when they are damaged. Both physiological cell death (apoptosis) and, in some cases, accidental cell death (necrosis) involve a two-step process. At a first level, numerous physiological and some pathological stimuli trigger an increase in mitochondrial membrane permeability. The mitochondria release apoptogenic factors through the outer membrane and dissipate the electrochemical gradient of the inner membrane. Mitochondrial permeability transition (PT) involves a dynamic multiprotein complex formed in the contact site between the inner and outer mitochondrial membranes. The PT complex can function as a sensor for stress and damage, as well as for certain signals connected to receptors. Inhibition of PT by pharmacological intervention on mitochondrial structures or mitochondrial expression of the apoptosis-inhibitory oncoprotein Bcl-2 prevents cell death, suggesting that PT is a rate-limiting event of the death process. At a second level, the consequences of mitochondrial dysfunction (collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione, and release of soluble intermembrane proteins) entails a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity (necrosis) and/or the activation of specific apoptogenic proteases (caspases) by mitochondrial proteins that leak into the cytosol (cytochrome *c*, apoptosis-inducing factor) with secondary endonuclease activation (apoptosis). The relative rate of these two processes (bioenergetic catastrophe versus protease and endonuclease activation) determines whether a cell will undergo primary necrosis or apoptosis (Arends *et al.,* 1990). The acquisition of the biochemical and ultrastructural features of apoptosis critically relies on the liberation of apoptogenic proteases or protease activators from mitochondria. The fact that mitochondrial events control cell death has major implications for the development of

cytoprotective and cytotoxic drugs (Kroemer *et al*., 1998). As mentioned, during apoptosis the cell fragments into apoptotic bodies which are then phagocytosed by macrophages or other neighboring cells. So, the cellular components of the cell do not leak out as the cell membrane is not damaged. Thus, the neighboring cells are not damaged with the proteases or other cellular enzymes. Apoptosis only affects the cell itself. However in necrosis the damaged cells swell and lyse. Therefore the cellular components spill to extracellular matrix causing inflammation, which doesn't occur in apoptosis. So, necrosis affects the neighboring tissues (Cotter *et al.,* 1990).

1.2.4.2.1 Apoptosis: Time for death

A healthy organism is an exquisitely integrated collection of differentiated cells, which maintain a balance between life and death. Apoptosis (also called programmed cell death; PCD) is a regulatory process which plays a critical role in normal tissues homeostasis and balance of regulating immune cells in circulation. Some cells are irreplaceable, some cells complete their functions and are then sacrificed, and some cells live a finite lifetime to be replaced by yet another generation. A failure of cells to fulfill their destiny has catastrophic consequences on an organism. PCD is the last phase of a cell's destiny (Steller, 1995).

Apoptosis is the controlled disassembly of a cell. It is defined as genetically programmed. When apoptosis occurs on schedule, neighboring cells and, more importantly, the organism itself, are not adversely affected. Apoptosis gone awry, however, has dire effects (Thompson, 1995). When apoptosis occurs in irreplaceable cells, as in some neurodegenerative disorders, functions critical to the organism are lost. When cells fail to undergo apoptosis after serving their purpose, as in some autoimmune disorders, escaped cells adversely affect the organism. When cells become renegade and resist apoptosis, as in cancer, the outlaw cells create a dire situation for the organism. Mistiming of, or errors in, apoptosis can have devastating

consequences on development. Apoptotic fidelity is, therefore, critical to the well-being of an organism.

There are three different mechanisms of apoptosis. The first is that apoptosis triggered by internal signals (the mitochondrial pathway). The second is that apoptosis triggered by external signals (the death receptor pathway) and the last one is that apoptosis triggered by reactive oxygen species (caspase independent pathway) (Fıgure 3).

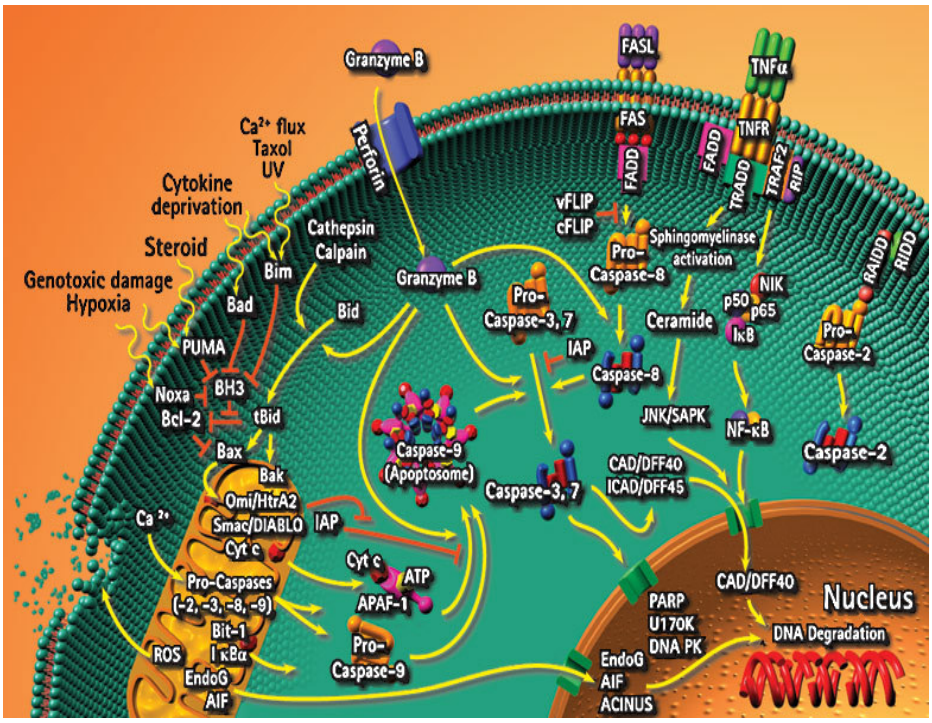


Figure 3 shows the overall apoptotic pathways, the death receptor pathway, the mitochondrial pathway, the ER pathway and caspase-independent pathways (Steller, 1995).

The first two mechanisms are under control of caspases which become activated upon different signals and pathways. Cytosolic Aspartate-Specific Proteases, called CASPases, are responsible for the deliberate disassembly of a cell into apoptotic bodies. Caspases are members of a cysteine protease family and they are able to cleave their substrates on the carboxyl side aspartate residues (Desagher *et al.,* 1999).

Caspases are synthesized as zymogens and they become active upon the cleavage of N-terminal prodomain at aspartate residues. Caspases may either self-activate themselves or activate other caspases (Salvesen and Dixit, 1997).

Mitochondria have crucial role in apoptosis. The mitochondrial pathway of apoptosis is triggered by either the internal signals such as DNA damage, cytoskeleton disruption and reactive oxygen species or by external signals such as death receptors. Release of cytochrome *c* from the mitochondria can trigger a series of events leading to the activation of effector caspases (Reed, 1997). For example, procaspase-9 is activated when complexed with dATP, APAF-1 and extramitochondrial cytochrome *c* (Li *et al.,* 1997). Following activation, caspase-9 can initiate apoptosis by cleaving additional caspases (Figure 3). Besides activating the mitochondrial pathway, death receptors may also trigger mitochondria independent pathway which involves the direct activation of initiator caspases through caspases 8 or caspase 10 (Luo *et al.,* 1998).

1.2.4.2.2 Necrosis

Necrosis normally results from a severe cellular insult. Both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosolic and organelle contents into the surrounding environment. Immune cells are attracted to the area and begin producing cytokines that generate an inflammatory response. Thus, cell death in the absence of an inflammatory response may be the best way to

distinguish apoptosis from necrosis (Darzynkiewicz *et al.,* 1997). Necrotic cells are characterized by cell and nuclear swelling patchy chromatin condensation, swelling of mitochondria, vacuoliation of cytoplasm and plasma membrane repture leading to the formation of "ghost-like" cells and, finally, dissolution of DNA (karyolysis). Other techniques that have been used to distinguish apoptosis from necrosis in cultured cells and in tissue sections include detecting PS at the cell surface with annexin V binding, DNA laddering, and staining cleaved DNA fragments that contain characteristic ends. At the extremes, apoptosis and necrosis clearly involve different molecular mechanisms. It is not clear if there is cellular death involving both the molecular mechanisms of apoptosis and those of necrosis. Cell death induced by free radicals, however, may have characteristics of apoptosis and necrosis (Kane *et al.,* 1993).

1.2.5 Genotoxicity

Genotoxicity is defined as DNA damage (Tennat *et al.,* 1987) and the purpose of genotoxicity testing in a screening sense are to identify agents, which can adversely affect the genetic material of the cells. This property may indicate the potential for a number of adverse effects in mammals, one of which is carcinogenicity. Many agents exhibit in mammals (Kier, 1988). Cytogenetic methodologies offer some of the most sensitive approaches for detecting genotoxic effects of mutagenic carcinogens.

The most common test is the Ames Salmonella typhimurium assay for evaluation of mutagenicity of chemicals in vitro (Ames *et al.,* 1975), as it is reproducible, and can be performed at a relatively low cost.

The most common test systems used to asses if humans are exposed to genotoxic compounds are scoring of either chromosome aberrations (chromatid breaks and chromatid fragments; interchange and interchange between

chromosomes; interchange and intrachange between chromatids; an increase or decrease in the chromosome number; ring chromosomes etc.), sister chromatid exchanges (SCEs) which is a sensitive and well established cytogenetic indicator of DNA damage or micronuclei as an indirect indicator of chromosome damage (Wulf *et al.,* 1990).

1.2.5.1. Sister Chromatid Exchanges (SCEs)

SCEs with its high sensitivity, is a widely accepted genotoxicity test, which is used as an indicator of mutagenic and carcinogenic potency of a given chemical. It yields few false positive or false negative results in vivo and in vitro and thereby provides valuable information on the genotoxic potential of a compound. Also an increase in SCEs is usually recorded at doses below those, which cause chromosomal aberrations (Latt *et al.,* 1982).

Although Taylor (1958) established the concept of SCE, recent staining techniques have greatly facilitated the performance of this assay. The general method relies upon the phenomenon of bromodeoxyuridine (BrdUrd) incorporation into DNA in place of thymidine. After two rounds of cell division, the two chromatids are asymmetrically labeled with BrdUrd which means M2 chromosomes posses one chromatid bifilarly (both DNA strands in one chromatid contain BrdUrd) substituted with BrdUrd and its sister unifiliarly (only one DNA strand in one chromatid contains BrdUrd) substituted with BrdUrd (Perry and Evan, 1975). Such chromatids stain differently with Giemsa (Ikushima and Wolff, 1974) the fluorochrome Hoechst 33258 (and various other fluorescent dyes like acridine orange and quinacrine) (Latt, 1973) or a combination of fluorochrome plus Giemsa- the FPG technique (Perry and Wolff, 1974). SCEs are visualized as light-dark exchanges between chromatids stained after two mitotic divisions in their metaphase stage. SCEs appear to respond to genotoxic compounds in a dose-related fashion and can offer clear evidence of DNA exposure (Wolff, 1977).

Among the staining techniques mentioned above the FPG technique seems to be the best one. The fluorescence technique for human chromosomes using the dye Hoechst 33258 was first described by Latt (1973). This dye normally fluoresces intensely when bound to poly (dA-dT), but when bound to poly (dA-BrdUrd) the fluorescence of the dye is quenched. After two rounds of replication in the presence of BrdUrd, followed by staining in Hoechst 33258, the unifiliarly-substituted chromatids fluoresced more brightly than the bifiliarly substituted chromatids.

Sister chromatid exchanges are readily observed. Though this technique provided degree of resolution for the detection of SCEs which is previously not possible, the dye demonstrated a remarkable degree of photo-instability, so that the image fades very rapidly. Perry and Wolff (1974) then demonstrated the FPG technique that has several advantages over the others. The number of SCEs observed when cells are stained with Hoechst 33258, with or without subsequent Giemsa staining, is greater than the number observed after staining in Giemsa alone or after autoradiography. In addition all chromosomes in a cell can be scored whereas after autoradiography only the larger chromosomes are Scrabble. The FPG technique, which is also used in this thesis offers all the advantages of the fluorescent technique discovered by Latt, but also has the additional advantages of producing non-fading permanently stained cells that do not require fluorescence microscopy and photography (Perry and Wolff, 1974). The molecular mechanism of the formation of SCEs is not known, but it is believed that SCEs are formed during DNA replication by a switch between parent and daughter DNA strands (Painter *et al.,* 1980). SCE involves a symmetrical exchange of one locus between sister chromatids, which does not result in an alteration of overall chromosome morphology. Such exchanges are originally demonstrated by Taylor (1958) in auto-radiographic studies of plant chromosomes and are seen as switch in radioactive label between chromatids at the second (M2) mitosis after H^3- thymidine labelling at the S phase of the M1 cycle. Later studies showed that many of the SCEs observed in auto-radiographic experiments are induced by the endogenous radiation from the incorporated tritium (Brewen and Peacok, 1969; Gibson and Prescott, 1972) that the frequency of SCE is increased in cells exposed to x-rays (Marin and Prescott, 1964; Gatti and Olivieri,

1974), ultraviolet light (Kato, 1973; Rommelare *et al.,* 1973) and certain chemical mutagens (Kato, 1974) and that a significant proportion of gross chromatid aberrations (including deletions) seem to involve a SCE event at the site of aberration (Heddle and Bodycote, 1970).

SCE analysis can be done using various cell types and animal strains both in vitro and in vivo. For in vitro SCE analysis, either monolayer cells (e.g., Chinese hamster ovary cells or human diploid fibroblasts) or human peripheral lymphocyte cultures are recommended. Among those human peripheral blood is easily obtained; the lymphocyte cultures are short-term and initially in a uniform Go state. The major purpose of mammalian cell SCE techniques in tissue culture is to obtain large number of cells with chromosomes containing differentially stained sister chromatids (Latt *et al.,* 1981).

In vivo analysis account for host mediation of the drug and thereby afford several advantages for mutagenesis trials. BrdUrd is administered either as tablet implantation to the lower lateral abdominal region of the animal or by continuous infusion which means a certain amount of BrdUrd is administered into the tail vein of animals by the help of a peristaltic pump for approximately a day. Bone marrow is considered the best tissue for routine chromosome analysis, primarily because of its high mitotic index for this purpose (Latt *et al.,* 1981).

It is shown that the incidence of SCE in human blood lymphocytes is relatively constant between individuals, and is independent of age and sex. An exception to this, the very high SCE incidence in cultured cells from patients with Bloom's syndrome, an increase which is not evident in cells from patients with other inherited chromosome instability syndromes (Perry and Evans, 1975).

Cell growth involves two easily recognized, coordinated events: the duplication of cellular DNA and the physical division of the cell into two daughter cells. If DNA synthesis and the cell division are considered the key events then the cell growth cycle can be divided into four periods: G_1, the gap between the previous

nuclear division and the beginning of DNA synthesis; S, the period of DNA synthesis; G_2, the gap between DNA duplication and nuclear division; and M, the period of mitosis, during which both of the chromosomes separate into the two daughter cells. Cell division is usually coordinated with nuclear division, but it may be delayed. Many non-dividing cells in tissues (resting fibroblasts, resting lymphocytes etc.) suspend the cell cycle after mitosis and just prior to DNA synthesis. Such "resting cells" which have exited from the cell cycle before the S phase, are said to be in the Go stage (Darnell *et al.,* 1990).

Lymphocytes are excellent sources of potentially dividing cells. The vast majority of the peripheral lymphocytes are normally non-dividing (Go) long living cells. Thus when they are isolated from the blood, they are quiescent. In vivo, the cells are capable of surviving in the non-dividing state for months or years. In culture, when confronted with a stimulus the resting lymphocytes undergo changes in every aspect of cellular metabolism culminating DNA synthesis and mitosis. The human lymphocytes are thought to spend their first 24 hours after mitogen stimulation in their Go stage after which they enter the S phase which will happen to be between 24-30 hours (Latt *et al.,* 1981).

1.2.5.2. Micronuclei Method with Preserved Cytoplasm

Micronuclei assay is one of the most sensitive markers for detecting DNA damage, and has been used in investigation of genotoxicity of variety of chemicals even some chemotherapeutic agents at molecular level. Micronuclei testing with interphase cells are more suited as a cytogenetic marker because it is not limited to metaphases, and has the advantage of allowing rapid screening of a large numbers of cells than in studies with sister chromatid exchanges (SCE) or chromosome aberrations (CA). The in vitro micronucleus test is a genotoxicity assay for detecting agents that induce structural or numerical aberrations of chromosomes. In comparison with the in vitro chromosome aberration test with mammalian or

human cells, the micronucleus assay is easier to perform, as the microscopic analysis is requires less training, and scoring can be quite quick. These characteristics have made the assay popular in genotoxicology laboratories, especially as that have divided once after the treatment, and where micronuclei could have been formed, can be identified as binucleate cells after cytokinesis has been blocked by cytochalasin B which disrupts the cytoskeleton of the cells(cytokinesis-block method). Thus, micronucleus formation in response to a genotoxic insult requires cell division. Micronuclei can result from clastogenic (chromosome breaking) or aneuploidogenic (or aneugenic) events, the former usually reflecting direct or indirect effect on DNA and the latter an influence on the mitotic apparatus such as the spindle. It is important to realize that the two basic mechanisms of micronucleus formation have different molecular targets, which must be considered in risk assessment of micronucleus formation have different molecular targets, which must be considered in risk assessment of micronucleus inducers. It also means that once an agent is positive in the micronucleus assay, the probable mode of action of the agent should be investigated.

Acentric chromosomal fragments resulting from breakage of chromatids or chromosomes are not attached to the mitotic spindle cells but lag behind when the rest of the chromosomes move towards the poles in anaphase. If the fragment is left far enough from both of the poles it will be included in neither of the daughter nuclei and will form a micronucleus. Characteristically, this type of micronuclei does not contain centromeric DNA sequences. Damage to the spindle, centromeres, or other components of the mitotic apparatus may cause failure of whole chromosomes to attach to the spindle. Such chromosomes lag behind in anaphase and may subsequently form micronuclei. In comparison with micronuclei containing fragments, micronuclei harboring whole chromosomes tend to be, on the average, larger and contain centromeric DNA.

Fragmented chromosome may be rebounded by newly synthesized cell membrane structure, which will be observed like micronuclei under light microscope. Micronuclei in human lymphocytes will be analyzed according to the

procedure of Högstedt, 1984. According to this method the cells will be analyzed with perverse cytoplasm, which allowed a more precise identification of micronuclei.

1.3 PURPOSE AND SCOPE

The purpose of this study was to examine biochemical, cytotoxic and genotoxic effects of aescin. Some original herbal compounds are used as a drug in the treatment of many diseases even cancer. These compounds have many effects on cells. Instead of trying such compounds on living organisms, it is much more convenient and safe to observe their effects on cell culture systems. The scope of this study is, therefore, limited to normal lymphocytes and HL-60 promyeloid leukemia cell cultures. The toxicity of such herbal compounds could be easily and efficiently followed by biochemical methods via observing detoxification enzyme activities. Such enzyme activities will increase/decrease in the presence of any toxic compound in the cell growth environment. The possible cytotoxic and genotoxic effects of these compounds could also be detected in a similar system by exposing the cells to their varying concentrations. This is very useful for the development of new therapeutic drugs especially for cancer treatment. If the compound is cytotoxic and/or genotoxic only for cancer tissues but not for normal cell lines, it could be considered as a good drug for cancer treatment. Induction of apoptosis, a form of programmed cell death, in cancer cells or malignant tissues could also be an efficient strategy for cancer chemotherapy.

There are many methods for the detection of cytotoxicity and genotoxicity of any compound. Apoptosis and necrosis detection methods, and cell viability tests are very useful tools for cytotoxicity detection. Sister Chromatid Exchange and Micronuclei formation are very useful and simple end point tests for genotoxic observations.

In order to evaluate the biochemical effects of aescin on all cell types used in this study, GSTs and NATs enzymes activities were followed spectroscopicly.

Cell viability technique, dye exclusion, was used as one of the cytotoxicity assays at the beginning of the construction of cell growth curves of each type used here. Then, to follow the cytotoxic effects of aescin, apoptosis and necrosis were supported by DNA fragmentation, cychrome *c* and COX IV blottings and, finally, immunostaining by NAPO antibody techniques. Genotoxic effects of aescin were observed by SCEs and Micronuclei formation techniques.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

RPMI 1640 HEPES modified, Fetal- Calf Serum (Heat-inactivated), Histopaque 1077-1, sodium pyruvate, L-glutamine, penicillin-streptomycin solution, 5-bromo-2-deoxyuridine (BrdUrd), bisbenzimide Hoechst No: 33258, tryphan blue, colchicine, steril phosphate buffered saline (sPBS), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight markers, ammonium persulphate (APS), bromophenol blue, coomassie brilliant blue R-250, N,N'-methylene-bisacrylamide (Bis), hydroxymethyl aminomethane (Tris base), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide, glycine, glycerol, sodium carbonate, sodium thiosulfate, Tween-20, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), agarose, proteinase K, β- aescin are purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Phytohemaglutinin L (PHA-L lymphocyte type) and fetal calf serum are obtained from Seromed, Biochrom K.G., Berlin, Germany. May-Grünward stain,

Giemsa stain, glacial acetic acid, hydrochloric acid, hydrogen peroxide, 2-mercaptoethanol, ultra pure methanol and Ciocalteu's Folin phenol reagent are purchased from E. Merck, Darmstadt, Germany.

HL-60 (promyelocytic leukemia cell line) [HÜKÜK-96041201] is purchased from Foot and Mouth Disease Institute, Turkey.

NAPO (Negative in apoptosis) antibodies are generous gift of Prof. Dr. Mehmet Öztürk, Head of Molecular Biology and Genetic Department, Bilkent University, who described NAPO and produced the antibodies in his laboratory.

QIAamp DNA Mini Kit is purchased from QIAGEN, Australia.

BD ApoAlertTM Cell Fractionation Kit, containing washing buffer, fractination buffer, DTT, Protease Cocktail, monoclonal antibodies against Cytochrome *c* Antibody and COX4 Antibody are purchased from Clontech Laboratories, U.S.A.

Amplified alkaline phosphatase goat anti-rabbit immun-blot assay kit and sequi-blot polyvinylidene difluoride membrane (PVDF) are purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Disposable syringe filter (pore size: 0.2 μm, diameter: 25 mm) is purchased from CHROMAFIL (MN). Cryovials (sterile, non-pyrogenic, DNAse and RNAse free) are purchased from Greiner bio-one, Germany.

All other chemicals are of analytical grade and are obtained from commercial sources at the highest grade of purity available.

2.2 Methods

2.2.1 Cell Viability in Artificial Conditions

In order to follow cell growth and cell viability in artificial cell culture conditions, Trypan blue-dye exclusion technique is performed.

Fifty micro liters of the cell suspensions are diluted with 50 µl Tryphan blue (1:1 ratio) (v/v) to get good results, the dye is diluted with sterile PBS (sPBS) (1:1 ratio) before use. This mixture is kept at room temperature for 3 minutes to allow the dye to penetrate through the membranes of the dead cells. Then the cells are observed and counted on hemocytometer with 25 squares under the light microscope (Olympus) at 40X magnification.

The number of living cells/ml is calculated by using the following formula:

Total counted living cells in 25 squares of hemocytometer X DF X 10^4.

2.2.2 Isolation of Lymphocytes from Blood

Human blood is obtained from a healthy donor at the METU Health Center. Lymphocytes are isolated according to density gradient centrifugation technique. Whole blood is diluted 1:1 with sPBS. The three parts of the diluted whole blood is layered on the one part of Histopaque 1077-1 without mixing the two layers. The cloudy layer containing lymphocytes is formed after the centrifugation of the test tubes at 800 rpm for 15 minutes.

The cloudy layer is removed by sterile Pasteur pipette without disturbing. Lymphocytes are washed away from Histopaque 3 times with sterile PBS by centrifugation at 800 rpm for 10 minutes each.

