1-2: History of Black Seed: -

Nigella sativa was discovered in Tutankhamen's tomb, implying that it played an important role in ancient practices (Nooruddin, 2003).

Although its exact role in Egyptian culture is not known, we do know that items entombed with a king were carefully selected to assist him in the after life. The earliest written reference on black seed is found in the book of Isaiah in the Old Testament Int.#1.

Easten's Babel Dictionary clarifies that the Hebrew word for the black cumin, "ketsah", refers to "without doubt to the *N. sativa*, a small annual of the order Ranunculaceae which grows wild in the Mediterranean countries, and is cultivated in Egypt and Syria for its seeds." Int.#1.

Dioscoredes, a Greek physician of the 1st century, recorded that black seeds were taken to treat headaches, nasal congestion, toothache, and intestinal worms. They were also used, he reported, as a diuretic to promote menstruction and increase milk production.

Ibn Sina(980-1037), most famous for his volumes called "The Canon of Medicine," regarded by many as the most famous book in history of medicine , East or West , refers to black seed as the seed "that stimulates the body's energy and helps recovery from fatigue or dispiritedness'." (Nooruddin, 2003).

Black Seed is also included in the list of natural drugs of Al-Tibb al-Nabawi, and, according to tradition, “hold onto the use of the black seed for it has a remedy for every illness except death.”

Its oil has been used to treat skin conditions such as eczema and boils and is used tropically to treat cold symptoms. The many uses of black seed have earned for this medicinal herb the Arabic approbation habbatul barakah, meaning “the seed of blessing” Int.# 2.

1-3: Classification:-

Kingdom: Plant Kingdom.
Subkingdom: Embryophyta.
Division: Spermatophyta
Subdivision: Angiospermae.
Class: Dicotyledonous.
Order: Ranales.
Family: Ranunculaceae.
Genus: Nigella.
Species: sativa.

According to (Al-Katib, 1988).

1-4: Other names for *Nigella sativa* : -

N. sativa and its black seeds are known by other names, varying between places of growth (Bedevian, 1994). In Arabic

known as black seed coequal names of its seed in arabic countries are Al-Habbah Al-Sawda, Kamoun Aswad, Scuniz and Kodria. In Pakistan, India and Srilanka it is called as Kalvanji, Azmut, Aof, and Aosetta; and in English language is known as black seed, black cumin and black carawy. While in Turkish called corekotu siyah (Ur-Rahman and Malik, 1985).

1-5: Description: -

Black seed belongs to dicot plants, belongs to the family Ranunculaceae (Townsend, 1980).

The plant has a rather stiff, erect, branching stem, bears- cut grayish- green leaves and terminal grayish blue flowers, followed by odd, toothed seed vessels, filled with small compressed seeds, usually three- cornered, with two sides flat and one convex, black or brown externally, white and oleaginous within, of a strong, an agreeable aromatic odour, like that of nut megs, and a spicy, pungent taste (Mukerji 1953, Townsend 1980). *N. sativa* propagates normally by itself and forms a fruit capsule consists of many white trigonal seeds. Once the fruit capsule has matured, it opens up the seeds contained within are exposed to the air, becoming black in color (black seed) (Mukerji, 1953) Int.#2.

Upon cultivation of *N. sativa* in Iraq, it was found that it could be cultivated in winter from October to March. The best period for its cultivation range from November to January (Al-Dajwee, 1996).

There are several types of this plant that belongs to one genus but they are different in morphology and in chemical composition, some of these are: *Nigella sativa, Nigella damascene, Nigella orietalis* and *Nigella arvensis* (Chakraverty ,1976 ; Srivastava and Chardra, 1983).

1-6: Biological activity of Black seed: -

The main inspiration of black seed comes from the famous saying (Hadith) of our Prophet Mohammed; (God peace be upon him), that "Habbat Al-soda is remedy for all disease except death."(Abu-Abdullah, 1984).

As a natural remedy people take *N. sativa* seed or oil as a promoter of good health and for the prophylaxis of common cold and asthma (Randhawa and Al-Ghamdi, 2002).

El-Kadi and Kandil investigated the effect of *N. sativa* on immune system and reported that the administration of 1g twice daily in human volunteers enhanced immune functions as manifested by improved helper T cell (T4) to suppressor T cell (T8) ratio and an improved natural killer cell activity (El-Kadi and Kandil, 1986).

The presence of saponins in black seed has several biological activities (Ansari *et al*., 1988), like antiinflammation (Sageska and Uemura, 1996), antitumor (Detommasi *et al*., 2000), and the action on relaxing of central nervous system (Park and Kim, 1974), also its activity against strok (Bingham *et al*.,1978), and decreasing sugar and cholesterol level in the blood (Poter *et al.*, 1975).

The ethyl- acetate chromatographic fraction of ethanolic extract of *N. sativa* had been reported to potenciate cellular immune responses (Swamy and Tan, 2000).

In Saudi Arabia and neighbouring countries *N. sativa* oil is used as a topical treatment for pain and stiffness in joints (Randhawa and Al-Ghamdi, 2002).

Al-Ghamdi, 2001 also reported an analgesic effect of aqueous suspension of *N. sativa* seeds, comparable to aspirin.

The anticancer activity of *N. sativa* was first revealed by El-Kadi and Kandil, 1986 who observed enhancement of natural killer (NK) cell activity ranging from 200-300% in advanced cancer patients receiving multimodality immunotherapy programme in which *N. sativa* was one of these components.

Thymoquinone and dithymoquinone, active principles of *N. sativa*, had cytotoxic effect against parental and multi-drug resistant human tumour cell lines which were over 10-fold more resistant to doxorubicin and etoposide (Worthen *et al*., 1998).

Aktar and Riffat, 1991 investigated the anti-cestodal effect of *N. sativa* seeds and its ethanolic extract when given orally to infected children. Both were effective in reducing the precentage of faecal eggs per gram counts and the effect was comparable to niclosamibe. Moreover, *N. sativa* methanol extracts (1 ml/kg) and powder (200 mg/kg) showed high efficacy.

Furthermore, (Bamosa *et al*., 1997) reported a significant decrease in blood sugar of healthy human volunteers treated with 1 gram of *N. sativa* a capsules twice daily.

The anti-bacterial effect of the phenolic fraction of *N. sativa* oil was first reported by Topozada *et al*., 1965. Thymohydroquinone was later isolated by El-Fatary, 1975 from the volatile oil of *N. sativa* and showed high activity against Gram-positive microorganisms.

Hanafi and Hatem, 1991 studied the antimicrobial effect of diethyl-ether extract of *N. sativa* and reported that it had a concentration dependent inhibition of Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aerogenosa* and *Escherichia coli*). In addition, the extract was found to have a concentration dependent inhibitory effect against the pathogenic yeast *Candida albicans.*

Recently, crude extract of *N. sativa* were reported to have a promoising effect on multi-antibiotic resistant microorganisms

including Gram-positive and Gram-negative bacteria (Morsi, 2000).

The phenolic compound that reacts against some microorganism because it has this ability to kill them and some of these phenols Thymol, Thymoquinone and Quinoine (Charles *et al*., 1969; Al-Solause, 1995).

Thymoquinone reduced the incidence and multiplicity of benzo-a-pyrene induced forestomach tumour in female Swiss albino mice by 70%-67%, respictively (Badary and Gamal El-din, 2001).

N. sativa oil also prevented liver damage induced by *Schistosoma mansoni* infection in mice (Mahmoud *et al*., 2002).

N. sativa oil has a protective effects against cryptosporidium infection in mice (Al-Azawi, 2003).

N. sativa extracts and some of its active principles, like thymoquinone, have been shown to possess protective effect against heamatological, hepatic, renal and other toxicities induced by anti-cancer drugs and some toxins (Randhawa and Al-Ghamdi, 2002).

In Arabian folk medicine whole seeds of *N. sativa* alone or in combination with honey or garlic are promoted for the treatment of hypertention, which drew the attention of El-Tahir *et al*., 1993 to investigate the effects of *N, sativa* on the cardiovascular system.

Al-Awadi and Gumma, 1987 have reported the use of a plant mixture containing *N. sativa*, Myrr, Gum *Olybanum*, Gum *Asafoetida* and *Aloe* by diabetics in Kuwait. They confirmed the blood glucose lowering effect of *N. sativa*, in combination with other herbs in rats.

However, (Al-Jishi, 2000) did not find any changes in blood cells when *N. sativa* was given to normal rats.

Thymol, thymoquinone, carvacol, anthole and terpinol are the active compounds of black seed oil, which acts as an antioxidant compounds in *N. sativa* (Burits and Bucar, 2000; Kruk *et al.*, 2000).

Black seed contains flavonoids, which have antimutagenic and free- radicals scavenging activities (Samajima *et al.*, 1995).

Radiation protection activity of *N. sativa* in mice against induction of chromosomal aberrations by gamma ray was also reported (Shubber *et al*., 2000).

1-7 : Chemical Constituents: -

1-7-1: Volatile oils:-

Volatile oils are a complex mixture of hydrocarbonic, oxygenated and aromatic compounds they are formed by metabolism, and found as an accumulated form in the tissue cells of several different organs in aromatic plants (Al-Shahit, 2000).

Volatile oil is characterized by its evaporation or volatile when exposed to air, and has strong odour and dissolved in ether and in an organic solvents and that is why called volatile because of its fast evaporation and it's odour (Al-Samarrae, 2003).

The volatile oil of black seed has pale yellow color and contains most of the important compounds in medicine like thymoquinone and its derivatives as shown in figure (1-1) which represent the basic compound in the oil, and there is another compound called Nigellon and these two compounds have the medicinal effect in the oil of black seed (Cowan, 1999).

Moreover, the volatile oil of black seed consists of cymene (32%), carbnyl (9.3%), carvone (4.05%) and limonene (4.27%) (El-Fatary, 1975; Jukneviciene and Dagyte, 1977; Al-Najar, 1997) shown in figure (1-2).

The yield of the essential oil of black seed (*N. sativa*) was the lowest among other studied plants as reported, about 0.40% and this essential oil was the most toxic extract (Hanefi *et al.*,2004).

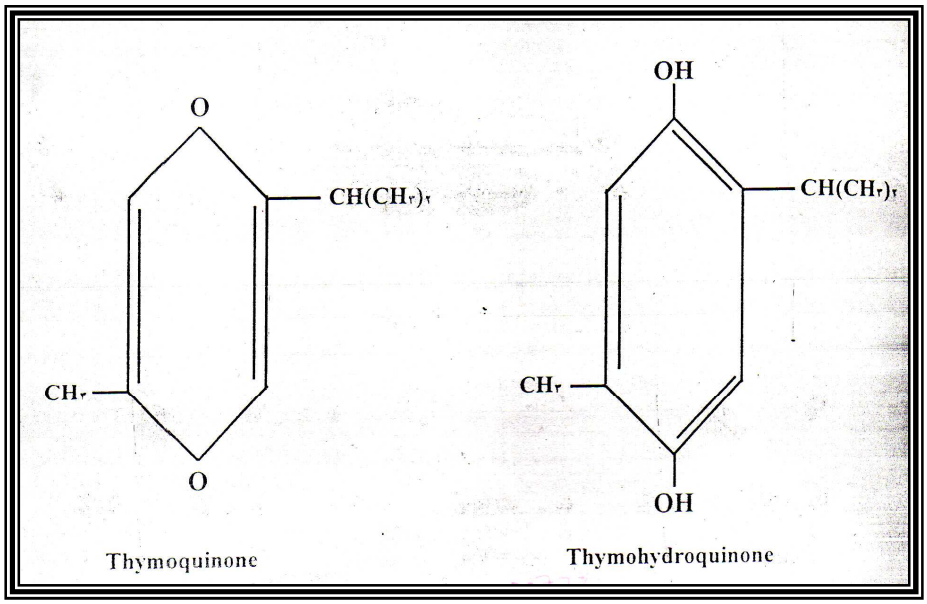
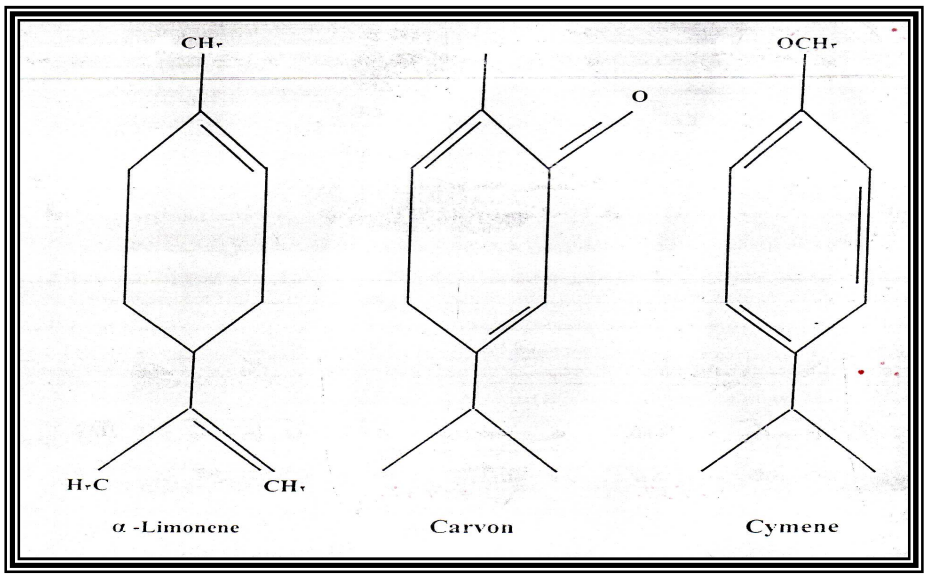


Figure (1-1): most important containts volatile oil in *Nigella sativa* extract .



Figure(1-2): Some containts of volatile oil in *Nigella sativa* extract .

1-7-2: Fixed oils: -

These oils represents the glycerids of fatty acids. Fixed oil characterized by that is not evaporated and cannot be volatile, also not distillated without its hydrolysis. Not dissolve in water, but dissolve in organic solvents, and it is sweet but doesnot have a strong volatile odour (AOAD, 1988). The percentage of fixed oil in black seed is about 30-38% (Al-Jassir, 1992; Al-Anee, ١٩٩٨).

The fatty acids that found in this oil are:

i) Un-saturated fatty acids:

The body cannot produce these acids but can get them from the meal like oleic acid (24.64%), linolic acid (56.12%), linolenic acid (0.70%) and elcosadienoic acid (2.53%) (Nergiz and Otles, 1993).

ii) Saturated fatty acids:

These acids have carbon chain without double bond and called "carboxylic acids" and contains: myristic acid (0.16%), palmetic acid (12.089%) and stearic acid (3.11%) (Babayan *et al*., 1978). Also behenic acid found about (0.19%) (Ustun *et al*., 1990).

iii) Sterols:

There are several types of sterols isolated from black seed oil including cholesterol, campesterol, stigmasterol, B-sitosterol and spinasterol (Salama, 1973).

pH number of fixed oil is about 7.01 and iodide number is about 136.5 (Sharif, 1989).

The volatile oils are more toxic than the fixed oils while the fixed oils of some plants like *Sesamum indicum* and *Urtica pilulifera* are completely non-toxic. Fixed oils are formed by the esterification of fatty acids with glycerin. By that means different fixed oils are formed from the essentially similar compounds (linoleic acid, oleic acid, etc) and these fixed oils like olive oil, corn oil, nut oil, etc constitute some of the basic elements of the human diet. In contrast volatile oils are quite distinct compounds except for those from plants of close kinds (Akgul, 1993).

1-7-3: Alkaloids: -

they are complex nitrogenic compounds that represent product of protein metabolism found in some animal and in some plants, and is used in medicine, pharmacy and its activity is related to that nitrogen-base that found in its amine structure (Tyler *et al*., 1988). It has found another special alkaloid in *N. sativa* is Nigellimine (Ur-Rahman and Malik, 1985).

Alkaloid not soluble in water but soluble in an organic solvents, has protection role in some plants and animals play role as a toxic compound and also represent as a storage molecules for nitrogen which is important to plants (Hugles and Genest, 1973;Al-Shamaa, 1989).

There are several alkaloids found in black seed such as Nigellimine, Nigellicine and Nigellone as shown in figure (1-3) (Al-Timemee, 2001).

