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

دراسة وراثية خلوية للسم المعوي الثابت حراريا (أ) المنتج من قبل بكتريا ايشيريشيا القولون السمية المعوية على مرضى سرطان القولون والمستقيم وتأثيره على الخلايا السرطانية

أطروحة

مقدمة الى كلية العلوم جامعة النهرين

كجزء من متطلبات نيل درجة الدكتوراه فلسفة

في التقانة الأحيائية

من قبل

مها فخري مجيد الطائي

بكالوريوس تقانة احيائية / جامعة النهرين/ ٢٠٠٠

ماجستير تقانة احيائية / جامعة النهرين/٣ ٢٠٠٢

محرم
١٤٢٨هـ

شباط
٢٠٠٧م

Dedication

To…

My lovely… … mother

My dearest… … father

My wonderful… … brothers and sister

Whom…

I still drink deep from the fountains of their

Love…

Warmth…

Comfort…

and Security…

Maha

Acknowledgement

At the beginning, thanks to great ***ALLAH*** *who gave me faith, health, energy, and strength to accomplish this work.*

I would like to express my sincere thanks and appreciation to my supervisors ***Dr. Abdul Wahid Al-Shaibany*** *and* ***Dr. Nawal Al-Khalidy*** *for their continuous support and valuable advice during the whole period of my study.*

A word of deep thanks, appreciation and gratitude to ***Dr. Mohammed Rafeeq****, for his distinctive handling with my thesis, his forbearance to reveal all the hidden points and for his specialist information in the tests and experiments.*

I owe my deep gratefulness to ***Dr. Suhair Al-Salihi****,* ***Dr. Samir Al-Jubory*** *and* ***Dr. Hazem Al-Ahmad,*** *for their great assistance and patience.*

With respects and regards to the ***Dean*** *of College* ***of*** *Science and to the* ***Chairman*** *and all* ***doctors and staff*** *in the* ***Biotechnology*** *department for their consent and support through my post graduate study.*

My great thanks to my colleagues ***Ranya****,* ***Zahraa****,* ***Maysaa****,* ***Ebtihal*** *and* ***Nada****.*

Finally, special thanks to all persons whom they could assisted me in any way and I can not remember them at this moment of written.

Maha

Supervisor Certification

We certify that this thesis was prepared under our supervision in Al-Nahrain University, College of Science as a partial requirements for the degree of Doctor of Philosophy in Biotechnology.

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Summary

This study was conducted for investigating the effect of heat stable enterotoxin a (STa) produced by enterotoxigenic *Escherichia coli* on the proliferation of colorectal cancer cells. A total of (25) stool samples were collected from patients suffering from diarrhea to isolate *E. coli* strains that produce STa, and after performing microscopic examination, cultural characterization and biochemical identification only (11) isolates showed positive *E. coli*. STa activity was estimated by using suckling mouse assay (SMA) and from these (11) isolates only (5) showed STa activity and the one with the highest STa activity was selected for large scale production of STa, which was followed by partial purification using ion-exchange chromatography, and after purification, the yield of toxin-protein was estimated as (1.08) mg/ml. The specific activity varied from (350) U/mg protein at the first step of purification to (2366.6) U/mg protein at the final step, while the final purification of the toxin was about (6.76) fold and with a yield of (18.25) %.

Partially purified STa was then undergoing three successive different experiments; and as follows:

***Experiment one*:**

Evaluating the cytogenetic effects of STa treatment by using five different doses (100, 200, 400, 800 and 1600µg/Kg) in comparison with negative (phosphate buffer saline / PBS) and positive (mitomycin C/ MMC, at doses of 2 and 5µg/Kg) controls on mouse bone marrow cells (*in vivo*) by employing the following parameters: mitotic index, chromosomal aberrations and micronucleus, also, the serum level of liver functional enzymes (GOT, GPT, ALP) after oral administration of STa for five successive days, was determined. In addition, lethal dose 50 (LD 50) with certain clinicopathological changes in five organs (colon, kidney, liver, stomach and lung) was also determined after

oral administration of STa for ten successive days and at two doses (500 and 1000 µg/Kg).

Results showed that, none of the five different doses of STa caused any significant changes in the three examined cytogenetic parameters in the mouse bone marrow cells; precisely, neither the low dose nor the high one of STa caused reduction or induction in these parameters. In fact, clear effect in decreasing mitotic activity and increasing spontaneous frequencies of both chromosomal aberrations and micronucleus was revealed after MMC treatment. Furthermore, significant differences in mouse serum level of the three enzymes were not seen at any doses of STa, while significant reduction in the levels of these enzymes was noticed after treatment with the two doses of MMC. In this study the LD 50 test was used to investigate the lethal effect of the partially purified STa, and it was shown to be not lethal to mice at both doses of (500 and 1000) µg/Kg, since death was not recorded, moreover, no clinicopathological effects were indicated in the all examined mouse tissues, however the only noticed clinical sign was diarrhea, which was observed after three days of STa treatment.

***Experiment two*:**

Evaluating the cytogenetic effects of STa treatment by using five different concentrations (100, 200, 400, 800 and 1600µg/ml) in comparison with negative (PBS) and positive (MMC, at concentration of 5µg/ml) controls on human blood lymphocytes (*in vitro*) obtained from both (10) normal healthy persons and (20) colorectal cancer patients by employing the following parameters: mitotic index, blast index, chromosomal aberrations and micronucleus.

On the human blood lymphocytes obtained from normal healthy persons, results showed that STa, and within all the different used concentrations, did not cause any significant cytogenetic changes in the all studied cytogenetic parameters. While on the human blood lymphocytes obtained from patients

with colorectal cancer, STa was shown to cause significant decrease in both mitotic and blast index, and especially at both concentrations of (800 and 1600µg/ml) and this decrease was concentration dependent, but at the same time non significant changes were seen in both chromosomal aberrations and micronucleus parameters and for the all used concentrations. However, reduced mitotic and blast index and induced chromosomal aberrations and micronucleus frequencies of human blood lymphocytes that were obtained from both normal healthy persons and colorectal cancer patients, were observed after treatment with MMC.

***Experiment three*:**

Evaluating the cytotoxic effect of STa treatment on primary cancer cell cultures, obtained from tumor samples that were collected from (13) cancer patients and as follows: (five colon cancer patients, two bladder cancer patients, two breast cancer patients, two stomach cancer patients and two lung cancer patients), and on normal cell line (rat embryonic fibroblast / REF) (*in vitro*) with the use of different concentrations starting from (1) mg/ml and ending with (0.0002) mg/ml by making two fold serial dilutions by using the 96- well microtiter plate, and in comparison with negative (PBS) and positive (MMC, at concentration of 10 µg/ml) controls .

Results showed that, after (24) hours of exposure to STa, the growth of all primary cancer cell cultures obtained from colon cancer patients was inhibited by STa treatment and this inhibition was concentration dependent. Also it was shown that the cytotoxic effect of the high concentration of STa was close to that seen after MMC treatment. While no differences were seen in the growth of all primary cancer cell cultures that were obtained from the other cancer patients, which mean that STa treatment neither inhibit nor enhanced their growth. At the same time STa did not show or has any cytotoxic effect on the normal cell line (REF).

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ALP	Alkaline Phosphatase
BI	Blast Index
CAs	Chromosomal Aberrations
D. W.	Distill Water
EMB	Eosin Methylene Blue
FCS	Fetal Calf Serum
GOT	Glutamate Oxaloacetate Transaminase
GPT	Glutamate Pyruvate Transaminase
hr	Hour
IP	Intraperitoneally
IV	Intravenously
KCl	Potassium Chloride
LD 50	Lethal Dose 50
LT	Heat Liable Toxin
MI	Mitotic Index
min	Minutes
MMC	Mitomycin-C
MR	Methyl Red
MTX	Methotrexate
NCI	National Cancer Institute
PBS	Phosphate Buffer Saline
PHA	Phytohaemagglutinine
SMA	Suckling Mouse Assay
STa	Heat Stable Enterotoxin a
TSI	Triple Sugar Iron
VR	Voges-Proskauer

Chapter one: Introduction and Literature Review

1.1 Introduction

Colorectal cancer is considered to be one of the main causes of death all around the world. Various methods and strategies have been used to treat such kind and others of cancer. The traditional methods include: radiation, chemotherapy and surgery (Crawford and Kumar, 2003). Recently new approaches have been suggested and developed; one of these using *Escherichia coli* toxin, for the treatment of colorectal cancer (Morgan, 2003; Web sites (1, 2)).

Pitari *et al.*, (2003) found that *E. coli* produces a toxin, which mimics a natural colon process and provoked diarrhea. However, the toxin also causes a flood of calcium into the affected cells, stopping colorectal cancer cells from replicating rapidly.

Three groups of *E. coli* are associated with diarrheal diseases. One of these groups is *E. coli* strains that produce enterotoxins, which is called enterotoxigenic *Escherichia coli* (ETEC) (Hales *et al.*, 1992).

Unfortunately there is unexplained inverse relationship between the incidence of colorectal cancer and ETEC infection. The toxin that produced by ETEC, which is heat stable enterotoxin a (STa), that cause one of the serious forms of food poisoning may be used in the treatment of one of the most deadly types of cancer (Ferlay *et al.*, 2001; Carrithers, 2003). The authors provided a convincing evidence for the presence of a novel intracellular signaling pathways initiated by STa that prevented the proliferation of colorectal cancer cells.

Chemically, STa binds to the guanylyl cyclase-C (GC-C) receptors specifically expressed in the intestinal cells. Ligand binding to GC-C activates

the intracellular synthesis of the second messenger cyclic guanosine monophosphate (cGMP) (Vaandrager, 2000). STa hyperactivates this signaling receptor causing large increases in the intracellular cGMP. In fact, GC-C and its ligands have been implicated in the regulation of the balance of proliferation and differentiation along the crypt-to-villus axis in the intestine. As a result, subsequent loss of the initiation of GC-C signaling may represent one key mutational event underlying neoplastic transformation in the colon (Qian *et al*., 2000; Pitari *et al*., 2001).

However, intestinal GC-C and its downstream intracellular effecter molecules are conserved in colorectal cancer. Thus the presence of STa / GC-C induced cGMP-dependent signaling pathway, through cyclic nucleotide-gated (CNG) channels and calcium was responsible for the anti-proliferation action of STa enterotoxin on human colon cancer cells because control using colon cancer cells devoid of GC-C were without effect (Waldman *et al*., 1998).

This study aims to open up a new approach in the development of anti-cancer drugs. An attempt to provide anti-cancer agent (STa) for the treatment of colorectal cancer with less or no cytotoxic effect on normal cells and with more cytotoxic effect on cancer cells. This approach could be reached by studying the following:

1. Studying the cytogenetic effects of STa on mouse bone marrow cells (*in vivo*), by using the following parameters: mitotic index, chromosomal aberrations and micronucleus. Also the study includes, determination of serum level of liver functional enzymes (GOT, GPT, ALP) and histopathological examination of some mouse tissues obtained from five organs (kidney, liver, colon, lung and stomach).

2. Studying the cytogenetic effects of STa in human blood lymphocytes culture (*in vitro*), by using the following parameters: mitotic index, blast index, chromosomal aberrations and micronucleus, that were obtained from both colorectal cancer patients and normal healthy persons.

3. Studying the cytotoxic effect of STa on normal cell line and primary cancer cell cultures that were obtained from different cancer patients.

1.2 Cancer

1.2.1 History of Cancer

Cancer has been recognized for more than 200 years, it was reported by Hippocrates and Galen. However, AL-Zahrawi was considered as the first physician who described cancer to look a little like crab because of "finger like projection" (Yaseen, 1990)

Cancer is a disorder of cell growth that leads to invasion and destruction of healthy tissue by abnormal cells. Although cancer has affected human since earliest time, it was a rare disease until the twentieth century. Cancer now ranks second only to heart disease as a major cause of death in the world (Sinclair *et al.*, 2000).

In Iraq, at the present time, cancer is considered as one of the most important causes of death especially after the Gulf war in 1991. For this reason, a large number of studies have been carried out on various forms of cancer with a view to understand the biology, diagnosis and treatment of this disease.

1.2.2 Characteristics of Cancer Cells

Cancer cells are shown to be undifferentiated cells (lack differentiation), while most normal cells are specialized; they have a specific form and function that suit them to the role they play in the body, in contrast cancer cells are non-specialized and have form and shape that are distinctly abnormal. They have uncontrolled growth pattern, in which they continue to divide and form an abnormal mass of cells called " tumor " (Devita *et al.*, 1993; Beketic *et al.*, 1995).

Cancer cells lack contact inhibition, and thus pile on top of one another and form disorganized multiple layers. They have abnormal nuclei (the nuclei

of cancer cells are enlarged and have abnormal number of chromosomes).

The chromosomes may be mutated, some parts may be duplicated and some may be deleted (Tailor and Parakarma, 1995; LaVecchia and Tavani, 1998).

1.2.3 Genetic Basis of Cancer

Malignancy occurs after a multi-steps process that disrupts the normal control of cellular growth and cause malignant transformation and unchecked proliferation in normal cells (Kelley and Johnson, 1994). Disrupted control can be caused by genetic alterations of growth-controlling genes, viral infections, increased stimulation by growth factors, or a combination of these factors (Weinberg, 1994; Moreno *et al*., 2001).

Biologists and geneticists have suspected that cancer may originate by an alteration in the genetic content of the somatic cells (Kundson, 1989). In addition, cancer cells frequently reveal abnormal cell division, suggesting that control of division must be abnormal. Thus it was concluded that cancer is a genetic disease (Harnder, 1984; Kundson, 1986; Bishop, 1987; Heim, *et al.*, 1988; Green, 1988).

Genetic alterations found in tumors occur in genes that can be grouped into two broad categories, tumor suppressor genes and proto-oncogenes (Kundson, 1986).

Tumor suppressor genes normally function to maintain the nonmalignant phenotype by limiting growth, invasiveness, and other properties found in malignant cells (Rhyu, 1995). Loss of this normal function, often caused by genetic alteration, leads to unregulated growth and /or tumor progression. Tumor suppresser gene mutations are usually recessive; that is, inactivation of both normal copies of the genes must occur before an alteration in the

phenotype of the cells occurs (Cavenee and White, 1995).

Evidence for the existence of tumor suppresser genes was initially suspected by loss of malignant characteristics in cellular hybrids formed between malignant and normal cells (Cavenee and White, 1995).

The second group of malignancy-associate genes is proto-oncogenes, were originally identified as the normal cellular homologues of the viral oncogenes of cutely transforming retroviruses. Proto-oncogenes have been implicated in oncogenesis because increase in their function lead to stimulation of growth or development of the malignant phenotype. Proto-oncogenes mutations are commonly dominant, requiring only one genetic alteration to result in an increase of abnormal function (Wallach, 1997).

1.2.4 Biological Basis of Cancer

Cancer cells behave as independent cells, growing without control to form tumor. Tumors grow in a series of steps. The first step is "hyperplasia", meaning that there are too many cells resulting from uncontrolled cell division. These cells appear normal, but changes have occurred that result in some loss of control of growth. The second step is "dysplasia" resulting from further growth, accompanied by abnormal changes in the cells. The third step requires additional changes, which result in cells that are even more abnormal and can now spread over a wider area of tissue. These cells begin to lose their original function; such cells are called "anaplastic". When the cells in the tumor metastasize, which means that they can invade surrounding tissue, including the bloodstream, and spread to other locations, this is the most serious type of tumor, but not all tumors progress to this point (Liotta, 1992; LaVecchia and Tavani, 1998).

The type of tumor that forms depends on the type of cells that was initially altered. There are five types of tumors: (Kathleen and Arthur, 1996; Yaseen, 1994)

- Carcinomas: result from altered epithelial cells, which cover the surface of human skin and internal organs. It is the most common type.
- Sarcomas: result from change in muscle, bone, fat or connective tissue.
- Leukemia: results from malignant white blood cells.
- Lymphoma: is the cancer of the lymphatic system cells that derive from bone marrow.
- Myelomas: are cancers of specialized white blood cells that make antibodies.

Although tumor cells are no longer dependent on the control mechanisms that govern normal cells, they still require nutrients and oxygen in order to grow (Franks and Teich, 1991). However, all living tissues are supplied with capillary vessels, which bring nutrients and oxygen to every cell. As tumor enlarges, the cells in the center no longer receive nutrients from normal blood vessels. To provide a blood supply for all the cells in the tumor, it must form new blood vessels to supply the cells in the center with nutrients and oxygen.

In a process called “angiogenesis”, tumor cells make growth factors, which induce the formation of new capillary blood vessels (Roger, 2000). Without the additional blood supplied by angiogenesis, tumor can grow no longer than about half a millimeter; also tumor cells can not spread, or metastasize to a new tissue (Sekido *et al.,* 1998).

1.2.5 Treatment of Cancer

Treatment of cancer is based on the removal and/or killing of the tumor cells while minimizing unwanted side effects on normal cells.

Surgery is often the first line of attack but it is becoming increasingly the practice to consider cancer as a systemic disease at the time of first detection and to give additional medical treatment such as chemotherapy and radiotherapy at the same time. Medical rather than surgical attack is used because of the inaccessibility or unknown site of metastatic spread (Cheresh, 1998).

The ideal treatment would be the one that remove all the cancer cells without affecting normal cells. Treatments are based on preventing growth; given that growth is such a general property of cells, methods designed to stop it will inevitably have side effects. Therefore, the objective of all current treatments is to maximize effects on the cancer whilst minimizing adverse side effects on normal tissue (Flanagan, 1998).

Drug treatment is the most widely used alternative therapy to surgery, the majority of drug treatments are designed to disrupt cell proliferation. The term "chemotherapy" is used to describe treatment based on drugs that have a broad cell specificity because they affect cell processes such as DNA synthesis and cell proliferation common to all cells. Compounds used for treatment are described as being cytotoxic or cytostatic depending, respectively, on whether they kill the cells or only stop their proliferation. Cytotoxic drugs have the potential to cure a patient whereas cytostatic drugs can prevent further growth but not always eliminate the cancer (Yu *et al*., 1997; Vivekan and Michael, 1997).

On the other hand, radiotherapy, or ionizing radiation is used in cancer treatment. X-rays causes irreversible DNA damage and are widely used in radiotherapy to kill cancer cells, often in conjugation with surgery.

However, ionizing radiation produces single- and double-strand breaks in the DNA, resulting in chromosome damage involving mainly deletion and rearrangement rather than the point mutations generated by chemical drugs. Another differences between the radiation and drugs, is that cells are most sensitive to radiation during the G2/M phases of the cell cycle whereas early S phase is the sensitive period for drugs. (Roger, 2000)

In addition to these three strategies, many attempts have been made for creating or finding new approaches to treat cancer with minimal or no side effects. One of these approaches includes using the heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli* (Morgan, 2003).

