Chapter Five

Discussion

Plants are a source of different chemical compounds, which make them of a medicinal importance. These compounds are divided into two types (inert and active constituents) depending on their activity. The inert constituents are defined as compounds that have no medicinal or physiological effects; for instance cellulose, lignin and subrine, while active constituents have these effects. The active constituents in turn are divided into other types (alkaloids, tannins, carbohydrates, volatile oils, saponines, steroids and flavonoids) depending on their chemical and physical characteristics (Hussien, 1981). Chemical analysis of sage (*S. officinalis*) extracts (aqueous and hexane) revealed some of these constituents (steroids, tanins, glycosides, flavonoids, saponines and terpens). Such findings are in agreement with the results of Chada (1976), who reported that the aqueous extract of sage contains these compounds with the exception of alkaloids. Additionally, the strong fragrance of both extracts suggests the presence of a large quantity of phenolic compounds and volatile oils in these extracts (Lu and Foo, 2000; Santos-Gomes and Fernandes-Ferreira, 2001). Accordingly, both extracts may serve as a good immune modulator and anti-mutagenic agents, especially if we consider the synergistic effects of their compounds (Elson *et al*., 1999; Derrida, 2003), and the present results can be justified in the light of these chemical constituents.

The results demonstrated that a treatment with sage extracts had no effect on total and differential count of leucocytes with the exception of neutrophils and eosinophils, which both showed a significant increase especially in animals treated with the aqueous extract. Neutrophils carry out important non-specific cellular defence mechanism, which is phagocytosis (Abbas and Lichtman, 2003). Such activity was significantly enhanced by both extracts, therefore the effect was functional. Several lines of evidence have suggested that phagocytosis can be modulated positively or negatively as a result of treatment

with different agents (Cole *et al*., 1998; Moller and Loft, 2002). In this regards, medicinal plants rich in flavonoids and glycosides have caused a significant elevation in the index of phagocytosis, for instance *Nigella sativa*, *Withania somnifera* and *Foeniculum vulgare* (Al-Zendi, 2006). These compounds may exert their effect on phagocytes through providing the cells with the energy required for such process, or enhance their killing ability; however, the latter function was not investigated in the present study. In agreement with this scope, it has been demonstrated that flavonoids and glycosides can modulate the macrophage function and enhance the cellular immune response, in addition to their contribution to the integrity of the immune system function through their antioxidant activity (Schoenherr and Jewell, 1997; Lu and Foo, 2000; Rao *et al*., 2003). Furthermore, aqueous extract of sage has been effective in stimulating the chemotatic activity, as well as, enhancing the production of 1L-12. Therefore, an enhancement of anti-inflammatory activity is pictured, and an engulfment of more yeast is then expected (Kang *et al*., 2000; Baricevic *et al*., 2001; Gabhe *et al*., 2006).

In addition to the innate immunity, the adaptive immunity was also explored in relation to treatments with aqueous and hexane extracts of sage through three assessments, which were Arthus reaction, delayed type hyper sensitivity and plaque forming cells. The first and third assessments aimed to evaluate humoral immune response, which is outcome in an antibody production. Anti-SRBC antibodies were significantly increased in the plaque forming cell assay, and the results of Arthus reaction support such finding. To explain such effects, it has been demonstrated that sage is able to increase the vitamin E concentration in serum, which is important stimulator of the adaptive immune response (Benich, 1988; Moller and Loft, 2002), therefore the effect could be indirect and as a result of vitamin E modulation. A further vitamin can also be involved, that is vitamin C. It has also been demonstrated that flavonoids extracted from sage exerted some positive effect on this vitamin in mice (Manach *et al*., 1996; Cook and Samman, 1996). Vitamin C protects plasma lipid and membrane lipid from the effect of oxidant compounds through either

increase the production of cytokines or interact with the formation of prostaglandins, and both consequences are in favour of immune system enhancement, especially the antibody production (Hughes, 2001).

The delayed type hypersensitivity reaction is a further test to assess the adaptive immunity with special reference to the cellular immune response (Jacayan *et al*., 2001). Both extracts of sage were effective in enhancing the cellular immune response against SRBCs. Francis *et al*., (2002) demonstrated that plants contain saponins can modulate the function of the immune system, due to their action in stimulating the cell-mediated immunity and activating the production of different cytokines, especially those involved in the cellular immune response. Therefore the present effect of sage extracts can be explained in this context, because sage is rich in saponins (Kavvadias *et al*., 2003).

A water-soluble polysaccharides complex from *S.officinalis* composed of galactose, glucose, mannose, xylose, and fructose have shown an immunomodulatory activity in the comitogenic thymocyte test which is interpreted as being an *in vitro* correlate of adjuvant activity in addition to their mitogenic activity (Capek and Hribalova, 2004). These polysaccharides from *S.officinalis* have previously shown to stimulate the immune function of bone marrow cells and in this regard several European demostic herbs have been analysed biologically for active polysaccharides components and reported that these polysaccharides are good modulators of the immune system (anti-cancer, anti-inflamantory, anti-ulcer, complement activating potency, macrophage phagocytosis stimulation and induction of cytokines) (Ebringerova *et al*., 2003). These augmentations of humoral and cellular immune responses involve mainly four immune cells (neurophils, macrophages and T- and B-lymphocytes), and the effect of sage extracts on these cells numerically or functionally can be explained in the light of active constituents that act either separately or synergistically in enhancing the responsiveness of these cells directly or indirectly (Gabhe *et al*., 2006).

The function of immune system is also genetically determined, and alternations (mutations) in the genetic make-up of animals do have their effect

on such function (Abbas and Lichtman, 2003). To explore the effects of sage extracts (aqueous and hexane) on the genetic make-up of mice directly or through interactions with the drug cytosar, two assessments were carried out; they were micronucleus formation in polychromtic cells of bone marrow and sperm-head abnormalities, which both are good parameters of mutagenic evaluations (Ghaskadbi and Viayda, 1991; Al-Rubaiey, 2000). The results of genetic evaluations showed that a treatment with sage extracts was associated with a significant reduction in micronucleus formation and sperm head abnormalities and such effect was dependent on dose and type of extract. Such findings can be considered important, especially if we consider that most cancers are preceded by mutations induced by different agents, especially those that have oxidant effects (Yassen, 1990; Ad'hiah *et al*., 2002). Numbers of studies have been conducted in regard to the antioxidant activity of sage, and in general their findings are in agreement with the present results. Wang *et al* (2000) found that rosmarinic acid extracted from sage possessed anti-oxidant activity and was free radical scavengers, while Miura *et al*., (2002) added another compound isolated from the plant and has anti-oxidant activity that is 12-O-methyl carnosol. Mimica-Dukic (2001) demonstrated that phenolic diterpenoids extracted from the plant showed a strong anti-oxidant activity, and Bozin *et al*., (2002) confirmed that, and suggested that these compounds are effective in scavenging free radicals. Such effects can be achieved through several metabolic pathways; for instances, inhibit the formation of free radicals, suppress chain initiation and/or breaking chain propagation reaction, increasing the activity of detoxifying enzymes such as glutathione transferase (GST) and superoxide dismutase (SOD) and *de novo* anti-oxidant and adaptation where the signal for the production and reaction of free radicals formation and transport of the anti-oxidant to the right site (Noguchi *et al*., 2000). Free radicals have the ability to cause damage to DNA and RNA and inhibit some enzymes from reacting with amino acids (Salganik, 2001).

The subject of anti-oxidant activity and ability of free radicals scavenging is further highlighted, if we consider other constituents of the plant that have

these effects. These constituents are glycosides, flavonoids and chlorophylls, which possess antimutagenic activity, in addition to their role in detoxification of mutagenic compounds (Kojima and Kuroda, 1992; Manach *et al*., 1996). These finding have also been further augmented to be involved in increasing the mitotic index of bone marrow and spleen cells, which was observed in the present study (Kojima and Kuroda, 1992; Manach *et al*., 1996; Cook and Samman, 1996). Enhancement of mitosis has been recently explored *in vitro*, and the conclusion was in favour of that sage can be considered as a mitogen (Capek et *al*., 2003).

The flavonoid compounds, in addition to their immune modulatory effect, serve other bio-functions, with special reference to antimutagenic activity. It has been demonstrated that the flavonoid compounds, 3-kameferolcoumate and luteolin, which were extracted from some plants of the family Lamiaceae (*S. officinalis*, *Thymus vulgaris* and *Mentha piperita*) inhibited the mutagenic activity of Trp-p-2, and the suggestion was that these compounds activate the metabolic inactivation by modifying the action of the enzyme cytochrome p450 enzyme (Samejima *et al*., 1995 ; Samejima *et al*., 1998). Such consequence can lead to a decrease in DNA strand breaks, lipid peroxidation and cellular oxidation (Miski *et al*., 1983; Kanazawa *et al*., 1998). By these pathways, the present extracts of sage could exert their protection effect against the drug cytosar, especially in the pre-treatment interaction. Such findings confirmed previous demonstrations related to the antimutagenic effects of sage, although different mutagen and biological system of assessment were employed; for instance, Vukovic-Gacic and Simic (1993) showed that UV light-induced mutations in *Echerichia coli* can be reduced to a significant level by extracts of sage.

The integrity of genetic make-up depends on several repair mechanisms; such as, excision repair, photoreactivation repair, post-replication repair, error-prone repair and error-free repair, and such mechanism may be considered as a target for medicinal plants or their products in terms of enhancement. In post-treatment interaction, both extracts showed a significant efficiency in reducing

the genetic effects of cytosar, and again these actions have been attributed to the chemical constituents of the plant with regard to the forthcoming repair mechanisms. It has been found that flavonoids of sage enhances the post-replication repair (Kourda *et al.*, 1992), while others have demonstrated that tannins, flavonoids and terpens stimulates the mechanism of error-free repair (Sasaki et *al*., 1998). Furthermore, terpens can activate recombinational repair mechanism, beside their action in activating the detoxification enzymes (Elosn and Yu, 1994; Burke *et al*., 1997).

The subject of antimutagenecity was further explored *in vitro* in blood cultures of acute lymphoid leukemia (ALL) patients and healthy controls through the assay of micronucleus formation. The results confirmed the *in vivo* findings in albino male mice, and a significant reduction in spontaneous, as well as, cytosar-induced formations of micronuclei was observed in a concentration-dependent manner. Such finding may further qualify the aqueous extract of sage as anti-mutagen. Again, the subject of chemical constituent is overwhelmed to explain such findings. In cultures, the antimutagenic effect of sage's aqueous extract has been ascribed to the low molecular-mass compounds contained in this plant but in relatively large amounts (phenolic acids, glycosides, diterpenoids and flavonoids), many of which possess a variety of related biological activities including: antioxidant, anti-platelet, anti-tumor and antiviral (Lu and Foo, 2001, 2002). Beside that, these compounds stimulate the function of immune system, inhibit the formation of DNA adduct with carcinogens, inhibit hormonal action and metabolic pathways association with the development of cancer and enhance the phase I or II detoxification enzymes (Steinmetz and Potter, 1991; Caragay, 1992; Cuvelier *et al*., 1994; Smith and Yang, 1996). Furthermore, investigations have shown that terpenoids of sage increase tumor latency and decrease tumor multiplicity (Pearce *et al*., 1992; Zheng *et al*., 1993; Elson and Yu, 1994).

The reduction in micronucleus formation in cultures treated with the aqueous extract was due to the polyphenolic compounds, flavonoid, present in the extract that have the ability to inhibit the reaction that induce a malignant

mutation by damaging the mutated DNA, and induce the activity of enzyme that protect DNA and repair the damage in the genetic material (Huk *et al*., 1998). Also polyphenolic compounds inhibit the growth of leukemic cells, and induce apoptosis (Helen *et al*., 2002). Szende *et al*., (2000) demonstrated that these compounds induce fast-signaling independent apoptosis in human monocytic leukemia and cause arrest in the S-phase prior to fast-independent apoptosis in acute leukemic cells and induce apoptosis in human premyelocytic leukemia (Surth *et al*., 1999; Bernhard *et al*., 2000). Further investigations on phenolic compounds showed that these compounds induce the accumulation of P53 and P21 proteins that slow down the cell cycle (Tze-Chen *et al*., 1999).

