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CYTOTOXIC AND GENOTOXIC EFFECTS OF *Datura stramonium* EXTRACTS ON CULTURED HUMAN LYMPHOCYTES

by

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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

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ABSTRACT

Datura stramonium is a plant in the *Datura* genus, within the *Solanaceae* family. The active ingredients are atropine, hyoscyamine and scopolamine which are classified as deliriants, or anticholinergics used as a hallucinogen and internally to treat madness, epilepsy, and depression. In this study, we aimed that determination of cytotoxic and genotoxic effects of *D. stramonium* methanolic seeds extract (DE) on human lymphocytes culture by using sister chromatid exchange(SCE) and TUNNEL test(genotoxicity test), Lactate dehydrogenease (LDH) and cell proliferation(WST-1) as a group of cytotoxicity test. Our results show that all DE do not have high cytotoxic effects (LDH assay) on human lymphocytes on the other part DE inhibited 24th and 48th cell proliferation significantly ($p<0.001$). Also tunnel assay results show that the concentration of DE cause DNA damage significantly ($p<0.001$). In addition that SCE frequency was increased when $125x10^3$ µg/ml and $50x10^3$ µg/ml concentration of DE added in the lymphocyte culture ($p<0.05$). All of the assay result suggested that the component of *D. stramonium* atropine and scopolamine which are classified anticholinergic agents have genotoxic and slight cytotoxic effects on human cultured lymphocytes.

Keywords: *Datura stramonium*, Lymphocyte, Sister Chromatid Exchange, Cytotoxicity, Genotoxicity.

Datura stromonium EKSTRAKLARININ İNSAN LENFOSİT KÜLTÜRLERİ ÜZERİNE SİTOTOKSİK VE GENOTOKSİK ETKİLERİNİN BELİRLENMESİ

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ÖZ

Datura stramonium, *Datura* cinsinden *Solanacea* familyasından bir bitkidir. Halüsinasyon, delilik, epilepsi ve depresyon tedavisinde kullanılan antikolinerjik ve deliriant olarak sınıflandırılan atropin, scopolamin ve hiyosiyamin aktif bileşenlerinden oluşmaktadır. Bu çalışmada *D. stramonium* metanolik tohumlarının insan lenfosit kültürü üzerindeki genotoksik ve sitotoksik etkileri kardeş kromotid değisimi(KKD), Tunnel, Laktat dehydrogenez(LDH) ve hücre büyümesi test (WST-1) yöntemleri kullanılarak belirlenmiştir. Sonuçlarımız *D. stramonium* ekstraktlarının (DE) lenfosit kültürü üzerinde yüksek toksik etkisi olmayıp konsantrasyonlara bağlı olarak 24. ve 48. saatlerdeki hücre büyümesinin belirgin bir şekilde sonlandırdığını göstermektedir ($p<0.001$). Ayrıca Tunnel deneylerinde, DE konsantrasyonlarının DNA hasarlarına yol açtığı görüldü ($p<0.001$). Bunun yanısıra lenfosit kültüründe DE konsantrasyonları $125x10^3$ µg/ml ve $50x10^3$ µg/ml olarak ilave edildiğinde Kardeş Kromotid Değişim frekansında artış görüldü ($p<0.05$). Yaptığımız calışmalar, antikolinerjik olarak sınıflandırılan *D. stramonium'*un atropin ve scopolamin bileşenlerinin, kültüre edilmiş insan kan hücrelerine genotoksik ve az da olsa sitotoksik etkilerinin olduğunu göstermektedir.

Anahtar Kelimeler: *Datura stramonium*, Lökosit, Kardeş Kromatid Değişimi, Sitotoksisite, Genotoksisite.

To my father Mürsel ÜLKER and my mother Fatma ÜLKER

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL	**ABBREVIATION**
BrdU	5-Bromo-2-deoksiüridin
CCl_4	Carbontetrachloride
DE	*Datura* Extract
Dw	Dry weight
EtOH	Ethanol
FBS	Fetal Bovine Serum
LDH	Lactate dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PHA	Phytohemaglutinin
PS	Penicilin-streptomycin
RPM	Revolutions per minute
SCE	Sister Chromatid Exchange
WST-1	Tetrazolium salt

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CHAPTER 1

INTRODUCTION

During the past 130 million years, medical plants have colonized practically every habitat on earth, from arid deserts, boggy meadows and windswept alpine summits. Medicinal plants are rich source of alkaloids content. One of the largest groups of chemicals produced by plants is the alkaloids. Many of these metabolic by-products are derived from amino acids and include an enormous number of bitter, nitrogenous compounds. More than 10,000 different alkaloids have been discovered in species from over 300 plant families (Raffauf 1996).

Alkaloids often contain one or more rings of carbon atoms, usually with a nitrogen atom in the ring. The position of the nitrogen atom in the carbon ring varies with different alkaloids and with different plant families. In fact, it is the precise position of the nitrogen atom that affects the properties of these alkaloids. Although they undoubtedly existed long before humans, some alkaloids have remarkable structural similarities with neurotransmitters in the central nervous system of humans, including dopamine, serotonin and acetylcholine. The amazing effect of these alkaloids on humans has led to the development of powerful pain-killer medications, spiritual drugs, and serious addictions by people who are ignorant of the properties of these powerful chemicals (Raffauf 1996).

The leaves, stem, root and fruits of *Datura stramonium* have tropane alkaloids, the most potent of which are atropine, hyoscyamine and scopolamine. These alkaloids affect the central nervous system and the autonomic nervous system. One autonomic response of atropine is the dilation of pupils. Depending on the dosages, several trapone alkaloids of *Datura* (when absorbed together) may have synergistic properties resulting in extreme hallucinations, delirium and death. Since the alkaloids are fat soluble they are readily absorbed through the skin and mucous membranes. Volumes have been

written about the uses and properties of *Datura* in the Middle-Ages. Most of the uses involved the consumption of potions or concoctions made from various parts of the plant (Schultes 1976).

In the light of these work the aim of this study is determination of cytotoxic and genotoxic effects of metanolic extract of *Datura stramonium* on human lymphocyte cell in the culture.

1.1 Species Description

Datura stramonium is a member of *Solanaceae* family also called Apple of Peru, Devil's Apple, Devil's Trumpet, Jamestown Weed, Mad-apple, Stinkweed or Thorn apple, is an erect, subherbaceous annual up to 1.5 m high with dark green or purplish leaves which are usually paler below. Flowers are white, mauve or purplish with a narrow funnel shape. The erect fruit capsules are brown in color, 50 mm long and covered with slender spikes up to 10 mm long (Henderson 1995). These capsules are filled with numerous brown to black, kidney shaped seeds of approximately 3 mm in length (van Wyk, van Oudtshoorn et al. 1997; van Wyk, van Heerden et al. 2002).

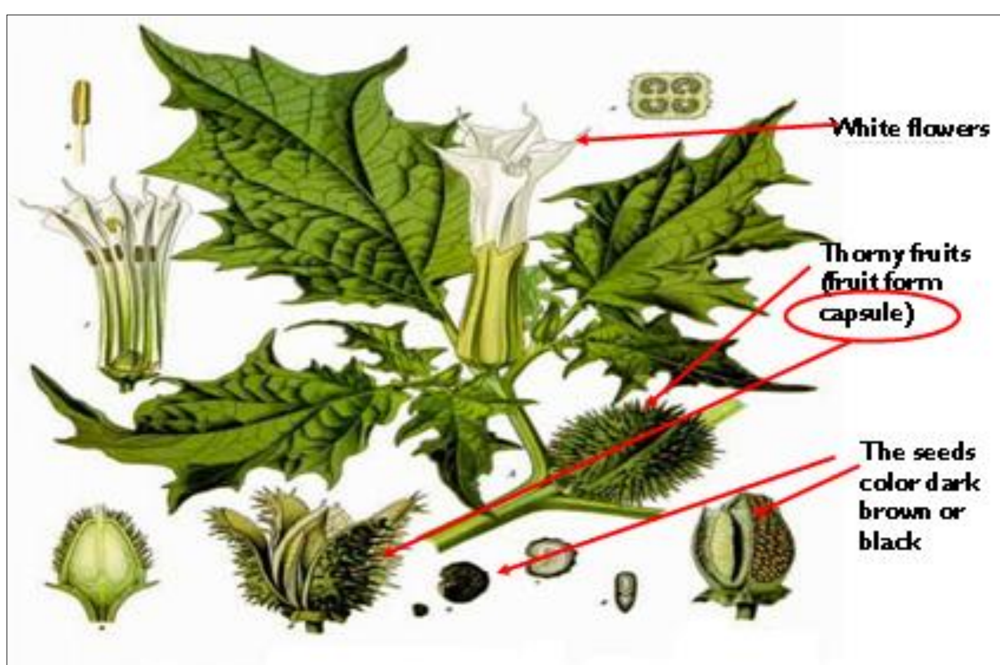


Figure 1.1:*D. stramonium* plant

Figure 1.2: Ripe fruit of *D. stramonium*

D. stramonium flowers from May to September, and the seeds, which include the most alkaloid, appear in the fall (Klein-Schwartz and Oderda 1984). The spiny pod contains between 50 and 100 brown-black seeds that are 2 mm to 3 mm in width. One hundred seeds contain the equivalent of approximately 6 mg of atropine (Klein-Schwartz and Oderda 1984). The plant is an annual that reaches five to six feet in height. It has dark green leaves and trumpet-shaped blue or white flowers (Vanderhoff and Mosser 1992). The most popular way to ingest this plant is to chew the seeds.

1.2 Natural History

The genus name is derived from dhatura, an ancient Hindu word for a plant. *D. stramonium* is originally from Greek, strychnosστρύχνος "nightshade" and maniakos μανιακός "mad" (University 2010).

D. stramonium is part of the *Solanaceae* family, which includes all the nightshades and agricultural plants such as eggplant, potatoes and tomatoes. *Solanaceae* has been broken up into about 90 genera and three sub-families (Solanaceae Source, 2004). There are around 3000-4000 different species in all (Bonde, Jakobsen et al. 1997). They are believed to have evolved primarily in tropical areas, specifically in Latin America, allowing the family to develop extensive adaptive variations, even before human exploitation for crops (IPANE 2004).

1.2.1 Historical Evolution

D. stramonium was used as a mystical sacrament in both possible places of origin, North America and South Asia. In Hinduism, Lord Shiva was known to smoke *Datura*. People still provide the small green fruit of *Datura* during festivals and special days as offerings in Shiva temples. Although lay devotees smoke marijuana as a devotional practice during religious festivals like Shivaratri (the Night of Shiva), they do not smoke *Datura*, whose effects can be unpredictable and sometimes fatal (Davis 1985).

In the United States the plant is called *D. stramonium,* or more rarely Jamestown weed; most common name is Jimson weed, a shortened version of 'Jamestown weed'. It was named after the first recorded accidental ingestion occurred in Jamestown, Virginia (USA), in 1676 (Caironi, Re et al. 1979; Beno, Osterhoudt et al. 2004).

Virginia, where British soldiers were drugged with it while attempting to suppress Bacon's Rebellion. They spent eleven days generally appearing to have gone insane. A thousand such simple tricks they played, and after eleven days returned themselves again, not remembering anything that had passed (Beverley 2008).

1.2.2 Solanaceae Family

The common names *of Solanaceae* are nightshade, potato or brinjal family. Although *Solanaceae* are found on most continents, the majority of the species in the family occur in Central and South America. Other centers of species diversity include Australia and Africa. *Solanaceae* are often found in secondary vegetation in disturbed areas, but species can occupy a variety of habitats, from deserts to tropical rainforests (Solanaceae Source, 2004).

The *Solanaceae* are a medium-sized family of flowering plants belonging to the Asteroids (Group 2003).

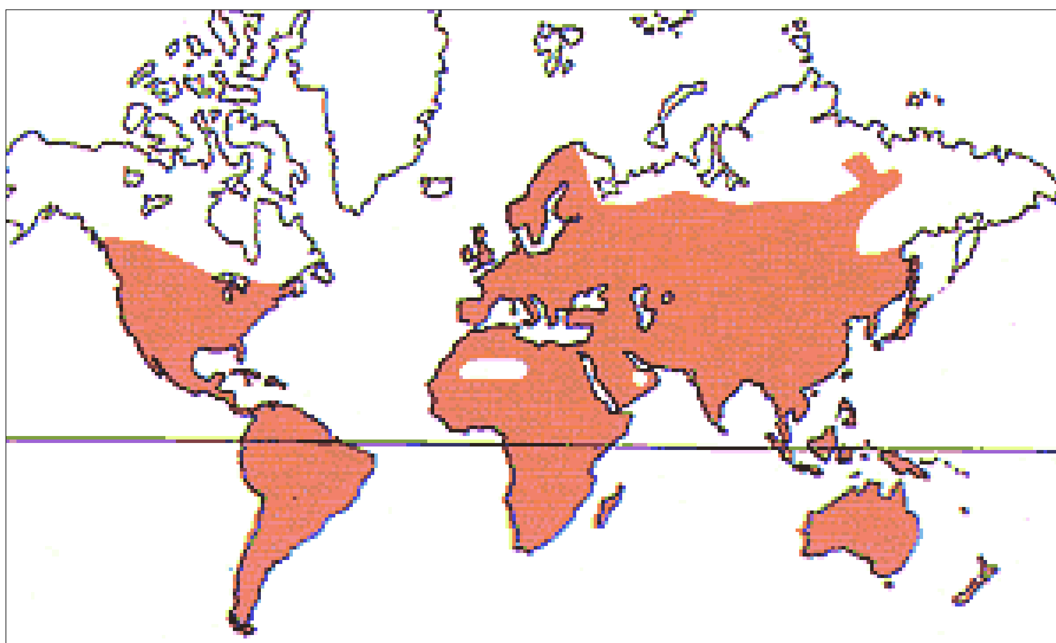
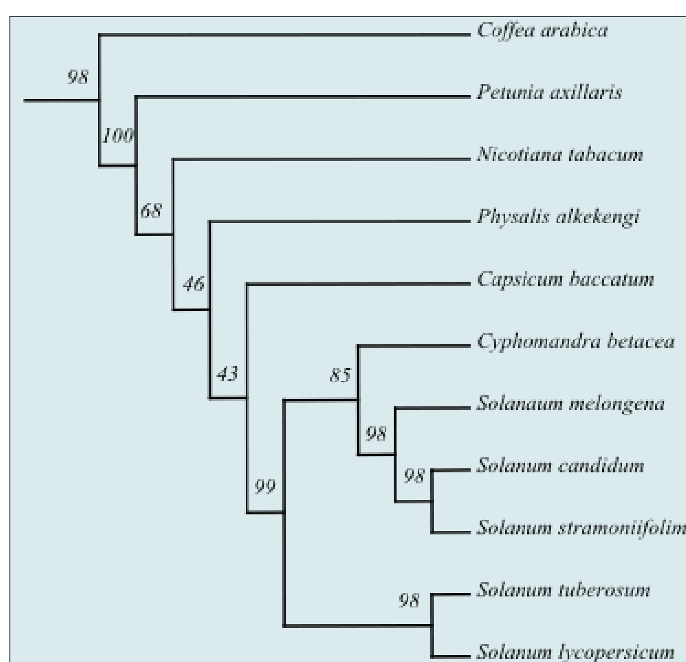


Figure 1.3:*Distrubition of Solanaceae family on the world(Heywood 1978)*

The family provides many products used by human beings for food, drugs and enjoyment. This includes edible species such as the potato, tomato, and eggplant (aubergine) and a host of minor fruit crops. Medicinal plants such as deadly nightshade, *D. stramonium*, tobacco, and henbane are the sources of drugs such as atropine, hyoscine, nicotine and other alkaloids (Solanaceae Source, 2004).

Table 1.1 Solanaceae family trees (Sol Genomics Network 2004)



1.2.3 Scientific Classification

A worldwide taxonomic of Solanaceae family in the genus Solanum which includes nightshades such as *D. stramonium.*

Table 1.2 Systematic of *D. stramonium* (Linnaeus 1753)

Kingdom	**Plantae**
Subkingdom	*Tracheobionta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Asteridae*
Order	*Solanales*
Family	*Solanaceae*
Genus	*Solanum*
Species	*Datura stramonium*

1.2.4 *D. stramonium* Origin and Distribution

Certain authors reported the origin of *D. stramonium* as uncertain (L. Henderson 1995), but most agreed that this plant originated from the tropical areas of Central and South America (Henderson 1995; van Wyk, van Heerden et al. 2002) and it is now a cosmopolitan weed in temperate regions.

D. stramonium is widely present across the United States. It has been reported in every state except Alaska and Wyoming (Figure 1.5) (NRCS 2005). Although most commonly found in agricultural areas, especially where grazing is present, the plant can thrive in a wide variety of climatic (from humid to arid conditions) and soil controls. In

non-agricultural areas it can be located on the side of roads, where the soil has been disturbed (FCES 1991).

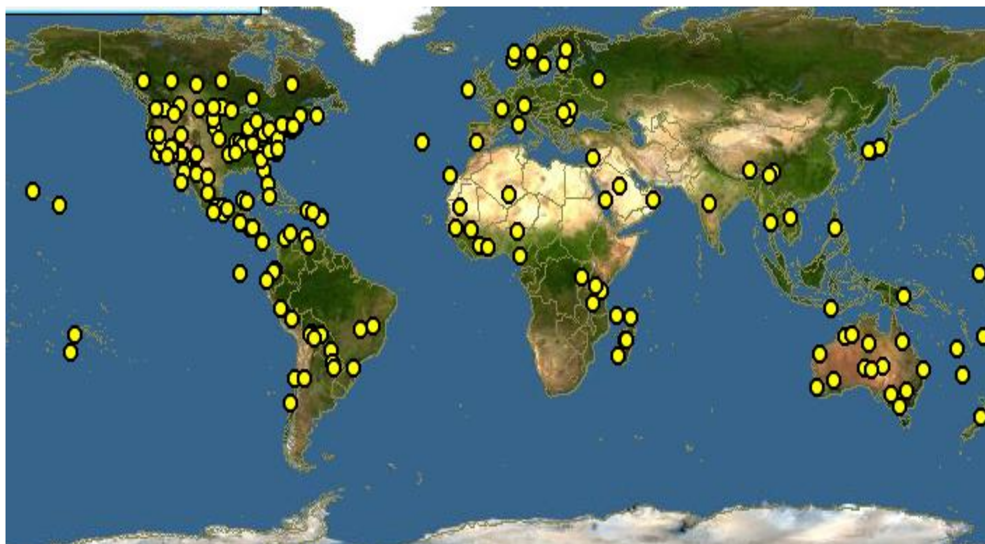


Figure 1.4: Distribution of *D. stramonium* in the world (Mapper 2008).

Today, it grows wild in all the world's warm and moderate regions, where it is found along roadsides and in dung heaps (Vanderhoff and Mosser 1992)

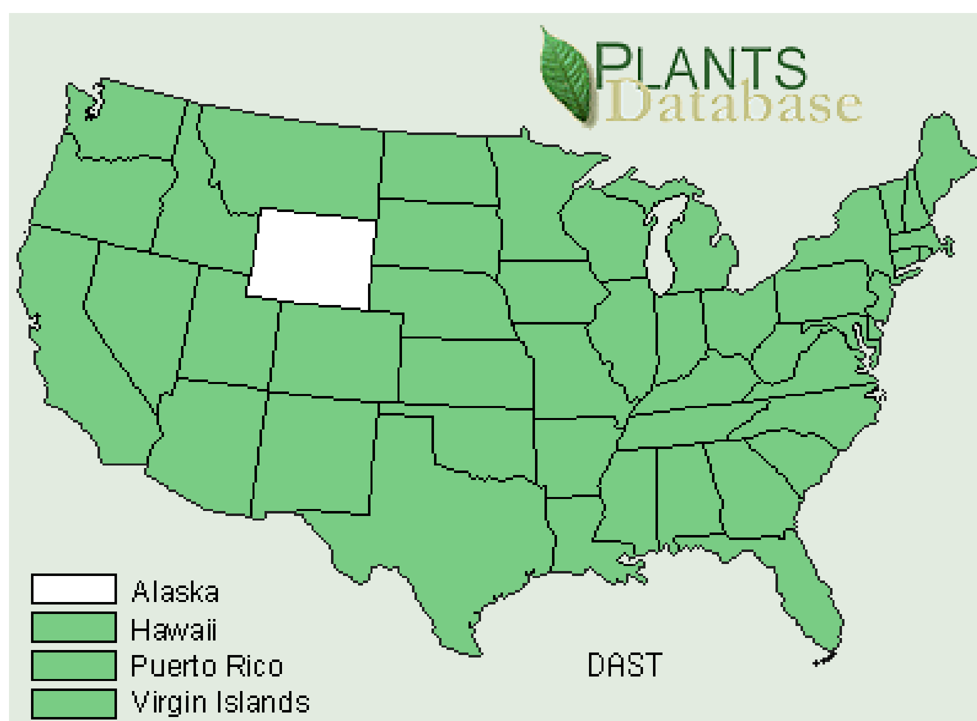


Figure 1.5: Distribution of *Datura stramonium* in the United States. http://plants.usda.gov

D. stramonium has an annual or short perennial life cycle, the seeds can remain viable for long periods of time, rhap sexplainingits wide distribution throughout the United States (Bonde, Jakobsen et al. 1997).

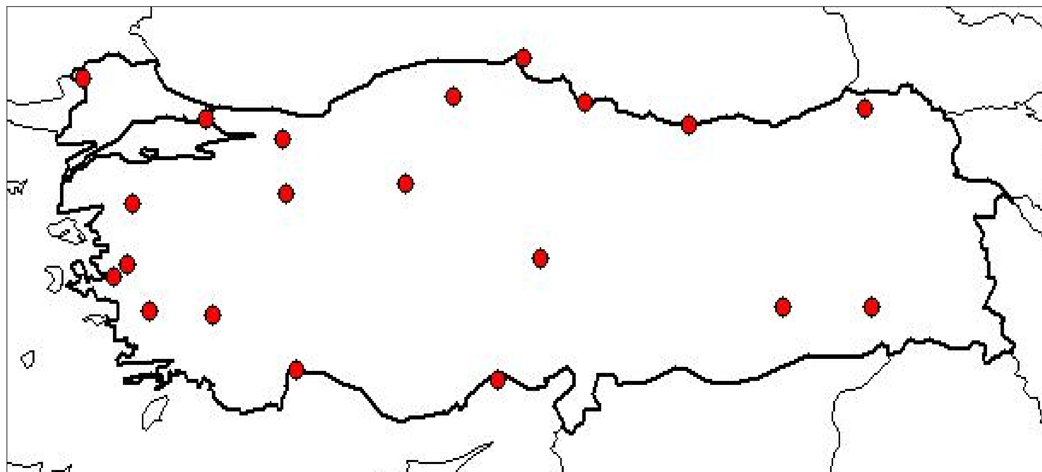


Figure1.6: Distribution of the Taxon over Turkey (TÜBİVES 2011).

Distribution of *D. stramonium* in Turkey: Istanbul, Siirt, Kastamonu, Ankara, Antalya, Artvin, Aydın, Denizli, Diyarbakır, Edirne, Eskişehir, Giresun, İçel, İzmir, Kayseri, Kocaeli, Manisa, Sakarya, Samsun, Sinop, Konya (TÜBİVES 2011).

1.3 Chemicals in *D. stramonium* seeds

Datura plants possess toxic and poisonous properties. These are basically due to the chemical compounds found in its parts especially the fruits. Chemical constituents found in the plant may produce sedative effects and submissive behavior and memory loss when taken internally in minor doses (Wahlstrom and Nordberg 1992).

The alkaloids in *Datura* are significant in respects other than their medicinal properties. They often leach from seeds into the surrounding soil. The resulting environment is toxic to some plants, but may be favorable to others due to decreased competition (Levitt and Lovett 1984).

Table 1.3 Chemical amount and names in *D. stramonium* seeds (J.Duke et al. 1992).

Chemicals	Part	Hi. (ppm)
Alkaloids	Seed	5,000 - 1,000
Fat	Seed	150,000 - 300,000
Hyoscine	Seed	1,200 - 5,000
Hyoscyamin	Seed	80 - 490
Linoleic-acid	Seed	22,500 - 45,000
Oleic-acid	Seed	93,000 - 186,000
Palmitic-acid	Seed	15,000 - 30,000
Protein	Seed	140,000 - 194,000
Scopolamine	Seed	53 - 3,050

1.4 Type of Toxin

The chemical toxins found in *D. stramonium* weed are classified as tropane alkaloids. Atropine, scopolamine, hyocyamine, and hyocine are found in this plant. The concentration of these components will vary with the species and the environment where it was cultivated. Therefore, the range of toxicity is unpredictable from plant to plant. The highest chemical concentration is located in the seeds (Miraldi, Masti et al. 2001).

1.4.1 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds which mostly contain basic nitrogen atoms (Luch 2009). This group also includes some related compounds with neutral (A. D. Mc Naught and A. Wilkinson 1998) and even

weakly acidic properties. Alkaloids contain carbon, hydrogen and nitrogen also oxygen, sulfur and more rarely other elements such as chlorine, bromine and phosphorus (Chemical Encyclopedia: alkaloids).

Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Rhoades 1979). Tropane alkaloids are a class of alkaloids and secondary metabolites that contain a tropane ring in their chemical structure. Tropane alkaloids occur naturally in many members of the plant family *Solanaceae (Laboratory 2005).*

The leaves, stem, root and fruits of *Datura* contain a battery of tropane alkaloids, the most potent of which are atropine, hyoscyamine and scopolamine. These alkaloids affect the central nervous system, including nerve cells of the brain and spinal cord which control many direct body functions and the behavior of men and women. They may also affect the autonomic nervous system, which includes the regulation of internal organs, heartbeat, circulation and breathing. One autonomic response of atropine is the dilation of pupils (Boumba, Mitselou et al. 2004).

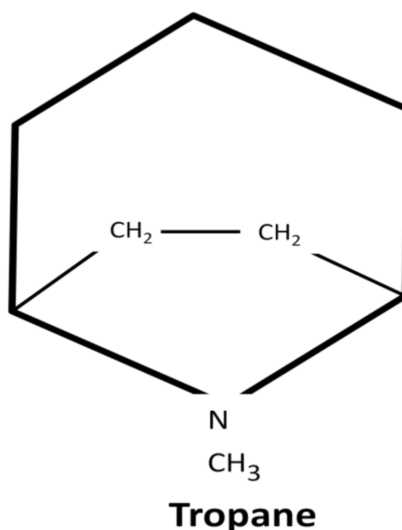


Figure1.7: Chemical structure of tropane

A common property of tropane alkaloids is a methylated nitrogen atom N-CH_3 at one end of the molecule (Figure 1.7). This chemical structure is also found in the neurotransmitter acetylcholine, which transmits impulses between nerves in the brain and neuromuscular junctions (Raffauf 1996).

Scopolamine, and hyoscyamine concentrations in various plant tissues of the most important *Datura* species, as reported by different authors to occur in contaminated feed materials, or which have led to intoxications in livestock. Individual authors reported data in various units. The given information did not allow a recalculation and harmonization of the units (Miraldi, Masti et al. 2001).