1.3 Colorectal Cancer

Colorectal cancer is a malignant neoplasm of epithelial cell origin affecting the large bowel. It is a common type of malignancies that affect gastrointestinal tract and it is of favorable prognosis provided that, it is diagnosed and treated in an early stage. Cancer detection at an early stage, and identifying susceptible individuals can result in reduced mortality from this prevalent disease (Cortan *et al.*, 1999).

1.3.1 Epidemiology of Colorectal Cancer

Colorectal cancer is the fourth commonest form of cancer occurring worldwide. The number of new cases of colorectal cancer has been increasing rapidly since 1975.

The peak incidence for colorectal cancer is 60 to 70 years of age, less than 20% of cases occur before the age of 50 years. Adenomas are the presumed precursor lesion; the frequency with which colorectal cancer arises from flat colonic mucosa remains undefined but appears to be low. Males are affected about 20% more than females (Crawford and Kumar, 2003).

Colorectal cancer has a worldwide distribution, with the highest incidence rates in the United States, Canada, Australia, New Zealand, Denmark, and other developed countries. It is incidence is substantially lower up to 30 fold-less in India, South America, and Africa (Carrithers, 2003)

In Iraq and according to the latest Iraqi Cancer Registry reports , the colonic malignancies represented about 4.7% of all primary malignant tumors registered during the period from 1995-1997 (2.2% in females and 2.5% in males). While rectal malignances represented about 3.4% (1.5% in females and 1.9% in males). The crude incidence rate in Iraq for colonic malignancies in 1997 was 1.30/100,000 of population, and that of colorectal malignancies was 0.83/100,000 of population.

In addition, the annual number of new cases of colorectal cancer in 1997 was 354 (193 in the colon and 161 in the rectum) (Iraqi Cancer Board, 1999).

1.3.2 Histological Typing of Colorectal Cancer

The World Health Organization (WHO) has classified colorectal carcinoma as follows: (Jass and Sobin, 1989)

- Adenocarcinoma; glandular epithelium.
- Mucinous adenocarcinoma; more than 50% extracellular mucin.
- Signet-ring-cell adenocarcinoma; more than 50% intracytoplasmic mucin.

- Squamous-cell carcinoma; exclusively squamous differentiation.
- Adenosquamous carcinoma; adenocarcinoma plus squamous-cell carcinoma
- Small-cell carcinoma (oat-cell carcinoma), similar to small-cell carcinoma of the lung (with neuroendocrine differentiation).
- Undifferentiated carcinoma; no glandular structure or other feature to indicate definite differentiation.

Colorectal adenocarcinoma and mucinous adenocarcinoma (sometimes still termed mucoid or colloid adenocarcinoma) account for 90-95% of carcinomas. All other types are uncommon (Hermanek, 1998).

1.4 Tumor Cell Lines

There has been a great deal of frustration among many investigators trying to establish cell lines of both human and animal tumor cells. The great difficulties in working, involving numerous unsuccessful attempts, many of which have remained unpublished, have lead to the conclusion that cell line establishment is controlled by the rare occurrence of a tumor with a built-in potential for long term *in vitro* growth and by the application of intriguing culture techniques. Therefore, in all fairness, many scientists attempt to establish continuous cell lines have failed (Freshney, 1986).

Fogh and Trempe (1975) stated that many factors must be considered in this respect, including the choice of materials , collection procedures, lapse of time between the clinical procedure and preparation for tissue culture, technical competence of assistants, and incidental factors known to everyone working in tissue culture. One thing, however, stands out as a conclusion; the careful

attention to all the minute details provided by the dedicated tissue culturist seems to be the most important of all these factors. Therefore, many investigators have encountered great difficulties in establishing cell lines from human and animal solid tumors and ascites fluids, in spite of numerous attempts (Perego *et al.,* 1994).

It is now apparent that some tumors can be cultured quite frequently, other types, however, having not yet yield cell lines (Wasserman *et al.,* 1992). Cultures derived from embryonic tissue will survive and grow better than those from adults which their initiation and propagation are more difficult, and their lifespan is often shorter. This presumably reflects the lower level of specialization and presence of replicating precursor or stem cells in the embryo (Simmons and Marmion, 1996; Young and Black, 2004; Zwaka and Thomson, 2005).

Normal tissue usually gives rise to cultures with a finite lifespan, while cultures from tumors can give continuous cell lines (Alley *et al.,* 1988; Young *et al.,* 2004).

Freshly isolated cultures are known as primary cultures until they are passaged or subcultured. They are usually hetero-geneous and have a low growth fraction, but are more representative of the cell type in the tissue from which they have been derived and in the expression of tissue specific properties (Freshney, 1986).

Primary cell cultures are quite short-lived , some cells frequently persist in culture for months , without dividing at all and then eventually die too. Still other cells begin to divide rapidly and continue to do so for some time, however, many of these cells also die after a period which varies from weeks to months (Nelson-Rees *et al.,* 1981; Dykes *et al.,* 1992).

If the cells multiply repeatedly for a long time they can often be "passaged", this is usually achieved by first obtaining the cells in suspension (trypsinization process) and then inoculating this into a new culture vessel along with a fresh medium. As soon as cells have been passaged in this way, the culture is designated as "primary cell line" (McLimans, 1979). Primary cell lines go on dividing at quite a high rate for a long time and can be passaged repeatedly. Even after quite a large number of passages some primary cell lines cease to proliferate and die out. However sometimes cell lines can be cultured for such a long time that they apparently have developed to be subcultured at least more than 50 passages at intervals of three days between each subculture , such cell lines are called "established cell lines" (Stanley and Parkinson , 1979).

Paul (1970), previously described the properties of established and continuous cell lines, which are these cell lines that behave in a remarkably similar manner whatever their origin , they have short doubling time , usually aneuploid or polyploid, also there is considerable variation in chromosomes number and constitution among cells in the population and cell line , have very similar nutritional requirements whatever their origin , grow to much higher densities than primary cells lines, often grow well from dilute inocula, loss of contract inhibition and most of them can be established in suspension culture (anchorage dependence), whereas it is exceptional for primary cell lines to grow in suspension

1.4.1 Applications of Tumor Cell Lines for Establishing the Effect of Secondary Metabolites on Tumors

To study the effect of any compound and to identify the mechanism through which that compound affect cancer cells, several requirements should

be available, and the most important one is tumor cell lines (Grafone *et al.*, 2003).

In 1990, The National Cancer Institute (NCS) in the United States put a new style to study the effect of different compounds on different tumor cell lines *in vitro*, through providing many tumor cell lines from different types of cancers, by using a simple method which makes it easy to deal with those lines and to give results which are so close to the nature (Boyed, 1998).

In general, some of these unknown compounds that are under study could have a cytotoxic or non-cytotoxic effect on cells, so several items have been employed to study the cytotoxic activity of the unknown compounds, which are: (Wilson, 2000)

- Discovering compounds which have an effective activity on cancer cells.
- Understanding the mode of action of these compounds on cancer cells.
- Predicting the anti-cancer activity for these compounds.
- Discovering the target cells for these compounds.
- Determining the optimal concentration for the active compound.
- Understanding the relation between the concentration and time of exposure of the cells to the active compound.

Recently, it has been shown that, the use of the cytotoxic assays became so basic and important for the discovering of a new active compounds for economic reasons and for reducing time that required when using laboratory animals. It has been discovered that, the cytotoxic activity for the unknown compounds on cancer cells could be either reversible or irreversible, and their effect could be immediate or extend for several weeks (Freshney, 2000)

The importance of tumor cell lines comes from realizing the biological

basis for cancer *in vitro*, in which these cells are available in a uniform shape which comes from a pure cell population that is free from any contamination (Tom *et al.*, 1976).

Testing of cytotoxic activity have many advantages; it gives us a statistical analysis that are simple and accurate , which makes no need for making duplicates , moreover, the relationship between the concentration and the time of exposure could be controlled more than *in vivo* , and with the possibility of controlling the physical , chemical and physiological environmental factors ,also it could be possible for doing a lot of steps through one experiment with simple requirements by using microtitration system. On the other hand, the limits for these cytotoxic assays involve; the difficulty for following up the pharmacokinetics of the compound *in vitro* as it *in vivo*, in which the metabolic activity *in vitro* depends on the target cell itself, while *in vivo* it depends on many cell types. The period of logarithmic phase of cancer cells *in vitro* is shorter than *in vivo* which in turn has a negative effect on the mechanism of action of the compounds that have been used against cancer. Also the permeability of cancer cell differs in glass than in *vivo* (Freshney, 2001).

1.5 Escherichia coli (E. coli)

Escherichia coli was first described by Theodore Escherich in 1885, after isolation from infant stool suffering from enteritis, and it was soon shown that this organism could also be isolated from the stool of healthy infants and adults (Edward and Ewing 1986).

The organism typically colonizes the infant gastrointestinal tract (GIT) within hours of life and, thereafter, *E. coli* and the host derive mutual benefit.

This organism is distributed in the environment as well as in the bowel of human and animals. It is also present in the water supplies as an indicator of a recent fecal contamination and the potential presence of enteric pathogens (Mackie and MacCartney 1996).

E. coli usually remains harmlessly confined to the intestinal lumen. However, in the debilitated or immunosuppressed host, or when gastrointestinal tract barriers are violated, even normal (non pathogenic) strains of *E. coli* can cause infection (Paton and Paton, 1998).

1.5.1 General Characterization of E. coli

Escherichia coli is a specie of the genus Escherichia within the family Enterobacteriaceae. It is a short, straight gram negative bacilli, usually motile with flagella, it occurs singly or in pairs in rapidly growing liquid cultures. It is often have capsule or microcapsule and a few strains produce profuse polysaccharide slime. All strains are non-spore forming bacilli that grow both aerobically and anaerobically on ordinary laboratory media (Prigent *et al.*, 2000; HaveLaar *et al.*, 2001). They are oxidase negative but catalase positive ; ferment glucose ; D-manitiol , and D-mannose ; do not ferment inositol ; give positive reaction in the methyl-red and indole tests but show negative reaction for the voges-proskaur , citrate , and phenylalanine deaminase tests ; generally urease negative ; do not produce H_2S in triple sugar iron agar (Macfaddin, 2000).

The optimal growth temperature is (36-37) C^0, though growth occurs over a fairly wide temperature rang (18-44) C^0. About 99% of strains recovered in clinical laboratory, ferment lactose and thus grow as a smooth, glossily, pink colonies on MacConkey agar, and strains that are capsulate, forming mucoid

colonies on solid media (Edward and Ewing, 1986).

1.5.2 Serotyping of E. coli

Serotyping of *E. coli* occupies a central place in the history of these pathogens. Prior to the identification of specific virulence factors in the diarrheagenic *E. coli*, serotypic analysis was the predominant means by which pathogenic strains were differentiated (Bettelheim and Thompson, 1987).

In 1933, Adaw showed that by serological typing strains of *E. coli* could be implicated in out breaks of pediatric diarrhea. In 1944, Kauffman proposed a scheme for the serologic classification of *E. coli* which still in modified form today (Nataro and Kaper, 1998). According to the modified Kauffman scheme, *E. coli* strains are serotyped on the basis of their O (cell-well lipopolysaccharide), H (flagellar protein), and K (capsular polysaccharide or envelope antigen) antigens. The O antigens are the heat-stable somatic antigens that are inactivated by heating at 100 or 121 C^0. The H antigens are the heat-liable protein antigens contained in the flagella of the bacteria. The K antigens are the heat liable somatic antigens of the bacteria (Bettelheim and Thompson, 1987; Frank and Belfort, 2003; Sharon *et al.*, 2005).

1.5.3 Common Themes in Diarrheagenic E. coli Virulence

Like most mucosal pathogens, *E. coli* is said to follow a requisite strategy of infection: colonization of mucosal site, invasion of host defense, multiplication, and host damage. The most highly conserved feature of diarrheagenic *E. coli* strains is there ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut (Gorbach *et al.,* 1998; Ogata *et al.,* 2004)

The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains, including non-pathogenic varieties. However, diarrheagenic *E. coli* strains have specific fimbrial antigen that enhance their intestinal colonizating ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Levine *et al*, 1984; Soto and Hultgren, 1999; Otto *et al.*, 2001).

Once colonization is established, the pathogenic strategies of the diarrheagenic *E. coli* exhibit remarkable variety, such as enterotoxin production, invasion, and intimate adherence with membrane signaling. The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence-related plasmids and chromosomal pathogenicity island (Goastra and Svennerholm, 1996; Qadri *et al.*, 2000).

As long as *E. coli*, as a common member of the normal flora of large intestine, does not acquire genetic elements encoding for virulence factors, they remain benign commensals. Strains that acquire bacteriophage or plasmid DNA encoding enterotoxins or invasive factors become virulent and can cause plain, water diarrhea or inflammatory dysentery (Nataro and Kaper, 1998).

Three major groups of *E. coli* are associated with diarrheal diseases; First, *E. coli* strains that produce enterotoxins which called (Enterotoxigenic *Escherichia coli* / ETEC), there are numerous types of these toxins some of them are cytotoxic, damaging the mucosal cells, whereas other are merely cytotonic, including only the secretion of water and electrolytes. The second group of *E. coli* strains has invasion factors and cause tissues destruction and inflammation resembling the effect of *Shigella*, called (Enteroinvassive *Escherichia coli* / EIEC). The third group called (Enteropathogenic *Escherichia coli* / EPEC) includes strains associated with outbreaks of diarrhea in newborn

nurseries but not producing recognizable toxins or invasion factors (Hales *et al.,* 1992).

1.6 Enterotoxigenic E. coli

Enterotoxigenic *E. coli* (ETEC), is an important cause of bacterial diarrheal illness. Infection with ETEC is the leading cause of traveler's diarrhea and a major cause of diarrheal disease in the undeveloping nations, especially among children (Rao *et al.,* 2003). ETEC is transmitted by food or water contaminated with animals or human feces. Although it causes a significant amount of illness worldwide, the infection will end on its own and is rarely life threatening (Sears *et al.,* 1995).

Strains of ETEC are characterized by the structural pili they produce. Whereas almost all *E. coli* strains produce common type mannose-sensitive pili, only ETEC produce specific type of mannose-resistant pili, these are important in facilitating colonization of the mucosal surface of the human intestine (Keshimaki, 2001).

ETEC strains produce two types of enterotoxins; heat liable-toxin (LT) and heat-stable toxin (ST). LT is a large oligomeric toxin that is closely related to cholera toxins. In contrast, ST is a small monomeric toxin that is closely related to *Shigella* toxins (Eisinghorst and Weitz, 1994).

1.6.1 Pathogenesis of ETEC

Diarrheal disease caused by ETEC is contracted orally by ingestion of food or water contaminated with pathogenic strain shed by an infected person. ETEC diarrhea occurs in all age groups, but mortality is most common in infants, particularly in the most undernourished or malnourished infants in developing countries (Olsvik *et al.,* 1991; Ohno *et al.,* 1997; Fegundes and

Scaletsk; 2000; Cohen *et al*., 2005).

The pathogenesis of ETEC diarrhea involves two steps: intestinal colonization, followed by elaboration of diarrheagenic enterotoxins (Firdausi *et al.*, 2005).

ST, can stimulate intestinal guanylyl cyclase, the enzyme that converts guanosine 5'-triphosphate (GTP) to cyclic guanosine 5'-monophosphate (cGMP). Increased intracellular (cGMP) inhibit intestinal fluid uptake resulting in net fluid secretion (Bakre and Visweswariah, 1997).

One method for testing suspect *E. coli* isolates for ST production involves injection of culture supernatant fluid into the stomach of infant mice and seeing whether diarrhea ensues (Giannella, 1976).

ETEC, also produce another type of enterotoxin, heat-liable toxin (LT). Its mechanism of action is similar to that of cholera toxin. It acts on the membrane bound adenylyl cyclase, the enzyme that converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). An excess production of (cAMP) lead in turn to hyper secretion of water and electrolytes into the bowel lumen (Dreyfus *et al.*, 1993).

LT production is demonstrated by serologic methods; testing for diarrheagenic activity in ligated rabbit intestine, and by testing for specific cAMP mediated morphological changes in Chinese hamster ovary cells (Guerrant *et al.*, 1974).

1.6.2 Heat-Stable Enterotoxin

The heat-stable enterotoxin (ST) is one of the two major types of enterotoxins produced by enterotoxigenic *Escherichia coli*, which causes diarrhea in neonatal animals, human infants, and travelers to undeveloped

countries (Lawrence *et al.*, 1983; Cohen *et al.,* 1993; Pitari *et al.*, 2003).

The heat-stable enterotoxins are a family of closely related peptides. They are classified into two structurally, functionally, and immunogenically unrelated types, namely STa and STb (Burgess *et al.*, 1978; Weikel and Guerrant, 1985; Clare *et al.*, 1999), which, respectively, are also known as STI and STII. STa is methanol-soluble and infant mice-active peptide toxin, while STb is methanol-insoluble and active in pigs, but inactive in infant mice. The toxic activity of STa is resistance to protease (Staples *et al.*, 1980; Dreyfus *et al.*, 1984), while that of STb is inactivated by treatment with trypsin (Whipp, 1987).

The biochemical, physiological and immunological properties and amino acid composition of STa enterotoxins characterized so for are remarkably similar (Yoshimura *et al.*, 1985; Yamanka *et al.,* 1997), they have a common, highly conserved region with ten amino-acids, including six cysteine residues, located in the same relative positions and linked intramolecularly by three disulphide bonds, which suggests that these enterotoxins have similar tertiary structures (Shimonishi *et al.,* 1987; Okamato *et al.,* 1995)

STa is translated as a precursor molecule of 72 amino acids and undergoes two cleavage events before the secretion of the mature form into the culture supernatant. Mature STa is a small peptide that ranges from 17 to 19 amino acids with a molecular weight ranging between (2000-4000) dl. The conserved region is essential for toxicity and the heat-stable nature of the toxin. However the gene for STa production is generally plasmid-mediated (Yamanka *et al.,* 1994; Giannella, 1995; Yamanka and Okamato, 1997).

1.6.2.1 Receptors for STa in Intestinal Tissue

The first step in the biological action of STa is its interaction with

specific high-affinity receptors. STa binds to a heterogeneous group of specific glycoprotein receptors on the brush borders of the intestinal epithelial cells (Rao *et al.,* 1980; Dreyfus and Robertson, 1984; Carrithers *et al.,* 1995), and this binding stimulates a membrane bound enzyme guanylyl cyclase, which in turn leads to an increase in the intracellular concentration of (cGMP), and this culminates in inhibition of Na^+ absorption and stimulation of Cl^- secretion (Jonge and Lohmann; 1985; Hirayama *et al.,* 1989; Kimberly *et al.,* 2000) .

Guanylyl cyclase is a family of enzymes that catalyze the conversion of GTP to GMP. The family comprises both membrane-bound and soluble isoforms. They are regulated by diverse extracellular agonists that include hormones, bacterial toxins, and free radicals (Behrends *et al*., 1995; Cagir *et al.,* 1999).