The results of micronucleus formation in untreated cultures of ALL patients revealed that the genetic make-up of patients underwent some alternations (i.e. mutations), and such consequences may lead to establish the leukemia disease. It was always augmented that leukemia requires a mutation before establishing a disease (Micallef *et al*., 2001), and such mutations can be assessed by several parameters including sister chromatid exchange (SCE) and micronucleus formation (Mertens *et al*., 1995). Such assessments are in agreement with present study, although different malignancies were investigated (Al-Amiry, 1999; Al-Abassi, 2001). In leukemic cells, these genetic alternations are outcome in loss of apoptosis and regulation of cell cycle progression (Kastan *et al*., 1995).

The present study also demonstrated that the anti-leukemia drug cytosar increased the frequency of micronucleus formation and sperm-head abnormalities, in addition to the reduction of metaphase index, and such findings suggest that such drug is a mutagen, as with other anti-cancer drugs. In this regard, McCowage *et al*., (1996) have demonstrated that anticancer drugs may cause abnormalities in lymphocyte receptors involved in mitogen recognition. Such effect may result in an inhibition of blastogenic index, mitotic index, and increase micronucleus formation and chromosomal aberrations. The action of these drugs may act on the repair systems inside the cells, and as a result the cells loss the ability to repair the damaged DNA (Uzeily *et al*., 1993).

1: Conclusions

1- The drug cytosar was immune suppressive and mutagenic agent as suggested by the results of total and differential counts of leucocytes, phagocytosis, Arthus reaction, delayed type hypersensitivity reaction, plaque forming cells, metaphase index, micronucleus formation and sperm-head abnormalities in mice.

2- The aqueous and hexane extracts of sage (*Salvia officinalis*) were effective in modulating the immune suppressive and mutagenic effects of cytosar, *in vivo* (albino male mice) and *in vitro* (acute lymphoid leukemia patients).

3- With respect to immunological functions and genetic effects, both extracts were effective in enhancing the immune response, and reducing the spontaneous formation of micronuclei and sperm-head abnormalities.

2: Recommendations

1. The immunological effects of aqueous and hexane extracts of sage require further investigations to determine the function of lymphocytes in terms of their CD markers and cytokine production. Furthermore, the function of phagocytes should also be investigated in this regard.
2. Further investigations are required to determine the genetic effects of both extracts, and interactions with other anti-cancer drugs should be investigated with other genetic assessments, especially at the molecular level.
3. Purification of the active compounds of both extracts to determine their immunological and genetic effects, as a strategy for a drug technology.

Table 4-2: Total leucocyte count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	7200 ± 821 a	-10.6
Negative Control (Distilled Water)		0.00	8050 ± 591 a	
Negative Control (Sunflower Oil)		0.00	9900 ± 208 b	
Sage Extracts	Aqueous	83.9	7050 ± 412 a	-12.4
	Aqueous	167.8	7100 ± 551 a	-11.8
	Aqueous	251.7	7150 ± 126 a	-11.2
	Hexane	83.9	8350 ± 550 b	-15.7
	Hexane	167.8	9650 ± 96 b	-2.25
	Hexane	251.7	6450 ± 222 a	-34.8

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-3: Total lymphocyte count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	4629 ± 589 a	-8.4
Negative Control (Distilled Water)		0.00	5054 ± 385 a	
Negative Control (Sunflower Oil)		0.00	6106 ± 164 b	
Sage Extracts	Aqueous	83.9	4134 ± 199 a	-18.2
	Aqueous	167.8	4089 ± 274 a	-19.1
	Aqueous	251.7	4005 ± 178 a	-20.8
	Hexane	83.9	5722 ± 386 b	-6.3
	Hexane	167.8	6512 ± 67 b	6.6
	Hexane	251.7	3783 ± 78 a	-38.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-4: Total neutrophil count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	1520 ± 125 a	-23.8
Negative Control (Distilled Water)		0.00	1996 ± 88 a	
Negative Control (Sunflower Oil)		0.00	2625 ± 110 b	
Sage Extracts	Aqueous	83.9	2520 ± 147 b	26.3
	Aqueous	167.8	2268 ± 237 ab	13.6
	Aqueous	251.7	2125 ± 87 a	6.5
	Hexane	83.9	1895 ± 118 a	-27.8
	Hexane	167.8	2293 ± 110 ab	-12.6
	Hexane	251.7	2151 ± 136 ab	-18.1

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-5: Total monocyte count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	784 ± 134 a	-7.7
Negative Control (Distilled Water)		0.00	849 ± 122 a	
Negative Control (Sunflower Oil)		0.00	968 ± 104 b	
Sage Extracts	Aqueous	83.9	265 ± 49 c	-68.8
	Aqueous	167.8	542 ± 90 d	-36.2
	Aqueous	251.7	736 ± 91 a	-13.3
	Hexane	83.9	669 ± 91 a	-30.9
	Hexane	167.8	797 ± 64 a	-17.7
	Hexane	251.7	455 ± 59 d	-53.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-6: Total eosinophil count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	251 ± 35 a	274.6
Negative Control (Distilled Water)		0.00	67 ± 44 b	
Negative Control (Sunflower Oil)		0.00	127 ± 49 c	
Sage Extracts	Aqueous	83.9	94 ± 37 bc	40.3
	Aqueous	167.8	186 ± 35 d	177.6
	Aqueous	251.7	232 ± 32 ad	246.3
	Hexane	83.9	43 ± 25 b	-66.1
	Hexane	167.8	25 ± 25 b	-80.3
	Hexane	251.7	48 ± 29 b	-62.2

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-7: Total basophil count (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm.blood)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	18 ± 18 a	-79.1
Negative Control (Distilled Water)		0.00	86 ± 34 b	
Negative Control (Sunflower Oil)		0.00	76 ± 25 b	
Sage Extracts	Aqueous	83.9	38 ± 22 a	-55.8
	Aqueous	167.8	15 ± 15 a	-82.6
	Aqueous	251.7	53 ± 18 ab	-38.4
	Hexane	83.9	22 ± 22 a	-71.1
	Hexane	167.8	25 ± 25 a	-67.1
	Hexane	251.7	15 ± 15 a	-80.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-8: Phagocytic index of peritoneal cells (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control) after 30 minutes incubation.

Groups		Dose (mg/kg)	Mean ± Standard Error (%)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	36.00 ± 1.68 a	-6.5
Negative Control (Distilled Water)		0.00	38.50 ± 1.85 a	
Negative Control (Sunflower Oil)		0.00	62.30 ± 0.48 b	
Sage Extracts	Aqueous	83.9	67.75 ± 0.48 c	76.0
	Aqueous	167.8	67.50 ± 1.55 c	75.3
	Aqueous	251.7	53.80 ± 1.11 d	39.7
	Hexane	83.9	66.50 ± 1.04 c	6.7
	Hexane	167.8	54.25 ± 1.11 c	-12.9
	Hexane	251.7	85.75 ± 0.85 e	37.6

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-9: Phagocytic index of peritoneal cells (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control) after 60 minutes incubation.

Groups		Dose (mg/kg)	Mean ± Standard Error (%)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	40.25 ± 2.14 a	-16.6
Negative Control (Distilled Water)		0.00	48.25 ± 4.00 b	
Negative Control (Sunflower Oil)		0.00	74.00 ± 1.68 c	
Sage Extracts	Aqueous	83.9	72.00 ± 0.41 c	49.2
	Aqueous	167.8	74.25 ± 1.25 c	53.9
	Aqueous	251.7	62.75 ± 1.11 d	28.5
	Hexane	83.9	71.25 ± 0.48 c	-3.7
	Hexane	167.8	62.75 ± 1.11 d	-15.2
	Hexane	251.7	91.75 ± 0.85 e	24.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-10: Metaphase index of bone marrow cells (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (%)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	2.35 ± 0.04 a	-21.7
Negative Control (Distilled Water)		0.00	3.00 ± 0.14 b	
Negative Control (Sunflower Oil)		0.00	4.33 ± 0.25 c	
Sage Extracts	Aqueous	83.9	3.43 ± 0.92 b	14.3
	Aqueous	167.8	3.84 ± 0.16 bc	28.0
	Aqueous	251.7	5.80 ± 0.15 c	93.3
	Hexane	83.9	5.54 ± 0.10 d	27.9
	Hexane	167.8	6.86 ± 0.13 e	58.4
	Hexane	251.7	7.58 ± 0.18 e	75.1

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-11: Metaphase index of spleen cells (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (%)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	0.83 ± 0.09 a	-37.6
Negative Control (Distilled Water)		0.00	1.33 ± 0.10 b	
Negative Control (Sunflower Oil)		0.00	2.56 ± 0.23 c	
Sage Extracts	Aqueous	83.9	2.01 ± 0.09 d	51.1
	Aqueous	167.8	2.53 ± 0.10 c	90.2
	Aqueous	251.7	2.74 ± 0.12 c	106.0
	Hexane	83.9	3.28 ± 0.09 e	28.1
	Hexane	167.8	3.76 ± 0.05 e	46.9
	Hexane	251.7	4.41 ± 0.21 e	72.3

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-12: Micronucleus formation in bone marrow cells (mean ± standard error) of albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (micronucleus/cell)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	0.0238 ± 0.0015 a	240.0
Negative Control (Distilled Water)		0.00	0.0070 ± 0.0004 b	
Negative Control (Sunflower Oil)		0.00	0.0060 ± 0.0004 b	
Sage Extracts	Aqueous	83.9	0.0040 ± 0.0004 c	-42.9
	Aqueous	167.8	0.0023 ± 0.0005 c	-67.1
	Aqueous	251.7	0.0030 ± 0.0004 c	-57.1
	Hexane	83.9	0.0030 ± 0.0005 c	-50.0
	Hexane	167.8	0.0050 ± 0.0003 c	-16.7
	Hexane	251.7	0.0033 ± 0.0006 c	-45.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-13: Sperm-head abnormalities (mean ± standard error) in albino male mice treated with aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (%)	Treatment Efficiency (%)
Positive Control (Cytosar Drug)		1.54	10.20 ±0.48 a	70.0
Negative Control (Distilled Water)		0.00	6.00 ± 0.63 b	
Negative Control (Sunflower Oil)		0.00	5.05± 1.12 b	
Sage Extracts	Aqueous	83.9	5.00 ± 0.22 b	-16.7
	Aqueous	167.8	5.50 ± 0.17 b	-8.3
	Aqueous	251.7	6.20 ± 0.18 b	3.3
	Hexane	83.9	6.63 ± 0.31 b	31.3
	Hexane	167.8	5.35 ± 0.13 b	5.9
	Hexane	251.7	6.12 ± 0.93 b	21.2

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-14: Total leucocyte count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	8650 ± 430 a	10225 ± 210 a		
Control II (Sunflower Oil-Cytosar)	8150 ± 435 a	7000 ± 510 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	8000 ± 337 a	9100 ± 92 c	-7.5	-11.0
Hexane Plant Extract (Ideal Dose)-Cytosar	6600 ± 216 b	8550 ± 250 d	-19.0	22.1

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-15: Total lymphocyte count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	5586 ± 305 a	7643 ± 163 a		
Control II (Sunflower Oil-Cytosar)	6899 ± 440 b	5955 ± 373 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	5000 ± 225 a	5436 ± 73 b	-10.5	-28.9
Hexane Plant Extract (Ideal Dose)-Cytosar	4782 ± 137 a	6195 ± 149 b	-30.7	4.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-16: Total neutrophil count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	2270 ± 111 a	1790 ± 76 a		
Control II (Sunflower Oil-Cytosar)	849 ± 33 b	610 ± 44 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	2162 ± 148 a	3186 ± 169 c	-4.8	78.0
Hexane Plant Extract (Ideal Dose)-Cytosar	1405 ± 104 c	1759 ± 208 a	65.5	188.4

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-17: Total monocyte count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	409 ± 10 a	536 ± 45 a		
Control II (Sunflower Oil-Cytosar)	343 ± 34 a	405 ± 67 ab		
Aqueous Plant Extract (Ideal Dose)-Cytosar	717 ± 19 b	250 ± 43 b	75.3	-53.4
Hexane Plant Extract (Ideal Dose)-Cytosar	328 ± 17 a	386 ± 76 ab	-4.4	-4.7

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-18: Total eosinophil count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	280 ± 16 a	154.7 ± 32.0 a		
Control II (Sunflower Oil-Cytosar)	38 ± 22 b	73.5 ± 34.8 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	80 ± 3 b	159.3 ± 43.2 a	-71.4	3.0
Hexane Plant Extract (Ideal Dose)-Cytosar	69 ± 40 b	148.5 ± 40.3 a	81.6	102.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-19: Total basophil count (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (cells/cu.mm.blood)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	152 ± 23 a	102.3 ± 2.1 a		
Control II (Sunflower Oil-Cytosar)	23 ± 23 b	57.0 ± 39.6 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	42 ± 24 b	68.8 ± 22.9 b	-72.4	-32.7
Hexane Plant Extract (Ideal Dose)-Cytosar	18 ± 18 b	63.0 ± 40.9 b	-21.7	10.5

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-20: Phagocytic index of peritoneal cells (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug after 30 minutes incubation.