2.2.3 Optimization of the Cell Growth in Culture Conditions

Optimisation of cell growth conditions is very important to get the correct results for further treatment of cells in culture. Sterility, temperature and humidity must be under control to get the best cell growth. To maintain the aseptic conditions, all materials used in the culture are sterilized by using autoclave (at 121 ^{o}C for 20 minutes) and UV. The UV sterile laminar flow of class II cabinet is used during performing all steps in cultivation and to the passage of over growth cultures (*Freshney, 1995*). The complete growth medium composed of HEPES modified RPMI 1640, 2.6% sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 12.5% heat inactivated fetal calf serum and 5 μg/ml PHA-L (antigen) is sterilized by passing through the sterile membrane (the pore size is 0.22 μm) each time after the preparation.

2.2.3.1 The Growth of Whole Blood Lymphocytes in Culture Medium

All procedures about the cell cultures and culture techniques are done under strict aseptic conditions.

Blood is withdrawn by venous puncturing from a healthy donor by a nurse at the METU Health Center; the cells are counted by Beckman-Coulter, Maxm.

An aliquot of blood containing $1x10^5$ lymphocytes/ml are inoculated into 25 cm^2 sterile cell culture flask containing 5 ml (final volume) complete culture medium containing HEPES modified RPMI1640, 2.6 % sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 12.5 % heat inactivated fetal calf serum. PHA-L is added at a final concentration of 5 μg/ml into the complete culture medium because lymphocyte cell does not divide if there is no

antigen. Then the cells are incubated for one day to allow the adaptation of the cells to their surrounding medium.

Next day, the cells are examined under the microscope. If the cells are healthy, meaning that there is no contamination in the culture flasks and no cell membrane distruption is detected, the cells are counted and then adapted healthy $1x10^5$ cells are inoculated into new 25 cm^2 sterile cell culture flask containing fresh 5 ml complete medium. At the next 24^{th} hour, fresh 5 ml complete medium is added into the flasks containing the cultured cells. Then the cells are incubated for 144 hours to construct the growth curve by sampling (50 µl of cell suspension) at every 24^{th} hour.

2.2.3.2 The Growth of Isolated Lymphocytes in Culture Medium

The isolated lymphocytes are counted and $1x10^5$ isolated lymphocytes/ml are inoculated into 5 ml complete culture medium containing HEPES modified RPMI 1640, 2.6 % sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 12.5 % heat inactivated fetal calf serum. PHA-L is added at a final concentration of 5 µg/ml of the complete culture medium and the cells are incubated for a day. Next day, cells are examined under microscope and counted. If they are healthy, the cells are counted and then adapted healthy $1x10^5$ cells are inoculated into new 25 cm^2 sterile cell culture flask containing fresh 5 ml complete medium. At the next 24^{th} hour, fresh 5 ml complete medium is added into the flasks containing the cultured cells. Then the cells are incubated for 144 hours by sampling (50 µl of cell suspension) at every 24^{th} hour for cell count and cell viability check to construct the growth curve of isolated lymphocytes.

2.2.3.3 The Growth of Human Myeloid Leukemia Cells in Culture Medium

HL-60 cell cultures are started by thawing the frozen cells. The thawing of the frozen cells has to be done as quickly as possible. The cells in cryovials are put into water bath at 37.5^{o}C directly. After dissolving the suspension, the cells are washed with 4-5 ml sPBS by centrifuging twice at 800 rpm for 5 minutes, immediately. Then, the cells are allowed to grow in complete medium for 24 hours. The cell maintenance should be checked at 24th hour and 48th hour. If no contamination and/or cell destruction is detected, the subculturing is performed.

1x10^{5} healthy adapted HL-60 are inoculated into 5 ml complete medium containing HEPES modified RPMI 1640, 2.6% sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 10% heat inactivated fetal calf serum. The cells are grown for 144 hours by sampling (50 µl of cell suspension) at every 24th hour.

2.2.4 Freezing of Cells for Storage

HL-60 cells are frozen and stored when they are not used. As the cell density reaches to approximately 2x10^{5} cell/ml 75 cm^{2} culture flasks, the cell suspension is taken into 50 ml sterile tubes and centrifuged at 800 rpm for 7 minutes. From this point forward, all steps should be done on ice. After discarding the supernatant, 5ml RPMI-1640 medium without FCS and antibiotics is added onto the pellet. The mixture is mixed gently on ice. Then, 500 µl FCS and 500 µl sterile glycerol are added on the mixture at 0^{o}C. After mixing the mixture very well, the suspension volume is divided into 1.8 ml sterile cryovials. The cryovials are stored in the freezing compartment (less than 0^{o}C) of the refrigerator for 60 or 90 minutes. Then, they are carried to the deep-freezer (-80^{o}C) for overnight. Next day, the cryovials are put into liquid nitrogen tank until they are thawed.

2.2.5 Treatment of cells with Aescin

Between 10^{-4} M to 10^{-9} M stock aescin concentrations are prepared in water because aescin could be dissolved in water. All stock aescin solutions are sterilized by 0.22 μm pore size filter membrane sterilization.

All the cell types in culture are treated with aescin at final concentrations ranging between 10^{-5} M to 10^{-10} M by the addition of 10 μl of aescin from 10^{-4} M to 10^{-9} M stock aescin concentrations, respectively for 10^{-5} M to 10^{-10} M aescin concentration within 5 ml complete medium at the 24^{th} hour of incubation. Control cultures are not treated with any chemical. After growing the cells as described for 96 hours, the effects of different aescin concentration on the cell growth and on the cell viability are detected by Tryphan blue-dye exclusion technique and/or the cells are harvested to prepare their cytosol for enzyme activity determination.

2.2.6 Preparation of Cytosol from Cells in Culture

The cells are harvested and then they are washed away from the medium 3 times with sPBS by centrifugation at 800 rpm for 10 minutes each.

The cells in the pellet after last washing step are counted by trphan blue and dissolved in 1 mM phosphate buffer (pH: 6.5) and might be stored in deep-freezer at -80^{o}C for later usage.

The cells are homogenized on ice by hand use small glass-teflon homogenizer with passing 10 to 20 times to disrupt the cell membrane. The glutathione-S-transferase and N-acetyl-transferase activity are determined, immediately.

2.2.7 Determination of GSTs Activity

GSTs activities are determined spectrophotometrically by monitoring the thioether formation at 340 nm as described by Habig *et al*., (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate.

The following solutions are prepared for the determination of GSTs activities toward CDNB as a general substrate.

The constituents of the incubation mixture for GSTs enzyme assays are shown in Table 3. The reactions are started by the addition of substrate, CDNB. Incubation mixtures without the enzyme source are used as blanks (nonenzymatic reactions). The increase in optical density at 340 nm is determined by following the reaction for 3 minutes. The concentrations of the conjugation products are determined from the slopes of initial reaction rates using the extinction coefficient of $9.6x10^3$ cm^{-1} M^{-1} at 340 nm (Habig et al., 1974). The GSTs activities are expressed as units/ cells. One unit of GST activity is defined as the amount of enzyme producing one μmol of CDNB conjugate in one minute.

Combination solution: 0.2 M potassium phosphate buffer pH 6.5, 50 mM GSH and water mixture in a ratio of 25: 1: 19 by volume.

Substrate solution: 20mM CDNB solution, prepared by dissolving the appropriate amount of CDNB first in pure ethanol then water is added in a ratio of 3:2 by volume.

Table 3 The constituents of the incubation mixture for GSTs enzyme assay with a common substrate, CDNB.

Constituents of The Reaction Mixture	Added volume (µl)	Final Concentration
Substrate Solution[a]	50	1mM
Combination Solution - Buffer[b] - 50.0 mM GSH - H_2O,	900	0.18M Buffer 45.0mM GSH
Enzyme Source[c] Lymphocyte Cytosol (2.0–12.0 mg/ml)	50	5µg-25µg

[a] CDNB is first dissolved in pure ethanol then H_2O is added in a ratio of 3:2, respectively to have a final 3%(v/v) ethanol in the reaction mixture.
[b.] 0.20 M Potassium phosphate buffer, pH 6.5.
[c.] The proper dilution is made to have 20 µg cytosolic protein in buffer for GSTs assay with CDNB.

2.2.8 Determination of NATs Activity

Acetyl coenzyme A dependent arylamine N-acetyltransferase activities are measured spectrophotometrically according to the method of Andres et al., (1985) with minor modifications. This spectrophotometric assay procedure can be divided into two parts. First part is completed by enzymatically-catalyzed reactions. 50 µl of regenerating system containing 2 mM EDTA, 2 mM DTT, 2.2u/ml CAT, 15 mM AcCAR and 50 mM Tris-HCl (pH: 8.0) and 10 µl of PABA from 0.1 mM stock solution as a substrate for NAT1 isozyme or 10 µl of SMZ from 0.1 mM stock solution as a substrate for NAT2 isozyme and 540 µl of 50mM Tris-HCl (pH: 8.0) are put on the bottom of 1.5 ml microcentrifuge tube. Then, 150 µl of cell fraction performed by homogenization on the ice is added to this tube. The tube is incubated

at 37^{o}C for 5 minutes. The addition of 60 µl of AcCoA started the reaction. The reaction is terminated by the addition 50 µl of 20% (w/v) trichloroacetic acid at the end of the second incubation period (5 and 15 minutes) at 37^{o}C. The precipitated protein is sedimented by centrifugation at 17,000 xg for 20 minutes. Second part of the procedure is done for the measurement of the enzymatic activity by determining the depletion of amine substrate which is reflected by decreasing Schiff's base formation with dimethylaminobenzaldehyde (DMAB). One hundred microliter 5% (w/v) DMAB in acetonitrile is added to samples, which are the recentrifuged for 30 minutes and incubated for 10 minutes at room temperature. Then, the absorbance reading is taken at 450 nm in Shimatzu double beam spectrophotometer. By using these standard conditions, with the extinction coefficient of 50X10^{3} cm^{-1} M^{-1} at 450 nm, the units of NAT activity is calculated as µmol/min/cell.

2.2.9 Protein Determinations

The protein concentrations in the prepared crude extracts are determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 to 0.5 ml of 1:20 diluted homogenated cytosol are taken into test tubes and are completed to a final volume of 0.5 ml with distilled water. Then, alkaline copper reagent is prepared by mixing 2 % copper sulfate, 2 % sodium potassium tartarate and 0.1 N NaOH containing 2 % sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent is added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1 N Folin Phenol reagent is added to each test tube, mixed immediately within 8 seconds by vortex and incubated 10 minutes at 50°C in water bath. The intensity of color developed in each tube is measured at 660 nm.

The protein concentrations in the crude extracts are calculated from a standard calibration curve that is constructed from the corresponding O.D$_{660nm}$ values

of BSA standards (0 to 200 μg). The protein concentrations in the prepared lymphocytes are found to be in the range of 2.0 to 12.0 mg/ml.

2.2.10 Immunodetection of NAPO antigen

The cells are grown as described in methods for 96 hours. All the cells types are added with different aescin concentrations ranging between 10^{-5} M and 10^{-10} M, within 5 mL complete medium, at the 24th hour of incubation. For the positive control, a group of the cells named Control II $_{H2O2}$ is forced to the induction of apoptosis with 15 mM H_2O_2 (oxidative stress).

The cells are fixed on coverslips with 4 %paraformaldehyde for 1 hour. The cells are permeabilized for 3 minutes with 0.1 % triton X-100 in 0.1 %sodium citrate. After saturation with 3% BSA in PBS-T (0.1%) for 15 minutes, the fixed cells are incubated with anti-NAPO antibody for 1 hour at room temperature. FITC-conjugated goat anti-mouse antibody (Dako) is used as the secondary antibody with 1/10.000 dilution. Nuclear DNA is visualized by incubation with 3 μg/ml Hoechst No: 33258 for 5 minutes in the dark. Cover slips are then rinsed with distilled water, mounted on a glass microscope slides in 50 % glycerol, and examined under fluorescent microscope (ZEISS).

2.2.11 Analysis of DNA Fragmentation

The method for DNA extraction from the cells and analysis of the DNA fragmentation, which is an esential change in apoptotic cells, is as follows.

All the cells types in culture are treated with different aescin concentrations ranging between 10^{-5} M and 10^{-10} M, within 5 mL complete medium, at the 24th hour of incubation. After growing the cells as described in methods for 96 hours, the cells

are centrifuged at 25000 rpm for 20 minutes.The supernatant is discarded and 2 ml sPBS is added onto the pellet. It is dissolved by vortexing gently for 15 seconds.

The number of the treated and untreated (negative control) cells is adjusted to 10.000/µl with PBS. Then, 300 µl of the cell suspension containing approximately $3X10^6$ cells is pipetted into the bottom of a sterile 1.5 ml microcentrifuge tube. Then, 30 µl of QIAGEN Protease (or Proteinase K) is added on the cells. This tube should be vortexed very well for a few seconds. Afterwards, 200 µl Buffer AL is added to the sample mix by vortexing for 15 seconds. In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogenous solution. The microcentrifuge tubes are incubated at 56^oC for 10 minutes and then, centrifuged briefly to remove drops from the inside of their lids. Next, 200 µl pure ethanol (96-100 %) is added to the sample and mixed again by vortexing for 15 seconds. After mixing, the microcentrifuge tubes are centrifuged briefly to remove drops from the inside of their lids. The mixture is carefully applied into the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim, the cap is closed and the microcentrifuge tubes are centrifuged at 8000 rpm for 1 minute. Then, the QIAamp Spin Column in a clean 2 ml collection tube, the old tubes containing the filtrate are discarded.

QIAamp Spin Column is carefully opened and 500 µl Buffer AW1 is added on the membrane withought wetting the rim. After closing the cap, the tubes are centrifuged at 8000 rpm for 1 minute. QIAamp Spin Column is placed in a clean 2 ml collection tube and the collection tubes containing the filtrate are discarded.

QIAamp Spin Column is carefully opened and 500 µl Buffer AW2 is added on the membrane without wetting the rim. After closing the cap, the tubes are centrifuged at 14000 rpm for 3 minutes. QIAamp Spin Column is placed in a clean 2 ml collection tube and the collection tubes containing the filtrate are discarded. There is an optinal step that is an extra centrifugation at 14000 rpm for 1 minute to remove all liquids from the membrane completely. QIAamp Spin Column is placed

in a clean 2 ml collection tube and the collection tubes containing the filtrate are discarded.

QIAamp Spin Column is carefully opened and 200 µl Buffer AE or distelled water is added on the membrane. The tubes are incubated at room temperature for 5 minutes that is required to increase DNA yield and centrifuged at 8000 rpm for 1 minute.

After DNA isolation, 8 µg of total DNA is loaded onto a horizontal agarose gel (1.5 %) for agarose electroforesis at 80 Volts for 1-1.5 hours.

Gel is stained with ethidium bromide in 0.5 µg Et-Br/ ml dH_2O and visualized under UV light (Vilber Lourtmat Gell Imaging System).

2.2.12 Western Blot Detection of Cytochrome c Oxidase Subunit IV (COX4) and Cytochrome c

2.2.12.1 Cell Fractionation to visualize the Cytochrome c Release

Mitochondrial and cytological suspension is prepared by ApoAlert Cell Fractination Kit and then, Immunostaining is carried out according to the instruction manual provided with the Amplified Alkaline Phosphatase (AP) Western Blotting Kit (Bio-Rad) that is used in the immunostaining of the electroblotted PVDF membranes. All of the incubations are performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

After growing the cells as described in methods for at 96 hours, the cells are centrifuged at 600 xg for 5 minutes at 4 ^{o}C. The supernatant is discarded and the pellet is re-suspended in 1 mL of ice-cold wash Buffer (ApoAlert Cell Fractination Kit). After resuspending the pellet completely, the cells are centrifuged at 600 xg for

5 minutes at 4 ^{o}C. The supernatant is discarded; the pellet is re-suspended in 0.8 mL of ice-cold Fractionation Buffer Mix. The tubes are incubated on ice for 10 minutes. The cells are homogenized in an ice-cold dounce tissue grinder on ice. The homogenate is transferred to a new 1.5 mL microcentrifuge tube, and is centrifuged at 700 xg for 5 minutes at 4 ^{o}C. The supernatant is transferred to a fresh 1.5 mL microcentrifuge tube, the tubes are centrifuged at 10, 000 xg for 25 minutes at 4 ^{o}C. The supernatant (this is the cytosolic fraction) is collected and the pellet is re-suspended in 0.1 mL of ice-cold Fractionation Buffer Mix (the mitochondrial fraction). After the protein concentration determination by the method of Lowry et al. (1951), 15 µg of each cytosolic and mitochondrial fraction isolated from induced (apoptotic) and uninduced (nonapoptotic) cells are loaded on a 12 % SDS-PAGE. Then, any standard Western blot procedure is performed by Cytochrome *c* Antibody (1:100 dilutions) and COX 4 Antibody (1:500).

2.2.12.2 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent sodium dodecyl sulfate (SDS), is performed on 4 % stacking gel and 12 % or 15 % separating gel in a discontinuous buffer system as described by Laemmli (1970). The seven proteins given below are used as molecular weight standards.

Table 4 The list of used molecular weight standards

- Bovine Albumin	(M_r 66000)
- Egg Albumin	(M_r 45000)
- Glyceraldehyde-3-Phosphate Dehydrogenase	(M_r 36000)
- Carbonic Anhydrase	(M_r 29000)
- Trypsinogen	(M_r 24000)
- Trypsin Inhibitor	(M_r 20100)
- α-Lactalbumin	(M_r 14200)

Preparation of Reagents

(A) Stock Separating Gel Buffer (1.5 M Tris-HCl, pH 8.8)

36.3 gm Tris base are dissolved in about 100 ml distilled water and pH 8.8 is adjusted with 1 M HCl. Finally, completed to 200 ml.

(B) Stock Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8)

12.1 gm Tris base are dissolved in about 100 ml distilled water and pH 6.8 is adjusted with 1 M HCl. Finally, completed to 200 ml.

(C) Stock Gel Solution (Acrylamide-BIS, 30 % A, 2.67 % C)

60.0 gm acrylamide are dissolved in about 175 ml distilled water and then 1.6 gm BIS (Bis-acrylamide) are added and solution is completed to 200 ml with distilled water. Finally, the solution is filtered through course filter paper.

Note: % A represents acrylamide monomer percent concentration and % C indicates the crosslinking monomer concentration, which are calculated as below:

% A = [(gm acrylamide/total volume)] X 100

% C = [gm BIS/(gm acrylamide + gm BIS)] X 100

(D) 10 % SDS Solution

10 gm SDS are dissolved in water with gentle stirring and completed to a final volume of 100 ml.

(E) Catalyst (10 % Ammonium Persulfate “APS”)

Prepared freshly by dissolving 100 mg ammonium persulfate (APS) in a final volume of 1 ml distilled water.

(F) Tracking Dye (0.05 % Bromophenol Blue)

Tracking dye solution is prepared by dissolving 5 mg solid bromophenol blue in a final volume of 10 ml.

(G) 5 X Electrode (Running) Buffer (25 mM Tris, 192 mM Glycine, pH 8.3 and

Stock running buffer solution is prepared by dissolving and completing 15 gm Tris base, 72 gm glycine to 1 liter distilled water. The pH of the buffer is not adjusted with acid or with base. This buffer is diluted 1:5 and 1 gm solid SDS is added to 1 liter of buffer before use.

(H) 4 X Sample Dilution Buffer (SDS Reducing Buffer)

0.25 M Tris-HCl buffer, pH 6.8 containing 8 % SDS, 40 % glycerol, 20 % 2-mercaptoethanol, 0.004 % bromophenol blue. It is prepared by mixing the following volumes of given solutions:

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	2-mercaptoethanol
0.4 ml	Tracking Dye
0.8 gm	10 % SDS
Distilled water to 10.0 ml	

Electrophoresis

Vertical slab gel electrophoresis is carried out using the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A) that can be used to run two gels simultaneously. The assembly of the glass plate cassettes (8.3 X 7.4 cm) and the process of gel casting are done according to instruction manual provided with the apparatus. Once the cassettes are properly assembled and mounted, the preparation of the separating and stacking gels is started.

The 12 % separating gel and 4 % stacking gel polymerizing solutions are prepared just before use by mixing the given volumes of stock solutions in the written order as given in Table 12. The separating gel solution is first prepared with the TEMED added just before casting the gel into the glass assembly from the edge of one of the spacers until the desired height of the solution (about 5 cm) is obtained. Then, the liquid gel is overlaid with distilled water (about 0.5 ml), without disturbing the gel surface, to obtain an even interface between the separating gel and the stacking gel. The gel is then allowed to polymerize at room temperature for a minimum of 30 minutes. After polymerization, the layer of water is removed completely using filter paper without hurting the gel surface. The stacking gel is then poured on the top of the resolving gel and the comb is inserted into the layer of the stacking gel without trapping air bubbles under the teeth of the comb. The gel is then allowed to polymerize for a minimum of 30 minutes.

After the gel is polymerized, the comb is removed carefully and the wells are washed with distilled water and filled with electrode buffer. At this point, the gel cassettes are removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside. When running only one gel, the blank plastic plate, provided with the system, is mounted in the place of the second cassette in the casting stand and in the running frame.

Aliquots from the protein samples to be analyzed and from the standards mixture are diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part sample buffer), to have the samples in 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue. Then the samples are placed in a boiling water bath for 5 minutes. Afterwards, protein samples (20 μg) and standards are loaded into different wells using a 25 μl Hamilton syringe with a tipped needle.

Table 5 Formulations for SDS-PAGE separating and stacking gels

	Separating Gel		Stacking Gel
Monomer Concentration	12 %	15 %	4 %
Acrylamide/bis	12.0 ml	15.0 ml	1.3 ml
distilled water	10.0 ml	7.0 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	----
0.5 M Tris-HCl, pH 6.8	----	----	2.5 ml
10% (w/v) SDS	300 µl	300 µl	100 µl
10 % APS	185 µl	185 µl	50 µl
TEMED	15 µl	15 µl	10 µl
Total monomer	30 ml	30 ml	10 ml

After loading the samples, the running buffer (135 ml) is added to the compartment formed by the running frame and the cassettes (the upper buffer compartment) and the system is checked for leakage. The running buffer (250ml) is then also added to the electrophoresis tank. Thereafter, the safety cover is replaced and the leads are plugged into the EC250-90 electrophoresis power supply. The power supply is adjusted to give a constant current of 15 mA when the samples are in the stacking gel and 30 mA when the samples passed to the separating gel. Under these conditions the voltage is about 250 V that took a total of about 1.5 hours.

The power supply is switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame is taken out and the buffer is removed from the upper buffer compartment. Afterwards, the clamps are detached and the cassettes are removed from the running frame. To gain access to the gels in the cassette, the glass plates are pried apart using a spatula taking care not to chip the edges of the glass plates. The left-top corner of each gel is cut to indicate the order of wells. The gels, usually adhered to one of the glass plates, are taken carefully using gloves and placed in the previously prepared appropriate solutions to stain the

samples which have been resolved on the gels, or to prepare the gels for subsequent blotting.

2.2.12.3 Electroblotting of the Gels from SDS-PAGE

Electroblotting is carried out using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.), and Polyvinylidene difluoride (PVDF) is used as a blotting membrane.

Prior to electroblotting, the gels taken from SDS-PAGE are placed for 30 min, with shaking, in the Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol) with shaking (Towbin *et al.*, 1979).

While the gels are incubated in the transfer buffer, the other system components and the transfer membrane are prepared. All of the electroblotting procedure is carried out wearing gloves. The PVDF transfer membrane, with the dimensions of the gel to be transferred, is soaked in 100 % methanol for 30 seconds with shaking, to overcome the hydrophobicity of the membrane. Then, the wet membrane is washed several times with distilled water and then with transfer buffer until it is equilibrated (submerged into the solution and not floating any more), the point at which the membrane is ready to bind the proteins in any blotting application. The membrane should not be allowed to dry, otherwise proteins will not bind to it, so if it does dry during the procedure, the wetting procedure should be repeated again. Afterwards, two pieces of filter paper, the Scotch Brite sponge pads, and the transfer membrane are soaked in the transfer buffer for 30 min with continuous shaking.