Table 1.4 Tropane alkaloid profile in *Datura* species originating from South Africa.

Species	**Hyocyamine**		**Scopolamine**		**Ratio:S/ H**
D. stramonium	**Average**	**Range**	**Average**	**Range**	
Purple stem	1046	531-2291	1063	296-2844	1.0
Green stem	587	491-742	525	249-1155	0.9
Seeds	557	273-908	587	254-800	1.1

Table 1.5 represent the average and range concentrations (mg/kg) detected in plant materials and in seeds. The ratio is calculated from S – scopolamine and H – hyoscyamine (Naudé 2007).

Table 1.5 Alkaloid contents in *Datura* stramonium (Miraldi 2001)

Samples	Young plants		Adult plants	
	Atropine	Scopolamine	Atropine	Scopolamine
Small leaves[b]	0.156 ± 0.008	0.073 ± 0.001	0.165 ± 0.006	0.016 ± 0.007
Medium leaves[c]	0.831 ± 0.014	0.047 ± 0.005	0.150 ± 0.002	0.022 ± 0.005
Big leaves[d]	0.228 ± 0.004	0.035 ± 0.009	0.134 ± 0.004	0.044 ± 0.006
Stems	0.915 ± 0.015	0.129 ± 0.014	0.001 ± 0.001	–
Roots	0.121 ± 0.015	0.014 ± 0.004	–	–
Flowers	Flower buds		Open flowers	
	0.299 ± 0.021	0.106 ± 0.031	0.270 ± 0.026	0.066 ± 0.004
Fruits	Immature fruits		Mature fruits	
Pericarp	0.001 ± 0.001	–	0.001 ± 0.001	–
Seeds	0.170 ± 0.003	0.012 ± 0.001	0.387 ± 0.015	0.089 ± 0.010

[a] Values are mean ± S.D. (n = 6); –, absent.
[b] Young, 1–5 cm; adult, 3–7 cm.
[c] Young, 5–9 cm; adult, 8–13 cm.
[d] Young, 9–11 cm; adult, 14–25 cm.

In addition to the major tropane alkaloids hyoscyamine and scopolamine, several minor tropane alkaloids have been identified in *Datura* species. Typical examples of minor alkaloids in *D. stramonium* are tigloidin, aposcopolamine, apoatropin, hyoscyamine N-oxide and scopolamine N-oxide (Romeike 1953)whereas substantial amounts of 6β-hydroxyhyoscyamine (anisodamine; 1.4-2.5% of total alkaloids) were found in flowers of *Datura metel* collected in China (He 1989) and 6β-[2-methylbutanoyloxy]tropan-3α-ol in *Datura ceratocaula***,** a species distributed throughout Mexico and Central America (Wooley, Baba et al. 1974). The presence of minor alkaloids might contribute to the toxic syndrome encountered following the ingestion of plant material including seeds (Simmat, Robert et al. 1983).

1.4.2 Adverse effects of tropane alkaloid

1.4.2.1 Mechanism of action

Tropane alkaloids are commonly described as anti-cholinergic compounds, due to their ability to bind to muscarinic acetylcholine receptors and hence acting as competitive antagonists at these receptors (Taylor, Tofler et al. 2006). According to the organ distribution, different subtypes of muscarinic receptors have been described, denoted M1 to M5, all belonging to the class of G-protein coupled receptors. M1

represent a population of receptors localized in the central nervous systems, as well as in gastric and salivary glands. M_2 receptors occur in the atria of the heart, at smooth muscles of the gastrointestinal tract as well as in the central nervous system. M_3 receptors dominate at exocrine glands including the salivary glands; occur in the gastro-intestinal tract as well as in the eye, and on the endothelium of blood vessels. M_4 receptors are predominantly found in the central nervous system and M_5 receptors are found especially in the *Substantia nigra* of the central nervous system, in the salivary glands and in the ciliary muscle of the iris of the eye. Atropine is a non-selective antagonist of all classes of muscarinic receptors, but known to have a stimulating effect on the central nervous system, whereas scopolamine is a depressant of the central nervous system, whereas scopolamine is a depressant of the central nervous system (Taylor 2006).

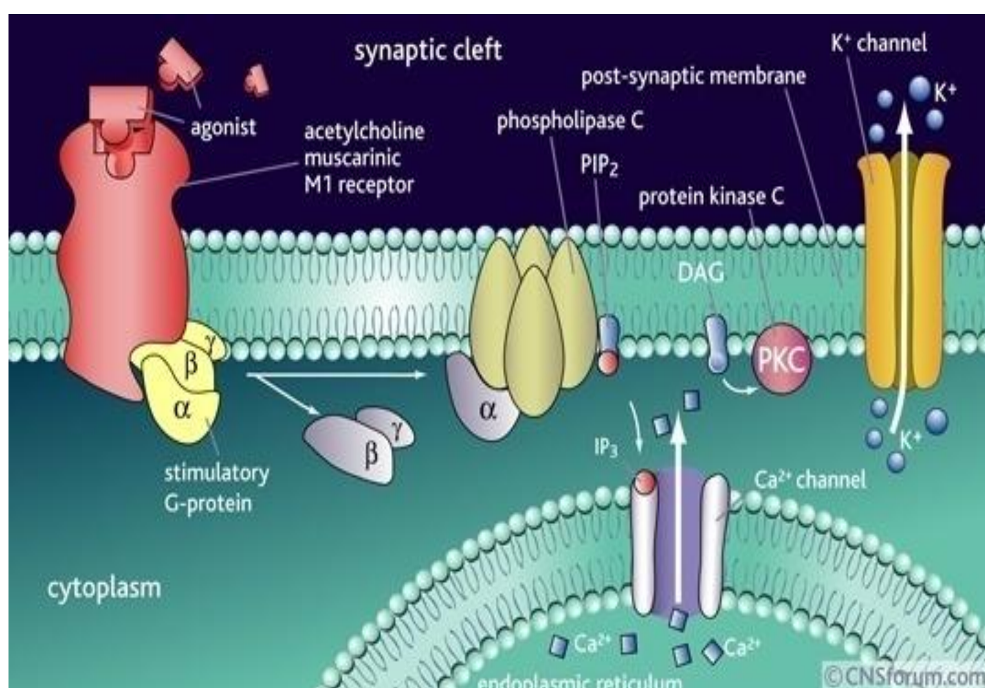


Figure 1.8: Muscarinic receptor agonist (Maslinski 1989)

1.5 Active Components and Pharmacological Effects

The anticholinergic toxic manifestation of seeds produced by the *Solanum* plant *D. stramonium* associated with the alkaloids atropine and scopolamine present in these seeds (Friedman and Levin 1989).

Atropine and scopolamine are competitive antagonists of muscarinic cholinergic receptors and central nervous system depressants (Brown and Taylor 2006). Approved

medications, these drugs are used for dilating pupils for fundoscopy and are used in anesthesia to treat/prevent bradycardia and to decrease bronchial and salivary secretions.

Atropine also is one of the medicines used for treating organophosphate poisoning and exposure to nerve agent chemical weapons. Scopolamine is a common treatment for motion sickness. In overdose, these drugs induce a toxic delirium marked by pronounced antergrade amnesia, confusion, dissociation, hallucinations, delusions, and an excited, giddy affect (Ardila and Moreno 1991).

Scopolamine and atropine are also described as euphorogenic, and their shamanic use has occurred amongst Native Americans as well as indigenous peoples throughout the world (Grinspoon, Bakalar et al. 1997).

1.5.1 Atropine

Atropine, an alkaloid obtained as the main component from *Atropa belladonna* L. and in much smaller quantities from *D. stramonium* and other *Solanaceae* is composed of approximately equal amounts of *d*- and *l*-hyoscyamine (Merck, Sonnenwald et al. 1989). Atropine is the best known of the anticholinergic or parasympatholyticdrugs, which inhibit the parasympathetic nervous system. Atropine is an extremely poisonous drug derived from a plant called belladonna (Miraldi, Masti et al. 2001).

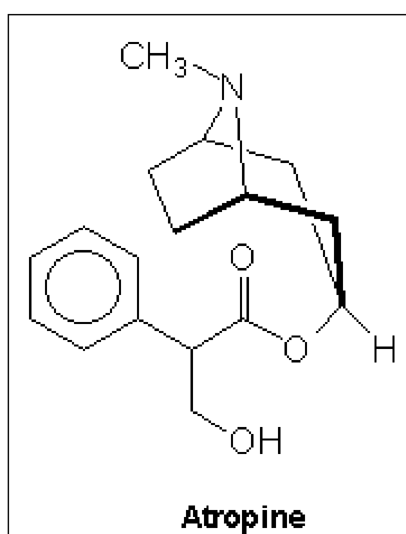


Figure 1.9: Chemical structure of atropine. Formula: $C_{17}H_{23}NO_3$, M_w: 289.369 g/mol.

Table 1.6: Effects of Atropine in Relation to Dosage (Brown and Taylor 2006)

Dosage	Effects of Atropine in Relation to Dosage
0.5 mg	slight decrease in heart rate, some dryness of mouth; inhibition of sweating
1.0 mg	definite dryness of mouth; thirst; increase in heart rate; mild dilatation of pupil
2.0 mg	rapid heart rate; palpitation; marked dryness of mouth; mydriasis; some cyclopedia
5.0 mg	all the above symptoms marked; speech disturbed; difficulty swallowing; restlessness, fatigue, headache; dry hot skin; difficulty in micturition; reduced intestinal peristalsis
10.0 mg	more marked symptoms; pulse rapid and weak; iris practically obliterated; vision very blurred; skin flushed, hot, dry, and scarlet; ataxia, restlessness and excitement; hallucinations, and delirium coma

1.5.2 Medicinal Uses of Atropine

Atropine (Atreza™, Sal-Tropine®, Isopto-Atropine®, others) is commonly used to manage terminal secretions. It may be administered by oral, intramuscular, intravenous, or subcutaneous, routes at a starting dose of 0.4mg every 4–6 hours as needed Management of Symptoms in the Actively Dying Patients (Sherman and Sherman 1999; AM. 1998).

Atropine 1% eye drops can be given orally to the back of the throat or sublingually to provide rapid relief of symptoms (Sorenson 2000). The initial recommended dose for the oral administration of eye drops is 1–2 drops every 4–6 hours as needed (Kaila, Korte et al. 1999).

Findings from a single unpublished study indicated that atropine ophthalmic solution administered sublingually was effective at reducing terminal respiratory

secretions (D'Anna, Frenna et al. 2006). Other studies have reported limited success using atropine drops for other hyper salivary conditions (Hyson, Johnson et al. 2002).

1.5.3 Other Effects of Atropine

Atropine has anticholinergic activity and causes blurred vision, suppressed salivation, vasodilatation, a relaxation of the bronchial smooth muscles, increase heart rate and delirium (van Wyk, van Oudtshoorn et al. 1997; van Wyk, van Heerden et al. 2002).

1.5.4 Scopolamine

Scopolamine is an antimuscarinic agent and a smooth muscle relaxant. It is used as an antispasmodic agent with antinauseant properties, and is extensively used for the prevention of motion sickness and in pre-operative medication (van Wyk, van Oudtshoorn et al. 1997; van Wyk, van Heerden et al. 2002).

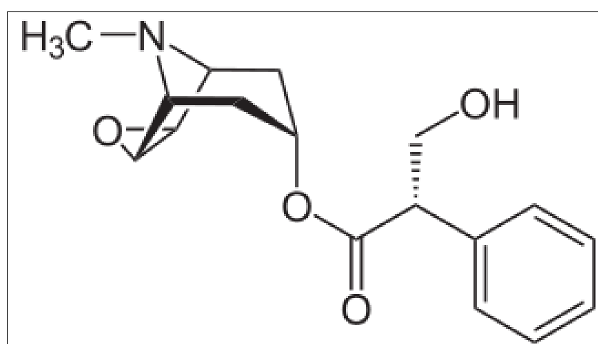


Figure 1.10: Chemical structure of scopolamine Formula: $C_{17}H_{21}NO_4$,M_w: 303.353g/mol.

1.6 Poisoning

Ingestion of *D. stramonium* flowers, leaves, or seeds can result in serious intoxication and even death. *D. stramonium* is easily available, acute intoxication may result from accidental ingestion of contaminated food or from ingestion with homicidal

intent. However, in the majority of cases, intoxication occurs following deliberate abuse as a hallucinogen (Romeike 1953).

1.6.1 Accidental Poisoning

Accidental *Datura* poisoning is quite common and various cases have been reported; Scopolamine poisoning occur from homemade ‘moon flower’ wine (Smith, Meloan et al. 1991). Atropine poisoning after drinking contaminated Indian tonic water (Boyd, Rintoul et al. 1997), eating chapattis contaminated with *D. stramonium* (Deery, Care et al. 2000), from "health tea" (Scholz, Kascha et al. 1980) and ,"Fifteen cases of atropine poisoning after honey (Ramirez, Rivera et al. 1999) ingestion. *The* toxicity of *D. stramonium* (thorn apple) to horse (Williams and Scott 1984), Many fatalities have occurred, especially among children who are attracted to the capsules and seeds. Not your everyday anisocoria: angel's trumpet ocular toxicity (Firestone and Sloane 2007).

Table 1.7: Summary characteristics of the four cases (Firestone and Sloane 2007)

Case	Intensive care unit admission	Month	Initial Glasgow Coma Scale	Halluci-nations	Agita tion	Pupil size	Psychiatric diagnosis	Days in hospital
1	yes	August	12	Yes	Yes	6mm	Hallucinogen abuse	2
2	No	September	5 to 15	Yes	Yes	5mm	Oppositional defiant disorder	2
3	No	August	13	Yes	Yes	5mm	Hallucinogen abuse	2
4	No	September	11	Yes	Yes	5mm	Polysubstance abuse	2

1.6.2 Deliberate

Intentional poisoning with *D. stramonium* has also been reported in several cases namely a fatal poisoning with *D. stramonium* (Boumba, Mitselou et al. 2004) and *D. ferox*, the smoking and ingestion of *D. stramonium* for its mind-altering properties (Guharoy and Barajas 1991) and the eating and chewing of *Datura* seeds in a suicide attempt (Simmat, Robert et al. 1983).

1.7 Anticholinergic Activities

D. stramonium contains a variety of alkaloids including atropine and scopolamine that can cause anticholinergic poisoning if taken in large doses (Friedman and Levin 1989). The seeds are responsible for the anticholinergic toxicity of the plant (Joshi, Wicks et al. 2003). These substances competitively block the binding point of acetylcholine in the central nervous system (CNS) and muscarinic receptors of parasympathetic postganglions and present an anticolinergic effect. The most common effect of anticolinergics depends on the antagonism of peripheral muscarinic receptors (Okeri and Alonge 2006).

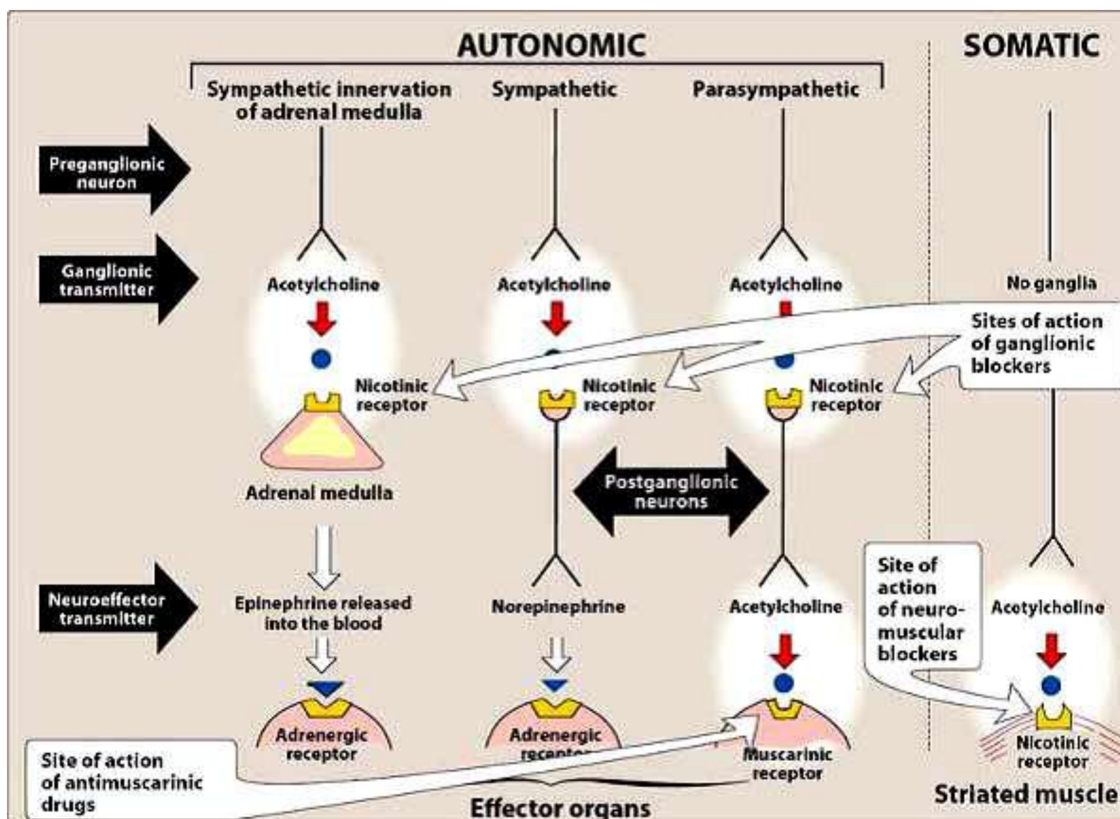


Figure 1.11: The site of action of anticholinergic drugs

Table 1.8 Atropine and scopolamine as a cholinergic receptor blocking drugs

Classify	Drugs
Antimuscarinic agents	
$M_{1,2}$R antagonist	Atropine, scopolamine,anisodamine
M $_1$R antagonist	Pirenzepine
M $_2$R antagonist	Gallamine

Numerous plants with anticholinergic properties can be included *D. stramonium*, *Salvia divinorum*, Angel's Trumpet (*Datura sauveolens*), Deadly Nightshade (*Atropa belladonna*) and Black Henbane (*Hyoscyamus niger*) (Kemmerer 2007).

Anticholinergic syndrome is a constellation of signs and symptoms that may be present in whole or in part in the poisoned patient. Central effects are dose-dependent and agent-dependent and include drowsiness, agitation, picking motions with the hands, hallucinations to seizures, and coma. Peripheral effects that are common to

anticholinergic agents include mydriasis with cyclopedia, dry mucous membranes, hyperflexia, flushed skin, diminished bowel sounds or ileus, urinary retention, tachycardia, and hypertension or hypotension (Caksen, Odabas et al. 2003; Kemmerer 2007).

Atropine and scopolamine act on the muscarinic receptors by blocking them (particularly the M_2 receptors) however, this will cause a continuous release in acetylcholine (ACh). ACh also act on nicotinic receptors; however, it is known that "over exposure" of nicotinic receptors may cause desensitization (Marx, Pretorius et al. 2006).

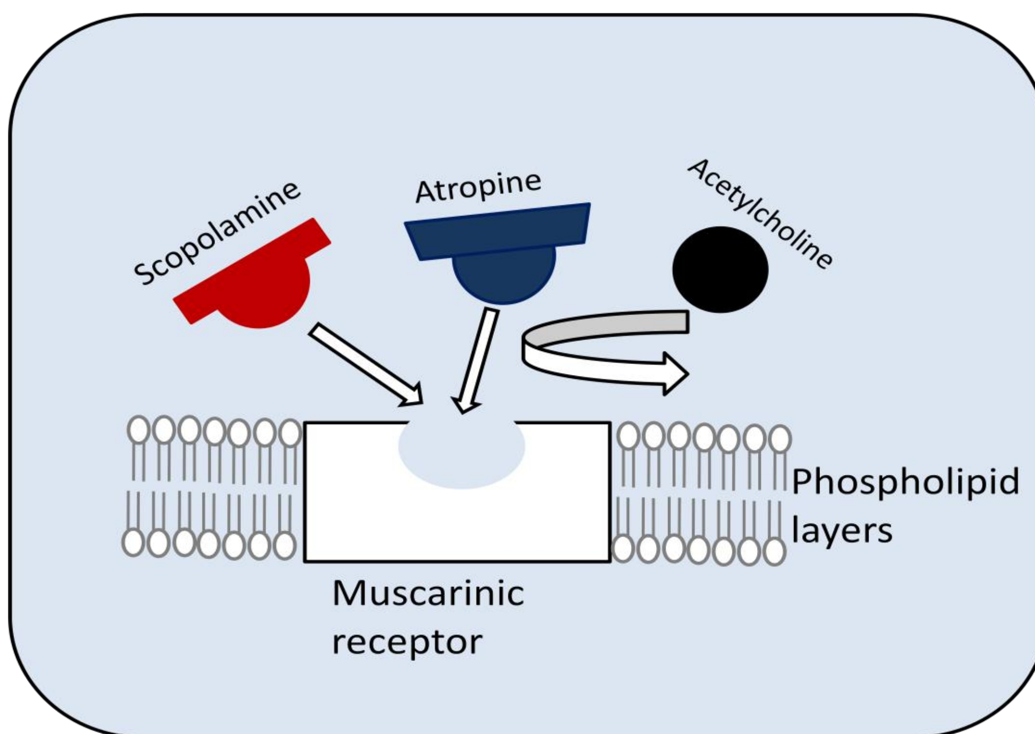


Figure 1.12: Competition of atropine and scopolamine with acetylcholine for the muscarinic receptor (Marx, Pretorius et al. 2006).

1.7.1 Presence of acetylcholine in lymphocytes

Lymphocytes contain both muscarinic receptors (mAChRs) and nicotinic receptors (nAChRs) (Bering, Moises et al. 1987; Razani-Boroujerdi, Boyd et al. 2007). The presence of both muscarinic and nicotinic ACh receptors in lymphocytes has been

shown by analysis of specific ligand binding, analysis of mRNA expression and immunocytochemical analysis using specific antisera against respective subtypes or subunits of ACh receptors. Moreover, using RT-PCR with human leukemic cell lines and MNLs, (Oka, Kameya et al. 1999) demonstrated for the first time that some lymphocytes have the potential to simultaneously express both muscarinic and nicotinic receptors. Reviews and overviews on cholinergic receptors in lymphocytes have been published by (Maslinski 1989; Wahlstrom and Nordberg 1992; Jansen, Homo-Delarche et al. 1994).

Table 1.9: *D. stramonium* uses in ancient remedy (Duke 1982)

Ethnopharmocological uses of *D. stramonium*			
Ache(Ear)	Carcinoma	Edema	Hallucinogen
Ache(Tooth)	Chorea	Epilepsy	Homicide
Anesthetic	Cold	Eruption	Hypnotic
Anodyne	Congestion	Expectorant	Inflammation
Antispasmodic	Cough	Fatality	Intoxicant
Asthma	Cyanogenetic	Felon	Madness
Boil	Dandruff	Fuel	Masticatory
Bruise	Dementia	Fumitory	Medicine
Burn	Demulcent	Fungicide	Mydriatic
Cancer(Breast)	Dye	Gum	Narcotic

1.8 LDH Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. With the use of the Cytotoxicity Detection Kit, LDH activity can easily be measured in culture supernatants by a single measurement at one time point. The use of a spectrophotometric microplate reader (ELISA reader) allows the simultaneous measurement of multiple probes and thereby guarantees the easy processing of a large number of samples.

An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant directly correlates to the amount of formazan formed during a limited time period. Therefore, the amount of color formed in the assay is proportional to the number of lysed cells. The formazan dye formed is water-soluble and shows a broad absorption maximum at about 500 nm, whereas the tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) shows no significant absorption at these wavelengths.

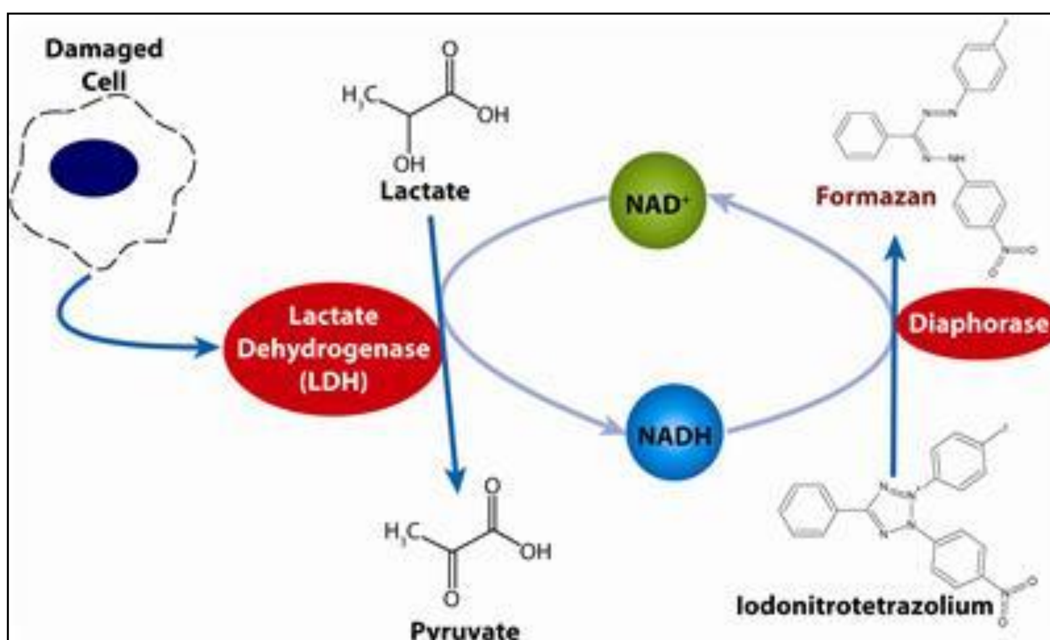


Figure 1.13: LDH assay Principe. In the first step, released lactate dehydrogenase (LDH) reduces NAD^+ to $NADH^+$ H^+ by oxidation of lactate to pyruvate. In the second

enzymatic reaction 2 H are transferred from $NADH^+ H^+$ to the yellow tetrazolium salt INT by a catalyst.

1.8.1 Relationship between cytotoxicity, apoptosis and necrosis

There are two experimentally distinguishable mechanisms of cell death: necrosis, the “accidental” cell death that occurs when cells are exposed to a serious physical or chemical insult, and apoptosis, the “normal” cell death that removes unwanted or useless cells.