Groups	Mean ± Standard Error (%)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	64.75± 1.66 a	25.00 ± 1.08 a		
Control II (Sunflower Oil-Cytosar)	36.25± 1.49 b	23.50 ± 1.44 a		
Aqueous Plant Extract (Ideal Dose)-Cytosar	59.25± 1.11 c	70.75 ± 0.85 b	-8.5	180.0
Hexane Plant Extract (Ideal Dose)-Cytosar	39.75± 0.85 b	40.00 ± 1.96 c	9.7	70.2

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-21: Phagocytic index of peritoneal cells (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug after 60 minutes incubation.

Groups	Mean ± Standard Error (%)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (DistilledWater-Cytosar)	62.00± 1.08 a	37.50 ± 2.60 a		
Control II (Sunflower Oil-Cytosar)	49.00± 0.41 b	44.25 ± 2.25 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	67.75± 0.85 c	76.00 ± 0.91 c	9.3	102.7
Hexane Plant Extract (Ideal Dose)-Cytosar	54.75± 1.25 d	58.25 ± 0.85 d	11.7	31.6

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-22: Arthus reaction (mean ± standard error) in albino male mice after type I- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)	Treatment Efficiency (%)
Aqueous Extract	Distilled water	0.00	1.28 ± 0.09 a	
	Cytosar	1.54	0.64 ± 0.06 b	-50.0
	Extract	83.9	1.48 ± 0.05 c	15.6
Hexane Extract	Sunflower Oil	0.00	1.11 ± 0.04 a	
	Cytosar	1.54	0.64 ± 0.06 b	-42.3
	Extract	83.9	1.18 ± 0.03 a	6.3

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-23: Arthus reaction (mean ± standard error) in albino male mice after type II- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)	Treatment Efficiency (%)
Aqueous Extract	Distilled water	0.00	1.25 ± 0.06 a	
	Cytosar	1.54	1.04 ± 0.07 ab	-17.0
	Extract	83.9	1.15 ± 0.06 ab	-8.0
Hexane Extract	Sunflower Oil	0.00	0.89 ± 0.04 b	
	Cytosar	1.54	1.04 ± 0.07 ab	16.9
	Extract	83.9	0.69 ± 0.04 b	-22.5

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-24: Delayed type hypersensetivity (mean ± standard error) in albino male mice after type I- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)	Treatment Efficiency (%)
Aqueous Extract	Distilled water	0.00	1.43 ± 0.13 a	
	Cytosar	1.54	0.58 ± 0.11 b	-59.4
	Extract	83.9	1.67 ± 0.11 a	16.8
Hexane Extract	Sunflower Oil	0.00	0.46 ± 0.06 b	
	Cytosar	1.54	0.58 ± 0.11 b	26.1
	Extract	83.9	0.83 ± 0.03 c	80.4

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-25: Delayed type hypersensetivity (mean ± standard error) in albino male mice after type II- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)		Treatment Efficiency (%)
Aqueous Extract	Distilled water	0.00	0.55 ± 0.06	a	
	Cytosar	1.54	0.89 ± 0.04	b	61.8
	Extract	83.9	1.20 ± 0.04	c	118.3
Hexane Extract	Sunflower Oil	0.00	0.58 ± 0.15	a	
	Cytosar	1.54	0.89 ± 0.04	b	53.4
	Extract	83.9	1.09 ± 0.04	c	87.9

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-26: Plaque forming cell (mean ± standard error) in albino male mice after type I- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)	Treatment Efficiency (%)
Aqueous extract	Distilled water	0.00	34.13 ± 0.43 a	
	Cytosar	1.54	9.38 ± 0.63 b	-72.5
	Extract	83.9	61.63 ± 2.25 c	80.6
Hexane Extract	Sunflower Oil	0.00	45.38 ± 0.63 d	
	Cytosar	1.54	9.38 ± 0.63 b	-79.3
	Extract	83.9	61.13 ± 0.83 c	34.7

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-27: Plaque forming cell (mean ± standard error) in albino male mice after type II- treatment with the ideal dose of aqueous and hexane extracts of sage leaves, distilled water and sunflower oil (negative controls) and cytosar drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error (mm)	Treatment Efficiency (%)
Aqueous Extract	Distilled water	0.00	29.38 ± 0.85 a	
	Cytosar	1.54	19.38 ± 1.03 b	-34.0
	Extract	83.9	50.25 ± 0.85 c	71.1
Hexane Extract	Sunflower Oil	0.00	39.38 ± 0.63 d	
	Cytosar	1.54	19.38 ± 1.03 b	-50.8
	Extract	83.9	53.25 ± 2.29 c	35.2

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-28: Metaphase index of bone marrow cells (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (%)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	post-treatment
Control I (Distilled Water-Cytosar)	2.01 ± 0.16 a	1.37 ± 0.11 a		
Control II (Sunflower Oil-Cytosar)	2.34 ± 0.14 a	2.11 ± 0.08 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	4.52 ± 0.16 b	3.63 ± 0.30 c	125.0	165.0
Hexane Plant Extract (Ideal Dose)-Cytosar	3.60 ± 0.09 c	3.30 ± 0.14 c	53.8	56.4

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-29: Metaphase index of spleen cells (mean ± standard error) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage and cytosar drug.

Groups	Mean ± Standard Error (%)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	1.08 ± 0.11 a	0.68 ± 0.09 a		
Control II (Sunflower Oil-Cytosar)	1.14 ± 0.05 a	1.47 ± 0.11 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	2.22 ± 0.16 b	1.44 ± 0.11 b	105.6	111.8
Hexane Plant Extract (Ideal Dose)-Cytosar	2.17 ± 0.09 b	2.14 ± 0.09 c	90.4	45.6

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-30: Micronucleus formation in bone marrow cells (mean ± standard error) of albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage leaves and cytosar drug.

Groups	Mean ± Standard Error (micronucleus/cell)		Treatment Efficiency (%)	
	Pre- treatment	Post- treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	0.0073± 0.0009 a	0.0160± 0.0009 a		
Control II (Sunflower Oil-Cytosar)	0.0238± 0.0015 b	0.0168± 0.0011 a		
Aqueous Plant Extract (Ideal Dose)-Cytosar	0.0013± 0.0003 c	0.0043± 0.0005 b	-82.2	-73.1
Hexane Plant Extract (Ideal Dose)-Cytosar	0.0123± 0.0011 d	0.0120± 0.0009 c	-48.3	-28.6

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table 4-31: Sperm-head abnormalities (mean ± standard error) of albino male mice after interactions (pre- and post-treatments) between the ideal dose (83.9 mg/kg) of aqueous and hexane extracts of sage and cytosar drug.

Groups	Mean ± Standard Error (%)		Treatment Efficiency (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control I (Distilled Water-Cytosar)	6.50 ± 0.21 a	8.15 ± 0.35 a		
Control II (Sunflower Oil-Cytosar)	12.56± 0.71 b	12.00 ± 0.53 b		
Aqueous Plant Extract (Ideal Dose)-Cytosar	5.25 ± 0.22 a	7.65 ± 0.25 a	-19.2	-6.1
Hexane Plant Extract (Ideal Dose)-Cytosar	6.75 ± 0.30 a	6.60 ± 0.27 c	-46.3	-45.0

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Table (4-32): Micronucleus formation in lymphocyte cultures (treated with sage leaves aqueous extract) of acute lymphoid leukemia patients and healthy controls.

Groups	Concentration (µg/ml)	Micronucleus/cell (Mean ± Standard Error)		Probability ≤
		Healthy Controls (No. = 5)	Leukemia patients (No. = 5)	
Negative Controls (Untreated)	0.0	0.0140±0.0011 A	0.0236±0.0023 A	0.05
Positive Controls (cytosar)	125.0	0.0180±0.0013 B	0.0230±0.0013 A	0.05
Sage (Aqueous Extract)	250	0.0104±0.0002 C	0.0144±0.0009 B	0.05
	500	0.0076±0.0002 D	0.0098±0.0009 C	0.05
	1000	0.0038±0.0005 D	0.0062±0.0004 D	0.05
Sage (Aqueous Extract) + Cytosar	250	0.0138±0.0007 A	0.0190±0.0007 B	0.001
	500	0.0110±0.0003 C	0.0148±0.0009 B	0.05
	1000	0.0086±0.0004 D	0.0108±0.0007 B	0.05

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Chapter One: Introduction

1-1: Introduction

Herbs are plants or plant parts that are valued for their medicinal and savory qualities. They contain and can produce a variety of chemical substances that have different biological actions, with a special reference to their medicinal importance. Therefore, they are employed by herbalists of different cultures, anciently and recently, to remedy peoples of their sicknesses (Anderson, 1977). This theme has encouraged scientists to investigate the medicinal uses of plants and their derivatives, and their efforts have been fruitful in producing drugs to treat a wide range of human diseases. Therefore, the list of drugs that are of a herbal origin is in an increase, and the World Health Organization.(WHO) has established that out of 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional uses as plant medicines by native peoples (Barens, 2002). In this respect, cancers are important target for such medicines due to the facts that the incidence of these diseases is increased in a way, which is a proportional with the complications of modern life (Sarasin, 2003).

The aetiology of cancer is multifactorial, and an interaction between genetic and environmental factors is required to initiate the disease (Goldman *et al*.,1999).The environmental factors are able to induce a mutation(s) in the genetic material of the cell, and such event may promote the cell to undergo a carcinogenic transformation (Raj *et al*., 1983). So, the mutation is a prerequisite for carcinogenesis, and any material that can interact with the mutation-induced agent or the genetic material may modulate the mutational effect. The medicinal plant and/or their derivatives may act on these pathways, therefore, they have been divided into desmutagens and bioantimutagens (Samejma *et al*., 1995), and their antimutagenic effects are exerted either on the mutagens to prevent or reduce the metabolic activation of the mutant, or the genetic material to prevent fixation of mutation (i.e. stimulating DNA repair mechanisms) (Samejma *et aI*., 1998).

Saliva officinalis is one of the medicinal plants, and researches have revealed its anti-mutagenic and anti-carcinogenic potentials, and such medicinal implications have been ascribed to more than 160 types of phenolic compounds that have been described in different species of the plant. The plant is known as sage in Western world, while in Arabian regions is known as Merameya. It is shrub-like plant, which is classified under the family Lamiaceae (Lu and Foo, 2002). Sage is a further subject of medicinal plant researches, and in folkloric medicine, the plant is advised to be used in the treatment of colds, abdominal pain and fever, while in anti-mutagen and anti-carcinogen researches, the plant chemical compounds have shown promising results (GaIi-Muhtasib *et aI*., 2000; Lima *et al*., 2005).

1-2: Aims of study

The present project was designed to evaluate the immunoIogicaI and cytogenic effects of two extracts (aqueous and hexane) of *S. officinalis* leaves in albino male mice. Interactions between the plant extracts and cytosar (anti-leukemia drug) were also made to evaluate their action in modulating the drug effects. The parameters of evaluation were:

- **Immunological parameters**
    1. Total and differential count of Leucocytes.
    2. Phagocytosis.
    3. Arthus reaction and Delayed type hypersensitivity.
    4. Plaque forming cells.
- **Cytogenetic parameters**
    1. Metaphase index.
    2. Micronucleus formation.
    3. Sperm-head abnormalities.

The assay of micronucleus formation was further evaluated in cultures of blood cells obtained from patients with acute lymphoblastic leukemia. However, only the aqueous extract was investigated in this respect.

Chapter Three

Materials and Methods

3-1: Materials

The general laboratory equipments and chemicals, which were employed in the present study, are presented in tables 3-1 and 3-2, respectively.