The blotting stack is assembled on the top of stainless steel grid cathode located in the trough of the frame stand of the Mini Blot Module, to which a small amount of transfer buffer is added.

The configuration of the assembly is as follows:

Top	Cover with Palladium Wire Anode
	Sponge Pad
	Sponge Pad
	Filter Paper
	PVDF Transfer Membrane
	Gel
	Filter Paper
	Sponge Pad
Bottom	Frame stand with Stainless steel Grid Cathode

After the above assembly is prepared, the cover of the electroblotting module is pressed onto the blotting stack and fixed with the clamps after turning assembled blotting module upright and then filled with the transfer buffer (about 100 ml). Thereafter, the fully assembled module is inserted into the outer tank and the safety cover with leads is replaced. The red lead is connected to the anode (+) and the black lead to the cathode (-), are the proteins will be transferred as anions to the direction of anode. The transfer process is performed at room temperature for 50 minutes using a constant voltage (20 V). When the blotting is finished, the PVDF membrane is immediately removed and placed in the blocking solution (%5 non-fat dry milk in TTBS), previously prepared.

2.2.12.4 Immunostaining of the PVDF Membranes

Immunostaining is carried out according to the instruction manual provided with the Amplified Alkaline Phosphatase (AP) Western Blotting Kit (Bio-Rad) that is used in the immunostaining of the electroblotted PVDF membranes. All of the incubations are performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

The electroblotted PVDF membrane is incubated in the blocking solution (5 % non-fat dry milk in TTBS buffer) for overnight. Afterwards, the membrane is incubated with the antibodies diluted in the blocking solution for 1 to 2 hours, where the monoclonal anti-cytochrome *c* (1/100 diluted) or the monoclonal anti-COX Subunit IV (1/500 diluted) are used. The membrane is then washed five times, each for 5 min with TTBS and incubated with the secondary antibody (biotinylated goat anti-rabbit 1/7,500 diluted in TTBS) for 1 to 2 hours.

During the secondary antibody incubation period, the streptavidin - biotinylated AP complex is prepared by the addition of streptavidin to biotinylated AP (both 1/7,500 diluted in TTBS) and allowed to stand at least 1 hour and not more than 3 hours at room temperature. After the incubation with secondary antibody, the membrane is washed again with TTBS (five times, each 5 min) and then incubated for 1-2 hours in the previously prepared streptavidin - biotinylated AP complex.

Afterwards, the membrane is washed again with TTBS (five times, each 5 min) and the AP color developing solution (BCIP/NBT) is added. The specific protein bands started to appear after 10-30 min. Finally, the membranes are carefully dried and the images are obtained using a scanner connected to the computer.

2.2.13 SCEs Assay

2.2.13.1 Preparation of Cell Cultures for Sister Chromatid Exchange Analysis

Although all cells types are grown same as described in methods for 96 hours, the only difference is that BrdUrd is added to all culture flasks (treated and untreated) as a final concentration of 10 μM (from 100 mM stock) at 24^{th} hour.

All the cells types are treated with different aescin concentrations ranging between 10^{-5} M and 10^{-10} M, within 5 mL complete medium, at the 24^{th} hour of incubation.

Complete darkness is necessary to protect the BrdUrd substituted DNA from degradation by light. The protection is achieved by wrapping the culture tubes with aluminium foil and working under dim yellow light. Four hours before harvesting the cells, 0.5 µg /ml colchicine (final concentration) is added to entrap the cells at metaphase of their mitotic division. Then, the cells are collected by centrifugation at 800 RPM for 15 minutes using a bench top centrifuge (IEC clinical centrifuge DAMON / IEC Division) at room temperature. After discarding the supernatant, 0.075 M hypotonic KCl pre-warmed to 37°C, is added on to the pellet and, mixed well with gentle shaking (without vortexing). Culture tubes are then returned back to the incubator for further 20 minutes in order to spread the chromosomes within the lymphocytes and to hemolyse the red blood cells by swelling the cells. Swollen lymphocytes are again collected by centrifugation at 500 g for 15 minutes. Supernatant is discarded leaving approximately 0.5ml of 0.075 M KCl on the pellet.

Then the cells are fixed by adding freshly prepared chilled methanol: acetic acid (3:1) on to the pellet drop by drop while mixing with vortex at low speed in order to prevent the aggregation of cells. Chromosomes within cells fixed that way are left at 4°C overnight and the next day fixation step is repeated two more times. Cells collected by centrifugation at 600 g for 15 minutes between the fixations are suspended in fresh fixative and the volume of the suspension is completed to 1 ml.

The cell suspension is dropped (5-7 drops/slide) on chilled wet slides, which are prepared by is sing with detergents and tap water and then left in the refrigerator in distilled water until use. Then the cells are left to dry at room temperature. The aged slides are then stained using the fluorescent plus Giemsa technique (Perry and Wolff, 1974) with some modifications in order to visualize Harlequin chromosomes under microscope.

2.2.13.2 Staining the Chromosomes with Fluorescent plus Giemsa (FPG) Technique

In order to visualize Harlequin chromosome i.e. the products of second cell division (one sister chromatid lightly stained, the other chromatid darkly stained), the dried slides of chromosome spreads prepared from whole and isolated lymphocytes are soaked in PBS for 3 minutes in Caplin jar. Then, they are stained with 5 µg/ml Hoeschst 33258 for 15 minutes. The fluorescent dye is prepared in PBS from a 100µg Hoeschst 33258 /ml stock. After staining, the slides are first rinsed in PBS and then in distilled water by shaking the Caplin jar gently. The slides are placed on a tray and the slide surfaces are covered with McIlvanes buffer (pH 8) prewarmed to 60 ^{o}C. The edges of slides are sealed with parafilm to prevent the evaporation of McIlvanes buffer from the surface of slides. Then, the slides are irradiated with short-wave UV (Ultraviolet Products Inc., Mineral KLight Lamp Model # C-81) for 20 minutes in a tray placed on water bath at 60 ^{o}C. The distance between UV-source and the slides is approximately 5 cm. After irradiation, the slides are rinsed with distilled water and incubated in 2X SSC for 10 minutes at 60 ^{o}C.Then, the slides are rinsed with distilled water thoroughly. Finally, the slides are stained with 2% Giemsa in 0.1 M Phosphate buffer, pH 6.8 for 15 minutes and rinsed well with distilled water again for few seconds and dried at room temperature.

2.2.13.3 Observation and Analysis

The slides are observed by using an Olympus binocular compound microscope. 10X magnification is used to determine the location of metaphase spreads and 100X oil immersion objective is used to analyze the metaphase chromosome. During analysis, metaphase chromosomes are divided into three as M1, M2 and M3. M1 metaphase chromosomes are the ones having unifiliarly substituted BrdUrd on their DNA strands and their both sister chromatids are darkly stained with

the above staining procedure. The chromosomes which are also called M2 metaphases on the other hand, have bifiliarly substituted BrdUrd on one sister Harlerquin chromosomes and unifiliarly substituted BrdUrd on the other, thus one sister chromatid stains darkly whereas the other stains lightly. Sister chromatid exchanges are scored from M2 metaphase spreads having 46±0 chromosomes. At this stage, an exchange in one chromatid can be easily observed as there will be dark stain on lightly stained chromatid and vice versa. M3 metaphases are ones having bifiliarly substituted BrdUrd on most of their DNA strands of both chromatids, so chromatids are uniformly lightly stained. In order to analyze cell cycle kinetics, more than 100 mitotic cells are scored for each treatment In order to analyze cell cycle kinetics, more than 100 mitotic cells are scored for each treatment and categorized as being M1, M2 and subsequent division metaphase chromosomes. An increase in the proportion of M1 cells with concomitant decrease in M2 and M3 cells as reflected by a decrease in mitotic index indicated a delay in the cell cycle progression in response to culture conditions such as BrdUrd concentration and treatments.

Mitotic index (MI) is calculated by using the formula:

$$\frac{M1X1+M2X2+M3X3}{M\ Total}$$

According to Sinha et al., (1988) in order to study the cell cycle delays following the treatments.

2.2.14 The Micronucleus Method with Preversed Cytoplasm

For micronuclei analysis the cells are grown for 96 hours and treated with same aescine concentrations as described in methods. At the end of incubation period, 6.4 µl of KCN is added from 3.3 mg/ml KCN stock to 10 ml cultures. The culture tubes are left at room temperature for 4 minutes and then centrifuged

at 600 xg for 10 minutes. The supernatant is discarded and pellet is suspended within a trace amount of medium left on the pellet. The cells are then smeared on wet chilled and left to dry at room temperature for 1-2 days.

The dried slides are stained by 10% May-Grünwald Giemsa stain for 15 minutes at room temperature. For the preparation of the stain, 5 ml of May-Grünwald from 100% stock is mixed with 5 ml of Giemsa from 100% stock and completed to 100 ml with distilled water. The stain preapared this way could be used for a week.

The analysis of the slides are done under 100X oil immersion objective. 1000 cells with and without micronucleus are counted for this purpose and the results are expressed as number of micronuclei/1000 cells.

2.2.15 Statistical Analysis

The results were presented as mean ±standart deviation (SD). Data were analyzed statistically by using ANOVA test in MİNİTAB computer program and the p values less than 0.05 were considered as statistically significant.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Evaluation of Cell Viability of HL60 cells, Isolated Lymphocytes and Whole Blood Lymphocytes

The biochemical, the cytotoxic and the genotoxic effects should be investigated on normal cells and cancer cells, if aescin were going to be used as an anticancer drug for chemotherapy. Isolated lymphocyte and whole blood lymphocyte were chosen for normal cell line culture and HL-60 cell was for cancer cell line culture.

In order to get reliable results when cell culture is used, the optimal growth and inoculation conditions have to be constructed for isolated lymphocyte, HL-60 cells and whole blood lymphocyte. The viability of each type of cell must be determined in order to be sure about the suitability of medium composition and other environmental conditions. All cells are sensitive to their environmental physical and chemical conditions even cancer cells although they are immortal. Nutrient requirements with respect to division time of cells should be under control. In order to get maximum growth, nutrient requirement and physical conditions have

to be supportive to obtain over %90 cell viability for studied cell types. Although RPMI 1640 is well established growth medium, L-glutamine and pyruvate have be added into culture medium as energy sources. Fetal Calf Serum contains high level of protein and growth factors to support growth. Normally, lymphocytes are non-dividing cells. Whenever, an antigen is found around them, they become the dividing cells. PHA-L is used as an antigen to induce cell division in lymphocyte cultures. Physical conditions besides nutrient requirements are also important. The most important physical requirement is the sterility. In medium, sterility is stabilized by adding of antibiotics such as penicillin-streptomycin solution. Although antibiotics are beneficial to protect cells from contaminations, they are harmful at high concentrations. In order to decrease the usage amount, cell growth has to be done under strict aseptic conditions, which was achieved by use of laminar flow, UV streilization in laminar flow, filter sterilization of all chemicals, autoclaving glass wares at 120^{o}C for 20 minutes.

In order to obtain the maximum cell number and growth over %90 cell viability, the optimal physical and chemical conditions were established for all used cell types.

The optimum incubation time with the well established culture medium used in this study, was determined as 96 hours to get maximum cell number and growth over %90 cell viability, based on the growth curves of all studied cell types. After 96 hours, the cell viability of the cells used in this study decreased sharply because of not only nutrient deficiency but also increasing toxic materials and related change in the pH of the culture medium.

This change in pH could be observed by naked eye with the help of phenol red as a pH indicator in RPMI medium. Phenol red is commonly used as a pH indicator. It is red at pH: 7.4, becoming yellow at pH: 6.5, lemon yellow below pH: 6.5. Another indicator of overgrowth, followed by naked eye was the cloudy appearance in the medium. The health of the used cells was controlled at least every 24 hours under the inverted microscope.

As shown in Figure 4 (a) and (b), healthy cells continue their division while retaining their shapes under well established culturing conditions. If cells are unhappy in their environment or are exposed to stress, as can be observed from their shapes, they are detached and died as seen in Figure 4 (c).

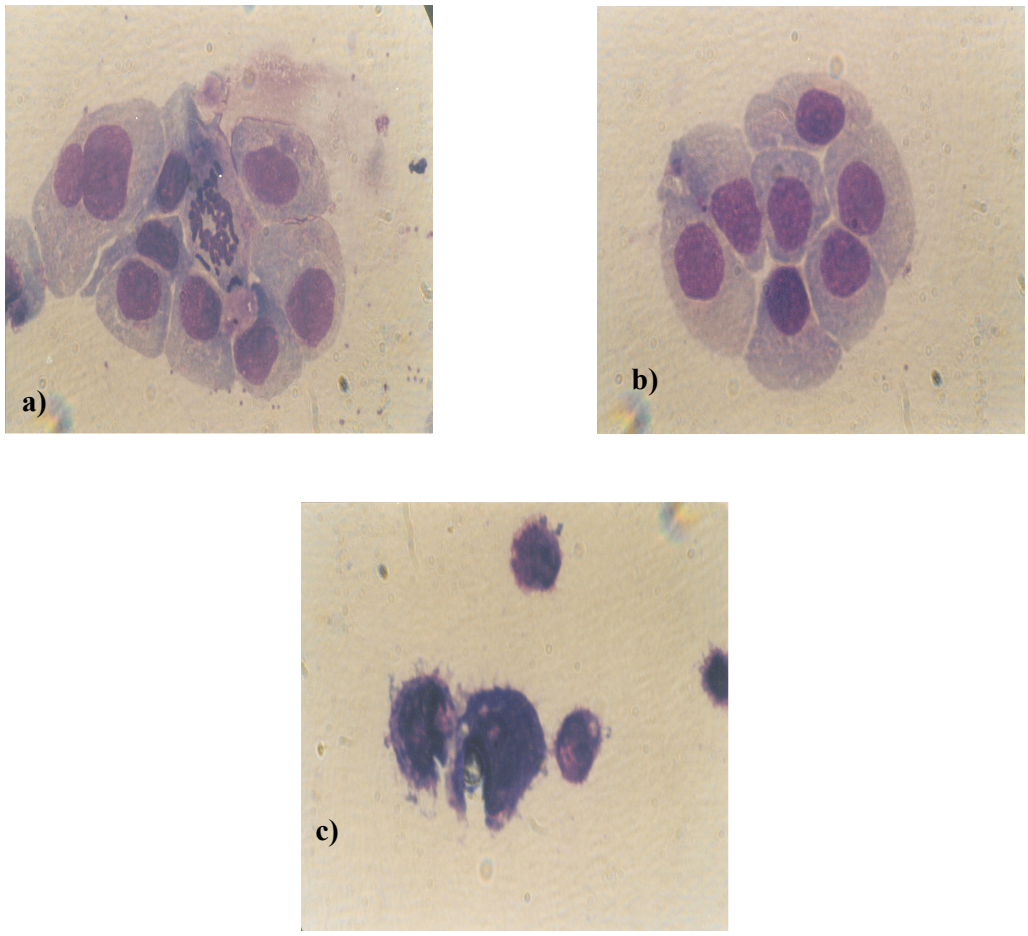


Figure 4 a) The healthy HL-60 cells and **b)** The healthy lymphocytes **c)** The HL-60 cells under the stress at 100X magnification.

A preliminary study had been conducted before getting the data in order to construct the growth curves and the time versus cell viability tables .Then, the incubation study was established to get reliable data by eliminating physical and chemical environmental effects. This preliminary study was carried out as follows; the lymphocytes which had been isolated from the whole blood and the HL-60 cells, which had been frozen at -180 ^{o}C in the cryovial containing approximately at least $2x10^5$ HL-60 cells/ml, were thawed and non-isolated lymphocytes at least a final density of $1x10^5$ cells/ml in whole blood were inoculated into the 5 ml complete medium in 25 cm^2 sterile culture flask at each 3 sets and incubated in few days. This preliminary study covered at least 24 hours for all used cell types and the aim was to understand the adaptation and survival of these cells in the new environmental conditions. After this preliminary study, the real study covers 144 hours. During this 144 hours incubation study, the growth curve had been established by using the data taken at every 24 hour intervals. The cell viability was determined by dye exclusion technique with the help of tryphan blue and hemocytometer (Figure 5 (a)) under light microscope by taking 50µl cell suspension from the culture flasks under aseptic conditions. The healthy cells were observed round and shiny without tryphan blue dye in their cytoplasm because they could pump out the dye by active transport system of their alive cell membranes (Figure 5 (b)). But the dead cells contained the dye in their cytoplasm because of failed active transport system of dead cells (Figure 5 (c)). After thawing the frozen HL-60 cells, high number dead cell was observed in the flasks under inverted microscope during the preliminary period. The reason of these cells' dead might be that some cells could not be adapted easily into the environment or not preserved efficiently although glycerol was used in order to preserve the cytoplasm of cells during freezing of HL-60 cells.

As represented 0 time in the tables of the incubation time versus the number of alive and death cells and in the growth curve diagrams for each type of all used cells, $1x10^5$ healthy cells/ml were inoculated one by one into the 5 ml complete medium in 25 cm^2 sterile culture flask at the beginning of the real study following the adaptation period. After the first 24 hour incubation of this study, 5ml fresh complete medium was added on the growing cells in 25 cm^2 sterile culture flask

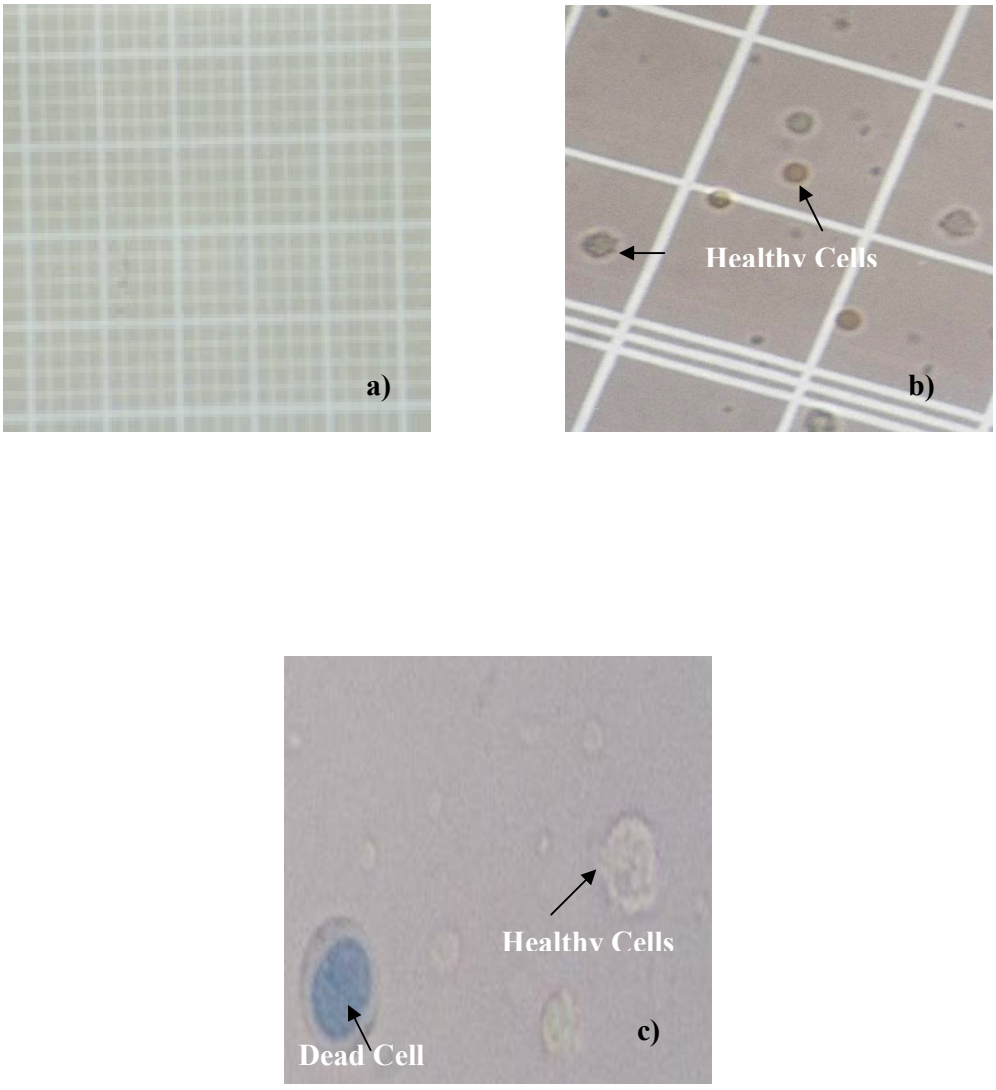


Figure 5 a) A view of the counted area of 25 squares on hemocytometer at 10X magnification **b)** The healthy HL-60 cells **c)** The alive and dead HL-60 cells were side by side at 40X magnification.

containing 5 ml complete medium. So, the cells in the flasks contained totally 10 ml complete medium, had grown until the end of 144 hours incubation from the first 24^{th} hour of this incubation.

Until 96^{th} hour, the percent viability of all used cell types was stabilized over 90 in the complete medium as the shown in Table 6, Table 7 and Table 8. However at 120^{th} and 144^{th} hour, the percent viability became decreasing under %90 viability due to the nutrient depletion of the complete medium and the worse changes of the environment such as changing of medium pH because of increasing cell amount and deposition of excreted materials.

The tables of cell viability for each cell types used in this study showed that within no more than every 3 days; the cells had to be sub cultured to provide the maintenance of their robustness. Otherwise, some occurred changes on their membrane could be observed under inverted microscope and related changes in their shapes, they died while detaching.

The growth curves show that the curves become steady state after 120 hours in all growth curves constructed for the used cell types.

Although cell viability was over 90% until the end of 96^{th} hour of isolated lymphocyte incubated in culture, the cell viabilities of isolated lymphocytes as seen in Table 7 were lower than that of HL-60 cells at each same time counting. The isolated lymphocytes were more sensitive than HL-60 cells even though sensitivity of cancer cells is higher than normal cells to environmental changes. Because isolated lymphocytes become more unprotected against to environmental factors in the absence of erythrocytes. The presence of erythrocytes in whole blood lymphocyte culture has beneficial effect on the cell viability of lymphocytes as observed in the tables of cell viability. Because erythrocytes have capacity of pH control and of shinking effect to get rid of the excreted toxic materials.

Table 6 The viability of HL-60 cells incubated for 144 hours: Number of alive and death cells was shown.

