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Some Genetic Changes in Lymphocytes and Bone Marrow Cells Associated with Yasmin Contraceptive Pills, Cafergot and Wild Carrot Seeds Oil in Women and Mice

A Dissertation

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صدق الله العظيم

الاهداء

الى وطني غارس العلم والمعرفة الانسانية في عقلي و قلبي

الى العلماء والمبدعين العراقيين الماضيين قدماً نحو قمم الحضارة

الانسانية المتجددة رغم الصعاب

الى الذين جاءوا بي الى هذه الدنيا وعلموني (ان العلم هو السبيل الى

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الى اساتذتي الاجلاء الذين ما انفكوا يتابعون خطواتي بحرص

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الى اخوتي ثروتي الاخرى في الحياةِ علياء,علي , حيدر , محمد, لينة, ميس

الى رفقةِ دربٍ لن انسى لهم ما قدموه لي ما حييتْ

الى كل هؤلاء اقدمُ جهدي الذي يشكلُ مستقبلَ حياتي

ورود

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Supervisor Certification

We, certify that this dissertation entitled **"Some Genetic Changes in Lymphocytes and Bone Marrow Cells Associated with Yasmin Contraceptive Pills, Cafergot and Wild Carrot Seeds Oil in Women and Mice "** was prepared by **Worood Kamil Shalash** under our supervision at the College of Science, Al-Nahrain University as a partial fulfillment of the requirements for the degree of Philosophiae Doctor in Science, Biotechnology.

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Summary

This study was undertaken to figure out the effects of contraceptives pills "Yasmin", fungal alkaloids "Cafergot" and wild carrot seed oil on apoptosis, DNA damage (using comet assay), cytogenetic analysis (using micronucleus assay) in blood lymphocytes of mice as well as on pregnancy rate of female mice. The oral effects of contraceptive pills (Yasmin) on apoptosis, DNA damage, cytogenetic analysis and aromatase activity of women blood lymphocytes were also investigated. Thirty five mice were divided into seven groups in antifertility experiment (3 females and 2 males per each group). The first group was regarded as a negative control group treated with normal saline, whereas groups II and III were treated with 30 and 60 µg/kg of contraceptive pills (COCPs) respectively. Groups IV and V were treated with 10 and 20 µg/kg of Cafergot pills respectively, and groups VI and VII were treated with 0.05 and 0.1 ml of wild carrot seed oil respectively. The pregnancy rate was higher ($P \leq 0.01$) in control and group IV as compared with the other groups. In apoptosis experiment, fifteen mice were used and divided into five equal groups. The first group was regarded as a control, while the second positive group was treated with 50 µg/kg of methotrexate (MTX). The remaining three groups were orally treated with 60, 20 µg/kg and 0.1 ml of COCPs, Cafergot and wild carrot seed oil respectively. The treatments were given for 5 successive days. Lower ($P \leq 0.05$) apoptotic percentage was observed in wild carrot seed oil that equal to the negative control group, whereas higher ($P \leq 0.05$) percentage was noticed in the MTX group, indicating that COCPs group is the most inducing contraceptive agents. Higher ($P \leq 0.05$) viable cells were noticed in Cafergot (99.54%) and wild carrot seed oil (99.6%) being similar with

negative control (99.66%) and greater than COCPs (97.83%) and MTX group. In comet assay, the same groups were used as mentioned in apoptotic experiment. Significant (P≤0.05) differences were observed among groups in tail length, being taller in MTX group (17.5%), followed by COCPs (12.18%) and lower in wild carrot seed oil group (1%). The percentage of DNA in the tails were 15.08% in COCPs while in Cafergot and wild carrot seed oil were 6.18% and 0.22% respectively. In control the percentage of DNA in the tails were 1.09% and 19.14% in MTX group. The tails moments for the exposed group with contraceptive pills were 25.04%, while in Cafergot and wild carrot seed oil were 5.09% and 0.36% consequently. In micronuclei investigating experiment, the wild carrot seeds oil and Cafergot recorded less (P≤0.05) percentage as compared with other groups being 2.75% and 3.5% respectively. In contrast, MTX recorded greater (P≤0.05) percentage of micronuclei namely 14.5% followed by COCPs group 10.75%. The percentage of apoptosis in women used Yasmin contraceptive pills were 7.08 and 12.2% for 2-5 and 6-8 years respectively as compared with the negative control (0.18%). Higher (P≤0. 05) viable cell percentage was noticed in negative control (99.54%) as compared contraceptive pill groups either for 2-5 years (92.18%) and 6-8 years (86.84%). The average tail lengths of comet in women used Yasmin pills were 19.13 and 25.08% for women used Yasmin pills for 2-5 and 6-8 years respectively and different (P≤0.05) from those in control group (0.8%). Furthermore, the percentage of DNA in comet tail were 20.12 and 28.63% for of women used Yasmin pills for 2-5 and 6-8 years respectively, being different (P≤0.05) from those in control group (1.6%). The tail moments of comet were 23.63 and 31.81% in women used Yasmin pills for 2-5 and 6-8 years respectively, being different (P≤0.05) from those in control women (0.01%). The average

numbers of micronuclei were 0.016 and 0.021% for women used Yasmin pills from 2-5 and 6-8 years respectively, seems higher ($P \leq 0.05$) than those in control group (0.004%). The negative control group of women exhibited the highest levels (9.303%) of aromatase activity in comparison with Yasmin pills groups used for 2-5 (6.113%) and 6-8 (5.248%) years. In conclusion, Yasmin pills was the most inducing drugs for apoptosis and DNA damage in comparison with Cafergot and carrot seed oil.

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List of Abbreviations

7-AAD	7-Amino-Actinomycin
BCP	Birth Control Pill
Bw	Body Weight
CBMA	Cytokinesis Block Micronucleus Assay
DMSO	Dimethylsulfoxide
DRSP	Drospirenone
DSG	Desogestrel
DVT	Deep Vein Thrombosis
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
FADD	Fas-Associated Protein With Death Domain
FITC	Fluorescein Isothiocyanate
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
hCG	Human Chorionic Gonadotropin
HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
LH	Luteinizing Hormone
LMA	Low Melting Agarose
LNG	Levonorgestrel
LTED cells	Long-Term Estrogen-Deprived Medium
MN	Micronucleus
MTX	Methotrexate
NaOH	Sodium Hydroxide
NMA	Normal Melting Agarose
OCPs	Oral Contraceptive Pills
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
PE	Pulmonary Embolism

PHA	Phytohaemagglutinin
PI	Propidium Iodide
PS	Phosphatidylserine
RBC	Red Blood Cell
SCGE	Single Cell Gel Electrophoresis
SPSS	Statistical Package Social Sciences
TBE	Tris-Borate EDTA
TMB	3,3',5,5'-Tetramethylbenzidine or
V-FITC	Fluorescein Isothiocyanate

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Chapter One

Introduction

1.1 Introduction

Contraception is prevention of an egg fertilization by a sperm (conception) or attachment of the fertilized egg to the lining of the uterus (Daniel and Mishell, 2011). Birth control is well documented in Mesopotamia and ancient Egypt. The Egyptian Ebers Papyrus from 1550 BCE and the Kahun Papyrus from 1850 BCE have within them some of the earliest documented descriptions of birth control by using of honey and acacia leaves, while in medieval Europe any effort to halt pregnancy was deemed immoral by the Catholic Church (Amy, 2010). Although birth control methods have been used since ancient times, but effective and safe methods only became available in the 20th century, while some cultures deliberately limit access to birth control because they consider it to be morally or politically undesirable (Hanson and Burke, 2010).

Birth control use in developing countries has decreased the number of maternal deaths by 40% and could prevent 70% if the full demand for birth control were met. Birth control also can improve adult women's delivery outcomes and the survival of their children by lengthening the time between pregnancies (Ahmed *et al.*, 2012). In the developing world women's earnings, assets, weight, and their children's schooling and health all improve with greater access to birth control which increases economic growth because of fewer dependent children, more women participating in the workforce, and less consumption of scarce resources (Canning and Schultz, 2012).

Gregory Pincus and John Rock with help from the Planned Parenthood Federation of America developed the first birth control pills (BCPs) in the 1950s, such as mestranol /norethynodrel, which became publicly available in the 1960s (Dudley, 2010). There are two types of oral birth control, the combined oral contraceptive pill (COCP) and the

progestogen-only pill which are available in a number of different forms, including oral pills, implants under the skin, injections, patches, and a ring. BCPs are currently available only for women (Christine, 2009), which work by inhibiting ovulation and fertilization (Nelson and Cwiak, 2011).

Combined hormonal contraceptives are associated with a slightly increased risk of venous and arterial blood clots (Brito *et al*., 2011), venous clots on average increase from 2.8 to 9.8 per 10,000 women years, and due to this risk, they are not recommend in women over 35 years of age who continue to smoke (Kurver *et al*., 2012), while progestin-only pills are not associated with an increased risk of blood clots and may be used by women with previous blood clots in their veins. In those with a history of arterial blood clots, non-hormonal birth control or a progestin-only method other than the injectable version should be used (Brito *et al*., 2011). Combined hormonal contraceptive effect on sexual desire is varied, with increase or decrease in some, but with no effect in most (Burrows *et al*., 2012). They reduce the risk of ovarian cancer and endometrial cancer (Havrilesky *et al.*, 2013), menstrual bleeding and painful menstruation cramps (WHO, 2011). Progestin-only pills may also improve menstrual symptoms and can be used by breastfeeding women as they do not affect milk production (Burke, 2011).

The Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Plants as an abortifacient and as contraceptive were well known to the ancient physicians of India. Various medicinal plant extracts have been tested for their antifertility activity both in male and female animal models activity and the active agents. Numerous herbs have been used historically to reduce fertility, and modern scientific research has confirmed anti-fertility effects in at least some of the herbs

tested. Herbal contraception may never reach the level of contraceptive protection of the pill, but it offers alternatives for women who have difficulty with modern contraceptive options or who just want to try a different way but with any method of contraception, there is some risk of pregnancy. Most modern forms of birth control are 70% to 99% effective depending on the method chosen. For women who can't use modern forms of contraception, herbs can offer alternatives, and reducing fertility would be better than no birth control. Some herbal contraceptives have a cumulative effect in the body so they need to be taken regularly to maintain the contraceptive effect (Umadevi *et al*., 2013). Medicinal plants in India have been screened for contraceptive potential and anti-fertility effects, since the country has always been concerned about the population explosion (Verpoorte, 2009).

Wild carrot (Queen Anne's lace) has long been used because of its contraceptive properties. The seeds made into a tea, have been used for centuries as a contraceptive. Wild carrot seed is also an early abortifacient, historically, sometimes used as a natural "morning after" contraceptive tea. Queen Anne's lace is a powerful uterine stimulant and is contraindicated in pregnant women. It acts via the pituitary gland, so may overstimulate women who suffer from hormone imbalance (estrogen dominance) or those with heavy menstrual bleeding (Cain, 2012).

Throughout history man has turned to nature for medicines. Microorganisms such as fungi offer a huge source of pharmaceutically useful molecules (Langley, 1998). The ergot alkaloids (EA) are among the most important natural pharmaceuticals and toxins in human history that is derived from the fungus *Claviceps purpurea* (Matossian, 1989). Ergot extract has been used in pharmaceutical preparations, including EA in products such as Cafergot (containing caffeine and ergotamine) (Black and Halmer, 2006).The EA have a high biological activity and a

broad spectrum of pharmacological effects; hence they are of considerable importance to medicine. They have adrenoblocking, antiserotonin and dopaminomimetic properties. The spectrum of effects depends on the agent, dosage, species, tissue, and experimental or physiological conditions (Langley, 1998). In 1582 Adam Loncier in Germany made the first note of ergot stimulating uterine contractions (van Dongen and de Groot, 1995). It was the most effective drug for this purpose at the time, resulting in a rapid and sudden termination of labor, with a delivery time lasting less than three hours. It was only during the 20th century that ergot was shown to be useful in the treatment of attacks of migraine. This would mainly involve the alkaloid of ergot, ergotamine (Boichenko *et al.*, 2001).

Aims of study

1. Investigating the oral effects of contraceptive pills (Yasmin), fungal alkaloids (Cafergot) and wild carrot seeds oil (*Daucus carota*) on apoptosis, DNA damage using comet assay and on cytogenetic effects using micronucleus assay in mice blood lymphocytes. Comparison among these contraceptive agents are also included.
2. Exploring the oral effect of contraceptive pills (Yasmin) on apoptosis, DNA damage using comet assay, on cytogenetic effects using micronucleus assay and on aromatase activity of women blood lymphocytes.
3. Studying the anti-fertility effects of three contraceptive types by examining the pregnancy rates of female mice.
4. Evaluate the influence of three contraceptive types in mice and women lymphocytes using micronucleus assay.

Chapter Two

Literature Review

2.1 Birth control pills (oral contraceptive)

Oral contraceptives (OCs) are oral medicines taken to prevent pregnancy. They are also known as the pills or birth control pills (BCPs). The OCs contain synthetic forms of two hormones produced naturally in the body; hormones are estrogen and progestin. Some types of oral contraceptives use progestin hormones only, but most use a combination of estrogen and progestin (Siberry, 2000). The progestin only pill is recommended for women who are breastfeeding because they does not affect milk production (estrogen reduces the amount of breast milk) and they are not associated with increased risks of deep vein thrombosis (DVT) or heart disease compared with non-users of hormonal contraception (Burke, 2011; Mantha *et al*., 2012).

The first approval for contraceptive use in the United States in 1960, and are a very popular form of birth control. They are currently used by more than 100 million women worldwide (Mosher *et al*., 2004). Contraceptive efficacy may be impaired by missing more than one active pill in a packet, delay in starting the next packet of active pills, intestinal malabsorption of active pills due to vomiting or diarrhea and drug interactions with active pills that decrease (contraceptive) estrogen or progestogen levels (Leon, 2005). These pills inhibit female fertility when taken by mouth every day (Trussell and Robert 2007), and they are usually taken for 21 days, then a seven-day gap during which a withdrawal bleeds occurs (Herceberg *et al*., 2010).

Most oral contraceptive pills contain a combination of estrogen (ethinylestradiol or mestranol) and progestin, but progestin-only preparations exist as well. Since they became available, considerable changes have been made in the estrogen and progestin content of combined oral contraceptives with regard to generic substance, dose, and

potency (i.e. The amount required to produce an effect of giving intensity) (Wilson *et al*., 2012).

2.1.1 Combined oral contraceptive pill formulation

The formulations of oral contraceptive pills (OCPs) have changed dramatically over the years. The first OCPs introduced in 1960, contained high doses of norethynodrel (progetin) and ethinyl estradiol or mestranol (estrogen). Norethynodrel is one of the first-generation progestins called "estranes". This class includes the current agent's norethindrone, norethindrone acetate and ethynodiol diacetate (Carr and Bradshow, 1998).

Levonorgestrel, a more potent, second- generation progestin, was developed in about 1970 (Darney, 1995).

Third -generation progestins from the gonane class were incorporated into OCPs formulations to reduce the androgenic and metabolic side effects that occur with older agents. These new progestins include desogestrel, gestodene and norgestomate (Abrams and Goldsmith, 2001).

Drospirenone is frequently referred to as a fourth generation progestin (Naomi and Tepper, 2012).

In hormonal contraceptives, progestins are the most important agent that suppresses ovulation through their anti-gonadotropic properties (Scott *et al.*, 1978). Progesterone has other effects that potentiate the antigonadotropic effect on contraception, which include changes in the quality of the mucus, endometrial changes and alteration in the motility of the fallopian tube (Erkkola and Landgren, 2005).

2.1.2 Types of combined oral contraceptives

Combination birth control pills are categorized according to whether the doses of hormones stay the same or vary as below:

- Monophasic oral contraceptives: use a fixed dose of both estrogen and progestin during the entire cycle.
- Biphasic oral contraceptives: use a constant amount of estrogen during the full cycle, but the amount of progestin is lower during the first half of the cycle and increases in the second half.
- Triphasic oral contraceptives: may vary both the estrogen and progestin levels at different times during the cycle.

The goal of the biphasic and triphasic formulations is to achieve adequate control of the menstrual cycle while using lower doses of both estrogens and progestins, thereby reducing the risk of adverse effects (Siberry, 2000).

2.1.3 Mechanism of action

Combined oral contraceptive pills were developed to prevent ovulation by suppressing the release of gonadotropins. They inhibit follicular development and prevent ovulation as a primary mechanism of action (Nelson and Cwiak, 2011).

Progestogen negative feedback decreases the pulse frequency of gonadotropin-releasing hormone (GnRH) release by the hypothalamus, which decreases the secretion of follicle-stimulating hormone (FSH) and greatly decreases the secretion of luteinizing hormone (LH) by the anterior pituitary. Decreased levels of FSH inhibit follicular development, preventing an increase in estradiol levels. Progestogen negative feedback and the lack of estrogen positive feedback on LH secretion prevent a mid-cycle LH surge. Inhibition of follicular development and the absence of a LH surge prevent ovulation (Vercellini *et al.*, 2010).

Estrogen was originally included in oral contraceptives for better cycle control (to stabilize the endometrium and thereby reduce the incidence of breakthrough bleeding), but was also found to inhibit follicular development and help prevent ovulation. Estrogen negative feedback on the anterior pituitary greatly decreases the secretion of FSH, which inhibits follicular development and helps prevent ovulation (Nelson and Cwiak, 2011).

Another primary mechanism of action of all progestogen-containing contraceptives is inhibition of sperm penetration through the cervix into the upper genital tract (uterus and fallopian tubes) by decreasing the water content and increasing the viscosity of the cervical mucus (Vercellini *et al*., 2010).

2.1.4 Benefits of oral contraceptive pills

The major benefit of hormonal contraceptives is reliable, reversible contraception. Hormonal contraceptives are the most effective methods of reversible contraception available to woman, being 97-99% effective if used properly. Furthermore, there are a number of non-contraceptive benefits associated with hormonal contraceptives. Because hormonal contraceptives decrease endometrial proliferation, they are beneficial for women who suffer from menorrhagia or dysmenorrhea, and they are particularly helpful for treating women with polycystic ovary syndrome. Decreased endometrial proliferation probably also underlies the positive effect on rates of endometrial cancer, and the reduction in risk is higher for those women who have used hormonal contraception the longest (Gemzell-Danielsson, 2010). Two large cohort studies published in 2010 both found a significant reduction in adjusted relative risk of ovarian and endometrial cancer mortality in ever-users of OCs compared to never-users (Martin *et al* ., 2010). The use of OCs for five years or more

decreases the risk of ovarian cancer in later life by 50%. In addition the hormones in "the Pill" have also been used to decrease the risk of colorectal cancer (Hansjörg and Ursula, 2007).

2.1.5 Side-effects and potential risks of contraceptive pills

Different sources note different incidences of side effects. The most common side effect is breakthrough bleeding (Serfaty, 1992).

The risk of venous thromboembolism (including deep venous thrombosis (DVT) and pulmonary embolism (PE) was increased (Blanco-Molina and Manuel, 2010). These risks are greatest in women with additional risk factors, such as smoking (which increases risk substantially) and long-continued use of pills, especially in women over 35 years of age (Rang *et al.*, 2012). The risk of thromboembolism varies with different types of BCPs. Compared with combined oral contraceptives containing levonorgestrel (LNG), and with the same dose of estrogen and duration of use, the rate ratio of DVT for combined oral contraceptives with norethisterone is 0.98, with norgestimate 1.19, with desogestrel (DSG) 1.82, with gestodene 1.86, with drospirenone (DRSP) 1.64, and with cyproterone 1.88 (Eichinger *et al.*, 2013). One study showed more than a 600% increased risk of blood clots for women taking COCPs with drospirenone compared to non-users, compared to 360% higher for women taking birth control pills containing levonorgestrel (Lidegaard and Milsom, 2012). Based on these studies in 2012 the Food and Drug Administration (FDA) updated the label for drospirenone COCPs to include a warning that contraceptives with drospirenone may have a higher risk of dangerous blood clots (Cibula *et al.*, 2010).

Report by International Agency for Research on Cancer (IARC) (2007) working group said COs increase the risk of cancers of the breast, cervix and liver. Two large cohort studies published in 2010 both found

no significant increase in adjusted relative risk of breast cancer mortality in ever-users of OCs compared to never-users (Martin *et al.*, 2010).

Low levels of serotonin, a neurotransmitter in the brain has been linked to depression. High levels of estrogen, as in the first-generation combined oral contraceptive, and progestin as in some progestin-only contraceptives have been shown to promote the lowering of brain serotonin levels by increasing the concentration of a brain enzyme that reduces serotonin (Trussell and Robert, 2007).

Combination hormonal contraceptives showed no large difference in weight when compared with no intervention groups. The evidence was not strong enough to be certain that contraceptive methods do not cause any weight change, but no major effect was found. This review also found "that women did not stop using the pill because of weight change (Gallo *et al.,* 2013).

In addition to other side effects progesterone can increase the risk of developing hypertension (Rose and Adams, 1972).

2.2 Combined oral contraceptives (Yasmin)

Yasmin is a combined oral contraceptive tablet containing the synthetic progestogen, drospirenone and the synthetic oestrogen, ethinyloestradiol.

The chemical name for ethinyloestradiol is 19-nor-17α-pregna-1, 3, 5(10)-trien-20-yne-3, 17β–diol while the chemical name for drospirenone is 6β, 7β, 15β, 16β-dimethylene-3-oxo-17α-pregn-4-ene-21, and 17-carbolactone and have the following structural formula (Figure 2-1):

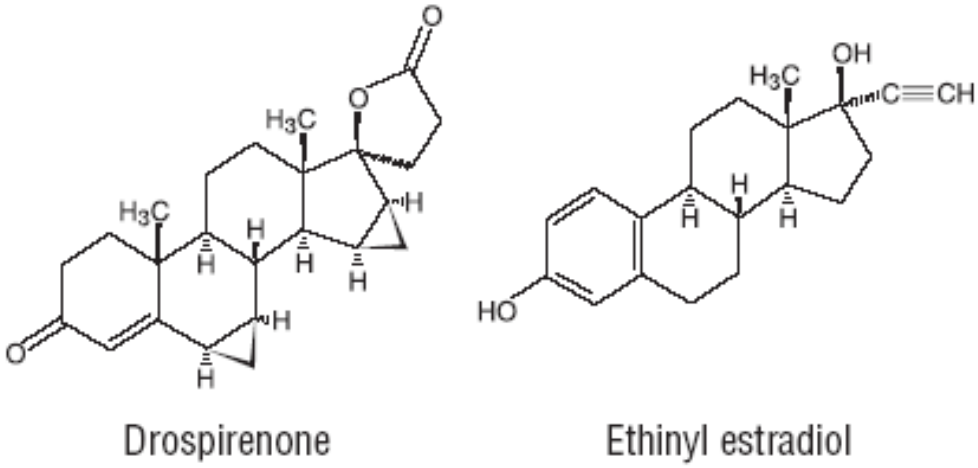


Figure (2-1): Chemical structure of the synthetic progestogen, drospirenone and the synthetic oestrogen, ethinyloestradiol.

Ethinyloestradiol is a white to creamy white, odorless, crystalline powder. It is insoluble in water and soluble in alcohol, chloroform, ether, vegetable oils, and aqueous solutions of alkali hydroxides, while drospirenone is a white to off-white crystalline powder. It is freely soluble in methylene chloride, acetone, methanol, sparingly soluble in ethylacetate and ethanol 96% (v/v) and practically insoluble in hexane and water (Jick and Hernandez, 2011).