Table 3-1: General laboratory equipments.

Equipments	Company / Country
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
incubator	Memmert / Germany
Digital camera	Mercury / China
Electric balance	Sartorius / Germany
Hemocytometer	Neubauer / Germany
Laminar air flow	Napco / France
Micropipette	Gilson / France
Microscope	Motic / Japan
Oven	Osaw / India
pH meter	Radiometer / Denmark
Rotary evaporator	Buchi / Switzerland
Soxhlet	Electrothermol / England
Vernier	Japan
Vortex	Griffin / England
Water bath	Gallenkamp / England

Table 3-2: Chemical materials.

Chemical Material	Company / Country
Agarose	Sigma / USA
Ammonium chloride	BDH / England
Calcium chloride	BDH / England
Chloroform	BDH / England
Citric acid	BDH / England
Dextrose	Fluka / Switzerland
Eosin	BDH / England
Ethanol	Ferak / Germany
Foetal calf serum	Sigma / USA
Giemsa stain	Fluka / Switzerland
Glacial acetic acid	Fluka / Switzerland
Glycerin	Fluka / Switzerland
Heparin	Leo Pharmaceutical /Denmark
Hepes	Sigma / USA
Hydrochloric acid	Sigma / USA
Magnesium sulfate di-hydrate	Fluka / Switzerland
Methanol	Fluka / Switzerland
Penicillin and Streptromycin	Sigma / USA
Potassium chloride	Sigma / USA
Potassium di-hydrogen Phosphate	BDH / England
Potassium hydroxide	Sigma / USA
Potassium monohydrogen phosphate	BDH / England
RPMI 1640 medium	Sigma / USA
Sodium bicarbonates	BDH / England
Sodium citrate	Sigma / USA
Sodium hydroxide	Sigma / USA
Trypan blue	Sigma/USA

3-2: The Plant Sage (*Salvia officinalis* L.)

3-2-1: Plant Collection and Identification

Dr. Khulood W. AL-Samarraei (Biotechnology Research Centre, Al-Nahrain University) supplied the aireal parts of plant (leaves) as dried material, which was imported from Jordan. She also identified the plant as *Salvia officinalis*.

3-2-2: Preparation of Plant Extracts

The plant was extracted with two types of solvents, which were distilled water or hexane. In both cases, the extracted part of the plant was leaves. The leaves were air-dried, and then powdered using a coffee grinder. Fifty grams of the leaf powder were extracted for three hours in 250 ml of the solvent (distilled water or hexane) using the soxhlet apparatus and the source of heating was a warm water bath (45°C). The leaf extract solution was then evaporated at 45°C using a rotary evaporator, and the resultant crude extract was frozen at -20°C until use to prepare the required doses and concentrations (Nadir *et al*., 1986).

3-2-3: Sage Doses and Concentrations

The plant extracts (aqueous and hexane) were assessed in two biological models, which were a mammalian model (albino mouse; *in vivo*) and an *in vitro* blood culture model (human blood cells). In the mammalian model, three doses of the two extracts were used (83.9, 167.8 or 251.7 mg/kg). These doses were correspondent to 10, 20 and 30%, respectively of the LD_{50} dose in mice (839 mg/kg) of the same genus (*Saliva*) but for a different species (*S. libanotica*) (Farahat *et al.*, 2001). To prepare these doses, the dried aqueous extract was dissolved in distilled water, while for hexane extract, the plant extract was dissolved in sunflower oil.

In human cultures, only the aqueous extract was assessed, in which, three concentrations (250, 500 or 1000 µg/ml) of the plant extract were tested. These

concentrations have shown immuno-modulatory activities using the *in vitro* mitogenic and comitogenic rat thymocyte tests (Ebringerova *et al*., 2003).

3-3: Solutions

1- Colchicine

One tablet (0.5 mg) of colchicine (Ibn Hayan/Syria) was dissolved in 1 ml of sterilized normal saline (Allen *et al*., 1977).The solution was freshly used.

2- Potassium chloride (KCl) hypotonic solution (0.075M)

Potassium chloride (5.75g) was dissolved in 1000 ml of distilled water, and then the solution was autoclaved (121°C, 1.05 pound / in^2, 20 minutes) and stored at 4°C (Allen *et al*., 1977).

3- Potassium chloride (KCl) hypotonic solution (0.1 M)

The solution was prepared by dissolving 7.45 g of KCl in 1000 ml of distilled water, and then autoclaved and stored at 4°C until used (Al-Sudany, 2005).

4. Leucocyte diluent

The solution was prepared by adding 2 ml of glacial acetic acid to 98 ml of distilled water, in addition to a few drops of methylene blue as a colour indicator (Sood, 1986).

5. Normal saline (0.9% NaCl)

A ready prepared solution (Jadda Company, Kingdom of Saudia Arabia) was used. The solution was supplied by the Baghdad Teaching Hospital.

5- Fixative solution

The solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid (Patton, 1967).

6- Sodium bicarbonate

Sodium bicarbonate (7.5 g) was dissolved in 100 ml of distilled water, and the solution was stored at 4°C (Allen *et al*., 1977).

7- Alsever's solution

Dextrose (20 g), sodium citrate (8 g), citric acid (0.55 g) and sodium chloride (4.2 g) were dissolved in 500 ml of distilled water, after adjusting the pH to 6.1, the volume was made up to 1000 ml, the solution was filter-sterilized, and stored at 4°C (Hudson and Hay, 1989).

8- Giemsa stain

Giemsa stock solution was prepared by dissolving one gram of Giemsa powder in 33 ml glycerin using water bath (60°C) for 2 hours with a continuous shaking. After cooling the solution for 30 min at room temperature, 66 ml of absolute methanol were added with a continuous mixing.The solution was then kept in a dark bottle at room temperature (Allen *et al*., 1977).

To prepare Giesma stain working solution, the following solutions were mixed:

- Giemsa stock solution: 1 ml
- Absolute methanol 1.25 ml
- Sodium bicarbonate solution 0.5 ml
- Distilled water 40 ml

9-Eosin stain

The stain was prepared by dissolving 1gm of eosin yellowish powder in 100 ml of distilled water. The stain was centrifuged (2000 rpm for 10 minutes) before use (Wyrobek and Bruce, 1975).

10-Ammonium chloride (NH_4Cl)

Ammonium chloride (8.7 g) was dissolved in 1000 ml of distilled water, and then the solution was autoclaved and stored at 4°C (Ad'hiah, 1990).

11-Antibiotic Solutions

Streptomycin solution was prepared by dissolving 1g of streptomycin sulphate in 10 ml of distilled water, while benzyl penicillin was prepared by dissolving 1000000 IU in 10 ml of distilled water. Both solutions were filter-sterilized (Millipore filter; 0.22 µm), and divided into aliquots (1 ml), and then stored at -20°C until use (Freshney, 2000).

12-RPMI – 1640 medium

RPMI-1640 medium base (10 g), sodium bicarbonate (2 g) and hepes (4 g) were dissolved in 500 ml of distilled water, and then 100 ml of heat inactivated foetal calf serum, 5 ml of penicillin (100000 IU/ml) and 5 ml of streptomycin (100 mg/ml) were added. The volume was made up to 1000 ml with distilled water, and the pH was adjusted to 7.2. The medium was filter-sterilized using Millipore filter (0.22 µm), and then divided into aliquots (2 ml) and stored in the freezer (4°C) until used (Nara and McCulloch, 1985).

13-Heat-killed Yeast Suspension

The yeast *Sacchoromyces cerevisiae* (Pakmaya Company, Turkey) was used to prepare the yeast suspension (Metcalf *et al*., 1986). The method is outlined as follows:

1- Ten grams of the yeast were suspended in 150 ml of sterile normal saline.
2- The suspension was heated in a boiling water bath for 60 minutes.
3- After cooling the suspension at room temperature, it was filtered using double layers of gauze.
4- The filtered cell suspension was assessed for yeast cell viability by dye exclusion test (trypan blue) to assure that all cells were dead.
5- The cell suspension was divided into aliquots (5 ml) after adjusting the cell count to 10^7 cell / ml, and stored at -20ºC until use.

14-Human Plasma

The National Blood Transfusion Centre in Baghdad supplied the human AB plasma. The plasma was transferred to the laboratory in an ice box.

In the laboratory, the plasma was divided into aliquots (5 ml) in sterile test tubes. Some of these tubes were stored in the freezer (-20°C) until use in the phagocytosis assay (Metcalf *et al*., 1986). The other tubes were placed in a water bath (56°C) for 30 minutes to inactivate the complement, and then stored at -20°C until use in the micronucleus assay (Schmid, 1976). In both cases, the plasma was filter-sterilized.

15-Gunia Pig Serum

The blood (5 ml) was collected from gunia pig by heart puncture in a sterile test tube using a disposable syringe, and left at 4°C for 30 minutes to agglutinate. Then the tube was centrifuged (2000 rpm) for 10 minutes, and the serum was collected and stored at -20°C until used. 0ne ml of the serum was diluted up to 10 ml with HEPES-BSS (Solution No. 17) in the plaque forming cell assay (Myres, 1995).

16-Sheep Red Blood cell (SRBC) Suspension

The procedure of Myers (1995) was followed to prepare the SRBC suspension.

1- The blood (5 ml) was collected in a sterile test tube from the jugular vein and diluted with an equal volume of Alsever's solution.
2- The diluted blood was left in the refrigerator (4°C) overnight.
3- The blood was centrifuged (1000 rpm) for 15 minutes, and the plasma and buffy coat were discarded.
4- The SRBC pellet was collected and washed with HEPES-BSS at least three times or until the supernatant was clear, and in each wash the centrifugation speed was 2000 rpm and for 5 minutes. After that, the deposited cells were suspended in HEPES-BSS as required, and stored in the refrigerator (4°C) until used.

17- HEPES-Buffered Balanced Salt Solution (HEPES-BSS)

Six stock solutions were prepared as follows:

- **First solution**: Phosphate buffer was made by dissolving 22.9 g of KH_2PO_4 and 19.5 g of K_2HPO_4 in 950 ml of distilled water. The pH was adjusted to 7.2 with KOH, and the volume was made up to 1000 ml.
- **Second solution**: HEPES buffer was prepared by dissolving 80 g of HEPES and 13.4 g of NaOH in 950 ml of distilled water. The pH was adjusted to 7.2 with NaOH, and the volume was made up to 1000 ml.
- **Third solution**: Sodium chloride (9.83 g) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Fourth solution**: Potassium chloride (12.5 g) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Fifth solution**: Calcium chloride (12.45 g) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Sixth solution**: Magnesium sulfate (41.3 g) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.

The HEPES-BSS working solution was prepared by adding the above stock solutions in the following proportions:

10 ml of first solution

30 ml of second solution

605 ml of third solution

20 ml of fourth solution

15 ml of fifth solution

5 ml of sixth solution

The solution was filter-sterilized (Millipore filter, 22 µm) and stored at 4°C (Myers, 1995).

18-Leishman Stain

The Institute of Sera and Vaccine (Baghdad) supplied a ready prepared stain kit.

19-Phytohemagglutinin (PHA)

The Iraqi Center for Cancer and Genetic Research (Al-Mustansryiah University) and the Agriculture Research Centre (Ministry of Biotechnology) supplied the PHA as a ready solution.

20-Heparin

The Baghdad Teaching Hospital supplied the solution of heparin (5000 IU/ml), which was the product of Leo Pharmaceutical (Denmark).

21-Trypan blue

One gram of trypan blue powder was dissolved in 100 ml of normal saline.The stain solution was filtred (Whattman filter paper No.3) before use (Ad'hiah, 1990).

22- Dose and Concentration of the Drug Cytosar

The Baghdad Teaching Hospital supplied the drug cytosar (Cytarabine), which was the product of Pharmacia Company (Belgium).

In the mammalian model, a dose of 1.54 mg/kg was tested. Such dose is the recommended dose in the treatment of leukemia in humans (Company Leaflet). While in human blood cultures, a concentration of 125 µg/ml was used. Such concentration has been found to be genotoxic in human blood cultures (Ramakers-van Woerden *et al*., 2000). In both cases, the drug solution was diluted with distilled water to prepare the required dose and concentration.