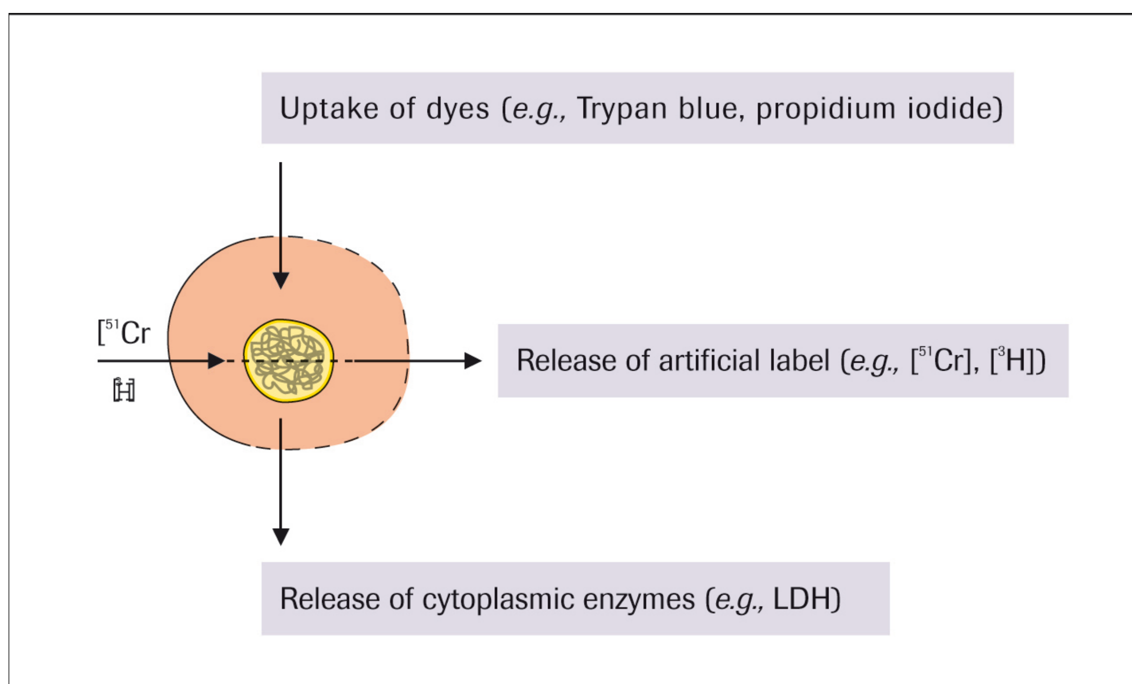


Figure 1.14: Schematic illustration of the three basic principles to assess plasma membrane leakage.

1.9 WST-1 Cell Proliferation Assay

The measurement of cell proliferation and cell viability has become a key technology in the life sciences. The need for sensitive, reliable, fast and easy methods has led to the development of several standard assays.

Proliferation assays have become available for analyzing the number of viable cells. The tetrazolium salts are cleaved to formazan by cellular enzymes and expansion

in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. Quantification of the formazan dye produced by metabolically active cells by a scanning multiwall spectrophotometer (ELISA reader). The absorbance of the dye solution is measured at appropriate wavelengths.

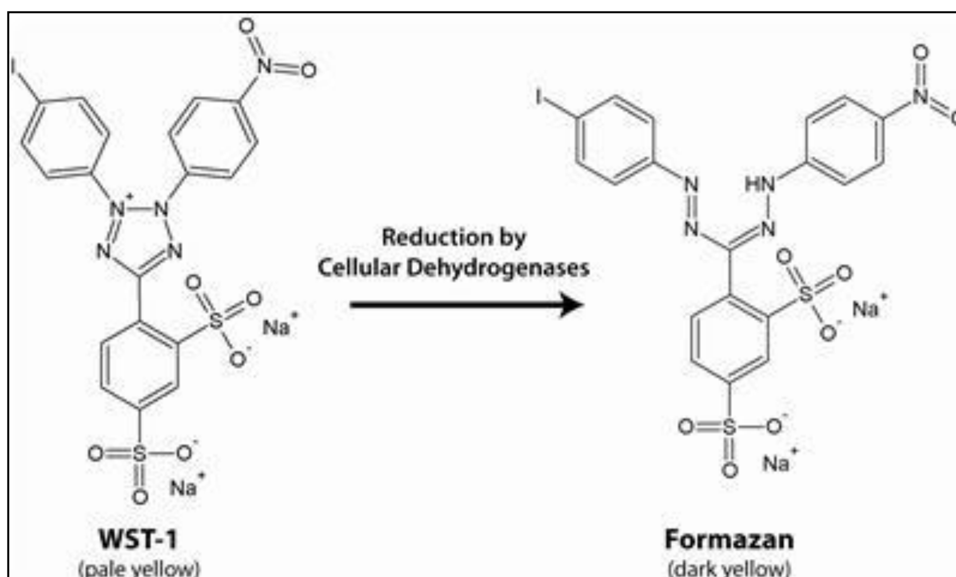


Figure 1.15: WST-1 Assay principle. Cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) to formazan. (EC = electron coupling reagent RS mitochondrial succinate-tetrazolium-reductase system).

1.10 TUNEL Assay

During apoptosis, DNAse activity not only generates double-stranded, low-molecular-weight DNA fragments (mono- and oligonucleosomes), but also introduces strand breaks ("nicks") into the high-molecular-weight DNA. These processes can be identified by labeling the free 3'-OH termini with terminal transferase (TdT), which attaches labeled nucleotides, to all 3´OH-ends (TUNEL reaction; TdT-mediated dUTP nick end labeling). This labeling is more sensitive than other methods, and is the method used by the Roche Applied Science In situ Cell Death Detection Kits.

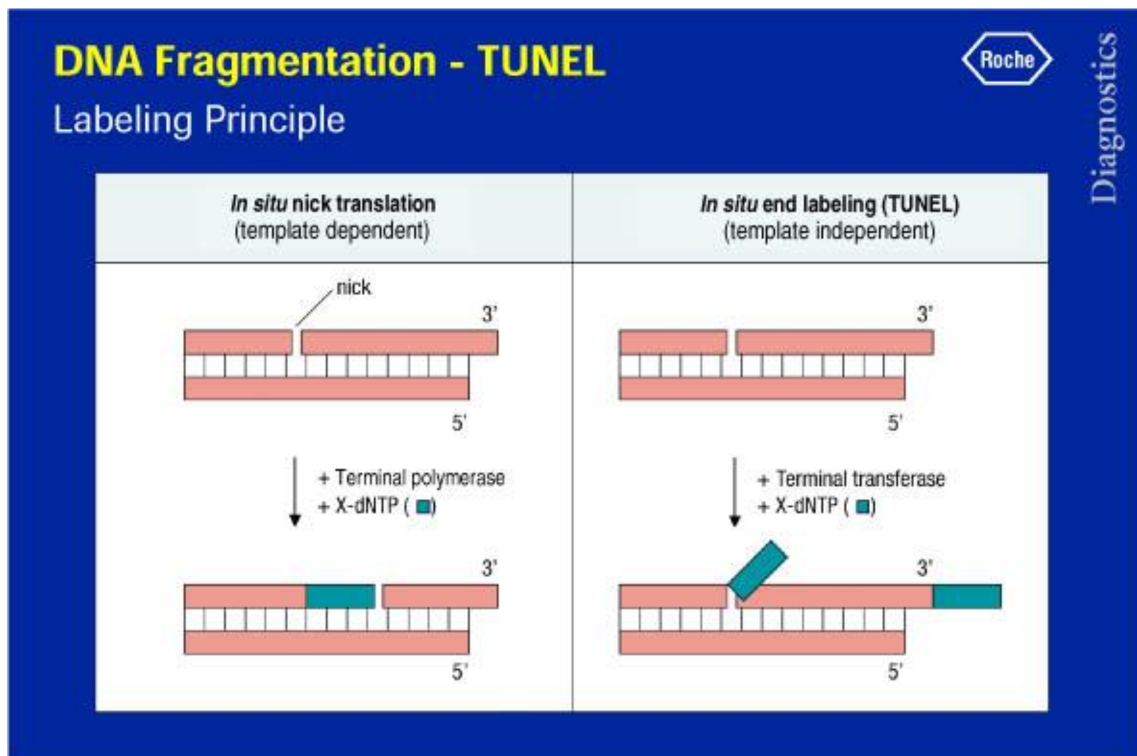


Figure 1.16: DNA Fragmentation-Tunel Labeling Principle

The Tunel method uses terminal dideoxynucleotidyltransferase (TdT) to incorporate hapten-tagged nucleotides into the 3'-strand breaks that occur in DNA during apoptosis (Gorczyca, Traganos et al. 1993). If these nucleotides are coupled to a fluorescent molecule, or if the hapten can be detected by a fluorescent secondary reagent, the apoptotic cells can be analyzed by fluorescence microscopy.

1.11 Sister Chromatid Exchange

Sister chromatid exchange (SCE) is an advanced cytomolecular technique that is commonly applied in a search for mutagenicity or genotoxicity. SCE analysis is show that whether the chromosomes and thus DNA of a particular interest group has undergone some genetic damage compared to a control group. Each chromosome comprises two sister chromatids which are genetically identical. In the SCE technique, one sister chromatid is stained dark and the other one pale. In a normal healthy person it is not unusual for the sister chromatids of one chromosome to break and swap pieces with each other. Therefore this is called sister chromatid exchange and providing the

number of SCEs do not go beyond a certain threshold, this is not considered to be harmful.

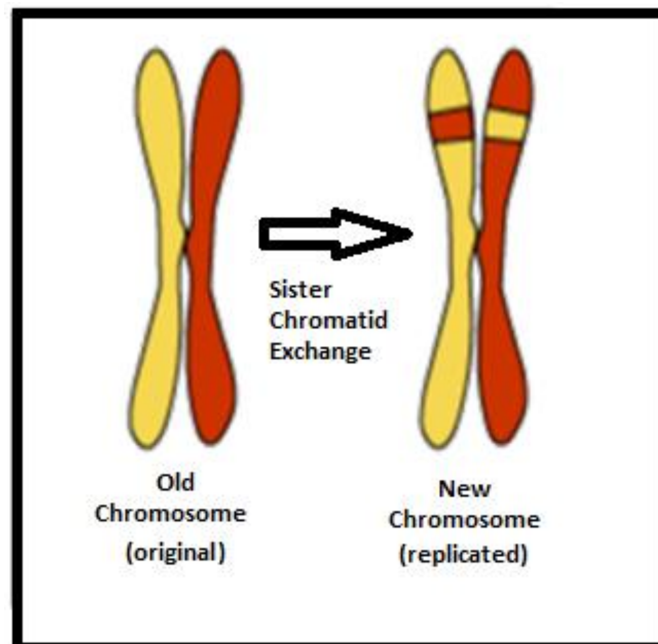


Figure 1.17: Sister Chromatid Exchange

SCE was first demonstrated in ring chromosomes and subsequently by autoradiography in rod chromosomes. Since then, the introduction of fluorescent dye/ Giemsa techniques has provided the degree of resolution and simplicity necessary for a reliable screening procedure for SCE. When mammalian cells are cultured through a single replication cycle in the presence of the thymidine analogue, bromodeoxyurine (BrdU), one DNA strand in each daughter chromatid is substitute with BrdU. After a second cycle of substitution, one chromatid contains one substituted DNA strand while both strands of its sister chromatid are substituted. The chromatids can be differentiated bye treatment with dye 33258 Hoechst, which fluoresces less when bound to DNA substituted with BrdU than when bound to unsubstituted DNA. At this stage the chromatids can be differentiated by fluorescence microscopy. Following photosensitization, which presumably leads to selective degradation of the highly substituted chromatid, and Giemsa staining, the sister chromatids can be observed by conventional light microscopy (Danford, Stancombe et al. 1984).

1.11.1 SCE Mechanisms

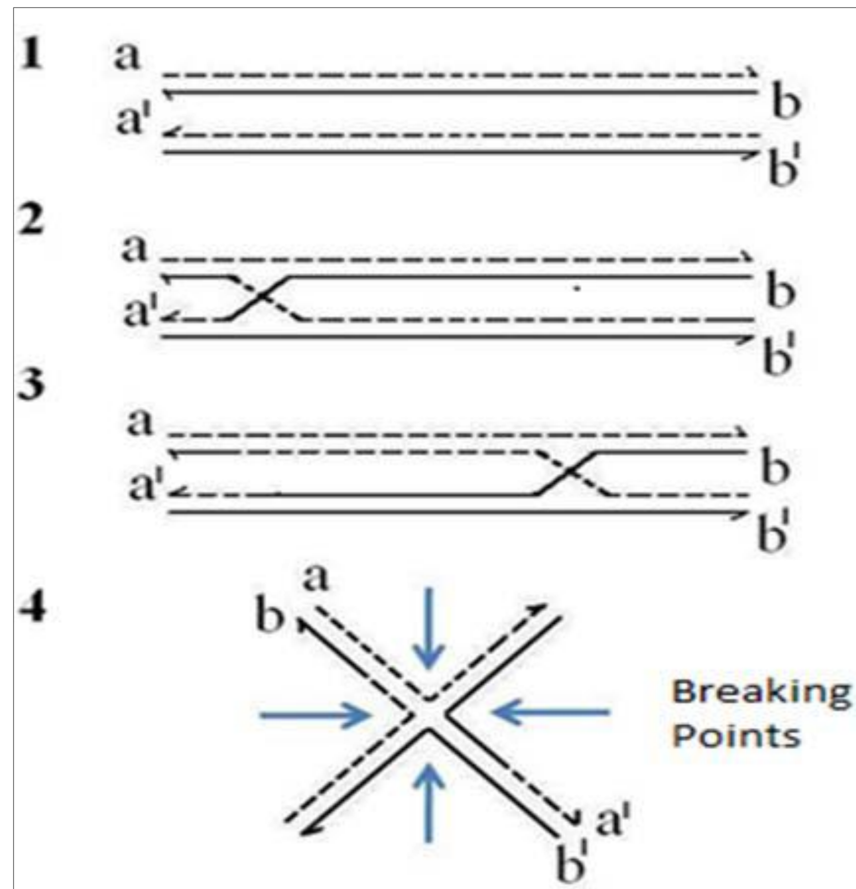


Figure 1.18: The mechanism of SCE formation – Steps 1-4 (Latt, Schreck et al. 1978)

1) Thymine containing template DNA, complementary DNA that is synthesized by taking BrdU instead of Thymine

2) Single strain breaks occur at each duplex and the joining of strands with the sister chromatid DNA duplex strands a result of crossing-over

3) The formation of heteroduplex containing Thymine and BrdU as a synthesis proceeds

4) The formation of the X form as a result of the rotation of the molecule around the crossing-over point.

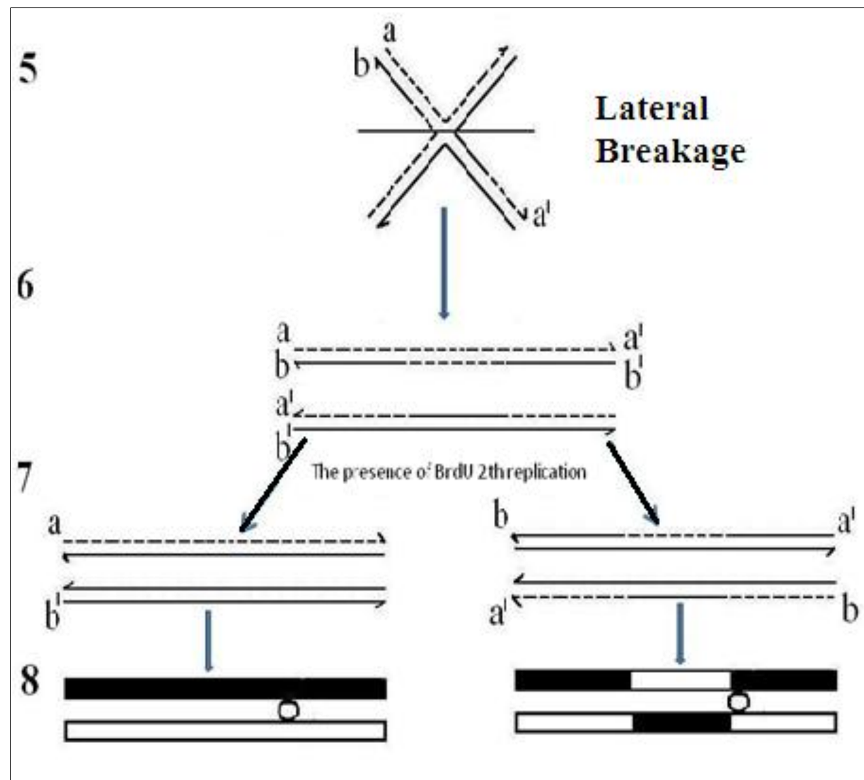


Figure 1.19: The mechanism of SCE formation – Steps 5-8 (Latt, Schreck et al. 1978)

5) The formation of lateral break on the X form of the molecule that is produce with the rotation of the molecule around the crossing-over point

6) The formation of regions on the duplex containing Thymine on one strand and BrdU on the other strand, as a result of combination of fragmented pieces produced by the breaks around rotation of crossing-over

7) In the presence of BrdU second replication passes, and complementary strand containing only BrdU is synthesized

8) The principle of this mechanism is observation of BrdU containing regions as faint colored and Thymine containing regions as dark colored indicating SCE

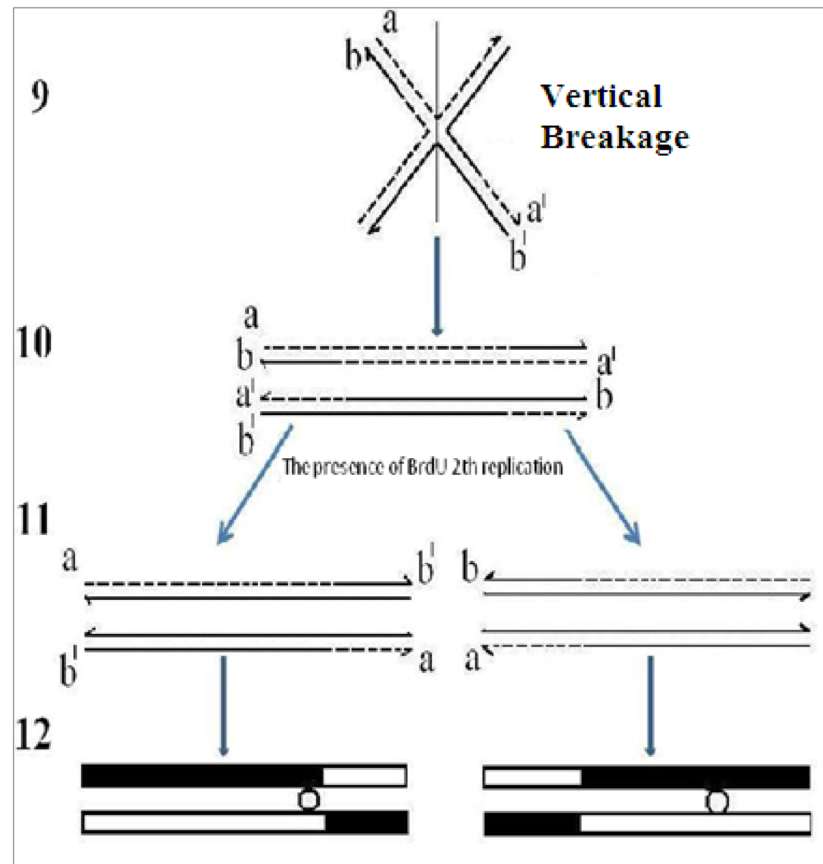


Figure 1.20: The mechanism of SCE formation – Steps 9-12 (Latt, Schreck et al. 1978)

9) The vertical breaking of the X form that is formed as a result of rotation of the molecule around the crossing-over point

10) The formation of regions on the duplex containing Thymine on one strand and BrdU on the other strand, as a result of combination of fragmented pieces produced by the breaks around rotation of crossing-over

11) In the presence of BrdU second replication passes, and complementary strand containing only BrdU is synthesized

12) The principle of this mechanism is observation of BrdU containing regions as faint colored and Thymine containing regions as dark colored indicating SCE Furthermore many environmental agents, at home or at work, can increase the frequency of SCE (e.g., UV light, X-rays) (Baysal, Sahin et al. 2003).

1.11.2. Effecting factors of SCE

• **Age:** According to Baysal et al 2003 adults have an increased frequency of SCE more than young (Baysal, Sahin et al. 2003).

• **Genetic:** Many inherited diseases are known to increase the frequency of SCE (Baysal, Sahin et al. 2003).

• **Diet:** Smoking and alcohol use is known to increase frequency of SCE (Gantt, Parshad et al. 1978).

• **Drug use:** Used in many of the drugs (such as penicillin Nitrofrontin) is an evidence to the increased frequency of SCE (Baysal, Sahin et al. 2003).

• **Diseases and infections:** Many chronic diseases, and viral infections are shown that to increase the frequency of SCE (Baysal, Sahin et al. 2003).

• **Runtime of blood:** Blood samples should be studied within 24 hours.

Furthermore many environmental agents, at home or at work, can increase thefrequency of SCE (e.g., UV light, X-rays) (Baysal, Sahin et al. 2003).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. General Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company (USA), Bender Med Systems (USA) Merck (Germany), Applichem (Germany).

Table 2.1: List of general chemical reagents and their brands.

Chemical Reagents	Brands
PBS	Biochrome AG
FBS	Biochrome AG
RPMI	Biochrome AG
Ficoll	Biochrome AG
Ethanol (96%)	Merck
Methanol (60%)	Merck
Sodium hydroxide	Merck
Sodium carbonate	Merck
Phytohaemaglutinin (PHA)	Biological Industries
L-Glutamine	Biological Industries
Penicillin	Biological Industries
Streptomycin (PS)	Biological Industries

Chemical Reagents	Brands
Colcemide solution (colchicine)	Biological Industries
Potassium chloride (KCl)	Merck
KH_2PO_4	Merck
Na_2HPO	Merck
Giemsa dye	Merck
Acetic acid	Merck
Hoechst 33258	Sigma
Cytotoxicity Detection Kit (LDH)	Roche (Germany)
In situ cell death detection kit	Roche (Germany)
WST-1 Kit	Roche (Germany)
5-Bromo-2-deoxyuridine (BrdU)	Sigma

2.1.2 Equipments

Table 2.2: List of general equipments and their brands.

Equipments	Models
Autoclave	CERTO CLAW A-4050 Traun, Austria
Centrifuge	Hettich, Mikro 22
Vortex IKA LABOTECHNIK	Vortex IKA LABOTECHNIK
Water Bath Nuve	Water Bath Nuve
Inverted Light Microscope Zeiss	Fluorescent Microscope Zeiss
Inverted Light Microscope Zeiss	Fluorescent Microscope Zeiss
CO2 Incubator Thermo, Sanko	CO2 Incubator Thermo, Sanko
CO2 Incubator Thermo, Sanko	CO2 Incubator Thermo, Sanko
Microfilter (1.0/0.45 µm) GF/PET	Microfilter (1.0/0.45 µm) GF/PET
Szhleicher & Schuell	Szhleicher & Schuell
Pipettor	Reddot Hirschmann Laborgerate

Equipments	Models
Centrifuge Tubes Falcon	Micropipettes Nichiryo
Serological Sterile, Plastic Pipets	(2 mL, 5 mL, 10 mL)
Grenier	Syringe Hayat Sırınga
Centrifuge Tubes Falcon	Micropipettes Nichiryo
Trypan blue	Sigma
Vacutainer	BD
Holder	Grenier
Rotavapor Buchi	Rotavapor Buchi
Micropipettes Nichiryo	Micropipettes Nichiryo
Laminar Flow Hood	Esco, Kotterman
Water Purification System	Millipore
96-well plates	Greiner

2.2 METHODS

2.2.1 Plant Extraction Process

D. stramonium were obtained from KONYA in 2010. 20 grams of cleaned DE seeds was crushed and mixed 1 to 4 with methyl alcohol. The mixed complex was set aside for 24 h in laboratory temperature. Then it was filtered three times through a mesh. The methanol was removed vacuum rotary at 50^0C until dryness. Then this portion was dissolved with 20 ml methyl alcohol. Finally, the extract was filtered through a GF/PET (glass fiber/polyethyleneterephtalate) 1.0/0.45 µm microfilter, and prepared for PBMC culture (Mustafa and Al-Khazraji 2008).

2.2.2 Isolation of PBMCs

The peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation with Ficoll. First, whole peripheral blood was collected into a heparinized Syringe. Using a wide centrifuge tube, blood was mixed with PBS onto an equal quantity. Taking another centrifuge tube filled with ficoll

an equal quantity with blood and PBS mix. Blood was slowly poured onto Ficoll, taking care not to disturb the interface between the blood and Ficoll. Tubes were centrifuged at 2500rpm for 25min at RT. After centrifugation, there are four different layers appear.

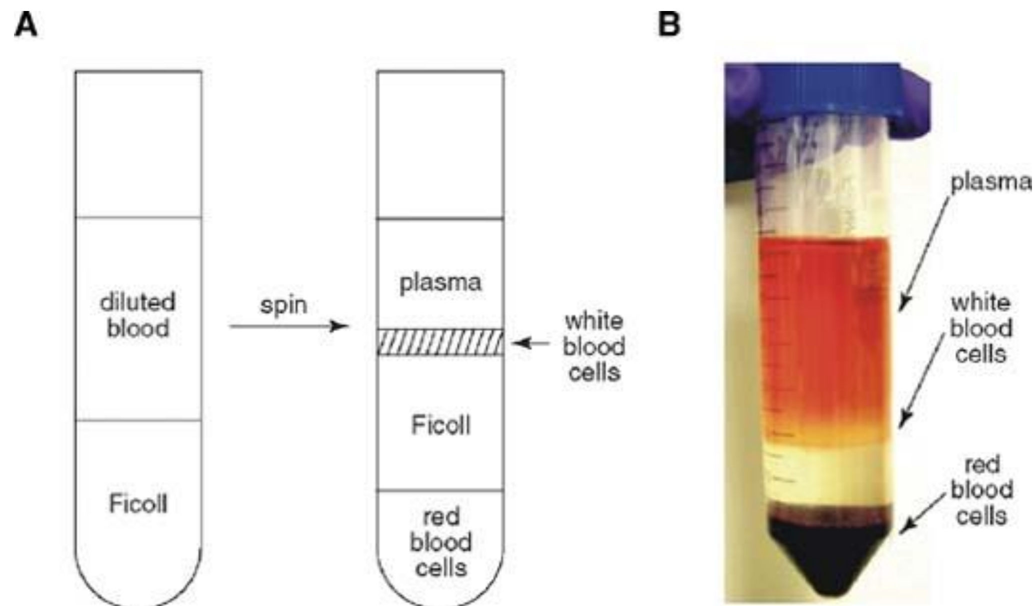


Figure 2.1: Isolations of PBMC populations with Ficoll density gradient centrifugation

Upper layer was removed then the lower layer was transferred into another empty Falcon and 10ml PBS mixed was with it. Tubes were centrifuged at 1700rpm for 7min. Then supernatant was discarded and the cell pellet was resuspended by firmly tapping the bottom of the tube. Tube was refilled with RPMI 1640 medium, centrifuged at 1700rpm for 7min. Then supernatant was discarded and the cell pellet resuspended as before. Volume was brought up to 5ml with complete culture medium (RPMI-1640 supplemented with 10% FBS in the tube). The tube mixed gently and using a sterile pipet tip 10µl sample was removed into tube. Then 20 µl trypan blue solutions were mixed with sample into the tube and mix loaded into haemocytometer. The cells were counted and calculated. The cell suspension was adjusted to 1x106 cell / ml equality (Larche 2000).

Counts can either be performed manually using a hemocytometer and for example trypan blue (Figure 2.2). This method is quick, inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to

determine the cell concentration (cell number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment.