Also it was found in the species *N. sativa* another kind of alkaloid known as damascenine that is produced from reducing methyl hydroxyl benzoic acid to methyl 2-methyl amino 3- methoxy benzoate and the last compound is damascenine (Al-Nadawee, 2003).

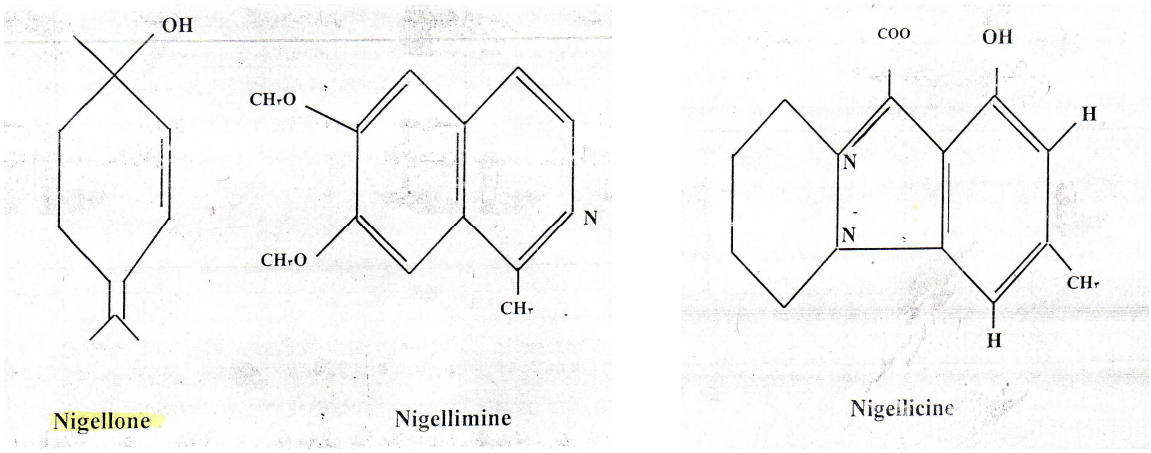


Figure (1-3): The active compounds in *Nigella sativa* seed extract.

1-7-4: Tannins: -

They are complex substances, they usually occur as mixtures of polyphenols they are difficult to separate because they donot crystallize. Some call them as a product of plant metabolism, with water form collodial solution acid with a stringent taste. Medicinally used as astringent compound for digestive system and also used in skin burning treatment. When it bound with protein of burning tissue making a simple protective layer and it is a role as a sterilizer growing beneath the new tissue, the phenol group has this role in stringent action and sterilizing, examples about tannin are Nut gall and Kino (Al-Shamaa, 1989).

1-7-5: Saponins: -

It is a glyceride consists of two parts sugar (glycon) and non sugar (a glycon) glycosides. Generally they are pungent in test and its ability to make foam in water. Some of saponins has ability to hydrolyse red blood cells and be toxic to cold blood organisms and its toxicity is related to its ability to decrease surface tention (Birk, 1969).

1-7-6: Carbohydrates: -

They are aldehyde or ketone compounds with several hydroxyl groups, represent the first compounds that is produced in plants by photosynthesis. These compounds are used as bacteriostatic and also as a feeding compounds (Tiwari and Singh, 1942).

The percentage rate of carbohydrates in black seed is about 19-20% and they are monosaccharides like glucose and disaccharides like sucrose (Al- Jassir, 1992).

1-7-7: Lectins: -

They are proteins contains polypeptides in single chain consists of 237 amino acids. Each of them has two sides one for binding with sugar and the other for binding with ions (Sharon, 1993).

The presence of lectins at high rate in seeds of the plants compared with its vegetative parts, the lectins have the ability to bind specifically with target sugars it binds with glycosidic compound that enters in structure of the cells and gives high ability to bind with normal or transformed cells (Abu *et al*., 1965).

Lectins have the ability to agglutinate with bacteria, yeast and virus if it is used to diagnose it to isolate pathogenic microorganisms by using lectin prob (Koshte *et al*., 1990).

It could be extracted and purified by using TLC method on coloum of sephadix gel G-50 (Al-Dwree, 1998). Its activity also provided as decreasing sugar and cholesterol levels in the blood of rabbit that have been induced with diabetes experimentally (Al-Assady, 2000).

1-7-8: Flavonoids: -

They are compounds generally found in plants, like saccharids structure and the hydroxyl group of phenol binds to sugar compounds. Flavonoids have several physiological importants like treatment of capillary fragility like Rutin and Hesprdin. Flavonoids that contain hydroxyl group characterized by their ability to prevent oxidizing of fatty compounds (Edward and Charles, 1983). Black seed contains flavonoids compounds known as antioxidant compounds like Quercetin and Kaempforol (Al-Najar, 1997).

1-7-9: Minerals: -

There are several essential minerals found in black seed including (mg/100g): P (449), K (789.30), Ca (140.40), Mg (118.65) and Na (171.15) and secondary minerals including (mg/100g): S (361.80), Fe (1.65), Zr (2.58), Cu (1.20), and Mn (1), and not contain any toxic minerals like Pd, Cd (Al-Jassir, 1992; El-Faham and Sawsan, 1994; Al-Anee, 1998).

1-7-10: Vitamins: -

There are several important vitamins found in black seed such as Thiamine-B1, Riboflavine-B2, Niacine-B5, Pyroxidine-B6 and Folic acid. Also Tocopherol found in black seed like: alpha-tocophrol, beta-tocopherol, gamma-tocopherol and delta-tocopherol (Nergiz and Otles, 1993).

1-8: The thymol:

Thymol is a volatile oil, thymoquinone is results from combination of two phenolic compounds, quinone and thymol, and these two phenolic compounds return to the phenolic compound 1,4-benzo quinone or what is called by para-quinone and from this drived thymoquinone (Charles *et al.*,1969).

Thymol discovered for the first time in 1719 and had been purified in 1853 (Felter and Lioyd, 1898). As shown in figure 1-4, there are several names of Thymol (Dickens, 2002): -

a) 2-isopropyl 5-methyl phenol,
b) 6-hydroxy-p- cymene,
c) 6-isopropyl-m cersol,
d) isopropyl- meta- cersol,
e) 5-methyl 2-1-(1 methyl ethyl) phenol,
f) 3-hydroxy-4-isopropyl-1-methyl benzene.

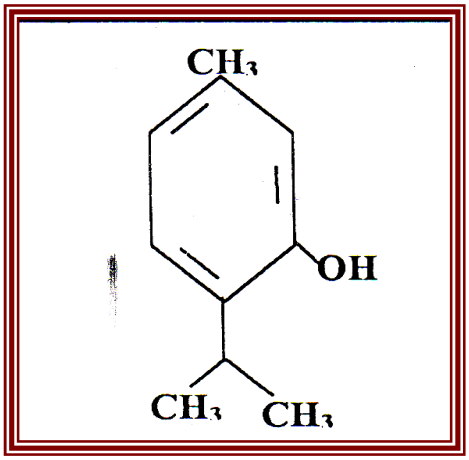


Figure (1-4): Thymol structure

The carvacol is the isomeric phenolic compound of the thymol its name is (iso propyl-ortho cersol) (Al-Niamey, 2004).

Thymol found in several plants including: *Thymus vulgaris, Nigella sativa, Carum ajawan, Ptychotis ajawan, Inula nervosa* (Jiang *et al*., 1990), also found in the oil of Eucalyptus (Federal, 2003).

Thymol is a crystal compound, colorless, its melting point at (48-52)ºc, while boiling point reaches 230ºc, soluble in ether and in alcohol also soluble in diluted alkali solution but not soluble in water (Al-Shamaa, 1989) and the metabolism of thymol is shown in figure 1-5 (Vernet *et al*., 1986; Linhart and Thompson, 1999).

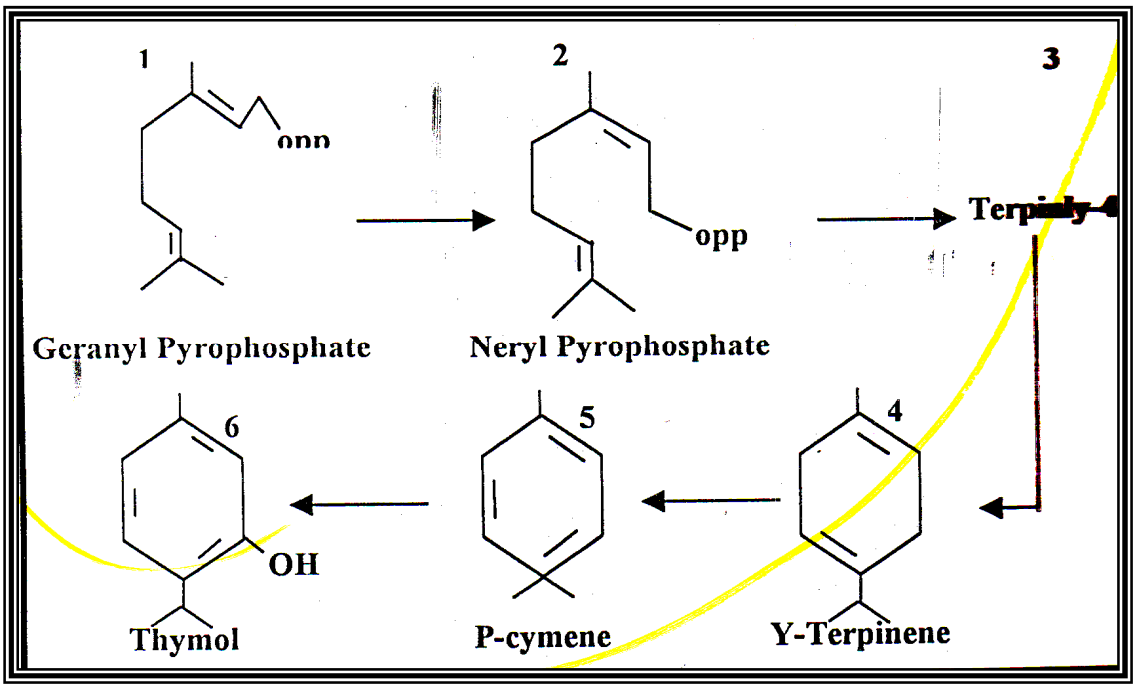


Figure 1-5: The metabolism of thymol.

Its role in the inhibition of arachidonic acid activity, which play role in platelet accumulation and then thrombosis occur. This activity of thymol is best than aspirin activity that is used in blood coagulation treatment (Enomoto *et al*., 2001).

The active part in thymol is the presence of oxidized benzyl ring (contain hydroxyl group) this cause the toxicity to microorganisms in a way of inhibition their enzymes by reactions with watery sulfate group or with other reactions not specific with proteins of microorganisms (Cowan, 1999).

The activity of thymol increased when the number of hydroxyl groups increased (Bruneton, 1999).

The phenolic compounds are present inside the cell vacuoles (Gayon, 1972; Harborne, 1973; Walker, 1975). The basic source of these phenolic compounds inside the plant cell is Shikimic acid compound this occur in way of aromatic amino acid production like phenyl alanine and tyrosine, this will enter the process of removing the amine group to produce compounds contains benzene ring and the phenolic compound is one of these compounds (Bruneton, 1999).

1-9: Plant Tissue Culture Techniques:-

The history of Plant Tissue Culture or some times known as Micropropagation or *in vitro* culture and finally called as Green Gold, go back to Haberlandt experiments (١٩٠٢), and represent the best important new biotechnique in the present time which has several techniques in different plant science (Al-Niamey, 1995).

The basic which plant tissue culture technique depends on is the definition Totipotency which means the ability of each cell in plant cells to divide and grown into complete plant like its mother if suitable conditions found like temperature, light and nutrients (Smith, 2000).

By this definition the scientists began to develop this technique to include several bio practice like Agriculture, or Industry, and also Trade to extract and produce compounds (secondary metabolites or active compounds) of several plants and this be important and could be used in several practice of tissue culture (Gras and Segura, 1988).

In vitro propagation of plants holds tremendous potential for the production of high- quality plant based medicines (Murch *et al*., 2000). This can be achieved through different methods including micropropagation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha,1994).

With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen- free material. Micropropagation of various plant species, including many medicinal plants, has been reported (Murashige, 1978; Withers and Anderson, 1986).

1-10: *In vitro* production of secondary compounds:

The using of plant tissue culture techniques make the easy of pharmaceutical compounds production instead of depending on the mother plant and become possible to produce these compounds at high amount and at high rate of pure may be over than these isolated from the complete mother plant, and its production may be quickly and independent on the season, also limit the surface area that is used in the medicinal plant culturing (Robins *et al*., 1985).

The production of medicinal plants by tissue culture describes its use instead of conventional culturing method, which provides industrial source of different necessary metabolic compounds, including alkaloid, phenol, terpenoid, vitamins and other of compounds which are necessary in medical uses (Shengwei and Jingsam, 2000).

It is necessary to say that the production of medicinal and pharmaceutical compounds and others by the using of tissue culture affected by several factors some of them are: natural like

the explant that is used in the culture and its source, the amount of explant that is used in the production of active compounds, the component of nutrient media that is used in the culture and the condition of storage culture like light, temperature and aeration (Mantell and Smith, 1984), and these will affect directly on the plants primary product and then affect on the secondary metabolic compounds which derived from primary metabolic compounds (Bhalsing and Maheshwari, 1998).

Propagation from existing meristems yields plants that are genetically identical with the donar plants (Hu and Wang, 1983).

Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona legeriana* and *Digitalis* spp (Paek *et al*., 1995; Pere *et al*., 2002)

Most of the research on plant cell tissue culture have emphasized the production of medicinals and drugs; however, food additives including flavors and essential oils may also become important products, and various other biochemicals and specially chemicals are likely candidates (Scott and Dougall, 1987).

The production of chemicals from plant tissue culture was first detailed by (Routien and Nickell, 1956). Their concept was based on the well- developed use of microorganisms for fermentation.

The art of plant tissue culture has been further developed by many other investigators, as of 1979, there were over 22 reports of cultures producing concentrations of chemical products that were equall to or greater than those found in the whole plant (Zenk, 1978; Dougall, 1979).

Plant cells in culture have some features that are similar to microbiological processes. For example, the product may be secondary metabolite that is excreted into the reaction media or the product may normally accumulate within the intracellular vacuole of the cells (Scott and Dougall, 1987).

Now there are reports of **ginsengosides** at 27% dry weight of *Panax ginseng* cultures (Zenk, 1978); **rosmarinic acid** at 23% of dry weight of *Coleus blumi* (Dougall, 1985); **shikonin (naphthaquinones)** at 12% dry weight in *Lithospermum erythrorhizon* (Zenk, 1978); **naphthaquinones** at 27% dry weight in *Morinda citrifolia* and 22% in *Galium glaucum* and *Rubia fructosa* (Dougall, 1985).

These yields are, in all cases, increased over those found in the whole plant by a factor of 5 or more. Thus, some tissue cultures may significantly enhance the yield of desirable plant phytochemicals (Scott and Dougall, 1987).

Scragg with his group isolated **quinoline** alkaloids in significant quantities from globular cell suspension culture of *Cinchona ledgeriana* (Scragg, 1992).

Ravishankar and Grewal reported that media constituents and nutrient stress influenced the production of **diosgenin** from callus cultures of *Dioscorea deltoidea* (Ravishankar and Grewal, 1991).

Parisi *et al*., 2002 obtained high yields of **proteolytic** enzymes from callus tissue culture of garlic (*Allium sativum* L.) on MS medium supplemented with NAA and BAP, (Pradel *et al*., 1997) observed that the biosynthesis of **cardenolides** was maximal in the hairy root cultures of *Digitalis lanata* compared to leaf.

Pande *et al*., 2002 reported that the yield of **lepidine** from *Lepidium sativum* Linn depends upon the source and type of explants.

1-11: Effect of auxins and cytokinins on callus induction:

Auxins and cytokinins are used almost in all plant tissue culture systems because of their effects on induction callus and inducing differentiation in vegetative parts and roots (Birsa and Segura, 1989; Kever *et al*., 1999).