3-4: Laboratory Animals

Albino Swiss male mice (*Mus musculs*) were the laboratory animals. They were supplied by the Biotechnology Research Centre (Al-Nahrain University). Their age at the start of experiments was 8-10 weeks, and their weight was 23-27 gram. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 23 – 25°C, and they had free excess to food (standard pellets) and water (*ad libitum*).

3-5: Experimental Design

The experiments were designed to evaluate (*in vivo*) the genetic and immunological effects of sage extracts (aqueous and hexane) in albino male mice, as well as, their role in modulating the genetic and immunological effects of the anti-leukemia drug cytosar. Therefore, such evaluations were carried out through three stages.

3-5-1: First Stage

In this stage, the genetic and Immunological effects of three doses of sage extracts (aqueous or hexane) and cytosar were investigated. Therefore, the animals were divided into five groups:

- **Group I**: The animals were treated with distilled water (negative controls of aqueous extract = 8 animals).
- **Group II**: The animals were treated with sunflower oil (negative controls of hexane extract = 8 animals).
- **Group III**: The animals were treated with cytosar at a dose of 1.54 mg/kg (positive controls = 8 animals).
- **Group IV**: The animals were treated with three doses of the aqueous extract (83.9, 167.8 or 251.7 mg/kg) (24 animals).
- **Group V**: The animals were treated with three doses of the hexane extract (83.9, 167.8 or 251.7 mg/kg) (24 animals).

The tested materials were injected subcutaneously as a single dose (0.1 ml) per a day and for 7 days. Then the mice were sacrificed in day 8 for laboratory assessments. The total number of mice in this stage was 72 animals.

3-5-2: Second Stage

In this stage, interactions (pre- and post-treatments) between the ideal dose of both extracts (83.9 mg/ kg) and cytosar (1.54 mg/kg) were carried out. The criterion of selection for the ideal dose was based on the approximation between the values of total leucocyte count in the extract-treated animals and negative controls.

- **In pre-treatment** interaction, the plant extract (aqueous or hexane) was given for 6 days (single dose/day), while cytosar was given in day 7, and then animals were sacrificed in day 8 for laboratory assessments. In both cases, the material was given subcutaneously

(0.1 ml). The total number of mice in this interaction was 32 animals.

- **In post-treatment** interaction, the animals was given cytosar on day one, while the plant extract (aqueous or hexane) was given in day 2 till day 7 (single dose/day), and then animals were sacrificed in day 8 for laboratory assessments. In both cases, the material was given subcutaneously (0.1 ml). The total number of mice in this interaction was 32 animals.

For both treatments, control groups were paralleled the two types of interactions, in which the plant extract was replaced by either distilled water (aqueous extract) or sunflower oil (hexane extract).

3-5-3: Third Stage

This experiment was carried out to assess three types of immunological reactions (Arthus reaction, delayed type hypersensitivity reaction and plaque forming cell assay) in mice that were immunized intraperitoneally with 0.1 ml of 10% SRBC suspension. These reactions were assessed in the animals after a subcutaneous injection (0.1 ml) of the plant ideal dose (83.9 mg/kg for both extracts), cytosar (1.54 mg/kg), distilled water and sunflower oil in two types of immunization-interaction regimes (type I and type II treatments). Details of the two types of treatments are given in tables 3-3 and 3-4, respectively.

Table 3-3: Immunization-interaction regimes between SRBC and the ideal dose of aqueous and hexane extract, cytosar, distilled water and sunflower oil in albino male mice (type I treatment).

Days	Material of Injection (M)
1-7	M (either aqueous extract, hexane extract, cytosar, distilled water or sunflower oil)
8	Sheep Red Blood Cells (SRBC)
9,10,11	No injection
12	SRBC
13,14	No injection
15	Arhtus Reaction
16	Delayed type hypersensitivity + plaque forming cells
Total number of animals = 20 mice (4 animal for each material of injection)	

Table 3-4: Immunization-interaction regimes between SRBC and the ideal dose of aqueous and hexane extracts, cytosar, distilled water and sunflower oil in albino male mice (type II treatment).

Days	Material of Injection (M)
1-4	M (either aqueous extract, hexane extract, cytosar, distilled water or sunflower oil)
5	M + SRBC
6-8	M
9	M + SRBC
10-11	M
12	Arhtus Reaction
13	Delayed type hypersensitivity + plaque forming cells
Total number of animals = 20 mice (4 animal for each material of injection)	

3-6: Laboratory Methods

3-6-1: Chemical Analysis of Plant Extracts

The chemical analysis of plant extracts (aqueous and hexane) were carried out at the College of Pharmacology (University of Baghdad) to detect the following compounds:

- Alkaloids, saponins, terpenes, flavonoids and glycosides (aqueous extract).
- Steroids (hexane extract)

3-6-2: Total Leucocyte Count

Blood samples were collected by heart puncture using a disposable insulin syringe (1 ml) precoated with heparin. The method of Haen (1995) was followed, in which, an aliquot of 0.02 ml blood was mixed with 0.38 ml of leucocyte diluent in a test tube, and left at room temperature for 5 minutes. A drop of the mixture was applied to the surface of Neubauer chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells. The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation:

$$\text{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Number of Cells Counted}}{4} \right) \text{x } 20 \text{ x } 10$$

3-6-3: Differential Count of Leucocytes

One drop of blood was smeared on a clean slide using another slide and left to dry at room temperature. The smear was stained with Leishman stain for 5 minutes and buffered for 10 minutes, and then washed with tap water. The slide was air-dried, and then examined under oil immersion lens (100X) (Haen, 1995). At least 100 leucocytes were examined, and the percentage of each type

was recorded, while the total count of each type was obtained using the following equation:

$$\text{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Percentage of Cells x Total Count}}{100} \right)$$

3-6-4: Phayocytic Index

The evaluation of phagocytosis was carried out on phagocytes obtained from the peritoneum of mice. The procedure of Metcalf *et al*., (1986) was followed with some modifications.

1- The animal was anaesthetized with chloroform, and then injected intraperitoneially with 3 ml of normal warm saline (37°).After that, the abdominal region was massaged for 3 minutes.
2- The animal was dissected, and the peritoneal cells were collected with a pasture pipette and transferred to a clean test tube.
3- The tube was centrifuged (2000 rpm/minutes) for 5 minutes.
4- The cells were suspended in 1 ml of normal saline, counted and their number was adjusted to 10^6 cell /ml. Also, the cell viability was assessed using trypan blue stain.
5- To carry out phagocytosis, 0.2 ml of cell suspension, 0.1 ml of heat-killed yeast suspension and 0.1 ml of human plasma AB were mixed in a test tube and incubated in a shaking water bath (37°C).
6- After 30 and 60 minute incubations, smears were made and the slides were air-dried, and then stained with Giemsa stain for 15 minutes.
7- The slides were examined under oil immersion lens (100X), and at least 100 yeast-phagocytic and non-phagocytic cells were randomly counted. The phagocytic activity was expressed as a phagocytic index, which was calculated using the following equation:

$$\text{Phagocytic Index (\%)} = \left(\frac{\text{Number of Phagocytic Cells}}{\text{Total Count}} \right) \text{x } 100$$

3-6-5: Metaphase Index Assay

Metaphase index was determined for cells obtained from bone marrow and spleen, following the procedure of Allen *et al*., (1977). Each animal was injected intraperitoneally with 0.25 ml of colchicine solution, and after 1.5 – 2 hours, the animal was sacrificed by cervical dislocation and then dissected to obtain femur bone and spleen. The femur bone was cut from both ends, and its cellular contents were collected in a test tube using a disposable insulin syringe (1 ml) and normal physiological saline (5 ml). The spleen was placed in a Petri dish containing 5 ml normal physiological saline, and then punctured several times with the needle of insulin syringe. The cellular content was obtained by repeated infusions of saline in the spleen, and by then the cell suspension (5 ml) was transferred to a test tube.

The cells of both organs were manipulated in a similar way, and as follows:

1- The cells were gently suspended using Pasteur pipette, and the tubes were centrifuged (2000 rpm/min) for 10 minutes.
2- The supernatant was discarded, and the cell deposit was suspended in 5 ml of a warm (37°C) hypotonic solution (KCl; 0.075 M). Then the tubes were incubated in a water bath (37°C) for 30 min with a gentle shaking every 5 minutes.
3- The tubes were centrifuged at 2000 rpm/min for 10 minute, and the supernatant was discarded.
4- Five ml of the fixative solution was added a drop-wise to the cell deposit with a gentle and a continuous mixing to make a homogeneous cell suspension. Then, the tubes were incubated in the refrigerator (0°C) for 30 minutes,
5- The tubes were centrifuged (2000 rpm/min) for 10 minutes, and step 4 was repeated two times.

6- The cell deposit was well-suspended in 2 ml of the fixative, and 4-5 drops of the cell suspension were dropped on a clean slide from a height of about two feet.

7- The slide was air-dried at room temperature, and by then it was stained with Giemsa stain for 15 minutes and rinsed with distilled water.

8- The slide was examined under oil emersion lens (100X), and at least 1000 cells were examined. The percentage of metaphase cells (metaphase index) was recorded using the following equation:

$$\text{Metaphase Index (\%)} = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}} \right) \text{x } 100$$

3-6-6: Micronucleus Formation Assay

To carry out the assessment of micronucleus formation, the procedure of Schmid (1976) was followed, which is outlined in the following steps:

1. The mouse was sacrificed by cervical dislocation, and then dissected to obtain the femur bone. After cutting both ends of the bone, it was grapped from the middle with a forceps in a vertical position over the edge of a test tube, and then the cellular content was collected with a heat inactivated (56°C for 30 minutes) human AB plasma (2 ml) using a disposable insulin syringe.
2. The test tube was centrifuged (1000 rpm) for 10 minutes, and the supernatant was discarded.
3. The cellular deposit was gently mixed, and a thin smear was made on a clean slide, and air-dried at room temperature.
4. The smear was fixed with absolute methanol for 5 minutes, and then air-dried at room temperature.
5. The smear was stained with Giemsa stain for 15 minutes, and rinsed with distilled water.

6. The slides were examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus formation. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus Index (micronucleus/cell)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}} \right) \text{x } 100$$

3-6-7: Sperm-head Abnormality Assay (SHA)

The mouse was sacrificed by cervical dislocation and then dissected to obtain the epididymis, which was collected in as Petri-dish containing 5 ml of normal saline. The epididymis was dispersed with a forceps and a scalpel to free the spermatozoa. The spermatozoa-containing saline was transferred to a test tube, which was centrifuged (1000 rpm) for 10 minutes. The supernatant was discarded, and the spermatozoa deposit was gently suspended in 1 ml of normal saline. A thin smear of the suspension was made on a clean slide, which was then air-dried at room temperature. The slide was stained with eosin for 5 minutes, rinsed with distilled water and air-dried. The slide was examined under oil immersion lens (100X), and at least 1000 spermatozoa were inspected for the morphology of their heads (Wyrobek and Bruce, 1975). The sperm-head abnormality (SHA) index was scored using the following equation:

$$\text{SHA Index (\%)} = \left(\frac{\text{Number of Spermatozoae with Abnormal Head}}{\text{Total Count}} \right) \text{x } 100$$

3-6-8: Arthus Reaction

After the immunization regimes presented in tables (3-3) and (3-4), the left foot pad was injected intradermally with 0.05 ml of 10% SRBC, while the right foot pad was injected with 0.05 ml of normal saline. After four hours, the thickness of both pads was measured using a vernier, and the difference

represented Arthus reaction index, which was given in units of millimeter (mm) (Triolo *et al*., 1989).

3-6-9: Delayed Type Hypersensitivity

The index of delayed type hypersensitivity (DTH) was carried out as outlined for Arthus reaction index, but the difference between the thicknesses of both pads was measured after 24 hours.

3-6-10: Plaque Forming Cell Assay

The procedure of Myers (1995) was followed to determine the plaque forming cells (PFC). After carrying out the DTH index, the mouse was sacrificed, dissected and the spleen was collected in a Petri-dish containing 5 ml of HEPES-BSS. The spleen was dispersed to free its cells, which were collected in a test tube. The test tube was centrifuged (2000 rpm) for 5 minutes and the supernatant was then discarded, and the cells were suspended in 10 ml of NH_4Cl. The cell suspension was incubated in a water bath at 37°C for 10 minutes, and then the cells were washed three times with HEPES-BSS, and in each wash the centrifugation speed was 2000 rpm (5 minutes). After the third wash, the cell deposit was gently suspended in 1 ml of HEPES-BSS, and the cell count was adjusted to 2.5×10^7 cell/ml. The cell suspension was kept in an ice bath until the determination of PFC, which required a preparation of two agarose solutions.