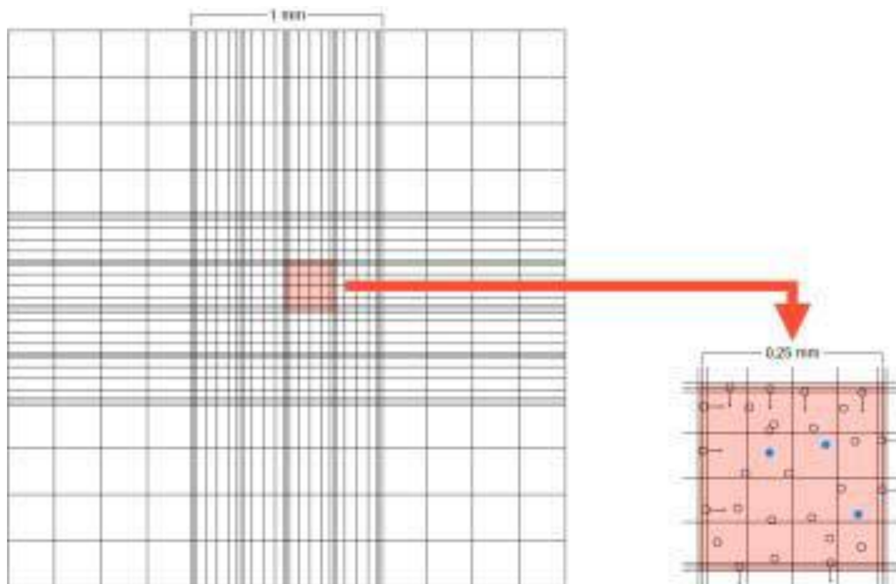
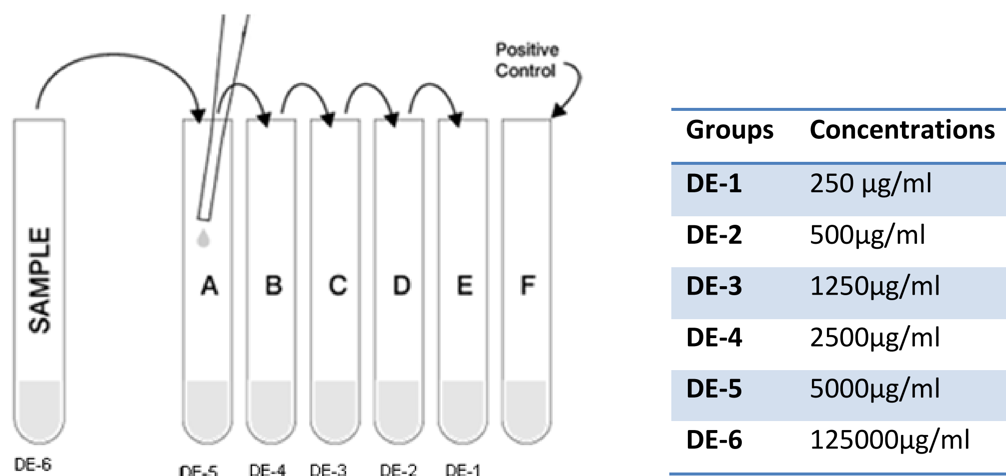


Figure 2.2: Measurement of proliferation by counting the cells with a hemocytometer. The addition of trypan blue helps to distinguish viable, unstained cells (0) from non-viable, blue-stained cells.

2.2.2.1 Adding Extract

The PBMCs were seeded as $2x10^6$ cell/ml equality and incubated with methanolic *D. stramonium* (DE) at different concentrations at 37°C and 5% CO_2 for 3 days. This proportion estimated with cytotoxicity tests and proliferation tests.



Groups	Concentrations
DE-1	250 µg/ml
DE-2	500µg/ml
DE-3	1250µg/ml
DE-4	2500µg/ml
DE-5	5000µg/ml
DE-6	125000µg/ml

Figure 2.3: Serial Dilutions of the different DE concentrations.

2.2.3 Cytotoxicity Tests

The cells were removed from the culture medium prior to the determination of LDH activity by centrifugation at about 250 × *g* for 10 min. Supernatant (100 µl/well) was removed carefully and transferred into 96-well flat bottom microplate (MP). Reaction mixture (described below) was added (100 µl to each well) and incubated for 30 min at RT. The MP was protected from light during this incubation period. The samples absorbance were measured at 490 nm with ELISA reader.

The lyophilisate (bottle 1, blue cap) was dissolved in 1 ml double distilled water for 10 min and mixed. The dye solution (bottle 2, red cap) was ready to use. The reaction mixture was mixed 250 µl of bottle 1 with 11.25 ml of bottle 2.

2.2.4 Proliferation Assay

Cells were cultured in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium in a humidified atmosphere (37°C, 5% CO_2). Cell Proliferation Reagent WST-1 was added in a 10 µl/well volume. Cells were incubated for 4h in a humidified atmosphere (37°C, 5% CO_2). The samples absorbance were measured at 420 nm with ELISA reader.

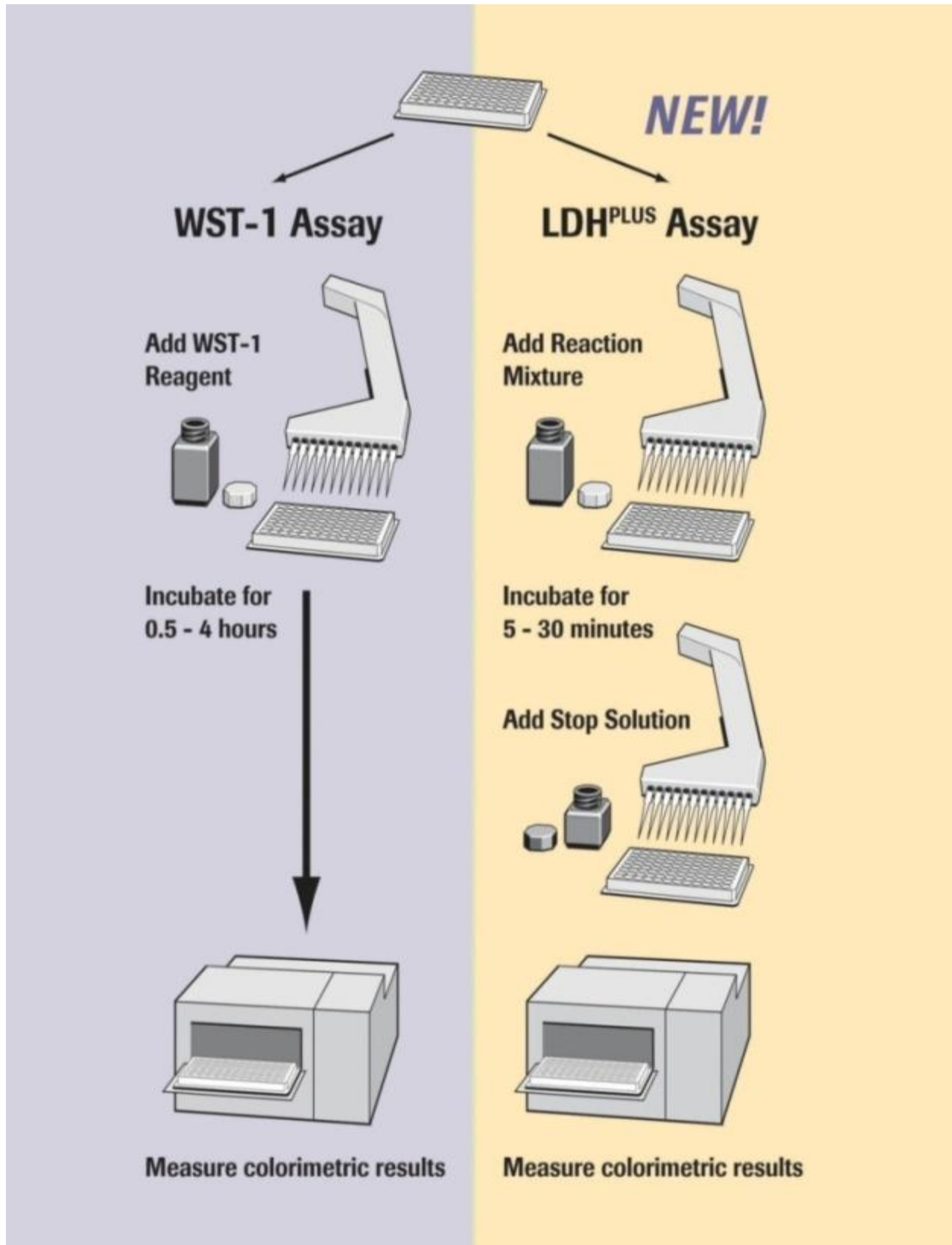


Figure 2.4: LDH and WST-1 assay experimental set up.

2.2.5 SCE Assay

2.2.6 Preparation of Solutions Used

2.2.6.1 Preparation of BrdU Solution

26 mg of powder BrdU was dissolved in 5 ml RPMI to prepare stock BrdUsolution (10-2 M). Then the stock solution was covered with aluminum foil to protect from light and storage at -20 °C.

2.2.6.2 Preparation of Hypotonic Solution

0.5592 gram of KCl was dissolved in 100 ml distilled water. The solution was incubated on 37 °C. The final concentration of our solution was 0.075 M.

2.2.6.3 Preparation of Solution Used for Fixation

The fixation solution was prepared by using both glacial ascetic acid and methanol 96%. After that the solution was stocked in fresh and cold conditions.

2.2.6.4 Preparation of Phosphate Buffered Saline (PBS) (pH: 7)

The PBS solution was prepared by mixing all of these chemicals; 8.0 grams of NaCl, 0.2 gram of KCl, 0.2 gram of KH2PO4 and 2.32 gram of Na2HPO4 (or 2.91 gram Na2HPO4.2H2O). All the chemicals were dissolved in 1 litter distilled water. The solution was finally stored at room temperature.

2.2.6.5 Preparation of Fluorescent dye

25 mg of Hoechst 33258 dye was dissolved in 5 ml distilled water and stored in-20 °C. The stock Hoechst dye was dissolved in distilled water in order to obtain 2.5mg/ml of Hoechst dye. The Hoechst 33258 dye prepared was mixed with 1 litter of PBS solution, covered with aluminum foil and stored in +4 °C.

2.2.6.6 Preparation of 2xSSC Solution

In order to prepare to 2xSSC Solution 1.74 gram NaCl was dissolved in 100 ml of distilled water. Then 0.882 gram of Sodium citrate was dissolved in 100 ml of distilled water. The same volume of both NaCl and sodium citrate solutions was taken and controlled with citric acid in order to obtain pH=7.2

2.2.6.7 Preparation of Giemsa dye Solution

The Giemsa dye solution was prepared by using two different solutions A and B. Solution A: 11.88 grams of Na_2HPO_4 dissolved in 1 litter of distillated water. Solution B: 9.08 grams KH_2PO_4 dissolved in 1 litter of distillated water. 5 ml of commercial Giemsa dye was added to the mix of the solutions A and B than stored at room temperature.

2.2.6.8 Establishment of the Experimental Groups

Samples of peripheral lymphocyte were obtained by venipuncture. We chose three (3 women) healthy individuals who were qualified, non-smoking and between the ages of 20-25. They were not undergoing any drug treatment and had not a viral infection or X-ray in the recent past, or hepatitis at any time. Also the last six months, for any reason, they did not define acute or chronic diseases that will be the designated start day of blood donors were tested. Each group includes different 8 cultures.

Previously prepared culture media at -20 °C was removed and come to the right to room temperature. The chemicals at room temperature are filtered and added to defined culture medium at planned rates. This experimental setup was repeated for a total of four donors. The experiment was performed on 8 groups as follows;

Table 2.3 Experimental groups of DE.

Groups	**Concentrations**
Culture 1	Blood samples (-) Control
Culture 2	Blood samples + 5 µM CCl4 (+)
Culture 3	Blood samples + 250 µg/ml DE-1
Culture 4	Blood samples + 500 µg/ml DE-2
Culture 5	Blood samples + 12500µg/ml DE-3
Culture 6	Blood samples + 25000µg/ml DE-4
Culture 7	Blood samples + 50000µg/ml DE-5
Culture 8	Blood samples + 125000µg/ml DE-6

2.2.6.9 Preparation and Incubation of Lymphocyte Culture

Lymphocyte medium were set up by adding; RPMI medium, L-glutamine, PS (1% penicillin- streptomycin), PHA (1% Phytohaemaglutinin) and FBS. Solution will be prepared in 8 ml of sterile cell culture tubes were shared. All cultures were maintained in darkness. Then they were stored -20 ºC until use.

Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to RPMI-1640 chromosome medium supplemented with 15% heat-inactivated fetal calf serum (FBS), 100 IU/ml streptomycin, 100 IU/ml penicillin, and 1% L glutamine. Lymphocytes were stimulated to divide by 1% Phytohemaglutinin. CCl4 (in concentration of 5 M, 100 µl), methanolic extract of *D. stramonium* (DE) (in concentrations of 250, 500, 12500, 25000, 50000 and 125000 µg/ml) were added to the cultures just before incubation. CCl_4 was used as a positive control.

2.2.6.10 Fluorescence plus Giemsa Staining Technique

For SCE demonstration, the cultures were incubated at 37 °C for 72 h, and BrdU at 8 mg/ml was added at the initiation of cultures. All cultures were maintained in darkness. Next, 0.1 mg/ml of colcemide was added 1 h prior to harvesting to arrest the cells at metaphase. Cultures that were prepared put inside centrifuge at 10 minutes on 1000 rpm and then part of supernatant separate for use biochemistry analyses. Cells were harvested and treated for 30 min with hypotonic solution (0.075 M KCl) and fixed in a 1:3 mixture of acetic acid / methanol (v/v). Bromodeoxyuridine-incorporated metaphase chromosomes were stained with fluorescence plus Giemsa technique as described by Perry and Evans (Perry and Evans 1975)

2.2.6.11 Sister Chromatid Exchange Counting

In SCE study, by selecting 20 satisfactory metaphases, the results of SCE were recorded on the evaluation table. For each treatment condition, well-spread second division metaphases containing 42–46 chromosomes in each cell were scored, and the values obtained were calculated as SCEs per cell.

2.2.7 Tunnel Assay

DNA fragmentation is an indicator for detecting the late apoptosis. TUNEL (Roche) is an assay that is composed of an enzyme solution and labeling solution which detects the nicks (single strand breaks) in DNA and binds to free 3'-OH ends. Adding of dUTPs to 3'-OH ends by terminal deoxynucleotidyl transferase cause labeling of DNA. Fluorescently labeled ends were detected by using fluorescent microscopy.

Preparation of cells and slides lymphocytes was isolated from whole blood. Lymphocytes were cytocentrifuged on glass slides and air-dried for at least 30 min. The following fixation protocols were investigated: (1) 3:1 Asetic acid -methanol for 10 min at 4° C 1200 RPM; (2) 2:1 acetic acid:methanol for 10 min at 4° C 1200 RPM; 4% *p*-formaldehyde/phosphate-buffered saline (PBS, 0.01 M, pH 7.4) for 5 min at room temperature followed by washing in PBS. Slides with cell or chromosome preparations were stored in plastic boxes until further processing.

Chromosome preparations stimulation of human lymphocytes was performed in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 5 µg/ml phytohemaglutinin (PHA) for 72 h at 37° C in a humidified atmosphere with 0.5% CO_2. After an 71-h incubation step with 40 µg/ml colcemide, metaphase chromosomes were prepared according to standard protocols (Moorhead, Nowell et al. 1960). Following experiment DNA fragmentation of cells were determined with the In Situ Cell Death Detection Kit, Fluorescein (ROCHE) according to the manufacture's instructions. The results were analyzed by fluorescence microscopy.

2.2.8 Statistical Analysis

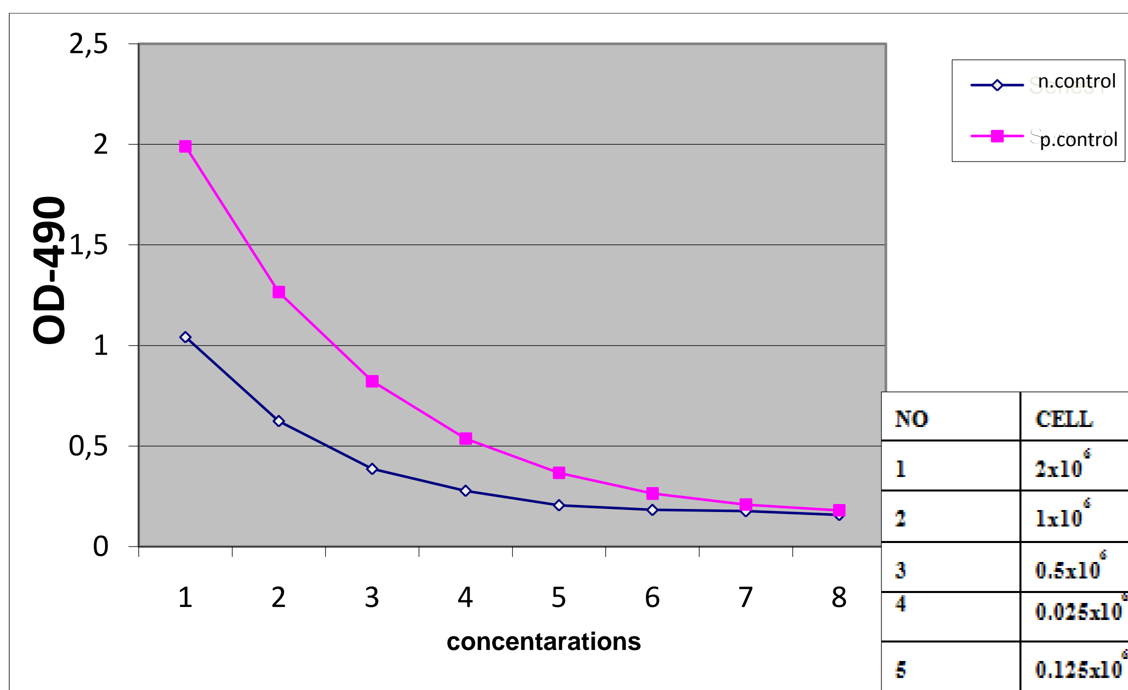
The statistical analysis of SCE, LDH and WST-1 values Mann– Whitney U-test was used. Also for Tunnel assay values One-way Anova was used. Statistical differences between time, dose and extract were analyzed. A value of P less than 0.05 was accepted as statistically significant. Results were expressed as mean ± SE. For these procedures, SPSS 11.5 version for Windows (SPSS Inc, Chicago, Illinois, USA) was used.

CHAPTER 3

RESULTS

3.1 Determination of the Optimal Cell Concentration for the LDH and WST-1 Assay

Different cell types may contain different amounts of LDH. So, the optimum cell concentration for lymphocytes should be determined in a preliminary experiment. In general, this cell concentration, in which the differences between the low and high control is at a maximum, should be used for the subsequent assay.



NO	CELL
1	$2x10^6$
2	$1x10^6$
3	$0.5x10^6$
4	$0.025x10^6$
5	$0.125x10^6$

Figure 3.1 Determination of optimum cell number in lymphocytes. The optimum cell number determined at 24 h was $2x10^6$µg/ml. Cells, seeded at $2x10^6$ refers a maximum differences in positive and negative control. Thus this cell number was chosen as an optimum for seeding in the further experiments.

3.2 LDH Cytotoxicity Assay

Lactate dehydrogenase activity of *D. stramonium* was measured with LDH cytotoxicity kit (Roche). Graphs observed in the figure represent two independent experiments performed in triplicate assays. Following treatment of lymphocytes with DE at determined concentrations, at 24 and 48 hours were analyzed with the LDH assay which is based on the detection of lactate dehydrogenase enzyme in the culture media, released from damaged cells.

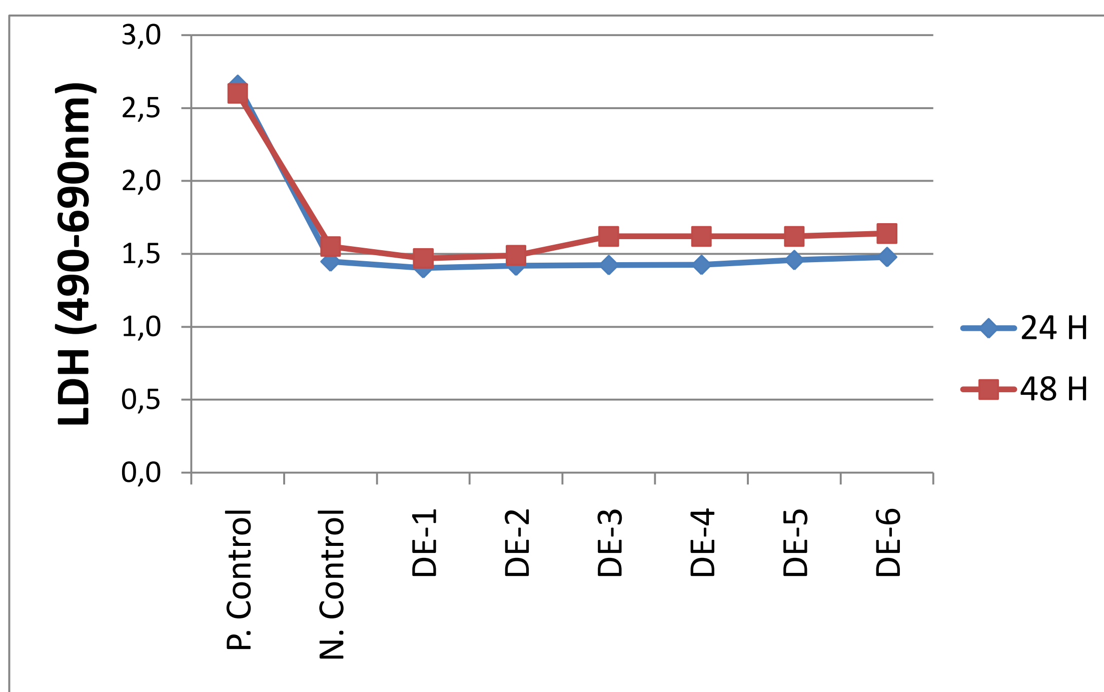


Figure 3.2 Results of the LDH assay treated for 24th and 48th hrs with different concentration of DE.

3.3 Determination of the Cytotoxicity Percentage

To determine the percentage cytotoxicity, calculate the average absorbance values of the triplicates and subtract from each of these the absorbance value obtained in the background control. The resulting values are substituted in the following equation:

$$\textbf{Cytotoxicity (\%)} = \frac{\text{exp. value - low control}}{\text{high control - low control}} \times 100$$

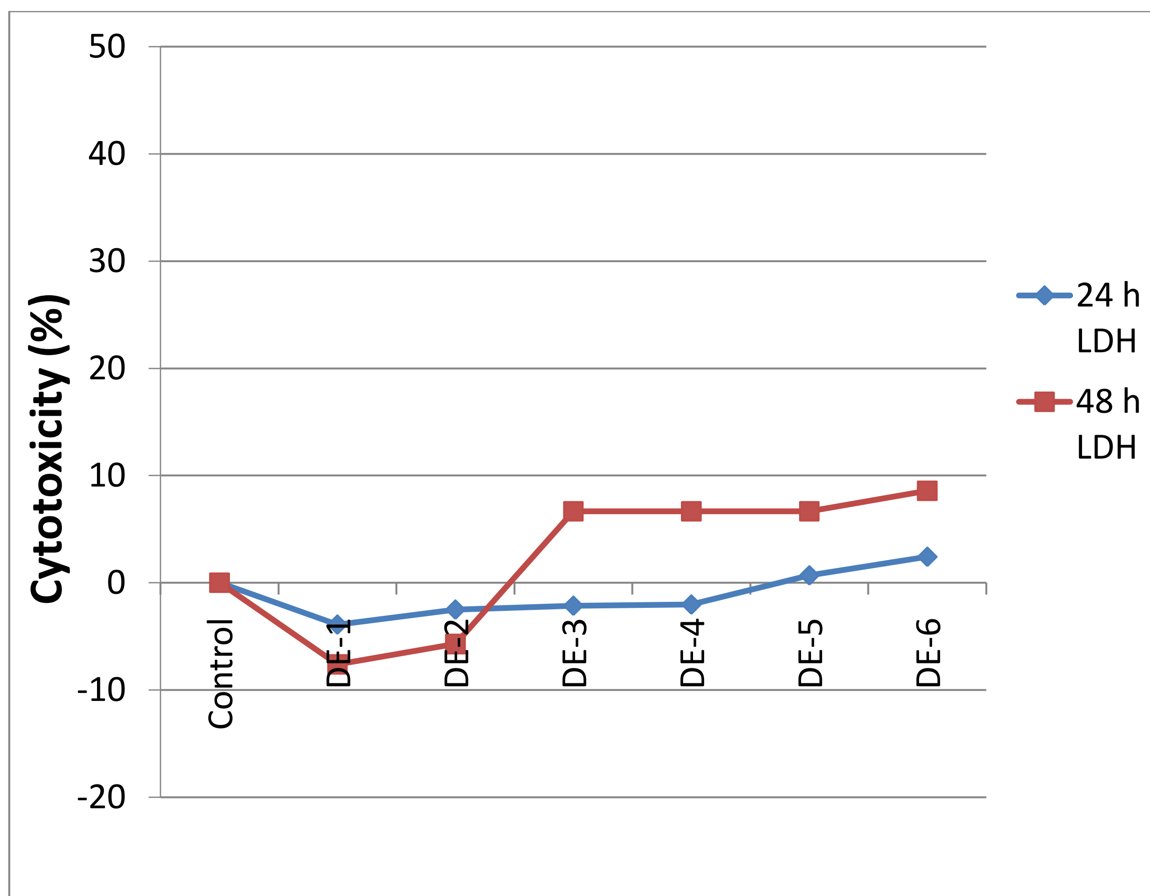


Figure 3.3 Determination of the Cytotoxicity (%) of different DE concentrations at 24th and 48th hours.

Table 3.1 DE different concentration on cell cytotoxicity (LDH)

GROUPS	24 H	48 H
P.Control	2.26±0.43[c]	2.17±0.46[d]
N.Control	1.16±0.32[a]	1.19±0.38[b]
DE-1	1.18±0.23[a]	1.15±0.34[b]
DE-2	1.21±0,23[a]	1.17±0.34[b]
DE-3	1.22±0,22[a]	1.26±0.38[b]
DE-4	1.24±0,20[a]	1.27±0.38[b]
DE-5	1.22±0,25[a]	1.28±0.37[b]
DE-6	1.30±0.20[a]	1.46±0.36[b]

[a]$p<0.05$ compare with positive control group 24 h; [b]$p<0.05$ compare with positive control group 48 h; [c]$p<0.05$ compare with negative control group 24 h; [d]$p <0.05$ compare with negative control group 48 h; Mann-Whitney test for repeated measurements.

LDH is a cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. DE doesn't cause any damage on cell membrane because the active molecules of *D. stramonium*, atropine and scopolamine, bind to muscarinic receptors in cell membrane. For that reason there were not any toxic effects observed.