Auxins are defines as that groups of organic acids with high molecular weight and used at low concentrations to make physiological effects on the cultured parts (Al-Shaigy, 2005). The

most frequently auxins used are NAA, IBA, 2,4-D and IAA (Conger, 1981), the last one represents the active auxin which proliferate callus growth more than the others (Al-Hadedy, 2002). When using 2,4-D at high concentration leads to inhibit root and vegetative parts to form callus (Schaufler and Walker, 1993).

While cytokinens are basic compounds with high molecular weight used at low concentration to give big effect on the cultured parts, the most usually used are BA, Kin, 2ip and Zeatin (Mohammed and Omar, 1990; Mohammed and Al-Yonise, 1991).

Auxin and cytokinen are the most frequently used in plant tissue culture (Smith, 2000; Raven *et al*., 2003), so there must be some balance between both rates, auxin play role in cell expanding which affects on cell wall flexibility in way increasing the activity of some enzymes (Collett *et al*., 2000; Lee *et al*., 2000), and cytokinins facilitate cell division (Ammirato *et al*., 1983).

So callus have irregular shape of paranchymatic cells and produced from meristematic cell of plant tissue (Mohammed and Omar, 1990; Caponetti, 1996).

Callus is produced on explants *in vitro* as a result of wounding and in response to hormones (either endogenous or supplied within the media) (Salmyan, 1988).

The induction of callus largely depends on the type of explants, composition of nutrient media and incubation conditions

(Al-Kanany, 1987). The callus needs 3-8 weeks to be subcultured in a new media after cutting it to several equal parts (Salmyan, 1988).

Leaveing the callus without subculture on the same media will leads to exhaust the media and the nutrient compound may be lost and toxic compounds will be accumulated and this will affects callus (Salmyan, 1988).

The differences in callus color (Dodds and Roberts, 1985), external shape and solidity of their tissues due to differences of the plantlets, the callus may be colorless or with pale green (contains chlorophyll) or yellow (contains carotenoid and flavoniods) or violate (contains anthocian pigments) (Salmyan, 1988), like khat callus culture produced dark colored pigment these pigment is a result of the high content of polyphenols (tannins) (El-Hag *et al*., 1999).

1-12: Plant tissue culture of *Nigella sativa*:

The *N. sativa* plant represents a good sample in the tissue culture, its vegetative parts have the ability to grow and forming callus and also have a special responsibility to almost known growth regulator and those synthetic and growth regulator like PDA and AHM (Banerjee and Gupta, 1974; Al-Baker, 2002).

In study (Chand and Roy, 1980) to compare between the responsible range of *N. sativa* explants (leaf and stem) for callus induction, they found that the responsible of leaf explant for callus induction was rapid than of stem explant so they proved by this study that the leaf represent the rapid source for callus induction.

However, the callus was induced from the three explants of *N. sativa* plantlet (leaf, stem and root) by culturing them on MS media in addition to NAA with coconut milk or MS with Kin and 2,4-D it was found that the stem tissue is the best in callus induction rate (Banerjee and Gupta, 1974; Chand and Roy, 1979 and Youssef *et al*., 1998).

Where as (Youssef *et al*., 1998) found that the three different parts (leaf, stem and root) of *N. sativa* plantlet have the ability to induce callus when culture them on MS media contained different concentrations of 2,4-D and Kin, the best concentration was MS with 10^{-6}M of 2,4-D and 5×10^{-6}M of Kin, and leaf callus gives

highest rate of fresh weight followed by stem callus and root callus.

In (Rasheed, 2000) callus induced from *N. sativa* plantlet by culturing (leaf, stem and root) explants on MS media in combination with Kin (0.1, 0.3 and 0.5 mg/L) and 2,4-D (0.2 and 0.3 mg/L) the result from this study was that the root gives the highest rate in callus induction.

The role of (2,4-D, IBA and NAA) as an auxin with different concentrations (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} M) for each of them with combination with BA to induce, growth and differentiate callus from stem explant of *N. sativa* plantlet in this study the best media for callus induction and callus growth which contains 10^{-6}M of 2,4-D alone which gives 44.42 g fresh weight of callus 100 day old (this represent the stander media), also in this study they found the best media for root differentiation was 10^{-3}M NAA, while the best differentiation for vegetative parts was $l0^{-10}$M 2,4-D with 10^{-6}M BA (Al-Baiker, 2002).

1-13: Effect of *Nigella sativa* on tumor cells:

The extract of *N. sativa* (black seed) has several biological activities that include antimicrobial, antiviral, antiinflammatory and immunomodulatory activities and anticarcinogenesis.

In addition to that, the volatile oil of *N. sativa* has efficacy as a chemotherapeutic agent. Evidence for this anticancer activity is provided by studies that indicate treatment with *N. sativa* extract inhibited dimethyl benz(a) anthracene (DMBA)/ croton oil-induced skin cancer and 2 O-methyl cholanthrene (MCA)- induced soft tissue sarcoma formation in mice (Salomi *et al.*,1991 ; El-Mofty *et al.*,1997).

Also, the extract of *N. sativa* at a concentration that does not compromise antitumor activity, reduces the incidence of cisplatin-induced myelo-suppression (Badary *et al.*, 1999) and nephrotoxicity (Nair *et al.*, 1991) and protect against doxorubicin-induced cardiotoxicity and nephrotoxicity (Al-Sabah *et al.*, 1998; Badary *et al.*, 2000).

Other studies demonstrated that *N. sativa* extract attenuates ifosfamide- induced Fancoi syndrom and improves the antitumor efficacy of this chemotheraputic agent in mice (Badary, 1999). The chemotherapeutic and chemoprotective effects of *N. sativa* extract may be due to quinones that include thymoquinone and dithymoquinone that are present in the oil of this seed,

thymoquinone suppresses benzo(a) pyrene(BP)- induced forestomach tumor formation (Badary *et al*., 1999), potentiates the antitumor activity of cisplatin, and improves its therapeutic index (Nair *et al*., 1991).

In vitro studies indicate that dithymoquinone is equally as cytotoxic as thymoquinone to several parental human tumor cell lines and their corresponding multidrug resistant (MDR) variants (Worthen *et al*., 1998).

There is a study suggested that thymoquinones kills cancer cells by a mechanism that involves apoptosis and cell cycle arrest at G1, and they found that there is Non-cancerous cells are relatively resist to thymoquinone (Ahmed *et al*., 2002).

Phenols that found in black seed have anti mutagenic activity by blocking the metabolic activation of the mutagens and scavenging the free radicals produced from mutagen metabolism. Phenols can also reduce the DNA-adduct formation by its binding to the target sites in the DNA to prevent the binding of the mutagen (Raj *et al.*, 1983).

1-14: Mitomycin-C (MMC):

An antitumor antibiotic isolated from *Streptomyces cupspitosus* in the late 1950s (Beijnen *et al*., 1987). Is a natural antitumor antibiotic and cytotoxic drug used in clinical chemotherapy regimens for the treatment of the various carcinomas (Verweij and Pinedo, 1990) as shown in the figure(1-6).

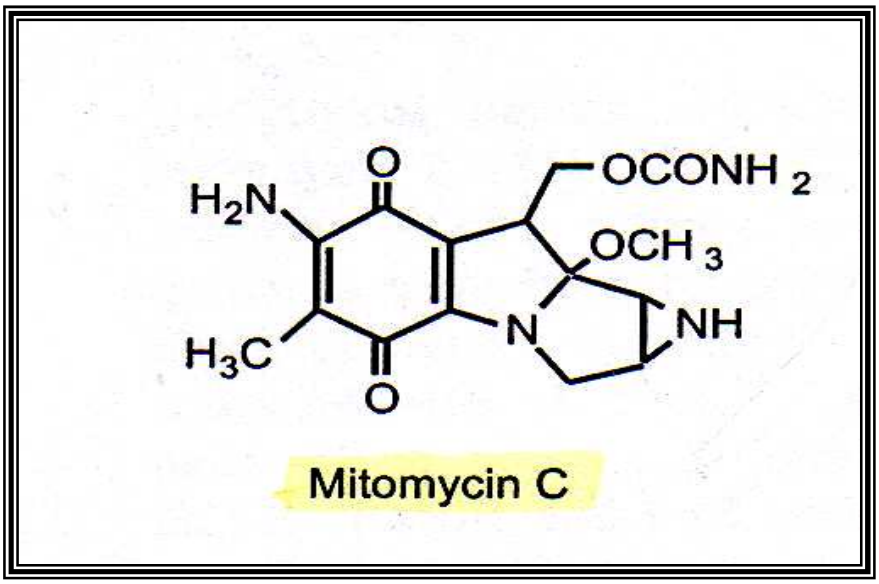


Figure (1-6): shows the structure of mitomycin

(Beal and Winski, 2000)

It interferes with the multiplication of cancer cells, slows or stops the growth and spread of cancer cells in the body, which interrupts DNA replication and inhibits mitosis (Costa *et al*., 1993). MMC shows activity against tumor of the breast, stomach, lung, pancreas, bladder and colon (Teicher, 1997).

There are number of quinone- containing antitumor agents that are used clinically including the anthracyclines- doxorubicin, daunorubicin and idarubicin- and the anthracenedione, mitoxantrone. These agents bind DNA in a non- covalent manner, and thus they are not considered alkylating agents, while mitomycin are quinone containing alkalyting agents and is currently approved for general use (Beal and Winski, 2000).

MMC is a very toxic drug (Pratt *et al*., 1994), and its principal toxicity, bone marrow suppression, is related to the total cumulative dose (Teicher, 1997).

Side effects depend on the site were given through:

*through a catheter into the bladder (in case bladder cancer) include:

- Decreased WBC counts and possibly reduced RBC and platelets counts.
- Discolored urine. The urine may be bluish green to purple. This is expected and can last for up to 2 days after each dose.

*given intravenously include:

- Nausea, vomiting and loss of appetite.
- Mouth sores and a sore throat.
- Hair loss.
- Liver may be temporally affected.
- Skin rash, sun sensitivity and easy burn.

- Lung damage that results in shortness of breath, cough and chest pain.
- Fever.

The cytotoxicity of MMC is due primarily to the formation of the DNA adducts-in particular DNA interstrand cross-links (ICL). (Lyer and Szybalski, 1963)

If MMC leaks to the tissue it may damage the tissue in that area Int.#3.

MMC is a carcinogenic in mice following its subcutaneous and intrapritoneal injection Int.#4.

1-15: Cytogenetic analysis:

Cytogenetic analysis has been proved to be good and reliable for the mutagen-carcinogen exposure and chromosomal aberration test (Nakanishi and Schneinder, 1979; Gebhart, 1981).

1-15-1: Mitotic Index (MI):

MI represents a ratio of somatic cells to interphase nuclei in a thousand cells:-

$$MI = \frac{\text{Number of dividing cells}}{\text{Total no. of the cells (1000)}} \times 100$$

(Stick and San, 1981)

The MI is used to detect the genotoxic effect of some mutagenic and carcinogenic agents that has chemical or physical nature in the cells, always these agents have effect on MI (King *et al.*, 1982).

Littlefield *et al.*, (1980) found the effect of MMC in inhibition of MI in human lymphocyte when treated with this drug.

Mitotic index increase by increasing colchicine concentrations but was unaffected by 5- bromodeoxy uridine (BrdUrd) concentrations in the medium (Shubber and Al-Allak, 1986).

1-15-2: Chromosomal aberrations (CAs):

Chromosomes are the structures that hold our genes. Genes are the individual instructions that tell our bodies how to develop and keep our bodies running healthy (Becher *et al*., 1983)

Living organisms may expose to different kinds of effects which may cause chromosomal abnormalities (or aberrations). CA considered as a genetic damage of chromosomal level observed as an alteration either in chromosome number or in chromosome structure (Catherine *et al*., 1998).

The increasing variety of chemicals, radiations and other physical agents we are exposed to nowaday has stimulated the development of many rapid, reliable assays for the detection of the mutagenicity or carcinogenicity of such agents. One of these

methods is the chromosome aberration assay (Lambert *et al*., 1978; Ardito *et al*., 1980).

The most important thing that the chromosomal structure abnormalities can be distinguished when the cell is in the metaphase of the mitosis (Evans, 1976).

Structural aberrations may be of two types, chromosome or chromatid. Structural aberrations of chromosomes are common in nature and have apparently played a significant role in evoluation. They occure spontaneously, but the frequency is increased by ionizing radiation and chemical mutagens. While chromatid structural aberrations induced by chemical mutagens (Evans and Oriordan, 1977).

The cytogentic examination of the bone marrow cells must be included in the study of the possible chromosome damaging effect of chemical agents *in vivo* because the bone marrow cells are very sensitive to chemicals, so they represent a good indicator for the effect of chemical oncogens and mutagens (Jensen and Nyfros, 1979).

This test is used to detect the mutagenetic effects of some drugs and used for cancer treatment. Miura *et al*., (1983) found that the MMC has the ability to induce chromosomal aberrations in the lymphocytes.

1-15-3: Micronucleus assay (MN):

Micronucleus is cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes lagging at anaphase or from a centeric chromosomal fragments, so these nuclei can easily distinguish it in cytoplasmic of polychromatic erythrocytes, this is because of the erythrocytes push its nuclei out at the end of transformation phase (Schmid, 1975; Mavourrin *et al*., 1990).

So the frequency of the occurrences of micronuclei in treated cells provides a comparatively rapid and sensitive indication to the genetic damage that is produced in hemopoietic system so this test is used toward physically and chemically agents which are genetically toxic.

Some study (Schroder, 1966; Schroder, 1970) recommended the use of bone marrow smears to detect *in vivo* damage from chemical mutagens and demonstrated the occurrences of micronuclei in bone marrow cells in connection with cytogenetic damage.

This increasing use of various micronucleus assays since 1973 undoubtedly seems mainly from the primary advantages of speed and simplicity (Heddle, 1973).

1-1: Introduction

Black seed (*Nigella sativa)* is considered to be one of the greatest healing herbs of all times. The herb has been used for millenniums to strengthen the immune system, cleans the body, purify the blood, protect against irritants and support healthy longevity Int.#1.

It is an annual herbaceous plant believed to be endogenous to the Mediteranean region but has been cultivated in other parts of the world including India and Pakistan (Al-Soltan,1993).

The first discovery of the plant in Iraq was made by the Indian scientist Chakraverty, this plant was grown in a wild area in the west –desert (Chakraverty, 1976).

Black seed oil contains about 0.5-1.5 % volatile oil including nigellone and thymoquinone used as anti-histanimic, antioxidant, antiinfective and bronchodilating effects (Randhawa and Al-Ghamdi, 2002).

Black seed contains essential (volatile) oils, organic compounds, minerals, protiens (21%), phytolipids (35%), carbohydrates (34%), sterols and others (Al-Dabee and Al-Khaledde, 1997).

Some scientists refer the inhibitory effects of black seed extracts against pathogenic microorganisms is due to its content of essential and fixed oils especially the active compound thymoquinone and its

derivatives (thymohydroquinone and dithymoquinone) (Charles *et al*., 1969; Al-Salouse, 1995).

Thymol, is one of the active compounds in *N. sativa* extract, plays important role in the inhibition of cancer cells, and can attach with the mutagenic substance, because thymol is one of the antioxidant phenolic compounds (Weisburger, 1996).

Also thymol compound is used as a food preservative because it represents as an antioxidant compound to phytolipids instead of using some chemical compounds which are added to the food like Butylated hydroxy tolouene (BHT) that has many side effects (Bullerman, 1974).

Plant tissue culture techniques inters in several applications like plant micropropagation, genetic study, plant improvement, study of plant cell physiology, the production of secondary metabolites in addition to production of viruse free plant diseases (bacterial and fungal) (George and Sherrington, 1984).

So, to increase the production of this compound (thymol) all the year round without depending on the mother plant, plant tissue culture techniques formed callus and then increased the production of thymol.

Mitomycin-C (MMC) is an antitumor antibiotic used specially in the treatment of cancer, it interferes with the multiplication of

cancer cells, it slows or stops the growth and spread of cancer cells in the body (Nissl, 2005).

The possibility that readily available natural substances from plants, vegetables, herbs and spices may be beneficial in the prevention of cancer (Abdullaev, 2002).

Many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another (Ernest and Pitteler, 2002).

The interactions between herbs and drugs may increase or decrease the pharmacological or toxicological effects of either component (Adriene, 2000).