Each light yellow active tablet contains drospirenone (3mg) and ethinyloestradiol (0.030 mg) and the excipients: lactose, maize starch, pregelatinised maize starch, povidone, magnesium stearate,

hypromellose, macrogol 6000, purified talc, titanium dioxide and iron oxide yellow (Parkin *et al.*, 2011).

For the majority of women, oral contraceptives can be taken safely. But there are some women who are at high risk of developing certain serious diseases that can be life-threatening or may cause temporary or permanent disability or death. The risks associated with taking oral contraceptives increase significantly if women:

- Smoke cigarette, smoking increases the risk of serious cardiovascular side effects from oral contraceptive use. This risk increases with age and with heavy smoking (15 or more cigarettes per day) and is quite marked in women over 35 years of age.
- Have high blood pressure, diabetes, high cholesterol, or are obese.
- Have or have had clotting disorders, heart attack.
- Women who currently have or have had breast cancer should not use oral contraceptives because breast cancer is a hormonally-sensitive tumor.
- Yasmin is different from other birth control pills because it contains the progestin drospirenone. Drospirenone may increase potassium. Therefore, Yasmin should not take if women have kidney, liver or adrenal disease because this could cause serious health problems.

No increased risk of birth defects in women who have used oral contraceptives prior to pregnancy. Studies also do not suggest a teratogenic effect, particularly in so far as cardiac anomalies and limb-reduction defects are concerned, when taken inadvertently during early pregnancy (van Hylckama Vlieg *et al*, 2009).

2.2.1 Dosage and administration

To achieve maximum contraceptive effectiveness, Yasmin (drospirenone and ethinyl estradiol) must be taken exactly as directed at intervals not exceeding 24 hours. Yasmin consists of 21 tablets of a monophasic combined hormonal preparation. During the first cycle of Yasmin use, the patient should be instructed to take one yellow Yasmin daily, beginning on day one (1) of the menstrual cycle (the first day of menstruation is day one). She should take one yellow Yasmin daily for 21 consecutive days. It is recommended that Yasmin be taken at the same time each day, preferably after the evening meal or at bedtime or it can be taken without regard to meals. If Yasmin is first taken later than the first day of the menstrual cycle, it should not be considered effective as a contraceptive until after the first 7 consecutive days of product administration. The possibility of ovulation and conception prior to initiation of medication should be considered. If spotting or breakthrough bleeding occurs while taking it, the patient should be instructed to continue taking it. This type of bleeding is usually transient and without significance; however, if the bleeding is persistent or prolonged, the patient should be advised to consult a physician (Lidegaard *et al.*, 2009).

2.3 The hormones used in formulation of contraceptive pills

2.3.1 Progesterone

Progesterone is the dominant ovarian hormone secreted during the luteal (second) phase of the menstrual cycle. Its main function is to prepare the uterus for implantation of an embryo. If pregnancy occurs, human chorionic gonadotropin (hCG) is released which maintains the corpus luteum, which in turn allows progesterone levels to remain raised, while progesterone levels decrease after delivery and during breastfeeding and they are low in women after menopause.

In males almost all progesterone is converted to testosterone in the testis (Kyle, 2008).

Progesterone also plays pivotal role in normal female reproductive functions in the uterus, ovary, mammary gland and the brain. Progesterone is important for normal functioning of several non-reproductive tissues such as the cardiovascular system, bone and central nervous system (Rocha and Soares, 2009).

2.3.2 Biosynthesis of progesterone hormone

Progesterone is also known as P4 (pregn-4-ENE-3, 20-dione) which is a steroid hormone essentially synthesized by the ovaries, to a lesser extent by testis and the adrenal glands, from the second trimester of pregnancy by the placenta, and to some extent from neurons (Schumacher *et al*., 2008).

Progesterone is synthesized from cholesterol molecule that comprising of three hexagonal cycles, A, B, C and one pentagonal cycle D (Figure2-2). Decimals enzyme converts cholesterol into Pregnenolone, which is then converted to progesterone by isomerase/dehydrogenase enzymes.

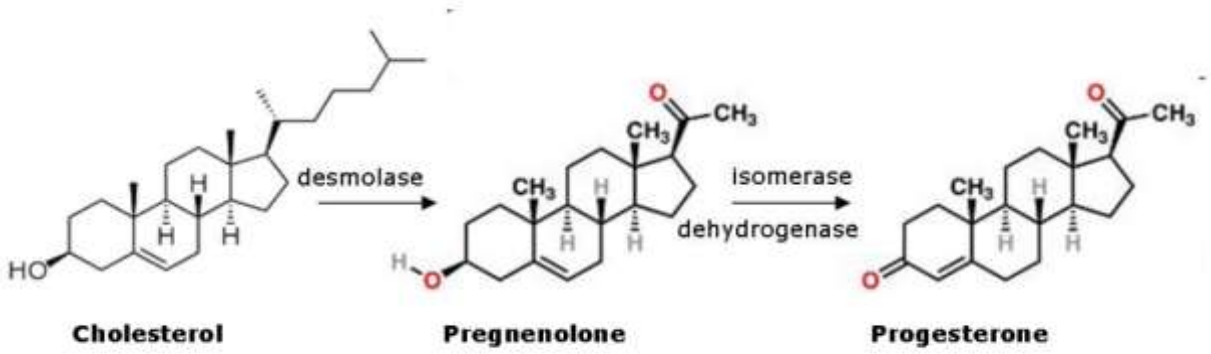


Figure (2-2): Biosynthesis of progesterone hormone.

Progesterone is synthesized from cholesterol by action of desmolase enzyme which converts cholesterol into Pregnenolone, which is then converted into progesterone by the action of isomers/dehydrogenase enzymes (Lu *et al.*, 2006).

2.3.3 Sources of progesterone

1-Animal

Progesterone is produced in the ovaries by the corpus luteum, the adrenal glands near the kidney, and during pregnancy by the placenta. Progesterone is also stored in adipose (fat) tissue.

In humans, increasing amounts of progesterone is produced during pregnancy, at first the source is the corpus luteum that has been "rescued" by the presence of hCG from the conceptus. However, after the 8th week, production of progesterone shifts to the placenta. The placenta utilizes maternal cholesterol as the initial substrate, and most of the produced progesterone enters the maternal circulation, but some are picked up by the fetal circulation and used as substrate for fetal corticosteroids. At term the placenta produces about 250 mg progesterone per day. An additional source of progesterone is milk products. After consumption of milk

products the level of bioavailable progesterone goes up (Goodson *et al.*, 2007).

Progesterone secretion is not constant during menstrual cycle. Just before ovulation, progesterone secretion is induced by LH secreted by the pituitary gland and is maintained till the second part of the menstrual cycle. Progesterone level diminishes near the end of cycle resulting in menstruation (Lu *et al.*, 2006).

Generally pregnant women with twins produce higher levels of hCG than those with single embryos (Prats *et al.*, 2012)

2-Plants

In at least one plant, *Juglans regia*, progesterone has been detected (Pauli, 2010).Progesterone-like steroids are also found in *Dioscorea mexicana*. It contains a steroid called diosgenin that is taken from the plant and is converted into progesterone (Noguchi *et al.*, 2006).

2.3.4 Estrogens

It is a main reproductive hormone affecting growth, development, maturation and functioning of reproductive tract, as well as the sexual differentiation and the behavior (Balthazart *et al*., 2009). They promote the development of female secondary sexual characteristic, such as breast, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle (Hill *et al*., 2004).

The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant estrogen during reproductive years, both in terms of absolute serum levels as well as in terms of estrogenic activity. During menopause, E1 is the predominant circulating estrogen while during pregnancy E3 is the predominant circulating estrogen in terms of serum levels. Though E3 is the most plentiful of the three estrogens it is also the weakest, whereas E2 is the strongest with a potency of approximately 80 times that of E3.

Thus, E2 is the most important estrogen in non-pregnant females who are between the menarche and menopause stages of life. Another type of estrogen called estetrol (E4) is produced only during pregnancy (Mikael, 2013).

Estrogen is also synthesized in the male reproductive system and it is found in high concentrations in rete testis and seminal fluids. It was reported that E_2 induces spermatogenesis in mice (Allan *et al*., 2010).

2.4 *Daucus carota* (wild carrot)

Daucus carota is a flowering plant in the family Apiaceae, native to temperate regions of Europe, southwest Asia and naturalized in North America and Australia.

Daucus carota common name's include wild carrot, (UK) bird's nest, bishop's lace, and Queen Anne's lace (North America).

Wild carrot was introduced and naturalized in North America, where it is often known as "Queen Anne's lace". Anne, Queen of Great Britain, and her great grandmother Anne of Denmark are taken to be the Queen Anne for which the plant is named (Cain, 2012).

2.4.1 Distribution

A native wild plant common everywhere in the British Islands. Near the sea in greatest abundance, superior medicinally to the cultivated kind, and near waste places throughout Europe, Asia, Russia, America and is even found in India.

Probably originally a native of the sea coast of Southern-Europe degenerated into its present wild state, but of very ancient cultivation. As with all herbal remedies and wild food gathering extra caution should be used especially since the wild carrot bears close resemblance to a

dangerous species, poison hemlock. Similar in appearance to the deadly poison hemlock (Cain, 2012).

The domesticated carrot (*D. carota sativus*) is grown around the world, while wild carrot (*D. carota carota*), also known as Queen Anne's lace is native to temperate regions of Europe and western Asia, and has been introduced into America, New Zealand, Australia and Japan (Rong *et al*., 2010).

Wild carrot is thought to have originated on the Iranian Plateau (an area which now includes Afghanistan, Pakistan and Iran). It occurs in free-draining and slightly acidic soils. It appears in a list of plants grown in the royal garden of Babylon in the 8th century BC. It was included in the list of aromatic herbs, so it is thought to have been grown for its fragrant leaves or seeds (Breckle and Rafiqpoor, 2010).

2.4.2 Description

Daucus carota is a biennial plant that grows a rosette leaves in the spring and summer, while building up the stout taproot that stores large amounts of sugars for the plant to flower in the second year.

Its root is small and spindle shaped, whitish, slender and hard, with a strong aromatic smell and an acrid, disagreeable taste, it penetrates some distance into the ground, having only a few lateral rootlets. A biennial herb is up to 150 cm tall with a grooved and bristly stem. The stems are erect and branched, generally about 2 feet high, tough and furrowed (Cain, 2012).

Leaves are finely divided giving a feathery appearance and they have a characteristic carrot odor .The leaves contain furocoumarins that may cause allergic contact dermatitis from the leaves, especially when wet. Later exposure to the sun may cause mild photo dermatitis. While the flowers are white to purple, borne in late summer in umbrella-like clusters (umbels) up to 7 cm in diameter. The umbels can be concave, flat

or convex. Central flowers of the umbel are sometimes dark purple. Flowering heads become concave (and are considered to resemble birds' nests) when they turn to seed. However, the fruits are splitting into two single-seeded portions, 2–4 mm in diameter, with spiny ridges. The spiny fruits attach to the fur of passing animals, aiding seed dispersal (Figure 2-3), (Breckle and Rafiqpoor, 2010; Cain, 2012).

Figure (2-3) *Daucus carota* (wild carrot) inflorescence (Breckle and Rafiqpoor, 2010).

2.4.3 Chemical Components

Several studies on the chemical characterization of carrot seed and seed oil have been carried out (Gonny *et al*., 2004).The chemical components can be described as the following:

A: Volatile oils

The thin oil is a pale yellow to amber or orange-brown color and it has a woody, dry aroma with fresh notes (Lis-Balchin, 2006).

Many components identified with relative composition vary between different cultivars. Various components include α-pinene, β-ipinene, geraniol, geranyl acetate, limonene, α-terpinen, *p*-terpinen, α-terpineol, terpinen-4-ol, *p*-decanolactone (monoterpenes) ; β-bisabolene, β-elemene, caryophyllene, caryophyllene oxide, carotol, daucol (sesquiterpenes) ; elemicin is a phenylpropene and asarone (phenylpropanoid derivative).

Carrot seed contains about (0.66-1.65%) of essential oil (Benecke *et al.,* 1987).Various compositions of *D. carota* essential oils have been characterized by the occurrence of a main component, pinene (up to 51%), geranyl cetate (up to 76%), elemicin (up to 35%), and carotol (up to 66%) (Marzouki *et al.*, 2010).

B: Flavonoids

1. Flavones:

Are a class of flavonoids that include apigenin, chrysin, and luteolin.

2. Flavonols:

Are a class of flavonoids that include kaempferol, and quercetin (El-Moghazi, 1980).

C: Minerals

The mineral contents of carrot seeds were determined and they were found rich in Ca, P, K, Na, Mg and Al (Özcan, 2004).

D: Other constituents:

Fatty acids that include butyric, palmitic, coumarin, and xylitol (Benecke *et al.*, 1987).

2.4.4 Medical use & pharmacological action

Women have used the seeds from *Daucus carota* for centuries as a contraceptive, the earliest written reference dates back to the late 5th or 4th century B.C. John Riddle writes in *Eve's Herbs*, that queen anne's lace seeds are one of the more potent antifertility agents available.

The seeds harvested in the fall are a strong contraceptive if taken orally immediately after coitus.

Research on small animals has shown that extracts of the seeds disrupt the implantation process, or if a fertilized egg has implanted for only a short period, will cause it to be released. The Chinese view Queen Anne's lace as a promising post-coital agent, " evidence suggests that terpenoids in the seed block crucial progesterone synthesis in pregnant animals and some herbalists have described it as having the effect of making the uterus "slippery" so the egg is unable to implant. Progesterone is essential for pregnancy to occur, progesterone's function is to prepare the uterine endometrium to receive an egg, if the endometrium isn't ready, the egg will find implantation very difficult. If the egg can't implant then the opportunity is missed, and the egg begins to break down and is no longer viable and menstruation arrives as usual (Riddle, 1997).

Plants with contraceptive properties were used in Ancient Greece from the 7th century BCE onwards and documented by Hippocrates, the use of Silphium, a plant well known for its contraceptive and abortifacient properties. Asafoetida, a close relative of siliphion was also used for its contraceptive properties. Other plants commonly used for birth control in ancient Greece include Queen Anne's lace (*Daucus carota*), willow, date palm, pomegranate, pennyroyal, artemisia, myrrh,

and rue. Recent studies have confirmed the birth control properties of many of these plants, confirming for example that Queen Anne's lace has post coital anti-fertility properties and is still used today for birth control in India (Humadee, 2013).

Clinical and pre-clinical studies on the hepatoprotective effects of *D. carota* has been conducted using an ethanol extract rather than the steam distilled essential oil. There are, however, shows that an animal study on the sesquiterpene, beta bisbolene, is regarded to have had potential hepatoprotective properties.

Antibacterial activity- *Daucus. carota* was examined along with 95 other essential oils for their antibacterial properties against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica. Daucus. carota* essential oil showed strong antibacterial effects against *Campylobacter*. *jejuni* (Friedman, 2002). This oil also showed that when exposed to ultraviolet, the antibacterial activity increased against certain pathogens (Gupta, 2003). The molecules those are responsible for this activity were identified as (E)-methylisoeugenol and elemicin (Luciani *et al.*, 2007).

Antifungal *D. carota* was evaluated for its antifungal activity against yeasts, dermatophyte and *Aspergillus* strains. While the various oils used to demonstrate different levels of antifungal activity, the researchers noted that the oils containing higher amounts of elemicin showed the strongest activity (Tavares *et al.*, 2008).

Essential oil is used for other medicinal purpose, such as diuretic, and stomachic (Mazzoni *et al*., 1999), and ntiulcer (Wehbe, 2009). Their high protein and oil contents along with their pleasant odor and taste suggest that this fruit can be used in the food industry (Schieber *et al*., 2001).

Carrots are credited with many medicinal properties; such as cleanse the intestines, antidiarrheal, an overall tonic and antianemic.

Queen Anne's Lace is a powerful uterine stimulant and is contraindicated in pregnant women. It acts via the pituitary gland, so may overstimulate women who suffer from hormone imbalance (estrogen dominance) or those with heavy menstrual bleeding (Cain, 2012).

The oil was reported to elicit CNS hypnotic effects in the rat, hypotension in the dog leading to respiratory depression at higher doses, *in vitro* smooth muscle relaxant activity, *in vitro* cardio tonic activity and vasodilation of coronary vessels of the isolated cat heart (Gambhir *et al*., 1966).

Fruit extracts may cause sensitivity reactions similar to those seen with celery. Excessive doses of the oil may cause renal irritation and may affect existing hypo- and hypertensive, cardiac and hormone therapies (Ceska, 1986).

2.5 Cafergot and ergot alkaloid

Cafergot is the proprietary name of a medication consisting of ergotamine tartrate and caffeine (Diener *et al*., 2002).

Ergot extract has been used in pharmaceutical preparations, including ergot alkaloids (EA) in products such as Cafergot (containing caffeine and ergotamine (Black and Halmer, 2006). Co-administration of caffeine increases the absorption of ergotamine through rapid dissolution and increased solubility of ergotamine. Therefore, the bioavailability of ergotamine present in Cafergot may be higher than that reported with ergotamine administration alone (Silberstein and McCrory, 2003).

Ergotamine was isolated from ergot by Prof. Arthur Stoll after his establishment of the pharmaceutical department of Sandoz (Switzerland) in 1918. It was introduced to the market under the trade name Gynergen in 1921 (Sandoz, 2010). Thus, this drug was introduced before any real effective regulations or authorities requiring controlled (randomized) clinical trials or safety evaluations were in place. This latter legislation

process started with the 1938 US Food, Drugs and Cosmetics Acts as followed by the Ergotamine tartrate is used in migraine. However, its adverse effects limit its use (Martindale, 2010). Ergotamine is commonly used for medicinal purposes as ergotamine tartrate (Bracher *et al.*, 2010).

EAs are indole-derived secondary metabolites synthesized by the phytopathogenic ascomycete *Claviceps purpurea* (Spiering *et al*., 2002), and have proven to be both pharmaceutically and agriculturally important (Panaccione, 2010). Most of the naturally occurring EAs show a tetracyclic ergoline ring system and more than 50 different ergot alkaloids have been identified in the past (Flieger *et al.*, 1997). The total EA-amounts and patterns vary between fungal strains, geographic regions and host plants (Krska and Crews, 2008).

The ergot fungus is a phytopathogenic ascomycete which infects the ears of several grasses, replacing the ovary and producing a hibernating structure, the so-called sclerotium, in which the ergot alkaloids are formed (Schardl *et al*., 2006). The ergots are common contaminants of cereals that must be diligently removed to prevent poisoning of food and feed grain supplies (DI Menna *et al.*, 2012). Historically ergotism has killed many thousands of people. For instance, over 40,000 deaths attributed to ergot poisoning were recorded in France in 943 (Prescott *et al*., 2005).

The main alkaloids in ergots from *C. purpurea* are ergocristine, ergotamine, ergocornine, α- and β-ergocryptine, ergometrine, ergosine, ergocristinine, ergotaminine, ergocorninine, α- and β-ergocryptinine, ergometrinine, and ergosinine; the alkaloid composition being highly variable (Appelt and Ellner, 2009).

2.5.1 Ergot alkaloids and pharmacological effects

EAs have been used for millennia to aid childbirth, birth control, treatment of migraines and, recently, treatment of parkinsonism and other CNS disorders (Di Menna *et al.*, 2012).

EAs have a number of effects on the reproductive process in rodents, including prevention of pregnancy by interfering with implantation, embryo toxicity, and inhibition of lactation. These effects have generally been observed at higher doses than the lowest observed levels in the repeat dose studies (Franzmann *et al.*, 2010a).

These substances show a high level of structural homology to some neurotransmitters like serotonin and dopamine and can therefore bind to the same receptors in the CNS, which is the basis for the application of ergot alkaloids in a variety of clinical conditions (Spiering *et al*., 2002).

Besides their role in life threatening epidemic food contaminations EAs show a broad spectrum of pharmacological effects and were used in medical applications for hundreds of years. EAs and EA-derived compounds were applied or tested for prolactin inhibition, treatment of Parkinsonism,cerebrovascular insufficiency, venous insufficiency, thrombosis, emboli, and stimulation of cerebral, and are still applied for migraine and uterine stimulation (Battilani *et al*., 2009).

In addition, LSD is a semi synthetic derivative of the EA-family. Legally introduced as a pharmaceutical in the mid-1950s and known for its potent psychoactive effects, it is an illegal drug of abuse today (Martindale, 2010).

The adverse effects of ergotamine tartrate may mainly be attributed either to its effects on the (CNS), or to vasoconstriction of blood vessels and possibly thrombus formation. The adverse effects have been reported

in the indicated frequency associated with the oral use of ergotamine tartrate in the recommended therapeutic dose range (Bracher *et al*., 2010).

Ergotamine tartrate is contraindicated during pregnancy. Women who may become pregnant or are pregnant are at risk from therapeutic doses of ergotamine tartrate because of its oxytocic effect on the uterus (causing contractions) and its vasoconstrictor effects. Thus during the entire pregnancy an increased risk for diminished placental blood circulation and premature labour is seen due to intake of ergotamine tartrate (Martindale, 2010).

Although the American Academy of Pediatrics includes ergotamine tartrate among those drugs that may be given with caution to breast-feeding mothers, it notes that maternal use in doses equivalent to those given for the treatment of migraine has been associated with vomiting, diarrhea, and convulsions in nursing infants (American Academy of Pediatrics, 2001). Product information says that ergotamine tartrate is contraindicated during breastfeeding, since the distribution of unchanged drug and metabolites into breast milk presents a risk of adverse effects (cardiovascular disorders, diarrhea, vomiting, cerebral convulsions) in the infant. Furthermore, repeated doses of ergotamine tartrate may impair lactation via inhibition of prolactin release from the pituitary gland (Anonymous, 2010).

2.6 Definition of Apoptosis

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms (Douglas, 2011). It is morphologically characterized by cell shrinkage, membrane blebbing, nuclear condensation, and formation of apoptotic bodies (Elmore, 2007).

PCD was already discovered by scientists more than 100 years ago and the German scientist Carl Vogt was first to describe the principle of apoptosis in 1842. In Greek, apoptosis means "dropping off" of petals or leaves of plants or trees (Susin *et al.*, 2000).

The apoptotic process is initiated by “death” signals, which trigger a complex series of events (Schimmer, 2008). A central step of the apoptotic process is the activation of caspases, a group of enzymes belonging to the cysteine protease family and enzyme activation led to cleave many vital cellular proteins, breaking down the nuclear scaffold and cytoskeleton, and subsequently leads to nuclear DNA degradation (Wong, 2011).

Based on the original source of “death” signals, caspase dependent apoptosis are classified into two pathways: intrinsic pathway, which is activated by modulators within the cell itself, and extrinsic pathway, which responds mainly to extracellular stimuli (Russo *et al.*, 2006). In the extrinsic pathway (also known as receptor-mediated death pathway), death ligands induce apoptosis by activating the death receptors at the cell surface. The activated death receptors, which leads to the recruitment of the adaptor proteins such as FADD (Fas-associated protein with death domain) and further chain activation of caspase-8 and -3 (Song *et al.*, 2008). While the intrinsic pathway, which is also known as mitochondria-mediated death pathway, is initiated by damage to the mitochondria, which results in the release of a series of proteins into cytoplasm including cytochrome C. (Beliz´ario *et al.*, 2007). For the intrinsic

pathway, the initial enzyme activated is caspase-9 (Long and Ryan, 2012).

Collectively, apoptosis is essential for the sophisticated architecture of life, including normal cell turnover, organ metamorphosis, tissue homeostasis, and embryonic development (Chao *et al*., 2012).