The cytotoxic effects of DE are given in the Figure 3.1. DE's had no significant cytotoxic effect on human lymphocyte cells in the culture 24th and 48th hours. Although there were some differences between DE-treated group and untreated group, they are not significant.

3.4 Proliferation Assay

For detection of viable cells in response to extract treatment, WST-1 cell proliferation assay was used. We established similar experimental set up with LDH assay for comparing the results. The anti-proliferative effects of DE at different concentration results are shown in Figure 3.2.

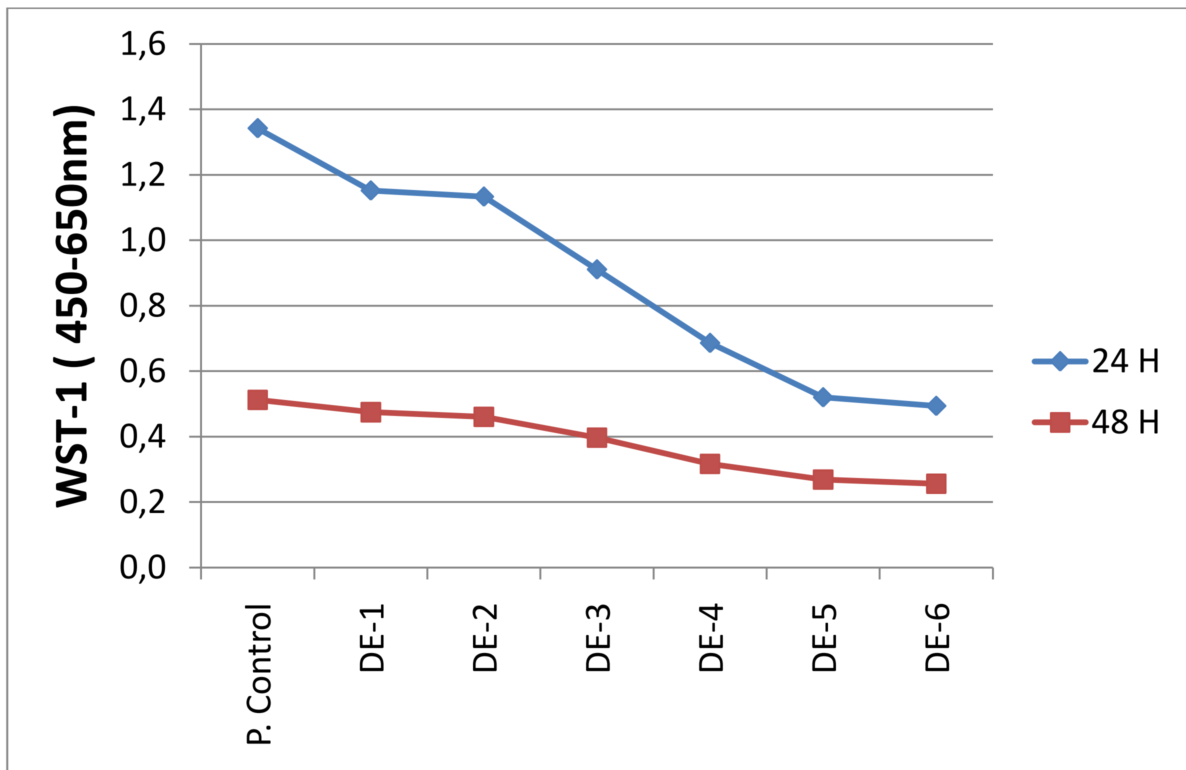


Figure 3.4 Results of the proliferation assay treated for 24 and 48 hrs with different concentration of DE.

3.5 Determination of the Cell Viability Percentage

To determine the percentage cell viability, calculate the average absorbance values of the triplicates and subtract from each of these the absorbance value obtained in the background control. The resulting values are substituted in the following equation:

$$(\%) = \frac{\text{exp. value - low control}}{\text{high control - low control}} \times 100$$

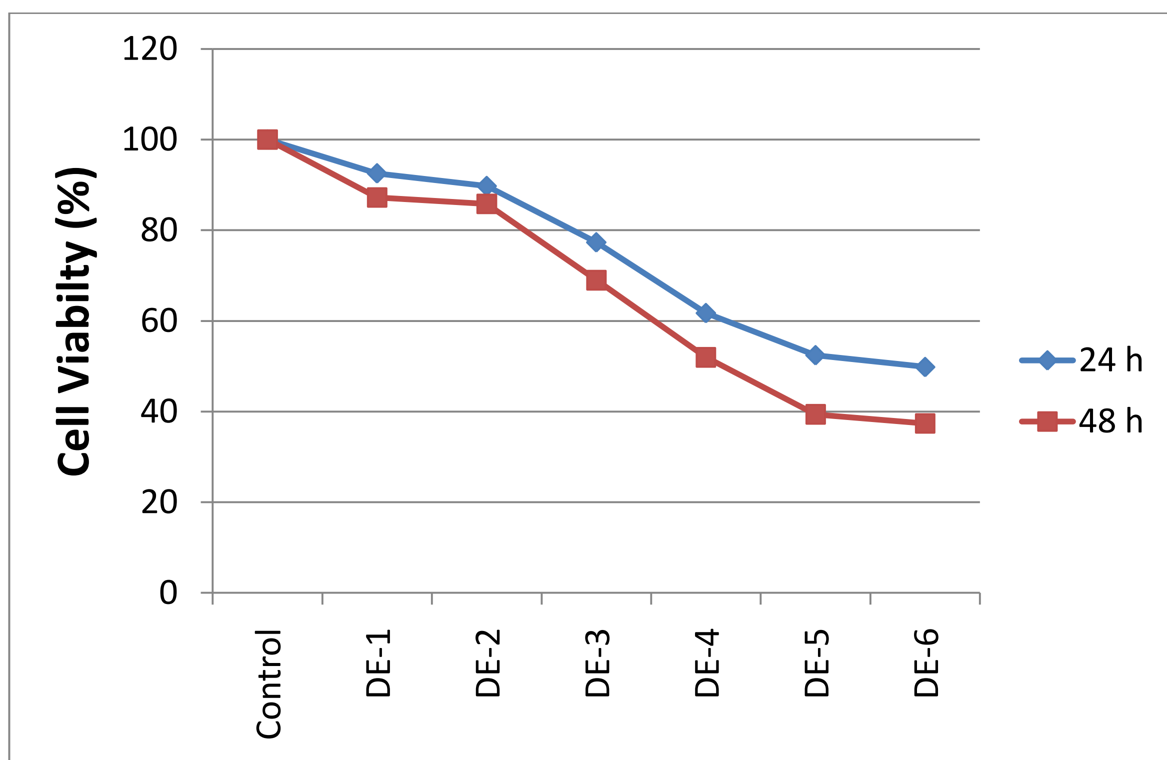


Figure 3.5 Determination of the Cell Viability (%) treated for 24 and 48 hrs with different concentration of DE.

Table 3.2 DE different concentration on cell proliferation (WST-1)

GROUPS	24 H	48 H
P.Control	1.13±0.24	0.62±0.12
DE-1	0.98±0.19	0.70±0.26
DE-2	0.92±0.23	0.67±0.22
DE-3	0.78±0.17	0.55±0.18
DE-4	0.67±0.04 [a,b,c]	0.47±0.18
DE-5	0.50±0.02 [a,c,d,e]	0.40±0.15
DE-6	0.48±0.01 [a,c,d,e]	0.36±0.12[f]

[a]p<0.05 compare with p.control group 24 h ; p[b]<0.05 compare with DE-1 group; p[c]<0.05 compare with DE-2 group 24 h ; p[d]<0.05 compare with DE-3 group 24 h ; p[e]<0.05 compare with DE-4 group 24 h ; p[f]<0.05 compare with p.control group 48 h ; Mann-Whitney test for repeated measures.

The effects of DE on cell proliferation were represented in Table 3.2 and Figure 3.2. DEs have anti-proliferative effect on human lymphocyte cell in the culture. Significant reduction in the cell proliferation were found in the DE-treated group

especially, 125000 and 5000µg/ml concentrations of DE when compared with untreated group as seen in the Table 3.2. ($p<0.001$ or 0.05).

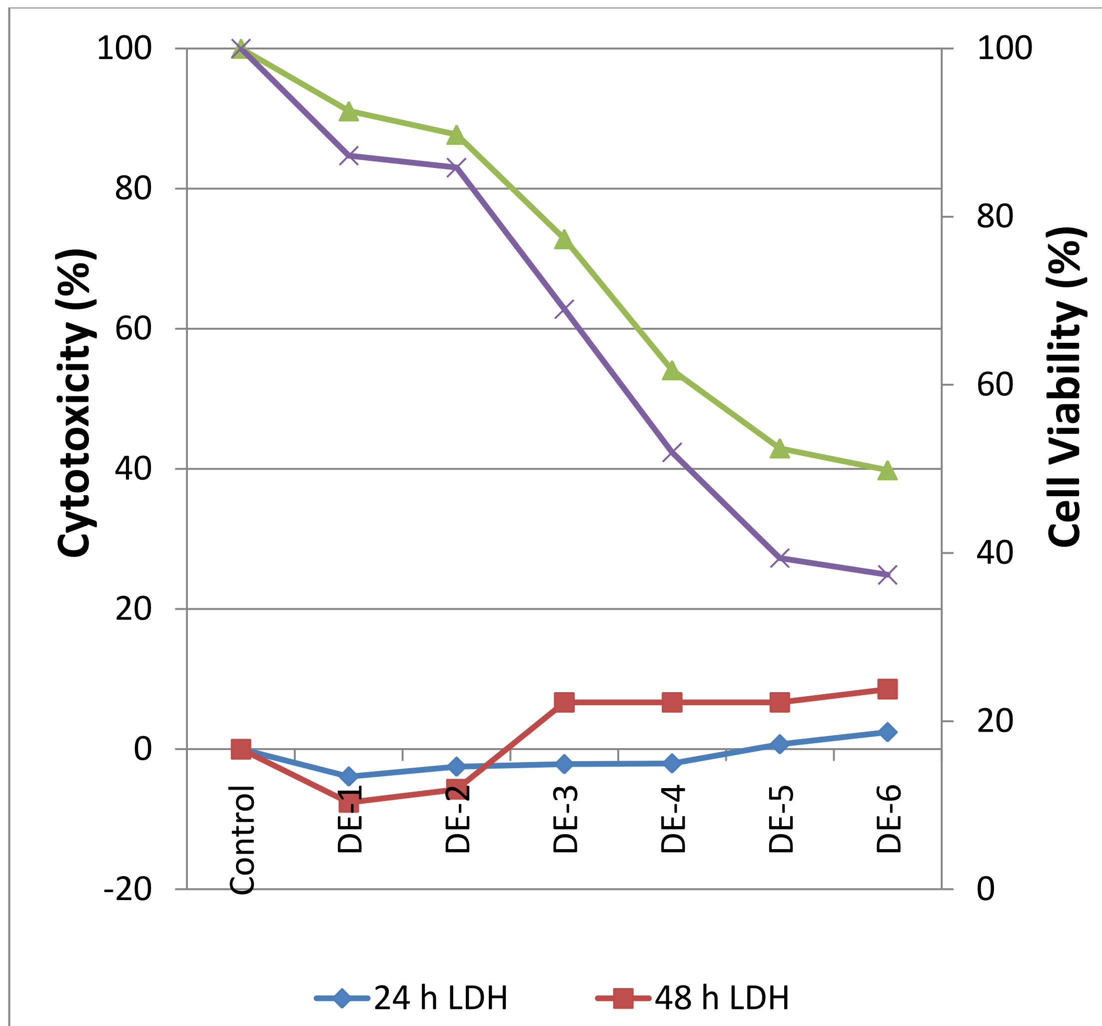


Figure 3.6 Comparison of Cytotoxicity (%) and Cell Viability (%) of DE on Cultured Human Lymphocytes

3.6 DNA Fragmentation (TUNEL Assay)

The detection of DNA fragmentation of *D. stramonium* different six concentration treated human cultured lymphocytes cells were assessed using the TUNEL assay.

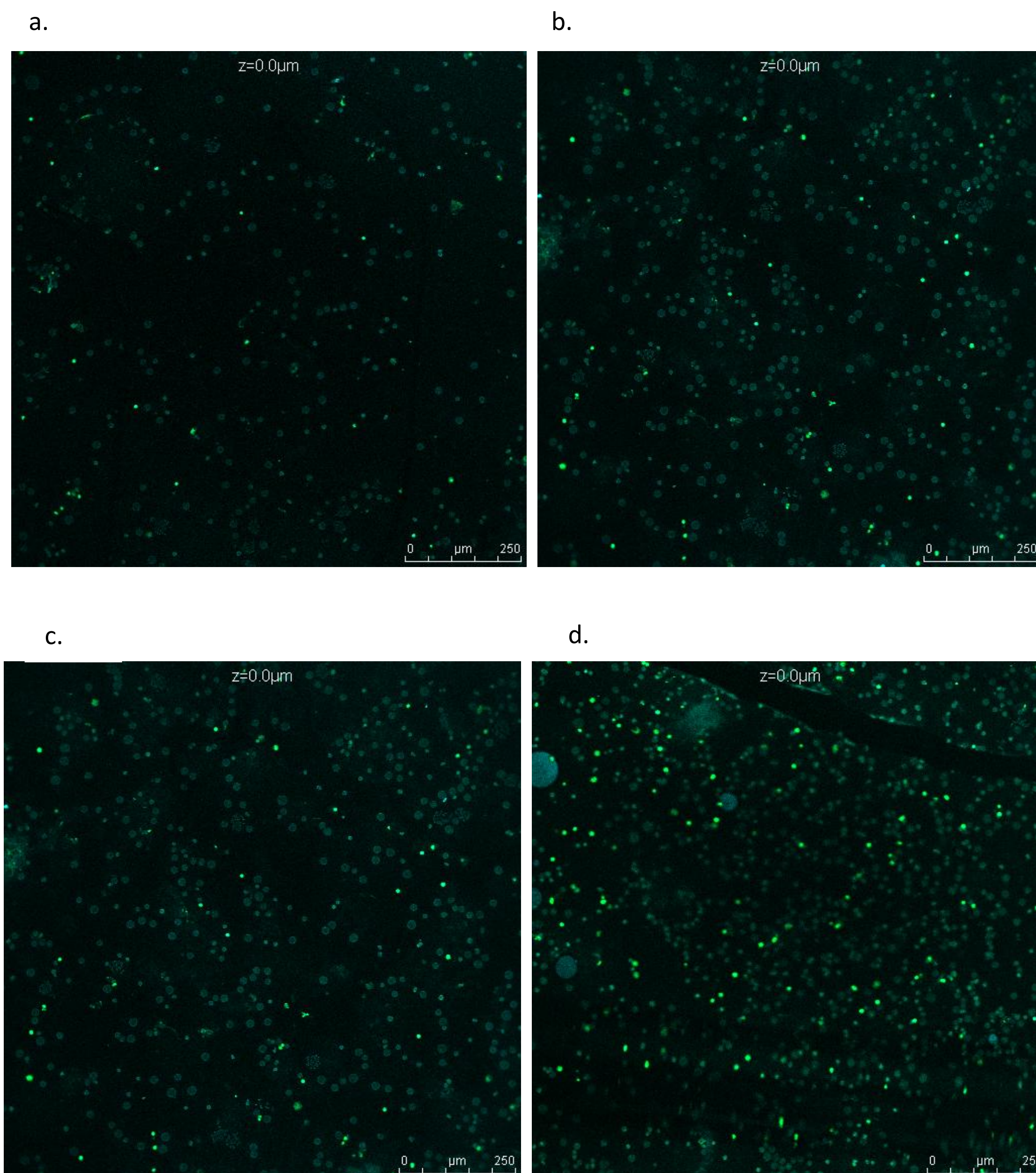


Figure 3.7: Lymphocytes observed under confocal microscope. The fluorescence images were obtained as a result of TUNEL assay at concentration of a.control, b.CCl_4, c. DE-1 d.DE-6. Images are at 10X.

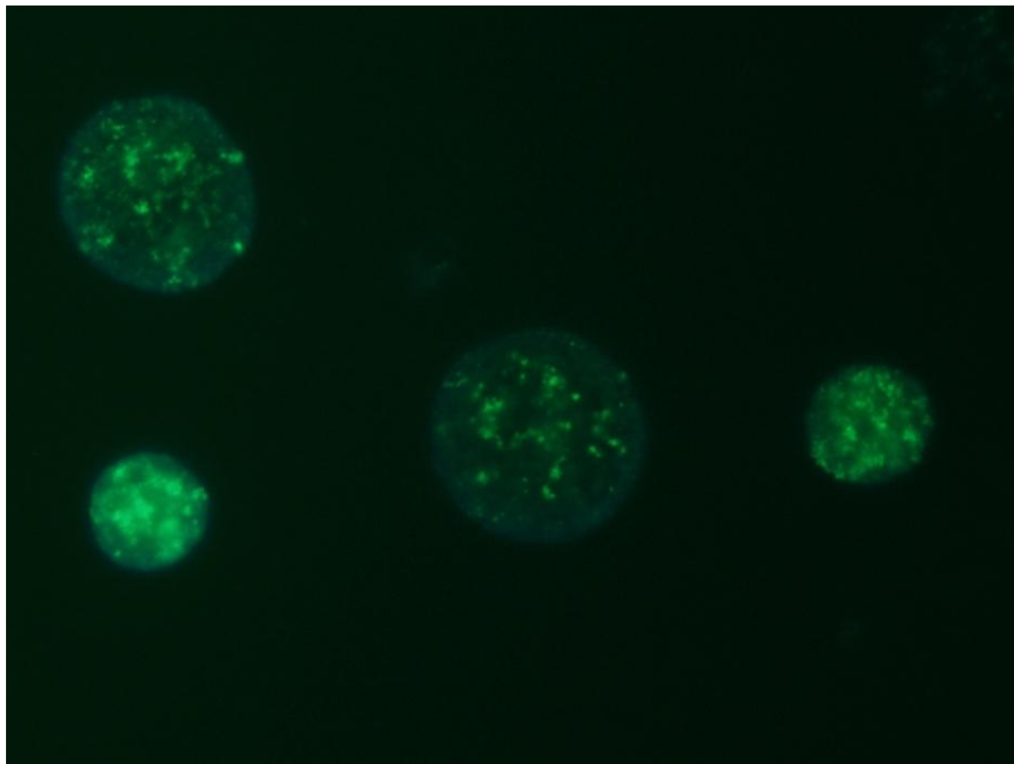


Figure 3.8 Tunel Assay of lymphocytes cells which are treated nothing (control) observed fluorescence microscope. Images are at 10X.

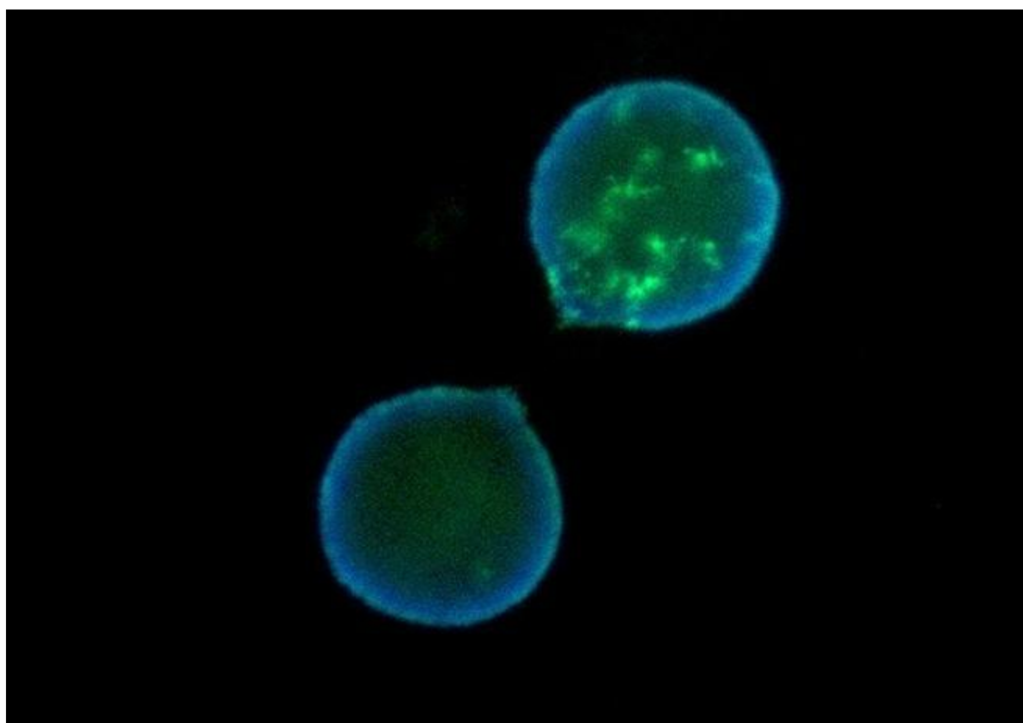
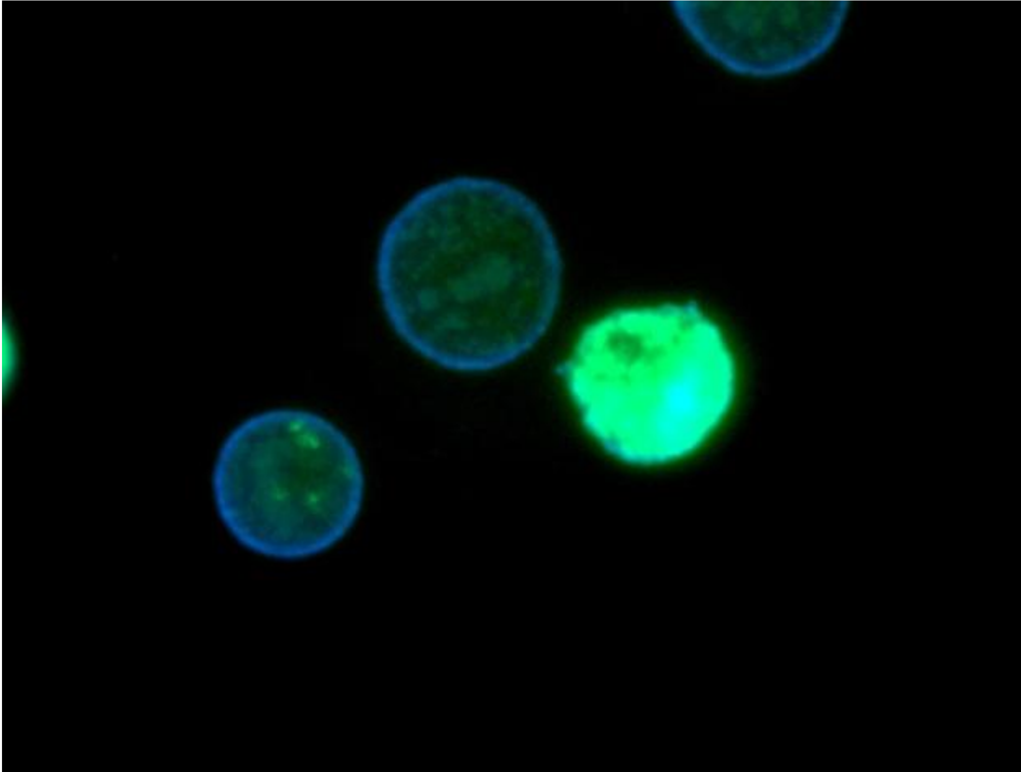


Figure 3.9 Tunel assay of lymphocytes cells which are treated CCl_4 observed fluorescence microscope. Images are at 100X.