The aims of the present study are:

- ✓ Using plant tissue culture techniques for callus induction from *N. sativa* explants and determination of the best combination of 2,4-D and Kin in callus induction from different *N. sativa* explants.
- ✓ Extraction of thymol from callus then detection it by using HPLC method.
- ✓ Investigating the ability of thymol in reducing the genotoxic effects produced by MMC by using (MI, MN and CAs) assays in mouse bone marrow cells (*in vivo*).

2-1: Materials

2-1-1: Equipments and apparatus

The following equipments and apparatus were used throughout the study:

Apparatus	Company
Analytical balance	Monobloc
Autoclave	Webeco Gmph (Germany)
Centrifuge	Beckman (England)
Cold incubator	Memmert (Germany)
Electric balance	Sartorius (Germany)
Laminar air flow cabinate	ESCO
Magnetic stirrer with heater	Triup international corp
Microscope	Motic (Japan)
Micropipette	Motic (Japan)
Oven	Sanyo
pH– meter	Radiometer (Denmark)
Vortex mixer	Griffin (England)
Versatile environmental test Chamber (light incubator)	Sanyo
Water bath	Gallenkamp (England)

2-1-2: Chemical materials

The following chemical materials were used in this study:

Material	Company
KH_2PO_4 , $MnSO_4.7H_2O$, $CuSo_4.5H_2O$, $CoCl_2.6H_2O$, Nicotinic acid , Kcl, glycerin , Giemsa stain , methanol , glacial acetic acid	Fluka / Switzerland
Thiamine _ HCl, pyridixne, myoinositol, NaOH , heparen , 2.4 – dichloro phenoxy acetic acid , kinetin, DMSO, Mitomycin-C.	Sigma / U.S.A
KI, EDTA $–Na_2$, H_3Bo_3, $ZnSo_4.7H_2O$, Benzene , sodium bicarbnat , chloroform , ammonium chloride , glycin , thymol, Agar – Agar.	BDH / England
KNo_3 , NH_3NO_2 , $MgSo_4.7H_2O$, $CaCl_2 .2H_2O$	Merck
Colchicine Human serum Ethanol Normal saline Sucrose	Ibn Hyyan pharmaceuticals/ Syria National center for blood Transfusion Ferak / German. Baghdad Teaching hospital Local market

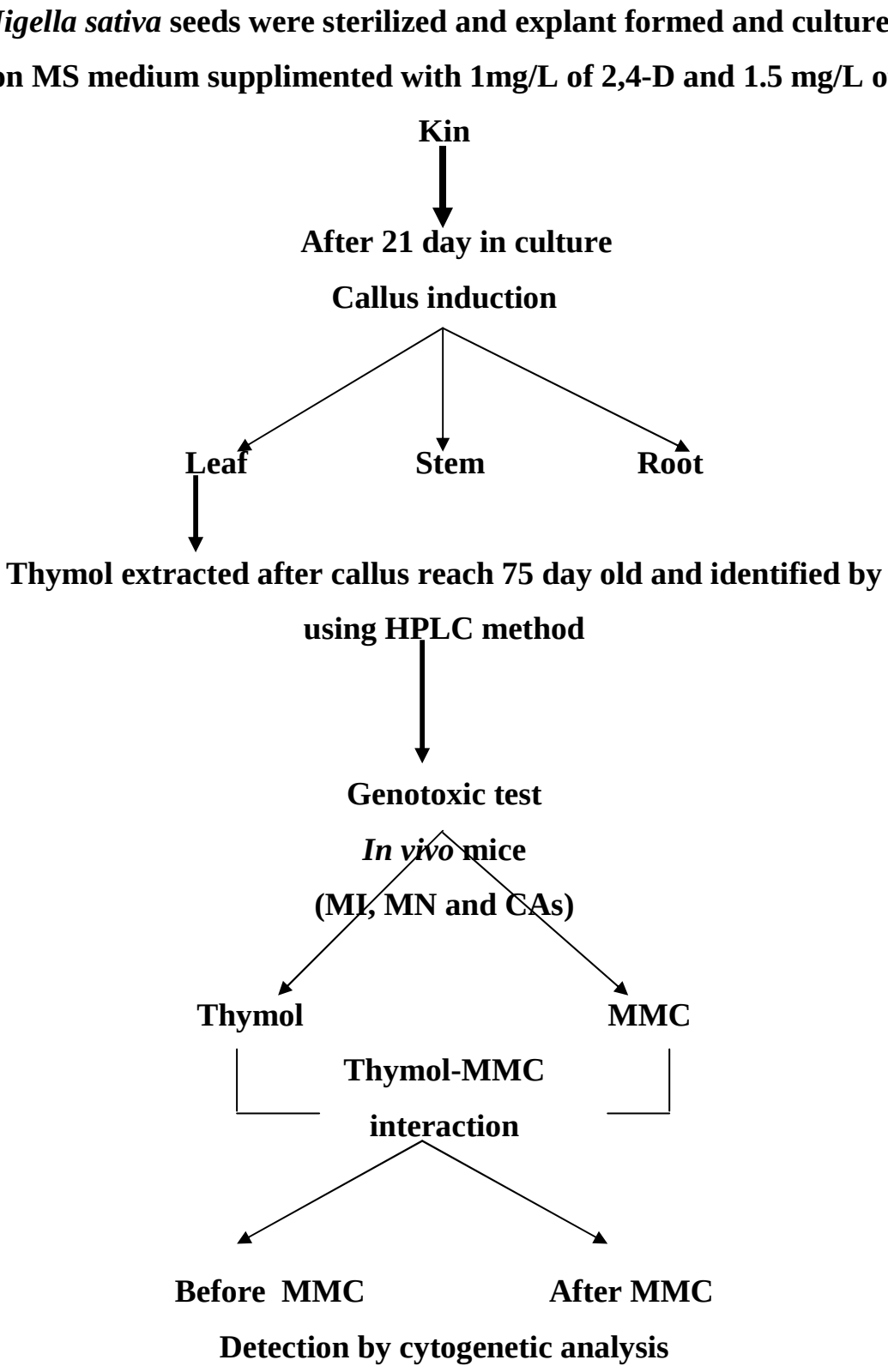


Figure (2-1): The research plan of this project.

2-2:Plant material:-

Black seed (*Nigella sativa*) shown in figure (2-2) used as a medicinal plant from Ranunculaceae family. The seeds gotten from Dr. Aws Al-Ani (Directorate of Agriculture Research and Food Technology/ Ministry of Science and Technology/ Baghdad/ Iraq) to be used.

Figure (2-2): *Nigella sativa* (black seed). (Adopted from www.uni-graz.at/katzer/eng /spice -photo html # nige-sar.)

2-3: Seed sterilization:-

Amount of (180) seeds from *N. sativa* were cleaned , washed with water to remove dust and any derties, then the seeds sterilized using normal bleach (6.2%) sodium hypochlorite concentration.

several volumes of bleach dilution were taken (0, 20, 40, 60, 80, 100 ml) to make (0, 1.2, 2.4, 3.6, 4.8 , 6.2 %) to make final concentrations respectively. The following table illustrates these dilutions (2-1).

Table (2-1): Different concentrations of bleach (NaOCl) used and its dilutions.

No.of tubes	Final concentration of bleach (%)
1	0
2	1.2
3	2.4
4	3.6
5	4.8
6	6.2

The seeds were divided into (6) equall parts each part contains (30) seeds and each group put in a specific concentration of bleach with continous shaking for (5) min, after that the seeds rinsed with sterile distilled water three times (5 min for each) to remove the effect of bleach .

2-4: Seed culturing:

In a laminar air flow cabinet (hood) sterile seeds cultured on petridishes contain sterilized distilled water about 5 ml in each plus a sterilized filter paper (sterilized in an autoclave at 121°c, pressure 104 Kg/cm² for 20 min.)

Five seeds cultured in each petridish with 4 replicates were used for each concentration of bleach; all cultures were incubated in an incubator 25 ± 1° c with light period 16 hour.

After that the percentage of seed germination were calculated using this equation:

$$\textbf{Percentage of seed germination} = \frac{\textbf{No. of seed germination}}{\textbf{Total of cultured seed}} \times 100$$

The notes were taken weekly about contamination rate and about seed germination for 4 weeks to calculate sterilization efficiency for each concentrations of bleach used, and after week from the culture uniform explants were chosed to be used for callus induction .

2-5: Nutrient media used in callus induction:-

Murashige and Skoog, (1962) MS medium was used. This medium is supplimented with inorganic salts in addition to growth regulators, vitamins, sucrose and agar.

Auxin (2,4-D) with concentrations (1, 2, 3 and 4 mg/L) and cytokinin (kinetin) with concentraions (1, 1.5, 2, 2.5, 3 and 5 mg /L)

The pH of the media was adjusted to 5.7 using 1 N of NaOH and 1N of HCL before autoclaving, then agar added to the media put on the magnetic stirrer (with heater) until mixed well and boild and then the media divided into small jars and sealed with metal covers which resist for the heat and sterilized in an autoclave for 20 min at 121 ^{o}c with pressure 1.04 kg/cm^{2} when the sterililzation process completed, these jars left at room temperature to cool and solidify the media until they use in culture .

Table (2-2) Mineral salts used in MS medium.

Stocks	Chemical structure	Amount (mg/L)
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.\ 2H_2O$	440
	$MgSO_4\ .\ 7\ H_2O$	370
	KH_2Po_4	170
Micronutrients	H_3Bo_3	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.\ 7H_2O$	8.6
	$CuSO_4\ .\ 5H_2O$	0.025
	$CoCl_2\ .\ 6H_2O$	0.025
	KI	0.83
	$Na_2Mo_4.\ 2H_2O$	0. 25
Chelated Iron	Na_2 -EDTA	37.3
	$FeSO_4\ .\ 7H_2O$	27.8

Table (2-3) Vitamins used in MS medium.

Organic component	Amount (mg /L)
Thiamine – HCL	0.1
Nicotinic acid	0.5
Pyrodoxine - HCL	0.5
Glycine	2.0
Myoinositol	100

2-6: Culture of explants:

Explants from seedling that grown on the plates has been chosed with uniform length, under sterile conditions (in the hood) these explants transferred to other plates contains sterile filter paper and partitioned into roots , stems and leaves pieces about (1cm) of length, these explants were cultured on prepared media of 5 replicates for each explant in each concentration and these were incubated at 25 $\pm$ 1 ^{o}c for 8 weeks .

2-7: Effect of light on callus induction:

All cultures were kept in an incubator at 25 $\pm$ 1 ^{o}c in long day (16 hour / day light and 8 hour / dark) with light intensity about 1000 lux. the cultures were then subcultured with fresh medium contains the same nutrient media.

Notes were taken weekly about the growth and callus development after 2-3 weeks from the culture, fresh weight of the calli were calculated to determine the optimum explant and the optimum hormone concentration for callus production .

2-8: Subculture of callus:

After determintion of the optimum concentration of plant growth regulator (2,4–D and kin) and the best plant part on the experiments which were done, all following media were prepared with 1.5 mg / L kin and 1 mg /L 2,4 –D to keep callus growing, and the leaf chosen to be best explant which gives

good yield callus induction and also give green callus color above other explants (stem and root).

2-9: Extraction of thymol from callus:

The method of Al-Shamaa, (1989) was used in the extraction of thymol from the callus with several modifications.

Solutions: -

a) NaOH (5%) solution (w/ v) prepared by dissolving 5 gm from NaOH in amount of distilled water then complete the volume to 100 ml.
b) Diethyl ether (DEE)

Procedure:

One g of callus was mixed with 30 ml of NaOH solution 5% and then DEE was then added in a ratio of (2:1) (v / v) and mixed well, this solution put on separation fennel left it for a while and then saparate the supernatent.

2-10: Detection of thymol using high performance liquid chromatography method (HPLC):

This was done in white field company. The separation of thymol accured on a reversed phase de base colum (MDB) 250 x 4.6 mm internal diameter (I.D)

The mobile phase that is used:-

Buffer phosphate: methanol (30:70), the pH was adjusted to 3.5

The detection occurred at UV light at 254 nm wave length, with flow rate 1 ml / min, the temperature was 30 ºc .

The concentration of thymol in the callus was calculated using this equation:

$$\text{Concentration of sample (mg /ml)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{concentration of standard}$$

The comparison between retention time (Rt) of standard thymol (gotten from college of pharmacology) and thymol extracted from callus.

2-11: Cytogenetic analysis:

Females (8-12 weeks old) balb/c– mice and weighting (23-27) g were used supplied by Biotechnology Research Center. They were maintained in control conditions of temperature (25 $\pm$ 1ºc) and light (14 and 10 hr. of light and dark condition respectively).

The animals were fed on a formula food pellets and supplied with water. Through out the experiment; 4 animals were housed in plastic cages containing hard –wood ship as bedding. The bedding was changed weekly to ensure a clean environment.

Component of fed:-

Product	Percentage %
Crushed barley	24.50
Crushed wheat	30.00
Crushed yellow corn	22.50
Soya bean	15.20
NaCl	0.45
Calce stone	0.20
Animal protein	7.15

Experiment no. I :-

The animals were divided into 3 groups:

Group 1: positive control injected intraperitoneally with mitomycin –C (2 mg/Kg) (Shubber *et al*., 1985).

Group 2: injected intrapertoneally with different concentrations of thymol (0.5, 1, 2, 4, and 8 mg / kg).

Group 3: negative control injected intrapertoneally with normal saline.

Animals sacrifice was carried out after 7 days of injections.

Using four animals for each group, two animals were used for estimating mitotic index and chromosomal aberration in bone marrow the other two animals were used for microuncleus test.

Experiment no. II :-

Interaction between thymol concentration and mitomycin-C.

The mice were divided into two groups:-

- group 1 :giving thymol 1mg/Kg intraperitoneally before giving MMC .
- group 2 :giving thymol 1mg/Kg intraperitoneally after giving MMC.

Animals sacrifices were carried out after 14 days of injections.

Using 6 animals for each group, 3 animals were used for estimating mitotic index and chromosomal aberration in bone marrow the other 3 animals were used for micronucleus test.

2-12: Cytogenetic Analysis *in vivo*:

2-12-1: Reagents and stains: according to (Allen *et al*., 1977)

- **Phosphate buffered saline (PBS)**:

was prepared by dissolving the following chemicals in distilled water bringing the final volume to 1 liter. This solution was stored at 4ºc.

-8 g sodium chloride (NaCl)

-0.2 g potassium chloride (KCI)

-1.15 Disodium hydrogen phosphate ($Na_2 HPO_4$)

-0.2 g potassium di hydrogen phosphate (KH_2PO_4)

- **Colchicine**: 1 tablet of 0.5 mg of colchicine was dissolved in 1 ml of distilled water and each animal was injected with 0.25

ml of this solution intraperioneally.This solution was prepared and used instantly

- **Hypotonic solution**: A 0.075 M KCL solution was prepared by dissolving 2.8 g of potassium chloride in 500 ml of distilled water.
- **Fixative solution**: 3 volumes of methanol were mixed with 1 volume of glacial acetic acid .
- **Sodium bicarbonate**: (0.75 % $NaHCO_3$) was prepared by dissolving 7.5 g of sodium bicarbonate in 1 L of distilled water and stored at 4 ^{o}c until used.
- **Stock Giemsa stain :**

One g of Giemsa stain powder was dissolved in 50 ml glycerine. The mixture was collected in stoppered brown bottle and placed in a 60^{o}c water bath for 2 hours with continuous shaking at half hour intervals.

The mixture was allowed to cool down and 50 ml of methanol added gradually with contineous shaking. This stain is stored at room temperature in the dark.

- **Working Giemsa stain :-**

The working stain was prepared by mixing the following compounds:-

40 ml distilled water

0.5ml $NaHCO_3$ solution

1.25 ml methanol

1 ml Giemsa stock stain.

- **Mitomycin –C stock 2mg/Kg : (mg / ml)**

It was prepared by dissolving 1mg from MMC drug in 1ml salin (stock) and injects 0.05 ml from this stock to animal.

2-12-2: Chromosome preparation from somatic cell of the mouse bone marrow.

The experiment was done according to (Allen *et al*., 1977) as follows:

1- The animals was injected with (0.25 ml) of colchicine with conecentration of (0.5 mg/ ml) intraperitoneally (I.P) 1.5 hours before scarifying the animals.