2.6.1 Apoptosis and contraceptive pills

Combination estrogen–progestin OC has a potent apoptotic effect on the ovarian epithelium mediated by the progestin component (Rodriguez *et al.,* 1998). Combination estrogen and progestin or progestin alone had a fourfold to sixfold increase in the proportion of apoptotic ovarian epithelial cells as compared with control or estrogen-only-treated monkeys. The apoptosis pathway is one of the most important *in vivo* mechanisms that function to eliminate cells that have sustained DNA damage and, thus, are prone to malignant transformation (Canman *et al*., 1994).

In addition, a number of well-known chemo preventive agents have been demonstrated to activate the apoptosis pathway in the target tissues that they protect from neoplastic transformation (Lotan, 1996*).* The finding that progestins activate this critical pathway in the ovarian epithelium suggests that the protective effects afforded by OCs against ovarian cancer may at least in part be caused by progestin mediated apoptosis. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer (Franko *et al*., 2000).

It was shown that only one month of a combined oral contraceptive was sufficient to significantly reduce markers of cell proliferation and increase the apoptotic index in the eutopic endometrium of patients with

endometriosis (Meresman *et al*., 2003). Excessive levels of gonadotropic hormones such as FSH and LH are also suggested to increase ovarian cancer risk through increased cell growth and inhibition of apoptosis (Konishi, 2006), and oral contraceptives are known to suppress pituitary gonadotropin secretion (Risch, 1998).

The synthetic progestin, levonorgestrel, may induce apoptosis in ovarian surface epithelium (Rodriguez *et al.*, 1998), ethinylestradiol plus levonorgestrel induces ovarian epithelial cell apoptosis in intact monkeys (Rodriguez *et al.*, 2002), while drospirenone was found to induce chromosome aberrations in human peripheral lymphocytes (Lidegaard *et al*., 2009). An experimental study by Rodriguez *et al.*(1998) revealed that progestin induced apoptosis in the ovarian epithelium of macaques, leading to the hypothesis that the progestin component may be the major mechanism underlying the protective effect of combined oral contraceptives.

2.6.2 Annexin V stain and detection of phosphatidylserine exposure in apoptotic cells

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. Inappropriately regulated apoptosis is implicated in disease states (Sylvia *et al*., 2008). Apoptosis is distinguished from necrosis, or accidental cell death, by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm, and loss of membrane asymmetry (Elmore, 2007). In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner

to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment (Niu and Chen, 2010).

The availability of Fluorescein isothiocyanate (FITC) labeled annexin V facilitated studies on PS exposure at the outer membrane leaflet, and provides not only a useful tool to measure the PS exposition of platelets and erythrocytes, but also in nucleated cells undergoing programmed cell death (Kheansaard *et al.*,2011). Using fluorescently labeled annexin V, it could be demonstrated that the apoptotic lymphocytes expose PS at their outer membrane early after onset of the execution phase of apoptosis (Bruchhaus *et al.*, 2007). PS appears on the outer leaflet of the plasma membrane, the integrity of which has not been compromised at this stage. PS exposure seems to last from the early execution phase of apoptosis until the final stage, at which the cell has broken up into apoptotic bodies (Kroemer *et al.,* 2009).

The annexin V-FITC apoptosis detection kit detects apoptotic cells by flow cytometry utilizing annexin V-FITC as a fluorescent probe to detect early apoptotic phosphatidylserine binding. Propidium iodide (PI) binding to DNA is also monitored to indicate compromise of the cell membrane and progression in apoptosis (Popescu *et al.,* 2011).

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as PI or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, FITC annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI (Martin *et al*., 1995).

2.7 Comet assay (Single cell gel electrophoresis)

The comet assay (single cell gel electrophoresis) is used for the detection of primary DNA damage induced in isolated cells or nuclei from multiple tissues of animals usually rodents (Rothfuss *et al*., 2010). It is one of the cheapest, reliable and rapid methods (Fellows and Donovan, 2007).The main advantage of the comet assay is its simplicity. Minimal training is required for clinicians to conduct this assay and the equipment's for the assay are cheap and available (Collins *et al*., 2008).

Muid *et al*. (2012) has pointed out that the comet assay is considered a suitable and rapid test for DNA-damaging potential in environmental and biomonitoring studies. The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in-vivo* biomarker assays, and the comet assay is gaining popularity and acceptance over other assays since its advantages include its sensitivity for detecting low levels of DNA damage (0.1 DNA break/109 Daltons) (Ali *et al*., 2008b).

The principle of the alkaline comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage (Lemay and Wood, 1999).

There are two conditions used in comet assay, alkaline and neutral conditions, the neutral comet assay will detect mainly double-strand breaks and can be useful for assessing the DNA fragmentation associated with apoptosis (Zainol *et al.*, 2009).While the alkaline comet assay can detect single and double stranded breaks resulting for example, from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair. Under certain modified conditions the assay

can detect DNA-DNA and DNA-protein crosslinking, and oxidized bases (Rothfuss *et al*., 2010).

The effect of the use of an oral contraceptive (OC) on the response in the alkaline comet assay (single-cell gel electrophoresis) was investigated in women taking contraceptive pills daily for 24 months and the increasing in the number of lymphocytes with DNA migration and an increased frequency of sister chromatid exchange per metaphase were observed in OC users as compared with their age-matched untreated controls. The fact that prolonged and extensive use of these drugs in our daily life may be hazardous and also, that OC users should be aware of multifactorial risk factors (environmental, genetic and life style patterns) that may be responsible for additional DNA damage (Biri *et al*., 2002).

2.8 Aromatase

Aromatase is an enzyme responsible for a key step in the biosynthesis of estrogens which is also called estrogen synthetase or estrogen synthase. It is a member of the cytochrome P450 superfamily, which are monooxygenases that catalyze many reactions involved in steroidogenesis (Ghosh *et al*., 2009).

Aromatase consists of 503 amino acids that are encoded by human CYP19A1 gene that is located on chromosome 15q21.1 (Toda and Shizuta, 1993) , and it consists of approximately130 kilo base pairs (Czajka-Oraniec and Simpson, 2010).

In particular, aromatase is responsible for the aromatization of androgens into estrogens and it can be found in many tissues including gonads, brain, adipose tissue, placenta, blood vessels, skin, and bone, as well as in tissue of endometriosis, uterine fibroids, breast cancer, and cancer. It is an important factor in sexual development (Ghosh *et al*., 2009).

Factors known to increase aromatase activity include age, obesity, insulin, gonadotropins, and alcohol ,while aromatase activity is decreased by prolactin, anti-Müllerian hormone and the common herbicide glyphosate. Aromatase activity appears to be enhanced in certain estrogen-dependent local tissue next to breast tissue, endometrial cancer, endometriosis, and uterine fibroids. It is also susceptible to environmental influences, particularly temperature (Duffy *et al.*, 2010).

2.8.1 Aromatase function and the production of estrogen

The cytochrome P450 that convert androgens to estrogens is CYP19, also known as aromatase. Aromatase has been recognized as a key enzyme steroidogenesis as it is the only enzyme responsible for the demethylation and subsequent aromatization of testosterone to estradiol and androstenedione to estrone (Chen *et al.,* 1998).

Aromatase is localized in the endoplasmic reticulum where it is regulated by tissue-specific promoters that are in turn controlled by hormones, cytokines, and other factors. Aromatase catalyzes the last steps of estrogen biosynthesis from androgens (specifically it transforms rostenedione to estrone and testosterone to estradiol). These steps include three successive hydroxylation's of the 19-methyl group of androgens, followed by simultaneous elimination of the methyl group as formate and aromatization of the A-ring (Figure 2-4).

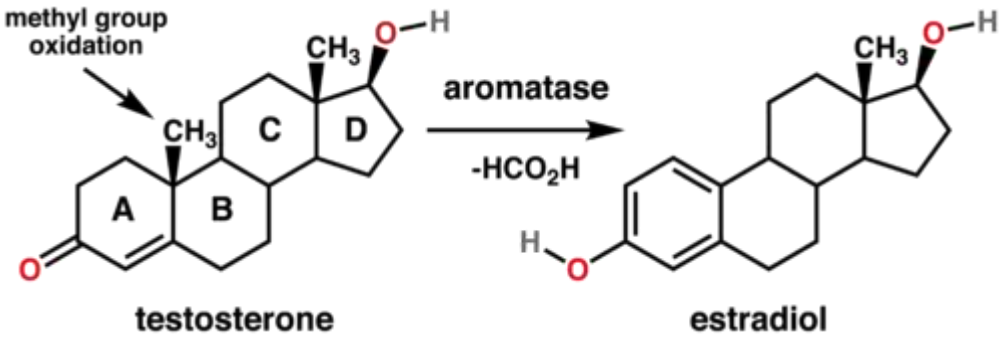


Figure (2-4): The conversion of testosterone to estradiol.

General reaction for the conversion of testosterone to estradiol catalyzed by aromatase. Steroids are composed of four fused rings (labeled A-D). Aromatase converts the ring labeled "A" into an aromatic state) (Vaz, 2003).

2.8.2 Assessment of aromatase in women using oral contraceptives

Combined oral contraceptives have been in use for about 50 years and are one of the most popular forms of contraception worldwide (Trussell, 2007).

Combined oral contraceptives are comprised of a synthetic estrogen and progestin. Ethinylestradiol is the most common estrogen component, but there are a number of different progestin types (Dinger *et al.*, 2011). *In vitro*, ethinylestradiol demonstrates inhibition of both phase I and phase II metabolizing enzymes including a major cytochrome P450 enzyme (Laine *et al.*, 2003). The inhibition of the enzyme leads to profound hypoestrogenism (low estrogen levels). Thus, aromatase inhibitors have become useful in the management of patients with breast cancer whose lesion was found to be estrogen receptor positive. Aromatase is the rate-limiting enzyme in estrogen biosynthesis. Estrogen plays an important role in breast cancer development. Upon binding to estrogen, estrogen receptor activates transcription of its target genes,

which are responsible for cancer cell proliferation in estrogen-dependent breast tumors (Ghosh *et al*., 2009).

Aromatase is expressed in both normal and malignant breast tissues and its activity in the breast varies widely (Yue *et al.*, 2001).Studies were carried out in an *in-vitro* model to investigate the potential role of estrogen in this regulation in which MCF-7 cells were cultured in long-term estrogen-deprived medium (LTED cells). It was found that long-term estrogen deprivation enhanced aromatase activity by three- to fourfold compared with that in wild-type MCF-7 cells. Re-exposure of LTED cells to estrogen reduced aromatase activity to the levels of wild-type MCF-7 cells (Tofteng *et al.*, 2004).

Changes in aromatase activity are also implicated in a wide range of human diseases, including Alzheimer's disease (Hiltunen *et al*., 2006), endometriosis (Fedele *et al*., 2008), and hepatic cancer (Miceli *et al.*, 2009).

2.9 Cytogenetic analysis

Cytogenetic analysis is a widely employed indication system for the evaluation of physically, chemically and biologically induced mutation that allow for objective evaluation of the genetic material damages and is a method that permit direct image analysis of the chromosome damage. These analyses have been carried out using *in vivo* and *in vitro* system and have been proved to be the best reliable of mutagen-carcinogen exposure and chromosomal aberration deduction (Gebhart, 1981). Chick embryo was the first to be utilized (*in vivo*) analysis (Bloom and Hsu, 1975), but later the mice became the animal most frequently used due to their fast reproduction, small size, and easy handling (Tice *et al*., 1989).

Cytogenetic analysis is a powerful tool used in the identification of chromosomal aberrations, with applications ranging from identification of pre‑natal birth defects to detection and prognostic evaluation of diseases

associated with sporadic or inherent karyotyping abnormalities. Such abnormalities include changes in karyotyping number, size and structure (Teixeira and Heim, 2011).

2.9.1 Micronucleus Assay

In vitro and *in vivo* tests measuring chromosomal aberrations in metaphase cells can be used to detect a wide spectrum changes in chromosomal integrity. Breakage of chromatids or chromosomes can result in micronucleus (MN) formation if an acentric fragment is produced; therefore, assays detecting either chromosomal aberrations or micronuclei can be used to detect clastogens. Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase; thus, MN tests have the potential to detect some aneuploidy inducers.

In regulatory testing, the rodent erythrocyte MN assay is the most common *in vivo* assay (ICH, 2011). The MN assay is a simple method that uses either bone marrow or peripheral blood to assess cytogenetic damage *in vivo* (Heddle, 1973).

The MN test detects mutagenic substances that break chromosomes or that interfere with mitotic spindle formation, thus altering the equitable distribution of chromosomes during cell division (Flores and Yamaguchi, 2008), and it is a widely used tool for assessing the safety of a substance, for classifying substances as carcinogenic or non-carcinogenic (Fenech *et al.*, 1999).

The DNA damage was evaluated by comet assays and MN which are the two sensitive, rapid and extensively used tools for detecting the mutagenic and genotoxic effects of chemicals in the environment, since Micronucleus (MN) assay is an easy and an ideal monitoring system that uses aquatic organisms to assess the genotoxicity (Ali *et al*., 2009; Bucker *et al*., 2012). The micronuclei in erythrocytes are easily viewed

and are strong indicators for measuring chromosomal aberrations (Flores and Yamaguchi, 2008).

The term "micronucleus test" was first suggested by Boller and Schmid in 1970 and later by Heddle in 1977(Evans, 1997).

Several genotoxic substances are known to be mutagenic and carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of human tumors or cancers (Fagr *et al.,* 2008). These genotoxicants have been reported to cause mutations because they form strong covalent bonds with DNA, resulting in the formation of DNA adducts preventing accurate replication (Obiakor *et al*., 2012).

An *ex vivo*/ *in vitro* analysis of lymphocytes in the presence of cytochalasin-B (added 44 hours after the start of cultivation), an inhibitor of actins, allows to distinguish easily between mononucleated cells which did not divide and binucleated cells, which completed nuclear division during *in vitro* culture. Indeed, in such conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations, accumulated *in vivo* and the frequencies of binucleated cells with MN, a measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis (Fenech *et al*., 2003).

Cytochalasin B, the name which comes from the Greek *cytos* (cell) and *chalasis* (relaxation) (Scherlach., *et al* 2010). Is a cell permeable mycotoxin. It was found that sub stoichiometric concentrations of cytochalasin B strongly inhibit network formation by actin filaments. Due to this, it is often used in cytological research. It inhibits cytoplasmic division by blocking the formation of contractile microfilaments. It inhibits cell movement and induces nuclear extrusion (Theodoropoulos *et al.,* 1994).

Chapter Three

Materials & Methods

3 Materials and Methods

3.1 Materials

3.1.1 Essential equipment

The experimental equipment and their sources are given in the Table (3-1).

Table (3-1): The experimental equipment and their sources.

Name of Equipment	Company	Country
Autoclave	P-Selecta/Medic lane	Spain
Sensitive balance	Sartorius	Germany
Balance	Sartorius	Germany
Centrifuge	Beckman	England
Incubator	Binder	U.S.A.
Digital camera	Sony	Japan
Light microscope	Olympus	Japan
Water bath	Memmert	Germany
Eppendorf bench centrifuge	Bionee	Germany
Flow cytometry	Apogee	England
Fluorescent microscope	Olympus	Japan

3.1.2 Essential chemicals

The used chemicals and their sources are given in table (3-2).

Table (3-2): The used chemicals and their sources.

	Name of chemical	Company	Country
1	Apoptosis assay kit-FITC (KIT-ANXF)	ExBio	Czech
2	Cytochalasin B	Sigma	U.S.A
3	Deionized water	Promega	Germany
4	Dimethylsulfoxide (DMSO)	Sigma	U.S.A
5	Enzyme-linked immune sorbent assay kit for aromatase	Uscn Life Science Inc.	China
6	Ethidium bromide (EtBr)	Sigma	U.S.A
7	Ethylene diamine tetra acetic acid (EDTA)	Sigma	U.S.A
8	Glacial acetic acid	Fluka	Switzerland
9	Low melting agarose (LMA)	Sigma	U.S.A
10	Methanol	Sigma	U.S.A
11	Phosphate buffered saline (PBS) (Ca++, Mg++ free)	Sigma	U.S.A
12	Normal melting agarose (NMA)	Sigma	U.S.A
13	Sodium chloride (NaCl)	Merch	Germany
14	Tris-HCl	Merck	Germany
15	Trise base	Sigma	U.S.A
17	TritonX-100	Sigma	U.S.A

3.2 Laboratory animals

A total of 80 Swiss albino strain BALB/c mice (14 males and 66 females), obtained from the Animal House of Biotechnology Research Center at Al-Nahrain University, were used. Their age ranged between 8-12 weeks and weight from 23-27 g. Mice were divided into 22 groups, each group was housed in a separated plastic cage (3mice/cage). The cages were put in a room temperature of 23-25°C and the animals were given an adequate allowance of feed and water.

Diet was composed of the following ingredients, (Table 3-3).

Table (3-3): Diet constituents of mice.

Ingredient	%
Grinding barley	24.50%
Grinding wheat	30.00%
Grinding yellow corn	22.50%
Soya bean	15.20%
NaCl	0.45%
Animal protein	7.15%
CaCO3	0.20%
CP	15.85%
Calories	3426.7 Kca/kg

3.3 Preparation of drugs

3.3.1 Preparation of combined oral contraceptive pills (Yasmin)

COCPs (Yasmin) used in this study obtained from Bayer Schering Pharmaceuticals Company/Germany. The active materials in each tablet are drospirenone and ethinylestradiol, constituting 3 drospirenone and 0.03mg ethinylestradiol for both materials respectively. Two doses of pills were prepared as 30 and 60 µg/kg body weight of the experimental mice.

3.3.2 Preparation of Cafergot pills

Cafergot pill is a registered trademark of Novartis Pharmaceuticals, constituting of 1 ergotamin tartarat and 100mg caffeine. Two doses of pills were prepared as 10 and 20 µg/kg body weight of the experimental mice.

3.3.3 Preparation of wild carrot seeds oil

Wild carrot seed oil was obtained from Janat Al-Ashab Herbarium that imported the plant extracts from the world of herbs/ Bahrain. Carrot seed oil was steam distilled from dried fruit (seeds). Two volumes were prepared as 0.05 and 0.1 ml.

3.3.4 Preparation of Methotrexate

Methotrexate (MTX) pills were obtained from KG. Pharmaceuticals /Austria. constituting 2.5 mg methotrexate. One dose was prepared as a positive control, 50 µg/kg body weight of the experimental mice.

3.4 Methods

3.4.1 Administration of drugs in laboratory animals

3.4.1.1 Antifertility efficiency experiment

Thirty five mice were used in this experiment, including 14 males and 21 females. Animals were divided into seven groups, six experimental groups with one negative control group (2 males and 3 females/group). Each experimental group was treated with the prepared doses of COCPs (Yasmin), Cafergot pills and wild carrot seeds oil for 21 successive days. Two males were entered to each cage at the 3rd day of treatment. It aims to select the most suitable concentration of antifertility drugs with the highest contraceptive efficiency to be used in the next experiment.

Seven groups of mice were used for this experiment and treated as follows:

- Group I: Negative control (3 female mice and 2 male mice). Female treated with (0.1 ml) of normal saline.
- Group II: COCPs treatment (3 female mice and 2 male mice). Female treated with (0.1 ml) of COCPs (30 µg/kg).
- Group III: COCPs treatment (3 female mice and 2 male mice). Female treated with (0.1ml) of combined oral contraceptive pills (60 µg/kg).
- Group IV: Cafergot pill treatment (3 female mice and 2 male mice). Female treated with (0.1 ml) of Cafergot pill (10 µg/kg).
- Group V: Cafergot pill treatment (3 female mice and 2 male mice). Female treated with (0.1 ml) of Cafergot pill (20 µg/kg).

- Group VI: Wild carrot seed oil treatment (3 female mice and 2 male mice).

  Female, treated with (0.05 ml) of wild carrot seed oil.
- Group VII: Wild carrot seed oil treatment (3 female mice and 2 male mice).

  Female, treated with (0.1 ml) of wild carrot seed oil.

The drugs were given orally for 21 successive days, and then the mice were diagnosed for pregnancy after 21 successive days along with a first control group of mice.

3.4.1.2 Apoptosis experiment

Fifteen female mice were used in this experiment and were treated with the highest concentration of contraceptives COCPs, Cafergot pills and wild carrot seed oil for 5 successive days. The apoptosis percentage was examined using flow cytometry.

Five groups of mice were used for this experiment and treated as follows:

- Group I: Negative control (3 mice).

  Treated with (0.1 ml) of normal saline.
- Group II: Positive control (3 mice).

  Treated with (0.1ml) of MTX (50 µg/kg).
- Group III: COCPs treatment (3mice).

  Treated with (0.1 ml) of combined oral contraceptive pills (60 µg/kg).
- Group IV: Cafergot pill treatment (3 mice).

  Treated with (0.1 ml) of Cafergot pill (20 µg/kg).
- Group V: Wild carrot seed oil treatment (3 mice).

  Treated with (0.1 ml) of wild carrot seed oil.

The drugs were given orally for 5 successive days. Blood samples were taken from the mouse's heart, and the analyses of apoptosis were carried out as described later (See 3.6).

3.4.1.3 Alkaline comet assay (single cell gel electrophoresis) (SCGE)

Fifteen female mice in this experiment were treated with the highest and most effective dose as the contraceptive of COCPs, Cafergot pills and wild carrot seed oil for 5 successive days. The main aim of this experiment was to evaluate the DNA damage percentage of the mice bone marrow cells by the drugs using the comet assay.

Five groups of mice were used for this experiment and treated as follows:

- Group I: Negative control (3 mice).
  Treated with (0.1 ml) of normal saline.
- Group II: Positive control (3 mice).
  Treated with (0.1ml) of MTX (50 µg/kg).
- Group III: COCPs treatment (3mice).
  Treated with (0.1 ml) of COCPs (60 µg/kg).
- Group IV: Cafergot pill treatment (3 mice).
  Treated with (0.1 ml) of Cafergot pill (20 µg/kg).
- Group V: Wild carrot seed oil treatment (3 mice).
  Treated with (0.1 ml) of wild carrot seed oil.

The drugs were given orally for 5 successive days, and then the mice were sacrificed after 5 successive days along with the first group of control mice. Bone marrow samples were taken from mice, and the analyses of DNA damage percentage using comet assay were carried out as described later (See 3.7).

3.4.1.4 Cytogenetic analysis

3.4.1.4.1 Micronucleus test (MN)

Fifteen female mice in this experiment were treated with the highest and most effective dose as the contraceptive of COCPs, Cafergot pills and wild carrot seed oil for 5 successive days. The main aim of this experiment was to evaluate the acute treatment effect of drugs by applying cytogenetic analysis and the induction of MN in bone marrow cells.

Five groups of mice were used for this experiment and treated as follows:

- Group I: Negative control (3 mice).
  Treated with (0.1 ml) of normal saline.
- Group II: Positive control (3 mice).
  Treated with (0.1ml) of MTX (50 µg/kg).
- Group III: COCPs treatment (3mice).
  Treated with (0.1 ml) of COCPs (60 µg/kg).
- Group IV: Cafergot pill treatment (3 mice).
  Treated with (0.1 ml) of Cafergot pill (20 µg/kg).
- Group V: Wild carrot seed oil treatment (3 mice).
  Treated with (0.1 ml) of wild carrot seed oil.

The drugs were given orally for 5 successive days, and then the mice were sacrificed after 5 days along with the first group of control mice. Bone marrow samples were taken from mice, and the analyses of MN were carried out as described later (See 3.10).

3.5 Collection of human samples

3.5.1 Patients group

Twenty five patients using contraceptive pills of Yasmin type from Al-Karama Hospital was taken. Their age ranged between 25-35 years old with treatment duration between 3-8 years.