Time in hour	# of alive HL-60 cells ($x10^4$)/ml of culture medium	# of death HL-60 cells ($x10^4$)/ml of culture medium	% Viability
0	10.0 ± 0.0	0.0 ± 0.0	100 ± 0.0
24	18.0 ± 0.7	1.0 ± 0.6	95.0 ± 3.2
48	34.0 ± 0.6	3.5 ± 1.0	93.0 ± 1.3
72	65.0 ± 0.5	4.5 ± 1.0	92.0 ± 1.0
96	129.0 ± 0.4	14.0 ± 1.6	93.0 ± 0.5
120	179.0 ± 0.6	31.0 ± 0.2	85.0 ± 0.7
144	187.0 ± 1.6	48.5 ± 1.7	78.0 ± 0.4

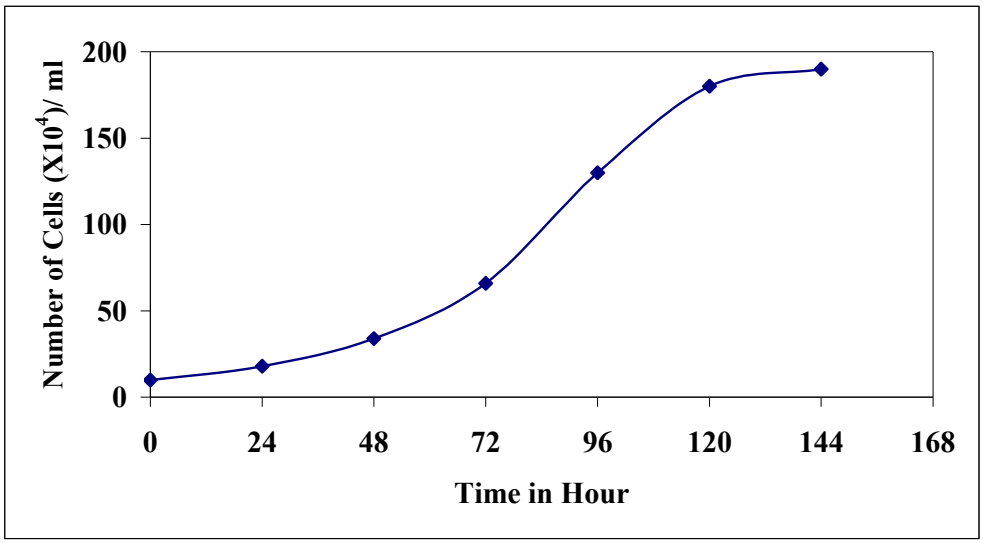


Figure 6 Growth curve of HL 60 cells based on the data given in Table 6.

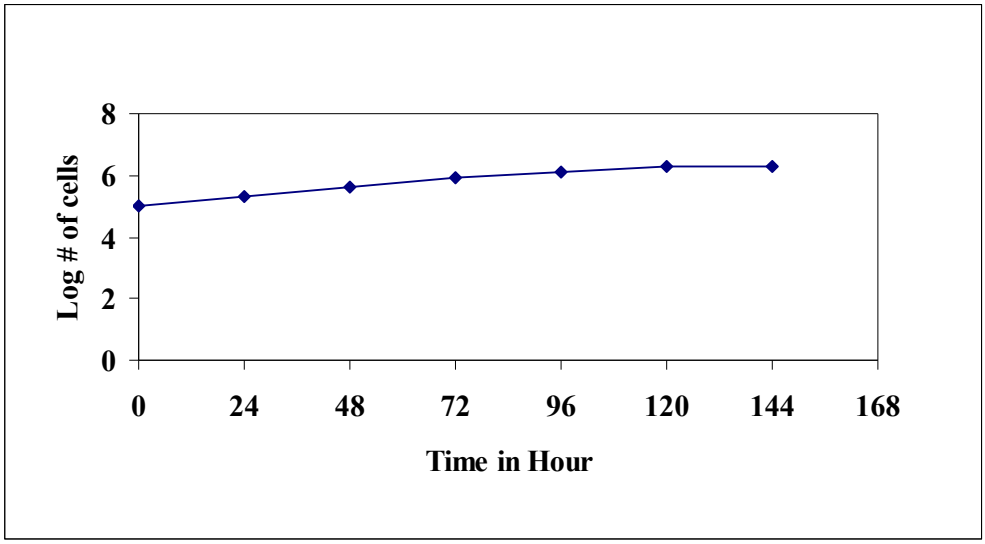


Figure 7 The logarithmic HL-60 cell number versus time(h) diagram.

Table 7 The viability of isolated lymphocytes incubated for 144 hours: Number of alive and death cells was shown

Time in hour	# of alive isolated lymphocytes ($x10^4$)/ ml of culture medium	# of death isolated lymphocytes ($x10^4$)/ ml of culture medium	% Viability
0	10.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
24	17.0 ± 0.6	2.0 ± 0.8	92.0 ± 0.8
48	32.0 ± 0.8	3.0 ± 0.6	90.0 ± 4.1
72	60.0 ± 2.2	5.0 ± 0.6	92.0 ± 1.5
96	118.0 ± 1.8	11.0 ± 0.6	90.0 ± 1.9
120	178.0 ± 2.2	32.0 ± 1.8	85.0 ± 0.8
144	187.0 ± 0.8	53.0 ± 0.9	77.0 ± 0.5

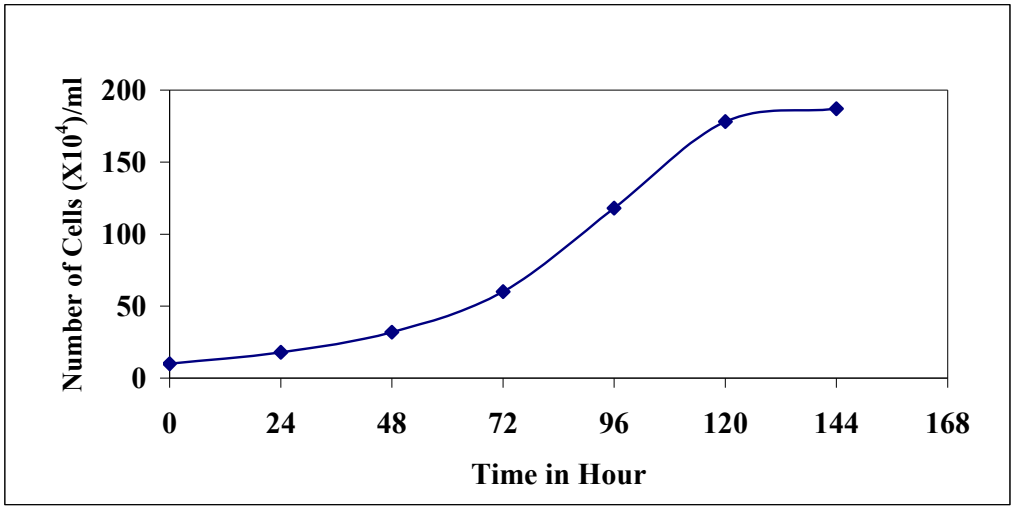


Figure 8 Growth curves of isolated lymphocytes based on the data given in Table 7.

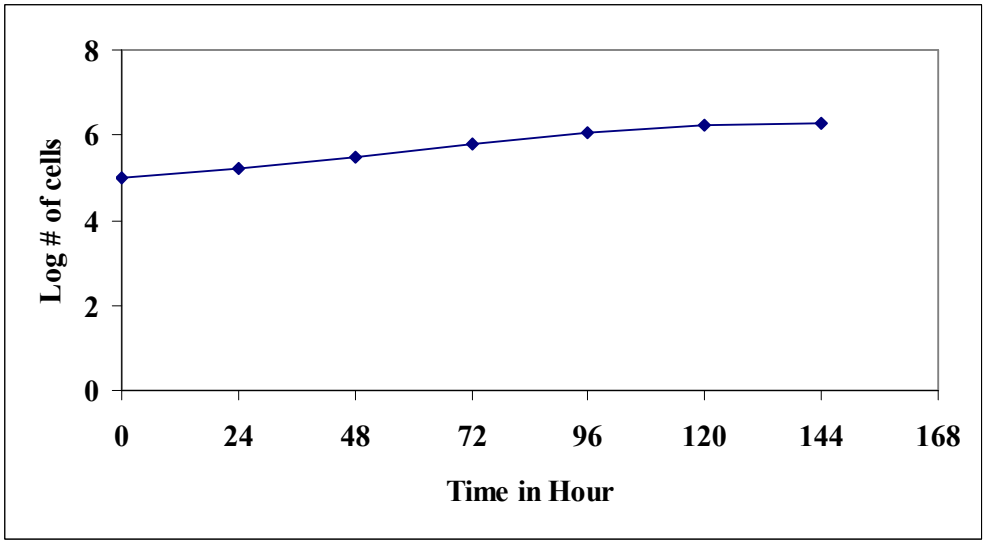


Figure 9 The logarithmic isolated lymphocyte number versus time(h) diagram.

Table 8 The viability of whole blood lymphocytes incubated for 144 hours: Number of alive and death cells was shown

Time in hour	# of alive whole blood lymphocytes ($x10^4$)/ ml of culture medium	# of death whole blood lymphocytes ($x10^4$)/ml of culture medium	% Viability
0	10.0 ± 0.0	0.0 ± 0.0	100 ± 0.0
24	18.0 ± 0.7	1.0 ± 0.6	95.0 ± 3.2
48	34.0 ± 0.8	1.0 ± 0.6	98.0 ± 1.4
72	64.0 ± 1.0	4.0 ± 1.4	94.0 ± 1.9
96	122.0 ± 1.3	13.0 ± 1.0	91.0 ± 1.0
120	176.0 ± 2.2	30.0 ± 1.7	86.0 ± 0.9
144	184.0 ± 2.5	48.0 ± 1.8	79.0 ± 0.5

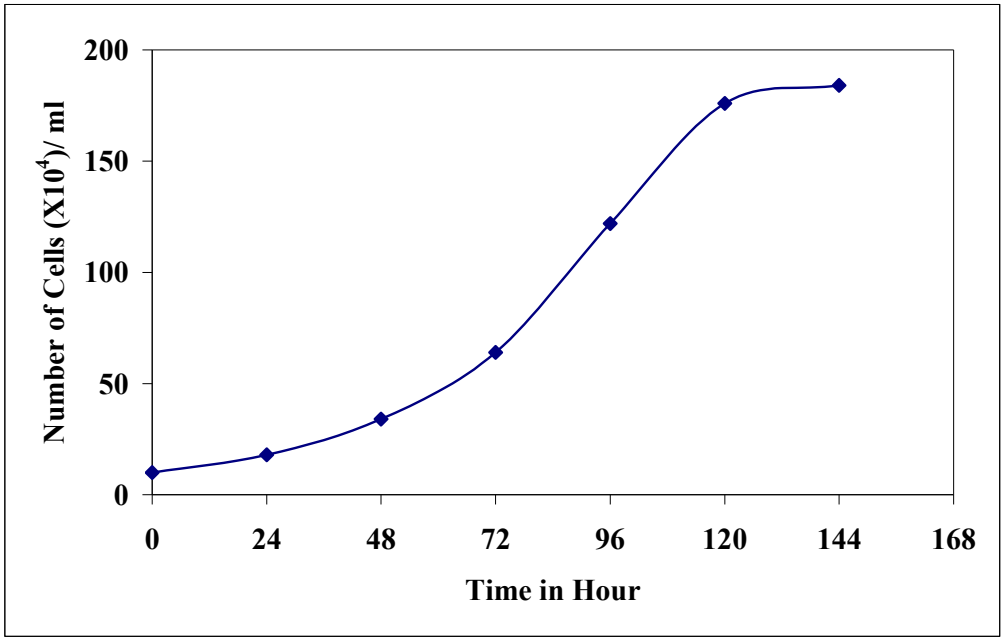


Figure 10 Growth curve of whole blood lymphocytes based on the data given in Table 8.

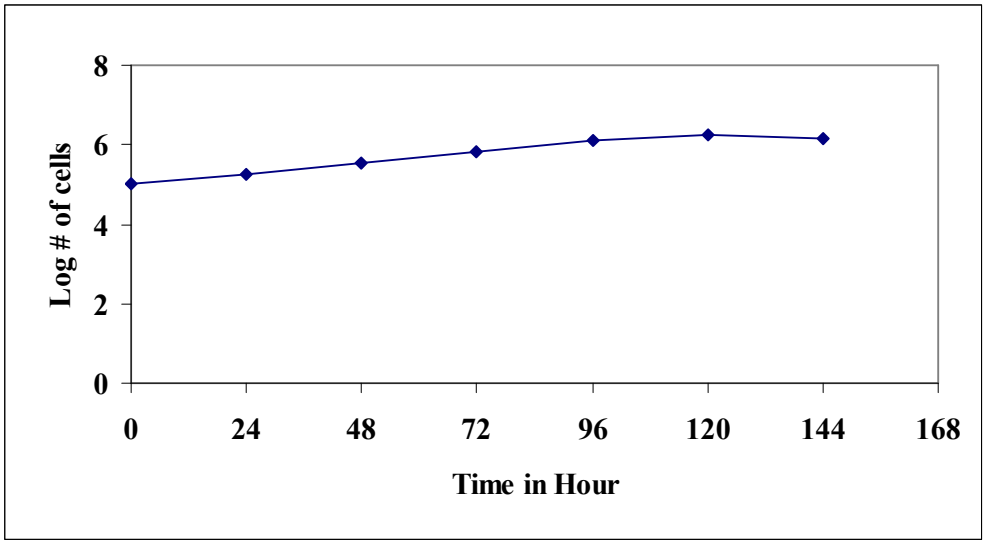


Figure 11 The logarithmic whole blood lymphocyte number versus time(h) diagram.

3.2 Effects of Aescin on Cell Viability

Many plants have been used as therapeutic regimen for thousands of years, based on experience and folk remedies. Horse chestnut is the most popular herbal treatment for venous insufficiency although whole horse chestnut is classified as an unsafe herb by the FDA. Eating the nuts or drinking a tea made from the leaves can cause horse chestnut poisoning, the symptoms of which include nausea, vomiting, diarrhea, salivation, headache, breakdown of red blood cells, convulsions, and circulatory and respiratory failure possibly leading to death (Chandler, 1993). Unprocessed horse chestnut seeds contain a toxin called esculin (also spelled aesculin). This toxin may increase the risk of bleeding due to its ability to stop blood clots forming (antithrombotic). Horse chestnut seeds are therefore processed to

remove the toxic component, resulting in purified horse chestnut seed extract (HCSE) (Brinker, 1998).

Horse chestnut extracts are said to have alternative analgesic, antipyretic, astringent, expectorant, narcotic, sternutatory, tonic and vasoconstricting properties. They have been used as cataplasm for mammary indurations and other types of topical ulcer and even cancer.

Aescin is the non-toxic part of the horse chestnut. Aescine is a saponin and has a reducing ability the rate of fluid leakage from stressed and irritated vessel walls. The exact mechanism is not known, but the most prominent theory proposes that aescin plugs leaking capillaries, prevents the release of enzymes that break down collagen and open holes in capillary walls.

In order to get an idea about the usage of aescin as an anticancer drug for chemotherapy, cytotoxic effect of aescin on normal cells and cancer cells should be investigated. The cytotoxic effect of aescin on cells in culture was examined by incubating the cells at different aescin concentrations varying from 10^{-5} M to 10^{-10} M. in culture medium. The aim of the different aescin concentration usage is to determine an aescin concentration not only minimum cytotoxic effect on normal cell but also maximum cytotoxic effect on cancer cells, in order to eliminate the cancer cells selectively.

Percent viability in control groups of all cell types has to be over %90 in cytotoxicity assay. In order to obtain data given in Table 9, the cells at 96^{th} hour were choosen to follow the cytotoxic effects of aescin on cells.

$1x10^5$ healthy and adapted cells/ ml was inoculated into 25 cm^2 sterile culture flask containing 5ml fresh complete medium and incubated for 24 hours. Six different aescin concentrations at the final concentrations varying from $2x10^{-5}$ M to $2x10^{-10}$ M in 5ml complete medium were added onto the growing cells at the 24^{th} hour of the incubation. By the addition of the second 5 ml complete medium

containing different aescin concentrations, total culture medium became 10 ml containing.10^{-5} M to 10^{-10} M aescin. Then, the cells were incubated for further 72 hours after the addition of aescin into the culture medium. The cells treated with different aescin concentrations were called treated cells while if the cells were not treated with any chemicals, they were called untreated cells or control group cells. To standardize the conditions for both treated and untreated cells, 5ml fresh medium was added into control group containing flask to get total 10 ml complete medium. Although the cells were cultured totally for 96 hours, the cells were exposed to aescin only for 72 hours and the data used in Table 9, was taken at the end of 96 hours incubation.

Table 9 illustrates that when the aescin concentration increased, the percent viability of all types of cells decreased. Toxicity of aescin on all used cell types shows that high aescin concentrations (10^{-5} M and 10^{-6} M) kill the cells by destruction the cell membranes because it is a saponin and has an effect on the cell membrane destruction. As shown in Figure 12, the cell membranes of the cells at high concentration at 10^{-6} M were affected and their cytoplasm was leaked out from their cell membranes. 10^{-8} M and 10^{-9} M have higher toxic effect on the viability of the isolated lymphocyte than on the viability of the whole blood lymphocytes. In whole blood lymphocytes, red blood cells have a sink effect. This sink effect has the protective capacity on lymphocytes against to toxicity of chemicals. HL-60 cells are more sensitive than whole blood lymphocytes but lesser sensitive than isolated lymphocytes. They are cancer and immortal cells. Their deaths were much more difficult than normal cells.

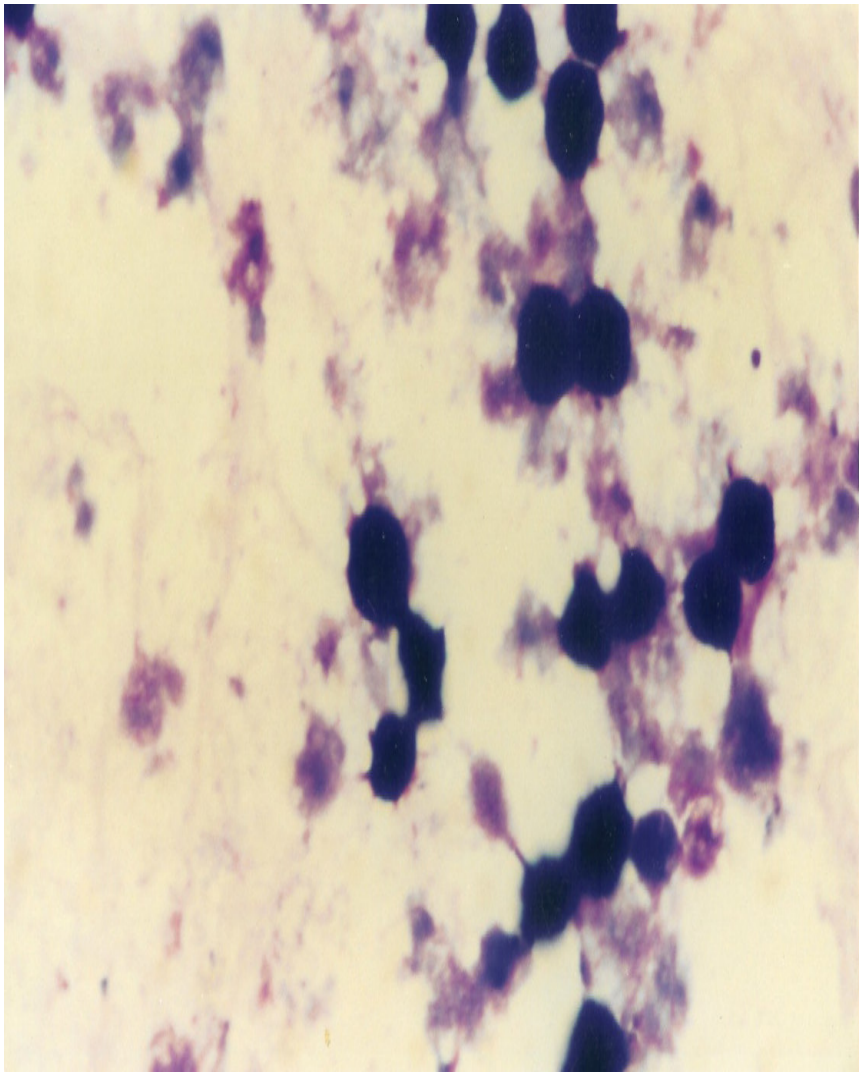


Figure 12 Overview of the cell membrane destruction of HL-60 cells with the treatment of 10^{-6} M aescin after 96 hours at 100X magnification.

Table 9 The effects of different aescin concentrations on the cell viability after 96 hours incubation of HL-60 cells, isolated lymphocytes and whole blood lymphocytes.

	# of alive cells (x10^4)/ ml of culture medium			# of death cells (x10^4)/ ml of culture medium			% Viability		
	Isolated Lymphocytes	**Whole Blood Lymphocytes**	**HL-60 Cells**	**Isolated Lymphocytes**	**Whole Blood Lymphocytes**	**HL-60 Cells**	**Isolated Lymphocytes**	**Whole Blood Lymphocytes**	**HL-60 Cells**
Control	59.0 ± 0.9	61.0 ± 0.9	65.0 ± 0.9	65.0 ± 0.9	2.0 ± 0.5	3.0 ± 0.5	91	97	94
10^{-10} M	25.0 ± 0.5	60.0 ± 0.7	52.0 ± 0.9	7.0 ± 0.3	10.0 ± 0.5	14.0 ± 0.3	82	86	79
10^{-9} M	18.0 ± 0.5	58.0 ± 0.9	50.0 ± 1.0	7.0 ± 0.7	16.0 ± 0.4	19.0 ± 0.6	74	78	73
10^{-8} M	14.0 ± 0.3	52.0 ± 0.8	43.0 ± 0.9	11.0 ± 0.5	24.0 ± 0.5	19.0 ± 0.7	70	68	67
10^{-7} M	10.0 ± 0.7	43.0 ± 0.9	36.0 ± 1.3	13.0 ± 0.4	32.0 ± 0.7	32.0 ± 0.6	54	57	53
10^{-6} M	7.0 ± 0.4	26.0 ± 0.6	28.0 ± 0.7	17.0 ± 0.6	37.0 ± 0.7	53.0 ± 0.9	29	41	34
10^{-5} M	2.0 ± 0.4	9.0 ± 0.6	6.0 ± 0.5	26.0 ± 0.7	56.0 ± 1.1	59.0 ± 0.6	7	14	9

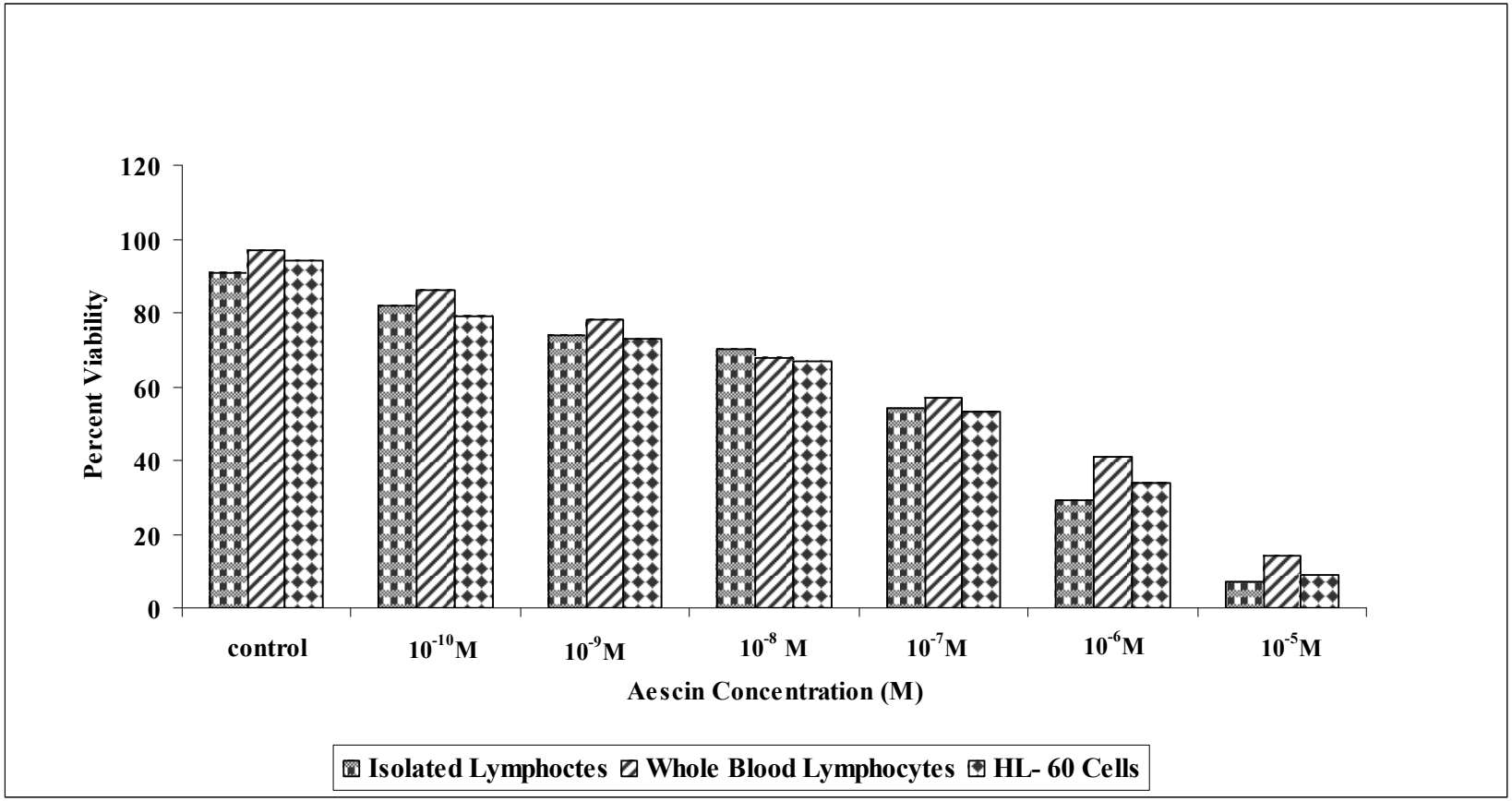


Figure 13 Percent Viability at different Aescin Concentrations.