2- The animals were sacrified by cervical dislocation.

3- The animal was fixed on it ventral side on the anatomy plate and the abdominal side of the animal and its thigh region was swabbed with 70 % ethanol.

4- The bone was taken and cleaned from the other 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 syring, (5 ml) of PBS was injected so as to wash and drop the bone marrow in the test tube .

5- The test tube were taken and put in the centrifuge at speed of 2000 rpm for (10 min).

6- The supernatant was removed and (5 ml) of potassium chloride (KCL) was added as a hypotonic solution at (0.075M),

and then the tubes were put in water bath at (37º c) with shaking from time to time.

7- The tubes were centrifuged at (2000 rpm) for (10min).

8- The supernatant was removed and the fixative solution was added (as drops) on the inside wall of the test tube with contineous shaking, the volume was fixed to (5ml) and the content was shaked well.

9- The tube were kept at (4 ºc) for 30 min. to fix the cells.

10- The tubes were centrifuged at 2000 rpm for (10 min).

- The process was repeated for 3 times and the cells were suspended in 2 ml of the fixative solution.

11- By a pasture pipette, few drops from the tube were dropped vertically on the chilled slide from a hight of 3 feet a rate of (4-5) drops to give the chance for the chromosomes to spread well.

12- The slides were dryed at room temperature, then stained with Gimsa stain and left for (15 min) then washed with distilled water.

13- Two slides for each animal were prepared for cytogenetic assay.

2-12-3: Micronucleus test in mouse bone marrow cells:-

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

1- The animals were sacrified by cervical dislocation.

2- The animal was fixed on its ventral side on the anatomy plate and 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, (2 ml) 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 a speed of 1000 rpm (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 slides then fixed with absolute methanol for (5 min), then stained with Giemsa stain and left to dry and then washed with distilled water and leave it to dry.

7- Two slides for each animal were prepared for micronucleus test.

2-13: Cytogenetic analysis test:

2-13-1: Mitotic index (MI) assay:

The slides were examined under light microscope with (40x) power, and (1000) of the divided and non divided cells were counted and the percentage rate was calculated for only the divided ones according to this equation:

$$\text{MI} = \frac{\text{No. of the divided cells}}{\text{Total no. of the cells (1000)}} \times 100\%$$

(Stick and San, 1981).

2-13-2: Chromosomal aberration (CA) assay:

The slides were examined under the oil immersion lens for 100 divided cells per each animal and the percentage of these aberrations was estimated.

2-13-3: Micronucleus test (MN):

The number of MN in 1000 cells of polychromatic erythrocytes (PCE) was scored under the oil immersion lens, and percentage of MN was calculated.

2-13-4: The protective value of thymol extract:

The protective value of thymol was calculated according to the following equation:

$$\textbf{P.V.} = \frac{\textbf{A - C}}{\textbf{A - B}} \times \textbf{100}$$

Were:

A: +ve control (treated with MMC only).

B: -ve control (treated with PBS only).

C: interaction groups (treated with MMC and thymol).

According to (Rawat *et al*., 1997).

2-13-5: Statistical analysis:

One way of analysis of variance was performed to test whether group variance was significant or not. The comparison between groups was used for analysis of variance test (ANOVA) (Al-Mohammed *et al*., 1986).

Results and discussion

3-1: Sterilization of black seed using sodium hypochlorite (NaOCl):

The results of seed germination after sterilization with different concentrations of commercial bleach (NaOCl) (0, 20, 40, 60, 80 and 100)% on a filter paper immersed on a sterilized distilled water as shown in figure (3-1).

Figure (3-1): growing seeds on filter paper soaked with sterilize distilled water.

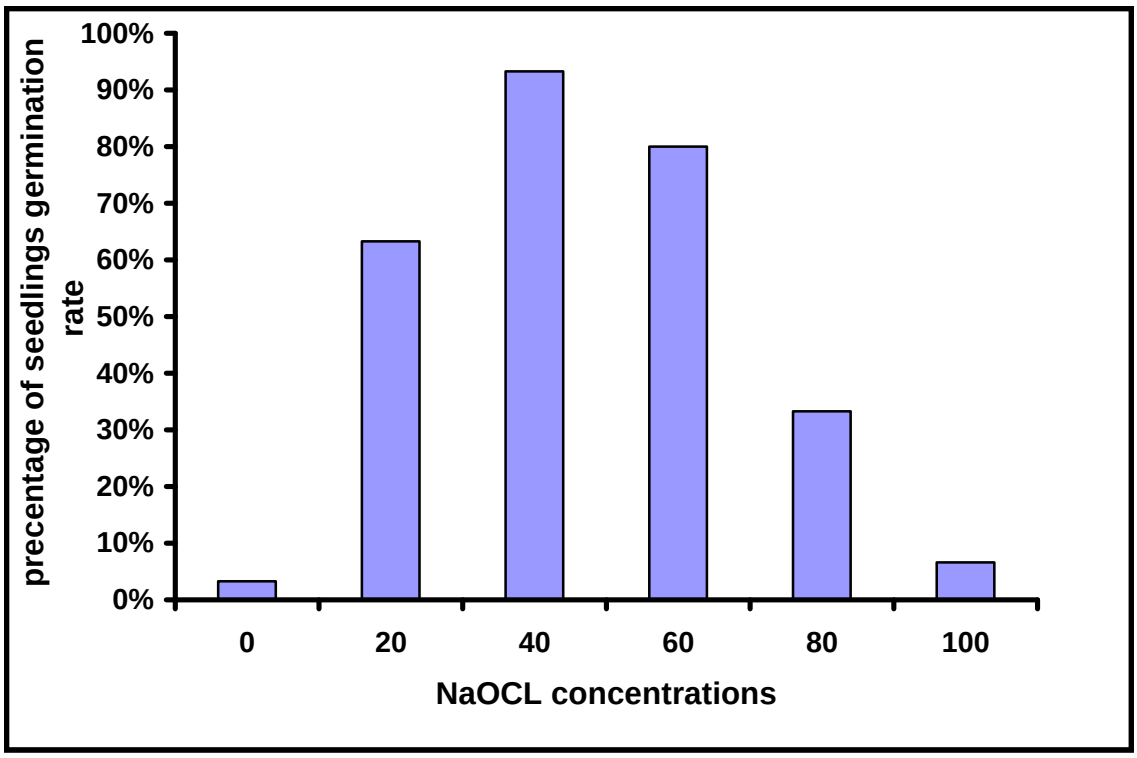
Table (3-1) indicates the highest germination rate which was 93.3% at the concentration 40% however, this growth began to decrease when increasing bleach concentration the germination rate was (80, 33.3 and 6.6%) at the concentrations (60, 80 and 100%) respectively. The germination rate at the

concentratons (0 and 20%) was (3.3 and 63.3%) respectively (figure 3-2).

The results indicate that increasing of hypochlorite concentrations may have toxic effect on seed embryo also increasing in germination rate of the growing seeds which treated at the concentration 40% related to that the NaOCl affects on the dissolving the hard seed wall of the seed and broken or damage it which easily permit the moisture to reach the embryo and induce germination compared with the control treatment 0% the seeds needs more time to saturate its solid wall and broken it (Salmyan, 1988; Mohammed and Omar, 1990; Al -Shaigy, 2005 and Al-Mafaragi, 2005).

Table (3-1): the precentage rate of germination seeds treated with different concentrations of NaOCl.

Concentration of NaOCL (%)	Number of cultured seeds	Number of germination seeds	Sterilization effeciancy (%)
0	30	1	3.3
1.2	30	19	63.3
2.4	30	28	93.3
3.6	30	24	80
4.8	30	10	33.3
6.0	30	2	6.6



Figure(3-2): the effects of different concentrations of NaOCl on the precentage of growth of *Nigella sativa* seeds.

In this way NaOCl may promote the embryo to grow at 40% concentration and increase its growth rate.

3-2: Induction of callus from black seed explants:

Calli were initiated from leaf, stem and root taken from *N. sativa* seedling and cultured on MS media with different concentrations of 2,4-D and Kinetin.

3-2-1: Response of leaf explants to different concentrations of 2,4-D and Kin:

The callus was induced from leaf explant and the results in table (3-2) showed that there were no significant differences when using different concentrations of Kin (0, 1, 1.5, 2, 2.5, 3 and 5 mg/L), however, there are significant differences when using different concentrations of 2,4-D (0, 1, 2, 3 and 4 mg/L).

The typical concentration which gave the highest mean weight of callus mass was 1 mg/L of 2,4-D which gave about 787.1 mg compared with other conentration of 2,4-D used.

As shown in table (3-2) there is decreasing in the mean weight of callus when increasing the concentration of 2,4-D over 1mg/L and this appears at the concentrations (3 and 4 mg/L) which didnt response to form callus and both concentrations give no callus induction and there are no significant differences between them, however, in the interactions between 2,4-D and Kin concentrations, the result showed significant differences between them.

The highest mean of callus weight appears in 1 mg/L of 2,4-D and 1.5 mg/L of Kin and gives about 1400mg which are significant compared with other concentrations as shown in the figure(3-3), this agreed with (Youssef *et al*., 1998) when they found an increasing of Kin concentration and 2,4-D concentration still constant this leads to decreasing the fresh weight of callus mass of *Nigella sativa* .

Also many scientists showed that the interaction between auxins and cytokinins concentrations lead to induce callus (Brisa and Segura 1989; Al- Hadedy, 2002).

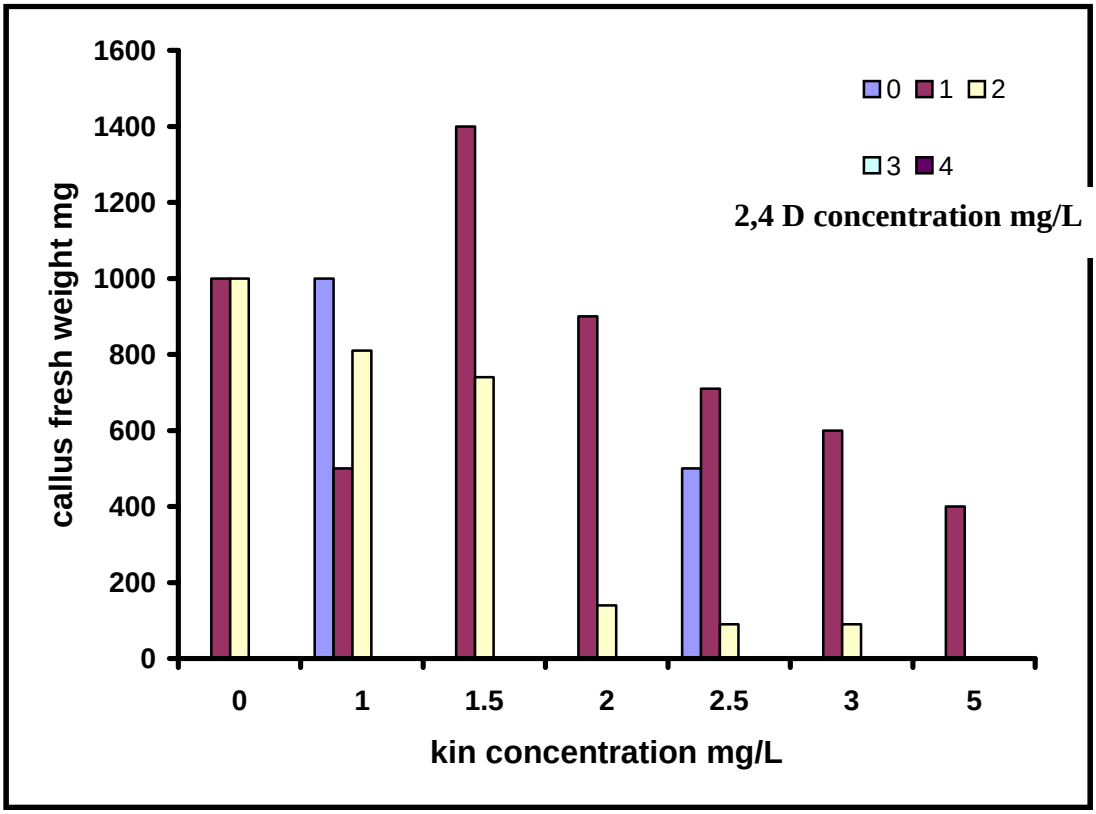


Figure (3-3): Effects of 2,4-D and Kin concentrations on callus fresh weight induced from leaf explant under light.

Table (3-2): Fresh weight of callus (mg) induced from leaf explant of *Nigella sativa* plantlet 75 day old .

3-2-2: Respone of stem explants to different concentrations of 2,4-D and Kin:

The results of the callus induction from stem explants are shown in table (3-3) when using different concentrations of 2,4-D and Kin. There was no significant differences were found when using 2,4-D concentrations were as a significant differences found when using different concentrations of Kin.

The highest mean weight callus mass presence in the control treatment (medium without hormones) about 660 mg compared with the using other concentrations (3 and 4 mg) of 2,4-D which gives the lowest mean about 30 mg as shown in the figure (3-4).

The reasons behind these respones may be explaind when using high concentrations of cytokinines especially Kin makes cells grow without division and this partialy related to water absorption because the formation of the reduced saccharides on the cells which is formed when lipid transformation occure in the presence of cytokinenes (Klis *et al*., 1973; Salahh,١٩٩٠)

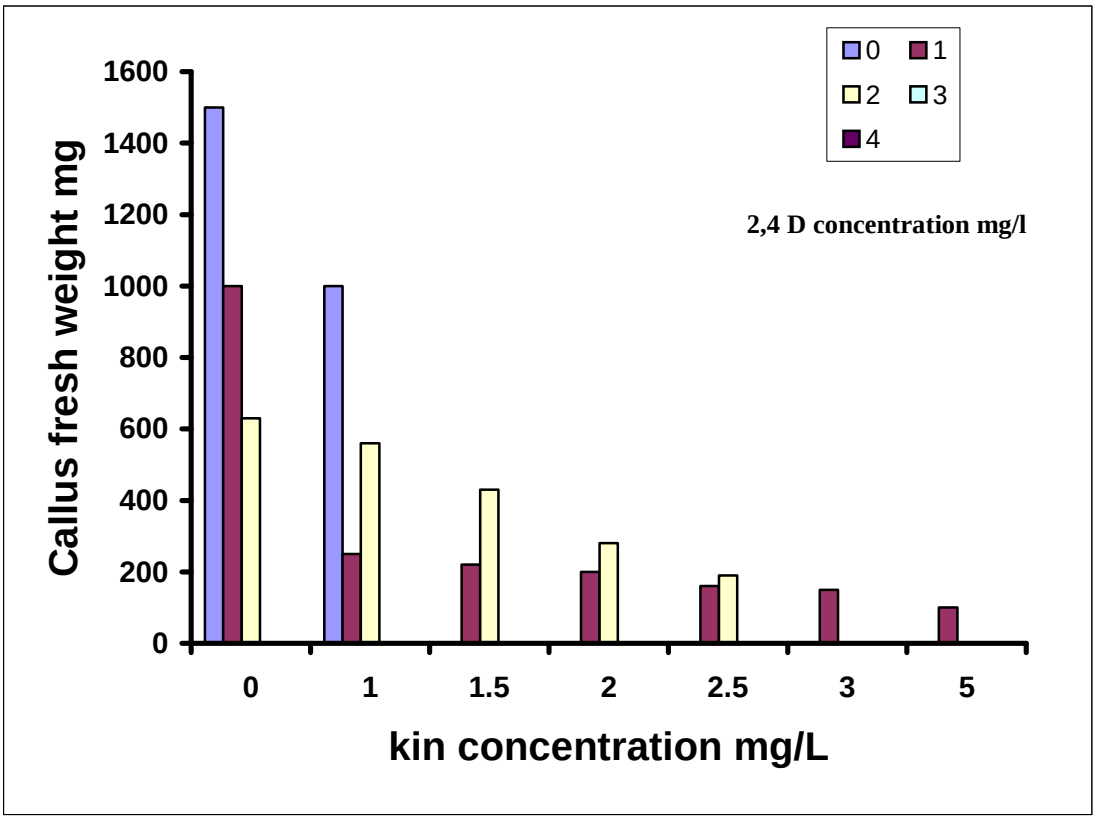


Figure (3-4): Effects 2,4-D and Kin concentrations on callus fresh weight induced from stem explants under light conditions.