Stimulation of guanylyl cyclase and the resultant accumulation of cGMP regulate complex signaling cascades through immediate downstream effectors, including cGMP-dependent protein kinase, cGMP-regulated phosodiesterases, and cyclic nucleotidegated ion channels. Guanylyl cyclase and cGMP mediated signaling cascades play a control role in the regulation of diverse (patho) physiological processes including; vascular smooth muscle motility and intestinal fluid and electrolyte homeostasis (Aparicio and Applebury, 1996; Ames *et al.,* 1999).

The receptor to which STa bind is called "guanylyl cyclase-C" (GC-C), disruption of the gene encoding GC-C in mice resulted in resistance to STa-induced diarrhea, demonstrating that GC-C is absolutely required for STa-induced intestinal secretion. High affinity receptors for STa are localized in the brush border membranes of enterocytes from the duodenum to the rectum (Krause et *al.*, 1994).

1.6.2.2 Relation between STa and Colorectal Cancer

Colorectal cancer exhibits low incidence in undeveloped countries, even though it is the third most common neoplasm worldwide (Greenlee *et al.*, 2001; Hawk, et *al.,* 2002)

This geographical imbalance suggests an environmental contribution to the resistance of endemic populations to intestinal neoplasia. Although the epidemiology of colorectal cancer remains poorly understood, there is clearly an unexplained inverse relationship between the incidence of colorectal cancer and ETEC infections (Ferlay *et al.*, 2001). Drawing from these observations the authors introduced an interesting hypothesis that specific peptide (STa) elaborate from ETEC may prevent the hyperproliferative and neoplastic development of intestinal epithelial cells that are associated with the initiation and progression of colorectal cancer (Carrithers, 2003).

Although a direct causal relationship between STa-mediated infectious diarrhea and low cancer rates in the undeveloped countries has not been proven, the authors provide convincing evidence of the presence of a novel intracellular signaling pathway initiated by STa that prevent proliferation of colon cancer cells (Pitari *et al.,* 2003).

Mechanistically STa binds to guanylyl cyclase-C (GC-C) receptors, and this binding activate the enzyme guanylyl cyclase, which in turn activates the intracellular synthesis of a second messenger cGMP, thus STa hyperactivates this signaling, causing large increases of cGMP level (Vaandrager, 2002). However, GC-C and its signaling pathway have been evolutionarily conserved in a wide variety of animal species, suggesting that it must play a role of an important aspect in intestinal physiology. In this way, STa represent molecular mimicry where in enterotoxigenic bacteria have evolved a strategy for its

transmission that exploits normal intestinal physiology (Uzzau and Fasano, 2000).

Indeed, STa is structurally and functionally homologous to the endogenous peptides, guanylin and uroguanylin, which mediate autocrine / paracrine control of intestinal fluid and electrolyte homeostasis (Forte, 1999).

In fact, GC-C has been implicated in the regulation of the balance of proliferation and differentiation along the crypt-to-villus axis in the intestine (Qian *et al.,* 2000; Pitari *et al.,* 2001).

Interestingly, expression of guanylin and uroguanylin is lost during colon cancer tumorgenesis (Cohen *et al.,* 1998; Steinbrecher *et al.,* 2000). In support of these studies, targeted inactivation of the mouse guanylin gene results in increased colonic epithelial proliferation (Kris *et al.,* 2002).

It was demonstrated that oral administration of uroguanylin suppresses the formation and progression of adenomatous polyps in mouse animal model of colorectal cancer, in which the uroguanylin gene had been inactivated (Yang *et al.,* 1997; Shailubhai *et al.,* 2000).

In fact, Pitari and his coworkers demonstrated the presence of unrecognized STa/GC-C induced cGMP-dependent signaling pathway, through cyclic nucleotide-gated (CNG) channels and calcium, responsible for the anti-proliferation action of bacterial enterotoxin on human colon cancer cells (Qiu *et al.,* 2000).

However it was shown that STa-mediated inhibition of DNA synthesis and cellular proliferation in colon cancer cells was GC-C dependent because controls using colon cancer cells devoid of GC-C were without effect (Waldman *et al.,* 1998). The essential role of Ca^{+2} influx is underscored by chelating of free cytosolic (Ca^{+2}), which reversed the anti-proliferative action of

STa. Also, depletion of (Ca^{+2}) abolished the ability of STa to inhibit cancer cell proliferation, whereas increase in (Ca^{+2}) restored the anti-proliferative effect of STa (Hoult *et al.,* 1988; Levine, *et al.,* 1991; Brayden *et al.,* 1993; Bhattacharya, 1998).

In conclusion, the signaling cascade suggested by the authors is: ETEC→ STa → CG-C→ cGMP→ CNG channel → Ca^{+2} entry → tumor growth inhibition.

1.7 Cytogenetic Analysis

Cytogenetic assays have been used since the early 1960s. Cytogenetic analysis is widely employed indication system for the evaluation of physically, chemically and biologically induced mutations. It allows for the objective evaluation of genetic material damages and it is a method that permits direct image analysis for the chromosomal damage (Lloyed *et al.,* 1998; Osuji, 2003).

Cytogenetic analyses have been proved to be good and reliable for the mutagen-carcinogen exposure and chromosomal aberration (Nakanishi and Schneider, 1979: 1984 Gebhart, 1981; Greulich *et al.,* 2000; Lindholm and Edwards, 2004).

Chicken embryos were the first to be utilized in *in vivo* analysis (Bloom and Hsu, 1975) but later mice became the animal most frequently used due to their fast reproduction, small size, easy handling and represent a typical mammalian system (Tice *et al.,* 1989; Haung *et al.,* 1990). Rabbits have also been used, but less frequently (Stetka and Wolf, 1976; Garriott *et al.,* 1995).

Concerning *in vivo* and *in vitro* analysis, there are obvious advantages of each approach for the screening of compounds. *In vivo* analysis stimulates human *in vivo* exposure and can detect compounds which require metabolic

activation and utilize normal cell population. While in the *in vitro* analysis; the delivery of the compound will not be affected by the catabolic and excretory mechanisms operative in the intact organisms (Schneider and Lewis, 1982; Wu *et al.*, 2003).

1.7.1 Blast Index (BI)

To measure cellular response to mitogens in term of induction of proliferation, a specific chain of morphological and biological changes occur in the lymphocyte when activated by specific mitogen, such as phytohaemagglutinine (PHA) and concavalin-A (Con-A) that activate T-cells, pock weed mitogen (PWM) that activates both T- and B-lymphocytes and lipopolysaccharid (LPS) that activate only B-cells (Soren, 1973; Nowell, 1980).

Activation occurs through four phases that begin with resting or silent phase (G0), the first growth phase (G1), protein and nuclei synthesis phase, and finally the second growth phase (G2). The resting phase begins when the specific mitogen binds through a specific ligand with a receptor on the surface of lymphatic cell (lymphocyte), which leads to the activation of the enzyme that responsible for the activity of the cell such as cyclic guanidine monophosphate (CGMP) and whose percentage increase during the first minutes of activation and thus leads to the appearance of the morphological changes as result of the transformation of rest, small cells (Lymphocytes) to blast cells called (Lymphoblast), where there is an increase in the size of lymphatic cells , increase in the number of vacuoles, and the nuclei become more visible inside the cells as a result of the accumulation of the nucleus's proteins (Stites, 1994).

1.7.2 Mitotic Index (MI)

Proliferating cells go through a regular cycle of events, the mitotic cell cycle, in which genetic material is duplicated and divided equally between two daughters. This is brought by the duplication of each chromosome to form two closely adjacent sister chromatids, which separate from each other to become two daughter chromosomes. These along with the other chromosomes of each set are then packaged into two genetically identical daughter nuclei. The molecular mechanism underlying the cell cycle is highly conserved in all organisms (Eva, 2001).

Mitotic abnormalities often arise directly from defects of centrosome and/or mitotic spindles, which then induce prolonged mitotic arrest or delayed mitotic exit and trigger induction of apoptosis (Mollinedo and Gajabe, 2003). Recent reports have demonstrated that entry into mitosis in the presence of damaged DNA leads to inactivation of centrosomes, formation of aberrant spindles and blockage of chromosome segregation , which consequently delays mitosis progression and induces mitotic abnormalities (Hut *et al*., 2003; Takada *et al*., 2003). In addition, chemical or pharmacological inhibition of the DNA damage checkpoint at the G2 stage induces premature entry into mitosis and subsequent initiation of apoptosis (Sampath and Plunkett, 2001).

An additional evaluation of the potential of physical and chemical agents in producing effects on cells can be carried out by analyzing the proportion of mitotic cells and calculating a mitotic index. Depression of the mitotic index is usually a consequence of a reduced rate of cell proliferation (mitotic delay) (Galloway *et al*., 1994).

The researches had indicated that this assay was effected by the mutagenic and carcinogenic materials in which all of these materials can affect

MI either *in vivo* or *in vitro* (King *et al*., 1982; Shubber and AL-Alak, 1986; Ekanem and Osuji, 2006; Guleray and Lokman, 2005).

1.7.3 Chromosomal Aberrations (CAs)

The increasing variety of chemicals, radiations and other physical agents we are exposed to now a day has stimulated the development of many rapid reliable assays for the detection of the mutagenicity or carcinogenicity of such agents. One of these methods is the chromosome aberration assay (Lambert *et al.,* 1978; Ardito *et al.,* 1980; Catherine *et al.,* 1998; Natarajan, 2002; Prakash *et al.,* 2005).

During the course of meiosis, portions of chromosome are often relocated, moving within the chromosome itself or between different chromosomes. This process produces changes in the morphology of the chromosome itself, which are referred to as chromosomal aberrations (CAs) (Savage, 1979).

Chromosomes are the structures that hold our genes. Genes are the individual instructions that tell our bodies how to develop and keep our bodies running healthy. In every cell of our body there are 20,000 to 25,000 genes that are located on the 46 chromosomes (Becher *et al.,* 1984; Holland, 2005).

CAs test serves to detect structural chromosomal aberrations, as may be induced via DNA breaks by various types of mutagens. Such DNA breaks may be either rejoin, such that the chromosome is restored to its original state, rejoin incorrectly or do not rejoin at all. These last two cases may be observable on microscopic preparations of metaphase cells (Moutschen, 1985; Savage, 1998; Hande *et al.,* 2003).

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

1976). The chromosomal studies are rapid, sensitive, reproducible, and yield quantitative measurements of breaks and gaps in mitotic chromosomes resulting from mutagenesis in G2 cells. The principle used consists of scoring microscopically identifiable breaks and gaps in mitotic chromosomes (Becher *et al.,* 1984; Prise *et al.,* 2001).

Numerical and structural aberrations are important in occurrence of both congenital abnormalities and cancer (Preston, 1987; Koshurnikova *et al.,* 2000; Gilbert *et al.,* 2000; IAEA 2001)

Therefore everyone should have 46 chromosomes in somatic cells of his body. If a chromosome or piece of a chromosome is missing or duplicated, there is missing or extra gene respectively. When a person has missing or extra informations (genes) problems can develop for that individual's health and development (Ishil and Watatani, 1983).

The *in vitro* chromosome aberrations were used to identify agents that cause structural chromosome aberrations in cultured mammalian cells (Galloway *et al.*, 1994). Structural aberrations may be of two types, chromosome or chromatid. Structural aberrations of chromosomes are common in nature and have apparently played a significant role in evolution. They occur spontaneously, but the frequency is increased by ionizing radiation. While chromatid structural aberrations are induce by chemical mutagens (Evans and Orirdan, 1977; Sachs *et al.,* 1997; Natarajan, 2005).

Most chemical mutagens and non-ionizing mutagenic radiations are unable to cause double strand breaks in DNA and act mainly in the single-strand DNA synthesis phase of the cell cycle, such agents produce mainly chromatid-type aberrations (OECD, 1997).

Cytogenetic monitoring of hospital workers exposed to low doses of ionizing radiation has been carried out by means of analyzing the frequencies of chromosomal aberration or centromere positive micronuclei (Hagelstrom *et al.*, 1995), comparing the results with those of a control group, a biologically significant result was obtained. Hande *et al.,* (2003), found a greater frequency of acentric chromosomes in workers occupationally exposed to radiation for radiological diagnosis, when compared with control individuals.

So according to Kasuba *et al.*, (1995), the evaluation of structural chromosomal aberrations of the general population is necessary for the interpretation and recognition of the pattern of responses in a population which is occupationally exposed to physical and chemical mutagens.

Techniques to prepare and stain chromosome have improved gradually in the last decades, parallel with increased ability to identify and quantify chromosome aberrations, thus leading to a better understanding of their origin (Obe and Natarajan, 2004).

The types of chromosomal aberrations that had been observed are: (Tamarin, 1996; Obe and Natarajan, 2004)

- Chromatid-type aberration: is a structural chromatid damage expressed as breakage of single chromatid and reunion between chromatids.
- Chromosome-type aberration: is a structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.
- Gap: is a chromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.
- Deletions: loss of segment, chromosome deletion occurs when apart of a chromosome (s) has been deleted. A deletion can occur on any

chromosome, and can be of any size (large or small).

- Ring chromosome: could happen in two ways:

  First the ends of the p and q arm break off and then stick to each other resulting in loss of informations. Second the ends of the p and q arm stick together (fusion) usually without loss of material.

- Acentric: fragment when a centromere is lost, an acrocentric chromosome is created which is usually lost during meiosis.

1.7.4 Micronucleus (MN)

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

As an alternative to classical metaphase analysis, the frequency of the occurrence of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosomal loss that lead to numerical chromosome anomalies (Roberts and Sturrock, 1973; Miller *et al.,* 1997; Miller *et al.,* 1998).

The first serious attempt to use micronuclei as a monitor of cytogenetic damage appears to be that reported by Evans *et al.,* (1959); they used the micronuclei frequency to measure the cytogenetic damage induced in root-tips by fast neutrons and X-rays in the presence and absence of oxygen. It was found that all chromosome and chromatid breaks, as well as asymmetrical and incomplete symmetrical exchange, will give rise to acentric fragments at mitosis, and that these fragments are frequently excluded from the daughter nuclei and appear in the following interphase as micronuclei.

Subsequently, Schroder (1966, 1970) recommended the use of bone

marrow smear to detect *in vivo* damage from chemical mutagens and demonstrated the occurrence of micronuclei in bone-marrow cells in connection with cytogenetic damage.

Beginning about 1970, Schmid and coworkers and Heddle initiated studies to determine which parameters might serve as the most useful indicators of cytogenetic damage in bone marrow *in vivo* (Schmid and Staiger, 1969; Boller and Schmid, 1970; Mather and Schmid, 1971; Matter *et al.,* 1973; Heddle, 1973).

This work led to the conclusion that the incidence of micronucleated polychromatic erythrocytes (PCEs) was a particularly useful index of *in vivo* bone –marrow cytogenetic damage. Others (Garriott *et al.,* 1995; Von *et al.,* 2000; Varga *et al.,* 2005) had also begun to explore the use of micronuclei in cytogenetic studies of mutagens, but the work of Schmid and coworkers is of particular historical importance because it led directly to the development of a simple *in vivo* test based on the identification of micronuclei in PCEs of mouse bone marrow. This assay is now in such wide spread use that is commonly referred to as "micronucleus test" (Schmid, 1976).

The increasing use of micronucleus assay undoubtedly stems mainly from the primary advantages of speed and simplicity. Heddle (1973) pointed out that scoring for micronuclei was considerably more than 10-folds faster than metaphase scoring at a similar power of test.

1.8 Chemotherapy drug (Mitomycin C)

Mitomycin C (MMC) is a mutagenic anticancer drug that is given as a treatment for several different types of cancer including; gastric, colorectal, lung, liver, uterine, breast, ovary and bladder cancer (Sartorelli *et al*., 1994;

Toshihiro, 2005). Mitomycin C is an antibiotic isolated from the fermentation of *Streptomyces capspitosus* that interrupts DNA replication and inhibit mitosis (Costes *et al*., 1993).

MMC is a very old established chemotherapy drug , it exert its action by combining with DNA in cancer cells and inhibiting DNA replications through the formation of cross-linkage to double stranded DNA , and thus the cell can not reproduce it self. It has been evidenced that cells in the later G1 phase of cell cycle through the early S phase of DNA synthesis are highly sensitive to mitomycin C (Finger *et al.,* 1998; Undeger *et al.,* 2004).

However, MMC has many side effects, the most common are:

- Lowered resistance to infection
- Bleeding
- Anemia
- Loss of appetite
- Tiredness and general feeling of weakness

While the less common side effects are:

- Nausea
- Changes in the lung tissue
- Hair loss
- Skin changes
- Changes in nails
- Kidneys may be affected (Cautrell *et al.,* 1985).

1.9 Enzymatic Assays (Liver functional enzymes)

Enzymes are proteins that carry out most of the catalysis in the living organisms; they catalyze all the biochemical reactions by forming enzyme-

substrate complex. Without assistance of enzymes most of the chemical reactions of metabolism would barely proceed at all (Philip and Donald, 1987).

Enzymatic assays are essential for estimating the amount of enzyme present in a cell or tissue after injury to help in the evaluation of the person's condition, whether he / she at a risk or not (Kraut, 1988).

However many reports are concentrated on studying the toxic effect of different drugs and chemical compounds on the liver, since the liver is the major organ in the body in which the metabolism and detoxification of different compounds occur, by evaluating the level of different liver enzymes especially when these compounds have different medical uses (Mathur and Dive, 1981; Al-Amiry, 1999).

Also, the elimination of the toxic effects of these drugs and chemical compounds by another different types of substances has been studied through the evaluation of liver enzymes level (Shamman *et al*., 1999; Hassan, 2002).

1.9.1 Glutamate Oxaloacetate Transaminase (GOT)

The enzyme GOT, also known as aspartate aminotransaminase, is widely distributed in the plants and animals, but it occurs in concentrated form in mammalian heart and liver (Tietz *et al*., 1986).

GOT catalyzes the following reaction:

$$\textbf{L-Asp + } \alpha\textbf{- Oxoglutarate} \xrightarrow{GOT} \textbf{Oxaloacetate + L-Glu}$$

GOT exists in two isoenzyme forms, the mitochondrial form (M-GOT) and the cytosol form (S-GOT). Serum GOT levels in healthy subjects are low, but the levels are significantly elevated in a number of clinical conditions such as acute and chronic hepatitis, obstructive jaundice, carcinoma of the liver and myocardial infraction. Therefore, determination of serum GOT level has great

clinical and diagnostic significance (Bergmeyer, 1974).

1.9.2 Glutamate Pyruvate Transaminase (GPT)

The enzyme GPT, also known as alanine aminotransaminase, is found in higher concentration in liver and in lower concentration in serum. The percentage of this enzyme differs from one person to another and from one tissue to another one (Wong *et al*., 2000).

GPT catalyzes the following reaction:

$$\textbf{L-Ala+ α- Oxoglutarate} \xrightarrow{GPT} \textbf{Pyruvate + L-Glu}$$

GPT is contained only in the cytosol of cells (S-GPT), serum values more than 15-fold above the upper normal limit always indicate an acute hepatocellular damage of viral, toxic or circulatory origin. In most types of liver diseases, GPT activity is higher than that of GOT, and GPT is more sensitive and specific in the detection of liver diseases (Pratt and Kaplan, 2001).