3.3 The Effect of Aescin on Glutathione S-Transferase Enzyme was Examined by Growing the Cells in the Presence of Varying Concentrations of Aescin in Culture Medium

In order to evaluate the biochemical effects of aescin on the cells, GSTs and NATs activities were investigated. It has been suggested that the glutathione S-transferases are the most important class of enzymes in the protection of the cell from the toxic effects of reactive electrophiles (Armstrong, 1991). Research has clearly established a role for these enzymes in mediating resistance to a wide variety of electrophiles, from the endogenous products of oxidative metabolism to environmental carcinogens and antineoplastic alkylating agents.

Studies on this enzyme family are clearly needed to obtain a better understanding of cancer prevention and therapy because the relationship between GSH transferases and drug resistance is well known. An increasing expression of activities GSTs in tumor cell indicates that GSTs play a role in the intrinsic and acquired resistance of tumors to anticancer drugs (Lewis *et al.*, 1989).

The enzyme activities for different aescin concentrations over all types of cells treated and untreated during the study were observed. In order to eliminate hemoglobin interference, whole blood lymphocytes were separated from whole blood cells by density gradient technique like in the procedure of lymphocyte isolation as described under “Methods” in section 2. Before homogenization of cells, cell count needed to be determined to calculate GSTs activities against CDNB as a common substrate per cell.

The average GSTs activities of 4 sets of all the studied cell types were given on Table 10. As observed from the Table 10, the GSTs activities were measured against CDNB in the cytoplasms of all used cell types in this study. GST activities increased not only with aescin but also with increasing of its concentration in all used cell

types. In HL-60 cells (tumor cells), GSTs activities were high even at control group when it was compared with other control groups in isolated lymphocytes and whole blood lymphocytes ($^{+}p< 0.0001$). The average specific activity against CDNB in HL-60 control group was calculated as 57.1 ± 0.6 μmole/min/cells ($X10^{-9}$) and in isolated lymphocyte control group 41.3 ± 0.7 μmole/min/cells ($X10^{-9}$) and in whole blood lymphocyte control group 40.4 ± 0.7 μmole/min/cells ($X10^{-9}$). Figure 14 illustrates percent activation in GSTs activity in all studied cell types. The effect of aescin on isolated lymphocytes were more significant than ($^{\delta}p< 0.05$) on whole blood lymphocytes in 10^{-5} M and 10^{-6} M aescin concentrations. In whole blood cultures, aescin concentration might be decreased by sinking effect of erythrocytes. So, the lymphocytes in the whole blood cultures might be less induced by aescin as compared to the lymphocytes in the isolated lymphocyte culture.

In order to evaluate whether the chemical effect of aescin on GSTs activity, isolated lymphocytes, HL-60 cells and whole blood lymphocytes are cultured for 96 hours without any treatment except the medium addition at the 24^{th} hour. For that purpose, the cells from control culture flasks could be used

Table 10 GSTs-CDNB activity determined in cell homogenate in the presence of varying aescin concentration after cell culturing of all cell types.

		GSTs Activity (µmol/min/cell x10-9) ± mean SE		
		Isolated Lymphocytes (n=4)	**Whole Blood Lymphocytes (n=4)**	**HL-60 Cells (n=4)**
Control		41.3 ± 0.7	40.4 ± 0.7	57.1 ± 0.6 $^{+}$
10^{-10}	**M**	43.2 ± 0.5	41.5 ± 0.7	60.3 ± 0.8** $^{+}$
10^{-9}	**M**	45.3 ± 0.5*	43.6 ± 0.5	66.2 ± 0.6*** $^{+}$
10^{-8}	**M**	46.9 ± 0.9**	45.1 ± 0.8*	69.8 ± 0.8*** $^{+}$
10^{-7}	**M**	51.3 ± 0.7**	48.3 ± 0.9**	73.4 ± 0.9*** $^{+}$
10^{-6}	**M**	55.2 ± 0.9***	51.4 ± 0.8*** $^{\delta}$	75.8 ± 1.0*** $^{+}$
10^{-5}	**M**	73.1 ± 1.3***	69.6 ± 0.9*** $^{\delta}$	76.2 ± 1.1*** $^{+}$

$^{*}p< 0.05$, significantly different from control

$^{**}p< 0.005$, significantly different from control

$^{***}p< 0.0001$, significantly different from control

$^{\delta}p< 0.05$, significantly different from Isolated Lymphocytes

$^{+}p< 0.0001$, significantly different from Whole Blood Lymphocytes and Isolated Lymphocytes

By decreasing the aescin concentrations, GSTs enzyme activities for all used cell types decreasing. In order to evaluate molecular action of aescin on GSTs enzyme, aescin was added on the cytoplasm of all used cell types, GSTs enzyme activities at the different aescin concentration were investigated. No change was observed when aescin was added on the cytoplasm of the cells, without incubation for 72 hours in cultures.

Table 11 Percent activation in GSTs activity. Values are calculated considering control activity as 100%.

	Percent Activation in GSTs activity		
	Isolated Lymphocytes	**Whole Blood Lymphocytes**	**HL-60 Cells**
Control	-	-	-
10^{-10} M	83	72	33
10^{-9} M	34	27	33
10^{-8} M	24	20	29
10^{-7} M	14	12	22
10^{-6} M	10	08	16
10^{-5} M	05	03	06

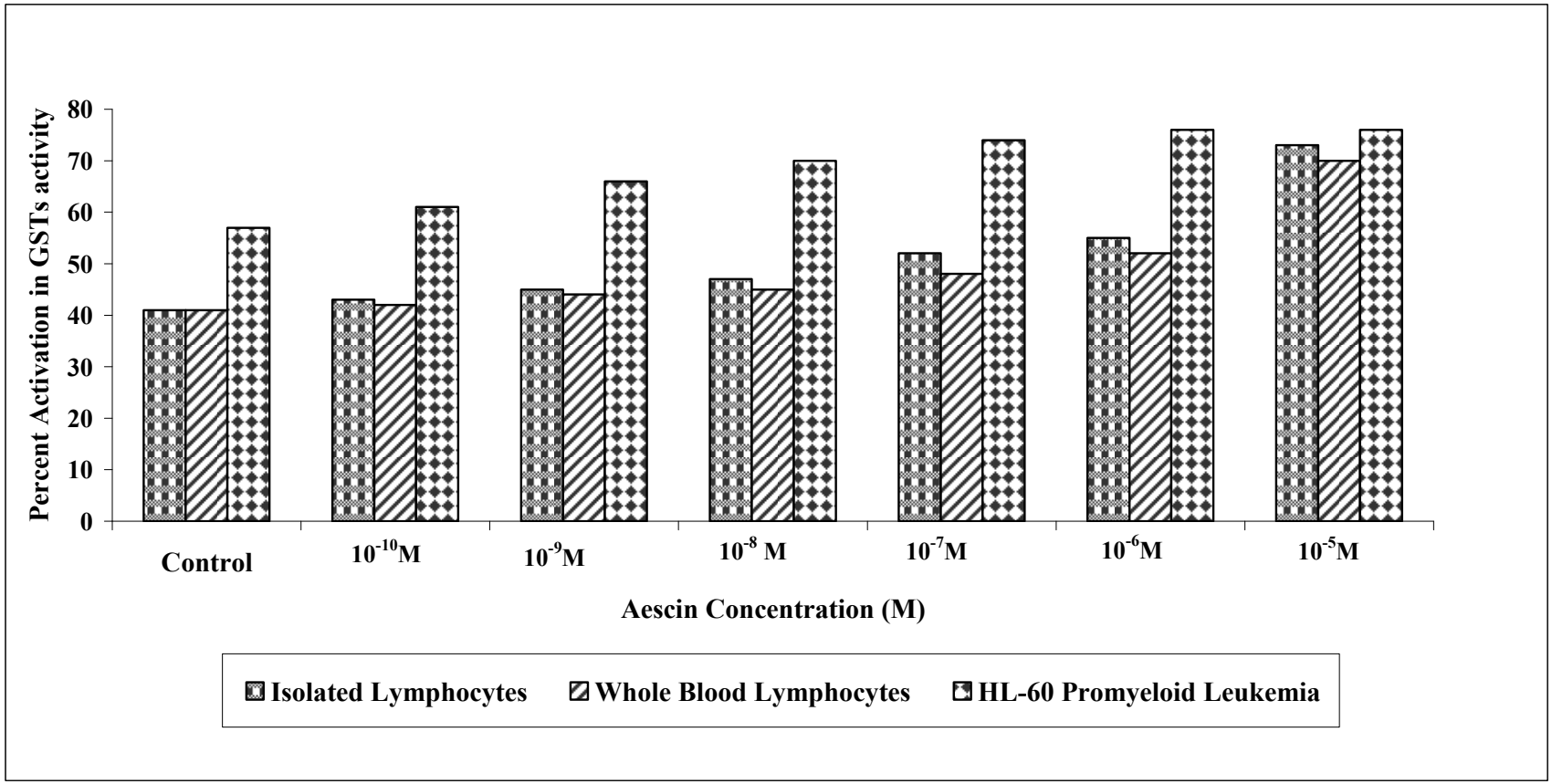


Figure 14 Percent activation in GSTs activity. Values are calculated considering control activity as 100%.

Table 12 GSTs-CDNB activity determined in cell homogenate in the presence of varying aescin concentration.

	GSTs Activity(µmol/min/cell x10^{-9}) ± mean SE		
	Isolated Lymphocytes.	**Whole Blood Lymphocytes**	**HL-60 Cells**
Control	42.1 ± 0.4	41.6 ± 0.4	56.9 ± 0.5
10^{-10} M	42.2 ± 0.5	41.6 ± 0.6	56.9 ± 0.5
10^{-9} M	42.2 ± 0.3	41.7 ± 0.6	56.9 ± 0.4
10^{-8} M	42.1 ± 0.5	41.6 ± 0.5	57.0 ± 0.5
10^{-7} M	42.2 ± 0.7	41.6 ± 0.5	56.9 ± 0.6
10^{-6} M	42.7 ± 0.6	41.9 ± 0.6	56.9 ± 0.5
10^{-5} M	42.7 ± 0.5	41.8 ± 0.6	57.2 ± 0.7

3.4 The Effect of Aescin on NAT1 and NAT2 was Examined by Growing the Cells in the Presence of Varying Concentrations of Aescin in Culture Medium

The enzyme activities of NAT1 and NAT2 for different aescin concentrations over all types of cells treated and untreated during the study were observed.

For enzyme assay, 2 sets of 25 cm^2 sterile culture flasks containing 5 ml complete medium and $1x10^5$ cells/ml were constructed. Each set includes untreated control group and six different aescin concentrations. At 24^{th} hour, cells were treated with different concentrations of aescin in 5 ml of fresh complete medium addition. The cells untreated (control group) and treated with aescin were incubated for 72 hours. Totally, at the end of 96 hours, all cell type cultures were harvested as described under "Methods" in section 2 except whole blood lymphocytes.

In order to eliminate hemoglobin interference, whole blood lymphocytes were subtracted from whole blood cells according to density gradient technique like in the procedure of lymphocyte isolation as described under "Methods" in section 2. Before homogenization of cells, cell count has to be carried out to calculate NAT1 and NAT2 activities per cell against PABA and SMZ as common substrates, respectively.

Another investigated detoxifying enzyme in this study was N- acetyl transferases (NATs); polymorphic enzyme. There are two isozymes with differential substrate specificity; NAT1 and NAT2. PABA (p-nitrobenzoic acid) was the substrate of NAT1isozyme and SMZ (sulfamethazine) was the substrate of NAT2 isozyme in this study. According to one study achieved by N. Atmane (Atmane *et al*, 2003), NAT1 activity decrease reversibly, due to the formation of stable sulfenic acid group at the active-site cysteine as a result of oxidative stress. The oxidative stress is elevated with membrane destruction by triterpene saponin (Valsala *et al*., 2004). So, aescin may cause the oxidative stress by disrupting the cells membrane. The results showed that NAT1 activities of all used cell types were decreased by aescine. NAT1 activity was higher in the control group of HL-60 cells than that of in isolated

lymphocyte and whole blood lymphocyte. In HL-60 cells, NAT2 activity was detected but in isolated lymphocyte and whole blood lymphocyte of the donor who was used in this study, NAT2 activity was not detected. The experiment was carried out two set by duplications but the donor was same. The average NAT1 activity against PABA in HL-60 control group was calculated as 5.8 ± 0.9 μmole/min/cells ($X10^{-9}$) and in isolated lymphocyte control group 4.0± 0.6 μmole/min/cells ($X10^{-9}$) and in whole blood lymphocyte control group 3.9 ± 0.5 μmole/min/cells ($X10^{-9}$). By decreasing the aescin concentrations, NAT1 activity was increased because of decreasing the oxidative stress. In all aescin groups and even the control group in HL-60 promyleoid leukemia, NAT activity was significantly higher than the control group in isolated lymphocytes and whole blood lymphocytes. In HL-60 cells, NAT activity decreased more significantly with the increasing aescin concentration.

Table 13 NAT1 and NAT2 response activity against PABA and SMZ, respectively of all the studied cell types untreated and treated by different aescin concentrations varying from 10^{-5} M to 10^{-10} M.

		NAT1 and NAT2 Activities (µmol/min/cells 10^{-9}) ± mean SE			
		Isolated Lymphocytes	**Whole Blood Lymphocytes**	**HL-60 Cells**	
		NAT1	**NAT1**	**NAT1**	**NAT2**
Control		4.0 ± 0.6	3.9 ± 0.5	5.8 ± 0.9$^{\delta\delta}$	2.2 ± 0.9
10^{-10}	**M**	3.0 ± 0.6	2.9 ± 0.9	5.0 ± 0.5$^{\delta\delta}$	1.8 ± 0.7
10^{-9}	**M**	2.9 ± 0.8	2.7 ± 1.2	4.6 ± 0.6$^{\delta}$	1.7 ± 0.8*
10^{-8}	**M**	2.6 ± 0.9*	2.5 ± 0.9*	4.1 ± 0.7*$^{\delta}$	1.6 ± 0.9*
10^{-7}	**M**	2.5 ± 0.9*	2.2 ± 0.8*	3.5 ± 1.0**$^{\delta}$	1.2 ± 1.0**
10^{-6}	**M**	2.3 ± 1.1*	1.9 ± 0.7**	3.2 ± 0.8**$^{\delta}$	1.0 ± 0.7**
10^{-5}	**M**	2.2 ± 0.4**	2.0 ± 0.9**	3.2 ± 0.9**$^{\delta}$	1.0 ± 0.5**

*p< 0.05, significantly different from control

**p< 0.005, significantly different from control

$^{\delta}$p< 0.005, significantly different from Isolated Lymphocytes

$^{\delta\delta}$p< 0.0001, significantly different from Isolated Lymphocytes

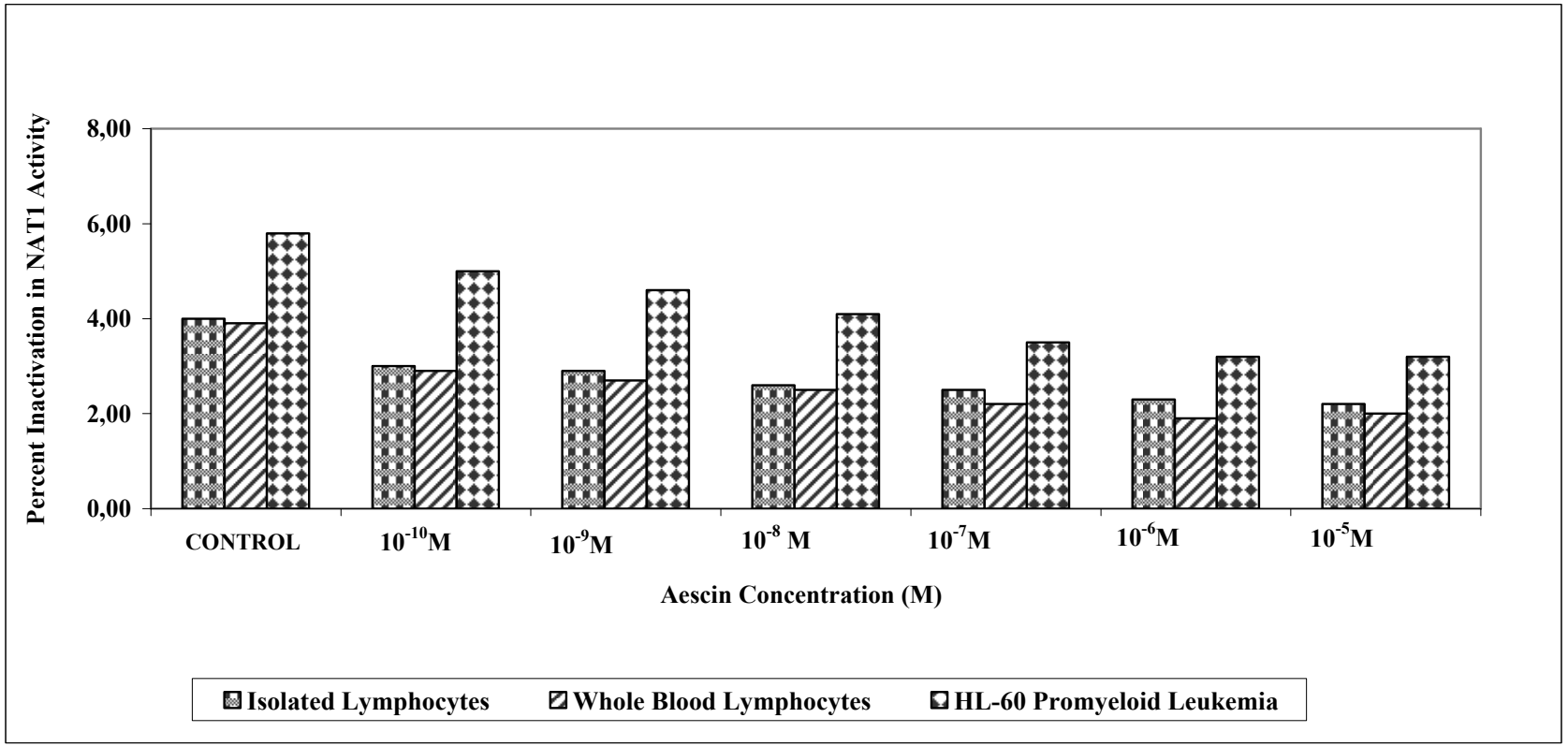


Figure 15 Percent Inactivation in NAT1 activity. Values are calculated considering control activity as 100%

Table 14 Percent inactivation in NAT1 and NAT2 activity. Values are calculated considering control activity as 100%.

		Percent Inactivation in NAT1 and NAT2 Activities (µmol/min/cells 10^{-9}) ± mean SE			
		Isolated Lymphocytes (n=2)	Whole Blood Lymphocytes (n=2)	HL-60 Cells (n=2)	
		NAT1	NAT1	NAT1	NAT2
Control		-	-	-	-
10^{-10}	M	25	25	14	18
10^{-9}	M	27	31	21	22
10^{-8}	M	35	36	29	27
10^{-7}	M	37	43	40	45
10^{-6}	M	42	51	45	50
10^{-5}	M	45	49	45	50

3.5 The Effect of Aescin on the Frequency of Sister Chromatid Exchanges (SCE) was Examined by Growing the Cells in the Presence of Varying Concentrations of Aescin in Culture Medium

In order to evaluate the genotoxic effect of aescin on isolated lymphocyte, whole blood lymphocyte and HL-60 cells, sister chromatid exchanges (SCE) and micronuclei (MN) assays were constructed.

In vitro induction of sister chromatid exchanges (SCEs) in cultured blood lymphocytes allow for the assessment of individual susceptibility to genotoxic effects of the compounds (Landi *et al*., 1996). In order to examine the genotoxic

effects of aescin in human lymphocytes, the whole blood, isolated human lymphocyte cultures and HL-60 cells were prepared as described under "Methods" in section 2. Human lymphocytes were treated with six different doses of aescin ranging between 10^{-5} M to 10^{-10} M concentrations. The lymphocytes were treated with different concentration of aescin after 24 hours initiation of culture.

Figure 16 shows that at **(a)** section, chromosomes at metaphase were illustrated under 10X magnification to find out their locations. At **(b)**, **(c)** and **(d)** sections, the chromosomes were seen at the first round mitosis (M1), at the second round mitosis (M2) and at the third round mitosis (M3). SCEs in cultured lymphocytes and HL-60 cells are evaluated by the chromosomes at M2 because of different color visualized sister chromatids by Fluoresence plus Giemsa stain technique. In order to calculate SCEs per cell, minimum 25 cells at the second round of mitosis was investigated.

Number of chromosomes at M2 decreased during either cell division has been stopped or slowed down. Mitotic Index (MI) is a valuable tool to detect change on cell division, since MI indicates cell division rate. The differences between SCEs values in whole blood, isolated lymphocytes and HL-60 cells against different concentrations of aescin were given in Tables 15, 16, 17.

Baseline SCEs value and MN value in HL-60 cells even in control group were high because the genome of HL-60 cell is transformed and these values in tumor cells are high. Mitotix Index of HL-60 cell was high with compared to isolated lymphocytes and whole blood lymphocytes, because it is cancer cell and the division rate is high in tumor cell. In whole blood culture, lymphocytes were protected and the medium was buffered by erythrocytes to support lymphocyte growth. Therefore, lymphocyte in whole blood culture grown better than lymphocyte in isolated culture. The table of SCE response in HL-60 cells showed that until 10^{-8} M aescin concentration from 10^{-5} M aescin concentration, SCE values were significantly different from the control group value ($p<0.0001$) and the Mitotic Index decreased sharply from 2.51 to 1.67 with 10^{-5} M aescin concentration. At 10^{-9} M aescin

concentration this difference decreased to $p<0.005$ significance and at 10^{-10} M aescin concentration to $p<0.05$ significance. With the decreasing used aescin concentration, this effect slowed down that means the effect was dose-dependent. Same dose-dependent effect was observed on isolated lymphocyte and whole blood lymphocyte.