Table (3-3):Fresh weight of callus (mg) of stem explant from *Nigella sativa* plantlet at 75 day old.

3-2-3: Response of root explants to different concentrations of 2,4-D and Kin:

When using different concentrations of Kin table (3-4) showed no significant differences in callus mean weight while there were significant differences found when using different concentrations of 2,4-D.

The concentration 1mg/L of 2,4-D gives highest mean weight of callus mass about 741.4 mg when compare with other concentrations of 2,4-D (3 and 4 mg/L) which not respond to callus induction, this becouse increasing auxin concentration may be toxic to callus cells and decrease or inhibit its growth figure(3-5) (Raven *et al*., 1986).

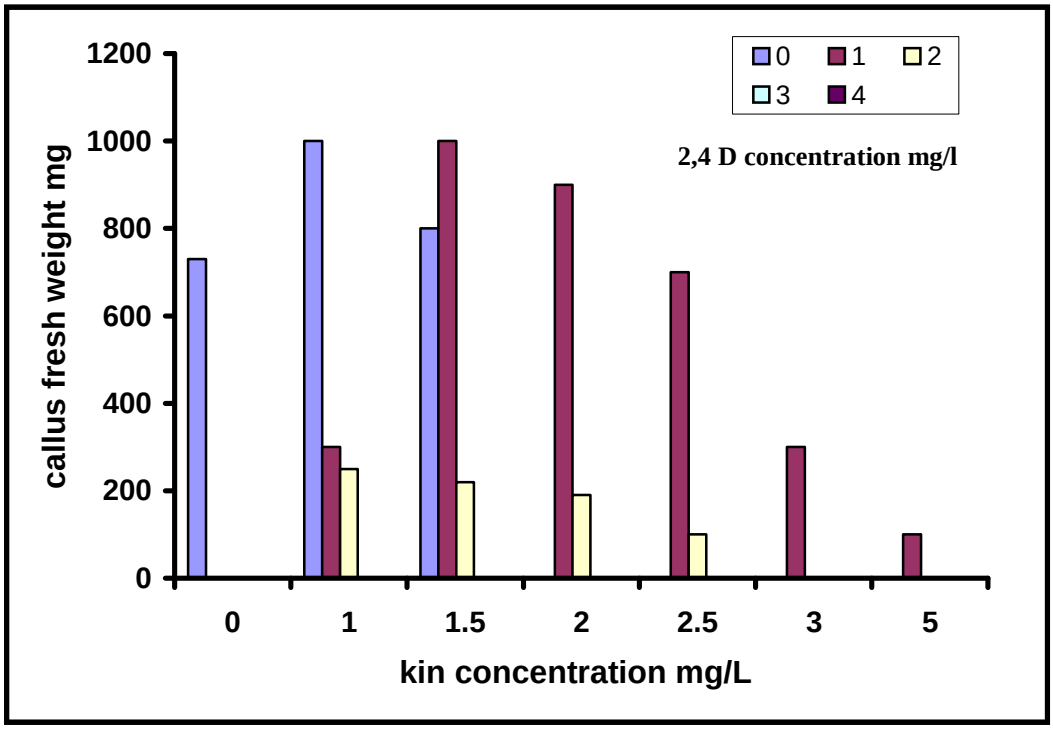


Figure (3-5): Effects 2,4-D and Kin concentrations on callus fresh weight induced from root explants under light conditions.

Table (3-4):Fresh weight of callus (mg) of root explant from *Nigella sativa* planlet at 75 day old.

Also Atea and Gadowaa, (1990) found there are decreasing in the mean weight of fresh callus weight which is induced when increasing 2,4-D concentrations which inhibit growth of the cells.

These results agreed with (Al-Shaigy, 2005) who studied the effect of different auxin concentration on *Thymus vulgaris* explants, and also agreed with (Dornelles *et al*., 1991) they induced callus from different classes of *Tricticum aestivum*.

Figures (3-6) and (3-7) show callus induced from different explants of *N. sativa* at different ages (21 and 75) days old with green callus color.

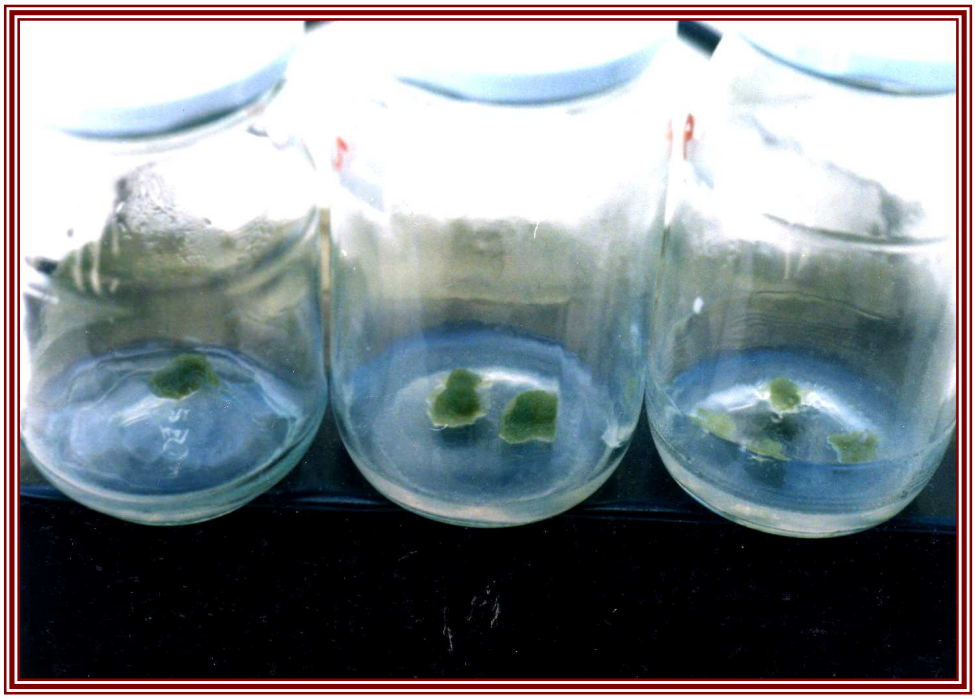


Figure (3-6): Callus induced from three explants of *N. sativa* at 21 days. Explants from left to right (leaf, stem and root) supplied with 1mg/L of 2,4-D and 1.5 mg/L Kin.

Figure (3-7): Callus induced from three explants of *N. sativa* at 75 days. Explants from left to right (leaf, stem and root) supplied with 1mg/L of 2,4-D and 1.5 mg/L Kin.

3-3: Detection of thymol using HPLC method:

The method of HPLC was used to detect the presence of thymol in the extract and quantitation of its concentration. The results presented in figuers (3-8) and (3-9) showed the similarity between standard and thymol isolated from leaf callus (75 day old) in the retention time between both samples (5.358) and (5.517) minutes, respectively. These results agreed with (Al-Timemee, ٢٠٠٤).

The concentration of isolated thymol was calculated as the equation shown in chapter two (2-10) and found that is equalls to 30.5 mg/ml in the leaves callus. However, (Al-Timemee, ٢٠٠٤) used Scanning Uv/ Spectrophotometer and applied Lamber- Beer Law to find the concentration of thymol from leaf callus at 75 day old and was equal to (19.87) mg/gm dry weight. Also in the same study, he found that when using the same hormone concentrations thymol extract from leaf calli with different ages was higher than thymol of root calli. This may be due to the differences of the endogenous hormones between the two explants (Phillips and Hubsterberger, 1985). The vegetative parts may represents the main center for anabolic metabolism in the plant (Lewin, 1976).

The reason behind not using stem explant in the extraction of thymol in our study was because when the stem callus becomes old age leads to decrease content and concentration of thymol while opposite appears when extract leaf explant the old

age of callus gives high contents of thymol this agreed with other studies (Dodds and Roberts, 1985; Al-Timemee, ٢٠٠٤).

The presence of the second peak as shown in figure (3-9) of the isolated thymol may be due to several reasons a) presence of isomeric thymol and these may be other phenolic compounds which has hydroxyl group in the sample or interacted with the solvent groups (Al-Timemee, ٢٠٠٤), b) the second reason may be separating another active compound from tissue culture or separating some pigments from the callus, c) the high sensitivity of the method used to lowest concentrations and finally d) some factors may also affect like temperature and pH (Harvath, 1980, Voet and Voet, 1991).

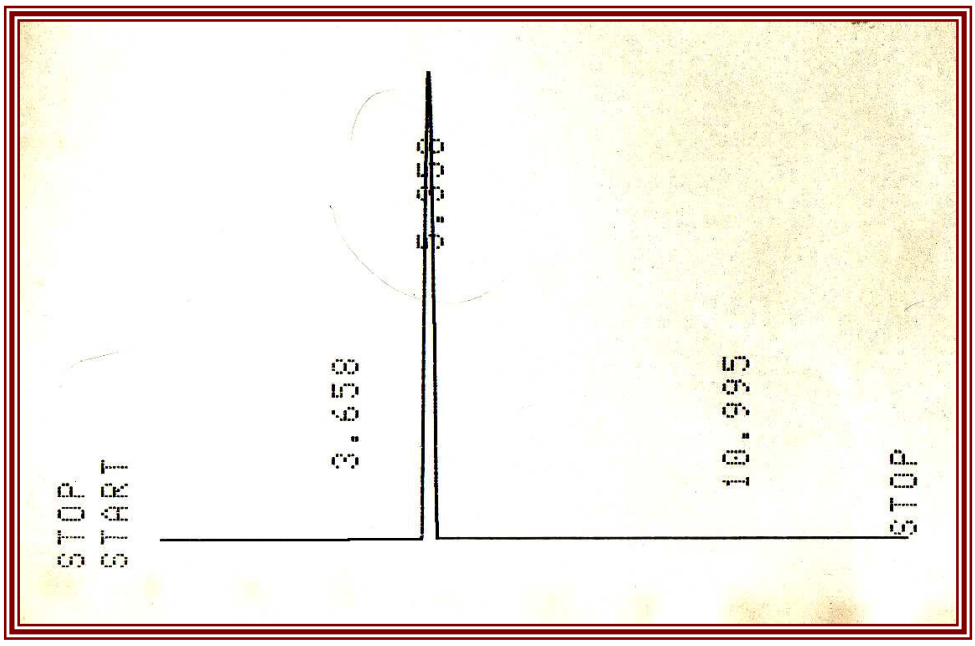


Figure (3-8): HPLC of the standard thymol.

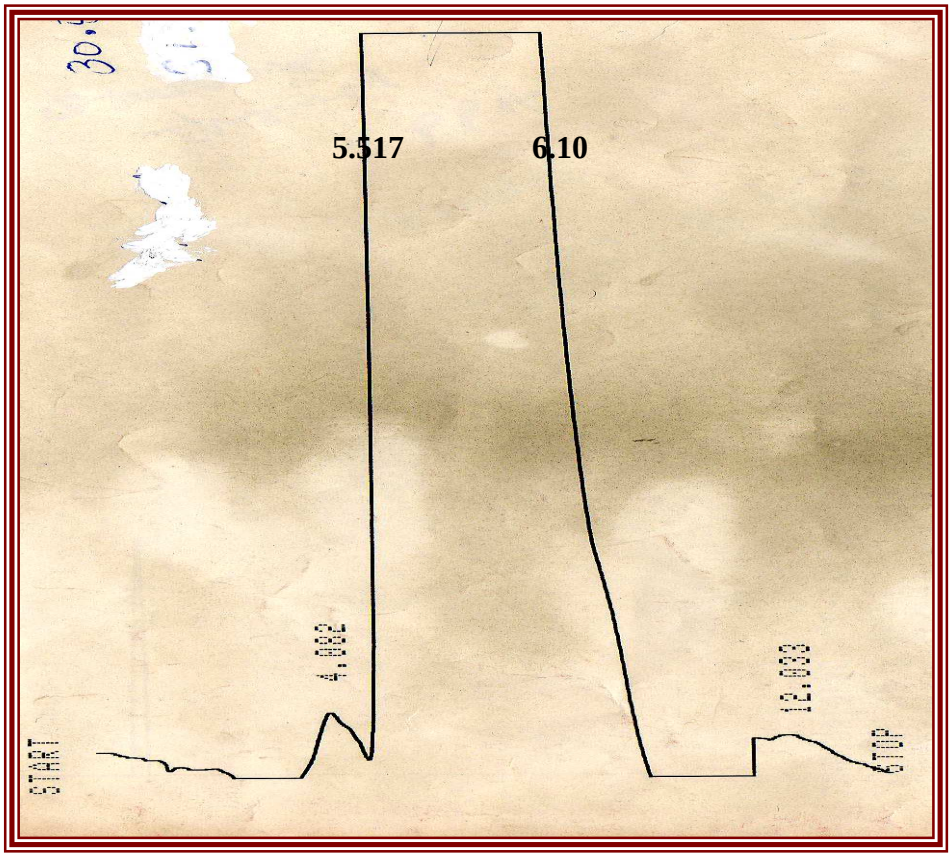


Figure (3-9): HPLC of isolated thymol.

3-4: Cytogenetic Analysis

3-4-1: Cytogenetic effects of MMC on mouse bone marrow cells

3-4-1-1: MMC effects on mitotic index (MI):

Normal white mice had a mitotic index of (6.2%) in their bone marrow cells (table 3-5) and this is considered as a negative control. While the mice treated with MMC caused a significant reduction ($p<0.05$) in MI (1.8%) in comparison with negative control.

This reduction is may be due to the proteins required for mitosis were not produced at the same quantities, or may be the code was not reach to the cell to induce it to proliferate, or may be the drug cause the death of bone marrow cells (Turner *et al*., 1988), another reason found that the mitotic activity of the cell may be affected with MMC could not repaired, or due to a defect occurred in the mitotic spindle composition during cell division (Shiraishi, 1978).

These results were agreed with the results of (Littlefield *et al*., 1980; Shubber, 1981; Shubber *et al*., 1985; Al-Khait, 1999 and Abdul- Hasan *et al*., 2006) who found that MMC had cause reduction in MI of bone marrow cells.

While disagreed with the result of (Al-Duliemy, 2005) who faild to demonstrate apotential for genetic damage *in vitro* which may due to tissue and /or species differences.

Table 3-5

There are some chemotherapeutic drugs that also cause inhibition in mitotic index value like Tamoxifen (TMA) (Al-Sudany, 2005), Metronidazol (MTZ) (Al-Romani, 2006), Methotrexate (MTX) (Al- Amiry, 1999 and Taj Al-Deen *et al*., 2003) and Cyclophosphamide (CP) (Al- Robaiey, 1996 and Hasan, 2002).