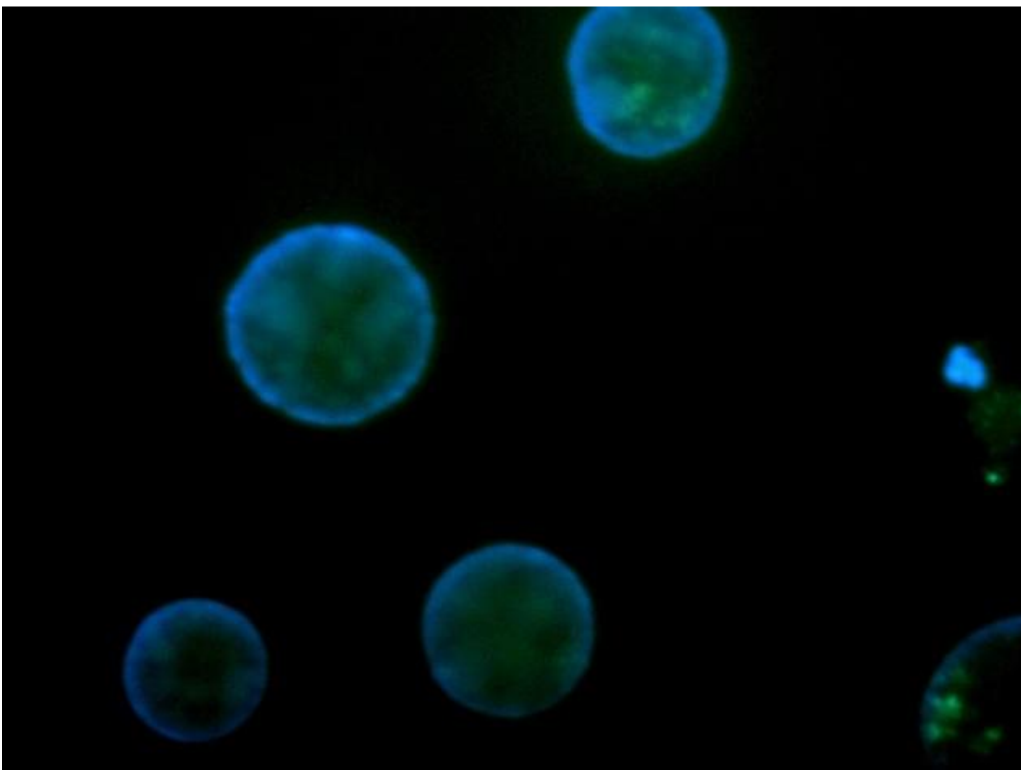
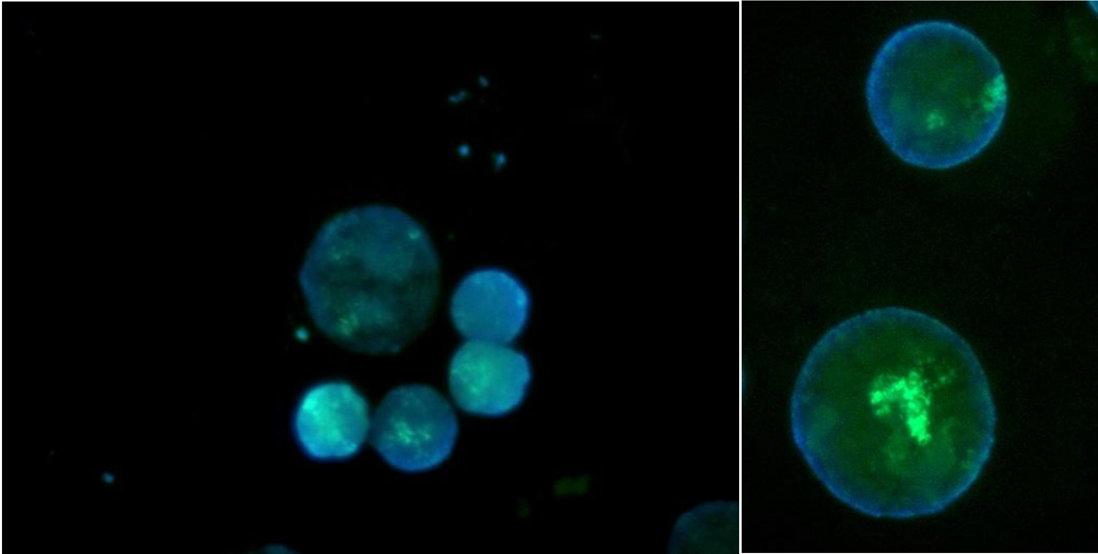
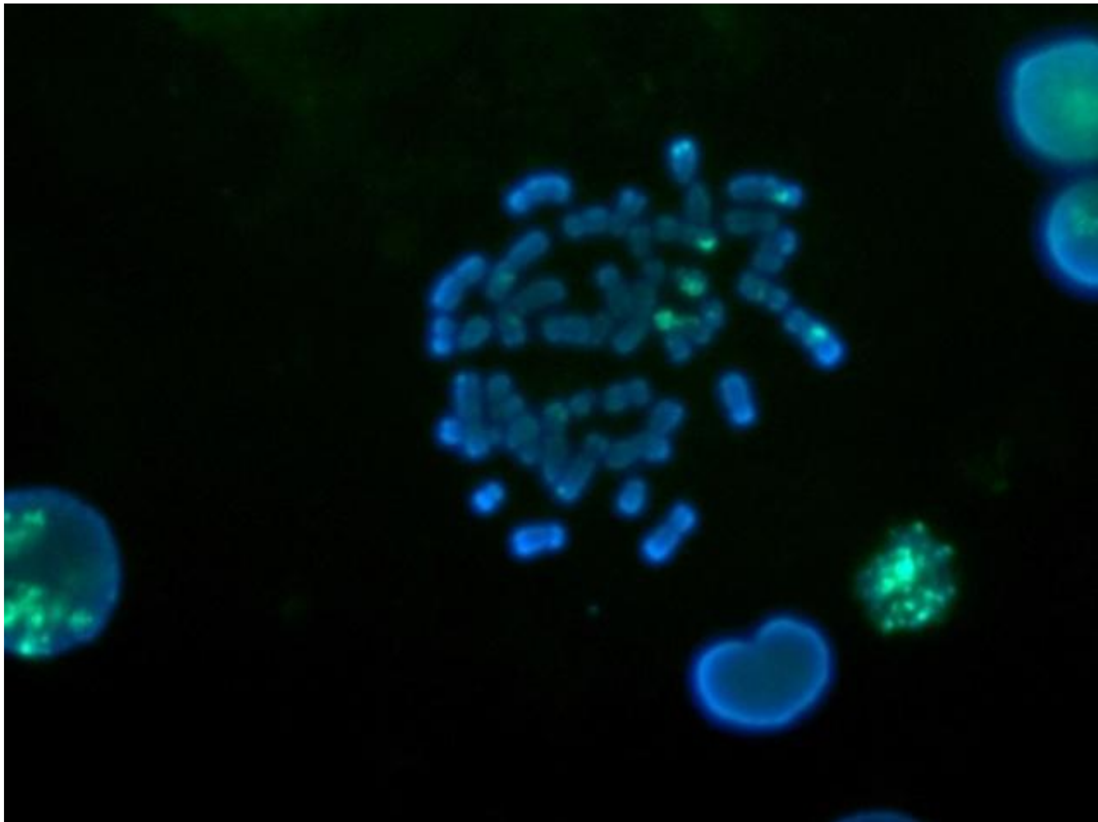


Figure 3.10 Tunel assay of lymphocytes cells which are treated DE-1 observed fluorescence microscope. Images are at 100X.





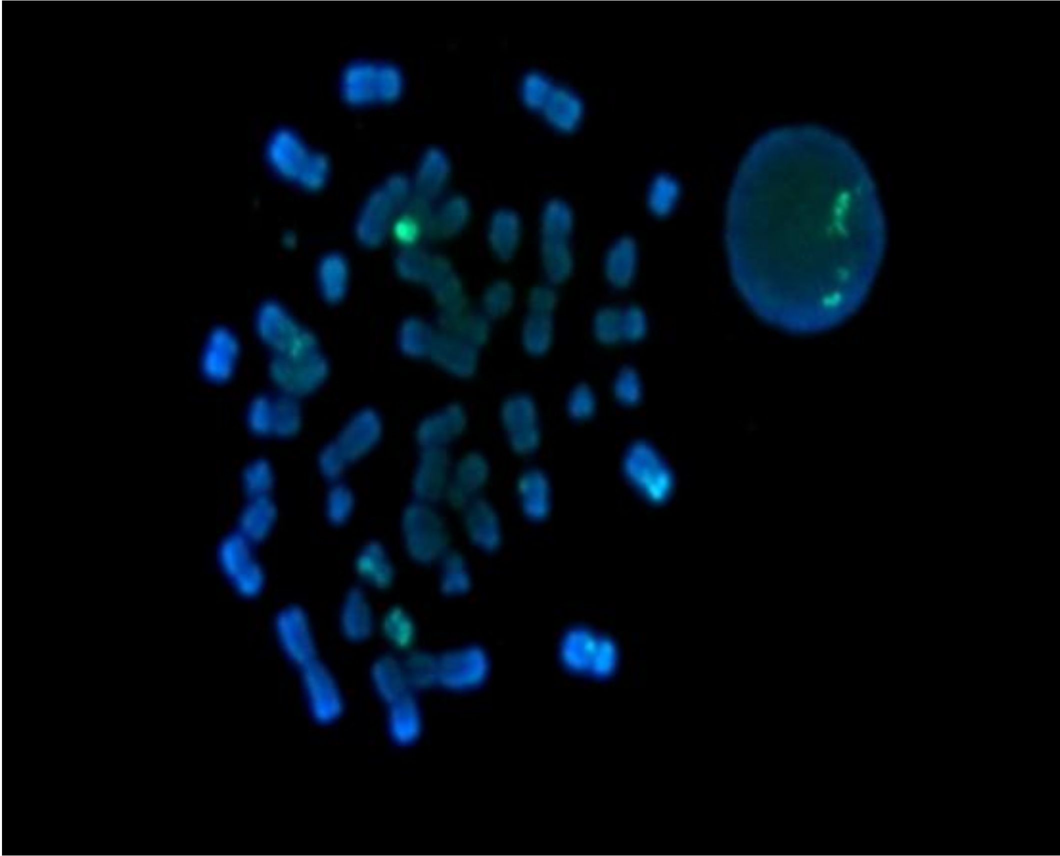
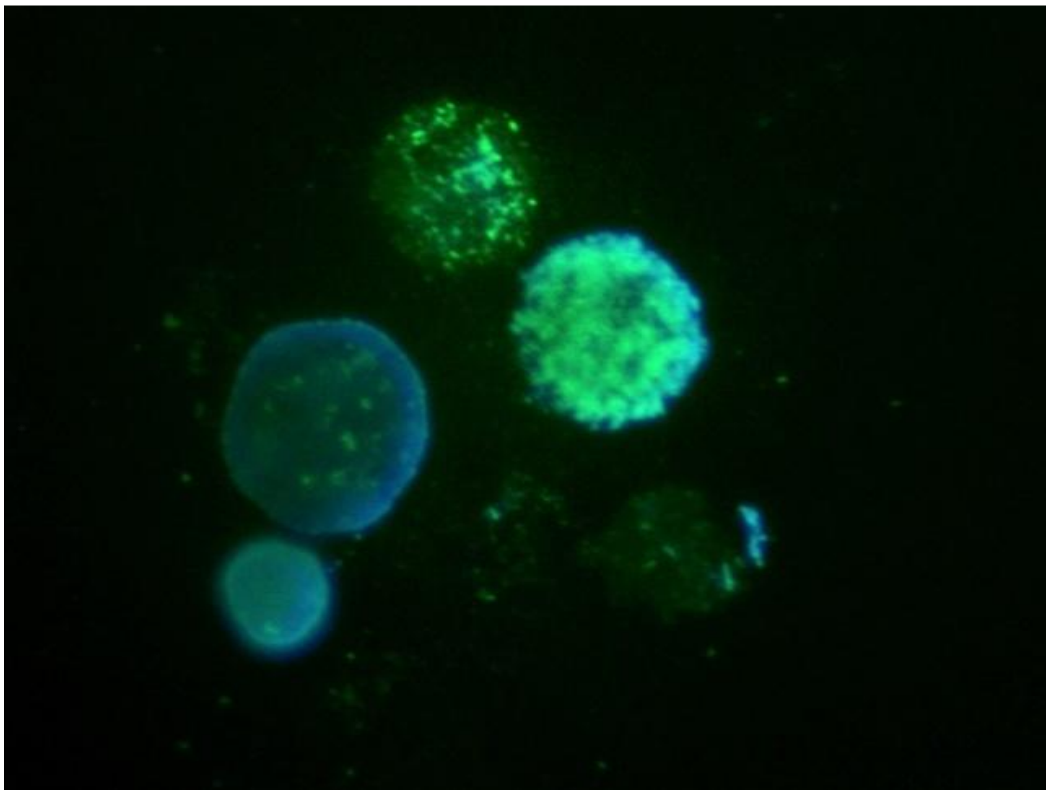


Figure 3.11: Tunel assay of lymphocytes cells which are treated DE-2 observed fluorescence microscope. Images are at 100X.



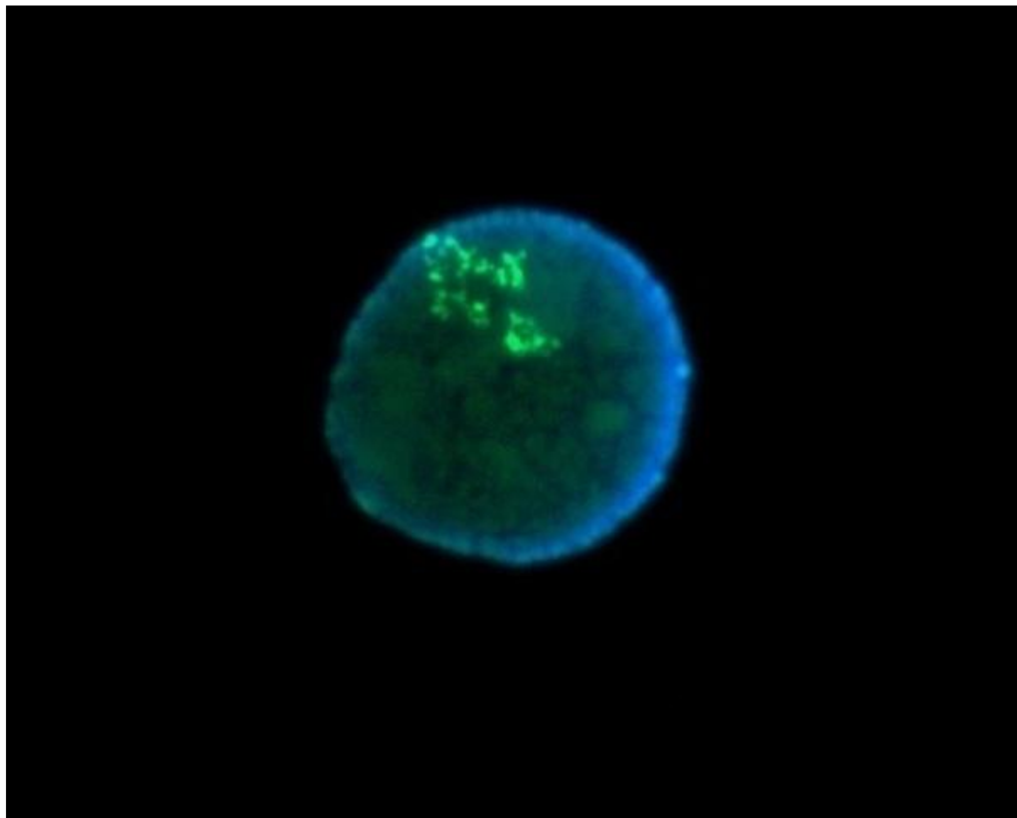
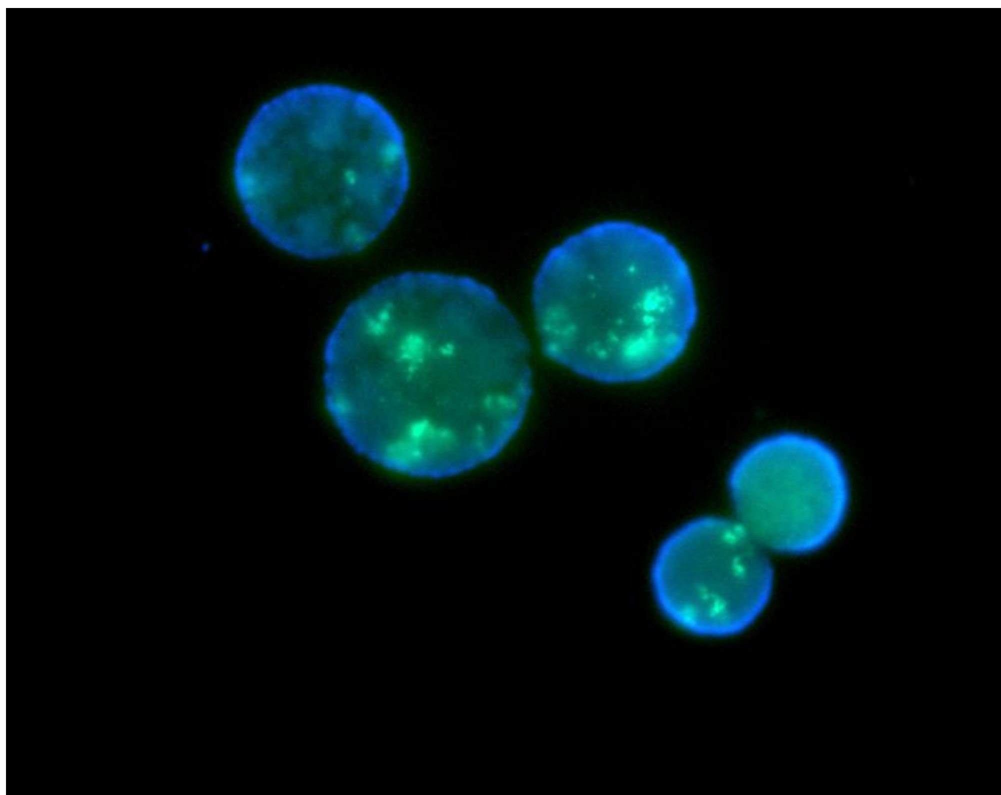


Figure 3.12: Tunel assay of lymphocytes cells which are treated DE-3 observed fluorescence microscope. Images are at 100X.



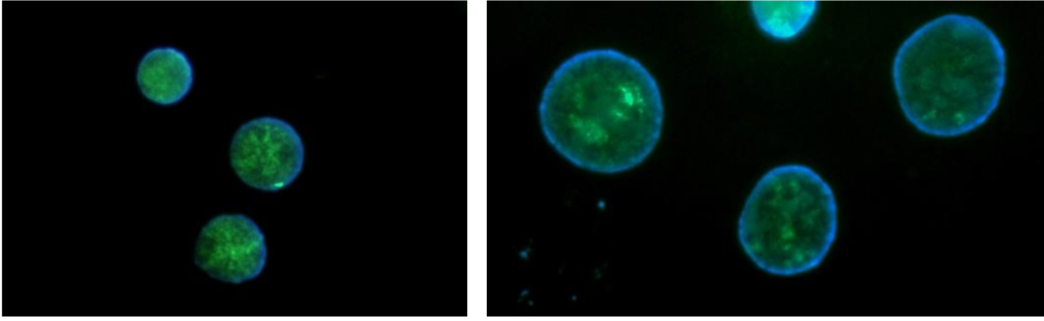


Figure 3.13: Tunel assay of lymphocytes cells which are treated DE-4 observed fluorescence microscope. Images are at 100X.

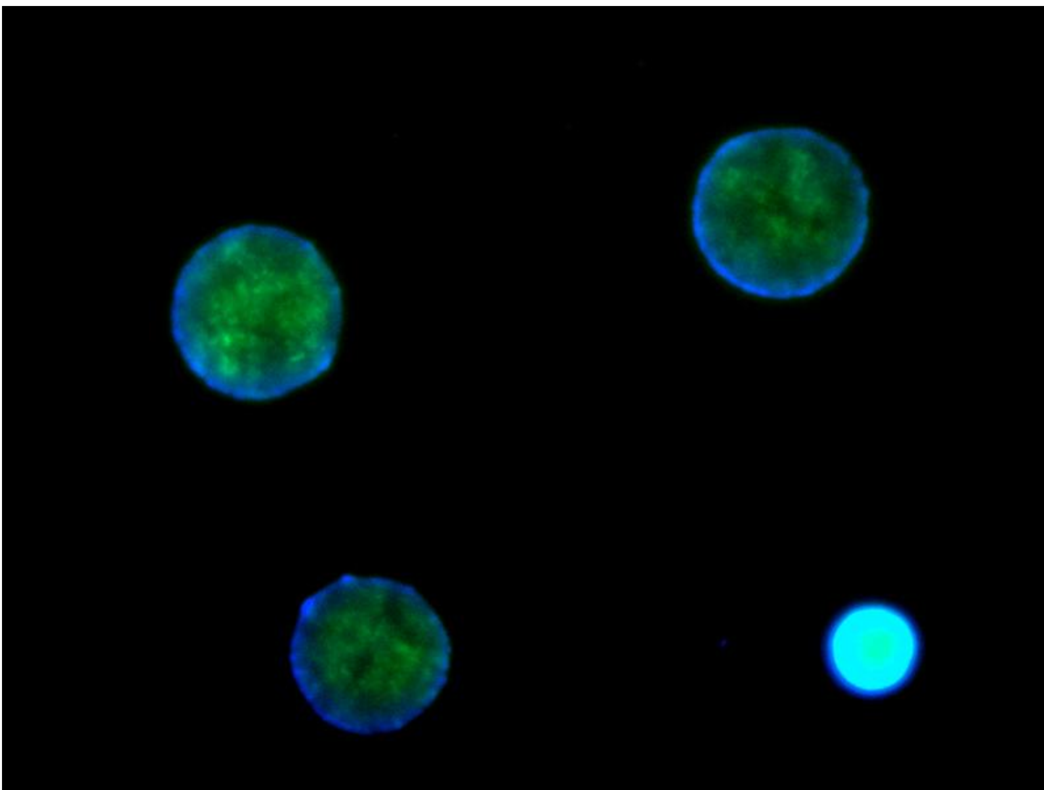


Figure 3.14: Tunel assay of lymphocytes cells which are treated DE-5 observed fluorescence microscope. Images are at 100X.

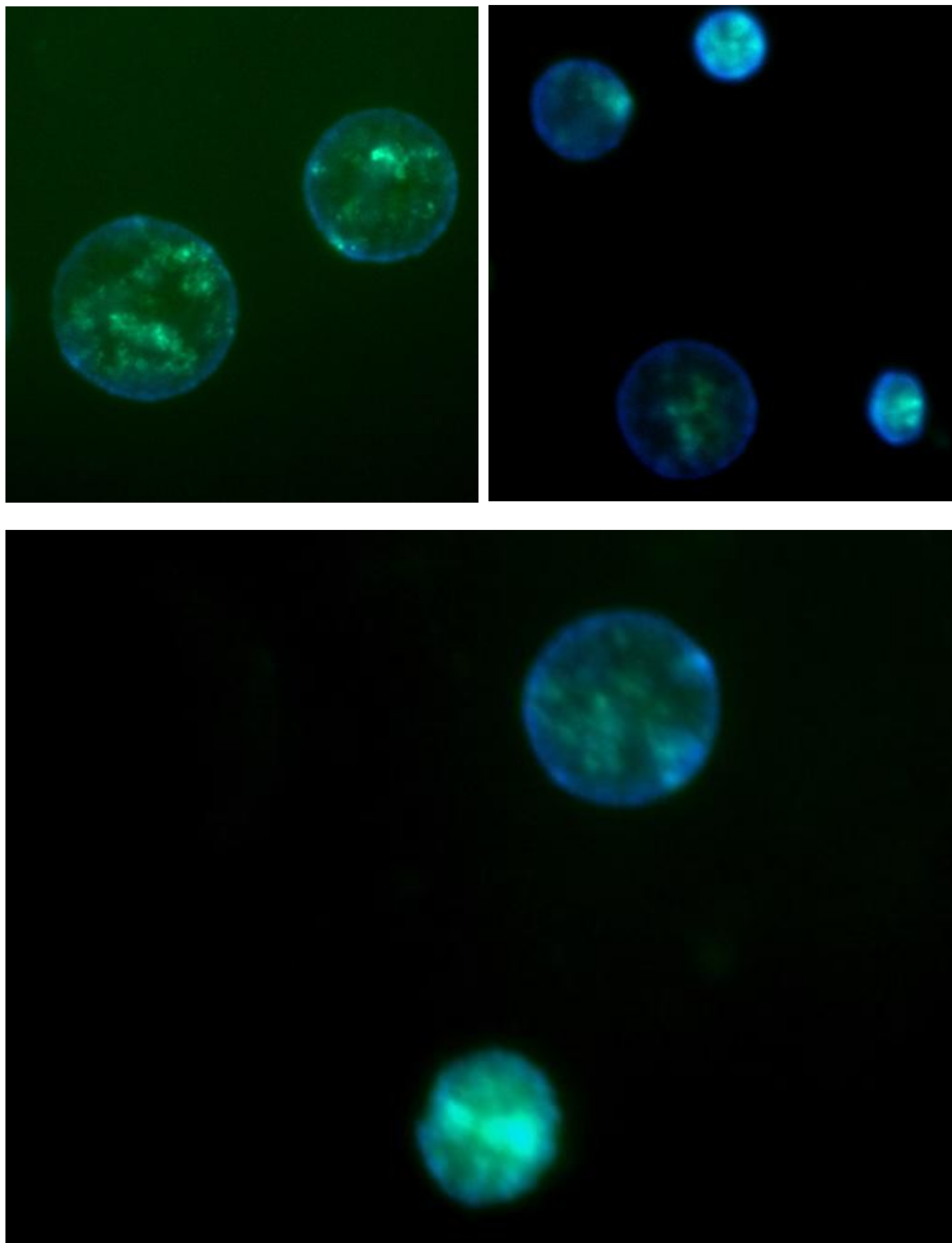


Figure 3.15: Tunel assay of lymphocytes cells which are treated DE-6 observed fluorescence microscope. Images are at 100X.

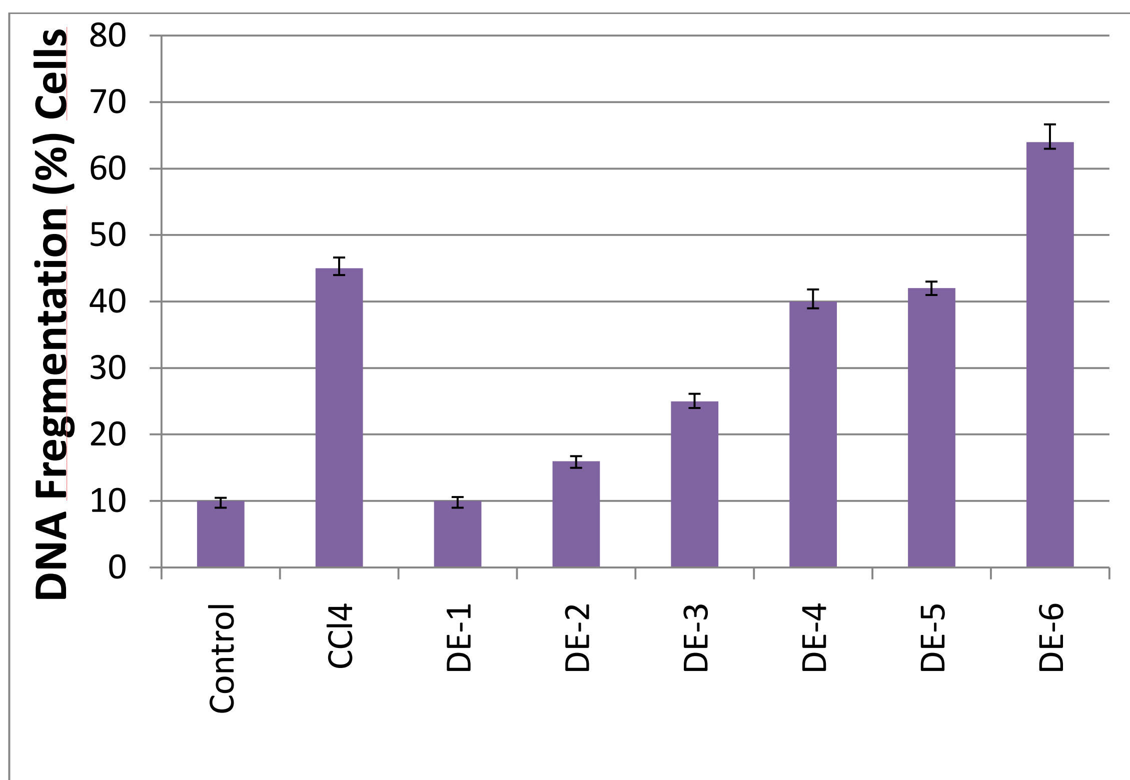


Figure 3.16: % DNA Fragmentation observed in different DE treated lymphocytes

Table 3.3 DNA Fragmentation values and **%** DNA Fragmentation in the cultures of human peripheral lymphocytes Analysis

GROUPS	Values	DNA Fragmentation% Cells
Control	10±0.49[b,e,f,g,h]	8.92
CCl_4	45±1.64[a,c,d,h]	69.2
DE-1	10±0.61[b,f,g,h]	13.15
DE-2	16±0.76[b,f,g,h]	25
DE-3	25±1.14[h]	26.2
DE-4	40±1.84[c,d,h]	39.25
DE-5	42±1.02[a,c,d,h]	50
DE-6	64±2.67[a,b,c,d,e,f,g,h]	59.26

[a]$p \le 0.05$ compared with control group; [b]$p \le 0.05$ compared with CCl_4; [c]$p \le 0.05$ compared with DE-1 group; [d]$p \le 0.05$ compared with DE-2 group; [e]$p \le 0.05$ compared with DE-3 group; [f]$p \le 0.05$ compared with DE-4; [g]$p \le 0.05$ compared with DE-5 group; [h]$p \le 0.05$ compared with DE-6 group; one way ANOVA test.

It is well known that increased DNA Fragmentation can origin by dead, dying cells or single and double-stranded breaks, incomplete repair sites that are potentially able to induce chromosomal damage and number.