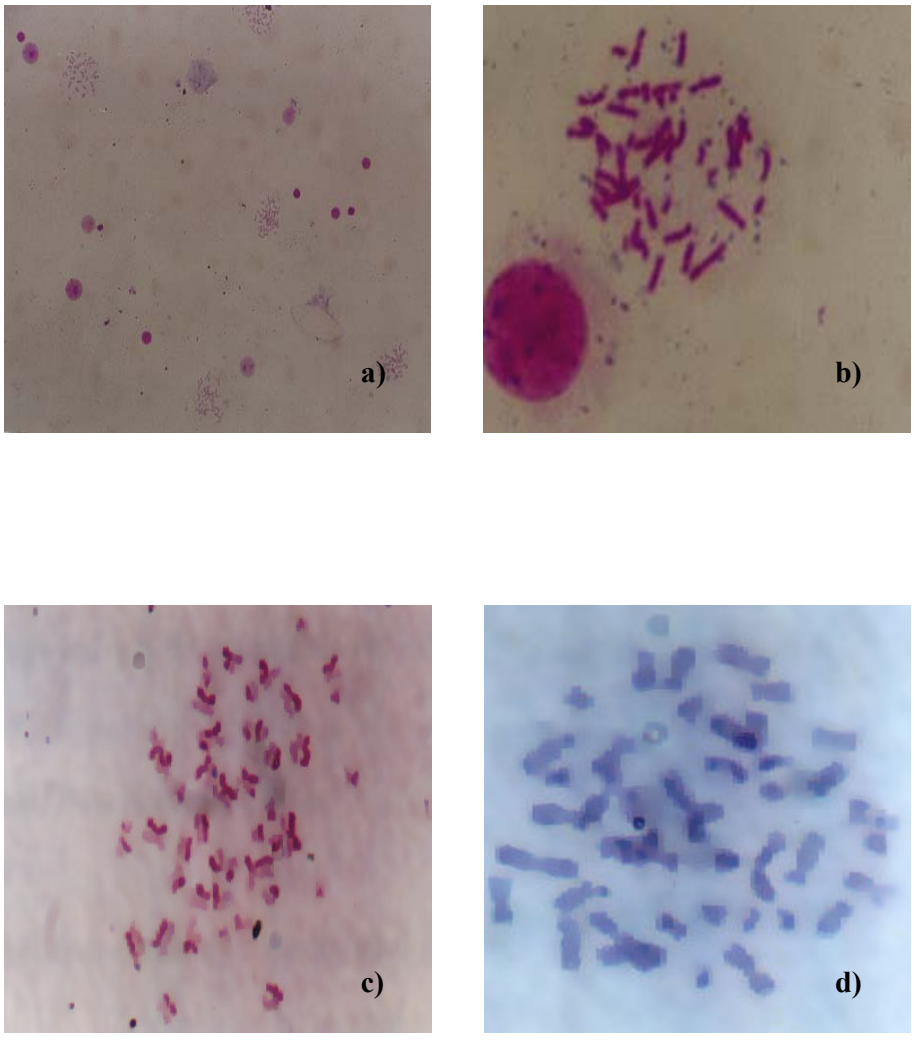


Figure 16 Plan views of chromosomes used in SCE detections under microscope **(a)** overview of chromosomes10X and **(b)** M1 chromosomes and **(c)** M2 chromosomes and **(d)** M3 chromosomes at 100X magnification.

Table 15 SCE response of isolated human lymphocytes to differing doses of aescin

		Total number of metaphases	# of M_1 cells	# of M_2 cells	# of M_3 cells	% of M_2 cells	Mean SCEs/cell (± SE)	Mitotic Index (MI)
Control		110	10	37	63	34	4.620 ± 0.306	2.30
10^{-10}	**M**	70	19	33	18	47	5.909 ± 0.283 **	1.99
10^{-9}	**M**	73	25	33	15	45	5.848 ± 0.247 **	1.94
10^{-8}	**M**	71	27	32	12	45	6.469 ± 0.280 *	1.79
10^{-7}	**M**	66	31	26	9	40	6.538 ± 0.352 *	1.67
10^{-6}	**M**	63	35	25	3	40	6.480 ± 0.289 *	1.49
10^{-5}	**M**	67	40	25	67	37	6.840 ± 0.340 *	1.41

*$p < 0.0001$, significantly different from control
**$p < 0.005$, significantly different from control

Table 16 SCE response of whole blood lymphocytes to differing doses of aescin

		Total number of metaphases	# of M_1 cells	# of M_2 cells	# of M_3 cells	% of M_2 cells	Mean SCEs/cell (±SE)	Mitotic Index (MI)
Control		97	10	38	49	40	2.895 ± 0.263	2.40
10^{-10}	**M**	112	12	47	52	43	2.467 ± 0.249	2.36
10^{-9}	**M**	111	10	45	50	41	2.711 ± 0.237	2.37
10^{-8}	**M**	139	45	45	48	32	3.067 ± 0.261	1.99
10^{-7}	**M**	114	47	35	32	31	4.257 ± 0.321**	1.87
10^{-6}	**M**	86	38	28	28	33	5.036 ± 0.410*	1.80
10^{-5}	**M**	88	41	30	30	34	5.33 ± 0.347*	1.73

*$p < 0.0001$, significantly different from control
**$p < 0.005$, significantly different from control

Table 17 SCE response of HL-60 cells to differing doses of aescin.

		Total number of metaphases	# of M_1 cells	# of M_2 cells	# of M_3 cells	% of M_3 cells	Mean SCEs/cell (± SE)	Mitotic Index (MI)
Control		211	10	39	119	39	6.143 ± 0.256	2.51
10^{-10}	**M**	156	15	98	43	62	6.89 ± 0.285 ***	2.20
10^{-9}	**M**	156	26	92	38	59	7.10 ± 0.272 **	2.08
10^{-8}	**M**	159	29	94	36	59	7.19 ± 0.376 *	2.04
10^{-7}	**M**	138	30	85	23	61	7.28 ± 0.287 *	1.94
10^{-6}	**M**	89	34	38	17	43	7.76 ± 0.192 *	1.81
10^{-5}	**M**	58	38	26	13	45	7.81 ± 0.266 *	1.67

*$p< 0.0001$, significantly different from control

**$p< 0.005$, significantly different from control

***$p< 0.05$, significantly different from control

Table 18 Effects of different aescin concentrations on SCE in all the studied cell types.

		Isolated Lymphocytes		Whole Blood Lymphocytes		HL-60 Promyeloid Leukemia	
		Mean SCEs/cell (± SE)	**Mitotic Index (MI)**	**Mean SCEs/cell (± SE)**	**Mitotic Index (MI)**	**Mean SCEs/cell (± SE)**	**Mitotic Index (MI)**
Control		4.620 ± 0.306+	2.30	2.895 ± 0.263	2.40	6.143 ± 0.256*	2.51
10^{-10}	**M**	5.909 ± 0.283+	1.99	2.667 ± 0.249	2.36	6.89 ± 0.285*	2.20
10^{-9}	**M**	5.848 ± 0.247+	1.94	2.711 ± 0.237	2.37	7.10 ± 0.272*	2.08
10^{-8}	**M**	6.469 ± 0.280+	1.79	3.067 ± 0.261	1.99	7.19 ± 0.376*	2.04
10^{-7}	**M**	6.538 ± 0.352++	1.67	4.257 ± 0.321	1.87	7.28 ± 0.287**	1.94
10^{-6}	**M**	6.480 ± 0.289++	1.49	5.036 ± 0.410	1.80	7.76 ± 0.192**	1.81
10^{-5}	**M**	6.840 ± 0.340++	1.41	5.33 ± 0.347	1.73	7.81 ± 0.266**	1.67

+p< 0.0001, significantly different from whole blood lymphocytes

++p< 0.005, significantly different from whole blood lymphocytes

*p< 0.0001, significantly different from Isolated lymphocytes

**p< 0.005, significantly different from Isolated lymphocytes

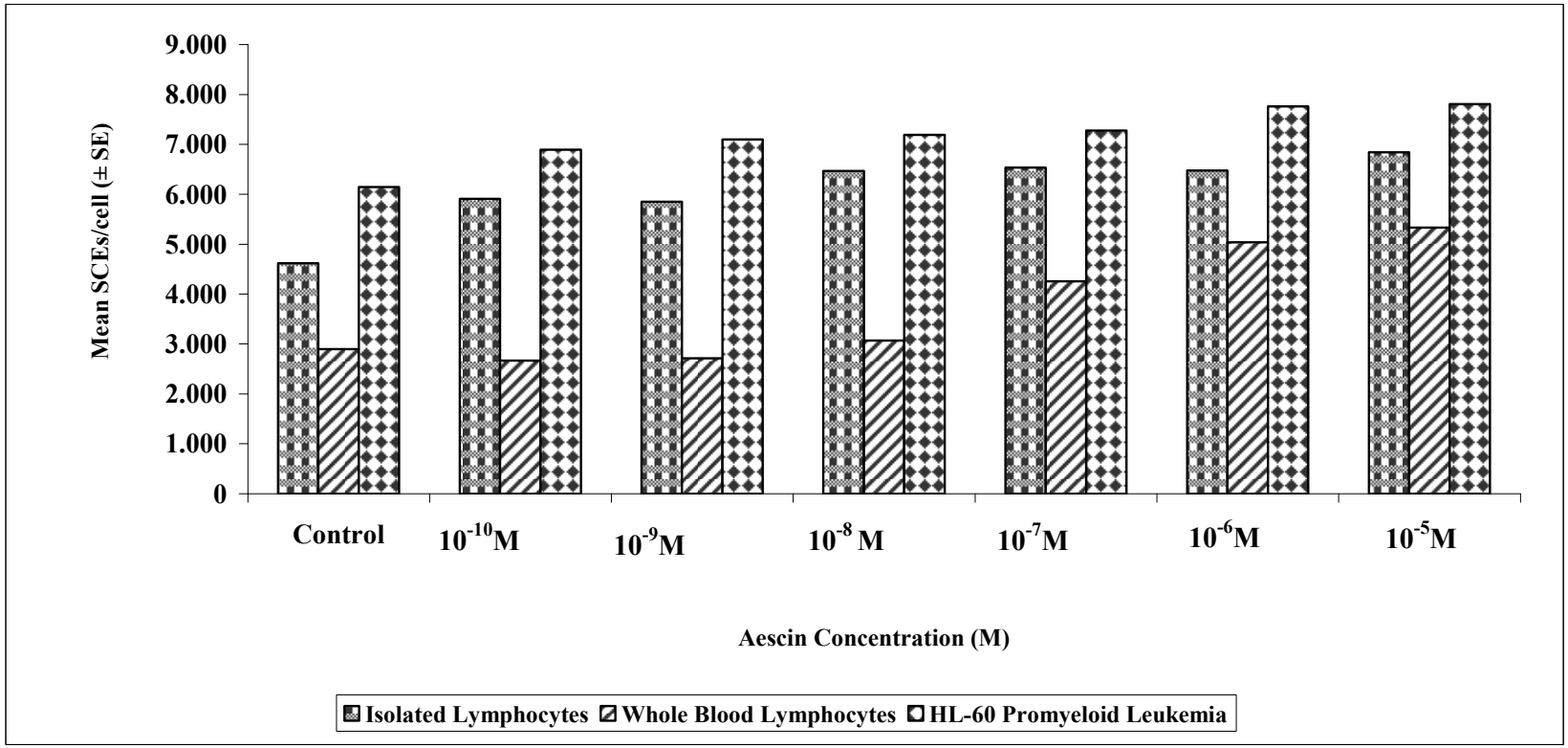


Figure 17 Effects of different aescin concentrations on SCE in all the studied cell types.

According to table 18 and figure 17, the mean of SCEs/cell was found as the lowest in the whole blood lymphocytes. The genotoxic effect between isoleted lymphocytes and the whole blood lymphocytes was significantly different. As mentioned before, the sink effect of erytrocytes was only significant in whole blood lymphocytes compared to isolated lymphocytes. But this difference was more significant between the whole blood and the HL-60, and it was not only due to existance of HL-60 cells in tumor cells, the tumor cells were already more sentitive to treatment with the chemicals. The genotoxic effect of aescin was observed at 10^{-7} M and at higher concentrations in whole blood lymphocytes. However, in isolated lymphocytes and HL-60 cells, the genotoxic effect was observed between concentrations of 10^{-10} M to 10^{-5} M aescin. So, even lowest concentration has a signifificant effect on the isoletd lymphocytes and HL-60 cells.

Same effect was also observed for mitotic index (MI). The effect of aescin was clearly observed on the MI. The cell division was inhibited or the cells were died in the presence and in the increasing aescin concentration for all studied cell types. But the serious effect was observed in isolated lymphocytes and HL-60 cells. The reasons were the same as described above.

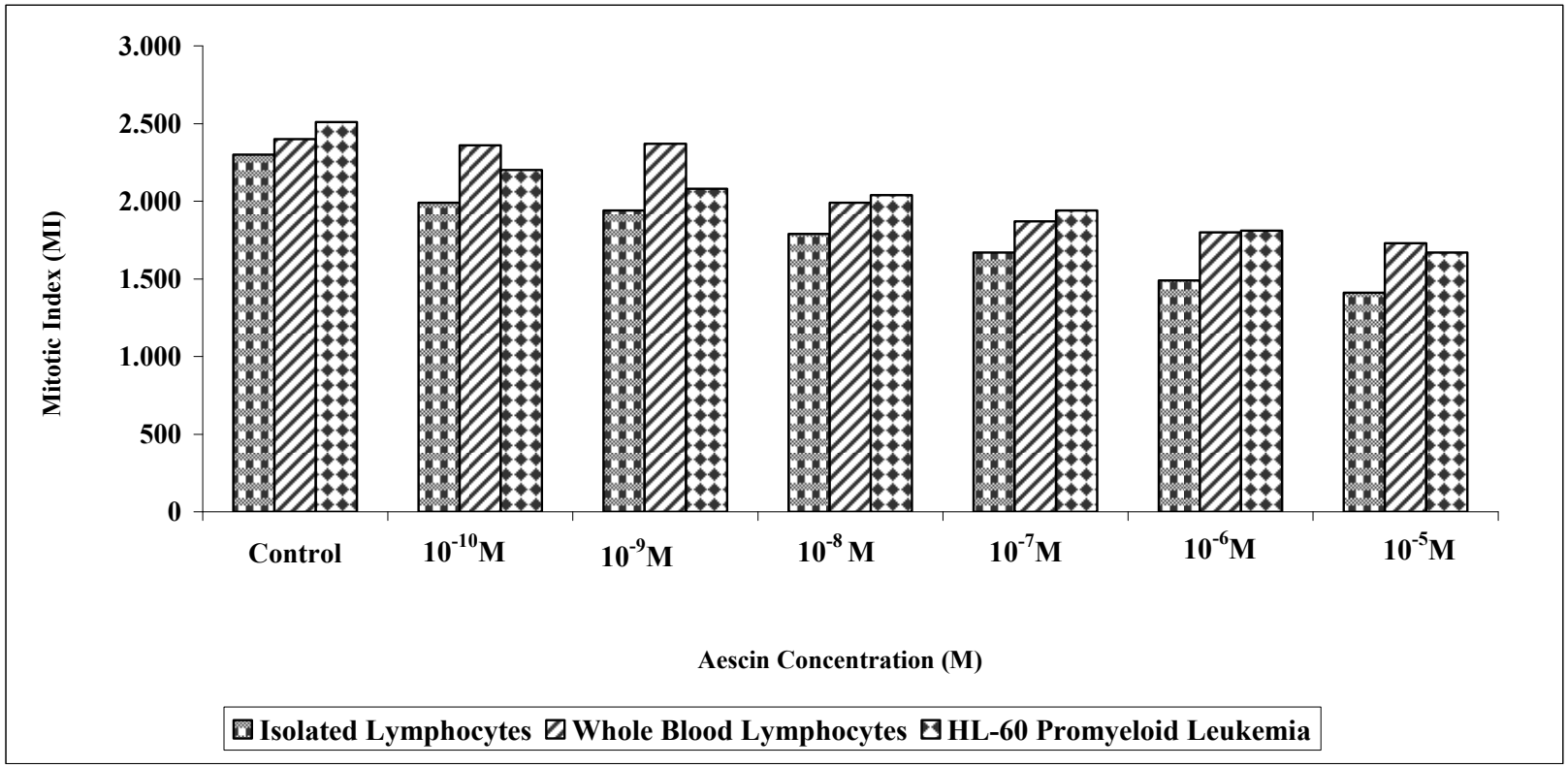


Figure 18 Effects of different aescin concentrations on mitotic index in all the studied cell types.

3.6 The Effect of Aescin on the Frequency of Micronuclei Formation was Examined by Growing the Cells in the Presence of Varying Concentrations of Aescin in Culture Medium

Another genotoxicity test used in this study was micronucleus formation because at least two genotoxicity tests have to be performed in order to evaluate genotoxic effect of any compound or any drug. As seen in SCE response table, baseline micronucleus formation was high in the control group of HL-60 cells because of their chromosomes and related change in their DNA. High concentrations varying from 10^{-5} M to 10^{-8} M aescin concentrations had high significant effect from the control group in HL-60. Same as in SCEs response table, micronuclei formation table represented that lymphocytes in whole blood cell culture were protected by the presence of erythrocyte sink effect. Lymphocytes in isolated lymphocyte culture were more sensitive than lymphocytes in whole blood cell culture.

Up to here, the previous results showed that 10^{-5} M and 10^{-6} M were the toxic concentrations of aescin for all used cell types. Although these both concentrations were toxic, 10^{-6} M aescin concentration had much more significant effect on the micronuclei formation in HL-60 cells than in the isolated lymphocytes and whole blood lymphocytes. This concentration was the second highest aescin concentration used in this study and had higher effect than10^{-5} M aescin concentration in HL-60 cells. The result might be effected from cell death consequently, from cell number. The cell number at 10^{-6} M aescin concentration was higher than at 10^{-5} M aescin concentration. So, 10^{-6} M aescin caused high genotoxicity and less cell death in stead of stricly killing the cells. But 10^{-5} M aescin caused cell death, significantly.

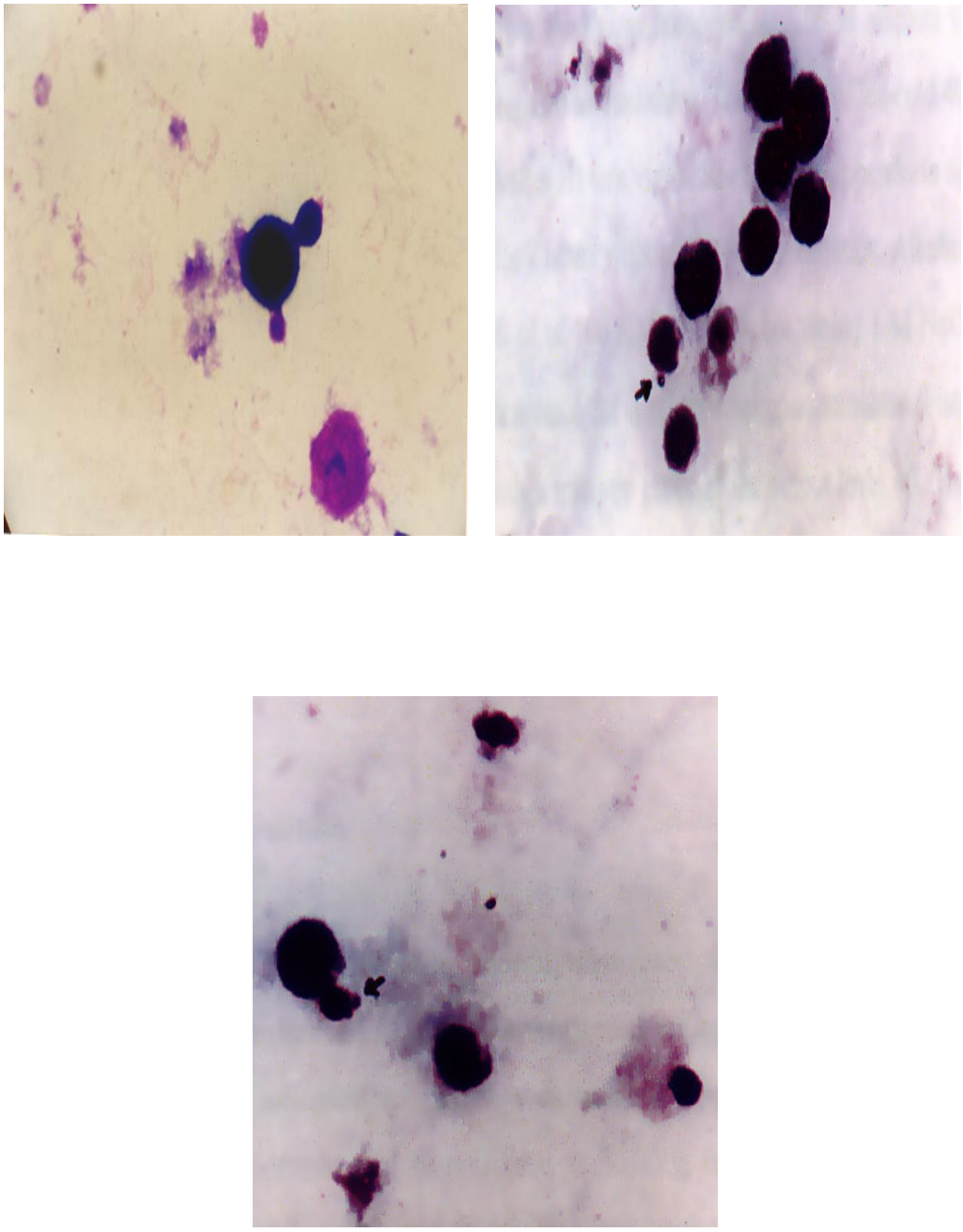


Figure 19 The overview of the formation of micronuclei in HL-60 cells at 100X magnification.