3.5.2 Healthy control group

Fifteen apparently healthy women who have not used the contraceptive pills were used. Their age ranged between 25-35 years old**.**

3.5.3 Blood sampling

Blood samples (5 ml) were collected by venipuncture from all patients and control groups. Blood was transmitted within few hours for comet assay, apoptotic analysis and within 24 hours for MN test using cooling container and stored in refrigerator at 4ºC. Samples for aromatase enzyme were kept in refrigerator freezer below -18 ºC, until all required samples were collected for one run. Each blood sample was divided into four tubes: heparinized tubes for apoptosis and MN, EDTA tubes for comet assay and serum tubes for aromatase enzyme activity.

3.6 Apoptosis

3.6.1 Annexin V-FITC (Fluorescein Isothiocyanate) detection

- **Principle**

Apoptosis is a regulated cell death process characterized by morphological and biochemical features occurring at different stages. One of the plasma membrane alterations during apoptosis was the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Detection of such membrane changes by Annexin V has been suggested as a suitable assay of early apoptotic cells as in (Figure 3-1).

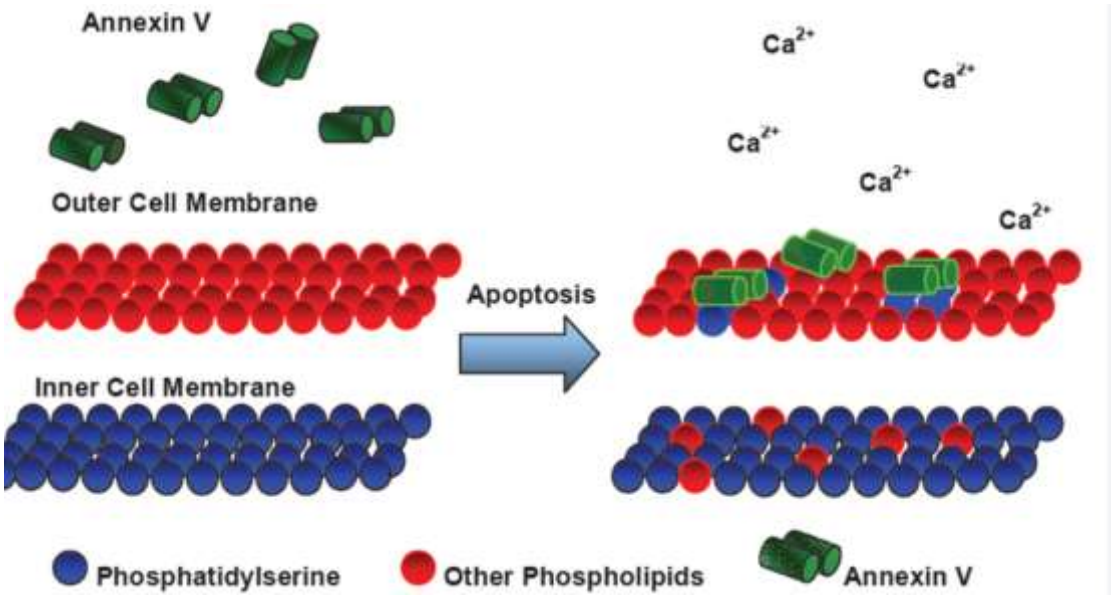


Figure (3-1): Schematic depiction of phosphatidylserine flipping during apoptosis and subsequent detection using Annexin V (Fadock *et al.*, 1992).

Annexin V was a 35 kDa protein and belongs to a member of a Ca^{2+} - depended phospholipids binding family of proteins. Fluorescently labeled Annexin V can be applied for direct quantification of apoptostic cell by using appropriate protocols. Since PS membrane translocation also occurs during necrosis, Annexin V was not an absolute marker of apoptosis.It was often used simultaneously with appropriate dyes such as propidium iodide (PI) to counter dead stained cells. Annexin V-FITC was employed in flow cytometry and performed according to the human Annexin V-FITC Kit-EXBio Praha. Reagents provided with the kit are binding buffer (10X concentrated), Annexin V-FITC, and PI (Dillon *et al.*, 2001; Jitkaew *et al.,* 2009).

3.6.2 Procedure

- **Preparation of reagent**
  - The Annexin V binding buffer was a (10x) concentrated and was diluted with deionized water prior to use in order to prepare 1x Annexin V binding buffer.

- **Procedure**

  1. Cells were harvested by centrifugation at 2,000 rpm for 5 min, the supernatant was discarded. Pellets were resuspended in cold PBS and cells were washed by gentle shaking or pipetting up and down. Re-centrifuged washed cells again and the supernatant was discarded.

  2. Pellet were resuspended with 1X binding buffer and a just cell density to 2-5 x 10^5 cells/ml. Preparing a sufficient volume of cell suspension (100µl/ assay).

3. Cells were stained with 5µl of Annexin V-FITC and (PI) and incubated for 15 minutes in the dark at room temperature.

4. After the incubation period, cells were harvested by centrifugation at 2,000 rpm for 5 min, pellets were resuspended in 100µl of binding buffer

5. The mixture was then analyzed by flow cytometry.

3.7 Alkaline comet assay (single cell gel electrophoresis) (SCGE)

The alkaline comet assay was used to investigate the possible DNA damage in peripheral blood lymphocytes from women used contraceptive pills previously in comparison with healthy controls. The comet assay was performed under alkaline conditions, according to Peggy and Banath (2006).

This protocol describes the single cell gel electrophoresis assay (also known as the comet assay) which is a simple, rapid and sensitive technique for analyzing and quantifying DNA damage in individual mammalian (and to some extent prokaryotic) cells. The more versatile alkaline method of the comet assay was developed by Singh and coworkers in 1988. This method was developed to measure low levels of strand breaks with high sensitivity.

The principle of the assay was based upon the cells, which were embedded in a thin agarose gel on a microscope slide. The cells were lysed to remove all cellular proteins and the DNA is subsequently allowed to unwind under alkaline/neutral conditions. Following unwinding, the DNA was electrophoresed and DNA stained with a

fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA) or relaxed chromatin migrates away from the nucleus. The extent of DNA liberated from the head of the comet is directly proportional to the DNA damage (Lovell and Omori, 2008).

3.7.1 Preparation of reagents

- **Agarose preparation**
    - **Low and normal melting point agarose**
    - Two water baths were equilibrated: one at 40 ºC and other at ~100 ºC.
    - 1% low melting point agarose was prepared by mixing powdered agarose (0.5g) with (50ml) distilled water in a glass beaker while 1.5% normal melting point agarose was prepared by mixing 0.75 g with 50ml distilled water.
    - Beakers were placed in the 100 ºC water bath (avoid vigorous boiling of the agarose and ensure that all agarose is dissolved).
    - Beakers were placed into a 40 ºC water bath.

- **Ethidium bromide Dye**

Ethidium Bromide (EtBr; 10X Stock - 20 µg/ ml): 10 mg was added in 50 ml dH_2O, store at room temperature 25 ºC. For 1X stock -1 ml mixed with 9 ml dH_2O.

- **Lysis buffer**
    - Ingredients per 1000 ml: 2.5 M NaCl 146.1 g.
    - 100 mM EDTA 37.2 g.
    - 10 mM Trise-base 1.2 g.

Ingredients were added to about 700 ml distilled water and begin stirring the mixture. PH was adjusted to 10.0 using 10 N NaOH and the volume completed with distilled water to 890 ml, fresh 1% Triton X-100 and 10% Dimethylsulfoxide (DMSO) will increase the volume to the correct amount.

- **Tris-Borate EDTA (TBE) electrophoresis solution**
  - Ingredients per 1000 ml: 10 mM Trise-base 10.8 g.
  - 100 mM EDTA 0.93 g.

The components were dissolved in 900 ml distilled water. The pH was adjusted to 13 (Alkaline pH) using 10 N NaOH and the volume completed with distilled water to 1000 ml and kept at 4^{o}C until used.

- **Lysis of human peripheral blood RBCs:**
  - The 10X RBC lysis buffer was diluted to 1X working concentration with distilled water. Warm the 1X solution to room temperature prior to use.
  - 5.0 ml of 1X RBC lysis buffer was added to each tube containing whole blood.
  - Gently vortex each tube immediately after adding the lysing solution. Incubated at room temperature, protected from light, for 10-15 minutes. Tubes were centrifuged at 1500 rpm for 2 min.

3.7.2 Preparation of samples and slides

1. Agarose slides were prepared by dipping the slides into normal molten 1.5 %(w/v) agarose.

2. Agarose was allowed to air dry to a thin film. Slides can be prepared ahead of a time and stored with a desiccant.

3. Slides were labeled on the end using a pencil, not a pen.

4. The cell suspension was centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the pellet washed once with ice-cold PBS (without Mg^{2+} and Ca^{2+}) and centrifuged at 1500 rpm for 2 min. Then supernatant was discarded.

5. A cell sample was combined with low melting point agarose at 1:10 ratio (V/V) and the mixture (75µl/ well) immediately was added into slide comet by pipette.

6. The slides were held horizontally, then transferred to 4ºC in a dark container for 30 min.

7. The slide was transferred to a small basin containing pre chilled lysis buffer, the slide was immersed in the buffer overnight (18-20 h) at 4ºC in the dark.

8. After overnight, the slides were immersed in an electrophoresis solution for 20 min.

9. The slides were holding horizontally, and then transferred to a horizontal electrophoresis chamber filled with a cold TBE electrophoresis solution, 24volt (V) /cm and 300 milliamps (mA) was applied to the chamber for 18 min.

10. The TBE electrophoresis solution was aspirated from the chamber and replaced with neutralization buffer, 0.4M of Tris-HCl solution (pH 7.5) for 5 min in order to neutralize the cells.

11. Diluted ethidium bromide dye 50 µl was added to each well of comet assay slide and incubated at room temperature for 15 min.

12. The slides were rinsed with distilled water to remove excess stain.

13. The slides were examined by fluorescence microscopy.

At least 50 randomly selected cells should be analyzed per sample. The quantification was done by using image analysis software comet score, the analysis software will calculate different parameters for each comet, three parameters were estimated to indicate DNA migration, tail length (distance from the head center to the end of the tail), mean tail moment and % DNA in tail (Azqueta *et al.,* 2009).

3.8 Aromatase enzyme

The kit is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of aromatase in human serum, plasma, tissue homogenates and other biological fluids.

- **Test principle**

The microtiter plate provided in this kit had been pre-coated with an antibody specific to aromatase. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to aromatase. Next, avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated. After the 3,3',5,5'-Tetramethylbenzidine or TMB substrate solution was added, only those wells that contain aromatase, biotin-conjugated antibody and enzyme conjugated avidin will exhibit changes in color. The enzyme – substrate reaction was terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of aromatase in the samples was then determined by comparing the optical density (O.D.) of the samples to the standard curves.

3.8.1 Samples collection and storage

A serum separator tube was used and samples allowed clotting for two hours at room temperature or overnight at 4 ºC before centrifugation for 20 minutes at approximately 1000xg. Assay freshly prepared serum immediately or store samples in aliquot at -20 ºC or -80 ºC for later use.

3.8.2 Reagent preparation

All kit components were provided from USCN China Life Science Company.

1. All kit components and samples brought to room temperature (18-25 ºC) before use.
2. Standard-reconstitute: the standard with 1ml of standard diluent were kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution was 100ng/ml. Firstly, the stock solutions were diluted to 10ng/ml and the diluted standard serves as the highest standard 10ng/ml. Then 7 tubes were prepared containing 0.5ml standard diluent and the dilutes standard were used to produce a double dilution series. Each tube was mixed thoroughly before the next transfer . 7 points of diluted standard were set up such as 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml,0.156ng/ml, and the last tubes with standard diluent was the blank as 0ng/ml as in (Figure 3-2).

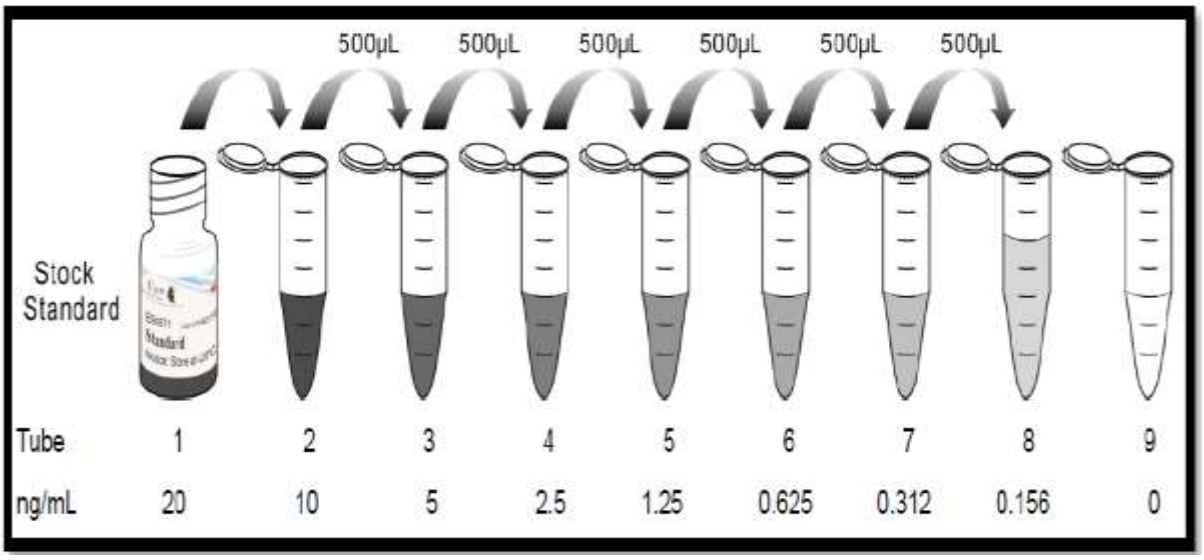


Figure (3-2): The dilution of stock standard.

3. Detection reagent A and detection reagent B: the stock detection A and detection B were centrifuged before use. The working concentration were diluted with assay diluent A and B, respectively (1:100).
4. Wash solution: 20ml of wash solution concentrate (30x) was diluted with 580ml of deionized or distilled water to prepare 600ml of wash solution (1x).
5. TMB substrate: the needed dosage of the solution was aspirated with sterilized tips and do not dump the residual solution into the vial again.

3.8.3 Procedure

1. Wells for diluted standard, blank and samples were determined. 7 wells were prepared for standard, 1 well for blank. 100µl was added to each of dilutions standard, blank and samples into the

appropriate wells. Covered with plate sealer and incubated for 2 hours at 37 ºC.

2. The liquid of each well was removed, didn't wash.
3. 100µl of detection reagent A working solution was added to each well and incubated for 1 hour at 37 ºC after covering it with the plate sealer.
4. The solution was aspirated and washed with 350µl of 1x wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto washer, and was let sit for 1-2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. Totally washed 3 times. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plat was inverted and plotted against absorbent paper.
5. 100µl of detection reagent B working solution was added to each well. Incubated for 30 minutes at 37 ºC after covering it with the plate sealer.
6. The aspiration wash process was repeated for total 5 times as conducted in step 4.
7. 90µl of substrate solution was added to each well. Covered with a new plate sealer and incubated for 15-25 minutes at 37 ºC (don't exceed the 30 minutes). Protected from light. The liquid will turn blue by the addition substrate solution.
8. 50 µl of stop solution was added to each well. The liquid will turn yellow by the addition stop solution. The liquid was mixed by tapping the side of the plate. If the color change doesn't appear uniform, gently tap the plate to ensure thorough mixing.

9. Any drop of water and fingerprint was removed on the bottom of the plate and confirm there was no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

3.9 Micronucleus assay in human blood lymphocytes culture

- **Principle (Fenech, 2000)**

Cytokinesis Block Micronucleus Assay (CBMA) in peripheral blood lymphocytes was used as a useful technique to evaluate cytogenetic damage. The *in vitro* MN assay is a mutagenic test system for the detection of chemicals that induce the formation of small membrane bound DNA fragments.

The purpose of the MN assay was to detect those agents that modify chromosome structure and segregation in such a way leading to the formation of micronuclei in cells at interphase phase. The principle of the test method is by adding cytochalasin B to the cell cultures. This block cytokines in cultured cells, which in turn allows cells to grow for a reasonable period of time. Consequently, this will allow for chromosomal damage leading to the formation of micronuclei in bi- or multinucleated cells at interphase. Harvested and stained these cells are then analyzed microscopically for the presence of micronuclei.

3.9.1 Preparation of stock solutions and media

3.9.1.1 PBL medium (PAA, 2013)

Modified RPMI 1640, Phytohaemagglutinin (PHA) high quality, pre - tested FBS, buffered with HCO3 with the antibiotics penicillin and streptomycin and L-Glutamine, were purchased from PAA Company and kept in a deep freeze at -20 ºC until use.

3.9.1.2 Hypotonic solution (0.075 M KCl)

The hypotonic solution (0.075 M KCl) was prepared by dissolving 2.8 g of KCl in 500 ml distilled water (D.W.).

3.9.1.3 Fixative solution

Three volumes of methanol were mixed with one volume of glacial acetic acid; the solution was prepared instantly before use.

3.9.1.4 Giemsa stain

Giemsa powder (2g) was dissolved in 100 ml of absolute methanol, stirred for two hrs at 60°C on a hot plate, then mixed well and filtered using Watman No.1. Aliquot of 4 ml of Sorenson buffer was then added to 1 ml of the stock solution. The stock solution was kept in a dark bottle at room temperature till use.

3.9.1.5 Cytochalasin B solution

It was prepped by dissolving 5mg of cytochalasin B powder in 1ml (DMSO) and then stored at -20^{0}C and divided to aliquots of 0.1ml and placed in Eppendorf tubes. The volume was completed to 1ml before use and added to the medium to give a final stock concentration of 500µg /ml, then stored at -20^{0}C.

3.9.1.6 Trypsin solution

It was prepared by dissolving 0.25 g of trypsin powder in 100ml of PBS, then distributed into sterile aliquots and kept on –20ºC till use.

3.9.2 Assay procedure

The experimental design was implemented according to Fenech (2000) as follows:

1. The culture tubes were prepared by placing 8ml of Quantum PBL medium in each tube.

2. An aliquot of 0.8ml of blood sample was added to each culture tube.
3. The contents of each culture tube were mixed gently by inverting for a few minutes, the culture tubes were then incubated for 44hrs at 37^0C in a slant position (this position creates more surface area between the liquid and gaseous phases and allow cells to settle over a larger area of the culture tubes which provide optimal culture conditions for cell growth and proliferation).
4. Aliquot of 600µl Cytochalasin B was then added to each culture at a 3µg/ml concentration to block cells cytokinesis and cultures re-incubated at 37^0C for 28 hrs. before cells harvest as in (Figure 3-4).

Note: All these steps were done under aseptic conditions.

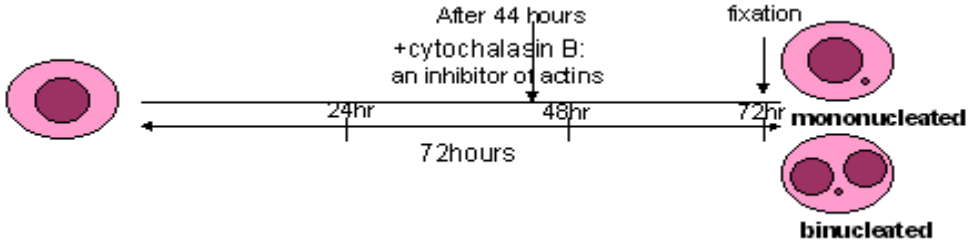


Figure (3-4): Cytochalasin-B (added 44 hours after the start of cultivation) (Fenech *et al*., 2003).

3.9.3 Cell harvest (Micale, 2010)

1. After 71.5hrs, the culture tubes were centrifuged at 10000 *rpm* for 10 mins.
2. The supernatant was discarded by pipetting the medium, leaving a little medium as possible over the cell pellet.
3. Cell pellets were re-suspended in 10ml of hypotonic warm solution of 0.075M KCl and incubated for 30min. in a water bath at $37\ ^0$C.

4. Tubes were centrifuged at 10000 rpm for 10 min.
5. The supernatant was discarded and the pellets were disturbed thoroughly by tapping on the bottom of the tubes, and the pellet was re-suspended in 5ml of fixative solution then the tubes were kept in a refrigerator for about 24hrs.
6. Again, the tubes were centrifuged; the supernatant was discarded. This step was repeated for three more times.
7. After a final centrifugation, the supernatant was aspirated and 1ml of the precipitate was shaken very gently, then the samples were dropped on clean slides from 30cm, the slides were left to dry then labeled.

3.9.4 Slides staining and preparation (Shapiro and Mandy, 2007)

1. Four Jars were prepared for staining:
   - The first jar was containing 10ml of trypsine solution at a concentration of 0. 25%, mixed with 90ml of normal saline.
   - The second jar contained 10ml of PBS pH 7.2, mixed with 90ml normal saline.
   - The third jar was consisted of 10 ml PBS pH 7.2, mixed with 90ml normal saline.
   - The fourth jar contained 10ml of Giemsa stain solution, mixed with 90ml normal saline.

2. A pair of slides was held with a forceps, placed in the first jar and shaken, then the second and so on. Slides were shaken in 1st jar that contained trypsine depending on antiquation time (one day equals one second).

3. The step 2 was repeated for the 2nd and 3rd jars and placed in the 4th jar that contained Giemsa stain for 7min., and then the slides were put on filter paper to dry.

3.10 Micronucleus assay in mouse bone marrow cells

The experiment was done according to Schimd, (1975) as follow:

1. The animals were scarified by cervical dislocation.
2. The animal was fixed on its ventral side on the anatomy plate, the abdominal side of the animal and its thigh region was swapped with 70% ethanol.
3.The thigh bone cleaned from tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe, (2ml) of human plasma was injected so as to wash and drop the bone marrow in the test tube.
4. The test tubes were centrifuged at speed of 1000rpm for (10 min).
5. The supernatant was removed, and one drop from the pellet was taken to make a smear on a clean slide. The slides were kept at room temperature to dry.
6. The slide were fixed with absolute methanol for 5 minutes, and then stained with Giemsa stain for 15 minutes, and left to dry. Finally washed slides with distilled water and leave it to dry.
7. Two slides for each animal were prepared for MN test.

3.11 Cytogenetic analyses test

3.11.1 Assessment of micronucleus frequency

At least 1000 cells were scored to assess the frequency of MN, the cells were classified as mononucleate, binucleates, trinucleates or titranucleates for human and 1000 cells were also scored to assess the frequency of mononucleate MN in mice (Kirsch-Volders *et al.*, 1997).

MN= [1(MN1) +2(MN2) +3(MN3) +4(MN4)/N]

MN, 1, 2, 3, 4=Number of micronucleus in cells.

N= Total number of cells (Lamberti *et al.*, 1983).

3.12 Statistical Analysis

Analysis of variance was performed to test whether group variance is significant or not, the comparison between groups were conducted using statistical package for the social sciences (SPSS) program.

Statistical model:

Yij= M + Ti+ eiJ

Where

Yij = Different of observations (comet assay, apoptosis, micronucleus and aromatase enzyme)

M=overall mean

Ti=Effect of treatment (Yasmin, Cafergot, wild carrot seeds oil and methotrexate)

erJ=error term

The chi-square test was used to compare among rates is Duncan.