The genotoxic effect of DEs on the human lymphocyte cells were given in Table 3.3 and Figure 3.12. According to our tunel assay results, there was a significant increasing in the DNA fragmentation. Increasing in the DNA fragmentation was observed after treatment with DEs significantly ($p<0.001$ or 0.05).

3.7 SCE Analysis

SCE frequency of the experimental groups pictures are given in below. SCE frequency in CCl_4-treated group was higher than the frequency in the control group. There was a significant decrease in the SCE frequency in CCI_4-treated group when compared with the groups receiving CCl_4 and DE.

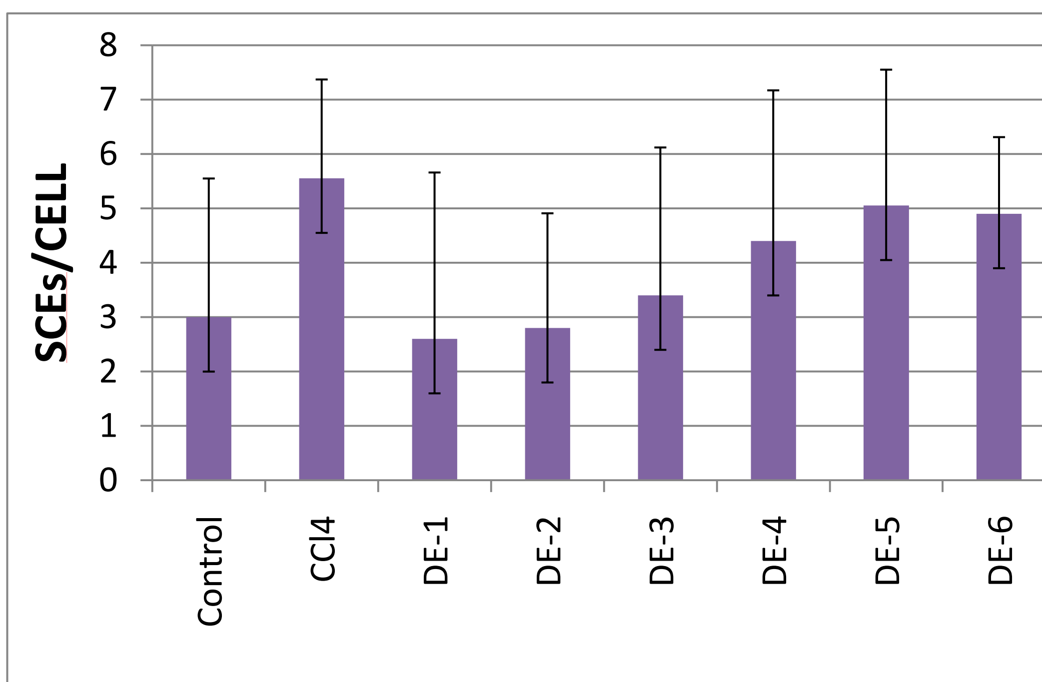


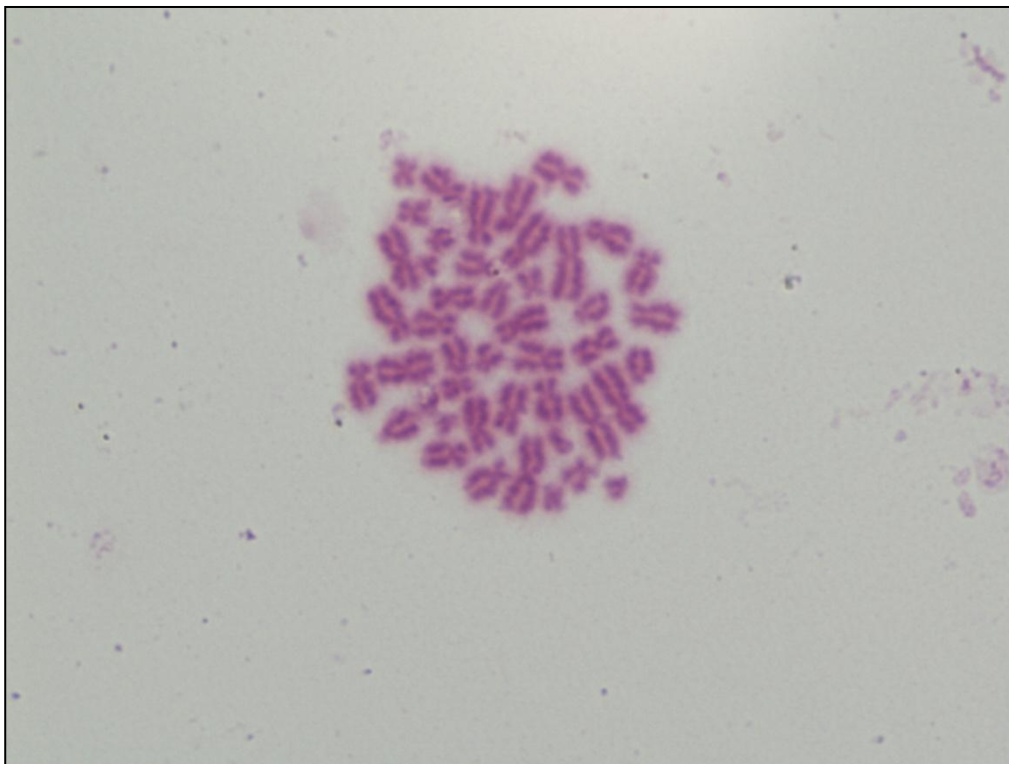
Figure 3.17 Comparison the effects on the number of sister chromatid exchanges (SCEs) different concentrations of DE.

Table 3.4 SCE groups and values are analysis in the cultures of human peripheral lymphocytes

Groups	SCE values
Control	3.00±2.55[b,f,g]
CCl_4	5.55±1.82[a,d,e]
DE-1	2.60±3.06[b,g]
DE-2	2.80±2.11[b,f,g]
DE-3	3.40±2.72[b,f,g]
DE-4	4.40±2.77[b,c,d,g]
DE-5	5.05±2.50[a,c,d,e]
DE-6	4.90±1.41[a,c,d,e]

[a]p≤0.05 compared with control group; [b]p≤0.05 compared with CCl_4; [c]p≤0.05 compared with DE-1 group; [d]p≤0.05 compared with DE-2 group; [e]p≤0.05 compared with DE-3 group; [f]p≤0.05 compared with DE-5; [g]p≤0.05 compared with DE-6.

The decrease in the SCE frequency was high in DE-5 and DE-6 concentrations. There was a significant decrease in the SCE frequency when different DE- treated groups were compared each other .Also used DE s have genotoxic effect when compared control group.



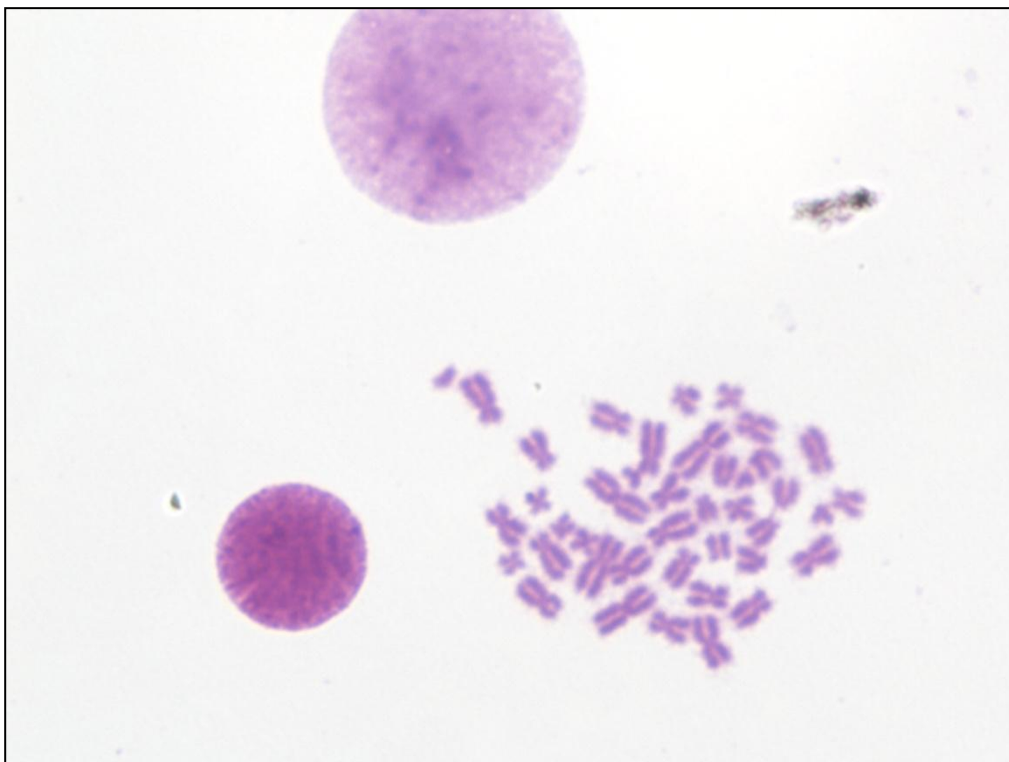
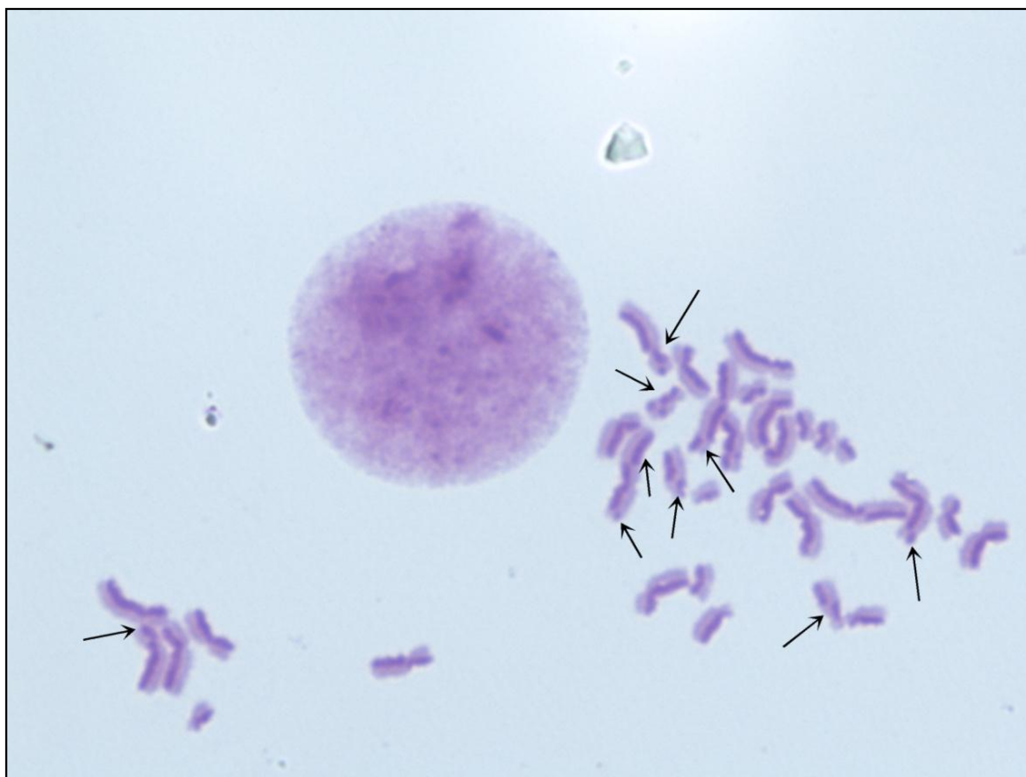


Figure 3.18: Culture 1: SCE on human peripheral lymphocyte cells, treatment with nothing Control (mag. 100X).



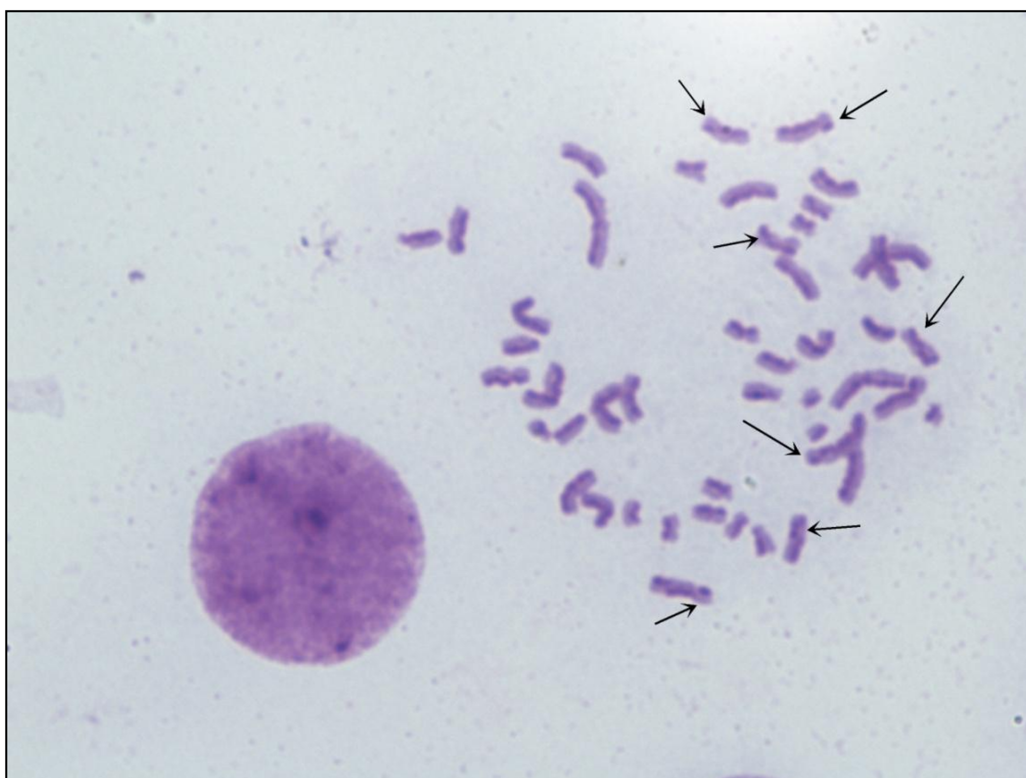
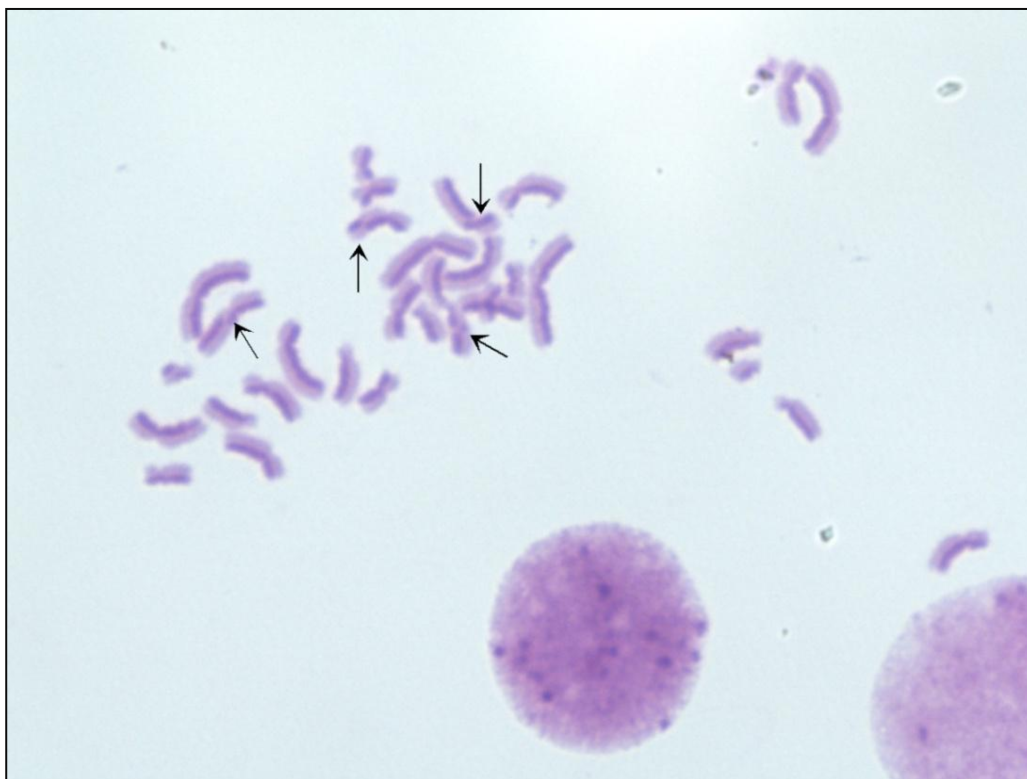


Figure 3.19: Culture 2: SCE on human peripheral lymphocyte cells, treatment with CCl_4 (mag. 100X)



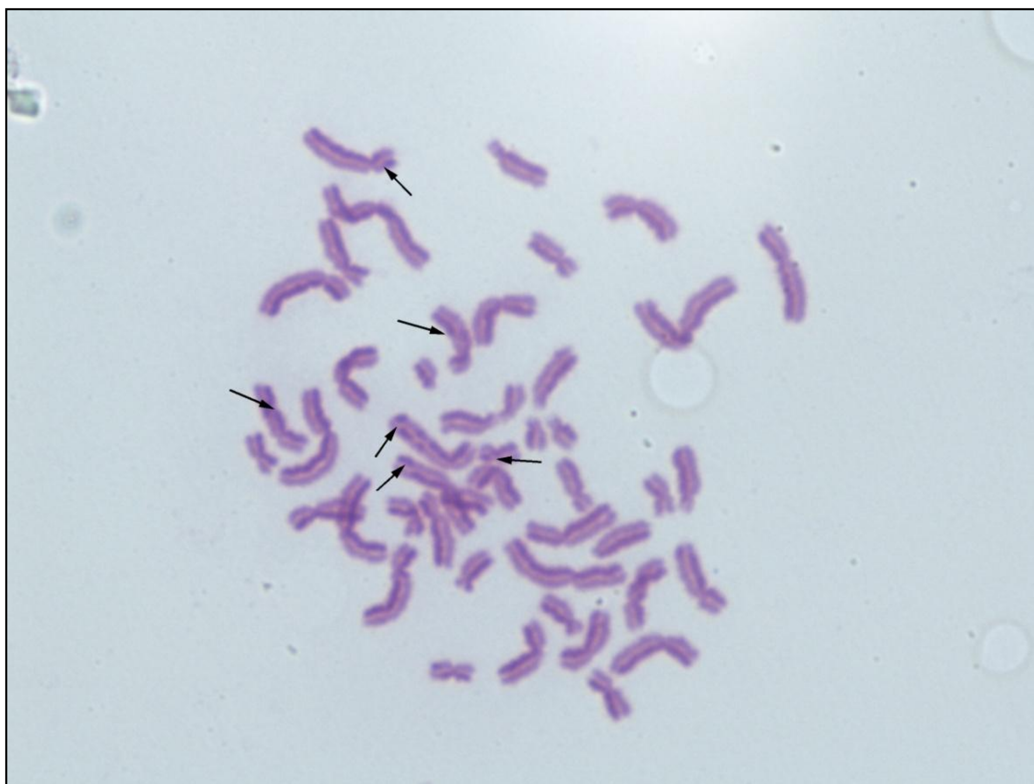
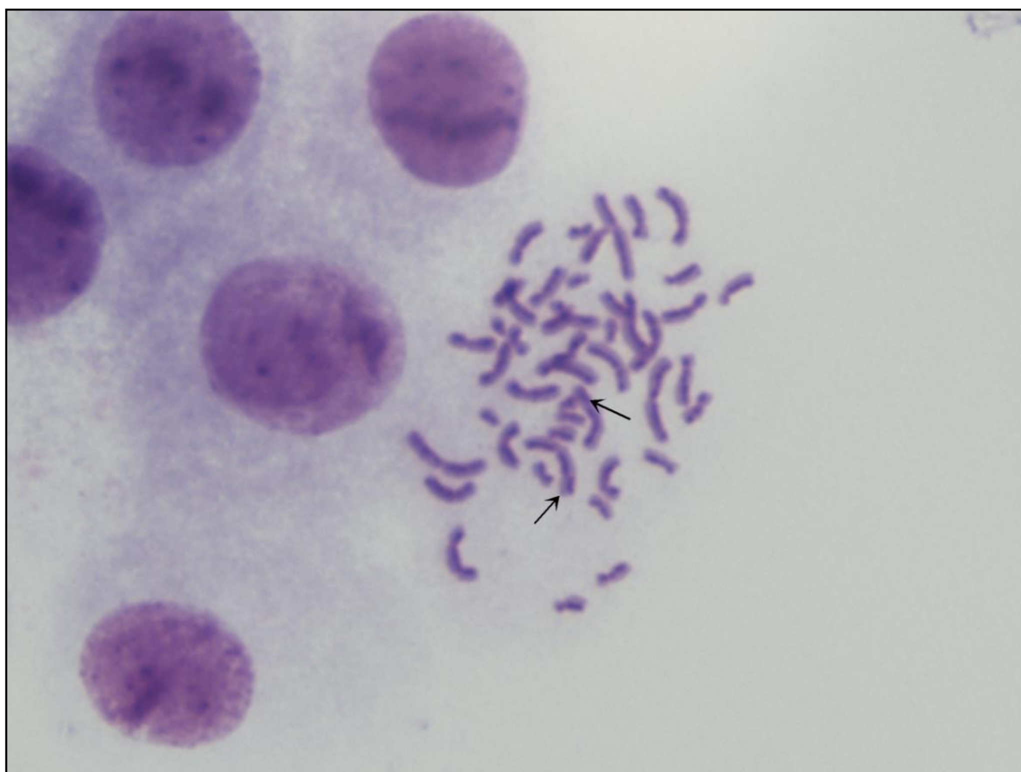


Figure 3.20: Culture 3: SCE on human peripheral lymphocyte cells, treatment with DE-1 (mag. 100X).



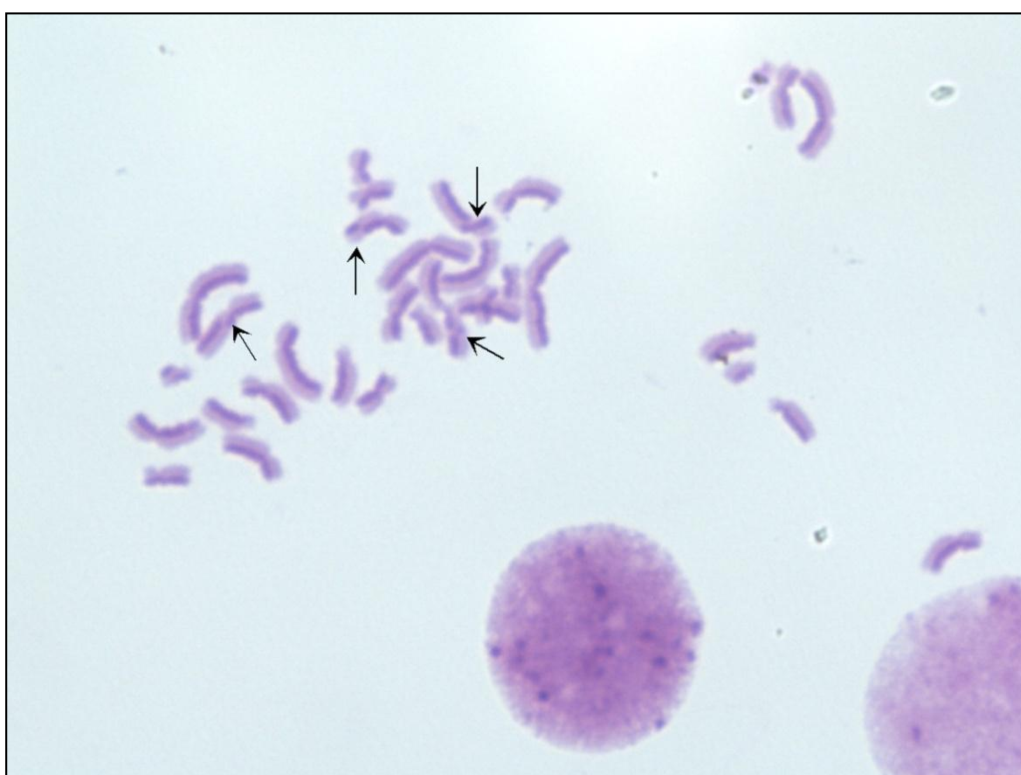
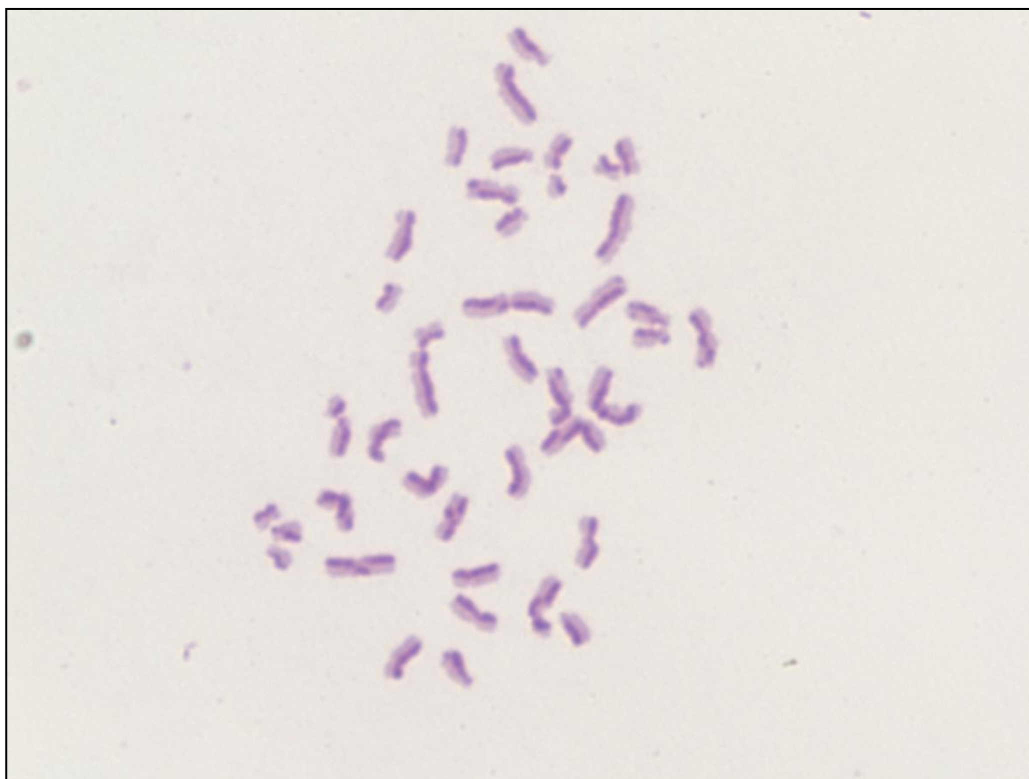


Figure 3.21: Culture 4: SCE on human peripheral lymphocyte cells, treatment with DE-2 (mag. 100X).