3-4-1-2: MMC effect on chromosomal aberrations (CAs):

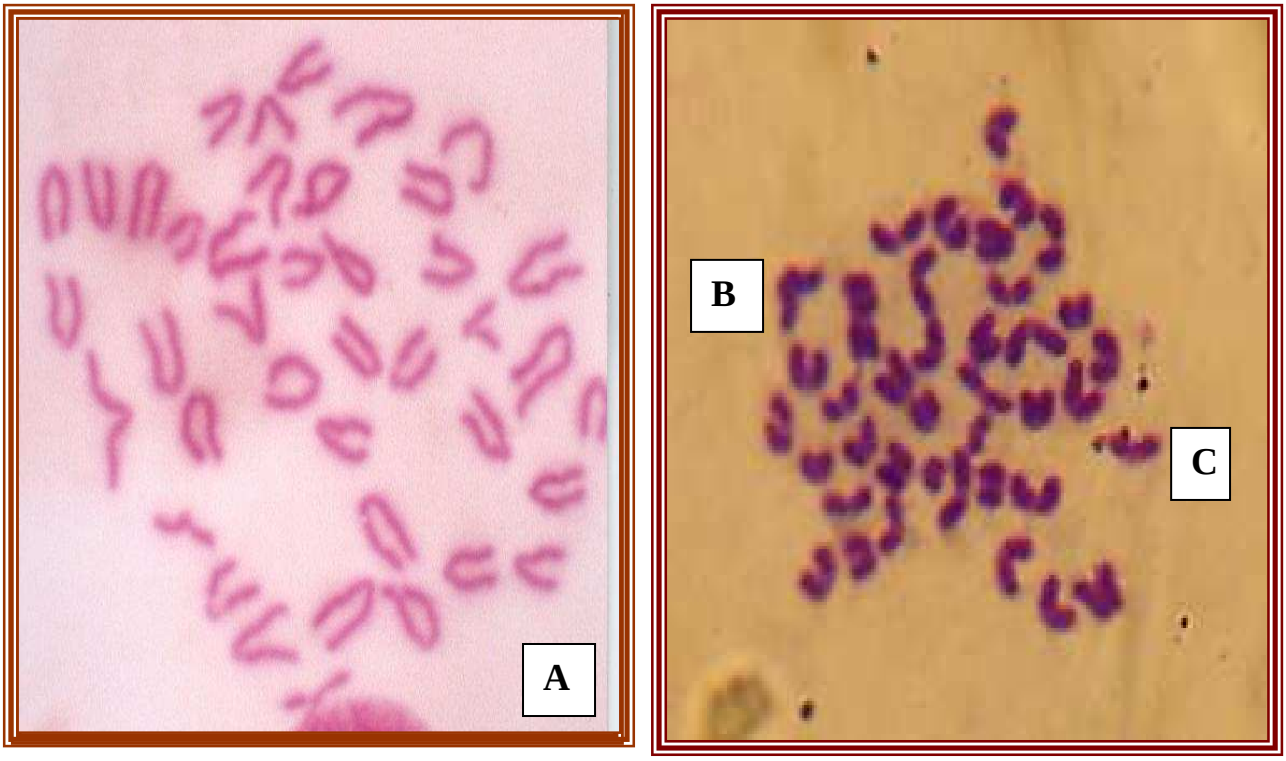
Several types of CAs were found and observed as ring, chromosome gap, dicentric, deletion, chromosome breaks and chromatid breaks as show in figure (3-10) and these were noticed in negative control and in mice treated with MMC as shown in table (3-5).

The spontaneouse frequancy of the chromosomal aberrations in control untreated mouse bone marrow cells was (1.42%) as shown in the table (3-5) and this precentage was increased in comparison with the result of CAs of the mice treated with MMC which was (4.12%).

Dicentric were significantly reduced ($p< 0.05$) to (0.12%) when compared with the negative control (0.26%).

While the other types of aberrations : chromatid break, chromosome break, chromosome gap, ring and deletion were significantly increased ($p<0.05$) to (0.81, 0.32, 0.83, 0.64 and 1.4%) respectively wen compared with negative contol which gives (0.19, 0.03, 0.13, 0.32 and 0.43%) respectivelly

This result agreed with (Shubber *et al*., 1985; Al-Khait, 1999; Al-Duliemy, 2005 and Abdul-Hasan *et al*., 2006) in which they noticed that CAs have been increased in bone marrow cells of treated mice with MMC.

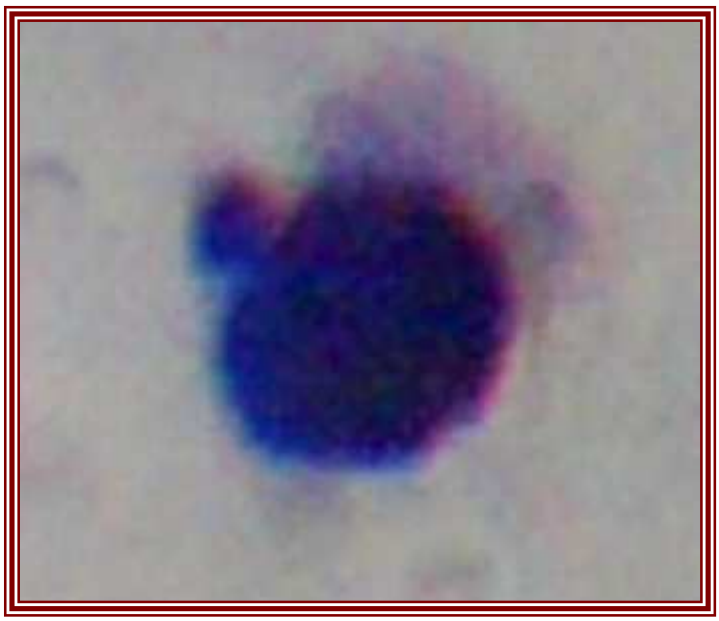


Figure(3-10):Cells in metaphase stage taken from mice treated with mitomycin-C, showing: normal chromosomes(A) dicentric (B), deletion (C) (1000 x).

3-4-1-3: MMC effect on micronucleus indection (MN):

Micronucleus frequencies of poly chromatic erythrocytes from negative control mice was (0.75%) as in table (3-5) and figure (3-11).

This precentage was increased when are treated the animals with MMC and became (3.13%). This increased was significant ($p<0.05$) from the negative control. These results are in agreement with those reported by (Kumpati *et al*., 2003) that mentioned MMC induced the MN in bone marrow cells.



Figure(3-11):Micronucleated bone marrow cell from mouse treated with MMC.

3-5: Cytogenetic effects of leaf callus extract (thymol) on mouse bone marrow cells

3-5-1:The effect of leaf callus extract (thymol) on mitotic index (MI):

Under normal experimental conditions, white mice have a mitotic index of (6.2%) and this considered as a negative control.

Table (3-5) pointed that the mitotic index of mouce bone marrow cells treated with five different doses of thymol has significant differences ($p<0.05$) which gave different precentages of cell division. The highest MI (20.4%) was seen in the bone marrow cells treated with 1mg/Kg thymol.

3-5-2:The effect of leaf callus extract (thymol) on chromosomal aberrations (CAs):

The CAs of normal mice was (1.42%) this considered as negative control. The result shown in table (3-5) tell as that thymol had the ability to reduce the spontaneouse frequencies of CAs with different doses. At dose (1mg/Kg) gives CA equall to (0.66%). This reduction in spontaneous frequency of CA was significant ($p<0.05$) in comparision with those of the control.

The CAs that were noticed are: chromatid break, chromosome break, chromosome gap, dicentric, ring and deletion which appeared when using different concentrations of thymol. These types of aberration were appeared in the negative control (0.19, 0.03, 0.13, 0.26, 0.32 and 0.43%) respectively, and this precentages were decreased when mice treated with all doses of thymol as shown in the table (3-5).

Black seed extracts and oil contain chemical compounds which may act to increase the activity of the detoxification enzymes, such as superoxide dismutase (SOD) and glutathione-S-transeferase (GST) that scavenging free radicals from the cells, or may act to decrease the activity of arylhydrocarbon hydroxylase (AHH) enzyme which has the ability to activate mutagens inside the cells. Those elemints are well known to participate in reduction of CA in living cells (Wang *et al*., 1989).

These results were in agreement with the results of (Al-Sudany, 2005), which indicated that the extract of black seed reduce the spontaneouse CAs in mouse bone marrow.

3-5-3:The effect of callus extract (thymol) on micronucleus induction (MN):

The spontaneouse frequency of MN in mouse bone marrow cells was (0.75%), thymol reduced this frequency according to its dose as shown in table (3-5).

The significant ($p<0.05$) decrease in MN fequency was observed after (1mg/Kg) which gave (0.46%) when compared with the negative control. This results agreed with (Al-Sudany, 2005).

3-6: Interaction between MMC and thymol on mouse bone marrow:

This experiment was designed to know the effect of plant extract on mutagenic effect of MMC which shows a high precentage of CAs, increase in MN and decrease in MI of bone marrow of mice. Therefore; we would like to know if callus extract has the ability to reduce the effect of MMC by injecting the animal before and after the time of MMC treatment.

3-6-1: Treatment with thymol before MMC:

The results of this experiment were represented clearly in table (3-6), which shows the ability of callus extract to reduce the effect of the drug in the mouse bone marrow on MI. This result may be attributed to the active compound (thymol) in the extract (Kanakis *et al*., 2005).

MI in mice treated with callus extract (thymol) before drug treatment was increased to reach (4.00%). This result was significantly different ($p<0.05$) from positive control (1.8%).

So leaf callus extract (thymol) provided (50%) protection against the genotoxic effect of MMC on MI as shon in figure (3-12).

The precentage of MN was reduced significant ($p<0.05$) when thymol was given before MMC treatment, which reached (2.06%) in comparison with the positive control (3.13%).

The pretreatment with the thymol provided a protection on MN (44.95%) aginst the effect of MMC figure (3-12).

The precentage of CAs was reduced significantly ($p<0.05$) when thymol used before MMC treatment, which reached to (2.03%) in comparison with positive control (4.12%).

Before-treatment with thymol provided a protection on CAs (77.24%) figure (3-12).

Thymol was considered as a desmutagens for its ability to decrease the effect of MMC if it was given before-treatment.

It could be seen that thymol proved to be protective agent against the genotoxic effect of MMC if it was given before the treatment. This might related to the chemical constituents of the black seed which may be linked with the drug or with its metabolites to form non- absorbable complexes (Negishi *et al*., 1994), or may inhibit the metabolic activation of the drug (Zhang *et al*., 1989)or may act as antioxidant. Those results were in agreement with (Al-Sudany, 2005) who concluded that pretreatment with black seed gave a protective effect against the genotoxicity of tamoxifene (TAM).

Table 3-6 interaction

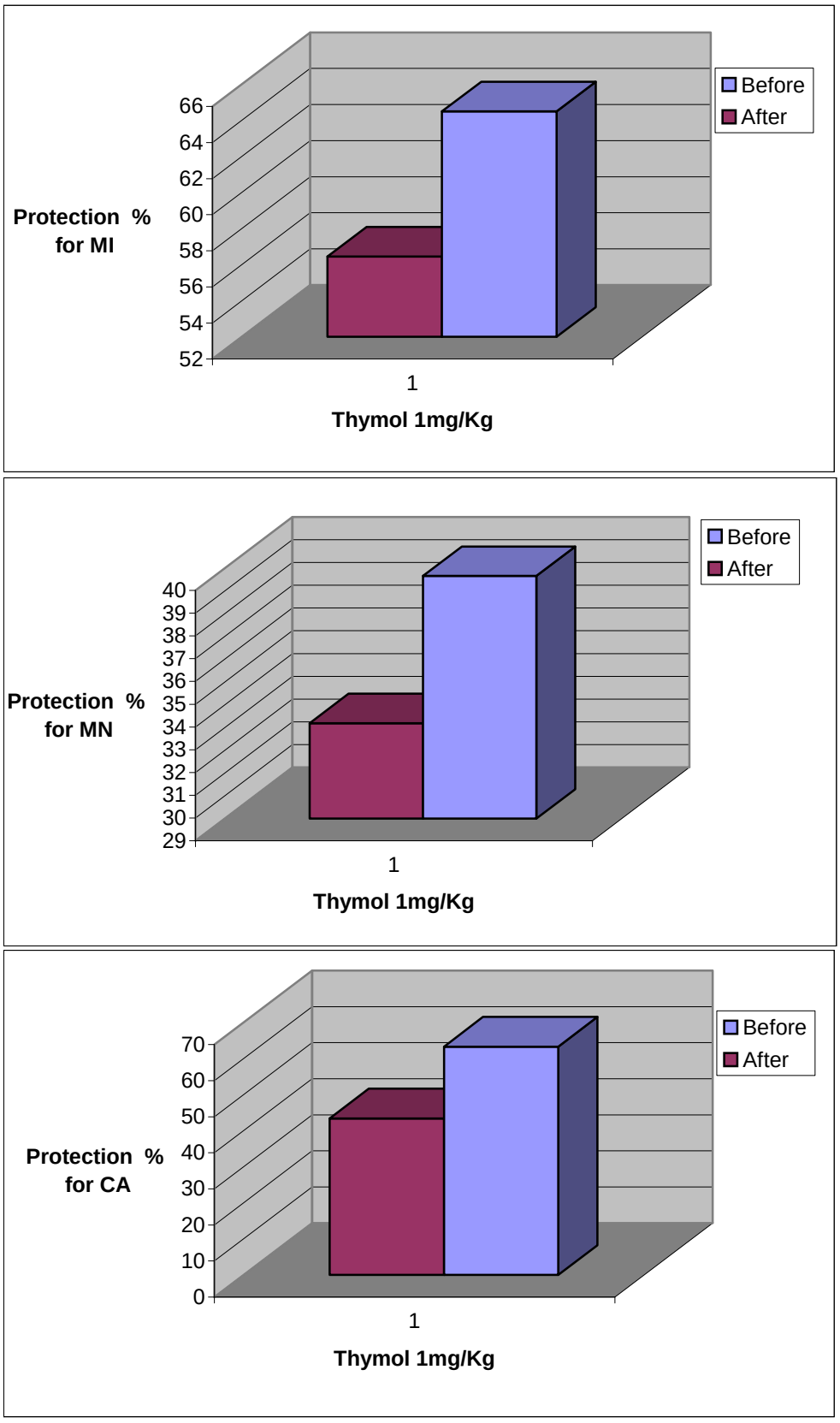


Figure (3-12): The protection ratios provided by thymol when giving before and after MMC treatment.

3-6-2: Treatment with thymol after MMC:

The results of this experiment were displayed in table (3-6). Post treatment with thymol caused a significant increase ($p<0.05$) in MI (3.5%) compared with the positive control (1.8%), so thymol provided (38.6%) protection from the effect of drug on MI figure (3-12).

It was found that thymol has the ability to reduce the effect of the drug, and its protection precentage provided by the thymol was about (38.6%).

The precentage of micronucleus cells was reduced to (2.46%) after treatment with thymol, which significantly different ($p<0.05$) from the positive control (3.13%).

The thymol provided (28.15%) protection from the effect of MMC on MN induction.

The precentage of CAs after treatment with thymol was (2.52%), which was significant ($p<0.05$) from the positive control (4.12%).

So, thymol treatment give protection (59.17%) from the effect of MMC on CA induction.

Conclusions

1. Leaf callus may be a good source for thymol since it has high quantities specially in 75 days old callus culture.
2. Leaf callus extract (thymol) was genotoxic at high concentration in bone marrow cells (*in vivo*).
3. Leaf callus extract (thymol) could be used for reducing the genotoxicity of MMC in bone marrow cells, which was represented by induction of MN and CAs and reduction of MI, for that thymol was considered as desmutagen in the first order and biomutagen in the second order.

Recommendations

1. Expanding in thymol producing from the callus especially leaves which gives best production.
2. Study the effect of the isolated thymol as antibacterial and antifungal and as anticarcinogenesis.
3. Further studies are required on thymol reduction of chromosomal abberations on tumor cells.

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5-BudR	**5-Bromodeoxyuridine**
2,4-D	**2,4-diclorophenoxyacetic acid**
2iP	**N-isopentenylamino purine**

AHH	**Aryl hydro carbon hydroxylase**
AHM	**Synthetic auxin**
BAP	**6-benzylamino purine**
BHT	**Butylated hydroxy tolouene**
BP	**Benzopyrene**
CA	**Chromosomal aberration**
CP	**Cyclophosphamide**
DMBA	**Dimethyl benz(a)anthracene**
GST	**Glutathione-S- transferase**
IAA	**Indol-3- acetic acid**
IBA	**3-indolbutyric acid**
ICL	**Interstrand croos-links**
KCL	**Potassium chloride**
Kin	**Kinetin**
MCA	**2 O-methylcholanthrene**
MDR	**Multi drug resistance**
MI	**Mitotic index**
MMC	**Mitomycin-C**
MN	**Micronucleus**

MS	**Murashige and Skoog medium**
MTX	**Methotrexate**
NAA	**1-naphthaleneacetic acid**
$NaHCO_3$	**Sodium bicarbonate**
NaOCL	**Sodium hypochlorite**
NaOH	**Sodium hydroxide**
NCI	**National Cancer Institute**
NK	**Natural killer cells**
SOD	**Super oxide dismutase**
PBS	**Phosphate buffer saline**
PCE	**Polychromatide erythrocyte.**
PDA	**Pentadienoic acid**
RBC	**Red blood cells**
TMA	**Tamoxifen**
WBC	**White blood cells**

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

دراسة بعض التأثيرات الوراثية لمستخلص من كالس نبات الحبة السوداء في خلايا نقي العظم في الفئران

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

زينب سعد عبد الغني الآوسي

بكلوريوس تقانة إحيائية جامعة النهرين ٢٠٠٣

ذو القعدة ١٤٢٧هـ

كانون الأول

٢٠٠٦م

Table (3-5): Cytogenetic effect of thymol concentrations on mouse bone marrow cells *in vivo*.