Chapter Four

Results & Discussion

4 Results and Discussion

4.1 Antifertility drugs efficiency experiment

Rising human population throughout the world in developing and underdeveloped countries has detrimental effects on the life supporting system on earth, so this study attempts to discuss the potentiality of medicinal plants (wild carrot seed oil), fungal alkaloids (Cafergot) and combined oral contraceptive pills (Yasmin) as a source of contraceptive principles in females.

The results indicated that the lower ($P \leq 0.01$) pregnancy rates (0%) were noticed in two COCPs groups (0.05 and 0.1 ml). Similarly, lower ($P \leq 0.01$) pregnancy rates (0%) were also observed in high dose of Cafergot group (20µg/kg) as well as in both wild carrot seed oil groups. Negative group exhibited greater ($P \leq 0.01$) pregnancy rates (66.66%) followed by low dose of Cafergot (10µg/kg) 33.33% (Table 4-1).

Table (4-1): Effects of contraceptive pills (Yasmin), Cafergot and wild carrot seeds oil on female mice pregnancy rate (%).

Groups	No. of male mice	No. of treated female (21 days)	No. of pregnant	Pregnancy rate (%)
Negative control	2	3	2	66.66
Contraceptive pills (30 µg /kg)	2	3	0	0
Contraceptive pills (60 µg /kg)	2	3	0	0
Cafergot (10 µg/kg)	2	3	1	33.33
Cafergot (20 µg/kg)	2	3	0	0
Wild carrot seeds oil (0.05 ml)	2	3	0	0
Wild carrot seeds oil (0.1 ml)	2	3	0	0
Chi-square value and level of significance				** 12.528

**** P≤0.01**

Combined hormonal contraceptives consist of an estrogen and a progestogen, and act primarily by preventing ovulation through the inhibition of FSH and LH. Another important strategy that hormonal contraceptives interfere with conception is progestogen component renders the cervical mucus relatively impenetrable to sperm and reduces the receptivity of the endometrium to implantation. These mechanisms render combined hormonal contraceptives very effective in the prevention of pregnancy.

Annual failure rates vary between 0.02% (two per 10.000 women /year) when full adherence to instructions for use is assumed (Ketting, 1988) and 5% for typical use (Fu *et al.,* 1999).

Plants are the source of medication for preventive, curative and protective purposes (N'guessan *et al.,* 2009). Chaudhury and Hag (1980 a), listed 11 plants with 100% antifertility activity in one species or the other viz., *Aristolochia indica, Curcuma longa, Cuminum cyminu, Daucus carota, Embelia ribes, Ensete superbum, Hyptis suaveolens, Mentha arvensis, Podocarpus brevifolia, Polygononum hydropiper* and *Sapindus trifoliatus*.

Evidence suggests that terpenoids in the seed block crucial progesterone synthesis in pregnant animals and some herbalists have described it as having the effect of making the uterus "slippery" so the egg is unable to implant. Progesterone is essential for pregnancy to occur, progesterone's function is to prepare the uterine endometrium to receive an egg, if the endometrium isn't ready, then the egg will find implantation very difficult. If the egg can't implant then the opportunity is missed, and the egg begins to breakdown and is no longer viable and menstruation arrives as usual (Riddle, 1997).

Sethi *et al.* (1990) administered the alcoholic extract of *Daucus carota* seed at different doses ranging from 50 to 250 mg/kg. bw after

coitus and showed a significant antifertility effect which was dose- dependent. The administration of this extract at a lower dose showed anti-implantational activity, whereas higher doses caused fetus resorption. Another study introduced by Garg and Garg (1971a) reported encouraging antifertility activity in the alcoholic and aqueous extracts of the seeds of this herb in female albino rats.

Ergot extract has been used in pharmaceutical preparations, including ergot alkaloids (EAs) in products such as Cafergot (containing caffeine and ergotamine (Black and Halmer, 2006). Caffeine is sometimes included oral and a rectal preparation of ergotamine to improve the latter's absorption (Martindale, 2010).

EAs have a number of effects on the reproductive process, including the prevention of pregnancy by interfering with implantation, embryotoxicity, developmental effects and inhibition of lactation. Subsequent studies confirmed inhibition of ovulation effect occurred in mice and hamsters and suggested that the alkaloids did not have a direct toxic effect, but interfered with implantation by inhibiting release of prolactin from the pituitary (Griffith *et al.*, 1978).

Ergotamine tartrate has a number of well-established effects on pregnant rats when orally injected with 10 mg/kg b.w. resulted in characteristic anomalies (shortening or absence of nails, phalanges and digits) (Schön *et al.,* 1975). In a subsequent study, women who may become pregnant or at risk from therapeutic doses of ergotamine tartrate because of its effect on the uterus (causing contractions) (Martindale, 2010).

4.2 Effect of different drugs on apoptosis in mice lymphocytes

Differentiating between apoptotic and necrotic cells was carried out using Annexin V-FITC kit. The different labeling patterns in this assay identify the different cell populations, e.g., region A: necrotic cells (PI-positive/annexin V-negative); region B: late apoptosis (secondary necrosis) (PI-positive/annexin V-positive) region C: vital cells (PI-negative/ annexin V-negative); region D: apoptotic cells (PI-negative/annexin V-positive).

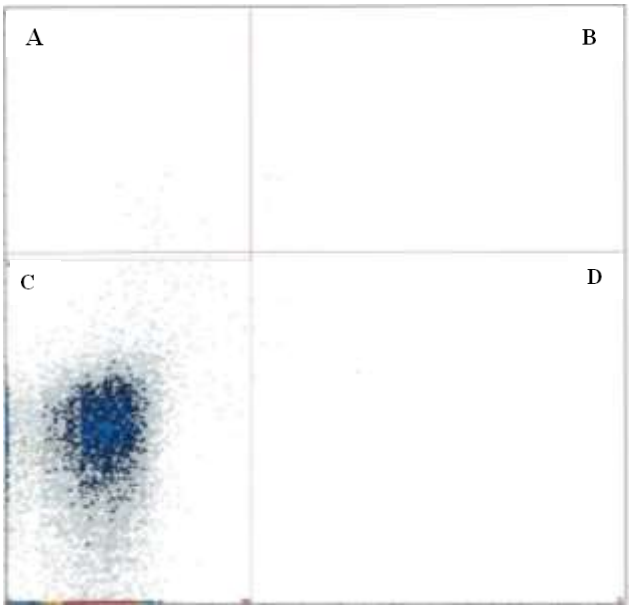
Results did revealed that the percentage of apoptosis using wild carrot seed oil were 0%, being the least value as compared with the other contraceptives and equal to the negative control 0%. On the other hand, there were no significant differences in the percentage of apoptotic cells with Cafergot treatment (0.1%) in comparison with the negative control. Yasmin pills treatment led to a significant increase ($P \leq 0.05$) in apoptotic percentage of 0.63%. This response also significantly increased ($P \leq 0.05$) in MTX treatment reaching 1.8%. These results indicated that the oral contraceptive pills (Yasmin) are the most inducing contraceptive drugs to apoptosis while Cafergot and wild carrot seed oil are less inducing as compared with the negative and positive control (Table 4-2, figure 4-1 and appendix 1).

Higher ($P \leq 0.05$) viable cells were noticed in Cafergot (99.54%) and wild carrot seed oil (99.6%) being similar with negative control (99.66%) and greater than COCPs (97.83%) and MTX group. The MTX group exhibited higher ($P \leq 0.05$) necrotic cell percentage (1.6%) followed by COCPs group (1.34%) as compared with the other groups. The differences among groups in late apoptotic cell percentage lacked significance (Table 4-2).

Table (4-2): Apoptosis percentage of lymphocytes isolated from mice by flow cytometry (Mean±SD).

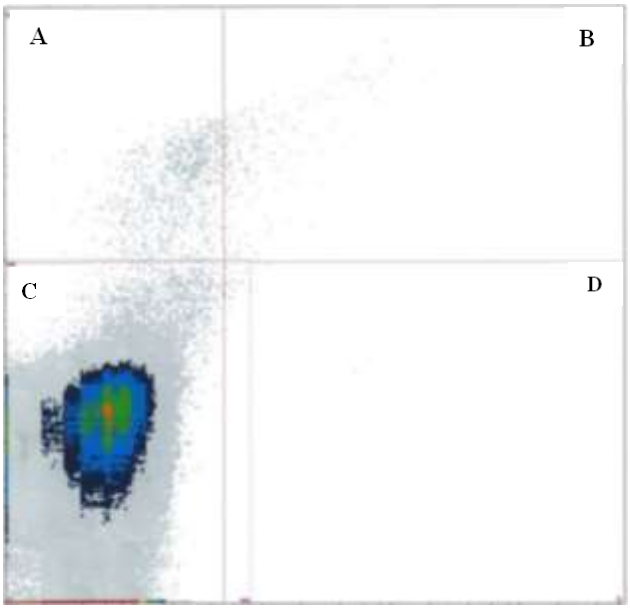
Groups	Necrosis (%)	Late apoptosis (%)	Viable (%)	Apoptosis (%)
Negative control	B 0.24±0.02	A 0.1±0.01	A 99.66±1.25	C 0.00±0.00
Positive control Methotrexate (50 µg/kg)	A 1.6±0.1	A 0.4±0.01	C 96.2±2.4	A 1.8±0.14
Contraceptive pills (60 µg /kg)	A 1.34±0.02	A 0.2±0.005	B 97.83±1.55	B 0.63±0.09
Cafergot (20 µg/kg)	B 0.26±0.01	A 0.1±0.01	A 99.54±3.17	C 0.1±0.01
Wild carrot oil (0.1ml)	B 0.24±0.01	A 0.16±0.02	A 99.6±2.00	C 0.00±0.00

Means with different superscripts within each column significantly different (P≤0.05).



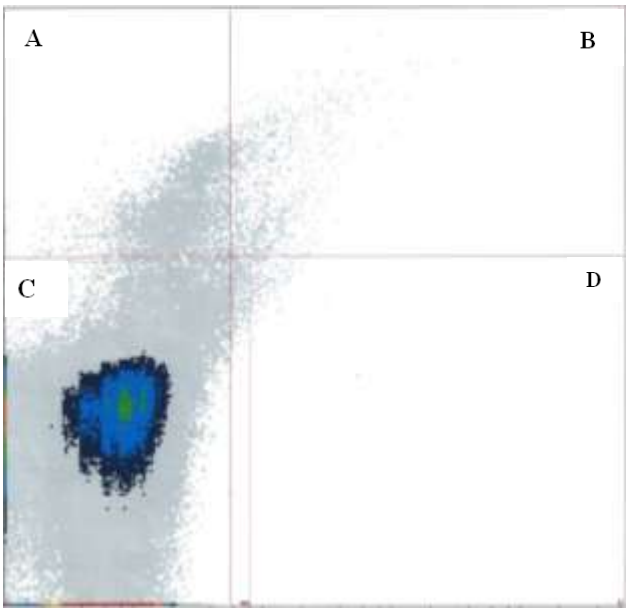
A

FL1-FL2-A	0.1%
FL1-FL2-B	0.0%
FL1-FL2-C	99.9%
FL1-FL2-D	0.0%



B

FL1-FL2-A	0.3%
FL1-FL2-B	0.1%
FL1-FL2-C	99.6%
FL1-FL2-D	0.0%



C

FL1-FL2-A	1.4%
FL1-FL2-B	0.3%
FL1-FL2-C	98.2%
FL1-FL2-D	0.1%

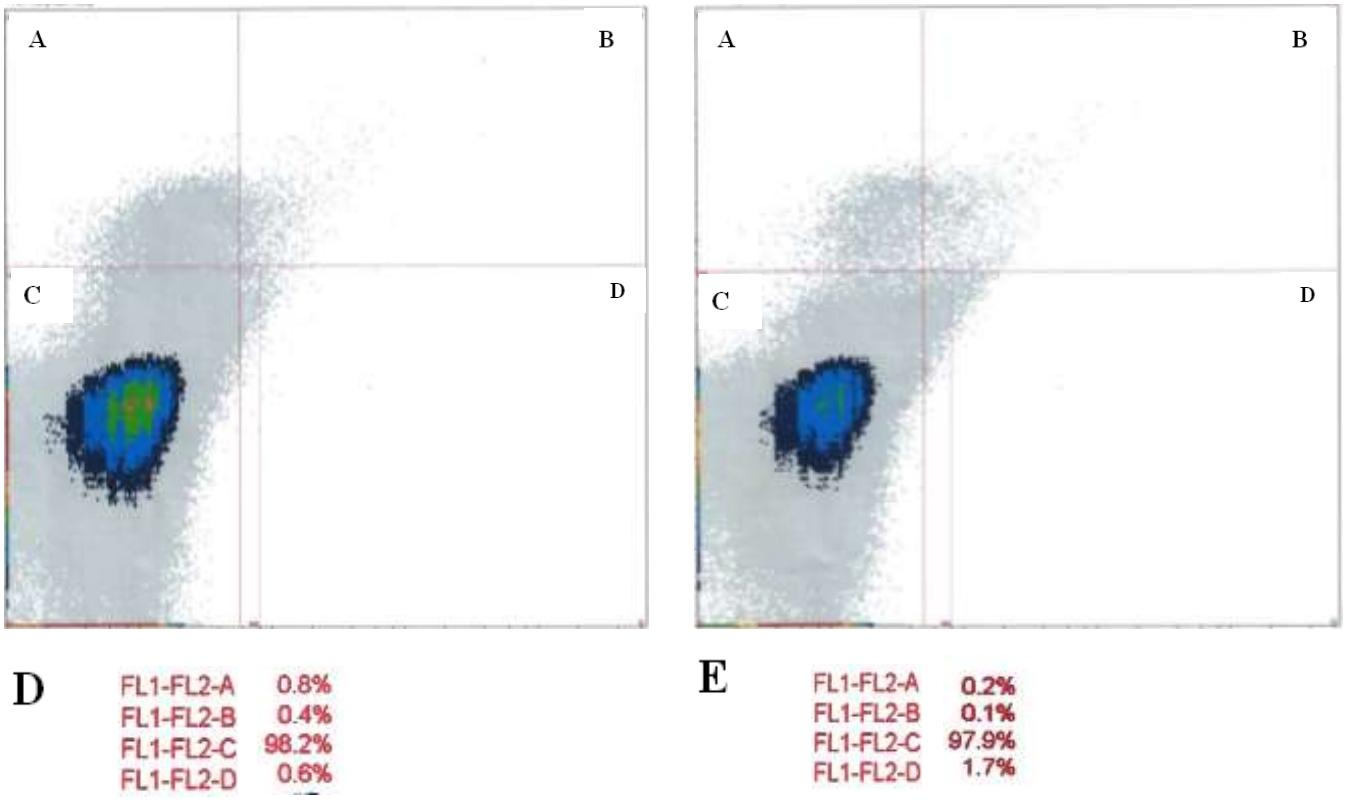


Figure (4-1): Annexin V expression for mice induced apoptosis analysis. The labeling patterns inside quadrants indicate the percentage of A: necrotic cells (PI- positive/ Annexin V- negative); B: late apoptosis (PI-positve/ Annexin V-positive); C: Vital cells (PI-negative/ Annexine V-negative); D: apoptotic cells (PI-negative/ Annexine V- positive). The labeling patterns outside quadrants indicate the percentage of apoptotic cells in control (A), wild carrot seed oil (B), Cafergot (C), contraceptive pills (D), and MTX (E).

Most oral contraceptive pills contain a combination of estrogen (ethinylestradiol or mestranol) and progestin (Wilson *et al*., 2012). Concerning the progestin component formulations with a dose equivalent to 0.30 mg norgestrel or more were categorized as high-progestin, while formulations with less than 0.30 mg norgestrel or its equivalent were categorized as low-progestin formulations. Yasmin contraceptive pills were belong to high-progestin formulations. An experimental study by Rodriguez *et al.* (1998) revealed that progestin induced apoptosis in the ovarian epithelium of macaques, leading to the hypothesis that the progestin component may be the major effect of combined oral contraceptives. Another study achieved by Gustavo *et al.* (2002) on monkeys showed a few apoptotic cells were noted in the ovarian epithelium from either the control or estrogen-only-treated monkeys. In contrast, in progestin-treated monkeys, either those treated with combination ethinyl estradiol and levonorgestrel or with levonorgestrel alone, the ovarian epithelium contained numerous brown-staining apoptotic cells.

Excessive levels of gonadotropic hormones such as FSH and LH are also suggested to increase cell growth and inhibition of apoptosis (Konishi, 2006). Oral contraceptives are known to suppress pituitary gonadotropin secretion. The primary mechanism of combined oral contraceptives is prevention of ovulation (Cibula *et al*., 2010). Combined oral contraceptives cause anovulation by inhibiting production and secretion of both FSH and LH through steroid feedback on the pituitary gland. The estrogen component inhibits the release of FSH thereby suppressing the development of the ovarian follicle, while the progestin component inhibits the release of LH whereby ovulation is prevented (Rivera *et al*., 1999).

At the cellular level, MTX is now firmly established to be a folate antagonist that inhibits DNA and protein synthesis by inhibiting, respectively, thymidylate synthetase (TYMS) and dihydrofolate synthetase (DHFR), as crucial enzyme for folate metabolism and purine synthesis, resulting in lymphocyte apoptosis (Genestier *et al*., 1998). Further evidence supporting such results observed by Wessels *et al*. (2008) who showed that MTX also inhibits T cell activation and induces T cell apoptosis.

4.3 Measurement of DNA damage of three drug types on mouse bone marrow by comet assay

Comet assays are one of the most common tests for genotoxicity. The technique involves lysing cells using detergents and salts. The DNA released from the lysed cell was electrophoresed in an agarose gel under alkaline pH conditions. Cells containing DNA with an increased number of double-strand breaks will migrate more quickly to the anode. This technique is advantageous in that it detects low levels of DNA damage, requires only a very small number of cells, cheaper than many other techniques, easy to execute, and quickly displays results. However, it does not identify the mechanism underlying the genotoxic effect or the exact chemical or chemical component causing the breaks (Tice, 2000).

Levels of DNA damage were determined using the alkaline comet assay. DNA damages were quantified by measuring the displacement between the genetic material of the nucleus and the resulting tails using an image analysis system. Three parameters were used as an indicator of DNA damage: i.e. tail length, percentage of DNA in the tail and tail moment. The current results of an alkaline comet assay in mouse bone

marrows handling with three contraceptive types were summarized in (Table 4-3).

Table (4-3): Tail length, tail moment and DNA% in the tail of mice lymphocytes (comet assay) treated with different types of contraceptives (Mean±SD).

Groups	Tail length	Tail moment	%DNA in tail
Negative control	**D** 2.8 ± 0.08	**E** 0.02 ± 0.004	**D** 1.09± 0.012
Positive control Methotrexate (50 µg/kg)	**A** 17.5±0.57	**A** 29.8±0.23	**A** 19.14±0.07
Contraceptive pills (60 µg /kg)	**B** 12.18 ±0.21	**B** 25.04 ± 0.17	**B** 15.08±0.07
Cafergot (20 µg/kg)	**C** 6.18± 0.06	**C** 5.09± 0.01	**C** 6.18±0.04
Wild carrot oil (0.1ml)	**E** 1.00± 0.21	**D** 0.36 ± 0.03	**E** 0.22±0.04

Means with different superscripts within each column differ significantly (P≤0.05)

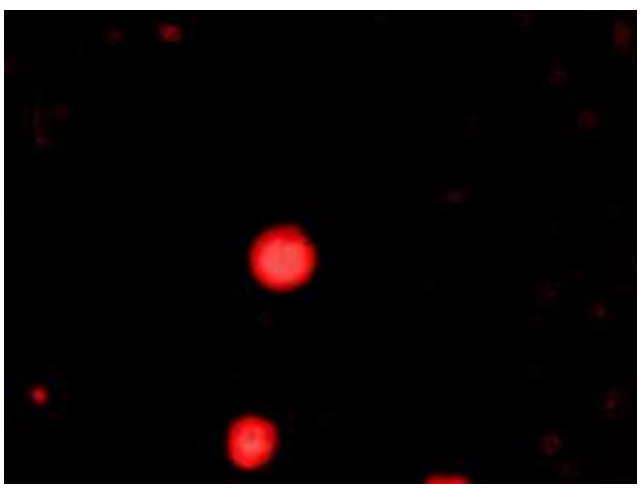
Significant (P≤0.05) differences were observed among groups in tail length, being taller in (MTX) group (17.5%), followed by COCPs (12.18%) and lower in wild carrot seed oil group (1%), (Table 4-3 , figure 4-2 and appendix 2).

The tails moments for the exposed group with contraceptive pills were 25.04%, while in Cafergot and wild carrot seed oil were 5.09% and 0.36% consequently. The observed values differed significantly (P≤0.05)

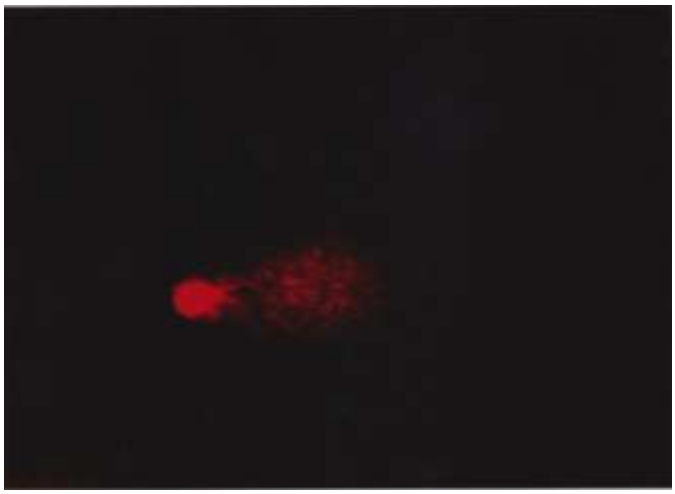
from those in negative control 0.02%, and positive control (MTX) 29.8%, (Table 4-3, figure 4-2 and appendix 2).

The percentage of DNA in the tails were (15.08%) in COCPs while in Cafergot and wild carrot seed oil were (6.18%) and (0.22%) respectively. In control the percentage of DNA in the tails were (1.09%) and (19.14%) in positive control (MTX). Differences observed between the exposed and control group were significant ($P \leq 0.05$), (Table 4-3, figure 4-2 and appendix 2).

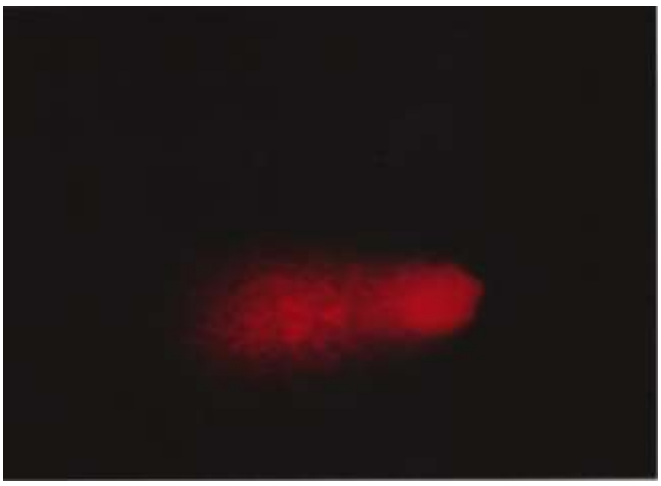
Kopjar and Garaj-Vrhovac (2001) used the alkaline comet assay to evaluate the genotoxicity towards peripheral lymphocytes of medical personnel regularly handling various drugs, including MTX.