1.9.3 Alkaline Phosphatase (ALP)

Alkaline phosphatases are a group of enzymes found primarily in the liver and bone. There are also small amounts produced by cells lining the intestine, the placenta, and the kidney (Sonoko *et al*., 2003; Malo *et al*., 2006).

GPT catalyzes the following reaction:

$$\textbf{Phanel phosphate} \xrightarrow{ALP} \textbf{Phenol + Phosphate}$$

The primary importance of measuring ALP is to check the possibility of bone diseases or liver diseases (Moss *et al*., 1989). When the liver, bile ducts or gallbladder system is not functioning properly or is blocked, this enzyme is not excreted through the bile and ALP is released into the blood stream. In addition

to that, an elevated serum ALP can be also due to bone cancers or rapid growth of bone, since it is produced by bone forming cells called "osteoblasts", and this explains why growing adolescents have much higher ALP level than a full grown adult because his/her osteoblasts are laying down bone very rapidly (Jennifer *et al*., 2005; Web site (3)).

Chapter Two: Materials and Methods

2.1 Materials:

2.1.1 Equipments and Apparatus:

The following equipments and apparatus were used throughout the study:

Equipments and Apparatus	**Company (Origin)**
Autoclave, Centrifuge Water-path, Electric oven Incubator	Gallenkamp (England)
Micropipettes	Gelson (France)
Compound light microscope	Olympus (Japan)
pH-Meter (Digital)	Orien Research (USA)
Laminar airflow	Metalab (France)
Shaking incubator	Merck (Germany)
Hot plate with magnetic stirrer	Retsch (Germany)
Microtiter plate	Lab-Tek (USA)
Electric balance	Metter (Sweden)
Spectrophotometer	Cecil (France)
Millipore filters (0.22µm, 0.45µm)	Difco (USA)

2.1.2 Biological and Chemical Materials:

Material	Company (Origin)
Casamino acids, Neutral red, Ammonium sulphate, Methyl red, Safranine, Trypan blue, Iodine, Agar, Sodium bicarbonate, Trypsin $CaCl_2$, $FeCl_2$, $MgCl_2$	BDH (England)
DEAE-Sephadex A50, Methanol, Ethanol, KCl, Yeast extract, Crystal violet, Glacial acetic acid	Pharmacia (Sweden)
Fetal calf serum, Mycostatine, Human serum, Bovine serum albumin (BSA), Versene, Na_2HPO_2, KH_2HPO_2	Sigma (USA)
Coomassie brilliant blue G250	LKB (Sweden)
Giemsa	Fisher (USA)
Colchicines	Houde (France) Ibn-Hayan (Syria)
Phytohaemagglutinine (PHA)	Sigma (USA)
Mitomycin C	Kyowa (Japan)

2.1.3 Media:

Media	Company (Origin)
Brain hart infusion broth, Kliger's iron agar, Eosin methylene blue agar, MacConkey agar, Nutrient broth	BDH (England)
Simmon citrate agar	Mast Diagnostic (UK)
RPMI-1640 with Hepes	Sigma (USA)
Urea agar base, Tryptone broth, Peptone broth, MR-VP broth	Biolife (Italy)

2.1.4 Kits:

Kits	Company (Origin)
Alkaline phosphatase (ALP)	Bio-Merieux (France)
Glutamic oxalocetic transaminase (GOT), Glutamic pyruvic transaminase (GPT)	Randox (England)

2.2 Methods:

2.2.1 Preparation of Buffers, Solutions and Stains

2.2.1.1 Solutions for Isolation and Identification of *E. coli*, and for Detection of STa Activity:

a. Peptone broth: (Mackie and MacCartney, 1996)

This solution was prepared by dissolving 5gm of peptone in 100ml of D.W., autoclaved at 121Cº for 15min. Then dispensed into 5ml aliquots and stored at 4Cº until use. It was used for the collection of stool samples.

b. Kovac's reagent: (Atlas *et al*., 1995)

This was prepared by dissolving 0.1gm of lactic acid in D.W. and the volume was completed to 250ml, then mixed thoroughly and kept in the refrigerator. This reagent was used as indicator in indole test.

c. Methyl red reagent: (Macfaddin, 2000**)**

It was prepared by dissolving 0.1gm of methyl red in 300ml of 96% ethanol, then 200 ml of D.W. was added. This reagent was used as indicator in methyl red test.

d. Voges-Proskauer reagent: (Macfaddin, 2000)

This regent was prepared as follow:

Reagent A: 5% α- naphthol in 96% ethanol.

Reagent B: 40% KOH in D.W.

These reagents were used as indicators in Voges-Proskauer test

e. Gram stain:

This stain was prepared according to Mackie and MacCartney (1996).

f. Evan's blue stain:

It was prepared by dissolving 0.2gm in 100 ml D.W., then dispensed into aliquots and store at 4Cº until use. It was used for detection of STa activity. (Giannella, 1976)

2.2.1.2 Solutions for Determination of Protein Concentration*:* (Bradford, 1976)

a. Coomassie brilliant blue G-250 stain:

It was prepared by dissolving 0.1gm of Coomassie brilliant blue G-250 in 50ml of 95% ethanol, then 100ml of 85% phosphoric acid was added with agitation and the volume was completed to one liter with D.W., then it was filtered through Wattman filter paper (No.1) and kept in a dark container.

b. Tris-HCl buffer (pH 7.5):

It was prepared by dissolving 0.3gm of Tris-HCl in D.W., then the volume was completed to 100ml with D.W. and the pH was adjusted to 7.5.

c. Bovine serum albumin (BSA):

It was prepared by dissolving 0.1gm of BSA in Tris-HCl buffer, then the volume was completed to 100ml with the same buffer.

2.2.1.3 Solutions for Partial Purification of STa: (Takeda *et al*., 1979)

a. Phosphate buffer (0.01M, pH 7.0):

It was prepared by dissolving 0.19gm of NaH_2PO_4 and 0.3gm of Na_2HPO_4 in one liter of D.W., then the pH was adjusted to 7.0.

b. Elution buffer:

It was prepared from the phosphate buffer (0.01M, pH 7.0) that contains different concentrations of NaCl ranging between (0.1- 1) M.

2.2.1.4 Solutions for Cytogenetic Study:

They were prepared according to the methods used by Yaseen (1990) and Yaseen and associates (1998).

a. Potassium chloride (KCl): (Hypotonic solution)

Two solutions (0.075 and 0.1) M of KCl were prepared by dissolving 5.587gm and 7.42gm, respectively, of potassium chloride in one liter of D.W., and the pH was adjusted to 7.2.

b. Phosphate buffer saline (PBS):

This solution was prepared by dissolving the following salts in one liter of D.W. and the pH was adjusted to 7.2, these salts are: -

(NaCl)	8 gm
(KCl)	0.2 gm
(NaH_2PO_4)	1.15 gm
(KH_2PO_4)	0.2 gm

This solution was sterilized by autoclaving at 121Cº for 15 min and stored at 4Cº until use.

c. Colchicine: Two colchicine products were used in this study

- Colchicine (Ibn-Hayan / Syria):

This solution was prepared by dissolving one tablet (0.5mg) of colchicine in 0.5ml PBS. It was used for mice injection, where each mouse was injected intraperitoneally (I.P) with 0.25ml of this solution.

- Colchicine (Houde / France):

This solution was prepared by dissolving one tablet (0.5mg) of colchicines in 10ml of D.W. to make a stock solution. This solution was stored at ˉ20Cº until use for human blood lymphocyte culture.

d. Fixative solution:

This solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid.

e. Giemsa stain:

Giemsa stock solution was prepared by dissolving 1gm of Giemsa powder in 33ml of glycerin and put it in water-bath at 60Cº for 2hr with

continuous shaking then left it for 30min at room temperature. After that 66ml of absolute methanol was added with continuous shaking by the aid of the magnetic stirrer. The stain solution was then filtered through Wattman filter paper (No.1) and stored at room temperature in dark bottles. This is considered as a stock solution.

For slide staining, Giemsa solution was prepared as follow:

Giemsa stain stock	1	ml
Absolute methanol	1.25	ml
Sodium bicarbonate solution	0.5	ml
D.W	40	ml

f. Sodium bicarbonate solution ($NaHCO_3$):

It was prepared by dissolving 4.4gm of ($NaHCO_3$) in 100ml of sterile D.W. This solution was kept at 4C^o until use.

g. Fetal calf serum (FCS):

Prepared by placing it in a water-bath at 56C^o for 30min to destroy the complement system. Then dispensed in 20 ml aliquots and stored at ˉ20C^o until use.

h. Antibiotic solution:

This was prepared by dissolving 10^6 unit of penicillin G and 1gm of streptomycin in 100ml sterile D.W. After distributed into 5ml aliquots, it was stored at ˉ20ºC. Then 1 ml of this stock solution was added to 100ml of the cell culture medium to give a final concentration of 10^4 unit of penicillin G and 10mg of streptomycin.

i. Mitomycin C solution:

It was prepared by dissolving one ampoule with 10mg mitomycin C in 10ml D.W. to obtain 1mg/ml stock solution.

2.2.1.5 Solutions for Detecting the Cytotoxic Effect of STa on Cell Cultures: (Freshney, 2000)

a. Antibiotic solution: as in item (2.2.1.4.h).

b. Mitomycin C solution: as in item (2.2.1.4.i).

c. Sodium Bicarbonate solution: as in item (2.2.1.4.f).

d. Fetal calf serum: as in item (2.2.1.4.g)

f. Phosphate buffer saline (PBS): as in item (2.2.1.4.b)

g. Neutral red stain:

It was prepared by dissolving 0.01gm of the stain in 100ml PBS, shaked well until it completely dissolved.

h. Neutral red extraction solution:

It was prepared by mixing equal volumes of PBS and absolute ethanol (1:1).

i. Hank's balanced salt solution (HBSS):

This solution was prepared as follows:

- **Stock solution A**

Ingredients 1:

NaCl	16	gm
KCl	8	gm
$MgSO_4.7H_2O$	2	gm
$MgCl_2.6H_2O$	2	gm
D.W.	800	ml

Ingredients 2:

$CaCl_2$	2.8	gm
D.W.	100	ml

These two sets of ingredients were mixed gently, then the volume was completed to one liter with D.W.

- **Stock solution B:**

Ingredients:

Na_2HPO_4	1.2 gm
KH_2PO_4	1.2 gm
Glucose	20 gm
D.W.	800 ml

After the ingredients were dissolved, 100ml of 0.4% phenol was added, and the volume was made up to one liter with D.W.

Complete HBSS:

Equal portions (100ml) of each stock solution (A and B) were added to 800ml of D.W., then sterilized by autoclaving at 121Cº for 15min and stored at 4Cº until use.

g. Trypan blue stain:

It was prepared by dissolving 1gm of the stain in 100ml of HBSS. Then filtered through Wattman filter paper (No.1) and it was kept at 4Cº until use, as a stock solution.

For working, the stock solution was diluted with HBSS at a ratio of (1ml :10ml).

h. Trypsin stock solution:

Trypsin solution was prepared by dissolving 1gm of trypsin powder in 100ml HBSS and sterilized by filtration, dispensed in 10ml aliquots and stored at ˉ20ºC.

i. EDTA (versene) solution:

This solution was prepared by dissolving 0.2gm of EDTA (diamine ethylene tetra acetic acid) in 400ml PBS and sterilized by autoclave. Then it was dispensed in 10ml aliquots and stored at ˉ20ºC.

j. Trypsin-Versene solution:

Equal volumes of trypsin solution and versene solution were mixed thoroughly and used.

2.2.2 Preparation of Culture and Diagnostic Media:

2.2.2.1 Ready-Prepared Media

Media used in this study (Listed in item 2.1.3) were prepared in accordance with the manufacture's instructions fixed on their containers. All the above media were sterilized in the autoclave at 121C^{o} for 15min, except the (RPMI-1640) culture medium, which has been sterilized by filtration through millipore filter (0.22µm).

2.2.2.2 Laboratory-Prepared Media

a. Casamino acids-Yeast extract salts (CA-YE) medium:

It was used for the production of STa, and it was prepared according to the method that was described by (John *et al*., 1977).

Casamino acids	20 gm
Yeast extract	1.5 gm
NaCl	2.5 gm
K_2HPO_4	8.71 gm
Trace salts solution	1 ml

All these ingredients were dissolved in D.W. and the pH was adjusted to 8.5 with NaOH and brought to a final volume of one liter with D.W.

Trace salts solution: it was prepared from the following ingredients

$MgSO_4$	0.5 gm
$MnCl_2$	0.05 gm
$FeCl_2$	0.05 gm

D.W.	10	ml

Few drops of concentrated H_2SO_4 were added to make the dissolution of these salts easier.

b. Preparation of cell culture medium for cytogenetic study:

This medium was prepared according to (Nara and McCulloch, 1985).

RPMI-1640	10.4	gm
Penicillin G	10^4	U/ml
Streptomycin	0.01	gm/ml
L-glutamine	0.5	gm
Sodium bicarbonate	1%	
FCS	10%	

All the ingredients were completely dissolved in one liter of sterile D.W., the pH was adjusted to 7.2 and the medium was sterilized by filtration through millipore filter (0.22µm) under aseptic conditions. After that 4ml of the medium was transferred into sterile tubes and kept at $^{-}20C^{o}$ until use.

c. Preparation of cell culture medium for transporting and washing of tumor tissue samples:

The preparation of this medium was performed following to the method taken from (Freshney, 2000)**.**

RPMI-1640	10.4	gm
Penicillin G	10^6	U/ml
Streptomycin	1	gm/ml
Mycostatine	250	U/ml

L-glutamine	0.5	gm
Sodium bicarbonate	1%	

All these ingredients were completely dissolved in one liter of sterile D.W., the pH was adjusted to 7.2 and the medium was sterilized by filtration through millipore filter (0.22µm) under aseptic conditions. After that 10ml of the medium was transferred into sterile tubes and kept at -20Cº until use.

d. Preparation of cell culture medium for the growth of cancer cells in the microtiter plate:

The preparation of this medium was also performed following to the method taken from (Freshney, 2000)**.**

RPMI-1640	10.4	gm
Penicillin G	10^4	U/ml
Streptomycin	0.01	gm/ml
Mycostatine	25	U/ml
L-glutamine	0.5	gm
Sodium bicarbonate	1%	
FCS	20%	

All these ingredients were completely dissolved in one liter of sterile D.W., the pH was adjusted to 7.2 and the medium was sterilized by filtration through millipore filter (0.22µm) under aseptic conditions**.** After that 20ml of the medium was transferred into sterile tubes and kept at -20Cº until use.

2.2.3 Collection of Samples

2.2.3.1 Collection of stool samples: (For isolation of ETEC)

A total of 25 stool samples were taken from patients suffering from watery diarrhea in AL-Yarmuk Teaching hospital / Baghdad. Each stool sample

was collected from the patient into a sterile tube containing 5ml peptone broth, and incubated at 37Cº for (18 to 24) hr before being plated on a screening medium (Sanderson *et al*., 1995).

2.2.3.2 Collection of blood samples: (For cytogenetic analysis)

A. Collection of blood from colorectal cancer patients

A portion of 5ml of peripheral blood was collected, by vein puncture into sterile tubes containing 0.05ml heparin, from 20 patients. Their ages were ranged between (25-55) years. These samples were obtained from Gastroenterology and Hepatology Teaching hospital.

B. Collection of blood from normal healthy persons

A portion of 5ml of peripheral blood was collected, by vein puncture into sterile tubes containing 0.05ml heparin, from 10 normal healthy persons. Their ages were ranged between (19-42) years,

2.2.3.3 Collection of tumor tissue samples: (For detecting the cytotoxic effect of STa on primary cancer cell cultures)

Tumor tissue samples of 5 different types of tumors (colon, stomach, lung, breast and bladder) were collected into sterile tubes containing a transport medium (2.2.2.2.c). They were obtained from the operations theatre of Gastroenterology and Hepatology Teaching hospital and Baghdad Teaching hospital.

2.2.4 Experimental Animals

Two groups of albino Swiss BALB/c mice, which were obtained from the Biotechnology Research Center / AL-Nahrain University, were used in this study.

Group A: consisted of 85 mice (females). They were used for the cytogenetic analysis, determination of liver enzymes level, and determination of lethal dose 50 (LD 50) in addition to histopathological examination. Their ages were ranged between (8-12) weeks and weighting (25-30) gm. They were divided into subgroups, and each group was putted in a separate plastic cage. The cages were kept in a room with (23-25) Cº temperature. The animals were fed with a suitable quantity of water and complete diet.

Group B: consisted of 100 suckling mice, which were used for the detection of STa activity. Their ages were ranged between (3-5) days. They were separated from their mothers and used immediately for the detection.

2.2.5 Administration of Experimental Animals

The animals in this experiment were treated with a cumulative dose of STa in a short time. The main aim of this experiment was to evaluate the acute treatment effect of STa in normal mice.

Ten groups of mice were used in this experiment and treated as follows: for the first seven groups only, one half of each group was used to determine BI and CA, while the other half was used for the determination of MN and liver enzymes level. These groups are:

Group I: Negative control (10mice), treated with (0.1ml) of PBS.

Group II: Positive control (20mice), (10mice) treated with (0.1 ml) of MMC (2µg /Kg) and (10mice) treated with (0.1ml) of MMC (5µg/Kg).

Group III: STa treatment (6mice), treated with (0.1 ml) of STa (100µg/Kg).

Group IV: STa treatment (6mice), treated with (0.1ml) of STa (200µg/Kg).

Group V: STa treatment (6mice), treated with (0.1ml) of STa (400µg/Kg).

Group VI: STa treatment (6mice), treated with (0.1ml) of STa (800µg/Kg).

Group VII: STa treatment (6mice), treated with (0.1ml) of STa (1600µg/Kg).

The PBS, MMC and STa were given orally for five successive days, and then the mice were sacrificed at the sixth day. Bone marrow and blood samples were taken and cytogenetic analysis, liver enzyme determination were carried out as described later.

Group VIII: STa treatment (10mice), treated with (0.1ml) of STa (500µg/kg).

Group IX: STa treatment (10mice), treated with (0.1ml) of STa (1000µg/kg).

Group IIX: Negative control (5mice), treated with (0.1ml) of PBS.

These three groups were used for the determination of the LD 50, which was assayed by orally giving (0.1ml) of STa and PBS for ten successive days. The mice were observed daily for hand-leg paralysis and death. Death between day 1 and 10 inclusively were tabulated, and then histopathological effects were detected by examining five organs (lung, liver, kidney, colon and stomach) (Strockbine *et al*., 1986). The LD50 was calculated by using the method of Reed and Muench, (1938) as follows:

$$\text{Proportionate distance} = \frac{\textit{mortality above } 50 \textit{ percent} - 50}{\textit{mortality above } 50 \textit{ percent} - \textit{mortality below } 50 \textit{ percent}}$$

Negative logarithm of LD50 titer = Negative logarithm of dilution above 50 percent mortality + proportionate distance

2.2.6 Isolation and Identification of *E. coli*:

After the collection of stool samples into tubes containing peptone broth, and incubation for (18-24) hr, a 1oopful from the culture was subcultured onto MacConkey agar plates, and incubated for overnight at 37Cº. Then the grown

colonies were individually identified both microscopically and by using classical biochemical tests (Macfaddin, 2000).