The agarose under-layer was prepared by dissolving 1.5 gm of agarose in 100 ml of HEPES-BSS, using a boiling water bath. Then, the solution was cooled to 45°C and divided into aliquots (2 ml) in test tubes. The agarose over-layer was prepared in a similar way, but the amount of agarose was 0.7 gm. Both solutions were autoclaved and then kept in the refrigerator (4°C) until use.

To carry out the PFC assay, the tubes of both agarose layers were transferred to a warm water bath (45°C) to melt the agarose. The agarose under-layer was poured in a disposable Petri-dish (1.5 x 6.0 mm) to make a thin

supporting layer, and then the dish was transferred to the refrigerator (4°C) to solidify the agarose. While, the agarose over-layer was mixed gently with 0.5 ml of spleen cell suspension and 0.2 ml of 15% SRBC suspension, and the mixture was applied to the surface of agarose under-layer in the Petri-dish. The dish was first incubated in the refrigerator (4°C) for 15 minutes, and then it was further incubated in a water bath (37°C) for 60 minutes. By then, 2 ml of diluted Guinea pig serum (1:10 with HEPES-BSS) were added, and a further 60 minutes incubation in the water bath was carried out.

The Petri-dish was examined under a 10X immersion lens, and an inspection of cells that lysed SRBC was carried out. These cells were recognized by a zone of lysis around them. At least, 100 cells (with lysis zone and without) were selected randomly for examination, and the percentage of cells that lysed SRBC (PFC) was scored using the following equation:

$$\text{PFC Index (\%)} = \left(\frac{\text{Number of Cells with Lysis Zone}}{\text{Total Count}} \right) \text{x}\,100$$

3-6-11: Micronucleus Formation in the Blood of Leukemia Patients

3-6-11-1: Subjects

The subjects of this assay were patients (5 subjects) with acute lymphoid leukemia (ALL), which were referred to the Baghdad Teaching Hospital for diagnosis and treatment. The diagnosis was based on a clinical examination and laboratory evaluations, which were carried out by the consultant medical staff at the hospital. The patients were Iraqi Arabs, and their age range was 25-40 years. They were firstly diagnosed (January-April, 2006), and none of them was under treatment. A further five healthy subjects (control group) were also investigated. They were university staff and students that had no history or signs of leukemia, and matched with patients for ethnic background and age.

Peripheral blood (5 ml) was obtained under aseptic conditions from each subject by a venepuncture using a disposable syringe precoated with heparin. The blood sample was placed in a cool box and transferred to the laboratory.

3-6-11-2: Treated Groups

This experiment was designed to assess *in vitro* the effects of three concentrations of sage aqueous extract (250, 500 or 1000 µg/ml) and one concentration of cytosar (125 µg/ml) in inducing micronucleus formation in the cultured blood cells of patients and controls. Therefore, eight cultures were set–up for each subject:

- **Culture Number 1**: The culture was treated with 250µg/ml of sage aqueous extract.
- **Culture Number 2**: The culture was treated with 500 µg/ml of sage aqueous extract.
- **Culture Number 3**: The culture was treated with 1000 µg/ml of sage aqueous extract.
- **Culture Number 4**: The culture was treated with 125 µg/ml of cytosar.
- **Culture Number 5**: The culture was not treated with any material (negative control).
- **Culture Number 6**: An interaction between sage aqueous extract (250 µg /ml) and cytosar (125 µg/ml) was made.
- **Culture Number 7**: An interaction between sage aqueous extract (500 µg /ml) and cytosar (125 µg/ml) was made.
- **Culture Number 8**: An interaction between sage aqueous extract (1000µg/ml) and cytosar (125 µg/ml) was made.

3-6-11-3: Micronucleus Test Cultures

The procedure of Al-Sudany (2005) was followed, in which, 2 ml of RPMI-1640 culture medium were supplemented with 0.1-0.3 ml of PHA, and

then 0.5 ml of blood was added to the culture tube (cultures number 4 and 5), together with 0.1 ml of the plant extract (culture numbers 1, 2, 3, 6, 7 and 8). The eight cultures were incubated at 37°C for 72 hours, while in cultures numbers 4, 6, 7 and 8, 0.1 of cytosar (125 µg/ml) was added after a period of 24 hours incubation, and, then the incubation continued up to 72 hours as in the rest of cultures.

After 72 hours incubation, the culture tubes were centrifuged (800 rpm) for 5 minutes, and then the supernatant was discarded and the cell deposit was gently suspended in 5 ml of a warm (37°C) hypotonic KCl solution (0.1M). The cell suspension was incubated in a water bath (37°C) for 30 minutes with a gentle mixing every 5 minutes.Then, the suspension was centrifuged (800 rpm) for 5 minutes, and the supernatant was discarded. The deposit was suspended in a few drops of a cold fixative (4°C), and the volume was made up to 5 ml with the fixative. The fixed cell suspension was incubated in the refrigerator (4°C) for 30 minutes, and after that, it was centrifuged (800 rpm) for 5 minutes. The process of fixation was repeated two times, and by then the cells was suspended in 1 ml of the fixative.The fixed cells were smeared on a clean slide, and left for air-drying. The slide was stained with Giemsa stain for 15 minutes, and then rinsed with distilled water, and finally it was air-dried.

The slide was examined under oil immersion lens (100X), and the cells were inspected for the formation of micronucleus. A total of 1000 cells was randomly examined, and the micronucleus index was scored using the following equation:

$$\text{Micronucleus index (micronucleus/cell)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of Cells}} \right) \text{x } 100$$

3-7: Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using the computer programme SPSS version 7.5.The difference was considered significant when the probability value was equal or less than 0.05.

A further estimation was also given; it was treatment efficiency (Perez-Serrano *et al*., 1997), which was calculated according to the following equation:

$$\text{Treatment efficiency} = \left(\frac{A - B}{B}\right) \times 100$$

A = Treated groups (plant extracts or cytosar).

B = Negative control groups (distilled water or sunflower oil).

Chapter Two

Literature Review

2-1: Medicinal Plants

Plants are the oldest friends of mankind. They not only provided food and shelter but also served the humanity to cure different ailments, and according to the world health organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for the health care of its people (Calixto, 2005). Historians from all around the world have produced evidence to show that apparently all primitive peoples used herbs, and often in a sophisticated way. Quinine from Cinchona bark was used to treat the symptoms of malaria long before the disease was identified, and the raw ingredients of a common or garden aspirin tablets were a popular painkiller for far longer than we have had access to tablet-making machinery (Pieters and Vlietinick, 2005). By the middle of the nineteenth century at least 80% of all medicines were derived from herbs. Even today, many pharmacological classes of drugs include a natural product prototype. Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine, and vinblastine are a few examples of what medicinal plants have given us in the past (Gurib-Fakim, 2005). Most of these plant-derived drugs were originally discovered through the study of traditional cures and folkloric knowledge of indigenous people and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Gilani and Atta-ur-Rahman, 2005). In this regard, sage (*Salvia officinalis*) is a further subject of medicinal plants, and the literatures are rich in overwhelming us with its medicinal uses (Bolta *et al*., 2000; Ninomiya *et al*., 2004; Lima *et al*., 2005).

2-2: The Plant Sage (*Salvia officinalis* L.)

Numerous species of the genus *Salvia* have been employed since ancient times in folkloric medicine and subjected to an extensive pharmacognostic

research to identify their biologically active compounds. Sage has a very long history of effective medicinal uses and is an important domestic herbal remedy for disorders of the digestive system. Its antiseptic qualities make it an effective gargle for the mouth where it can heal sore throats and ulcers. The leaves applied to an aching tooth will often relieve the pain. The whole herb is antihydrotic, antiseptic, antispasmodic, astringent, carminative, stimulant, tonic and vasodilator. Sage is also used internally in the treatment of excessive lactation, night sweats, excessive salivation (as in Parkinson's disease), profuse perspiration (as in tuberculosis), anxiety, depression, female sterility and menopausal problems (Phillips and Foy, 1990). Externally, it is used to treat insect bite, skin, throat, mouth and gum infections and vaginal discharge. The essential oil from the plant is used in small doses to remove heavy collections of mucous from the respiratory organs and mixed in embrocations for treating rheumatism. In larger doses, however, it can cause epileptic fits and giddiness. The leaves make excellent tooth cleaners, have antiseptic properties and can heal diseased gums (Genders, 1994).

2-2-1: Common Names and Classification

In English literature, several common names are used to describe *S. officinalis*, for instance, sage, garden sage, narrow-leaved white sage, meadow sage, true sage, scarlet sage, broad-leaved white sage and kitchen sage (Simon *et al*.,1984). However, from the point view of taxonomists, the plant follows the following classification (Internet I):

Kingdom:

Plantae

Sub-kingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Sub-class: Asterdia

Order: Lamiales

Family: Lamiaceae (labiates)

Genus: *Salvia*

Species: *Salvia officinalis* L.

2-2-2: Plant Description and Distribution

Sage is a herbaceous annual plant with a strong root. The stem is sturdy and branched with length between 15-30 cm. Leaves are gray to silver-green with a velvety texture. The flowers are white, blue or purple, which bloom from late winter to early summer. Each flower has four corolla and a calyx smaller than the corolla with purple tints, while the ovary has two fusion parts and the peel consists of four nutlets, each one contains one seed (Figure 2-1) (Jones and Luchsinger, 1987).

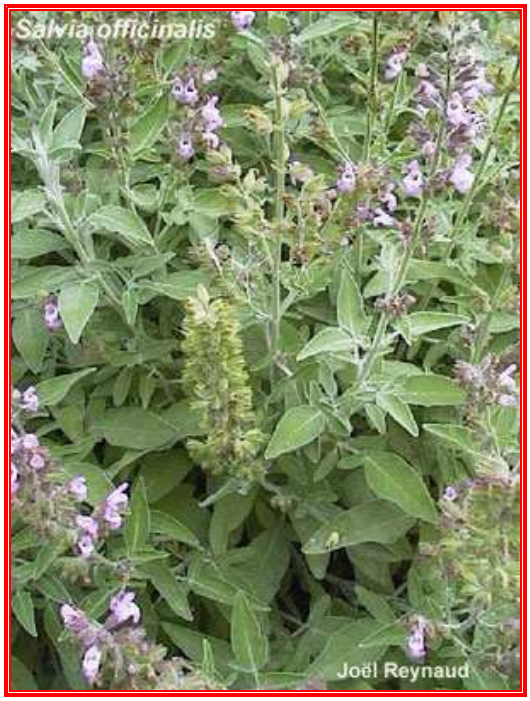

Figure 2-1: The plant sage (*S. officinalis*) (Internet II).

The plant is distributed in tropical regions and it is indigenous in Southern Europe, in which it is planted for its medicinal and garden purposes. The plant is also cultivated and collected from the wild in Albania, Turkey, Italy, Greece, Spain and the United States of America. In Iraq, the plant is distributed in

different regions, but it is more common in Western regions (Chada, 1976; Simon *et al*., 1984).

2-2-3: Chemical Constituents

Chemical analyses of sage revealed that the plant is rich in the following constituents:

- **Essential oil**s: The principal components are monoterpenoids, such as α-thujone, β-thujone, camphor and 1,8-cineole, together with sesquiterpenes such as α-humulene, β-caryophyllene and viridiflorol (Pitatevic *et al*., 1984; Lawrence, 1992).
- **Hydroxycinnamic acid derivatives**: Principally, they are presented by caffeic acid dimer rosmarinic acid. Caffeic acid trimers (melitric acid A, methyl melitrate A, sage coumarin and salvianolic acid K) and a tetramer (sagerinic acid) have also been isolated. Collectively, these and similar compounds are sometimes described as “tannins”. However, they are not genuine tannins in the sense of condensed tannins (proanthocyanidins) or hydrolysable tannins (gallo- and ellagitannins). Other hydroxycinnamic compounds are also present, including 6-feruloyl-glucose and a polyalcohol derivative of it, three hydroxycinnamic esters of disaccharides and free caffeic acid (Lu *et al*., 1999).
- **Phenolic diterpenes**: Carnosic acid, a tricyclic diterpene, occurs in the fresh leaves and to some extent in the dried leaves and certain types of extracts. However, carnosic acid is fairly unstable and readily auto-oxidises to form lactones, especially the bitter-tasting lactone carnosol. In turn, carnosol can degrade further to produce other phenolic diterpenes with lactone structures, such as rosmanol, epirosmanol, 7-methoxyrosmanol and galdosol, which have been identified in sage leaves (Kavvadias *et al*., 2003).