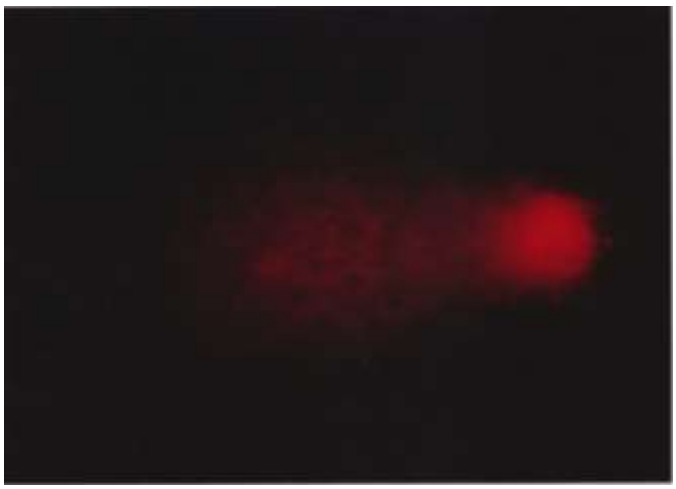
(A)



(B)



(C)



(D)

Figure (4-2): Comet assay in mice bone marrow cells examined by florescent microscope (400X) of the control group (A), showing fluorescent spheres without DNA damage (no tail), Cafergot group (B), contraceptive pills group (C), and MTX group (D), showing a lot of fluorescent heads with tails indicating DNA damage (ethidium bromide stain).

4.4 Evaluation of micronucleus of different drug types on mouse bone marrow

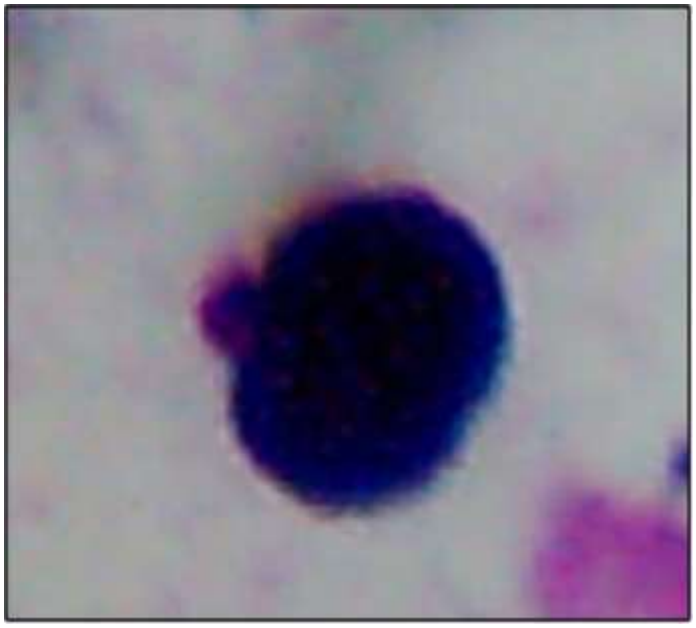
The micronucleus assay is based on the loss of chromosomes or chromosome fragments during meiosis, which are not reincorporated into the nucleus after cell division and, therefore, are transformed into a smaller nucleus or micronucleus (Grisolia, 2002). The induction factors were calculated by computing the percentage of micronuclei from exposed cells to the percentage of micronuclei in the negative controls. Results were recorded as the percentage of cells containing micronuclei compared to the total number of counting cells. Defining the micronucleus frequency was done by analysis of 1000 mononucleate cells of each mouse as shows in (Table 4-4).

Table (4-4): Micronucleus frequency of mouse bone marrow (Mean±SD)

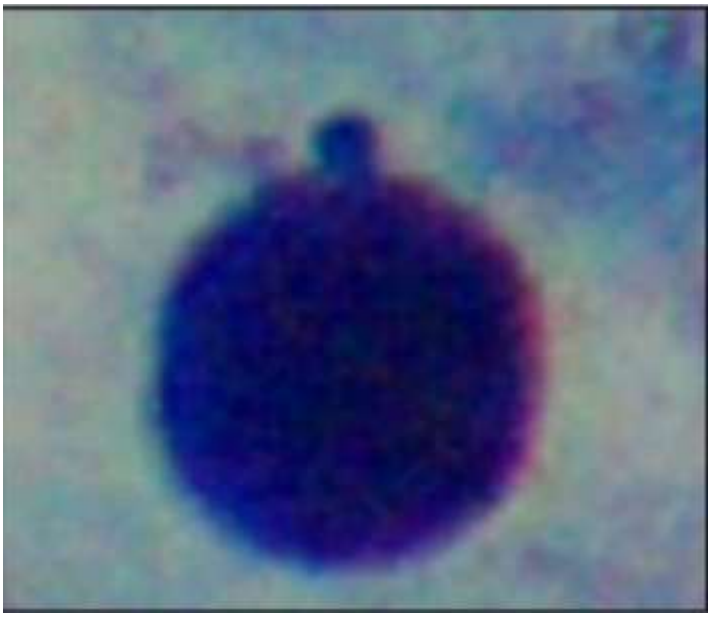
Groups	MN%
Negative control	**C** 4.25 ±2.58
Positive control Methotrexate (50 µg/kg)	**A** 14.5±5.77
Contraceptive pills (60 µg /kg)	**B** 10.75±9.57
Cafergot (20 µg/kg)	**D** 3.5±5.77
Wild carrot oil (0.1ml)	**D** 2.75±5.00

Means with different superscripts within each column differ significantly (P≤0.05)

MN was investigated following the orally administered by contraceptives in mice. The results indicated that wild carrot seeds oil and Cafergot recorded less ($P \leq 0.05$) percentage of micronuclei as compared with other groups being 2.75% and 3.5% respectively. In contrast, MTX recorded greater ($P \leq 0.05$) percentage of micronuclei namely 14.5%, followed by COCPs group 10.75% (Table 4-4, figure 4-3 and appendix 3).



(A)



(B)

Figure (4-3): Micronucleus from mice bone marrow of contraceptive pills (A) and MTX (B) by light microscope (100 X).

Further evidence supporting such results observed in mouse bone marrow by Al-Khraquly (2006) who showed a significant increase in MN rate in three groups of mice treated with OCP.

A series of published studies reported that steroid hormones induced dose-dependent micronuclei increases and aneuploidy (Ahmed *et al*., 2000). Gestogens as steroid hormones affect target cells by binding their steroid receptors (Klotzbucher *et al*., 1997). Then hormone-receptor (H-R) complex interacts directly with DNA chromatin. Consequently, H-R complexes act until target cells achieve free receptors while the dose dependent increase of MN frequency appears with the increase of therapeutic gestogen doses.

The results of the study reported by Shahin *et al*. (2001) clearly showed that MTX is a genotoxic drug and the frequency of micronuclei was significantly increased in bone marrow cells as well as in peripheral blood cells of MTX-treated rats and rheumatoid arthritis patients, respectively. Moreover, several *in vivo* studies have indicated that multiple doses of MTX induced more micronuclei when compared to a single treatment in male mice. The MTX-induced micronuclei formation might be explained by the intracellular accumulation of the drug resulting in a continuous inhibition of deoxyribonucleotide triphosphate (dNTPs) synthesis, subsequently causing genetic lesions due to the inhibition of DNA repair (Kasahara, 1992).

Some investigations showed that MTX could induce increased frequencies of micronulei and CAs in both rats and humans (Shahin *et al*., 2001).

4.5 Assessment of apoptosis in women using oral contraceptives

Differentiating between cells of either apoptosis or necrosis was carried out using Annexin V-FITC kit. The different labeling patterns in this assay identify the different cell populations, e.g., region A: necrotic cells (PI-positive/annexin V-negative); region B: late apoptosis (secondary necrosis) (PI-positive/annexin V-positive) region C: vital cells (PI-negative/ annexin V-negative); region D: apoptotic cells (PI-negative/annexin V-positive).

Concerning use of oral contraceptives, the women were asked whether they had ever used combined oral contraceptives for at least one month. Color photographs of all brands that were on the market in Iraq were used to obtain detailed information about oral contraceptive use. During the interview, the woman was asked to recall in which period and for how long she had used a specific brand. If she could not remember the name of the specific brand, photographs of brands marketed in the relevant period were shown to the woman and women who had a mixed use, i.e. women who used different kinds of combined oral contraceptives, and women who did not remember the brand they had used were excluded. The total sample numbers were 40 (25 for contraceptive users and 15 for control). Contraceptive pills users were divided into two groups depending on the period of treatment to 2-5 years and 6-8 years.

The percentage of apoptosis in women who used Yasmin contraceptive pills for 2-5 years was 7.08%. On the other hand, there was observed increase in the percentage of apoptotic cells to 12.2% in women used Yasmin contraceptive pills for 6-8 years as compared with the negative control 0.18%. The apoptosis percentage in contraceptive users were significantly different ($P \leq 0.05$) in comparison with those of the

control group. The conclusion from these results show that the prolonged use of these drugs led to increase in apoptosis percentage as compared with the negative control (Table 4-5, figure 4-4 and appendex 4).

Higher (P≤0.05) viable cells percentage was noticed in negative control (99.3%) as compared contraceptive pill groups either for 2-5 years (92.18%) or 6-8 years (86.84%). The differences among groups in necrotic or late apoptotic cells lacked significance (Table 4-5, figure 4-4 and appendex 4).

Table (4-5): Apoptosis percentage in women blood lymphocyte used contraceptive pills for different periods (Mean+SD).

Groups	Necrosis (%)	Late apoptosis (%)	Viable (%)	Apoptosis (%)
Negative control	**A** 0.22±0.09	**A** 0.24±0.06	**A** 99.36±0.44	**C** 0.18±0.09
Contraceptive pills (2-5 years)	**A** 0.36±0.03	**A** 0.38±0.003	**B** 92.18±1.75	**B** 7.08±0.55
Contraceptive pills (6-8 years)	**A** 0.54±0.021	**A** 0.42±0.035	**C** 86.84±2.47	**A** 12.2±0.93

Means with different superscripts within each column differ significantly (P≤0.05).

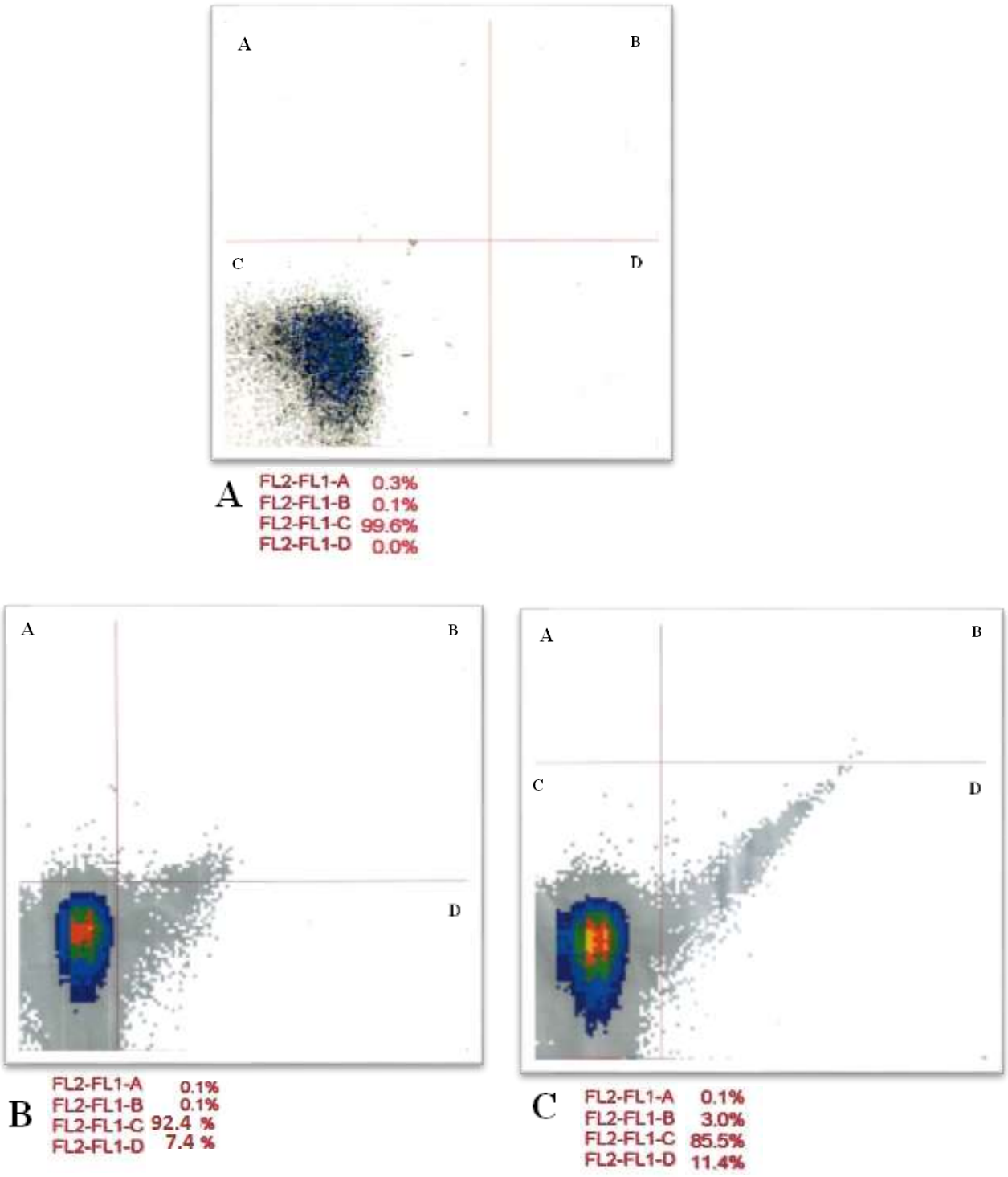


Figure (4-4): Annexin V expression for mice induced apoptosis analysis. The labeling patterns inside quadrants indicate the percentage of A: necrotic cells(PI- positive/ Annexin V- negative); B: late apoptosis(PI-positve/ Annexin V-positive); C:Vital cells(PI-negative/ Annexine V- negative); D: apoptotic cells (PI-negative/ Annexine V- positive).The labeling patterns outside quadrants indicate the percentage of apoptotic cells control (a), contraceptive pills treatment duration (2-5) years (b), contraceptive pills treatment duration (6-8) years (c).

Compatible with our findings, Rodriguez *et al*. (1998) found that the combination estrogen–progestin OC has a potent apoptotic effect on the ovarian epithelium mediated by the progestin component and the inducing ability on apoptosis percentage increased more than sixfold by levonorgestrel alone and almost fourfold by the contraceptive combination of ethinylestradiol and levonorgestrel.

It was shown that only one month of a combined oral contraceptive was sufficient to significantly reduce markers of cell proliferation and increase the apoptotic index in the eutopic endometrium of patients with endometriosis (Meresman *et al.*, 2003).

The synthetic progestin, levonorgestrel, may induce apoptosis in ovarian surface epithelium (Rodriguez *et al*., 1998), ethinylestradiol plus levonorgestrel induces ovarian epithelial cell apoptosis in intact monkeys (Rodriguez *et al*., 2002), while drospirenone was found to induce chromosome aberrations in human peripheral lymphocytes (Lidegaard *et al*., 2009).

The estrogen component inhibits the release of FSH and thus suppresses the development of the ovarian follicle, while the progestin component inhibits the release of LH thereby preventing ovulation. They also reduce the volume of the cervical mucus, increase its viscosity and cell content, and alter its molecular structure, making it less suitable for sperm penetration (Cibula *et al*., 2010).

4.6 Assessment of DNA damage in women using oral contraceptives by using the comet assay

The alkaline comet assay was used to evaluate the genotoxicity towards human peripheral blood lymphocytes. The DNA damage was quantified by measuring the displacement between the genetic material of the nucleus (comet head) and the resulting tail. At least 50 randomly selected cells should be analyzed per sample. The quantification was done by using image analysis software comet score, the analysis software will calculate different parameters for each comet, and three parameters were estimated to indicate DNA migration, tail length (distance from the head center to the end of the tail), mean tail moment (appropriate index of induced DNA damage in considering both the migration of genetic material as well as the relative amount of DNA in the tail) and % DNA in tail (Azqueta *et al.*, 2009). Peripheral blood lymphocytes from the subjects were embedded in agarose on a microscope slide and lysed; the DNA was unwound and subjected to electrophoresis at pH13.Staining with a fluorescent dye was used to identify cells with DNA damage, as judged by increased migration of genetic material from the cell nucleus. The presented results of an alkaline comet assay on peripheral blood lymphocytes obtained from 25 subjects handling contraceptive pills drug and 15 controls. The samples were divided into two groups according to the period of contraceptive pills treatment which was from (2-5) years and (6-8) years, (Table 4-6).

Table (4-6): Tail length, tail moment and DNA% in the tail (comet assay) of women lymphocytes treated with contraceptives for different periods (Mean ± SD).

Groups	Tail length	Tail moment	%DNA in tail
Negative control	**C** 0.8 ± 0.1	**C** 0.01 ± 0.003	**C** 1.6 ± 0.387
Contraceptive pills (2-5 years)	**B** 19.13± 0.01	**B** 23.63± 0.07	**B** 20.12± 0.03
Contraceptive pills (6-8 years)	**A** 25.08± 0.04	**A** 31.81± 0.18	**A** 28.63± 0.13

Means with different superscripts within each column differ significantly (P≤0. 05).

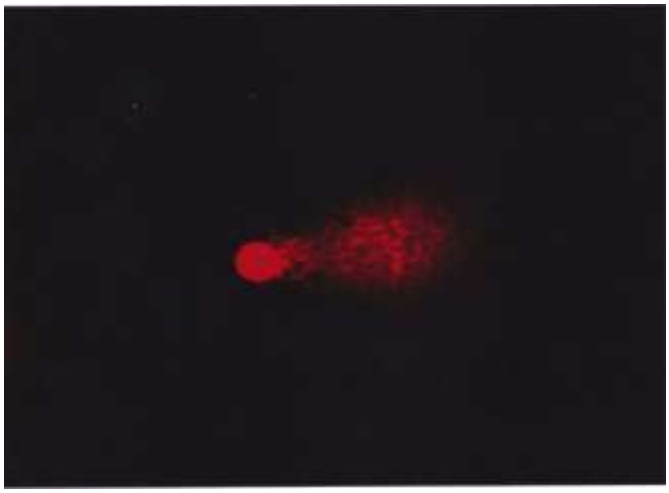
The mean of tail lengths of comet in women used birth control pills for (2-5) years were 19.13%, while for those used birth control pills (6-8) years were 25.08%. The observed values were significantly different (P≤0.05) from those in control subjects, being (0.8%), (Table 4-6,f igure 4-5 and appendix 5).

The percentage of DNA in the tail of comet in women used contraceptive pills for (2-5) years were 20.12%, while in those contraceptive pills treatment for (6-8) years were 28.63%. The observed values were significantly different (P≤0.05) from those in control subjects, being (1.6%), (Table 4-6,figure 4-5 and appendix 5).

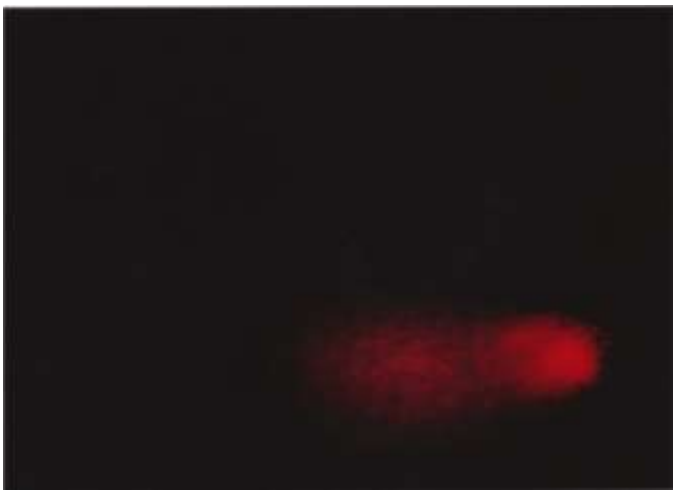
The tail moments of comet in women with contraceptive pills for (2-5) years were 23.63%, while in those treated with contraceptive pills (6-8) years were 31.81%. The observed values were significantly different (P≤0.05) from those in control subjects, being (0.01%), (Table 4-6,figure 4-5 and appendix 5).

All the measures of DNA damage in peripheral lymphocytes taken from the women were significantly greater than in controls.

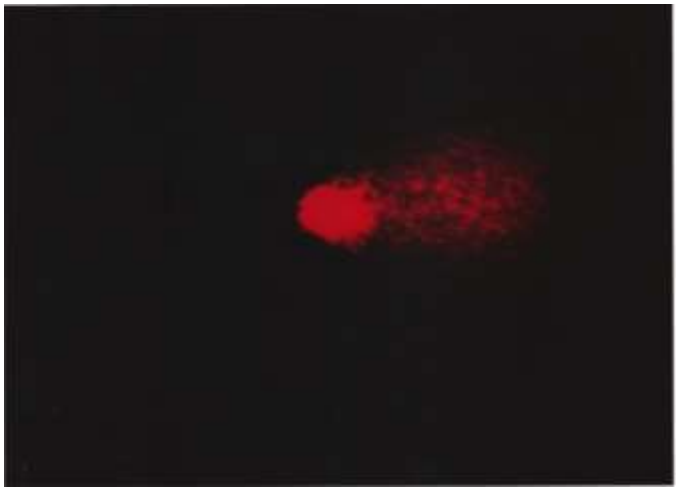
(A)



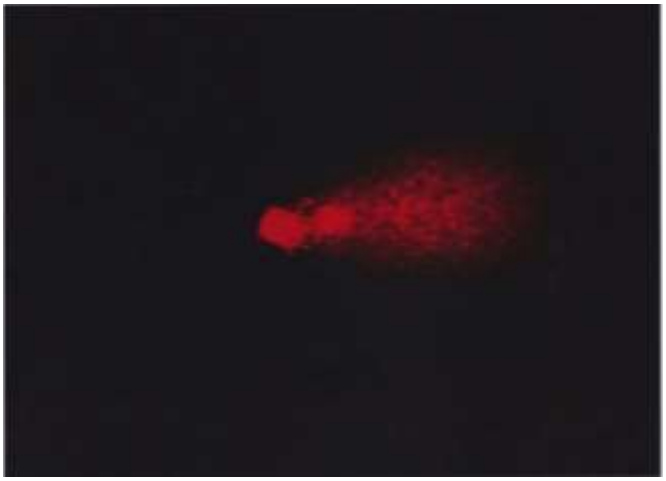
(B)



(C)



(D)



(E)

Figure (4-5): Comet assay in women peripheral blood lymphocytes examined by florescent microscope (400X) of the control group (A), showing fluorescent spheres without DNA damage (no tail) ,contraceptive pills treatment group (2-5 years) (B and C),and contraceptive pills treatment group (6-8 years) (D and E), showing a lot of fluorescent heads with tails indicating DNA damage (ethidium bromide stain).

The effect of using OC on the frequency of sister chromatid exchanges (SCEs) and on the response in the alkaline comet assay (single-cell gel electrophoresis) was investigated in 18 women taking contraceptive pills daily for 24 months by Biri *et al*. (2002). A significant increase in the number of lymphocytes with DNA migration and an increased frequency of sister chromatid exchange per metaphors was observed in OC users as compared with their age-matched untreated controls. As higher incidences of spontaneous SCEs in peripheral blood lymphocytes have been reported to occur in females during pregnancy due to profound changes in the levels of certain sex hormones such as progesterone and estrogen, particularly during the last trimester, 17 pregnant women served as positive controls in this study in order to test the rate of genetic damage due to those changes. Higher frequencies of SCEs and comet responses were observed in pregnant women than in their matched controls. However, no statistically significant difference in DNA damage was observed among OC users and pregnant women ($P \leq 0.05$).