MacConkey agar is a differential plating medium for the selection and recovery of Enterobacteriaceae. Bile salts and crystal violet are included to inhibit the growth of gram-positive bacteria.

In addition to MacConkey agar, eosin methylene blue (EMB) agar has been used as a selective medium for *E. coli*. The aniline dyes (eosin and methylene blue) in this medium combine to form a precipitate at acidic pH, which appearing as a metallic green sheen (Atlas *et al*., 1995).

2.2.6.1 Gram Staining:

Dry heat fixed smears were taken from the colonies and placed on microscopic glass slides to examine the morphology of the bacteria (Atlas *et al*., 1995).

2.2.6.2 Biochemical Tests

2.2.6.2.1 Indole Test:

Tryptone broth was inoculated with a young culture of each suspected isolate and incubated at 37Cº for 24hr. Kovac's reagent (0.5ml) was added directly to the culture tube and if the culture produces tryptophanase, indole ring will appear at the tope of the broth (Macfaddin, 2000).

2.2.6.2.2 Methyl Red Test:

MR-VP broth was inoculated with a young culture of each suspected isolate and incubated at 37Cº for 24hr. Five drops of methyl red reagent were added, mixed, and result was read immediately. A bright red color indicates a positive reaction (Macfaddin, 2000).

2.2.6.2.3 Voges-Proskauer Test:

MR-VP broth was inoculated with a young culture of each suspected isolate and incubated at 37Cº for 24hr. One ml of 40% KOH and 3ml of 5% solution of α -naphthol were added. A positive reaction was indicated by the development of a pink to red color in 2-5 min (Macfaddin, 2000).

2.2.6.2.4 Simmon's Citrate Test:

Simmon's citrate slant was inoculated with a young culture of each suspected isolate and incubated at 37Cº for 24hr. Growth of the microorganism on this medium results in a rise in the pH which causes the pH indicator to turn form green into blue, which indicate a positive reaction (Macfaddin, 2000).

2.2.6.2.5 Urease Test:

Urea agar slant was inoculated heavily over the entire slant surface and incubated at37Cº for 24hr. Urease test is positive if the indicator was changed to purple-pink color (Macfaddin, 2000).

2.2.6.2.6 Triple Sugar Iron (TSI) Test:

Heavy inoculum was streaked over the surface of the slope and stabbed into the butt of the triple sugar iron agar slant. Incubated at 37Cْ for 24hr. Results were recorded as follows: (Macfaddin, 2000)

Slant/Butt	**Color**
Alkaline / Acid	Red/yellow
Acid / Acid	yellow / yellow
Alkaline / Alkaline	Red /Red
H_2S	Black precipitate

2.2.7 Production of STa:

The production of STa was done according to the method of (Gomes *et al*., 1979). Suspected isolates that were collected from stool and which has been identified as *E. coli* by biochemical tests, were used for the experiment regarding production of STa, and they were considered as stock cultures. The starter culture was loop-inoculated from the stock cultures into a tube containing 10ml brain heart infusion broth (for bacterial activation), then incubated at 37C° for 18hr. After that 0.1ml from the starter culture was inoculated into 10ml CA-YE medium in a 100ml flask capacity, then incubated at 37C° for 18hr in a shaking water bath at 140 shakes per min. The culture was then centrifuged at 6,000rpm for 30min, and the respective supernatant was carefully separated and filtered through millipore filter (0.45µm) before testing the STa activity. The *E. coli* isolate that showed the highest STa activity were used for the large scale production of STa by inoculating 2.5ml from the starter culture into 250ml CA-YE medium in 2000ml flask capacity, then incubated at 37C° for 18hr in a shaking water bath at 140 shakes per min. The culture was then centrifuged at 6,000rpm for 30min, and the respective supernatant was carefully separated and filtered through millipore filter (0.45µm) and kept at 4C° to be ready for the purification.

2.2.8 Detection and Determination of STa Activity :

This was done according to the method used by (Giannella, 1976).

Suckling mouse assay (SMA) has been used for the detection of STa activity, and 4 mice were used for each test. Newborn Swiss albino suckling mice (3-5) days old were separated from their mothers immediately before use. Each mouse was inoculated (intragastric, percutaneous injection) with 0.1ml of crude culture filtrate containing 2 drops of 2% Evan's blue stain per ml. After 3-4 hr the mice were killed by cervical dislocation. The abdomen was opened,

and the entire intestine (excluding the stomach) was removed with forceps. The intestines and carcasses of the four animals were pooled, and weighed, and the ratio of the intestine weight to remaining carcass weight was calculated and referred to as intestine to body-weight (IW / BW) ratio. The assay was considered as positive if the (IW/BW) ratio is above 0.085. The activity was determined by using the bovine serum albumin (BSA) standard curve by plotting the (IW/BW) ratio against the corresponding concentrations of BSA.

Animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded.

2.2.9 Determination of Protein Concentration:

Protein concentration was determined according to (Bradford, 1976), and as follows:

2.2.9.1 Preparation of bovine serum albumin standard curve:

A standard curve of bovine serum albumin was carried out by using different concentrations (0, 20, 40, 60, 100, 120, 140µg/ml) from BSA stock solution (as prepared in 2.2.1.2 C. Then to test tubes containing 0.1ml of each concentration, 0.4ml Tris-HCl buffer and 2.5ml of Coomassie brilliant blue G-250 stain was added, mixed well, and left to stand for 5min. The optical density at 595nm for each tube was measured using UV-Vis spectrophotometer and the relationship between the optical density and BSA concentrations was plotted to determined the standard curve of BSA (figure 2-1).

2.2.9.2 Estimation of protein concentration for the unknown samples:

The concentration of protein was estimated by mixing 0.1ml from the unknown solutions with 0.4ml Tris-HCl and 2.5ml of Coomassie brilliant blue G-250 stain, then mixed well and left to stand for 5min. The optical density was

measured at 595nm and the protein concentration of the unknown samples was calculated from the standard curve as shown in the figure (2-1).

The specific activity has been calculated according to the following equation :(Whitaker and Bernard, 1972)

$$\text{Specific activity} = \frac{enzyme\ activity\ (Ulml)}{protein\ concentration\ (mg/ml)}$$

(U/mg)

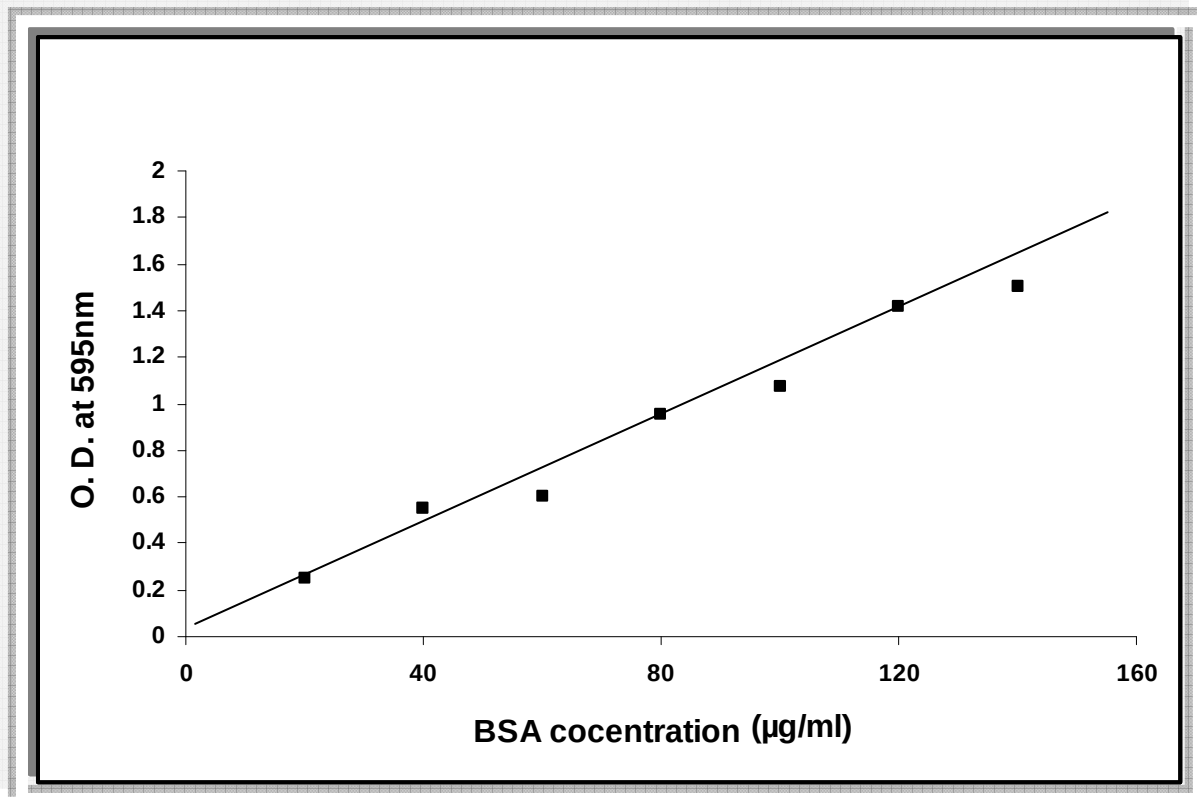


Figure (2-1): Standard curve of bovine serum albumin.

2.2.10 Partial purification of STa: By using Ion-Exchange Chromatography

2.2.10.1 Preparation of crude STa:

After collecting culture supernatant and detecting STa activity and estimating its concentration, crude toxin was prepared, and this was done depending on the method used by (Takeda *et al*., 1979).

Crystalline ammonium sulfate (50mg/100ml) was added to 250ml supernatant of bacterial lysate and kept at 4Cº for 2hr. Then the resulting precipitate was discarded after centrifugation at 6,000 rpm at 4Cº for 15min. Then the supernatant was filtered through millipore filter (0.45µm) and the filtrate was concentrated in the oven at 45Cº until its volume became (5ml).

After that, the concentrated filtrate was filtered through 0.45µm and then 0.22µm millipore filters. Then the concentrated material was dialyzed against 0.01M phosphate buffer using membrane tubing which cuts off molecules with molecular weight of less than 1,000 dl .The dialyzed material were used as a crude toxin, and stored at ¯20Cº until use. Both the activity and the concentration of the toxin were determined.

2.2.10.2 Preparation of DEAE-Sephadex A-50 column:

This was performed according to the method that was depended by (Roby and White, 1987).

DEAE-Sephadex A-50 column was prepared by dissolving 3gm of the resin in 250ml D.W., then it was kept in a shaker water-bath at 85-90Cºfor (1-2) hr. After cooling, it was washed several times with phosphate buffer (0.01M, pH .7.0) until the pH become near (7.0). Gas bubbles were degassed using vacuum pump. Then it was poured accurately on the inner side of the column (2 x 10 cm) and equilibrated with the phosphate buffer (0.01 M, pH 7.0) until the pH was about (7.0).

2.2.10.3. Purification procedure:

The purification of STa had done according to the method described by (Takeda *et al*., 1979).

Crude toxin 5ml was added gently to DEAE-Sephadex A-50 column that has been previously equilibrated with the phosphate buffer (pH 7.0) .The fractions have been collected at a flow rate of 30 ml/hr and with a fraction size of 3 ml/tube. Then 50ml of (0.1) M phosphate buffer was added to the column until the absorbency at 280nm reached the base line, and that represent the washing part. After that proteins were eluted using 200ml of (0.01) M phosphate buffer (pH 7.0) that contains gradient of NaCl (0.1-1.0) M and that represent the elution part. Protein contents were traced in accordance to absorbency at 280nm and plotted as absorbency versus the elution volume. STa activity and concentration were also determined.

2.2.11 Cytogenetic Experiments

2.2.11.1 Cytogenetic analysis in mouse (*in vivo*)

2.2.11.1.1 Chromosomal preparation from somatic cells of the mouse bone marrow:

This experiment was done according to (Allen *et al*., 1976) as follows:

a. Each animal was injected with 0.25ml of colchicine with a concentration of (1mg/ml) intraperitoneally (I.P) 2hr before sacrificing the animal.

b. The animal was sacrificed by cervical dislocation.

c. Then the animal was fixed on its ventral side on the anatomy plate and the abdominal side of the animal and its thigh region were swabbed with 70% ethanol.

d. The femur bone was taken and cleaned from the other tissues and muscles, then gabbed from the middle with a forceps in a vertical position over the

edge of the test tube, and by sterile syringe 5ml of PBS were injected so as to wash and drop the bone marrow in the test tube.

e. The test tube was taken and centrifuged at speed of 2000 rpm for 10min.

f. The supernatant was removed and 5ml of potassium chloride (0.075) M was added as a hypotonic solution, then the test tubes were left for 30min in the water bath at 37C º and shaked from time to time.

g. The tubes were centrifuged at 2000 rpm for 10min.

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

i. The tube was kept at 4Cº for 30min to fix the cells.

j. The tubes were centrifuged at 2000 rpm for 10min. The process was repeated three times and the cells were suspended in 2ml of the fixative solution. By a pasture pipette, few drops from the tube were dropped vertically on the chilled slides from a height of 3 feet at a rate of (4-5) drops to give the chance for the chromosomes to spread well. Later the slides were kept to dry at room temperature.

k. The slides were stained with Giemsa stain and left for 15min, then washed with D.W.

l. Two slides per each animal were prepared for cytogenetic assays.

2.2.11.1.2 Micronucleus test in mouse bone marrow cells:

This assay was adapted from that described by (AL-Sudany, 2005).

a. The femur bone was cleaned from tissue and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube, and by a sterile syringe 1ml of human serum was injected so as to wash and drop the bone marrow in the test tube.

b. The test tube was centrifuged at 1000 rpm for 5min.

c. The supernatant was removed, and a drop from the pellet was taken to make a smear on clean slides .The slides were kept at room temperature for 24hr.

d. The slides were fixed with absolute methanol for 5min, then stained with Giemsa stain for 15min, then washed with D.W. and left to dry.

e. Two slides for each animal were prepared for micronucleus test.

2.2.11.2 Cytogenetic analysis in human blood lymphocytes (*in vitro*) (For both normal healthy persons and colorectal cancer patients)

2.2.11.2.1 Chromosomal preparation from human blood lymphocytes culture:

This experiment was done according to (Shubber and Al-Shaikhly, 1988) as follows:

a. Human blood was collected into a sterile test tube containing 0.05ml heparin.

b. 0.25ml of peripheral blood was added into test tube containing 2ml of culture medium (RPMI-1640, as prepared in 2.2.2.2.b).

c. PHA (0.25) ml was added, the components were mixed very well and transferred to 37C^{o} incubator.

d. After 24hr of incubation, 0.1ml of different concentrations of STa (100, 200, 400, 800 and1600 µg/ml) was added to each test tube. Also 0.1ml of PBS was added as a negative control, and 0.1ml of MMC at a concentration of (5) µg/ml as a positive control.

e. The test tubes were put back in the incubator at 37C ^{o} and shaked gently each 24hr one try at least. The incubation period was completed to 72hr.

f. A portion of 0.2ml of colchicine was added to each tube half an hour before harvesting the cells, and gently shaked each 10min.

g. The test tubes were centrifuged at 2000 rpm for 10min.

h. The supernatant was removed and 5ml of pre-warmed (0.075) M potassium chloride (KCl) was added to each tube as a hypotonic solution, then the test

tubes were left for 30min in the incubator at 37Cº and shaked from time to time.

i. The tubes were centrifuged at 2000 rpm for 10min.

j. The supernatant was removed and the fixative solution was added as drops on the inside wall of the test tube with continuous shaking, then the volume was fixed to 5ml. the contents were shaked well and the tubes were kept at -4Cº for 30min to fix the cells.

k. The tubes were centrifuged at 2000 rpm for 10min.This process was repeated for three times and the cells were suspended in 2ml of the fixative solution.

l. By a pasture pipette, few drops from the tube were dropped vertically on the chilled slides from a height of 3 feet at a rate of (4-5) drops to give the chance for the chromosomes to spread well. Later the slides were kept to dry at room temperature.

m.The slides were stained with Giemsa stain and left for 15min, then washed with D.W.

n. Two slides for each concentration were prepared for cytogenetic assays.

2.2.11.2.2 Micronucleus test in human blood lymphocytes culture:

This assay was adapted from that described by (AL-Sudany, 2005).

a. Human blood was collected into a sterile test tube containing 0.05ml heparin.

b. A portion of 0.25ml of peripheral blood was added into a test tube containing 2ml of culture medium (RPMI-1640, as prepared in 2.2.2.2.b).

c. Then 0.25 ml of PHA was added, the components were very well and transferred to 37Cº incubator.

d. After 24hr of incubation, 0.1ml of different concentrations of STa (100, 200, 400, 800 and1600 µg/ml) was added to each test tube. Also 0.1ml of PBS as a negative control and 0.1ml of MMC at a concentration of (5)µg/ml as a positive control was used.

e. The test tube was put back in the incubator at 37Cº, and gently shaked each 24hr one try at least. The incubation period was completed to 72hr.

f. Test tubes were centrifuged at speed of 800 rpm for 5min.

g. The supernatant was removed and 5ml of pre-warmed (0.1) M potassium chloride (KCl) was added as a hypotonic solution, then the test tube was left for 30min in the incubator at 37Cº and the tube was shaked from time to time.

h. The tube was centrifuged at 800 rpm for 5min.

i. After that the supernatant was removed and the fixative solution was added as drops on the inside wall of the test tube with continuous shaking, and then the volume was fixed to 5ml and the contents were shaked well.

j. The tube was kept at -4Cº for 30min to fix the cells.

k.The tubes were transferred into the centrifuge at 800 rpm for 5min. The process was repeated for three times and after that, the supernatant was discarded and one drop of the pellet was smeared on a clean slides and left to dry at room temperature.

l. The slides were stained with Giemsa stain and left for 15min, then washed with D.W.

m. Two slides for each concentration were prepared for micronucleus assay.

2.2.12 Cytogenetic Parameters Analysis

2.2.12.1 Mitotic Index (MI) Assay:

The slides were examined under the high dry power (40X) of the compound light microscope and (1000) of divided and non-divided cells were counted and the percentage rate was calculated for only the divided ones according to the following equation:

$$\text{MI} = \frac{nomber\ of\ divided\ cells}{total\ number\ of\ the\ cells\ (1000)} X\ 100$$

2.2.12.2 Blast Index (BI) Assay:

The slides were examined under high dry power (40X) of the compound light microscope and (1000) cells were counted to calculate the percentage rate of the blast cells according to the following equation:

$$BI = \frac{number\ of\ blast\ cells}{total\ number\ of\ cells\ (1000)} X100$$

2.2.12.3 Chromosomal Aberrations (CAs) Assay:

The prepared slides were examined under the oil immersion lens for 100 divided cells per each animal or blood lymphocyte culture, and the cells should be at the metaphase stage of the mitotic division, where the chromosomal aberrations are clear, and the percentage of these aberrations was estimated.