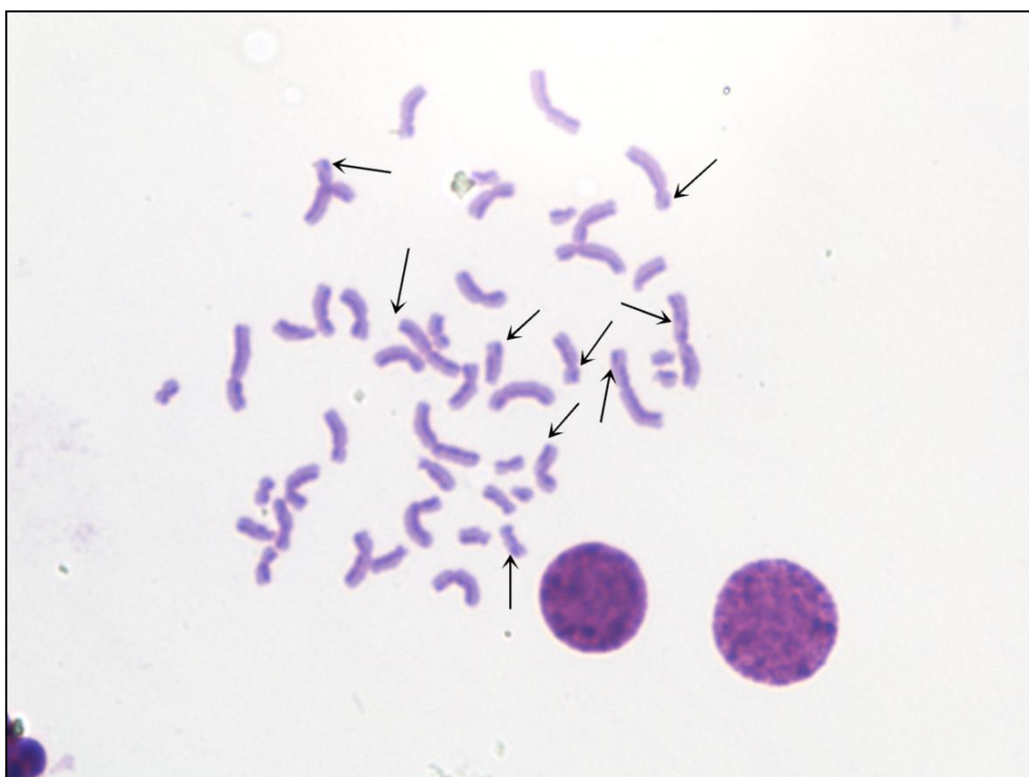
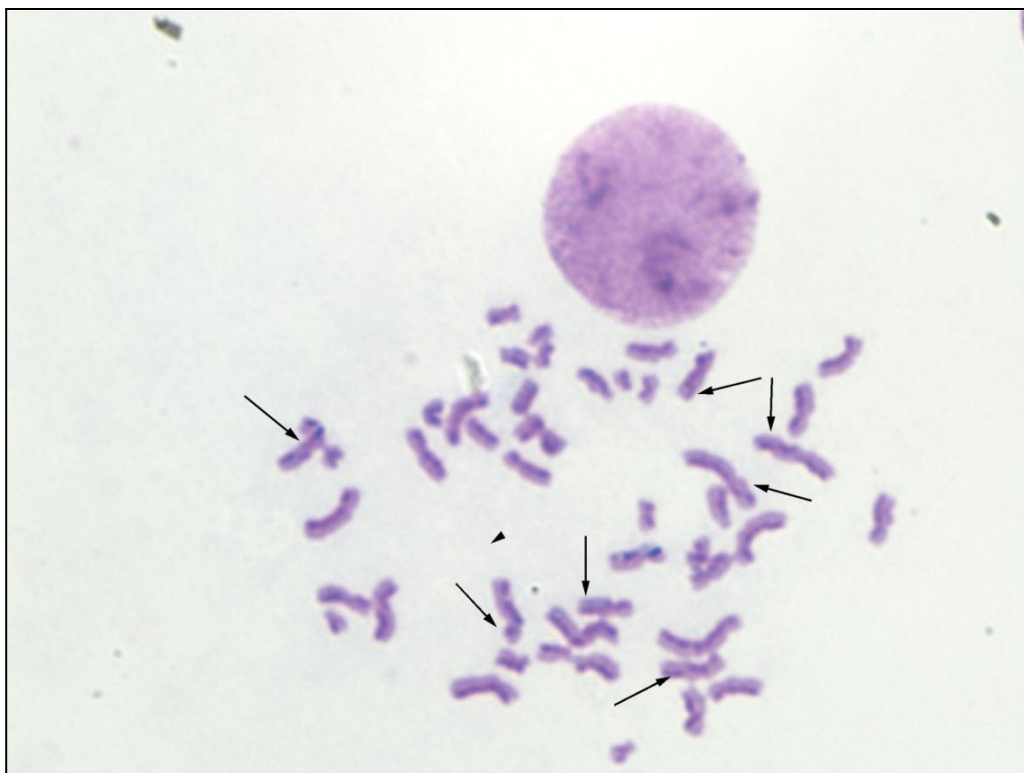


Figure 3.22: Culture 5: SCE on human peripheral lymphocyte cells, treatment with DE-3(mag. 100X).



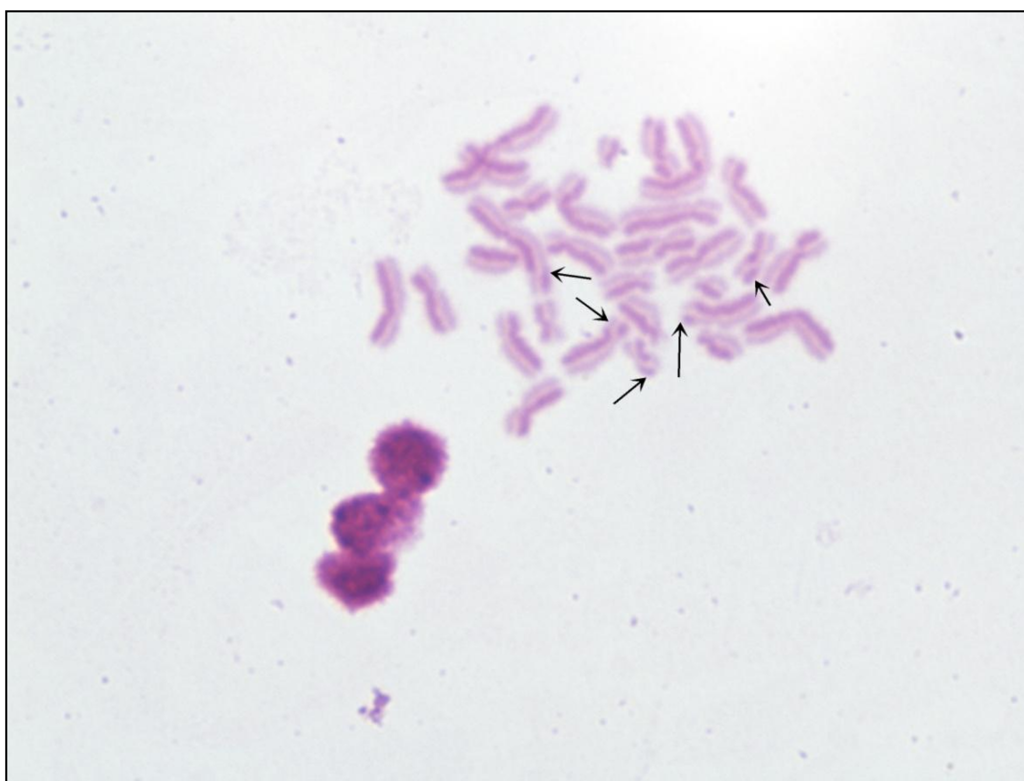
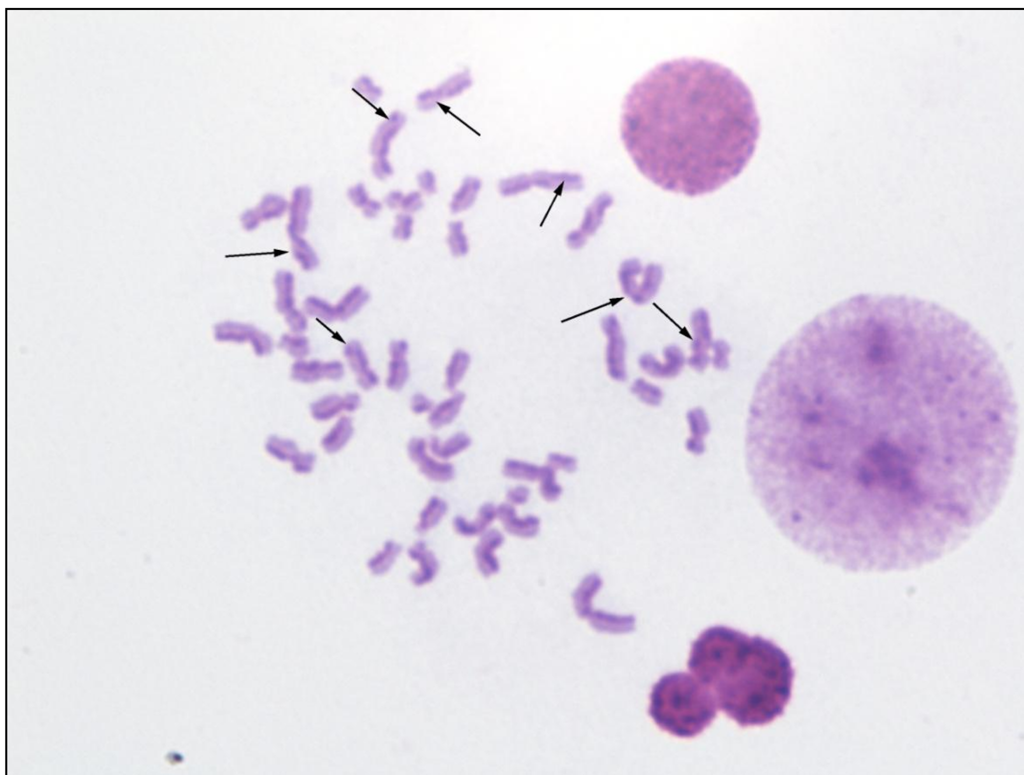


Figure 3.23: Culture 6: SCE on human peripheral lymphocyte cells, treatment with DE-4(mag. 100X).



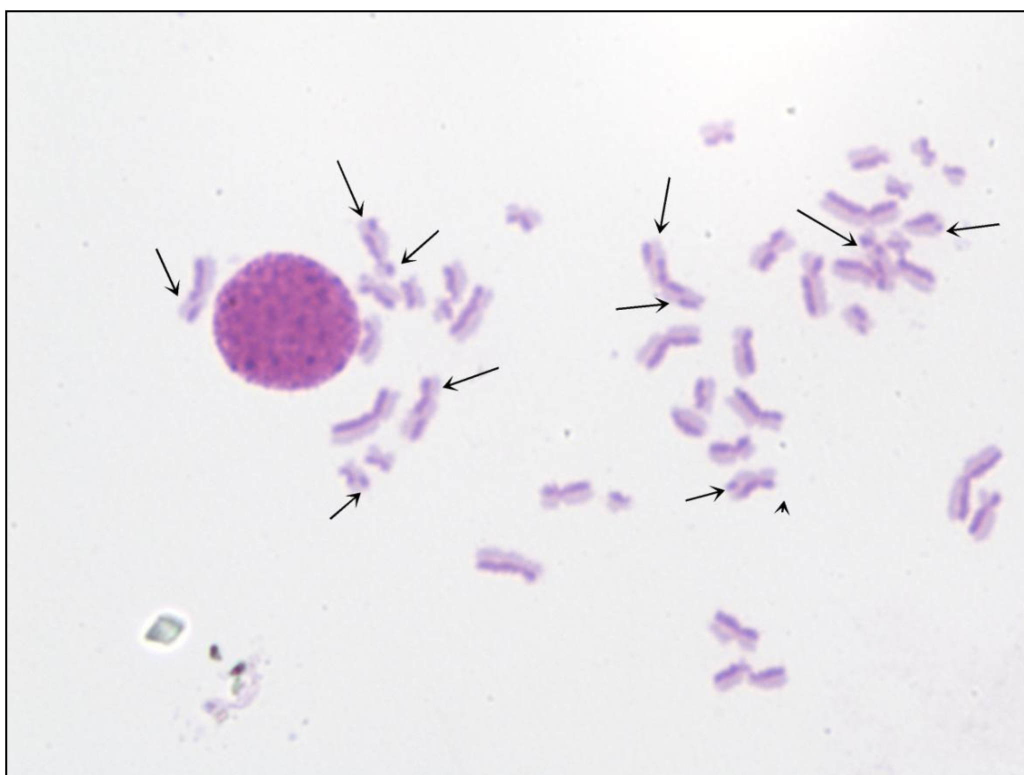


Figure 3.24: Culture 7: SCE on human peripheral lymphocyte cells, treatment with DE-5 (mag. 100X).

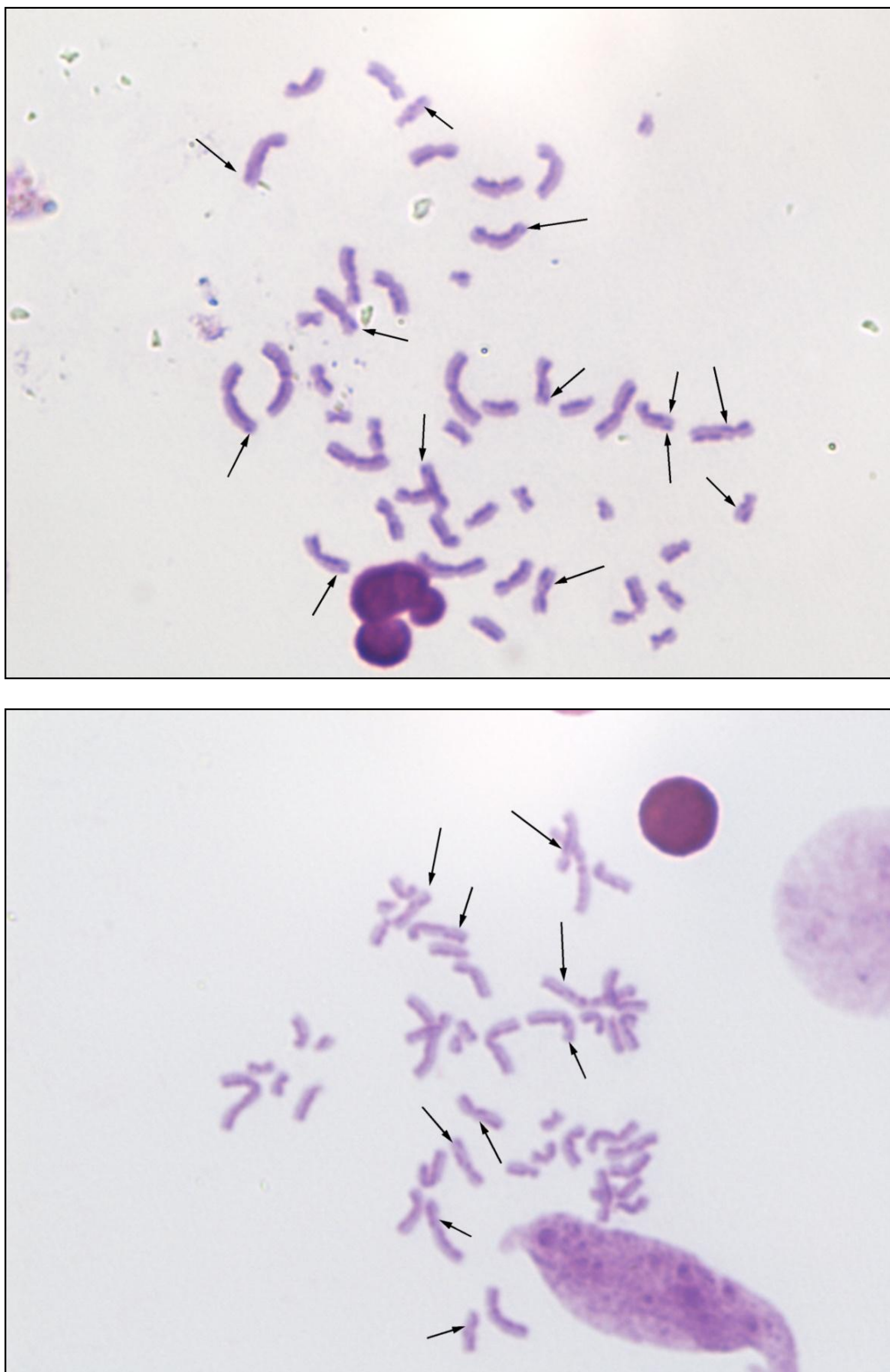


Figure 3.25: Culture 8: SCE on human peripheral lymphocyte cells, treatment with DE-6 (mag. 100X)

CHAPTER 4

DISCUSSION

The main alkolololoids of *D. stramonium,* atropine and scopolamine, which are inhibitors of muscarinic receptors caused a slight but significant increasing of the frequency of binuclear interphase cells and also of the frequency of cells in late telophase and early G_1 that had not completed cleavage. Binuclear cells are prone to inhibition of next genertaion of cells with highly aberrant chromosome numbers convenient to acetylcholine blocking agents as atropine and scopolamine (Reuterwall, Aringer et al. 1991).

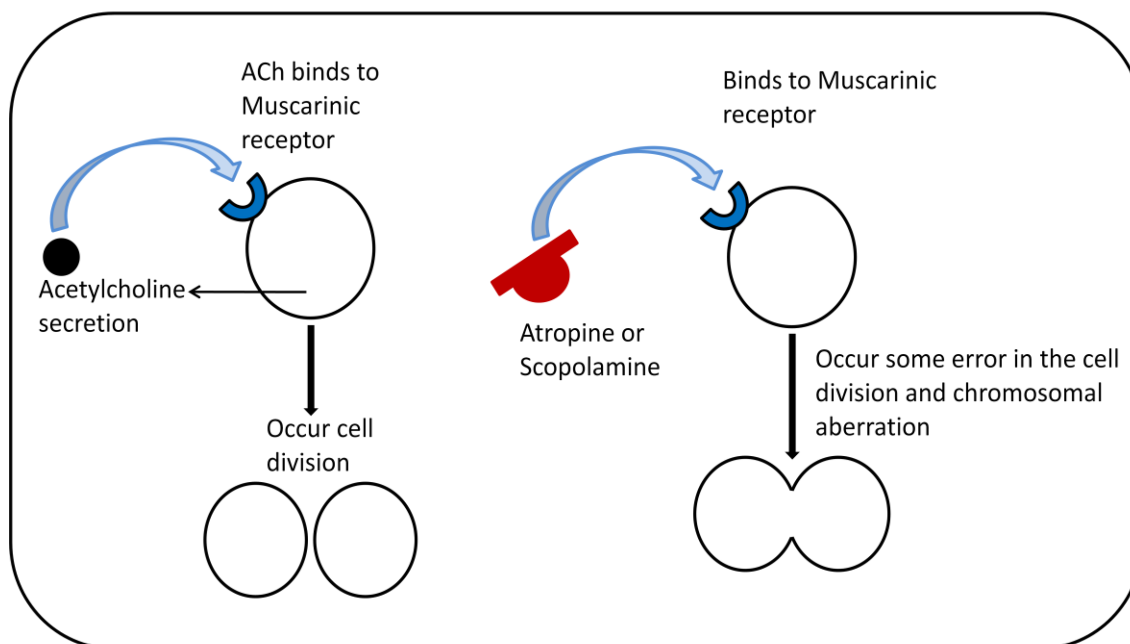


Figure 4.1: Atropine and scopolamine binding mechanism to its specific receptor

Acetylcholine (ACh), a classical neurotransmitter in both the central and peripheral nervous systems, is synthesized by choline acetyltransferase (ChAT, EC 2.3.1.6) fromacetyl coenzyme A and choline However, both muscarinic and nicotinic

ACh receptors have been identified by specific receptor-binding assay and immunecytochemical, analysis in lymphocytes isolated from thymus, lymph node, spleen, and peripheral blood (Dolezal and Tucek 1982).

Lymphocytes and accessory cells express muscarinic and nicotinic receptors (Richman and Arnason 1979; Rinner, Porta et al. 1990). And functionally respond to cholinergic stimulation, e.g. with an increasing of cytotoxic activity or specific antibody producing cells (Rinner and Schauenstein 1991). The number of cholinergic receptors on lymphocytes is increased in patients with progressive multiple sclerosis (Anlar, Karaszewski et al. 1992) in rats with adjuvant polyarthritis (Maslinski, Laskowska-Bozek et al. 1992) and decreased in Alzheimer's disease patients (Adem, Nordberg et al. 1986). Despite the presence of acetylcholine esterase activity in plasma, considerable amounts of acetylcholine are found in the plasma of humans and animals (Kawashima, Oohata et al. 1987).

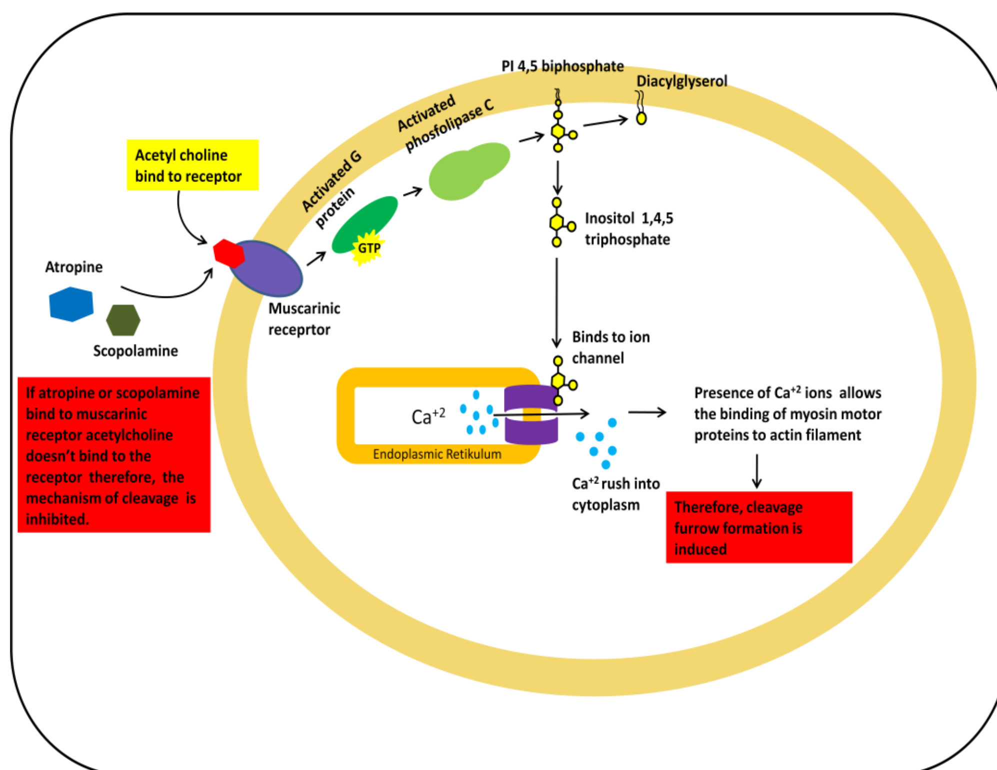


Figure 4.2: Ach, atropine and scopolamine pathway on human lymphocytes

In normal cell cycle Ach bind to M1 type muscarinic receptor. This receptor is found mediating slow excitatory postsynaptic potential (EPSP) at the ganglion in the

postganglionic nerve (Messer and I'Anson 2000) is common in exocrine glands and in the CNS (Richelson and Souder 2000).

It is predominantly found bound to G proteins of class Gg which use upregulation of phospholipase C and therefore inositol triphosphate and intracellular calcium as a signalling pathway. Activation can induce calcium-release from intracellular strores, presence of Ca^{+2} ions allows the binding of myosin motor protein to actin filament. Therefore, cleavage furrow formation is induced. If Atropine or scopolamine binds to musacarinic receptore instead of Ach, the mechanism of cleavage is inhibited (Rinner and Schauenstein 1991).

Datura contains hyoscyamine and the tropane alkaloid atropine. Both chemicals selectively block certain acetlycholine receptors; atropines are anti-inflammatory, and hyoscine prevents secretions and is a component of commercial pre-anaesthetic drugs and Omnopon-Scopolamine. Small doses of these chemicals, present in most *Datura* species, may produce a sedative effect and cause submissive behavior and memory loss (Fernald and Kinsey 1958; Mann 1992).

Muscarinic acetylcholine receptors, which are ion-channel-coupled receptors on skeletal muscle and nerve cells that can be activated by the binding of nicotine, as well as by acetylcholine (Alberts, Johnson et al. 2008).

Many effects of Ca^{+2}, however, are more indirect and are mediated by protein phosphorylations catalyzed by a family of serine/threonine protein kinases called Ca^{+2}/calmodulin-dependent kinase (CaM-kinases). One of the best studied CaM-kinase is CaM-kinase II, which is found in most animal cells especially enriched in the nervous system. CaM-kinase II has special properties. It can function as a moleculer memory device, switching to an activate state when exposed to Ca^{+2}/calmodulin and then remaining activate even after the Ca^{+2} signal has decayed. This is because the kinase phosphorylates itself. CaM-kinase II activation can serve as a memory trace of a prior Ca^{+2} pulse, and it seems to have a role in some types of memory and learning in the vertebrate nervous system (Alberts, Johnson et al. 2008).

There is no literature about the genotoxic and cytotoxic effects of *D. stramonium* seeds extract on cultured human lymphocytes. In our work, we have demonstrated that *D. stramonium* methanolic seed extract and its components atropine and scopolamine have cytotoxic and genotoxic effects on human cultured lymphocytes by confirming with four different methods: LDH, WST-1, TUNEL and SCE assay.

Based on our results, it can be concluded as DE quantities at concentrations are 50000µg/ml and 125000µg/ml because of genotoxic effect on human cultured lymphocytes significant decrease occurs in the frequency when we compare experiments with each others.

Tunnel assay confirmed the SCE result on chromosome level, shows the green fluorescence signals in the cells due to the increasing *D. stramonium* concentrations. Chromosomal damage and abnormal chromosome number are result of DNA fragmentation.

WST-1 results show that cell proliferation was inhibited by DE at concentrations 50000 µM and 125000 µM. And lastly LDH experiments show that in all different DE concentrations, there were not any toxic effects.

CHAPTER 5

CONCLUSIONS

In this thesis, we have demonstrated that different DE concentrations have not significant cytotoxic effect on human cultured lymphocytes. On the other hand all DE concentrations inhibited cell proliferation on human cultured lymphocytes. On the genotoxic effects we have showed that all DE concentrations especially at concentrations 50000µg/ml and 125000µg/ml cause DNA fragmentation on cultured human lymphocytes by confirming SCE assay result. Based on our results, it can be concludede that different DE concentartions have not cytotoxicty effect but its have genotoxic effect on human cultured lymphocytes by confirming our results with WST-1,LDH,Tunel and SCE assay.

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