The fact that prolonged and extensive use of these drugs in our daily life may be hazardous and also, that OC users should be aware of multifactorial risk factors (environmental, genetic and lifestyle patterns) that may be responsible for additional DNA damage.

During the last few years, several studies described the influence of hormonal therapy on the human genome (Loncar, 2007). Since oral contraceptive pills have been continually consumed for a longer period, so it is very important to explore long-term genetic effects after using these medications (Speroff and De Cherney, 1993).

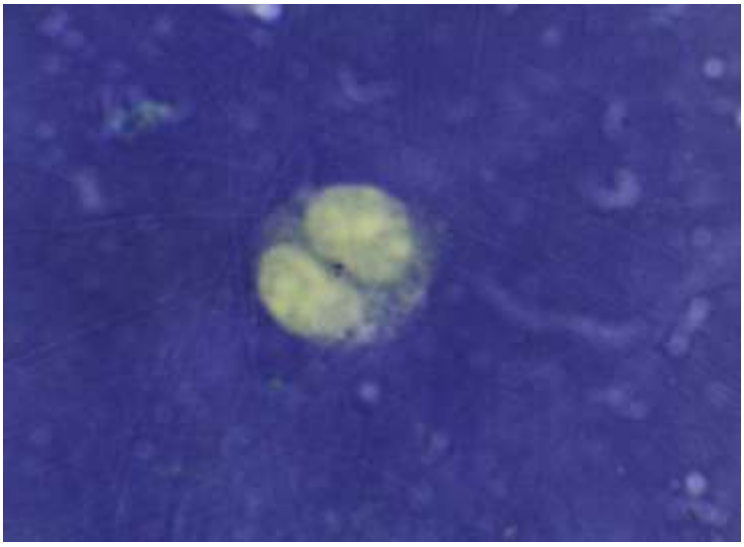
4.7 Measurement of micronucleus in women using oral contraceptives

Defining the micronucleus frequency was done by analyzing 1000 cells of each patient. The average number of analyzed micronuclei was (0.016%) of women who used the oral hormonal contraceptives from (2-5 years), while the average number was (0.021%) of women who used the oral hormonal contraceptives from (6-8 years). The results of the study showed a significant increase ($P \leq 0.05$) in micronuclei number from those in control, which were (0.004%), as shown in (Table 4-7, figure 4-6 and appendix 6).

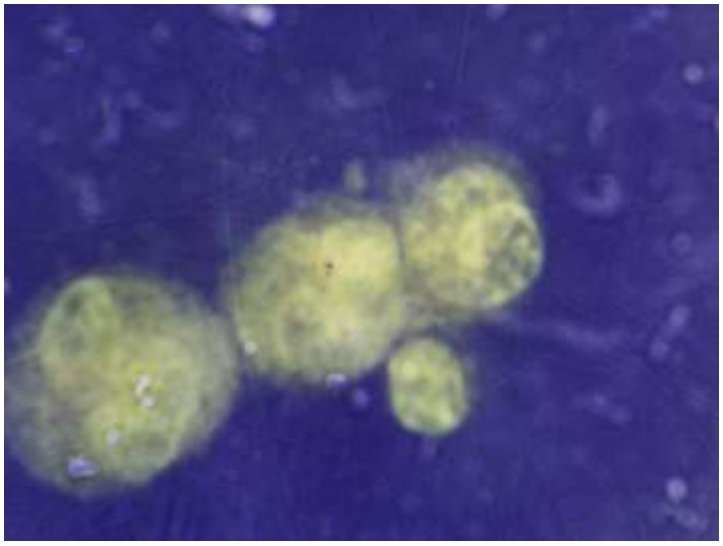
Table (4-7): Micronucleus assay on women peripheral blood lymphocytes that used the oral hormonal contraceptives.

Groups	Distribution of MN in cells (Mean±SD)				No. of cells with MN	No. of MN
	0	1	2	3		
Negative control	**B** 996.24 ± 1.20	**B** 2.88 ± 0.0781	**C** 0.88 ± 0.0781	**B** 0.00 ± 0.00	**C** 3.76 ± 1.0208	**C** 0.004 ± 0.0018
Contraceptive pills (2-5 years)	**A** 988.8 ± 1.30	**A** 7.4 ± 0.8944	**B** 2.0 ± 0.0000	**A** 1.8 ± 0.0836	**B** 11.2 ± 1.304	**B** 0.016 ± 0.0027
Contraceptive pills (6-8 years)	**A** 986.17 ± 0.75	**A** 8.5 ± 1.0488	**A** 3.33 ± 0.0516	**A** 2.0 ± 0.6325	**A** 13.83 ± 0.753	**A** 0.021 ± 0.0009

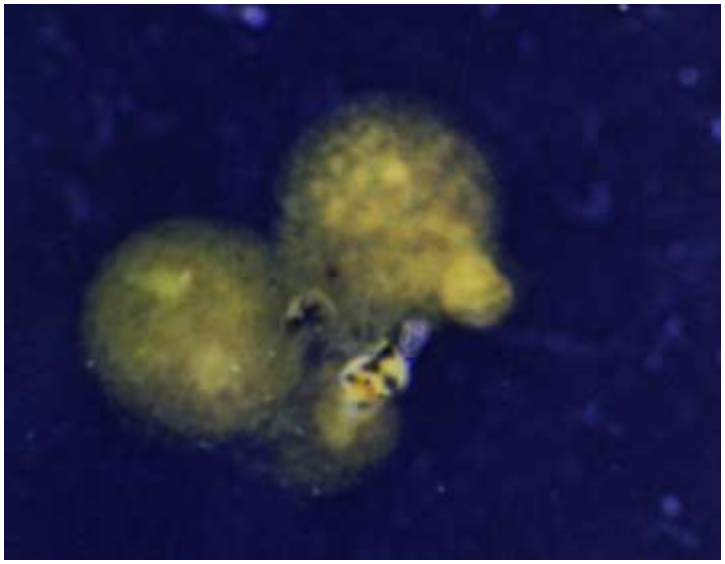
Means with different superscripts within each column differ significantly (P≤0. 05).



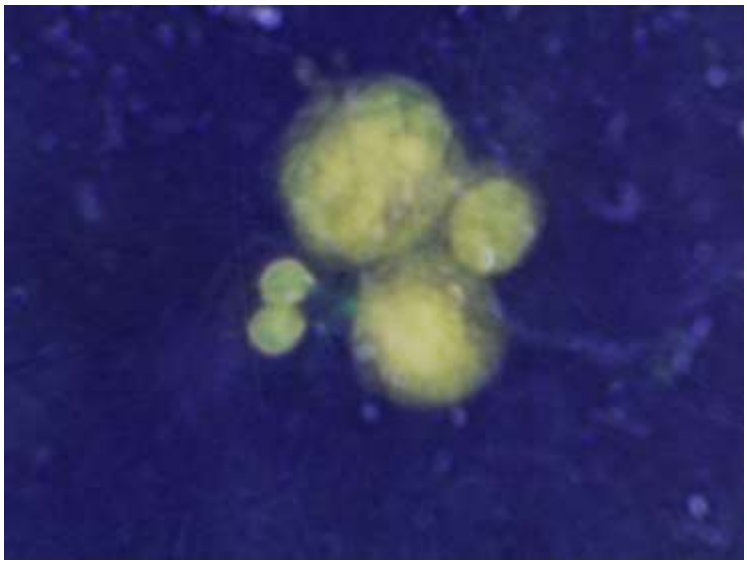
(A)



(B)



(C)



(D)

Figure (4-6): Micronucleus assay of women peripheral blood lymphocytes who used the oral hormonal contraceptives of the binucleated cell control (A) , binucleated cell with one micronuclei (B), binucleated cell with two micronuclei (C), and binucleated cell with three micronuclei (D).

Further evidence supporting such results observed on human blood lymphocytes treated with OCP by Al-khraquly (2006) which showed a significant increase in MN rate as compared with the negative control.

During the last three decades many researches of the influence of hormonal contraceptive pills on metabolism, cardiovascular system and psychological behavior of the patients have been conducted and the results indicated that the low-dosed contraceptives reduced the risk of complications which appeared during the use of earlier high- dosed contraceptive pills while small number of studies about the way that oral hormonal contraceptives affect the human genome has been published. Both components, estrogen and progesterone, after joining with cytosolic or nucleic receptor, join with wanted DNA cells (Grujicic, 1999). This method means stopping the cell division blockage of cytokinesis while the division of the core was in progress. This effect was achieved by cultivating the cells in medium to which cytochalasin B was added and that was how binucleate cells originated (Müller and Streffer, 1990).

GrujicÏicÂ *et al.* (1999) showed that at the beginning, and even before therapy, pregnant women with an indication for hormonal therapy had ~2-fold higher MN frequency, in comparison with the control group of pregnant women.

BukvicÂ *et al.* (2000) reported that the highest hormone concentrations increased the number of highly damaged cells and a reduced mitotic index. The cited therapeutic doses for therapy in patients induced remarkable changes in genetic material, which we detected as the greatest average MN frequency.

In the studies achieved by Furberg *et al.* (2003) indicated that women who were administered combined oral contraceptives appear to have sustained genetic alterations. It should be recognized that the observed effects of combined oral contraceptives could have been the result of a

direct genotoxic effect of the hormonal preparation or could have been an indirect effect of hormonal influences on cellular functions, most notably cell proliferation, mediated by receptor or non-receptor-linked mechanisms. It is therefore appropriate not to over interpret these observations as evidence of a direct genotoxic effect.

4.8 Aromatase activity in women using contraceptive pills

The obtained results of this experiment are shown in (Table 4-8). The results showed that the negative control group showed highest levels of aromatase activity 9.303% when compared with contraceptive pills users from 2-5 years and contraceptive pills users since 6-8 years reaching to 6.113% and 5.248% respectively.

Table (4-8): Aromatase activity in women using contraceptive pills (Mean+SD).

Groups	Aromatase (ng/ml) activity
Negative control	**A** 9.303+0.296
Contraceptive pills (2-5 years)	**B** 6.113+0.124
Contraceptive pills (6-8 years)	**B** 5.248+0.034

Means with different superscripts within each column differ significantly (P≤0.05).

A standard curve was drawn by using absorbance for 7 wells of standard and blotting the O.D value of the standard (X-axis) against the known concentration of standard (Y-axis), (Figure 4-7).

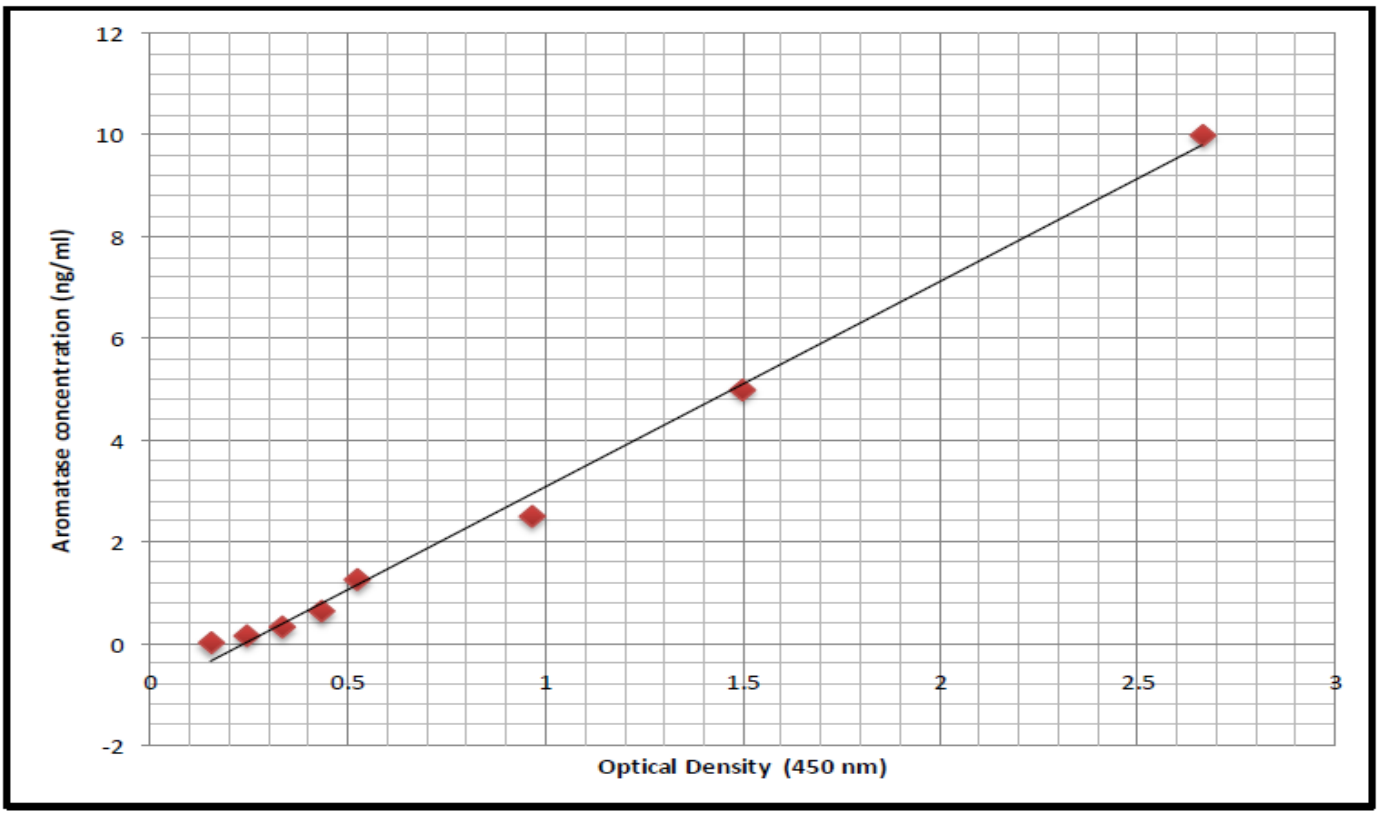


Figure (4-7): Typical standard curve for human aromatase activity.

Little is known about the effect of hormonal therapy on aromatase (CYP 19), which is responsible for the synthesis of estrogens. Aromatase is expressed in both normal and malignant breast tissues. Studies were carried out in an *in-vitro* model by Yue *et al.* (2001) in which MCF-7 cells were cultured in long-term estrogen-deprived medium (LTED cells). It was found that long-term estrogen deprivation enhanced aromatase activity by three- to fourfold compared with that in wild-type MCF-7 cells. Re-exposure of LTED cells to estrogen reduced aromatase activity to the levels of wild-type MCF-7 cells.

The absorption rates of orally administered estrogens and progestogens are usually rapid; peak serum values are observed between 0.5 and 4 h after intake. Serum concentrations rise faster with multiple treatments than with single doses and achieve higher steady-state levels, which are still punctuated by rising after each daily dose. The rise in steady-state levels with multiple doses may reflect the inhibitory effect of both estrogens and progestogens on cytochrome P450 (CYP) metabolic enzyme activities (IARC, 1999).

Endocrine disruptors can act as oestrogens, anti-oestrogens, androgens or anti-androgens, depending on the mechanism and site of action (Sumpter, 1995).

Conclusions
&
Recommendations

Conclusions

1. The apoptotic index of isolated lymphocytes induced in mice treated with contraceptive pills and women was used the pills, while fungal alkaloids (Cafergot) and wild carrot seed oil have no effects on apoptosis index that evaluated by flow cytometry.

2. DNA damages were detected by the comet assay and MN test in mice treated with contraceptive pills and women was used the pills. Contraceptive pill (Yasmin) is the most inducing contraceptive drugs to DNA damage while Cafergot and wild carrot seed oil are the least effective as compared with the negative control. The prolonged use of contraceptive pills led to increase the probability of DNA damage.

3. Contraceptive pills have an inhibitory effect of aromatase activity in women who used the pills as compared with negative control.

Recommendations

1. Completing the antifertility experiment by histologic detection of ovary and endometrium (histochemical) morphometric analysis.

2. Effect of contraceptive pills on immunological like phagocytosis.

3. Estimation of plasma P4 and E2 concentartions of cafergot and oil.

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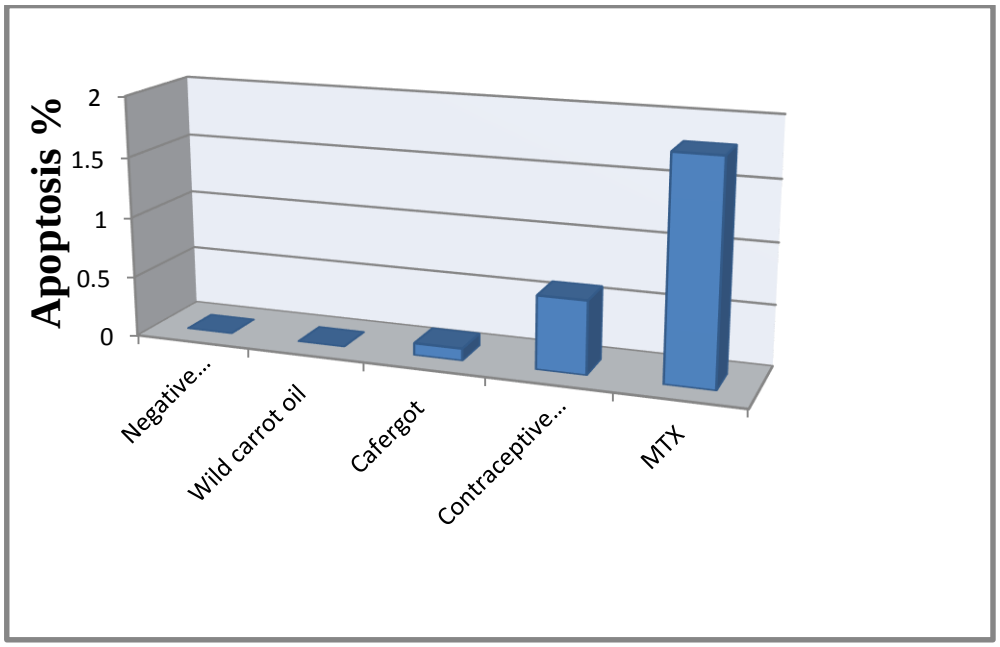
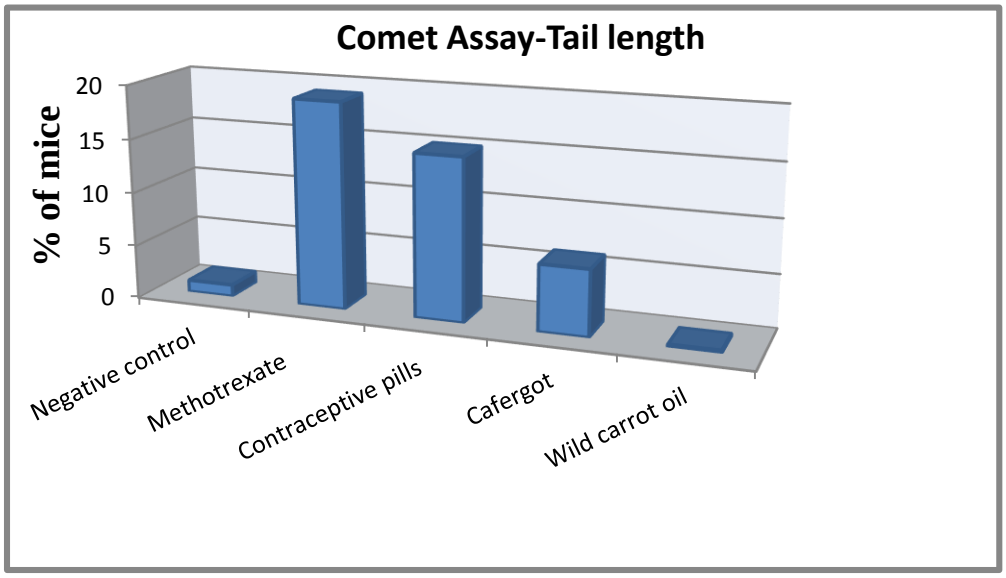
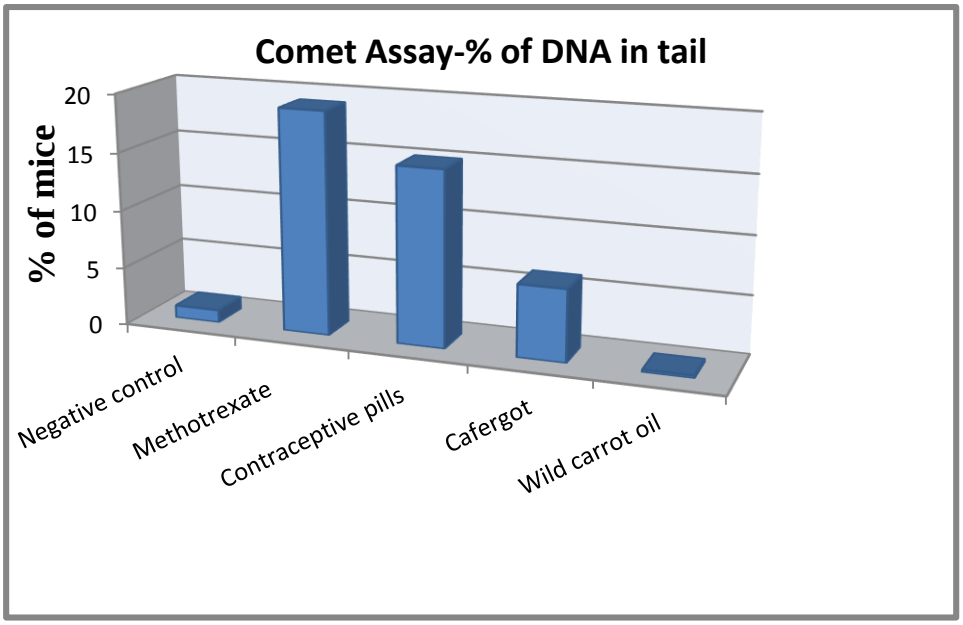
Appendices



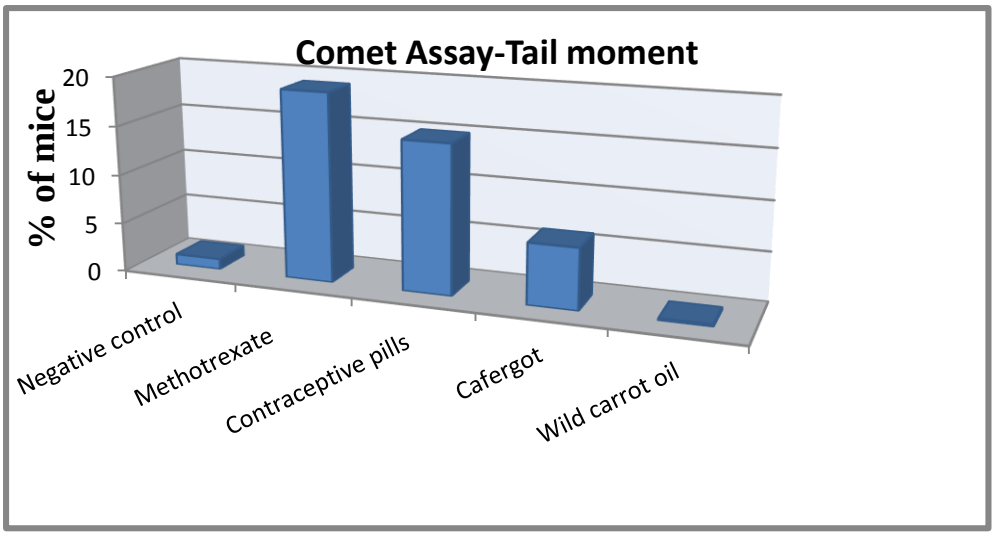
Appendix 1: The lymphocyte apoptotic percentage in mice treated with different contraceptive agents.