Groups	Mitotic Index % μ±SE	Micronucleus% μ±SE	Chromosomal aberration % μ±SE						
			Chromatid Break	Chromosome Break	Chromosome Gap	Dicentric	Ring	Deletion	Total of CAs
Negative control	A 6.2±0.723	A 0.75±0.028	A 0.19±0.004	A 0.026±0.004	A 0.133±0.054	A 0.261±0.014	A 0.32±0.011	A 0.43±0.014	A 1.415±0.044
Positive control MMC 2mg/Kg	E 1.8±0.152	C 3.13±0.185	B 0.81±0.037	B 0.32±0.011	B 0.83±0.03	B 0.122±0.03	B 0.64±0.02	B 1.4±0.023	B 4.122±0.02
Thymol 0.5mg/Kg	AC 8.3±0.405	B 1.42±0.25	A 0.19±0.004	A 0.025±0.001	A 0.13±0.004	A 0.26±0.011	A 0.21±0.001	A 0.42±0.011	A 1.235±0.04
Thymol 1mg/Kg.	B 20.4±1.342	A 0.46±0.07	B 0.11±0.017	B 0.01±0.001	B 0.02±0.004	AC 0.17±0.017	B 0.12±0.004	C 0.23±0.025	C 0.66±0.028
Thymol 2mg/Kg.	CD 9.33±0.338	B 1.17±0.193	B 0.12±0.017	B 0.01±0.001	B 0.02±0.004	AC 0.32±0.005	A 0.21±0.002	AB 0.34±0.02	B 0.931±0.055
Thymol 4mg/Kg	CD 10.06±0.554	AB 0.94±0.082	B 0.095±0.001	B 0.02±0.001	B 0.03±0.002	C 0.32±0.003	B 0.101±0.01	C 0.29±0.02	C 0.767±0.01
Thymol 8mg/Kg.	D 11.93±1.039	AB 0.8±0.054	B 0.091±0.001	B 0.01±0.001	B 0.01±0.004	B 0.22±0.005	C 0.098±0.02	C 0.28±0.02	C 0.7±0.028

Differences A,B,C are significant ($p<0.05$) to comparesion rows and columns.

Table (3-6): Interaction between thymol and MMC *in vivo*.

Groups		Mitotic Index % μ±SE	Micronucleus % μ±SE	Chromosomal aberration % μ±SE						
				Chromatid Break	Chromosome Break	Chromosome Gap	Dicentric	Ring	Deletion	Total of CAs
Negative control		A 6.2±0.723	A 0.75±0.028	A 0.19±0.004	A 0.026±0.004	A 0.133±0.054	A 0.261±0.014	A 0.32±0.011	A 0.43±0.014	A 1.415±0.044
Positive control MMC 2mg/Kg		E 1.8±0.152	C 3.13±0.185	B 0.81±0.037	B 0.32±0.011	B 0.83±0.03	B 0.122±0.03	B 0.64±0.02	B 1.4±0.023	B 4.122±0.02
Thymol 1mg/Kg.		B 20.4±1.342	A 0.46±0.07	B 0.11±0.017	B 0.01±0.001	B 0.02±0.004	AC 0.17±0.017	B 0.12±0.004	C 0.23±0.025	C 0.66±0.028
Interaction	**Before**	A 4.0±0.115	A 2.06±0.088	A 0.24±0.005	A 0.29±0.014	C 0.29±0.014	C 0.44±0.0011	C 0.26±0.005	C 0.511±0.014	C 2.031±0.05
	After	A 3.5±0.208	A 2.46±0.233	C 0.41±0.17	C 0.07±0.002	D 0.3±0.002	D 0.68±0.085	D 0.4±0.002	C 0.66±0.008	D 2.52±0.05

Differences A,B,C are significant ($p<0.05$) to comparesion rows and columns.

Republic of Iraq
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Al-Nahrain University
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Cytological effects of *Nigella sativa* (black seed) callus extract on mouse bone marrow cells

A thesis
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By

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Table (3-2): Fresh weight of callus (mg) induced from leaf explant of *Nigella sativa* plantlet 75 day old .

2,4-D mg/L / Kin mg/L	0	1	2	3	4	μ ± SE
0	A 0.0±0.0	B 1000±104	B 1000±99.9	A 0.0±0.0	A 0.0±0.0	A 400±244.9
1	A 1000±144.3	B 500±95.2	AB 810±212.2	C 0.0±0.0	C 0.0±0.0	A 462 ±204.8
1.5	A 250±50.3	B 1400±359.2	AB 740±151.4	A 0.0±0.0	A 0.0±0.0	A 478+267.1
2	A 0.0±0.0	B 900±45.8	C 140±26.4	A 0.0±0.0	A 0.0±0.0	A 208±175.1
2.5	AB 500±202	A 710±200.1	B 90±28.8	B 0.0±0.0	B 0.0±0.0	A 260±145.6
3	A 0.0±0.0	B 600±5.77	C 90±40.9	A 0.0±0.0	A 0.0±0.0	A 138±116.8
5	A 0.0±0.0	B 400±120.9	A 0.0±0.0	A 0.0±0.0	A 0.0±0.0	A 80±80
μ ± SE	A 250±144.3	B 787.1±129.7	AB 410±159	A 0.0±0.0	A 0.0±0.0	

Differences A,B,C are significant (p<0.05) to comparesion rows and columns.

Table (3-3): Fresh weight of callus (mg) induced from stem explant of *Nigella sativa* plantlet 75 day old .

2,4-D mg/L / Kin mg/L	0	1	2	3	4	μ ± SE
0	A 1500±173.2	AB 1000±125.8	B 800±246.6	C 0.0±0.0	C 0.0±0.0	A 660±292.5
1	A 1000±388.3	B 250±28.8	AB 560±20.8	B 0.0±0.0	B 0.0±0.0	AB 362 ±189.8
1.5	A 0.0±0.0	AB 220±30	B 430±160.9	A 0.0±0.0	A 0.0±0.0	B 130±86.2
2	A 0.0±0.0	B 200±36	B 280±90.7	A 0.0±0.0	A 0.0±0.0	B 96±60.1
2.5	A 0.0±0.0	AB 160±45.8	B 190±80.2	A 0.0±0.0	A 0.0±0.0	B 70±43.1
3	A 0.0±0.0	B 150±28.8	A 0.0±0.0	A 0.0±0.0	A 0.0±0.0	B 30±30.1
5	A 0.0±0.0	B 150±28.8	A 0.0±0.0	A 0.0±0.0	A 0.0±0.0	B 30±30
μ ± SE	A 357.1±236.9	A 304.2±116.8	A 322.8±111.6	A 0.0±0.0	A 0.0±0.0	

Differences A,B,C are significant ($p<0.05$) to comparesion rows and columns.

Table (3-4): Fresh weight of callus (mg) induced from root explant of *Nigella sativa* plantlet 75 day old .

2,4-D mg/L / Kin mg/L	0	1	2	3	4	μ ± SE
0	A 710±149.7	B 0.0±0.0	B 0.0±0.0	B 0.0±0.0	B 0.0±0.0	A 142±141.9
1	A 1000±144.3	B 300±28.8	B 250±76.3	C 0.0±0.0	C 0.0±0.0	A 310 ±183.3
1.5	A 933.3±33.1	B 1000±57.7	B 220±30	C 0.0±0.0	C 0.0±0.0	A 430.7±222.7
2	A 0.0±0.0	B 900±86.6	C 190±37.8	A 0.0±0.0	A 0.0±0.0	A 218±174.4
2.5	A 0.0±0.0	B 700±208.1	A 100±25.6	A 0.0±0.0	A 0.0±0.0	A 160±136.4
3	A 0.0±0.0	B 300±140	A 0.0±0.0	A 0.0±0.0	A 0.0±0.0	A 60±60
5	A 0.0±0.0	B 100± 80	A 0.0±0.0	A 0.0±0.0	A 0.0±0.0	A 20±19.9
μ ± SE	AB 377.6±236.9	B 471.4±149.1	AB 108.5±42.1	A 0.0±0.0	A 0.0±0.0	

Differences A,B,C are significant (p<0.05) to comparesion rows and columns.

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Summary

The aim of this study was to initiate callus from black seed *Nigella sativa* to extract thymol substance and then study its potency to inhibit the cytogenetic effects of mitomycin-C (MMC) on laboratory mice (*in vivo*).

Establishment of *Nigella sativa* (black seed) seedlings in tissue culture was achieved in our laboratory by using Murashige and Skoog medium, (1962) (MS) and optimization of cultural medium using a combination of 2,4-D and Kin concentrations.

The seeds were cultured in sterilized distilled water (after their sterilization) at different concentrations of sodium hypochlorite.

Then different explants (leaves, stems and roots) were cultured on MS medium supplied with different concentrations of growth hormons including 2,4-D at (0.0, 1.0, 2.0, 3.0 and 4.0 mg/L) and Kin at (0.0, 1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 mg/L).

Data and observations were recorded on callus growth through measuring its mean fresh weight under light conditions.

Results revealed that best callus initiation was observed on explants cultured on 1mg/L of 2,4-D and 1.5 mg/L of Kin. Leaves were superior in callus production as compared to stem and root explants. Significant differences were observed among the explants in their response to callus production.

Thymol was extracted and detected and semipurified by using HPLC (High Performance Liquid Chromatography) method to measure its concentration in callus induced from leaves after 75 days in culture, which observed that retention time of standard thymol was identical with retention time of thymol substance and it was (5.3 and 5.5 min.) respectively. The concentration of thymol substance in callus was 30.5 mg/ml.

While, the cytogenetic study was aimed to investigate the role of the isolated thymol in reducing the cytogenetic effect of MMC in mice.

Mitotic index, chromosomal aberrations and micronucleus assay in mouse bone marrow were examined.

The cytogenetic effects of the drug and callus extract were investigated 7 days after treatment of mice with 5 different doses of thymol (0.5, 1.0, 2.0, 4.0 and 8.0 mg/Kg) and 2 mg/Kg for MMC.

An interaction between thymol and MMC was carried out through two types of treatment (before and after treatment thymol) by determine the activity of *Nigella sativa* leaf callus extract in preventing or reducing the drug side effects *in vivo*.

The results indicated that MMC has clear effects in reducing mitotic activity, increased spontaneous chromosomal aberration and increased micronucleus in mouse bone marrow cells (*in vivo*), these effects suggested that the drug has a genotoxic effect.

Thymol had genotoxic effects at high doses over 1 mg/Kg in mouse bone marrow cells (*in vivo*), while at dose 1 mg/Kg show a protective value against the genotoxic effect of MMC.

In mouse bone marrow, this effect was more observed in pre-treatment than in post-treatment.

Thymol extracted from initiated leaf callus was considered as desmutagen in the first order and biomutagen in the second order, as a result for its ability to increase mitotic activity, decrease micronucleus and repair chromosomal aberration in mouse bone marrow cells.

الأهداء

الى روح النبي العربي محمد المصطفى (صلى الله عليه و اله وسلم)

الى رمز العزة والشموخ .....و المضحي الكبير.....والدي الحبيب

الى شلال العطاء الذي لا ينضب.....و التي بدعائها أسير.....والدتي الغالية

الى من هي عنوان فخري.....ويوم وجعي تكون راحتي.....أختي دياري

الى كل الطيبين الذين أوقدوا شمعة أنارت دربي

اليكم جميعا أهدي ثمرة جهدي

زينب

(وَأَنزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ
وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ
اللَّهِ عَلَيْكَ عَظِيمًا)

صدق الله العظيم
سورة النساء الاية ١١٣

الخلاصة

هدفت هذه الدراسة إلى استحداث الكالس من نبات الحبة السوداء *Nigella sativa* لاستخلاص مادة الثايمول وبعد ذلك دراسة فعالية هذه المادة لمنع التأثيرات الوراثية الخلوية لعقار MMC على فئران المختبر (داخل الجسم).

تم الحصول على بادرات الحبة السوداء بالزراعة النسيجية (داخل الزجاج) باستعمال وسط MS (Murashige and Skoog, 1962) وتحقيق افضل التراكيز الزرعية من منظمي النمو 2,4-D و Kin .

زرعت البذور على ورق ترشيح معقم و مغمور جزئيا في الماء المقطر المعقم بعد تعقيمها بتراكيز مختلفة من مادة هايبوكلورات الصوديوم NaOCl .

كما تم زراعة الأجزاء النباتية (أوراق، سيقان و جذور) في وسط غذائي MS يحوي تراكيز مختلفة من منظمي النمو 2,4-D تراوحت بين (٠، ١، ٢، ٣ و ٤) ملغم/لتر، و Kin تراوحت بين (٠، ١، ١,٥، ٢، ٢,٥، ٣ و ٥) ملغم/لتر.

البيانات و الملاحظات سجلت عن نمو الكالس من خلال قياس معدل الوزن الطري تحت ظروف الضوء.

و بينت النتائج بأن التركيز ١,٥ ملغم/ لتر من ال Kin و ١ملغم/ لتر من ال 2,4-D كان الأفضل في تشجيع نشوء الكالس. تفوقت الأوراق في استحداث الكالس

مقارنة بأجزاء الساق و الجذر فضلا عن وجود فروقات معنوية بين الأجزاء النباتية في استجابتها لتكوين الكالس.

كما تم الكشف عن مادة الثايمول باستخدام طريقة كروموتوغرافيا السائل عالي الكفاية (HPLC) للكشف عن مادة الثايمول و معرفة تركيزه في الكالس المستحدث من الأوراق بعمر ٧٥ يوم إذ تطابق وقت ظهور المركب القياسي مع وقت ظهور مادة الثايمول و بلغ (٥,٣ و ٥,٥) دقيقة على التوالي. كما بلغ تركيز مادة الثايمول من الكالس المستحدث (٣٠,٥ ملغم/مل).

وهدفت الدراسة أيضا لتحري دور الثايمول المعزول في تثبيط تأثير MMC في الصفات الوراثية الخلوية في الفئران.

تم أتباع ثلاثة فحوص في الفئران و هي دراسة معامل الانقسام الخيطي، التغيرات الكروموسومية و تكون النوى الصغيرة.

التأثيرات الخلوية للعقار و لمستخلص الكالس درست بعد ٧ أيام من المعالجة في الفئران لخمس جرع من مادة الثايمول (٠,٥، ١، ٢، ٤ و٨ ملغم/ كغم) و ٢ ملغم /كغم لل MMC.

أجري التداخل بين الثايمول و ال MMC من خلال نوعين من المعاملات لتحديد فعالية مستخلص كالس الورقة لنبات الحبة السوداء في منع أو تثبيط الآثار الجانبية للعقار MMC داخل الجسم.

لقد أظهرت النتائج أن العقار MMC له تأثيرات سلبية تمثلت بانخفاض معامل الانقسام الخيطي، زيادة التغيرات الكروموسومية و زيادة نسبة تكوين النوى الصغيرة في خلايا نقي العظم في الفئران، هذه التأثيرات أشارت إلى امتلاك هذا العقار تأثيرات وراثية خلوية.

أما الثايمول فقد اظهر تأثيرات وراثية خلوية في التراكيز التي تكون أعلى من التركيز ١ ملغم/كغم في خلايا نقي العظم للفئران (داخل الجسم)، بينما في التركيز ١ ملغم/ كغم فقد اظهر نسبة حماية ضد التأثيرات السامة للعقار MMC في خلايا نقي العظم للفئران، هذا التأثير لوحظ عند المعاملة قبل العقار اكثر من المعاملة بعده.

لذلك عد مستخلص الكالس من أوراق الحبة السوداء *Nigella sativa* من المثبطات المباشرة للعقار بالدرجة الأولى ومن المثبطات الحيوية من الدرجة الثانية كنتيجة لقابليته من رفع قيمة معامل الانقسام الخيطي و إصلاح التشوهات الكروموسومية و خفضه نسبة تكوين النوى الصغيرة في خلايا نقي العظم للفئران.

ث

Chapter One

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and

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Chapter Three

Results And Discussion

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References

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Zaynab

Supervisor Certification

We certify that this thesis was prepared under my supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

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We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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