- **Triterpenes**: They occur as pentacyclic triterpene acids, which are mainly ursolic acid and oleanolic acid, and the triterpene alcohols α- and β-amyrin (Wang *et al*., 2000).
- **Flavonoids**: Principally, they are flavones and their glycosides (Lu and Foo, 2000).
- **Phenolic glycosides**: They represent a diverse range including, picein (4-hydroxyacetophenone glucoside), 4-hydroxyacetophenone 4-(6′-apiosyl)-glucoside, *cis*- and *trans*-*p*-coumaric acid 4-(2′-apiosyl)-glucoside, isolariciresinol 3-glucoside and 1-hydroxypinoresinol 1-glucoside (Wang *et al*., 2000).
- **Polysaccharides**: Crude fractions rich in water-soluble arabinogalactans, high molecular weight pectin and glucuronoxylan-related polysaccharides have been isolated from the aerial parts of sage (Capek *et al*., 2003).
- **Other constituents** include small amounts of benzoic acid derivatives (*p*-hydroxybenzoic, gentisic, syringic and other acids) and phytosterols (β-sitosterol and stigmasterol) (Kennedy *et al*., 2006).

2-2-4: Biological Potentials

Several biological potentials have described for different extracts of sage or its natural products. They can be summarized in the following:

- **Antioxidant activity**: The leaf extracts exhibit strong antioxidant activity, largely attributable to various phenolic constituents including phenolic diterpenes such as carnosol and hydroxycinnamic acid derivatives, notably rosmarinic acid (Lamaison *et al*., 1991; Wang *et al*., 2000). In a carotene bleaching test, the antioxidative activity of a dry acetone extract from leaves was found to be 101-116% of that of the synthetic antioxidant butylated hydroxytoluene (Dapkevicius *et al*., 1998). Lipid peroxidation in both enzyme-dependent and enzyme-independent test systems were

inhibited more effectively by a dry 50%-methanolic extract from aerial parts of leaves than by α-tocopheryl acid succinate (as a positive control).The antioxidant activity was attributed mainly to phenolic compounds, rosmarinic acid being the main contributor due to its high concentration in the extract (Hohmann *et al*., 1999; Zupko *et al*., 2001). Recently, it has been demonstrated that the leaf aqueous extract of sage can improve the antioxidant status of livers in mice and rats (Lima *et al*., 2005).

- **Antimicrobial activity**: Antiviral, antibacterial and antifungal activities of sage have been demonstrated. With respect to antiviral potentials, extracts of the plant were effective in the treatment of hepatitis B virus (Xiong, 1993), vesicular stomatitis viral infection (Sivropoulou *et al*., 1997) and *Herpes labialis* (Saller *et al*., 2001). As antibacterial, different extracts of sage were effective against different Gram-negative, as well as, Gram-positive bacteria (*Bacillus subtilits*, *Echerichia coli*, *Staphylococcus aureus, Salmonella typhimurium*, *Proteus mirabilis* and *Morganella morganii*) (Hammer *et al*., 1999; Bariceviv *et al*., 2001; Pereira *et al*., 2004; Rios and Recio, 2005). Similarly, different fungus species (*Botrytis cinerea, Ehrysanthemum morifolium, Trichophyton mentagrophytes* and *Epidermophyton floccosum*) showed a sensitive response to the volatile oils extracted from sage (Catra *et al*., 1996; Al-Khaphagi, 2000).
- **Antimutagenic and antitumor activities**: *Salvia* is considered as plant that has chemical constituents with antimutagenic and antitumour potentials. In this regard, 18 compounds were isolated from the species *Salvia miltiorrhizae* and tested for their cytotoxicity *in vitro* against cancer cells from colon, lung, ovary and skin. The results revealed that such compounds were effective in inhibiting the proliferation of these cells (Sato *et al*., 1992: Ryue *et al*., 1997). Such findings were further confirmed on mice with induced skin tumor, and the conclusion

favoured that the plant compounds are cytostatic rather than cytotoxic (Gali-Muhtasib and Affra, 2000). However, cytotoxic and DNA-damaging effects of diterpenoid quinones from the roots of sage on cultures of colonic and hepatic human cells have been demonstrated *in vitro* (Santos-Gomes *et al*., 2002). Furthermore, sage extracts with in vivo antimutagenic effects have also been reported (Vujosevic and Blagojevic, 2005).

- **Immunological activity**: Immunostimulant and anti-inflammatory properties have been suggested *in vitro* and *in vivo* for extracts and compounds of sage. It was demonstrated that sage extracts (hexane or chloroform) inhibit induced ear edema in mice by Croton oil, therefore anti-inflammatory effects were suggested (Baricevic *et al*., 2001). Isolated polysaccharides from sage leaves were effective in enhancing the lymphocyte transformation (Capek *et al*., 2003), while Ebringerova *et al*., (2003) considered the sage polysaccharides as immune modulators with a high adjuvant activity. In a further study, the plant aqueous extract of aerial parts showed mitogenic and comitogenic activities on human peripheral blood lymphocytes and rat thymocytes (Capek and Hribalova, 2004).

2-3: Leukemia

Leukemias are group of malignant disorders of the haematopoietic tissues that are characteristically associated with increased numbers of leucocytes in bone marrow and/or peripheral blood. The course of leukemia may vary from a few days or weeks to many years, depending on the type of disease (John, 2000).

2-3-1: Incidence of Leukemia

The worldwide incidence of all types of leukemia in the populations is approximately 10 per 1000000 per year. Males are affected more frequently than female at ratio of 3:2 in acute leukemia and 2:1 in chronic leukemia. Acute lymphoid leukemia accounts for 20-25% of all reported cases of leukemia, and 60-70% of these cases occur in children, while only 20% of cases occur in adults. In contrast, acute myeloid leukemia is almost a disease of adults (Haen, 1995; Pekham *et al*., 1995; Behrman *et al*., 1998; Edward and Boucher, 1999).

2-3-2: Aetiology

The aetiology of leukemia is unknown but several lines of evidence do suggest that the disease is a result of interactions between several factors:

I: Viral infections: Virally induced tumors are well-recognized, and shared expressed antigens by tumors induced by the same virus have been documented. For instance, when mice are injected with killed cells from a particular polyoma-induced tumor, the recipients are protected against a subsequent challenge with live cells from any polyoma-induced tumor. Additionally, in human Burkitts lymphoma, cells have been shown to express nuclear antigen of Epstein-Barr virus (Ricard, 2000)

II: Ionizing radiations: X-ray and other ionizing radiations are the first agents, which are associated with an increased risk of leukemia. This was apparent in the survivors of atomic bomb explosion in Hiroshima and Nagasaki, furthermore, an evidence of low dose irradiation induced leukemia has been presented (Henshaw *et al*., 1990). Moreover, patients who received radiotherapy for malignant disease may have a greater chance to develop leukemia (Granfield and Bunch, 1995).

III. Chemicals: Different types of chemical have been suspected as a cause of leukemia. For instance, benzene and petroleum derivatives, alkylating agents, paints, herbicides and pesticides have been associated with increased risks of leukemia (Freibert and Susan, 1998)

IV-Genetic factors: Family based evidences have suggested that identical twins have much more chance to develope the disease when compared with the general population (Behrman *et al*., 1998). In agreement with this scope, genetic studies of several leukemias have identified a number of genes that must be mutated in order to trigger the development of leukemia or maintain the growth of malignancy. In this regard, the term oncogenes has been introduced, and the products of such mutated DNA sequence play a role in the formation and growth of malignant tumors (Shovlin *et al*., 1999). Proto–oncogenes are defined as normal cellular genes that are involved in a normal cell differentiation, and have the potential to contribute to the induction of malignant tumors when their structure is altered due to:

1. Mutation in DNA that either induced or spontaneous (Martein *et al*., 1999).
2. Chromosome rearrangements like translocations or inversions (Croce and klein, 1984).
3. Gene amplification that involves oncogenes (Alitalo *et al*., 1983).

2-3-3: Classification

Leukemia is clinically classified into two categories; acute rapidly progressing disease (the history is usually brief and life expectancy, without treatment, is short) and chronic slowly progressing multi-staged disease (the patient may have been unwell for months, and survival is usually measured in years). Undifferentiated, proliferating and immature cells characterize acute leukemia, whereas chronic leukemia involves more differentiated cells. Chronic leukemia is subdivided into chronic lymphoid leukemia (CLL), which results form proliferation of non-functional lymphocytes, and chronic myeloid leukemia (CML), which is a malignant transformation of the myeloid stem cell. Neither terms refer to severity of the disease, but depend on the type of cell involved in the malignancy (Goldman and Tarig, 1999; McGuire and Kazakoff, 1999; Sarasins, 2003)

As in chronic leukemia, acute leukemia is also subdivided into lymphoid (ALL) and myeloid (AML) (Richmann, 2000). The former leukemia was investigated in the present study.

2-3-4: Acute Lymphoid Leukemia

Acute lymphoid leukemia (ALL) is a malignant transformotion of lymphoblasts, characterized by excessive accumulation of lymphoblasts and their progenitors in the blood. The disease is thought to arise from a clonal proliferation of a precursor cell that had undergone a genetic alternation leading to an unregulated growth and arrested differentiation (Richmann, 2000). The malignant cells in ALL are lymphoid precursor cells (lymphoblastic) that replace the normal marrow elements resulting in marked decrease of normal blood cells, therefore, anemia, thrombocytopenia and neutropenia are occurred with varying degrees (Seiter, 2001).

2-3-4-1: Incidence

The incidence of ALL varies, and depends on age, sex and race; however, it is more common in children (80% of ALL) than adults (20% of ALL), and boys have much more chance to develope the disease than girls. The survival outcome depends on race, and in this respect it has been demonstrated that Negro children have a lower survival rate than Caucasian children (Friebert and Susan, 1998). In Iraq, it has been recorded that the incidence of ALL is increased during the period 1991-1998, especially in the ages between 1-10 years (Mukheef, 1999).

2-3-4-2: Aetiology:

There is no specific factor which can be considered as an aetiological agent of ALL. Several observations may suggest the role of genetics, environment or an interaction between them. Genetically identical twins have shown much higher concordance rate to develop leukemia than dizygotic twins (Friebert and

Susan, 1998). Some environmental factors like maternal and paternal exposure to radiation have also been suggested (Uckumn *et al*., 1998). However, the most accepted theory is the interaction between environmental factors and genetic make-up that cause new DNA sequences or rearrangements that contribute to the development of malignant conditions such as ALL (Goldman and Tarig,1999).

2-4: Antineoplastic (Chemotherapeutic) Agents

Antineoplastic agents (cytotoxic drugs) are used in the treatment of malignant diseases when radiotherapy and surgery are not possible or they are not effective. The aim of cytotoxic therapy is to induce a remission (absence of any clinical conventional laboratory evidence of the disease) and to eliminate the hidden leukemia cell populations by courses of a consolidation therapy (Reynolds, 1989)**.**

The chemotherapeutic agents have been categorized by Laurance (1996) into three groups:

1. Cycle specific agents, which kill only cells that are actively cycling such as antimetabolite agents.
2. Phase-specific agents, which kill only cells that are at a particular phase of the active cycle.
3. Cycle-non specific agents, which kill cells wheather resting or actively cycling such as alkalyting agents.

In leukemia, the main treatment is chemotherapy, and one of these therapies is the drug cytosar, which is grouped under the antimetabolite agents. It is a pyrmidine antagonist, which is known with other names, for instance cytarabine and cytosine arabinoside (Nandy *et al*., 1999).

Cytosar is a cytotoxic agent to a wide variety of proliferating mammalian cells in culture. It inhibits cell cycling phases and specifically G1-phase to S-phase and it has been suggested that cytosar acts through the inhibition of DNA

polymerase. However, the balance of kinase and deaminase level may be important factors that determine the sensitivity and resistance of the cell to cytosar (Frei and Bodey, 1973).