2.2.12.4 Micronucleus (MN) Test:

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

2.2.13 Enzymatic Assays:

2.2.13.1 GOT and GPT Tests:

According to Reitman and Frankel (1957), blood was collected from the mice by heart puncture. Blood serum was separated by centrifuging at5000 rpm for 10min. Then, the serum was treated as follows:

Two test tubes were used for each sample, the first contained the blank reagent and the second contained the sample. These samples were treated as in the following:-

Tubes / Reagent	GPT	GOT
Reagent 1	1 ml	-----
Reagent 2	-----	1 ml
Incubated for 5min at 37C^{o}		
Serum	0.2 ml	0.2 ml
Mixed and incubated at 37C^{o}	--------- 1 hour	------- 30 min
Reagent 3	1 ml	1 ml
Mixed. Let stand for 20 min at room temperature		
NaOH	10	10
Mixed. Waiting for 5min and measure under conditions identical to those used for the standard curve.		

Wave length: 505 nm (490-520nm).

Activities of these two enzymes in the serum were estimated from the activity table attached with the kit of each enzyme.

2.2.13.2 ALP test:

Sample used in this test was the same serum sample used for GOT and GPT tests. To estimate the activity of ALP enzymes, procedure for Kind and King (1954) was used:

Four test tubes for each sample were prepared, the first contained the sample, the second contained the blank sample, the third contained the standard sample and the fourth contained the blank reagent, as shown below:

Tubes / Reagents	Serum Sample	Serum Blank	Standard	Reagent Blank
Reagent	2 ml	2 ml	2 ml	2 ml
Incubated for 5 min at 37Cº				
Serum	50 ml	-------	------	------
Reagent 2	------	-------	50 ml	------
Incubated for exactly 15 min at 37Cº				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Mixed well or preferably vortex.				
Reagent 4	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Serum	------	50 ml	------	------
D.W.	------	------	------	50 ml
Mixed. Let to stand for 10 min in the dark then measured at 510nm				

$$\text{Calculation} = \frac{OD\ serum\ sample - OD\ serum\ blank}{OD\ s\tan dard} X\ enzyme\ activity\ (U/L)$$

2.2.14 Histopathological Examination:

This was performed by using method of (Bancroft and Stevens, 1992)

2.2.14.1 Preparation of histological sections:

At the time of death, mouse organs including (lung, liver, kidney, colon and stomach) were taken for histopathological examination. After removal they were immediately preserved in 10% formaldehyde. Then after washing with tap water, they were fixed and processed with a set of increasing alcohols concentrations (70%, 80%, 90%, and 100%). After that, tissues were embedded in paraffin blocks and sectioned at (5-6) µm for all oranges except the kidney,

which was sectioned at 4µm. All the sections were stained with hematoxyline and eosin stain, and histopathological changes were observed.

2.2.14.2 Hematoxyline-Eosin Method:

Histological sections were placed in the following solutions and reagents as follows:

a. Xylol for 5min.

b. Absolute alcohol for 1min.

c. Grade of series of ethanol (80%, 70%, 50%, and 35%) to be dried and then rinsed in D.W. for 1min.

d. Iodine for 1min.

e. Sodium-hydrosulphate to erase the iodine and turn the color of tissue to white and rinsed in tap water for few min.

f. Hematoxyline for (1-5) min, then rinsed in tap water to get rid of the excess dye.

g. Acid alcohol was added until the color turned to pink then rinsed in tap water.

h. Sodium -bicarbonate was added until the color turned to blue, then rinsed in tap water.

i. Eosin for 5min, then rinsed in tap water.

j. Xylol for 3min, then mounted in Canada balsam.

2.2.15 Detecting the Cytotoxic Effect of STa on Primary Cancer Cell Cultures and Normal Cell Line:

2.2.15.1 Cytotoxic effect of STa on primary cancer cell cultures

This procedure was adapted from that described by **(**Freshney, 2000).

2.2.15.1.1 Management of tumor tissue samples to obtain primary cancer cell cultures:

All the work was done under sterile conditions in the laminar airflow hood as follows:

a. The collected tumor tissue sample was put in a sterile pettry dishs and all the necrotic tissue and fat were removed.

b. The tissue was washed at least five times with the culture media (RPMI-1640, as prepared in 2.2.2.2.c).

c. Then the tissue was transferred into a sterile tube that contains the culture medium (RPMI-1640, as prepared in 2.2.2.2.d) and minced very carefully by a sharp curved seasor, with maintaining the tissue in the culture media.

d. The tube was centrifuged at 1000 rpm for 5min, to get rid of the large tissue pieces.

e. The supernatant was then transferred into another sterile tube and before seeding into a 96-well microtiter plate, the number of the cells must be counted and the number of cells at seeding should be 10^7 cell /ml.

2.2.15.1.2 Viable cell count:

Cells were counted by using trypan blue stain. Only the dead cells will take up the dye and appear blue under the microscope while viable cells exclude the dye and appear white, which make it very easy to distinguish dead cells from viable cells, and this done by mixing 0.2ml of cells and 0.2ml of the stain with 1.6ml of PBS. Then after mixing well sufficient volume was aspirated to fill the Neubaur haemocytometer.

2.2.15.1.3 Cytotoxic assay:

a. 50µl of complete culture media (RPMI-1640, as prepared in 2.2.2.2.d) was added to each well in the microtiter plate, except the first vertical line.

b. then 100µl of STa was added to first well in the first and second vertical line only at a concentration of (1) mg/ml. 6 repetitives were made. Mixed well and then 50µl from the first well in the second vertical line was transferred to the second well and two-fold serial dilutions were made until the well number (12).

c. A positive MMC (at a concentration of 10µg/ml) and negative PBS controls was added to each well in the seventh and eighth line of the microtiter plate, respectively.

d. Then 150µl from cell suspension (cancer cells suspension), after their counting, was added into each well in the microtiter plate and incubated at 37Cº in 5% CO2 incubator.

e. After 24hr of exposure time, the microtiter plate was removed from the incubator, and 50µl of neutral red dye was added, then incubate at 37Cº for 2hr. The viable cells will acquire the dye, while the dead cells will not.

f. After that, all the contents of the microtiter plate were removed and the cells were washed with PBS.

g. Then 50µl from neutral red extraction solution (ethanol : PBS) at a ratio (1:1) was added. This solution elutes the dye from the viable cells that taken the dye. The absorbency was determined by using the ELISA Reader at 492 nm.

All the previous steps were done with all tumor types (colon, stomach, lung, breast and bladder) that were used in this study.

The cytotoxic concentration 50 (CC50) was determined by plotting the STa concentrations against the absorbency.

2.2.15.2 Cytotoxic effect of STa on normal cell lines

The detection of cytotoxic effect of STa on normal cell line was done according to the method of (Abdul-Majeed, 2000). It was carried out on the normal cell line Ref (Rat embryo fibroblast), at passage (52) that was provided

by Dr. Ahmed M. AL-Shamery / Iraqi Center for Cancer and Medical Genetic Research/ Baghdad, and as follows:

a. 2ml of trypsin-versene solution was added to tissue culture bottle (falcon) of 25cm^3 that contain the cells (Ref) after the removal of old tissue culture media and washing with PBS, the bottle was shaked smoothly and then inoculated at 37C^0 for 2min to disaggregate cells and obtain more single cells as much as possible

b. After that, cells were suspended in a new fresh tissue culture media and counted at a concentration of 10^5cell/ml by trypan blue as mentioned previously in (2.2.15.2.1)

c. 50µl of complete culture media (RPMI-1640, as prepared in 2.2.2.2.d, but with 5% FCS) was added to each well in the microtiter plate, except the first vertical line.

d. then 100µl of STa was added to first well in the first and second vertical line only at a concentration of (1) mg/ml. 6 repetitives were Made. Mixed well and then 50µl from the first well in the second vertical line was transferred to the second well and two-fold serial dilutions were made until the well number (12).

e. A positive MMC (at a concentration of 10µg/ml) and negative PBS controls was added to each well in the seventh and eighth line of the microtiter plate, respectively.

f. Then 150µl from cell suspension, after their counting, was added into each well in the microtiter plate and incubated at incubated at 37C° in 5% CO_2 incubator.

g. After 24hr of exposure time, the microtiter plate was removed from the incubator, and 50µl of neutral red dye was added, then incubate at 37C° for 2hr. The viable cells will acquire the dye, while the dead cells will not.

h. After that, all the contents of the microtiter plate was removed and the cells were washed with PBS.

i. Then 50µl from neutral red extraction solution (ethanol : PBS) was added at a ratio (1:1). This solution elutes the dye from the viable cells that taken the dye. The absorbency was determined by using the ELISA Reader at 492 nm.

The cytotoxic concentration 50 (CC50) was determined by plotting the STa concentrations against the absorbency.

2.3 Statistical Analysis

A one-way analysis of variance was performed to test whether group variance was significant or not. Data were expressed as mean±standard deviation and statistical significances were calculated using ANOVA test (AL-Mohammed *et al*, 1986).

Chapter Three: Results and Discussion

3.1 Isolation and Identification of *E. coli* from Stool Samples

A total of (25) stool samples were collected from patients suffering from diarrhea form AL-Yarmuk Teaching hospital in Baghdad. *E. coli* was isolated from only (11) samples, while most of the rest contained other enteric pathogens belonging to *Salmonella* spp. and *Shigella* spp.

Isolation of *E. coli* was done by using selective enrichment technique including culturing of stool on MacConkey agar, which contains bile salts and crystal violet that promote growth of Enterobacteriaceae and related enteric gram-negative rods and suppresses growth of gram-positive bacteria and some fastidious gram-negative bacteria (Atlas *et al*., 1995).

To obtain pure culture, single colonies from the surface of MacConkey agar were selected and subcultured on the surface of other MacConkey plates.

Isolates were first identified depending on their gram staining and microscopic characteristics. *E. coli* isolates were found to be gram negative, short bacillus, non-spore forming, occur singly under the light microscope as were also described by (Prigent *et al*., 2000; HaveLaar *et al*., 2001; Todare, 2002). Accordingly, (21) isolates were suspected to belong to *E. coli*.

After that, identification of the isolates were done depending on the colonial shape and form on the surface of both MacConkey and EMB (eosin methylene blue) agars. On MacConkey agar, *E. coli* colonies were glossily, pink, smooth with an entire edge, and some of the colonies were mucoid, such characteristics were concede with (CFSAN, 2002). Accordingly, (15) isolates were suspected to belong to *E. coli*.

On the other hand, EMB agar had been used as a selective medium for *E. coli* (Web site (4)). The aniline dyes (eosin and methyl blue) in this medium combine to form a precipitate at acidic pH and appearing as a metallic green sheen, thus serving as indicators for acid production. Therefore, *E. coli* colonies

appeared very dark, almost black, when observed directly against the light. By reflecting light, a green sheen can be seen due to the highly amount of acid produced by fermentation, and they usually appeared as avoid or circular smooth colonies as described by (Atlas *et al*., 1995). Upon this, only (11) isolates were suspected to belong to *E. coli*.

Further identification of *E. coli* was also achieved by the six biochemical tests used for this purpose. *E. coli* showed positive reaction for both indole and methyl red tests, but negative reactions for voges-proskauer, citrate and urease tests. In triple sugar iron (TSI) test, *E. coli* isolates turned the color of both the slant and butt to yellow with bubbles formation (Macfaddin, 2000). Results of the previous biochemical tests insured what was already found by culturing on EMB agar in that only (11) isolates were identified as *E. coli.*

Enterobacteriaceae (enterics) members are gram negative bacteria that grow in the intestinal tract of human and animal (Silverblatt and Weinstein, 1979; Brenner, 1984). The IMViC tests (Indole, Methyl red, Voges-Proskauer and Citrate) are frequently employed for identification of this group of microbes which includes organisms like *Klebsiella*, *Enterobacter* and *E. coli* (Venkateswara *et al*., 1996). The presence of *E. coli* is used by public health officials as an indicator of fecal contamination of food and water supplies (Cakir *et al*., 2002). While the presence *of Enterobacter* and *Klebsiella* does not necessarily indicate fecal contamination because they usually widespread in soil and grass. The IMViC tests can be used to differentiate these three organisms from each other (Harrigan, 1988; Venkateswara *et al*., 1996).

In the indole test, the ability to hydrolyze tryptophan to indole is a characteristic of certain enteric bacteria that possess the enzyme tryptophanase; an enzyme that cleaves tryptophan producing indole, pyruvic acid and water. Indole positive bacteria such as *E. coli* produce tryptophanase, and when

Kovac's reagent was added to a broth containing indole, a red ring will be formed at the tope of the broth (Regina *et al*., 1979).

The methyl red (MR) and Voges-Proskauer (VP) tests are read from a single inoculated tube of MR-VP broth, and after incubation, the broth was split into two tubes. The MR-VP media contain glucose and peptone. All enterics oxidize glucose for energy. However, the end-products vary depending on bacterial enzymes. Both the MR-VP tests are used to determine what end-products result when the organism degrades glucose. *E. coli* is one of the bacteria that produce acids, causing the pH to drops down below (4.4).

Upon the addition of methyl red (as indicator) to this acidic broth, its color turned from yellow to bright red, indicating a positive MR test. Other enterics produce more pH-neutral products from glucose, which not inhibit growth of the bacteria. Thus the bacteria will be able to attack the peptone in the broth, causing the pH to rise above (6.2). At this pH, the broth did not turned to red and remind yellow after the addition of methyl red indicator indicating MR-negative test (Regina *et al*., 1979).

After adding the reagents of VP test, the color of the broth was changed to pink-red due to neutral pH. Therefore, *E. coli* show a VP- negative test (Edwards and Ewing, 1972).

Citrate test utilizes the citrate in Simmon's citrate medium to determine if a bacterium can grow on it as a sole carbon and energy source. Simmon's medium contains bromthymol blue, a pH indicator with a range of (6.0) to (7.6). Bromthymol blue is yellow at acidic pH and gradually changes to blue at more alkaline pH. Uninoculated Simmon's citrate agar shows an intermediate green color. Growth of bacteria in this medium leads to change its color, which indicates a positive citrate test. *E. coli* is unable to utilize citrate in this medium and thus showed a negative citrate test (Edwards and Ewing, 1972).

In the urease test, the enzyme urease catalyzes the breakdown of urea, and the bacteria that can produce this enzyme are able to detoxify the waste products and to derive metabolic energy from its utilization which change the medium color from yellow to purple-pink, indicating urease positive test. *E. coli* produce no urease enzyme, giving urease negative test (Chomrarin *et al*., 2006).

Regarding TSI test, its medium may differentiates genera of Enterobacteriaceae from each other based on their carbohydrate fermentation patterns and H_2S production. TSI agar slants contain 1% lactose, 1% sucrose and 0.1% glucose. The pH indicator (phenol red) changed the medium color from orange-red to yellow in the presence of acids. TSI agar also contains sodium thiosulfate, a substrate for H_2S production, and ferrous sulfate which produces black precipitate to differentiate H_2S producing bacteria from others. *E. coli* produced an acid slant (yellow) and acid butt (yellow) accompanied by gas production, but without black precipitate formation, which indicated that lactose and/or sucrose fermentation had occurred and no H_2S was produced (Atlas *et al.*, 1995).

3.2 Production of STa

Secretary diarrhea of human and other mammalian neonates was often induced by enterotoxins from enterotoxigenic strain of *E. coli* (ETEC)**.** Most of the ETEC isolated from cases of diarrhea produce a distinct class of enterotoxins of low molecular weight, referring to a heat-stable enterotoxin (ST) (Levine *et al*., 1977; Martinez *et al*., 1980; Thompson, 1987; Fasano, 1998; Fabio *et al*., 2000). There are two types of ST, STa or STI produced by *E. coli* strain isolated from human, and STb or STΠ produce by *E. coli* strains isolated from porcine (Burgess *et al*., 1978; Mullan *et al*., 1978; Weikel and Guerrant, 1985; Clare *et al*., 1999).

Synthetic media have been effectively employed for the production of STa from human strains of *E. coli* (Saeed *et al*., 1983).however the yield of toxin was shown to vary from medium to medium (Starvic *et al*., 1978). More than three media were used by authors for the production of STa, including; the trypticase soy (TS) broth (Merson *et al*., 1979), the casamino acids-yeast extract (CA-YE) medium (Evans *et al*., 1973), and the brain heart infusion (BHI) broth (Whipp, 1990).

In this study two media were used for the production of STa, including; BHI broth and CA-YE medium. The results showed that, CA-YE medium is more effective for the production than BHI broth. These results came in agreement with those obtained by (Dorner *et al*., 1976; Giannella, 1976; Alderete and Robertson, 1977; Johnson *et al*., 1978; Takeda *et al*., 1979; Gomes *et al*., 1979; Lallier *et al*., 1980; Thompson *et al*., 1986). They had compared between TS broth, BHI broth and CA-YE medium for the production of STa, and they showed that CA-YE medium was shown to be highly recommended for the production. It yield more toxin with less protein contamination than did other media, beside each of its components was tested for its contribution to the growth and toxin production. The trace salts in this medium (Mg+2, Mn^{+2}, Fe^{+3}) were shown to play an important role to the growth STa producing *E. coli* strains, since elimination of these salts resulted in poor growth. Also STa that had been detected when using CA-YE medium , was not detected when other media were used especially when these media contain vitamins or glucose , which shown to reduce STa production (Alderete and Robertson, 1977).

But at the same time, these results disagreed with those obtained by some other authors. Merson *et al*., (1979) and Naline *et al*., (1978) used TS broth for the production of STa. While, Whipp, (1990), used BHI broth for the production. Those authors showed that, these two media were more effective than CA-YE.

However, other investigators used more different types of media rather than the three previously mentioned media. Staples *et al*., (1980) used the asparagines-salt medium, while, Kapitany *et al*., (1979) and Jacks and Wu, (1974) used the syncase broth.

The using of different types of media for the production of STa could be explained by the presence of close relationship between serotypes of ETEC and enterotoxin production, beside that, the genes for enterotoxins are carried on the plasmid and not on the chromosome (Orskov and Orskov, 1977; Rowe *et al*., 1977; Merson *et al*., 1979).

3.3 Detection of STa Activity

Eleven *E. coli* isolates were tested for STa production by using suckling mouse assay (SMA), and from which only five isolates were shown to produce STa.