A

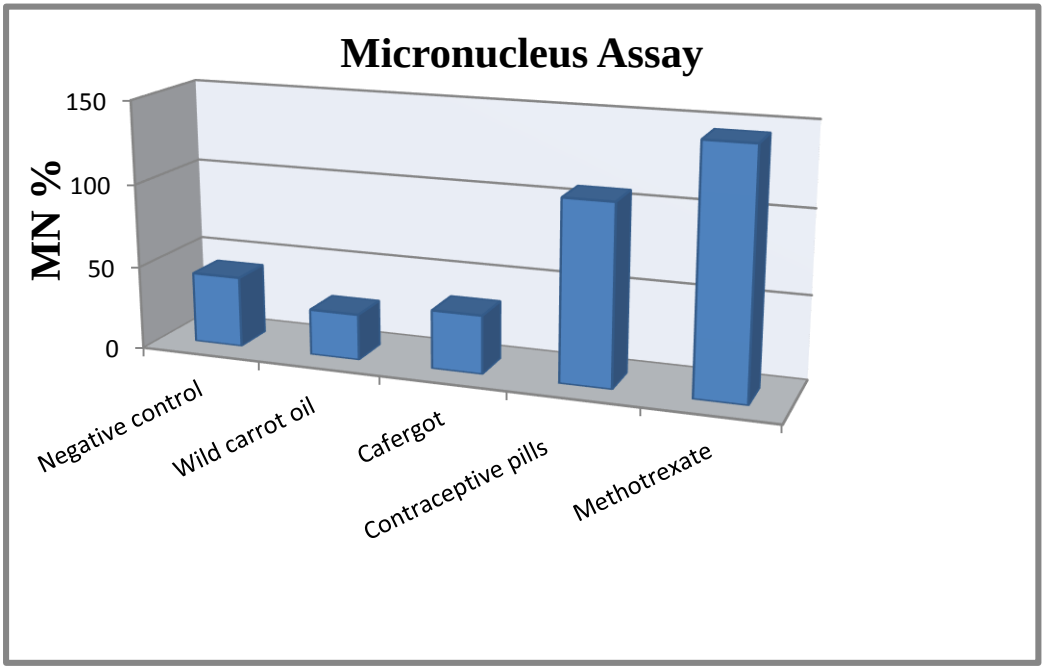


B

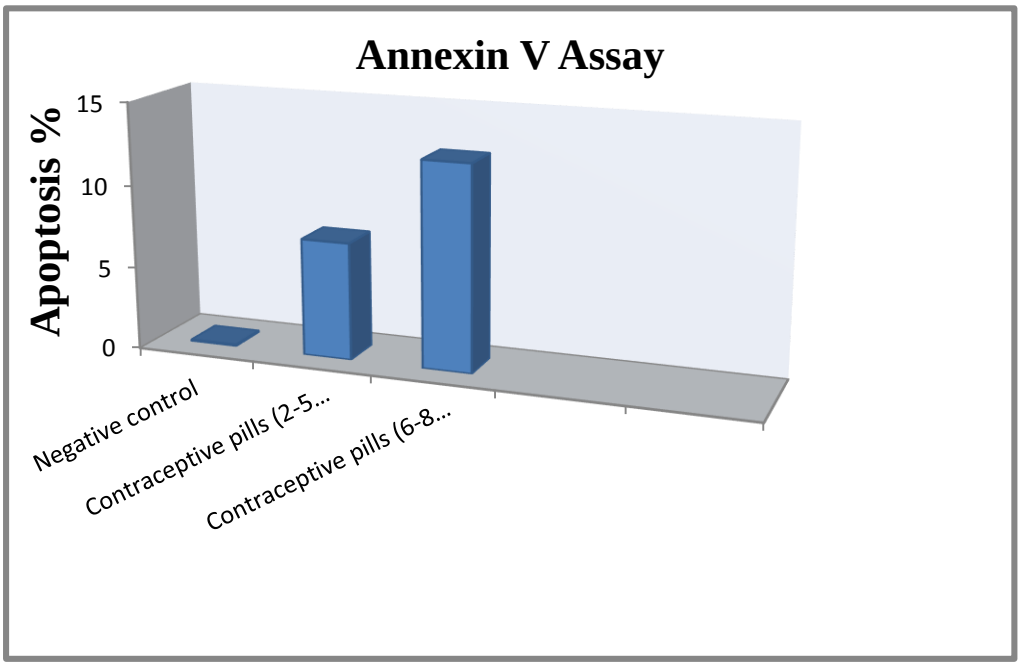


C

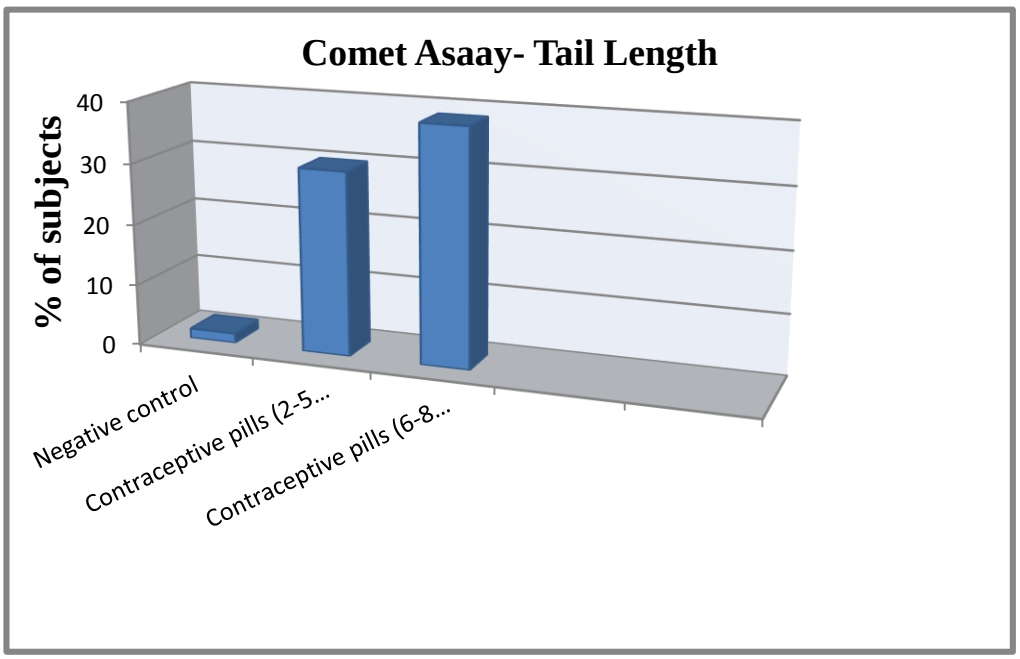
Appendix 2: Tail length **(**A**),** % DNA in tail (B) and tail moment (C) of mice lymphocyte (comet assay) treated with different types of contraceptives.



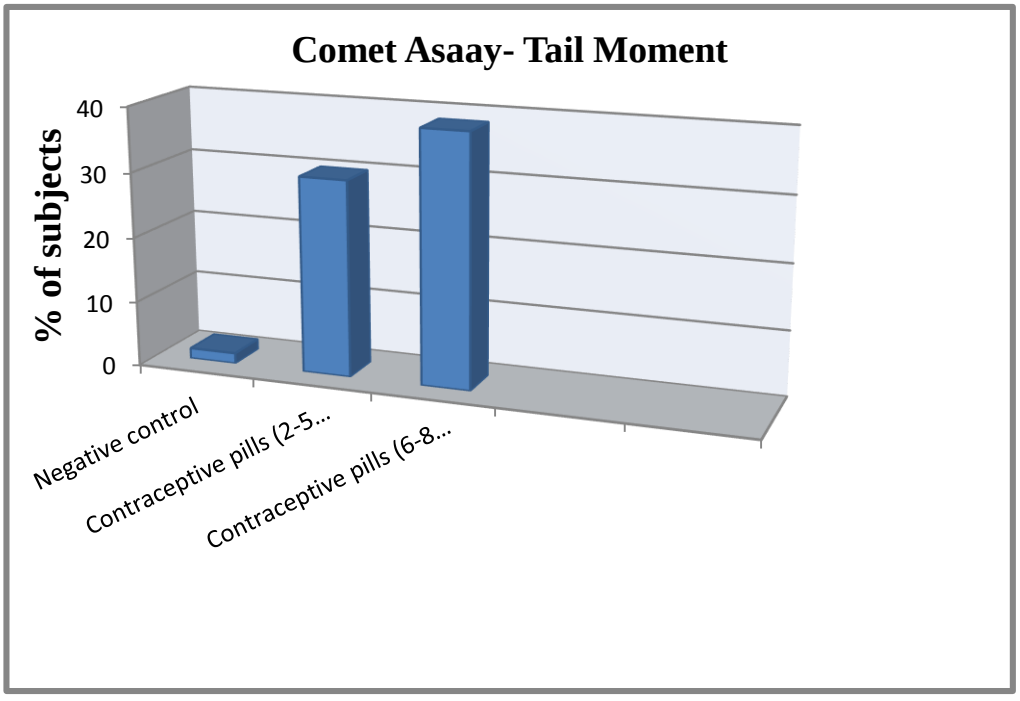
Appendix 3: Micronucleus percentage in mouse treated with different contraceptives drugs.



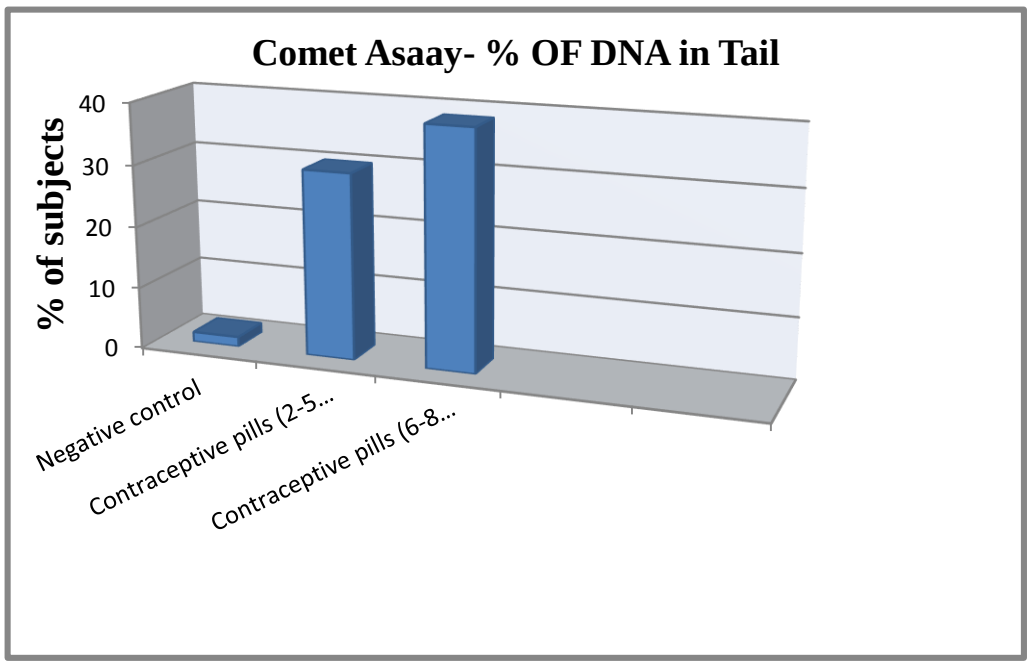
Appendix 4: The apoptotsis percentage in women blood lymphocyte by Annexin V assay.


Comet Asaay- Tail Length
% of subjects
40
30
20
10
0
Negative control
Contraceptive pills (2-5...
Contraceptive pills (6-8...

A

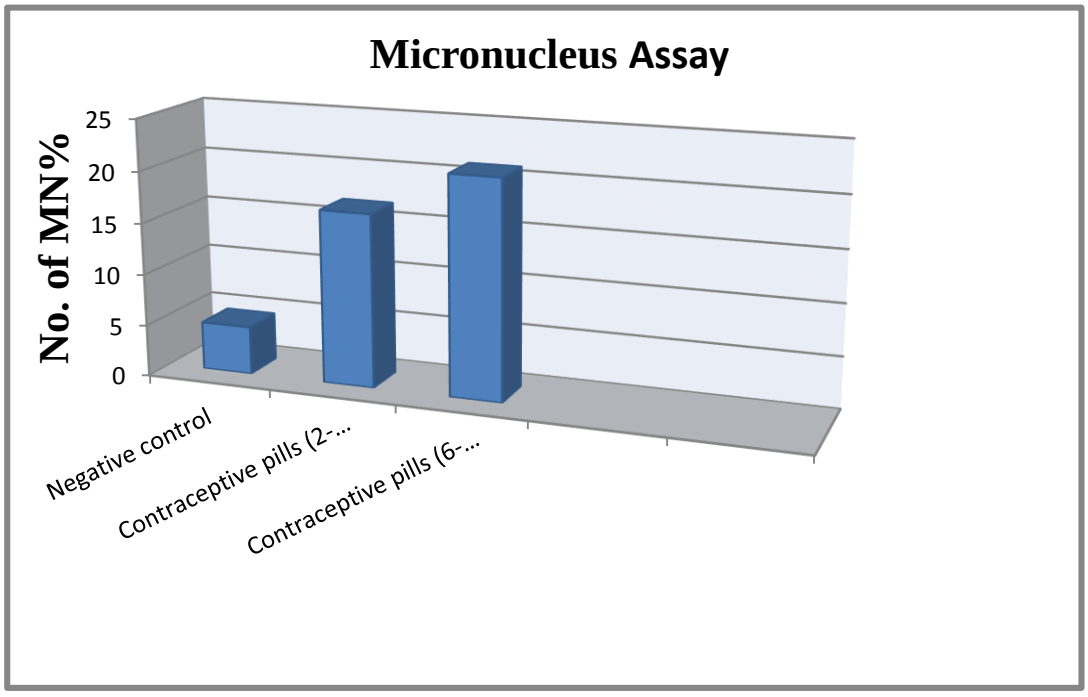

Comet Asaay- Tail Moment
% of subjects
40
30
20
10
0
Negative control
Contraceptive pills (2-5...
Contraceptive pills (6-8...

B

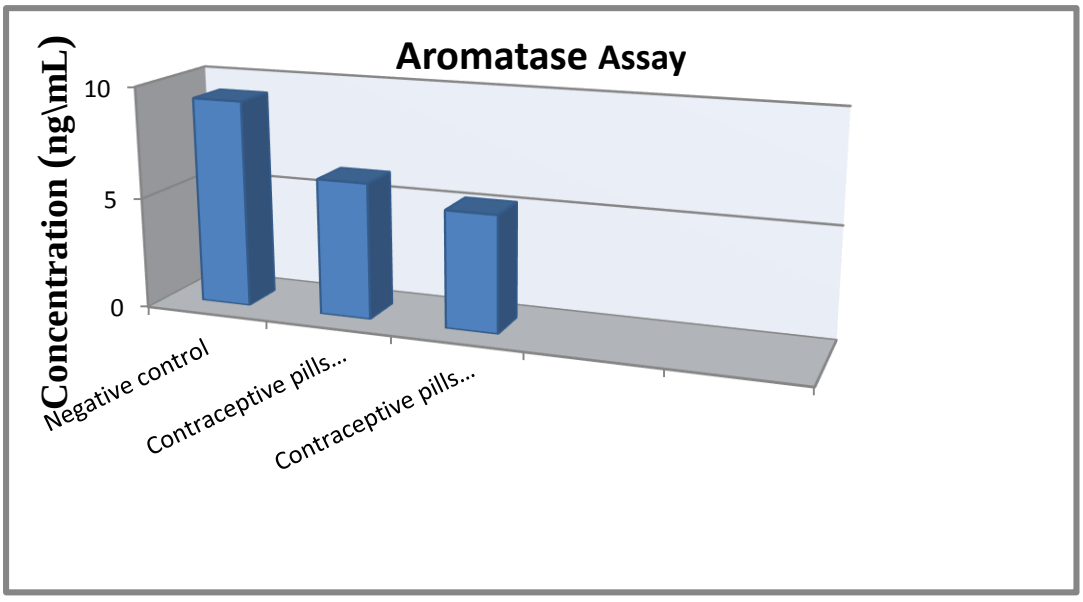


C

Appendix 5: The mean values for tail length (**A**), tail moment (**B**) and DNA % in tail (**C**) of comets in women treated with contraceptive pills for different periods.



Appendix 6: The micronucleus assay of women peripheral blood lymphocytes used the oral hormonal contraceptive pills during different periods.



Appendix 7: Aromatase activity of women peripheral blood lymphocytes that used the oral hormonal contraceptives of the contraceptive pills treatment for different ages.

الملخص

أجريت هذه الدراسة لمعرفة تأثيرات حبوب منع الحمل "ياسمين"، القلويدات الفطرية "Cafergot" و زيت بذور الجزر البري على موت الخلايا المبرمج، الضرر الحاصل في الـ DNA (باستخدام مقايسة المذنب)، التحليل الوراثي الخلوي (باستخدام فحص النواة الصغرى) في الخلايا الليمفاوية لدم الفئران بالاضافة الى معدل الحمل في إناث الفئران. وقد تم التحقيق أيضا من التأثير الفموي لحبوب منع الحمل (ياسمين) على موت الخلايا المبرمج، الضرر الحاصل في الـ DNA ، التحليل الوراثي الخلوي وفعالية انزيم الاروماتيز لخلايا الدم الليمفاوية في النساء. تم تقسيم خمسة وثلاثين فأرة إلى سبع مجموعات في تجربة مضاد الخصوبة (3 إناث و 2 ذكور في كل مجموعة). اعتبرت المجموعة الأولى كمجموعة سيطرة سالبة تعامل مع محلول ملحي، في حين أن المجموعة II و III عوملت مع 30 و 60 ميكروغرام / كغم من حبوب منع الحمل على التوالي. المجموعة الرابع والخامس عوملت مع 10 و 20 ميكروغرام / كغم من Cafergot على التوالي، و المجموعة السادس والسابع عوملت مع 0.05 و 0.1 مل من زيت بذور الجزر البري على التوالي. وكان معدل الحمل أعلى ($P\leq0.01$) في السيطرة السالبة ومجموعة IV بالمقارنة مع المجموعات الأخرى. في تجربة موت الخلايا المبرمج، استخدمت خمسة عشر فأرة قسمت إلى خمس مجموعات متساوية. اعتبرت المجموعة الأولى كسيطرة سالبة، في حين السيطرة الموجبة عوملت مع 50 ميكروغرام / كغم من الميثوتريكسيت (MTX). المجموعات الثلاث المتبقية جرعت فمويا مع 60، 20 ميكروغرام / كغم و 0.1 مل من COCPs، Cafergot و زيت بذور الجزر البري على التوالي. أعطيت المعاملات لمدة 5 أيام متتالية. وقد لوحظ ادنى ($P\leq0.05$) نسبة موت خلوي في زيت بذور الجزر البري و المساوية للمجموعة السالبة، في حين اعلى نسبة ($P\leq0.05$) لوحظ في مجموعة MTX، مشيرا إلى أن مجموعة COCPs هي الاكثر تحفيزاً. وقد لوحظت اعلى نسبة ($P\leq0.05$) للخلايا الحية في Cafergot وزيت بذور الجزر البري ٪99.54 99.6٪ والمماثلة للسيطرة السالبة (99.66٪) وأكبر من COCPs ٪97.83، ومجموعة MTX. في مقايسة المذنب، استخدمت نفس المجموعات على النحو المذكور في تجربة الموت الخلايا المبرمج. وقد لوحظت فروق معنوية ($P\leq0.05$) بين المجموعات في tail length اذ يكون أطول في مجموعة MTX ٪17.5، تليها COCPs ٪12.18 وأقل من ذلك في مجموعة زيت بذور الجزر البري (1٪). وكانت نسبة tail DNA ٪15.08 في COCPs بينما في Cafergot و زيت بذور الجزر البري كانت ٪6.18 و ٪0.22 على التوالي. في السيطرة السالبة كانت نسبة tail DNA

1.09٪ و 19.14٪ في مجموعة MTX. كانت tail moment للمجموعة المعاملة مع حبوب منع الحمل 25.04٪، بينما في Cafergot و زيت بذور الجزر البري كانت 5.09٪ و 0.36٪ على التوالي. في تجربة الانوية الصغرى سجل زيت بذور الجزر البري وCafergot أقل (P≤0.05) نسبة بالمقارنة مع المجموعات الأخرى وصولا 2.75٪ و 3.5٪ على التوالي. في المقابل، سجلت MTX أكبر (P≤0.05) نسبة من الانوية الصغرى وهي 14.5٪ تليها مجموعة COCPs ٪10.75. نسبة الموت الخلوي المبرمج في النساء اللواتي يستعملن حبوب منع الحمل (ياسمين) 7.08 و 12.2٪ لمدة 2-5 و6-8 سنوات على التوالي مقارنة مع السيطرة السالبة (0.18٪). وقد لوحظ اعلى (P≤0. 05) نسبة من الخلايا الحية في السيطرة السالبة (99.54٪) بالمقارنة مع مجاميع حبوب منع الحمل لمدة 2-5 سنوات (92.18٪) و6-8 سنوات (86.84٪). كان متوسط tail length في النساء اللواتي يستخدمن حبوب منع الحمل ياسمين 19.13 و25.08٪ لمدة 2-5 و6-8 سنوات على التوالي ومختلفة (P≤0.05) عن السيطرة السالبة (0.8٪). وعلاوة على ذلك، كانت نسبة tail DNA 20.12 و28.63٪ للنساء اللواتي يستعملن حبوب منع الحمل ياسمين لمدة 2-5 و6-8 سنوات على التوالي، و المختلف (P≤0.05) عن تلك الموجودة في المجموعة السالبة (1.6٪). كانت tail moment 23.63 و31.81٪ في النساء اللواتي يستخدمن حبوب منع الحمل ياسمين لمدة 2-5 و6-8 سنوات على التوالي، والذي يختلف (P≤0.05) عن تلك الموجودة في المعاملة السالبة (0.01٪). وكان متوسط عدد الانوية الصغرى 0.016 ,0.021٪ بالنسبة للنساء اللواتي يستخدمن حبوب منع الحمل ياسمين 2-5 و 6-8 سنوات على التوالي، والذي يبدو أعلى (P≤0.05) من تلك الموجودة في المجموعة السالبة (0.004٪). أظهرت مجموعة السيطرة السالبة للمرأة أعلى المستويات (9.303٪) من نشاط هرمون الاروماتيز بالمقارنة مع النساء اللواتي يستخدمن حبوب ياسمين لمدة 2-5 (6.113٪) و6-8 (5.248٪) سنة. نستنتج من هذا ان حبوب منع الحمل ياسمين أكثر الأدوية المسببة لموت الخلايا المبرمج و الضرر الحاصل في الـ DNA بالمقارنة مع Cafergot وزيت بذور الجزر.

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

بعض التغييرات الوراثية في الخلايا اللمفاوية وخلايا نخاع العظام المرتبطة بحبوب منع الحمل ياسمين و Cafergot وزيت بذور الجزر البري في النساء والفئران

رسالة

مقدمة الى كلية العلوم/ جامعة النهرين
كجزء من متطلبات نيل درجة الدكتوراه في علوم التقانة الاحيائية

من قبل

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