Metabolically, cytosar is converted intracellulary to nucleoside triphosphate, which is an inhibitor of DNA polymerase and is also incorporated into DNA, where it terminates the strand elongation (Kufe *et al* ., 1984). Such activation is carried out by two intracellular enzymes; cytidine deaminase and deoxycytidylate deaminase. The activity of cytosar depends on the intracellular phosphorylation by the enzyme cytoplasmic deoxycytiidine kinase, which affects the formation of cytosar nucleoside triphosphate (Hande and Chabner, 1997; Beutler *et al.*, 2001).

Acquired cytosar resistance in experimental leukemias results from the loss of deoxycytidine kinase, which is the initial activating enzyme in the cytosar pathway (Owens *et al*., 1992). Other changes are also implicated in experimental tumors including decreased drug uptake, increased deamination and increased pool size of competitive deoxycytidine triphosphate (Hannun, 1997). Such drug-resistance studies have paved the way to seek alternative medicines as a treatment of leukemia, and the medicinal plants and their natural products are candidates in this regard, which have been the subject of an intensive research, moreover, most chemotherapies including cytosar are mutagens that have the potential to cause chromosomal aberrations and other structural changes in the DNA material (Umemoto *et al*., 2000; Mukherjee *et al*., 2001).

2-5: Investigated Parameters

Cytogenetic analysis have been employed widely to detect the effect of mutagens and carcinogens on the genetic make-up. This is carried out using *in vivo* and *in vitro* systems. Chick embryos were the first to be utilized *in vivo* analysis (Bloon and Hsu, 1975), but later mice became the animals of choice in such experiments due to their fast reproduction, small size and easy handling, moreover, they represent a typical mammalian system (Haung *et al*., 1990). *In*

vitro studies generally employ live cells of a mammalian origin, and in this regard the lymphocytes are the cells of choice in investigating the mutagenic effects of some agents (Nath *et al*., 1988).

2-5-1: Mitotic Index

Many studies that determine the activity of immune system and the effect of different agents on it depend on the ability of lymphocytes to proliferate in lymphoid organs and/or depend on dividing cells of bone marrow because it is the source of all blood cells (Hughes, 2001). Mitotic index assay is defined as the ratio of the numbers of cells in a population undergoing mitosis to the total numbers of cells (Gosh *et al*., 1991). Therefore, by the employment of this assay the effect of different physical and chemical agents on the mitotic response can be detected, and studies have revealed that the mitotic index can be affected negatively or positively by chemicals, radiations, drugs and medicinal plants (Ad'hiah *et al*., 2001a; Ad'hiah *et al*., 2002; Ad'hiah *et al*., 2004; Ad'hiah *et al*., 2006).

2-5-2: Micronucleus Formation

Micronuclei are cytoplasmic chromation masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from acentric chromosomal fragments (Tian *et al*., 2003).

Evans *et al*., (1959) employed the micronucleus assay to measure the cytogenetic damage induced in the dividing cells of root tips by fast neutrons and X-ray in the presence and absence of oxygen. It was found that all chromatid and chromosome breaks will give rise to acentric fragments at mitosis, and these fragments are excluded from the daughter nuclei and appear in follwoing interphase as micronuclei.

Schroder (1966, 1970) recommended the use of bone marrow smears to detect *in vivo* genetic damage induced by chemical mutagens and demonstrated

the occurrence of micronuclei in bone marrow cells in connection with cytogenetic damages.

von Ledebur and Schmid in 1973 reached the conclusion that the incidence of micronucleated polychromatic erthrocytes (PCE) was particularly useful index of an *in vivo* bone marrow cytogenetic damage, and such finding formed the basis to develop a simple *in vivo* assay based on an identification of micronuclei in PCE of mouse bone marrow. Since then, many researchers have employed this assay for the assessment of mutagenic effects induced by different mutagens (Martino-Roth *et al*., 2003; Ad'hiah *et al*., 2006).

2-5-3: Sperm-head Abnormality Assay

The entire process of sperm formation, from spermatogonia to spermatozoa is reffered to as spermatogenesis, which occurs in the seminiferous tubules of the testis (Seely *et al*., 1996). The stem cell (spermatogonia) is divided mitotically to give primary spermatocytes that undergo mieosis I to give rise to haploid secondary spermatocytes that undergo mieosis II to produce spermatids. The spermatids are then transformed to spermatozoa by a series of morphological changes (Hafez and Hafez, 2000). These events are under a genetic control, especially the morphological changes involved in sperm head formation. Therefore, an assay was developed to detect genotoxic effects induced by physical and chemical agents. The assay scores the percentage of sperms with abnormal head morphology. Such morphological changes are dependent on the stage of spermatogenesis. If the abnormality appeared at the end of first week post-treatment, it means that the genetic abnormality occurred in the stage spermatid formation, while if the abnormality appeared after 3-5 weeks post-treatment, it means that the abnormality occurred in the stages of spermatocytes or spermatogonia. Therefore, this test has been qualified to assess the mutagenic effects of chemical and physical mutagens *in vivo* (Topham, 1980; **Al-Rubaiey**, 2000).

2-5-4: Total and Differential Counts of Leucocytes

Leucocytes are considered as the active cells in carrying out the functions of the immune system, both non-specifically and specifically, and their count may give a general picture about the function of the immune system (Lydyard and Grossi, 1998).These cells are originated in the bone marrow through two cell lineages; myeloid progenitor and lymphoid progenitor, which give rise to the five types of leucocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Each type of these cells is commenced to carry out an immune function. The neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (Kramer, 2003). Due to these diverse immunological functions, the normal counts of leucocytes (total and differential) can be deviated by infections, radiations, environmental pollutants, drugs and products of medicinal plants (Ad'hiah *et al*., 2001b; Ad'hiah *et al*., 2002; Ad'hiah *et al*., 2004; Ad'hiah *et al*., 2006).

2-5-5: Phagocytosis

Phagocytosis represents the non-specific cellular immune response, which is carried out mainly by neutrophils and monocytes. These cells function to engulf and attack particles in the host that have been signaled for removal by various mechanisms (Asmis and Jelk, 2000). Both cells can migrate from the blood stream to the site of infections in response to some chemo-attractants (i.e. C3a and C5a), but the monocytes (also known as macrophages in tissues) can migrate back to the secondary lymphoid organs where they act as professional antigen presenting cells. Phagocytes are then motile cells and roam throughout the bloodstream, the lymphatic system and non-vascular tissue in search of

particles to engulf. When a non-self particle is encountered, it is taken into the phagocyte, combined with destructive compounds and destroyed (Hughes, 2001). This process, termed phagocytosis**,** involves several discrete steps**:**

1. Detection of the foreign particle and movement of the phagocyte to the area as a response to chemo-attractants.
2. Attachment of the foreign particle to the phagocyte. Such mechanism is enhanced by IgG and C3b (opsonins), because, the phagocyte expresses surface receptors for them.
3. Engulfment or ingestion of the foreign particle.
4. Fusion with lysosome and formation of the phagolysosome.
5. Intracellular killing and digestion, which are carried out through two chemical pathways; oxygen-dependent and oxygen-independent killing.
6. In the case of macrophages, epitopes of the engulfed object are presented to the lymphocytes in the secondary lymphoid organs.

Several investigations have suggested that phagocytosis can be modulated positively or negatively as a consequence of infections or treatments with drugs, chemicals, natural products and vitamins (Ad'hiah *et al*., 2002; Al-Keenani, 2005; Abdullah, 2005; Maiekan, 2006).

2-5-6: Arthus Reaction

Arthus reaction is a type III hypersensitivity reaction, which is mediated by immune complex formation in a second challenge with the same antigen. The immune complexes are formed between antibodies and the challenged antigen in the area of injection. Such formation leads to the activation of the classical pathway of complement system, which in turn leads to the generation of chemo-attractants factors (C3a and C5a) that enhance the migration of neutrophils to the area of injection. As a consequence, a local inflammatory response is generated with the manifestation of a local erythema and oedema after 3-4 hours of the

injection. Therefore, this assay can be successively employed to assess the humoral immune response, as well as, the role of some agents in modulating such response *in vivo* (Szalai *et al*., 2000; Cruse and Lewis, 2000).

2-5-7: Delayed Type Hypersensitivity Reaction (DTHR)

Delayed type hypersensitivity reaction represents the fourth type of hypersensitivity reaction (Type IV), which differs from Arthus reaction in the immunological constituents that participate in its generation. It is cell-mediated reaction, in which a specifiv T-helper lymphocyte, called T_{DTH}, plays a major role in its initiation together with macrophages. It occurs locally after 24-48 hours of a second challenge with the same antigen. Such time is required to activate the T_{DTH} by the antigen that is presented by macrophages, a process that requires the production of cytokines (IL-2, IFN-γ and tumour necrosis factor-β). These cytokines stimulate the migration of more macrophages to the area of injection, which in turn produce extracellular lysozymes that are responsible for the inflammatory reaction in the area of injection. Therefore, the DTHR is employed to assess the cellular immune response *in vivo* (Moore *et al*., 1999; Jacayan *et al*., 2001).

2-5-8: Plaque Forming Cells (PFC)

Jerne and Nordin developed the PFC assay in 1963 with the aim to detect antibody-producing lymphocytes *in vitro*. In this assay, mice are immunized with a large inoculate of sheep red blood cells (SRBC) to elicit a primary antibody response. Lymphocytes then become sensitized to the large number of antigenic determinants on the SRBCs, antibody production begins, and the titer of IgM antibodies rises over a period of about four days (direct method). A second challenge with SRBCs promotes the antibody-producing cells to produce IgG (indirect method). The immunized mice are then sacrificed, the spleen is removed, and a single-cell suspension of spleen cells is made. These cells are mixed with a suspension of SRBCs. The mixture is incorporated into agarose

support medium and poured as a thin layer in a Petri dish. After an incubation period at 37°C, sensitized lymphocytes secrete antibodies that diffuse through the agarose and react with the indicator cells (SRBCs). The formed immune complexes (antibody-SRBC) are able to activate the classical pathway of the added complement. A clear area of lysis surrounding the antibody producing cell is then produced and called a plaque. Therefore, this assay is a good parameter to estimate the antibody producing cells, and to test the effect of medicinal plants and their products on this mechanism (Myers, 1995; Cruse and Lewis, 2000; Rahim *et al*., 2003).

Supervisor Certification

We certify that this thesis was prepared under our 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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Committee Certification

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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List of abbreviations

S.officinalis	*Salvia officinalis*
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
WHO	World Health Organization
PCE	Polychromatic Erythrocytes
PFC	Plaque Forming Cells
SRBc	Sheep Red Blood Cells
DTHR	Delayed Type Hypersensitivity Reaction
S.libanotica	*Salvia libanotica*
INF-γ	Interferon- γ
PHA	Phytohaemoagglutinin
SHA	Sperm Head Abnormality
SOD	Superoxide Dismutase
GST	Glutathione Transferase
UV	Ultraviolet

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
C3a	Complement Component C3a
C3b	Complement Component C3b
C5a	Complement Component C5b
IgG	Immunoglobulin G
IL-12	Interleukin-12
SCE	Sister Chromatid Exchange
HEPES-BSS	HEPES-Buffered Balanced Salt Solution
TLC	Total Leucocytes Count
ppt	Precipitate
rpm	Revolution Per Minute
LD50	Lethal Dose 50

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Immunological and Cytogenetic Effects of Sage (*Salvia officinalis*) Leaves Extracts on Albino Male Mice and Acute Lymphoid Leukemic Cells

A Thesis
Submitted to the College of Science / AL-Nahrain University in Partial Fulfillment of the Requirements for the Degree of M.Sc. in Biotechnology

By

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B. Sc. Biotechnology. Al-Nahrain University (2003)

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جمهورية العراق

وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائيه

التأثير المناعي و الوراثي لمستخلصات أوراق نبات الميراميه (*Salvia officinalis*) في ذكور الفئران البيض و خلايا ابيضاض الدم اللمفي الحاد

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

رقيه محمد إبراهيم العزي

بكالوريوس تقانة إحيائية جامعة النهرين ٢٠٠٣

ذو القعدة **كانون الأول**

١٤٢٧ هـ **٢٠٠٦**

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