It is increasingly evident that enterotoxin producing strains of *E. coli* play an important role in human acute diarrheal illnesses (Gorbach, 1971; Okamato *et al*., 1988; Conway, 1995). In 1967, Sakazaki *et al*., observed that many strains of *E. coli* associated clinically with acute diarrhea are noninvasive. This was followed by observations that noninvasive strains of *E. coli* associated with acute diarrhea in man produce enterotoxins which elicit a secretary response in the intestine (Banwell *et al*., 1971; Formal *et al*., 1971; Gerady *et al*., 1984; Eisinghorst and Weitz, 1994). As a result, various models were successfully employed for characterizing the biological activities of *E. coli* enterotoxins, one of these including; SMA for detecting STa activity (Jacks and Wu, 1974).

However, the biological activity of STa is mediated by stimulation of intestinal guanylyl cyclase, which occurs after binding to high-affinity receptors, guanylyl cyclase-C (GC-C) (Field *et al*., 1978; Frantz *et al*., 1984; Visweswariah *et al*., 1992; Nandi *et al*., 1997; Scott *et al*., 2002).

At first, different methods were used to detect STa activity, including; dog loop assay (Naline *et al*., 1978), intestinal loop assay in adult mouse (Takeda *et al*., 1979), and rabbit ligated intestinal loop assay (Pierce and Wallace, 1972), in addition to suckling mouse assay.

In this study, SMA was used and it was shown to be effective for detecting STa activity, although comparing with other assays was not done, but it was shown to be also more effective model by many other investigators comparing with previous assays (Jacks and Wu, 1974; Alderete and Robertson, 1977; Johnson *et al*., 1978; Kapitany *et al*., 1979; Lallier *et al*., 1980; Saeed *et al*., 1983; Lawrence *et al*., 1983), since dog loop assay requires concentrated culture supernatant, adult mice do not response effectively as suckling mice, and rabbits are not easy to handle comparing with suckling mice assay which require unconcentrated supernatant, response more effectively and mice are more easy to handle.

Later, more advanced methods were used for detecting STa activity such as radioimmunoassay (RIA) (Frantz and Robertson, 1981) and enzyme-linked immunosorbent assay (ELISA) (Thompson *et al*., 1984; Handl *et al*., 1988). Although these two assays are more sensitive and easy, but they are time consuming and expensive comparing with SMA.

3.4 Partial Purification of STa

3.4.1 Preparation of crude STa

Preparation of crude STa was done from the *E. coli* isolate that showed the highest STa activity (0.102) U/ml. However, after extraction of STa; protein concentration, STa activity then specific activity were determined after each step of the purification. Results showed that bacterial supernatant had (0.16) mg/ml of crude STa with an activity of (56) U/ml which had a specific activity

of (350) U/mg after one fold of purification for (250) ml of bacterial supernatant as shown in table (3-1).

The purification step was started with precipitation by saturated ammonium sulfate then by concentration in the oven until the volume of the culture supernatant became (5) ml. Protein concentration, STa activity and specific activity in (5) ml were recorded to be (0.41) mg/ml, (594) U/ml and (1448.7) U/mg respectively, with a purification fold of (4.13) and a yield of (21.21).

Unlike many other proteins, STa was collected from the supernatant and not from the precipitate, after precipitation by ammonium sulfate, from which most of the proteins were usually collected (Takeda *et al*., 1979).

Concentration of STa was done by authors usually by using an Amicon-filter in the ultrafiltration-cell. In this study, concentration by oven was used, since it was not easy to obtain an Amicon-filter, depending on the heat-stable nature of the STa (STa can withstand boiling at 100C^{o} for up to 30min). STa activity was detected and it was shown to be not affected by concentration in the oven.

Purification table (3-1)

3.4.2 Purification by using DEAE-Sephadex A-50

Partial purification of STa was done by ion exchange chromatography using DEAE-Sephadex A-50 column as described in (2.2.10.3). The concentrated sample from the previous step was passed through the DEAE-Sephadex column. Results shown in figure (3-1) indicated that washing with (50) ml of (0.01) M phosphate buffer (pH 7.0) allowed the presence of one peak which was represented by fractions (6-13). Then after elution with (200) ml of (0.01) M phosphate buffer (pH 7.0) containing gradient of NaCl (0.1-1.0) M, three peaks were obtained which were represented by fractions (21-31), (31-35) and (36-41). Each peak was tested for STa activity. Accordingly, only one peak in the elution part (21-31) was able to reflect STa activity.

After that, the fractions of this peak were tested for STa activity, and only nine fractions (21-30) were showed STa activity. Then, these fractions were pooled and collected into a clean container. Protein concentration, STa activity and specific activity were determined, and results shown in the table (3-1) indicated a protein concentration of (0.04) mg/ml with STa activity of (95) U/ml and a specific activity of (2366.6) U/mg with a purification fold of (6.76) and a yield of (18.25) in (27) ml.

Partial purification of STa by ion-exchange chromatography was applied by many authors, and all of them indicated the presence of STa activity in the elution part only, and no activity was detected in the washing part (Takeda *et al*., 1979; Burgess *et al*., 1980; Chan and Giannella, 1981; Ronberg *et al*., 1983; Gerady *et al*., 1984). In addition, they declared the presence of STa activity in the first peak only of the elution part, except with (Burgess *et al*., 1980) who indicated STa activity in two peaks.

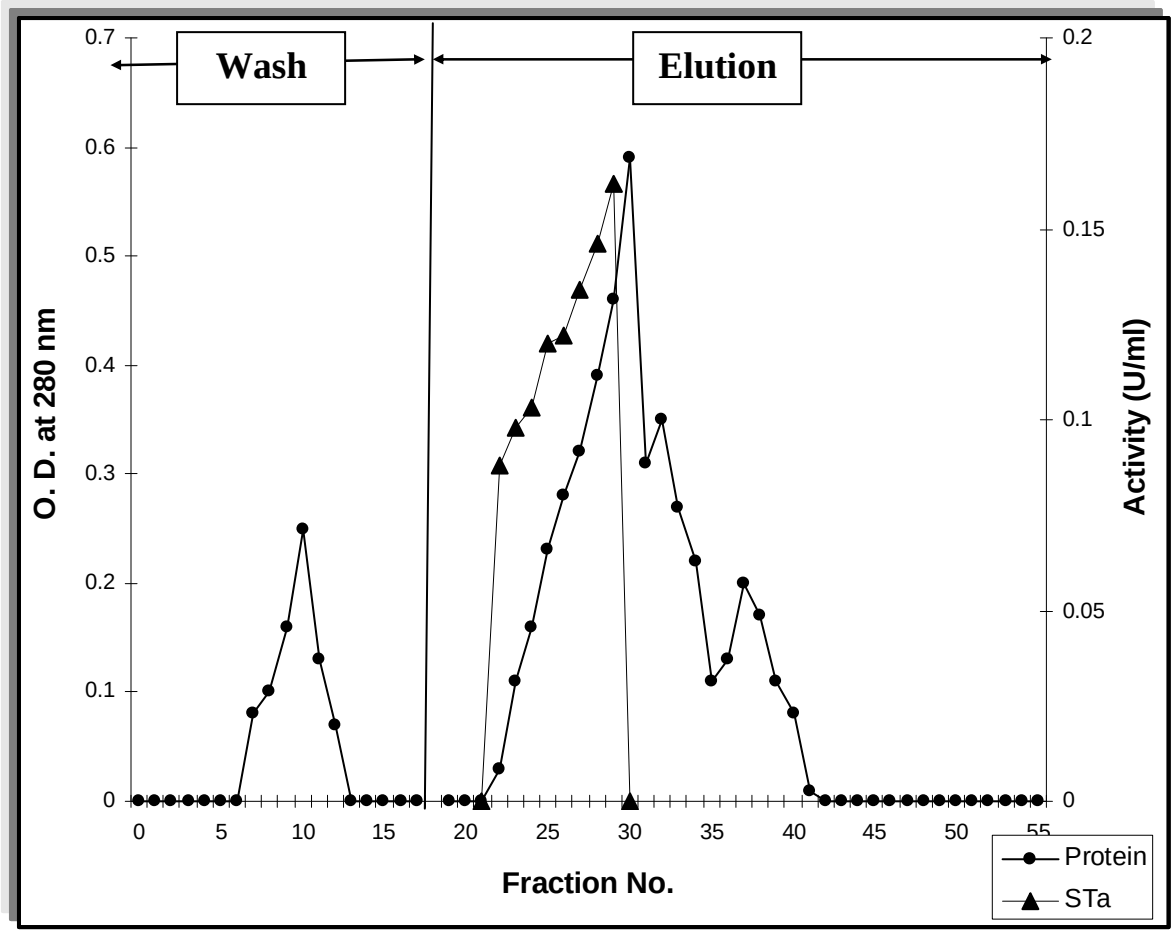


Figure (3-1): Purification of STa by ion-exchange chromatography (DEAE-Sephadex A-50) column (2x10 cm).

3.5 Clinicopathological Effects of STa on Mouse (Mouse lethality assay)

In this study the LD 50 test was used to investigate the lethal effect of the partially purified STa , and it was shown to be not lethal to mice at both doses of (500 and 1000 µg/Kg), since death was not recorded. Ten mice were used for each dose and after ten successive days, the earliest, dominant and only clinical sign was diarrhea which was noticed after three days of oral inoculation, while mice that were orally inoculated with PBS (negative control) survived without developing any significant symptoms. This result came in agreement with (Manninen *et al*., 1982), who found that *E. coli* (that secreting STa) treated mice suffered from diarrhea but without death in comparison with *Compylobacter jejuni* treated mice which suffered from diarrhea that followed by death.

Sections from (colon, kidney, liver, stomach and lung) were taken from both STa treated mice and controls. No histopathological abnormalities were detected in any of these tissues as shown in figures (3-2, 3-3, 3-4, 3-5 and 3-6) respectively. The lack of significant pathological changes in kidney, liver, stomach and lung can be explained by the absence of GC-C receptors in these organs (Krause *et al*., 1994; Nandi, 1997), and since STa bind only to these receptors (Vaandrager, 2002), so no pathological changes were seen. But the only explanation for the lack of significant pathological signs in the colon after STa treatment may be due to that STa caused only functional abnormalities (diarrhea) in this tissue without visible pathological disturbance.

These results have documented that STa has no pathological effect and can be used in a safe way and unlike other *E. coli* toxins, such as verotoxin, which has been invistigated by some investigators in a try to use it in the treatment of brain cancer (Heil, 1999; Brown, 1999). But it was shown to be lethal for mice (Naahma, 2004; Suzuki *et al*., 2000).

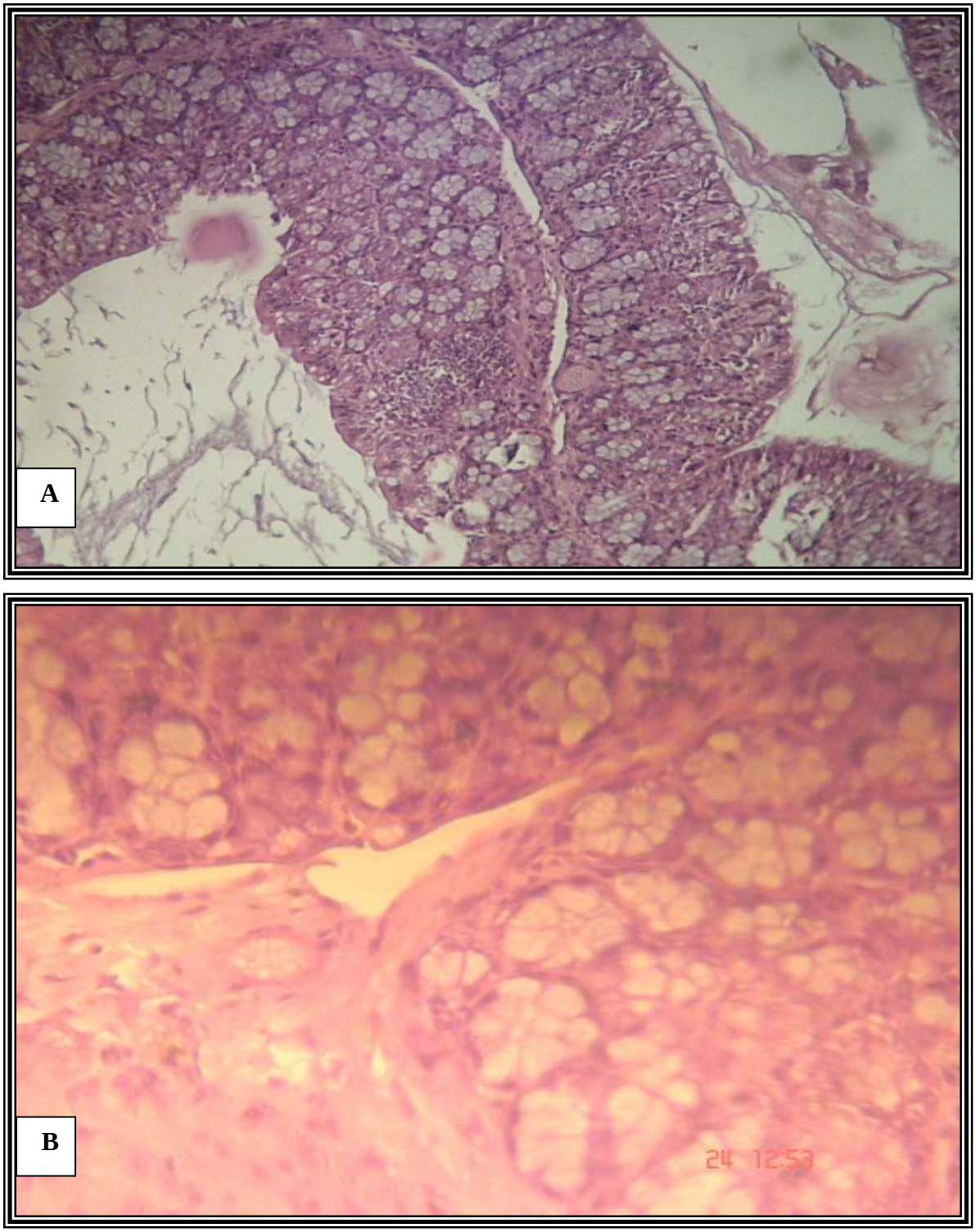


Figure (3-2): Histological sectioning showing normal (unaffected) mouse colon tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) (A): at 100X (B): at 400X

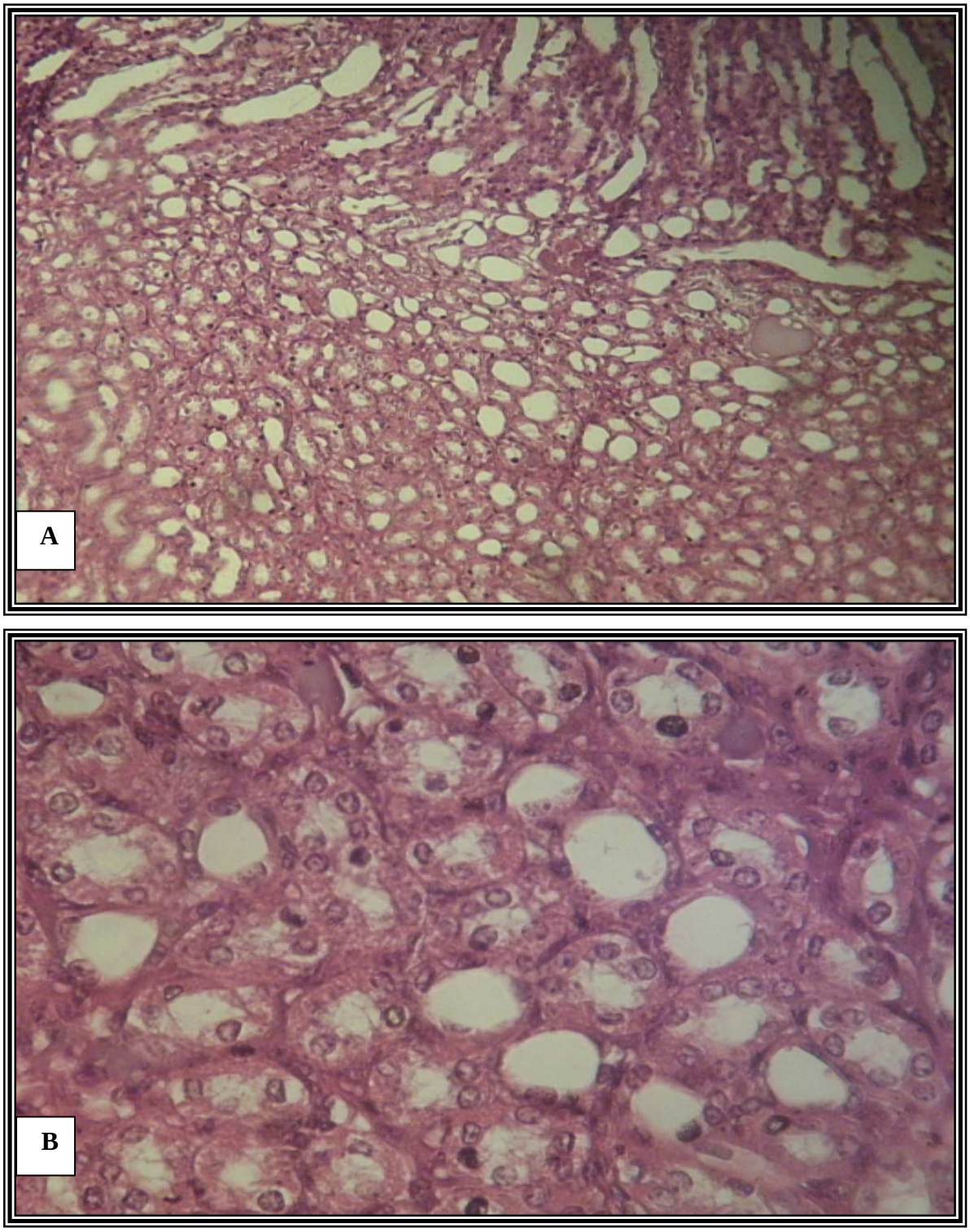


Figure (3-3): Histological sectioning showing normal (unaffected) mouse kidney tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) (A): at 100X (B): at 400X