Table 19 Micronucleus response of isolated lymphocytes to the different doses of aescin

	# of cells	# of micronucleus	# of micronucleus per 1000 cells
Control	**1213**	**89**	**73**
10^{-10}M	**1038**	**89**	**86****
10^{-9}M	**1009**	**98**	**97***
10^{-8}M	**1015**	**99**	**98***
10^{-7}M	**1013**	**97**	**96***
10^{-6}M	**979**	**100**	**103***
10^{-5} M	**673**	**72**	**107***

*p< 0.0001, significantly different from control

**p< 0.005, significantly different from control

Table 20 Micronucleus response of whole blood lymphocytes to the different doses of aescin

	# of cells	# of micronucleus	# of micronucleus per 1000 cells
Control	**1018**	**65**	**64**
10-10M	**1029**	**69**	**67**
10-9M	**1013**	**72**	**71**
10-8M	**1101**	**79**	**72**
10-7M	**1089**	**91**	**83****
10-6M	**902**	**78**	**87***
10-5 M	**874**	**80**	**91***

*p< 0.0001, significantly different from control

**p< 0.005, significantly different from control

Table 21 Micronucleus response of HL-60 cells to the different doses of aescin

	# of cells	# of micronucleus	# of micronucleus per 1000 cells
Control	**1575**	**134**	**85**
10-10M	**1198**	**129**	**108****
10-9M	**1085**	**116**	**107****
10-8M	**1212**	**135**	**111***
10-7M	**1121**	**132**	**118***
10-6M	**1002**	**133**	**133***
10-5 M	**987**	**119**	**121***

*p< 0.0001, significantly different from control

**p< 0.005, significantly different from control

Table 22 Micronucleus response of isolated lymphocyte, whole blood lymphocyte and HL-60 cell to the different doses of aescin

		Number of micronucleus per 1000 cells		
		Isolated Lymphocytes.	**Whole Blood Lymphocytes**	**HL-60 Cells**
Control		**73**	**64**	**85****
10^{-10}	**M**	**86++**	**67**	**108***
10^{-9}	**M**	**97++**	**71**	**107***
10^{-8}	**M**	**98+**	**72**	**111***
10^{-7}	**M**	**96++**	**83**	**118***
10^{-6}	**M**	**103+**	**87**	**133****
10^{-5}	**M**	**107++**	**91**	**121***

+p< 0.05, significantly different from whole blood lymphocytes

$^{++}$p<0.005, significantly different from whole blood lymphocytes

*p< 0.005, significantly different from whole blood lymphocytes

**p< 0.001, significantly different from whole blood lymphocytes

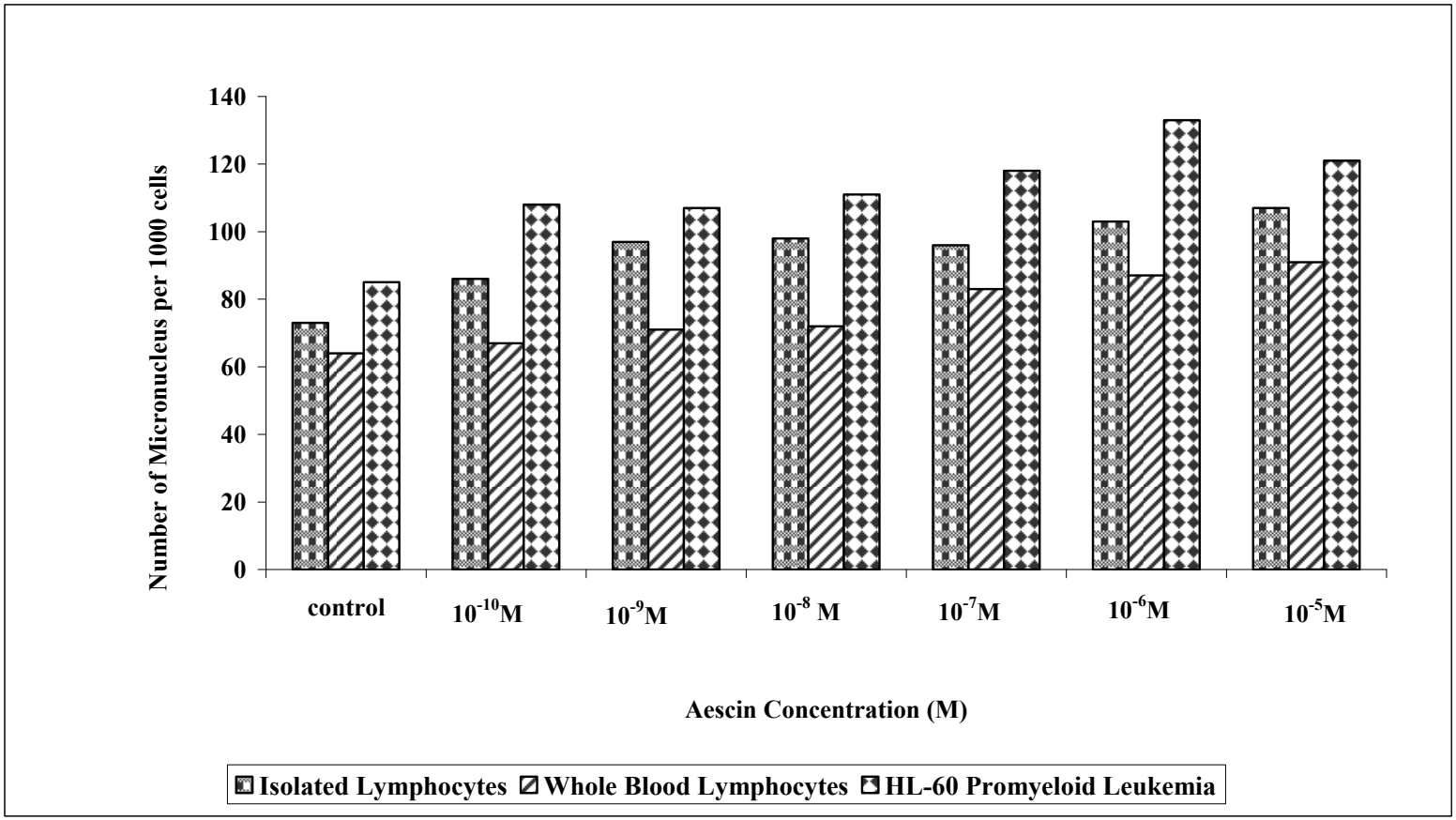


Figure 20 Effects of different aescin concentrations on micronucleus formation in all the studied cell types.

3.7 The Effects of Aescin on HL-60 Cells

3.7.1 Induction of DNA Fragmentation in HL-60 Cells

The idea at the beginning of this study was the finding of a concentration of aescin which was used to cure tumor cells without the damaging of normal cells. In addition to the idea thaf chemotherapy must create a lethal injury to DNA to produce malignant cell death was the mechanism of apoptosis. A dose of chemotherapy which did not produce necrosis could trigger apoptosis, either immediate or delayed. It is clear that an endonucleolytic pathway is activated in apoptosis and results in the cleavage of the cell genome, first into large DNA fragments that vary from 300 kb to 50 kb in size, then into 180 bp multiples (Walker *et al*., 1994). Together with morphological changes characteristic of the process of apoptosis; 180 bp periodicity of the DNA fragments visible on agarose gels stained for DNA is a telltale sign of this mode of cell death. This technique is also known "DNA Ladder".

In order to make comparison between the positive and the negative effect, a positive control group was constructed. H_2O_2 was used in this study due to apoptose induction ability of oxidative stress in all cell types (Shinji *et al*., 1999). As figure 21 showed that as in positive control group of HL-60 DNA fragmentations, the cells treated with the different aescin concentrations varying from 10^{-6} M to 10^{-10} M has similar DNA fragmentation. Negative Control group untreated HL-60 cells had intact DNA without fragmentation. If there was necrotic effect on HL-60 cells, DNA would be observed as smear due to random DNA fragmentation in necrosis on agarose gel electrophoresis. In isolated lymphocyte, aescin effect on DNA fragmentation showed that there was necrosis at high concentrations varying from 10^{-6} M to 10^{-8} M aescin concentrations. Whole blood lymphocyte had been affected by aescin at high concentrations (10^{-6} M and 10^{-7} M) and induced to apoptosis but at low concentrations (from 10^{-8} M to 10^{-10} M), lymphocytes were not induced. As shown in Figure 21, there is a break in genomic DNA structure.

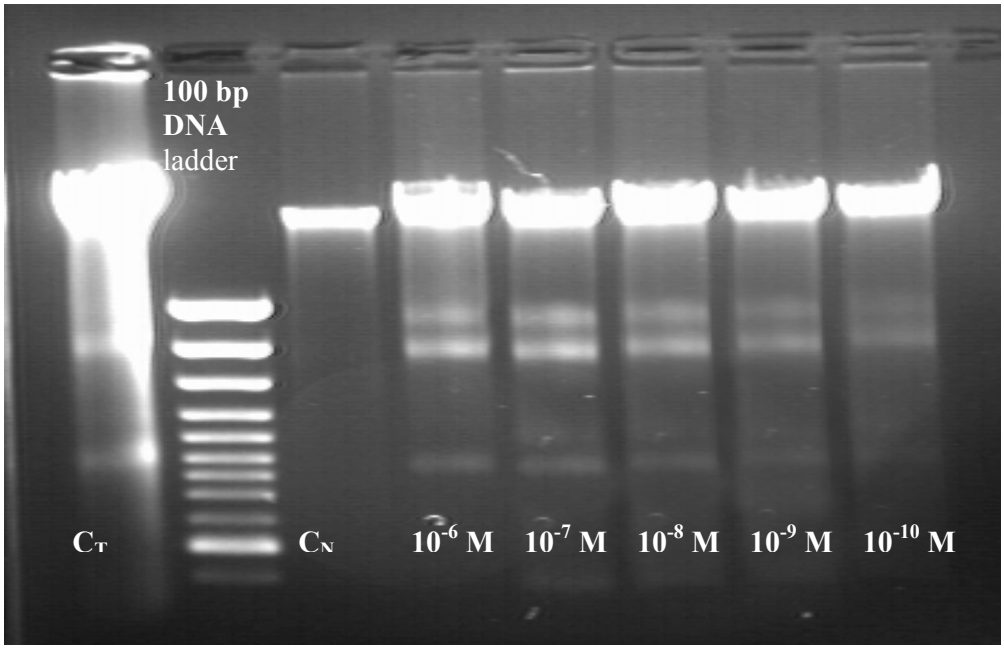


Figure 21 Apoptosis and necrosis identified by agarose gel electrophoresis.

The effects of different concentrations of aescin on DNA of HL-60 cells.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: 100 bp DNA ladder.

Lane 3: Negative control (untreated control (CN)).

Lane 4 to lane 8: HL-60 cells were treated by 10^{-6} M to 10^{-10} M aescin addition.

Same as in genotoxic tests, apoptosis also has to be examined by at least two separate techniques. For that purpose, cytochrome *c* release characteristic of apoptosis was chosen to support the first data.

3.7.2 Induction of Cytochrome c Release from Mitochondria of HL-60 Cells

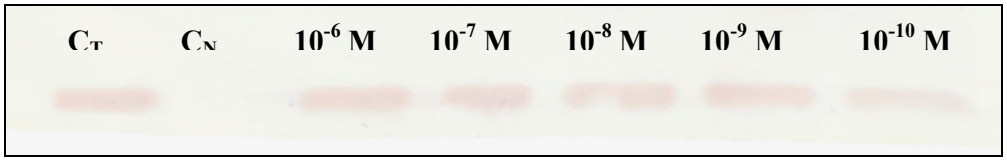


Figure 22 Presence of cytochrome *c* in cytoplasm in HL-60 cells

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: HL-60 cells were treated by 10^{-6} M to 10^{-10} M aescin addition.

Figure 23 Presence of cytochrome *c* in mitochondria in HL60 cells.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: HL-60 cells were treated by 10^{-6} M to 10^{-10} M aescin addition.

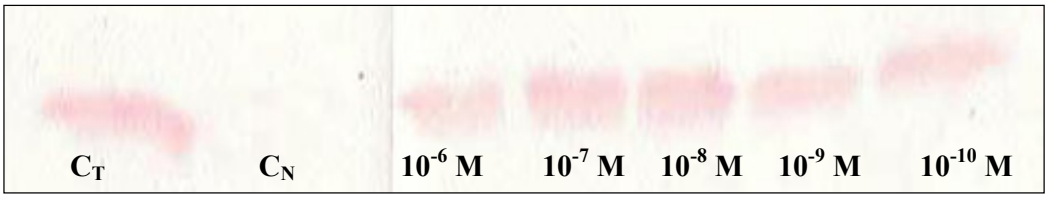


Figure 24 Presence of COX4 in mitochondria in HL-60 cells.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control(C_N)).

Lane 3 to lane 7: HL-60 cells were treated by 10^{-6} M to 10^{-10} M aescin addition.

3.8 The Effects of Aescin on Isolated Lymphocytes

3.8.1 Induction of DNA Fragmentation in Isolated Lymphocytes

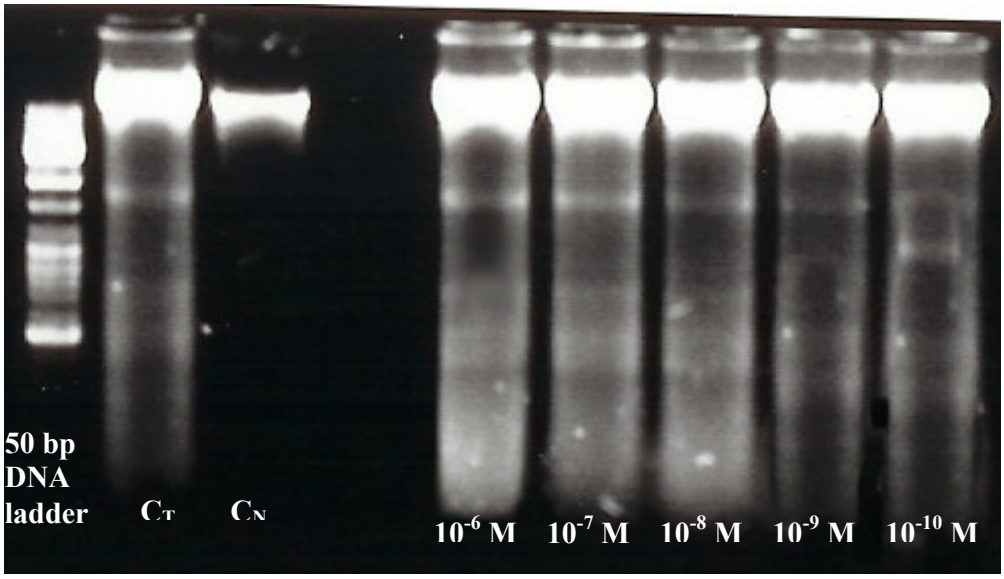


Figure 25 Apoptosis and necrosis identified by agarose gel electrophoresis

The effects of different concentrations of aescin on DNA of isolated lymphocytes.

Lane 1: 50 bp DNA ladder.

Lane 2: H_2O_2 treated positive control (CT).

Lane 3: Negative control (untreated control (CN)).

Lane 5 to lane 9: Isolated lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

3.8.2 Induction of cytochrome c release from mitochondria of isolated lymphocytes

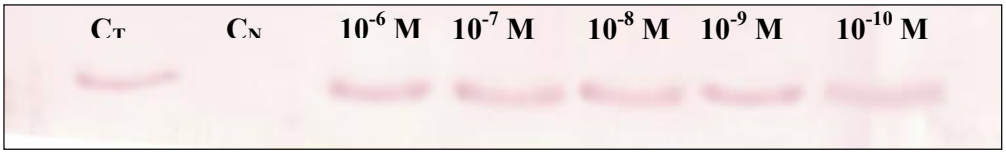


Figure 26 Presence of cytochrome *c* in cytoplasm in isolated lymphocyte.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: Isolated lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

Figure 27 Presence of cytochrome *c* in mitochondria in isolated lymphocytes.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: Isolated lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

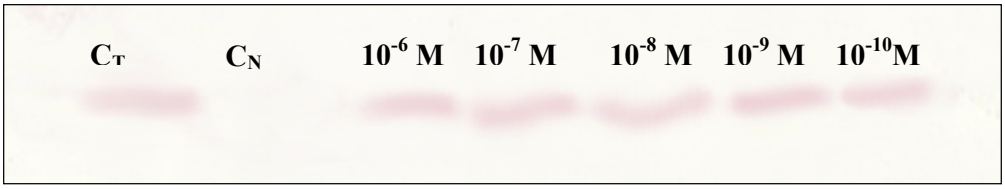


Figure 28 Presence of COX4 in mitochondria in isolated lymphocytes.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: Isolated lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

3.9 The Effects of Aescin on Lymphocytes in Whole Blood Culture

3.9.1 Induction of DNA Fragmentation in Whole Blood Lymphocytes

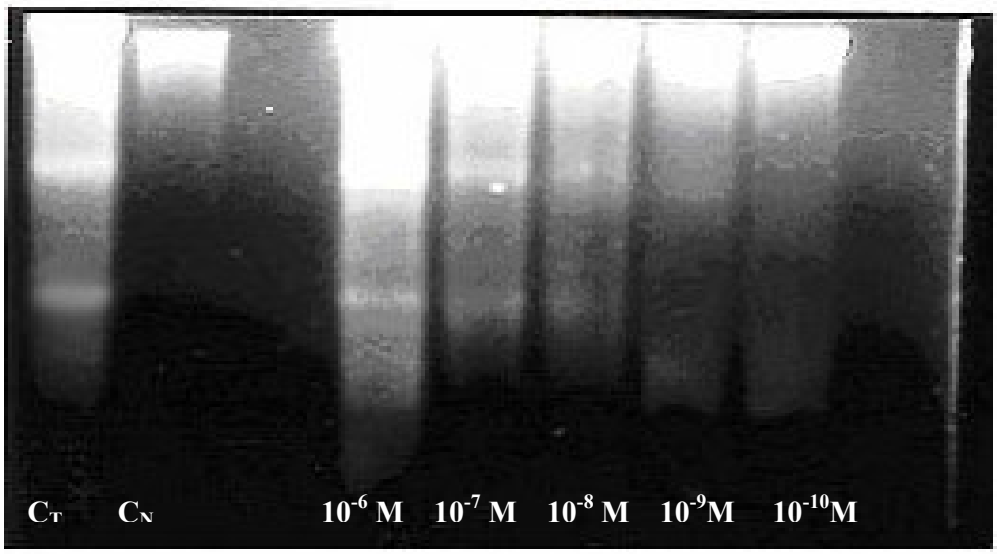


Figure 29 Apoptosis and necrosis identified by agarose gel electrophoresis.
The effects of different concentrations of aescin on DNA of whole blood cells.
Lane 1: H_2O_2 treated positive control (CT).
Lane 2: Negative control (untreated control (CN)).
Lane 4 to lane 8: whole blood lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

3.9.2 Induction of Cytochrome c Release from Mitochondria of Lymphocytes in Whole Blood Culture

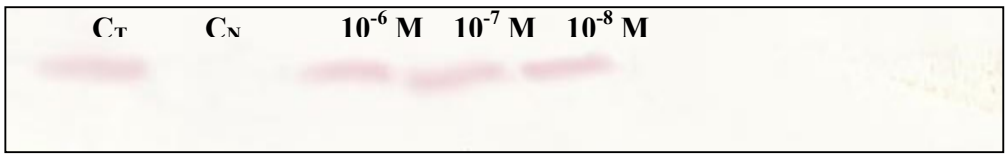


Figure 30 Presence of cytochrome *c* in cytoplasm in whole blood lymphocytes.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: whole blood lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

Figure 31 Presence of cytochrome *c* in mitochondria in whole blood lymphocytes.

Lane 1: H_2O_2 treated positive control (CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 7: whole blood lymphocytes were treated by 10^{-6} M to 10^{-10} M aescin addition.

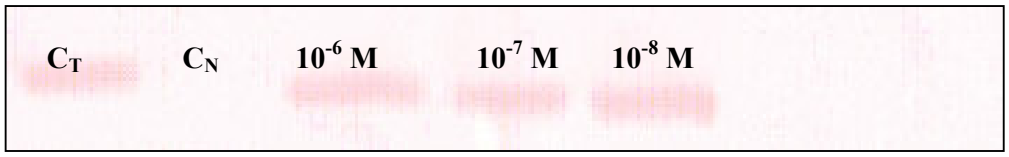


Figure 32 Presence of COX4 in mitochondria in whole blood lymphocytes.

Lane 1: H_2O_2 treated positive control(CT).

Lane 2: Negative control (untreated control (CN)).

Lane 3 to lane 5: whole blood lymphocytes were treated by 10^{-6} M to 10^{-8} M aescin addition.

3.10 NAPO staining results

3.10.1 Immunostaining of HL-60 Cells by NAPO

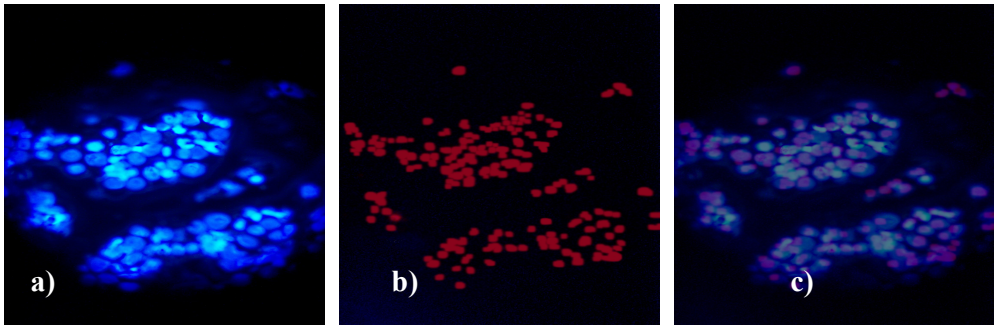


Figure 33 Immunostaining of HL-60 cells (untreated) negative control:

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

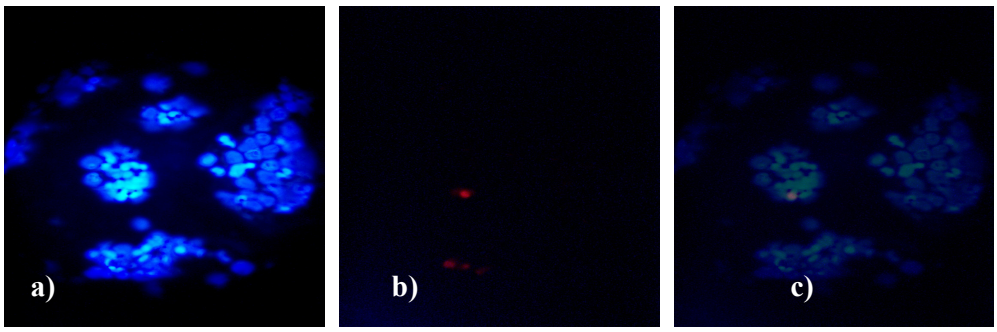


Figure 34 Immunostaining of HL-60 cells (treated with15µM H_2O_2/ml) positive control:

a) indicates Hoechst 33258 counterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

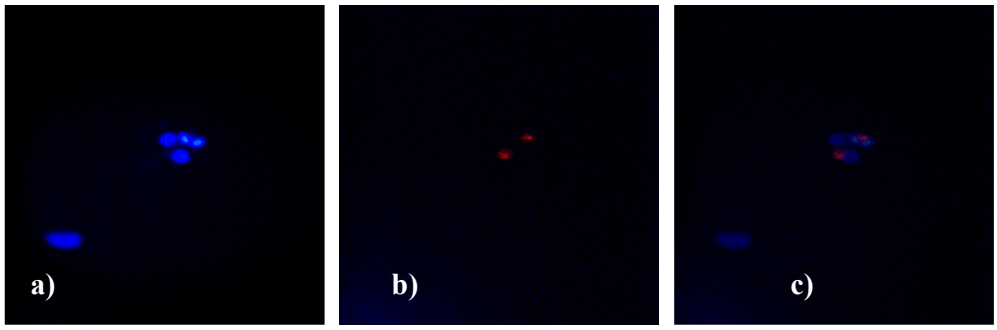


Figure 35 Immunostaining of HL-60 cells treated with 10^{-5} M aescin concentration:

a) indicates Hoechst 33258 counterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

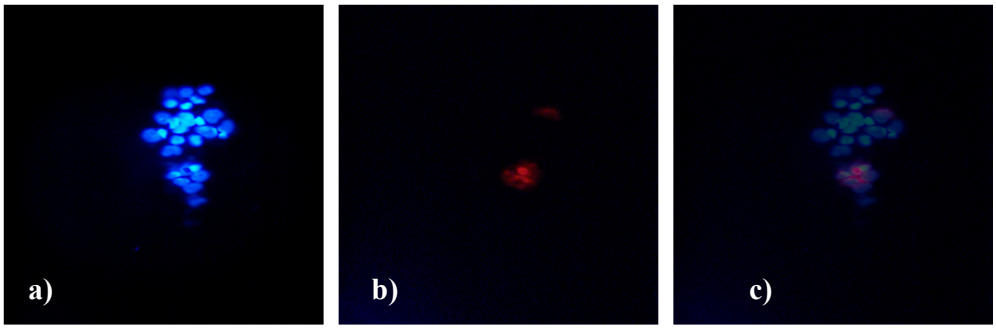


Figure 36 Immunostaining of HL-60 cells treated with 10^{-6} M aescin concentration:
a) indicates Hoechst 33258 counterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

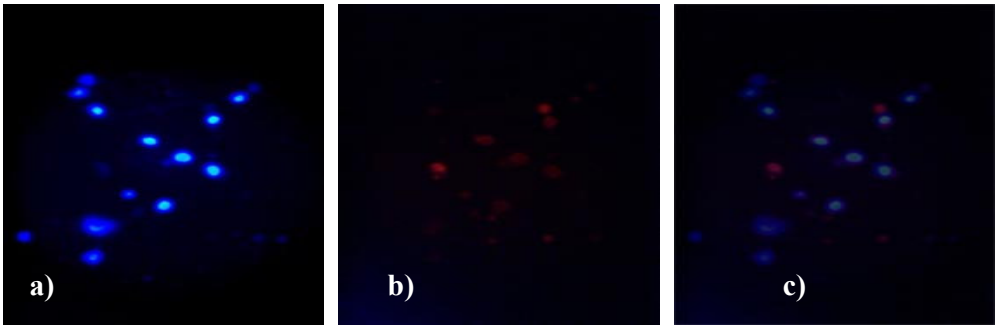


Figure 37 Immunostaining of HL-60 cells treated with 10^{-7} M aescin concentration:
a) indicates Hoechst 33258 counterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

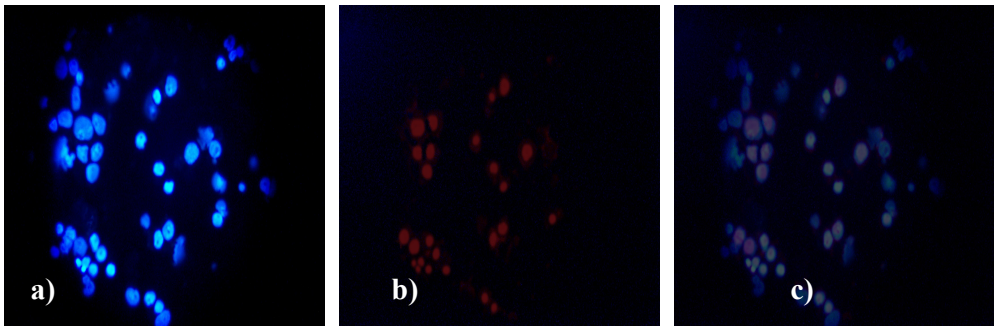


Figure 38 Immunostaining of HL-60 cells treated with 10^{-8} M aescin concentration:
a) indicates Hoechst 33258 counterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

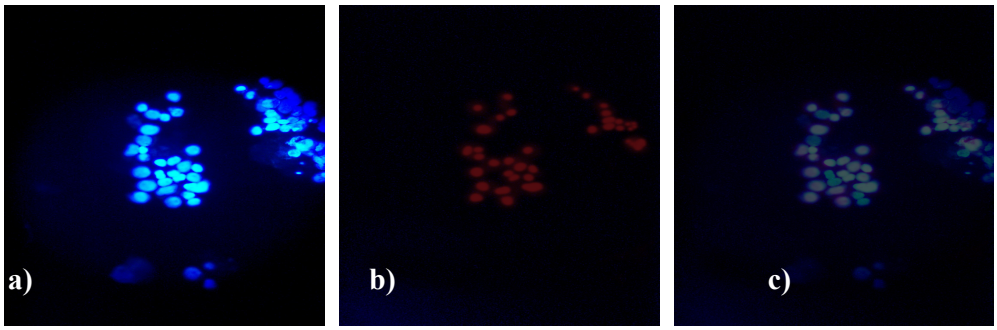


Figure 39 Immunostaining of HL-60 cells treated with 10^{-9} M aescin concentration:
a) indicates Hoechst 33258 counterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

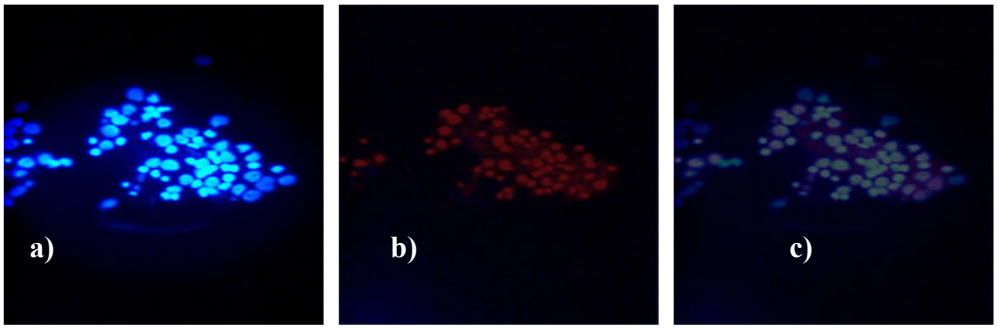


Figure 40 Immunostaining of HL-60 cells treated with 10^{-10} M aescin concentration:
a) indicates Hoechst 33258 counterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

3.10.2 Immunostaining of Isolated Lymphocytes by NAPO

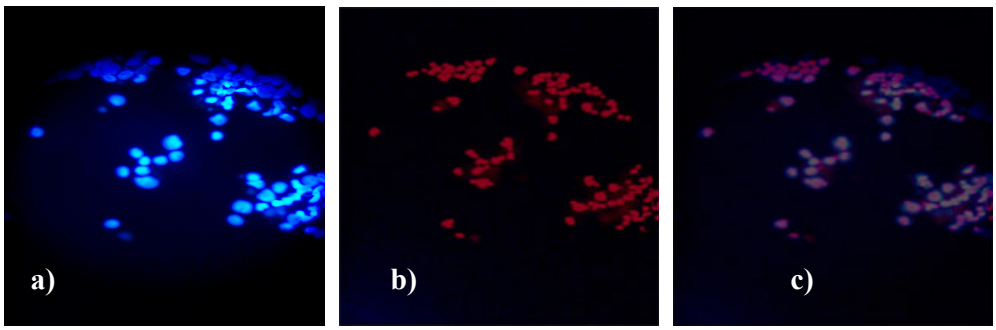


Figure 41 Immunostaining of isolated lymphocytes (untreated) negative control:
a) indicates Hoechst 33258 countercounterstaining;
b) indicates anti-NAPO staining; and
c) indicates overlapping of both stainings.

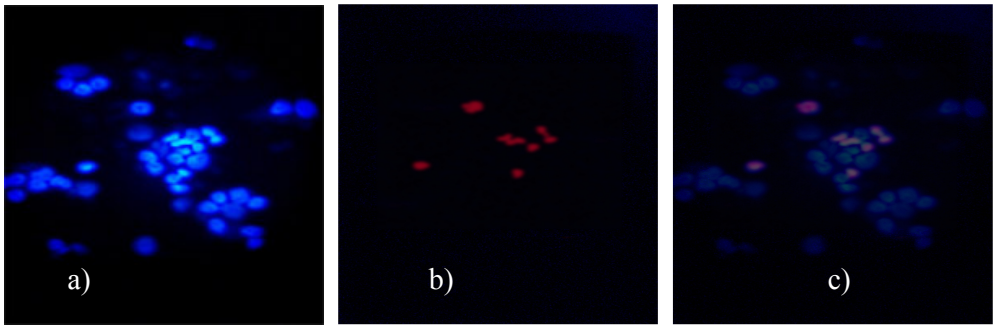


Figure 42 Immunostaining of isolated lymphocytes (treated with 15µM H_2O_2) positive control:

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

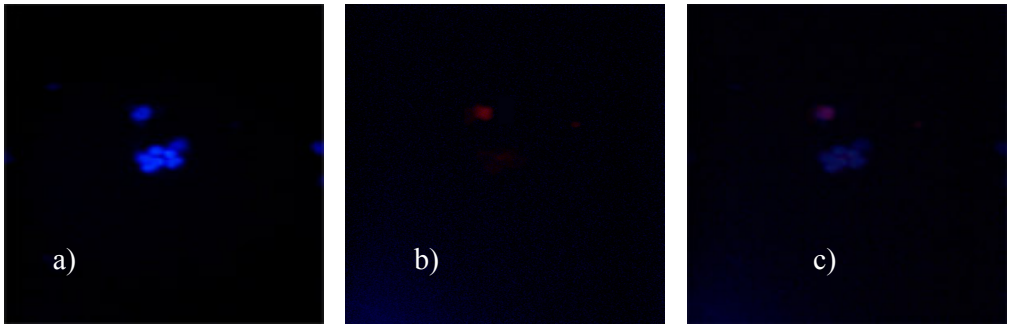


Figure 43 Immunostaining of isolated lymphocytes (treated with 10^{-5} M aescin concentration) positive control:

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

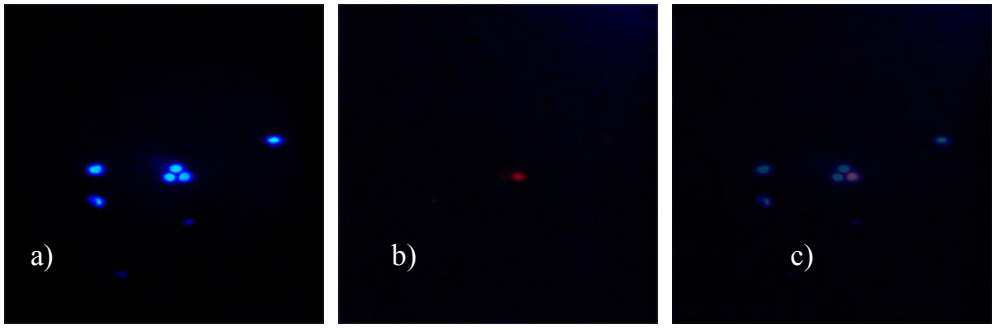


Figure 44 Immunostaining of isolated lymphocytes (treated with 10^{-6} M aescin concentration):

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

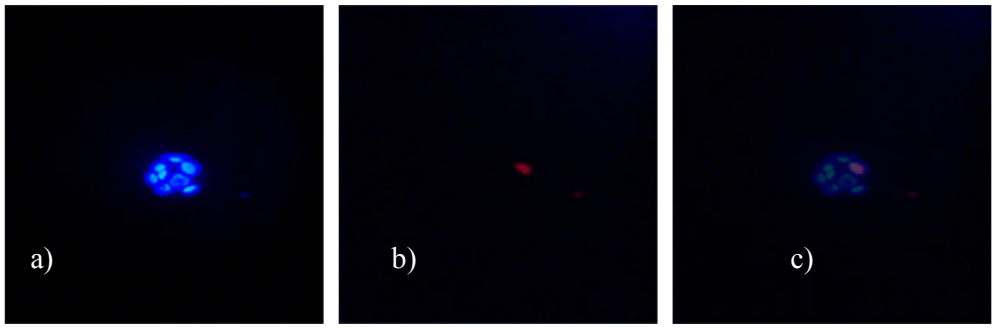


Figure 45 Immunostaining of isolated lymphocytes (treated with 10^{-7} M aescin concentration):

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

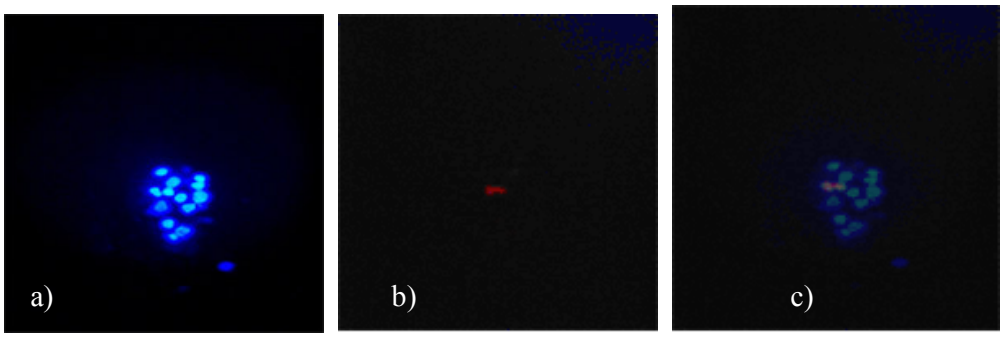


Figure 46 Immunostaining of isolated lymphocytes (treated with 10^{-8}M aescin concentration):

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

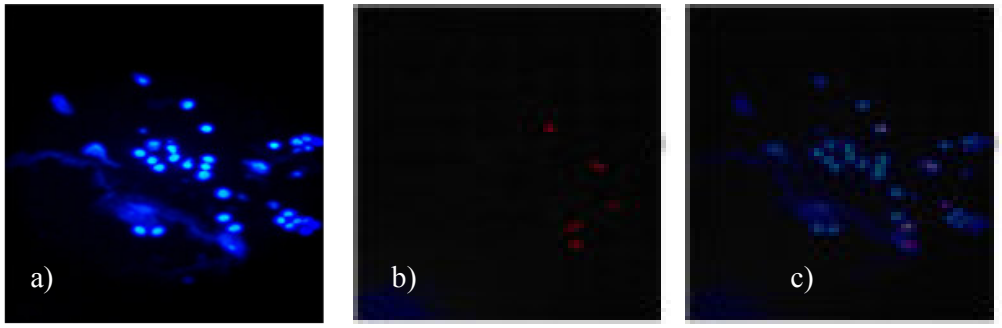


Figure 47 Immunostaining of isolated lymphocytes (treated with 10^{-9} M aescin concentration):

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

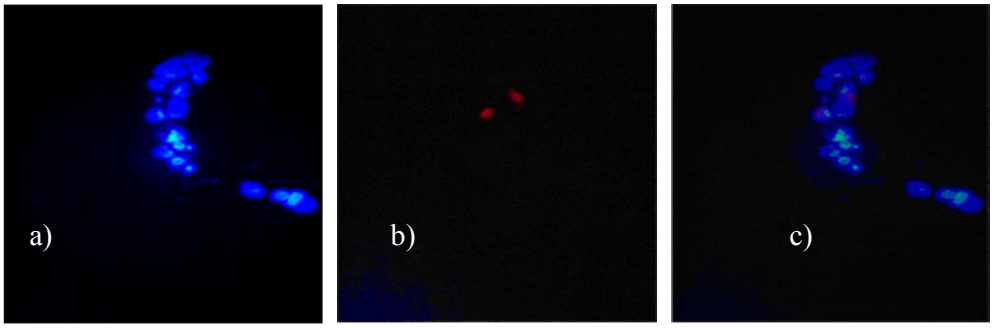


Figure 48 Immunostaining of isolated lymphocytes (treated with 10^{-10} M aescin concentration):

a) indicates Hoechst 33258 countercounterstaining;

b) indicates anti-NAPO staining; and

c) indicates overlapping of both stainings.

CHAPTER IV

CONCLUSION

The maximum number of cells were attained and over 90 % viability was sustained in cell cultures for 96 hours. As different aescin concentrations were applied over cell lines, it was observed that 10^{-5} M is the most toxic concentration that lead to death of all the cells. The 10^{-10} M concentration was observed to be effective over all cell lines being less toxic over whole blood lymphocytes and HL-60 cells. This can be attributed to the protective effect of whole blood erythrocytes on lymphocytes and to the resistance of HL-60 cell line against aescin.

Aescin treatment resulted an increase in GST enzyme activity to a varying extend in all cell lines. In order to reveal whether the increase in GST levels are related to the chemical nature of aescin, it was directly added to the reaction medium during activity determination using cell homogenates as enzyme source. Accordingly, even at the highest aescin concentrations, there was no increase observed at the enzyme activity levels, indicating an induction of GSTs by aescin treatment.

Aescin treatment resulted in a decrease in NAT activity in response to increasing concentrations in all cell types.

As evient by SCE results, genotoxic effect of aescin was higher over isolated lymphocites compared to other cell lines. The reason for this was believed to be the protective effect of erythrocytes over whole blood lymphocytes. The 10^{-8} M and lower concentrations of aescin were not toxic over whole blood lymphocytes, whereas this concentration is considerably toxic for isolated lymphocytes and for HL-60 cell lines. The results of the micronucleus assay was also supporting this observation.

NAPO-immuno staining is performed but due to the reason why the material tested is a saponin and the cell membrane is damaged the results are not evaluted due to lack of reliability of the results.

All the aescin concentrations tested resulted in apoptosis over HL-60 cell lines, without necrotic cell death. However, isolated lymphocytes exhibited necrosis, besides apoptosis, at higher concentrations. But, at 10^{-9} M and 10^{-10} M concentrations, only apoptosis was observed. In addition to these, at 10^{-8} M, 10^{-9} M and 10^{-10} M aescin concentrations, whole blood lymphocytes showed apoptosis without leading to necrosis.

It can be revealed from all the above results that at low: 10^{-8} M, 10^{-9} M and 10^{-10} M concentrations, aescin can be considered as a promising chemotheraupetic agent and can be medically tested for cancer treatment.

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Education

1997-2005 Middle East Technical University, Ankara, Turkey
PhD. in Biochemistry

"Biochemical, Cytotoxic and Genotoxic effects of Aescin on Human Lymphocytes and HL-60 promyeloid Leukemia Cell Line"

1994 - 1997 Middle East Technical University, Ankara, Turkey
M. S. in Biochemistry

"Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes"; September, 1997

1987 - 1993 Middle East Technical University, Ankara, Turkey
B. S. in Biology

Completed Projects

DPT: 2001-01-03-dpt/01K/12100: "Biyolojik Aktif maddelerin bitkilerden özütlenmesi ve katakterizasyonu: At kestanesi özütlerinin kanser üzerine etkileri".

AFP: BAP-2002-07-02-00-66: "Esin'in lenfosit hücreleri ve HL-60 hücre hattı üzerine: Biyokimyasal, Sitotoksik ve Genotoksik etkileri".

AFP: BAP- ""Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes".

Working Experiences

01.04.2005 - Department of Mining Analysis and Technology, Subdivision of Environmental Research
General Directorate of Mineral Research an Exploration (MTA)
06520 ANKARA

26.03.2004-01.04.2005 : International Projects and Foreign Affairs
General Directorate of Mineral Research an Exploration (MTA)
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03.2003 -26.03.2004 : Department of Metabolism
Hacettepe University- İhsan Doğramacı Children Hospital
Sıhhiye/ ANKARA

2002- 03.2003 : Department of Molecular genetic- cytogenetic laboratory
Hacettepe University- İhsan Doğramacı Children Hospital
Sıhhiye/ ANKARA

1993 – 2002 : Department of Biology
Middle East Technical University, Ankara, Turkey

- ***Teaching and Research Assistant.***

  In Cytology and Physiology Student Laboratories.
  In Biochemistry Laboratory.
  In Molecular Biology for Molecular Biology Students

- ***In Cytology Student Laboratory;***

  The Following Experiments are carried out;
    Staining Techniques
    Polytene Chromosome Staining
    PAS Staining
    Feulgen Staining
    Cell Fractionation, Mitochondria Staining
    Permanent Slide Preparation
    Lymphocyte Cell Cultures
    Cell and Tissue Differentiation under Microscope (From Permanent Slides).

- ***In Physiology Student Laboratory;***

  The Following Experiments are carried out;
    Muscle Experiments; Skeletal, Smooth and Cardiac Muscles
    Blood Experiments; White blood- Red blood Counting, Hemotocrite, White Blood Cells Differentiation.
    And Other Experiments; Respiratory, Circulation in Man (EKG etc.).

- ***In Biochemistry Research Laboratory;***

  The Following Experiments are carried out;
    Chemical Carcinogenesis, Environmental and Molecular Toxicology.
    The effect of Glutathione S-transferase theta on methylene chloride genotoxicity is worked out on lymphocyte cell cultures.

Computer Knowledge

Frequent user of windows and MS office applications.

Foreign Language

Fluent in English all in spoken, written and read.

Summer Practice

August-September, 1992; *ROCHE Pharmaceutical*, Quality Control Laboratory.

The following experiments were done;

Microbiological testing of water,
Microbiological assay of D-biotin, vitamin B_{12} and Folic acid,
Microbiological testing of raw materials and finished products,
LAL test,
Total viable aerobic count,
Test for specified micro-organism,
Gram staining,
Sterility test of preparation,
Microbiological assay of Virginiamycin,
Microbiological assay of Tetracycline Hydrochloride,
Biotest.

Occupations

Undergraduate Research Project: "Typing of Zooplankton and Phytoplanktone in Eymir Campus Lake".

Undergraduate Special Project: "Partial purification of xanthine oxidase from sheep liver".

Fellowships received

From Turkish Petroleum Company, during BS education (4 years) in METU.

FEBS Youth Travel Fund and FEBS for FEBS Advanced Course on "An introduction to cellular and molecular techniques for molecular biologist" July 2 - 17, 2002, Dublin, IRELAND.

European Science Fundation ULTRA PROGRAMME: SUMMER SCHOOL- "Theory and Experiment in Ultrafast Processes" Summer School (with International Participation), June 12-16, 2002, Algarve, PORTUGAL.

FEBS Youth Travel Fund and FEBS for FEBS Advanced Course on "Meiosis Meeting" September 18-22, 1999, Obertraun, AUSTRIA.

ISSX Grant, "7th European ISSX Meeting" August 22-26, 1999 in Budapest, HUNGARY.

FEBS Youth Travel Fund and FEBS for FEBS Advanced Course on "Techniques in Cell Biology" June12-21, 1995, Aarhus, DENMARK.

FEBS Youth Travel Fund and FEBS for FEBS Advanced Course on "An introduction to animal cell culture techniques for biochemists" June2-July 6, 1994, Dublin, IRELAND.

Awards Received

Several honour and high-honour awards in all stages of education. Ranked third among the graduating class

Interest And Activities

Researching about environmental and molecular toxicology, human health and diseases, chemical carcinogenesis and biochemistry.

As a special assistant to NATO Advance Study Institute on Molecular & Applied Aspects of Oxidative Drug Metabolising Enzymes; August 31- September 11, 1997; Tekirova, Antalya, Turkey.

As a participant to XIIIth National Congress of Biochemistry (with International Participation); March 26-30, 1996; Dedeman Hotel, Antalya, Turkey.

Poster Presentations

TOPSOY, S., İŞCAN, M., GÜRAY, G., 12-16 June, 2002; "Biochemical, Cytotoxic and Genotoxic Effects Aescine on Normal and Cancer Cell Cultures", "Theory and Experiment in Ultrafast Processes" Summer School (with International Participation), Algarve, Portugal. Book of Abstracts & Lecture Notes.

TOPSOY, S., İŞCAN, M., GÜRAY, G., 12-16 April, 2000; "Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes", IVth Xenobiotic Metabolism and Toxicity Workshop of Balkan Countries (with International Participation), Antalya, TURKEY. Book of Abstracts & Lecture Notes.

TOPSOY, S., İŞCAN, M., GÜRAY, G., 18-22, Septemberl, 1999; "Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes", FEBS Advanced Course on "Meiosis Meeting" in Obertraun, AUSTRIA. Book of Abstracts & Lecture Notes.

TOPSOY, S., İŞCAN, M., GÜRAY, G., August 22-26, 1999 "Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on

induction of SCE in lymphocytes", "7th European ISSX Meeting" in Budapest, HUNGARY. Book of Abstracts & Lecture Notes.

Oral Presentations

TOPSOY, S., İŞCAN, M., GÜRAY, G., 12-16 April, 2000; "Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes", IVth Xenobiotic Metabolism and Toxicity Workshop of Balkan Countries (with International Participation), Antalya, TURKEY. Book of Abstracts & Lecture Notes.

TOPSOY, S., İŞCAN, M., GÜRAY, G., 8-11 July, 1998; "The Biochemistry graduate at Middle East Technical University (METU)", National Congress of Biochemistry and Symposium on Problems and Solutions of Biochemistry Education in next millennium (with International Participation), Büyük Efes Hotel, İzmir, TURKEY. Book of Abstracts & Lecture Notes.

TOPSOY, S., İŞCAN, M., GÜRAY, G., 28-31 October, 1997; "Genotoxicity of methylene chloride: effects of glutathione S-transferase theta polymorphism on induction of SCE in lymphocytes", XIVth National Congress of Biochemistry and Symposium on Automation in Clinical Laboratory (with International Participation), Antalya, TURKEY. Book of Abstracts & Lecture Notes.

İŞCAN, M., ABU-HIJLEH, A. A., BOZCAARMUTLU, A., TOPSOY, S., GÜRAY, G., 28-31 October, 1997; "Biological significance of glutathione S-transferase theta isoenzyme", XIVth National Congress of Biochemistry and Symposium on Automation in Clinical Laboratory (with International Participation), Büyük Efes Hotel, İzmir, TURKEY. Book of Abstracts & Lecture Notes.

Hobbies

Playing tennis, gardening, travelling, dealing with computers, swimming, running.