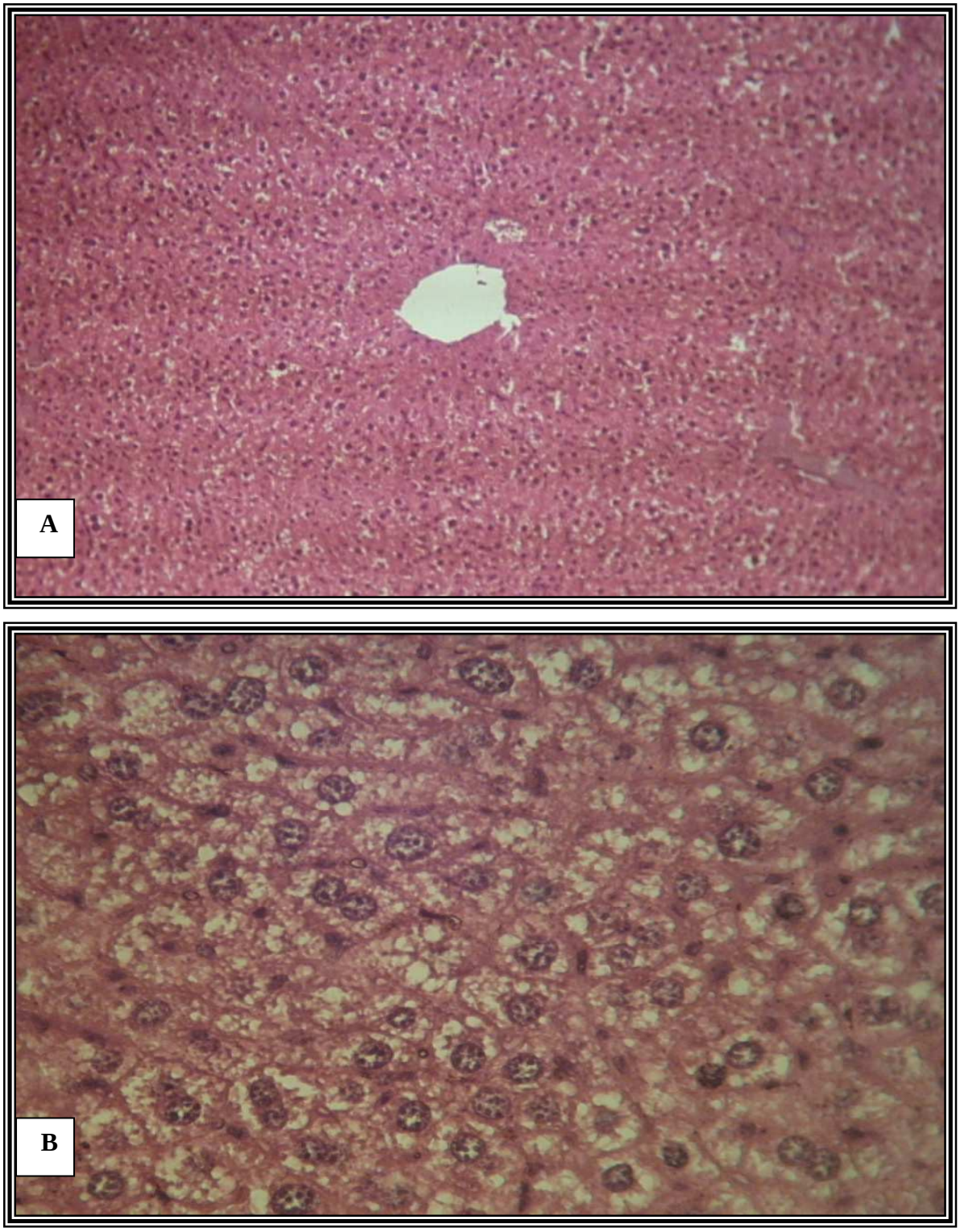


Figure (3-4): Histological sectioning showing normal (unaffected) mouse liver tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) (A): at 100X (B): at 400X

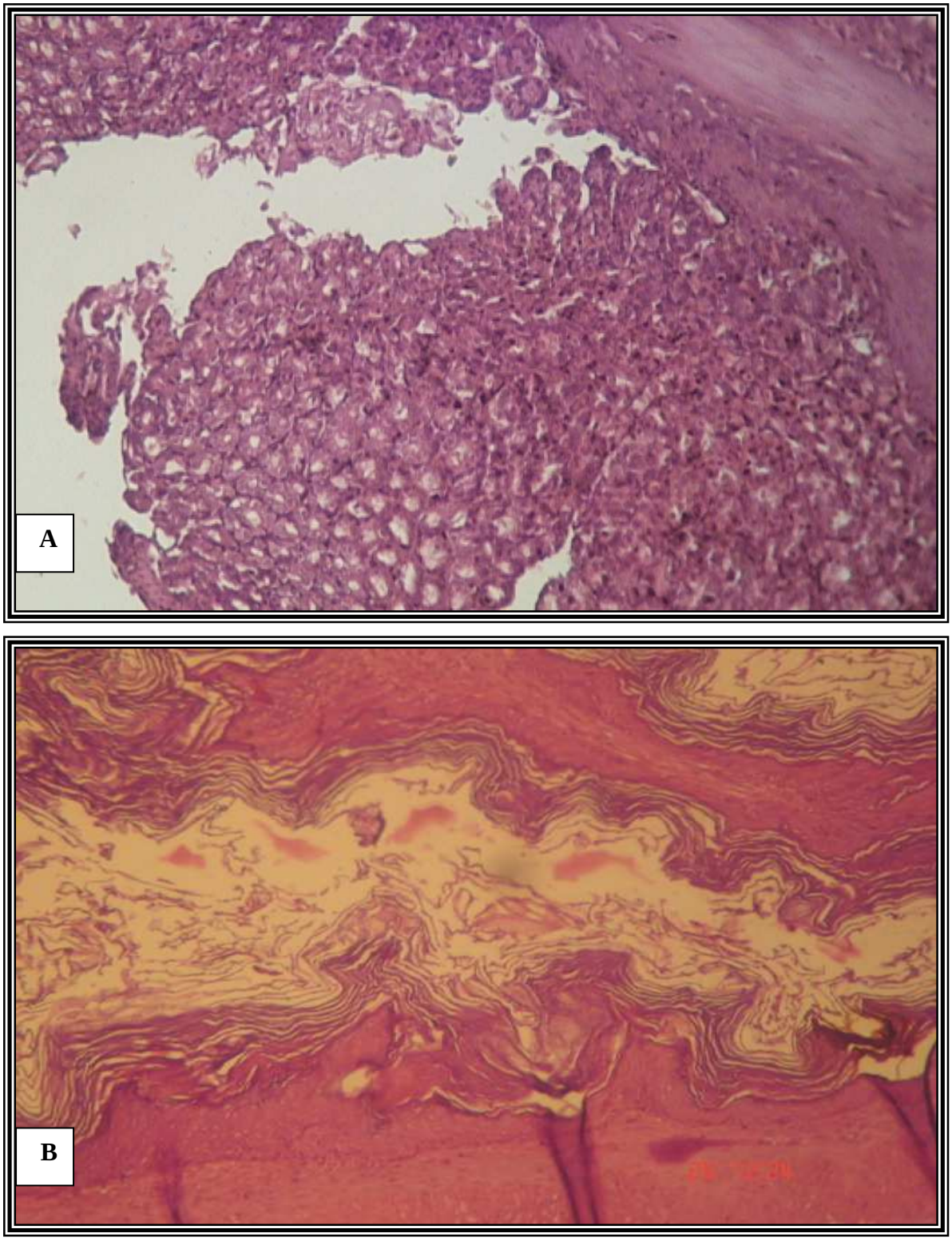


Figure (3-5): Histological sectioning showing normal (unaffected) mouse stomach tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) (A): at 100X (B): at 400X

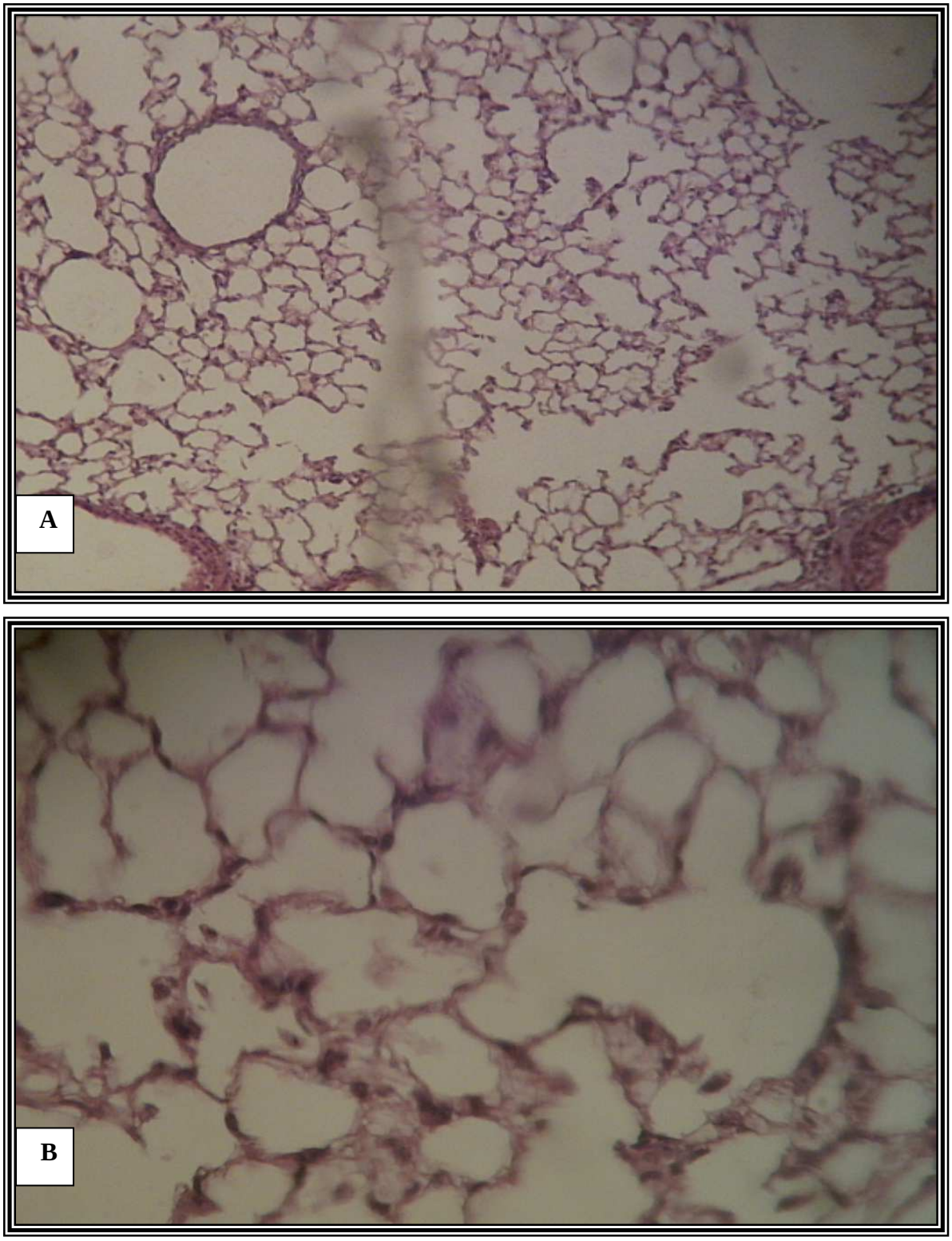


Figure (3-6): Histological sectioning showing normal (unaffected) mouse lung tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) (A): at 100X (B): at 400X

3.6 Cytogenetic Analysis

3.6.1 Cytogenetic effect of STa on mouse bone marrow cells

3.6.1.1 STa effect on mitotic index (MI)

The treatment effect of five doses of STa and two doses of MMC (as a positive control) in addition to PBS (as a negative control) on mitotic index of mouse bone marrow cells was shown in table (3-2). There were no significant differences ($P>0.05$) for all STa doses after five days of treatment (6.933, 6.133, 6.30, 7.0 and 6.233%) at doses of (100, 200, 400, 800 and 1600µg/Kg) respectively as compared with negative control (6.60%). However, a significant decrease ($P<0.05$) in the MI was seen after treatment with two doses of MMC (2.866 and 0.942%) at doses of (2 and 5µg/Kg) respectively. The reduction in the MI after MMC treatment has been also demonstrated by many authors (Littlefield *et al*., 1980; Shubber *et al*., 1985; Shubber, 1987; Papachristou *et al*., 2006). There are other chemotherapeutic drugs that also cause MI reduction, like methotrexate (MTX) (AL-Amiry, 1999; Taj *et al*., 2003) and tamoxifen (TAM) (Sayhood, 2000).

Reduction in the MI may be related to several factors: first of all, the proteins required for mitosis may not be produced in the same quantities, or the code did not reach the cells to induce it for proliferation or the drugs may caused death of bone marrow cells (Turner *et al*., 1988) or due to the effect occurred in the mitotic spindles composition during cell division (Shiraishi, 1978). At the same time, MI was shown to increase after treatment with other kinds of agents, such as antioxidants (e.g., vitamin C which is the active constituent of many plants), they act by inducing cell division by acting as mitogens (Ketterer, 1988; Travis, 1995).

The non significant differences in MI that were seen after STa treatment, may be due to that STa does not have any effect on the proteins or enzymes that

have correlation with cell division, or it does not have the activity of many mitogenic agents.

3.6.1.2 STa effect on micronucleus (MN) induction

The mouse bone marrow micronucleus assay holds a key position in all schemes for detecting potential carcinogens and mutagens. Micronucleus frequency of polychromatic erythrocytes from negative control mice was (0.566%) (table 3-2, figure 3-7).This percentage was non significantly differed ($P>0.05$) after treatment with STa (0.30, 0.533, 0433, 0.50 and 0.590%) at doses of (100, 200, 400, 800 and 1600µg/Kg) respectively, as compared with the negative control. A significant increase ($P<0.05$) in MN frequency was detected after treatment with MMC (2.733 and 5.133%) at (2 and 5µg/Kg) respectively as represented in table (3-2). However, similar results for induction of MN frequency in MMC-treated mice were denoted by (Majone *et al*., 1983; Nakagawa and Mori, 2003). The increase in MN number may be suggested that, micronuclei may be originated from acentric chromosome fragments, either from the double-stranded DNA damage before cell division or after breakage of anaphase bridges (Schmid, 1982). Miller *et al*., (1997), pointed that MMC act as a clastogenic agent and a micronucleus inducer in both mouse and human.

Although, there are agents that cause increased MN frequency, at the same time several plant extracts cause decreased MN frequency. A number of plants were shown to contain active constituents such as rutin and quercetin which protect DNA from damage by their antimutagenic and detoxification activities (Weitbery, 1987; Ahmed, 2000). STa neither causes DNA damage nor protects DNA from damage and therefore, no significant differences in MN frequency were reported after treatment with it.

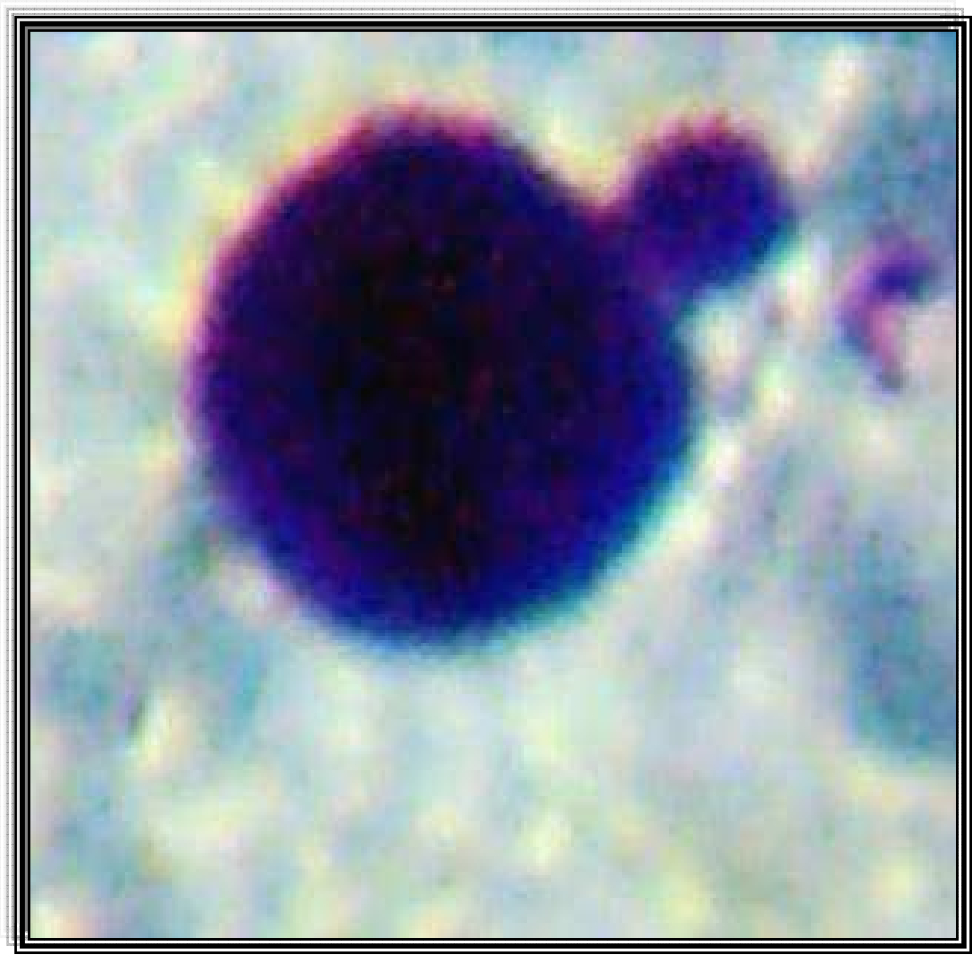


Figure (3-7): Micronucleated bone marrow cell from mouse

Table 3-2 mice cyto

3.6.1.3 STa effect on chromosomal aberrations (CAs)

The spontaneous frequency of chromosomal aberrations in untreated mouse bone marrow cells was (0.276%) which considered as negative control (table 3-2, figure 3-8). In this study, the types of chromosomal aberrations which have been observed were; ring, gap, acentric, dicentric, chromosome break, chromatid break and deletion. Neither the low nor the high doses of STa caused a significant differences (P>0.05) in total and in all types of CAs frequency in comparison to the negative control. Results of table (3-2) indicated that treatment with different doses of STa did not reduce or induce spontaneous CAs (0.325, 0.309, 0.286, 0.274 and 0.322%) at doses of (100, 200, 400, 800 and 1600µg/Kg) respectively as compared with negative control.

A significant increase in total and in all types of CAs was seen after treatment with MMC (0.907 and 1.558%) at doses of (2 and 5µg/Kg) respectively when compared with the negative control, except with chromosome break which showed non significant difference with the (2) µg/Kg dose only (table 3-2, figure 3-8). These results came in agreement with Majone *et al*., (1983), who had reported that CAs frequency increased in mouse bone marrow cells after treatment with different doses of MMC, and it was dose dependent. MMC was shown to increase CAs frequency through inhibition of DNA repair system, or it may act on topisomerase II and leading to more DNA damage (Koomen *et al*., 2002; Prasher *et al*., 2005; Papachristou *et al*., 2006).

On the other hand, the reduction in CAs frequency was noticed after treatment with plant extracts that contain vitamins which act as scavengers for free radicals in the cells and they may also act as antimutagens for many mutagenic drugs in mouse bone marrow cells (Kola *et al*., 1989; Ghaskadbi *et al*., 1992).

STa neither caused an increase in CAs frequency because it does not bind to DNA or affect DNA repair system, nor caused a decrease in CAs frequency because it does not have scavenger or antimutagenic properties.

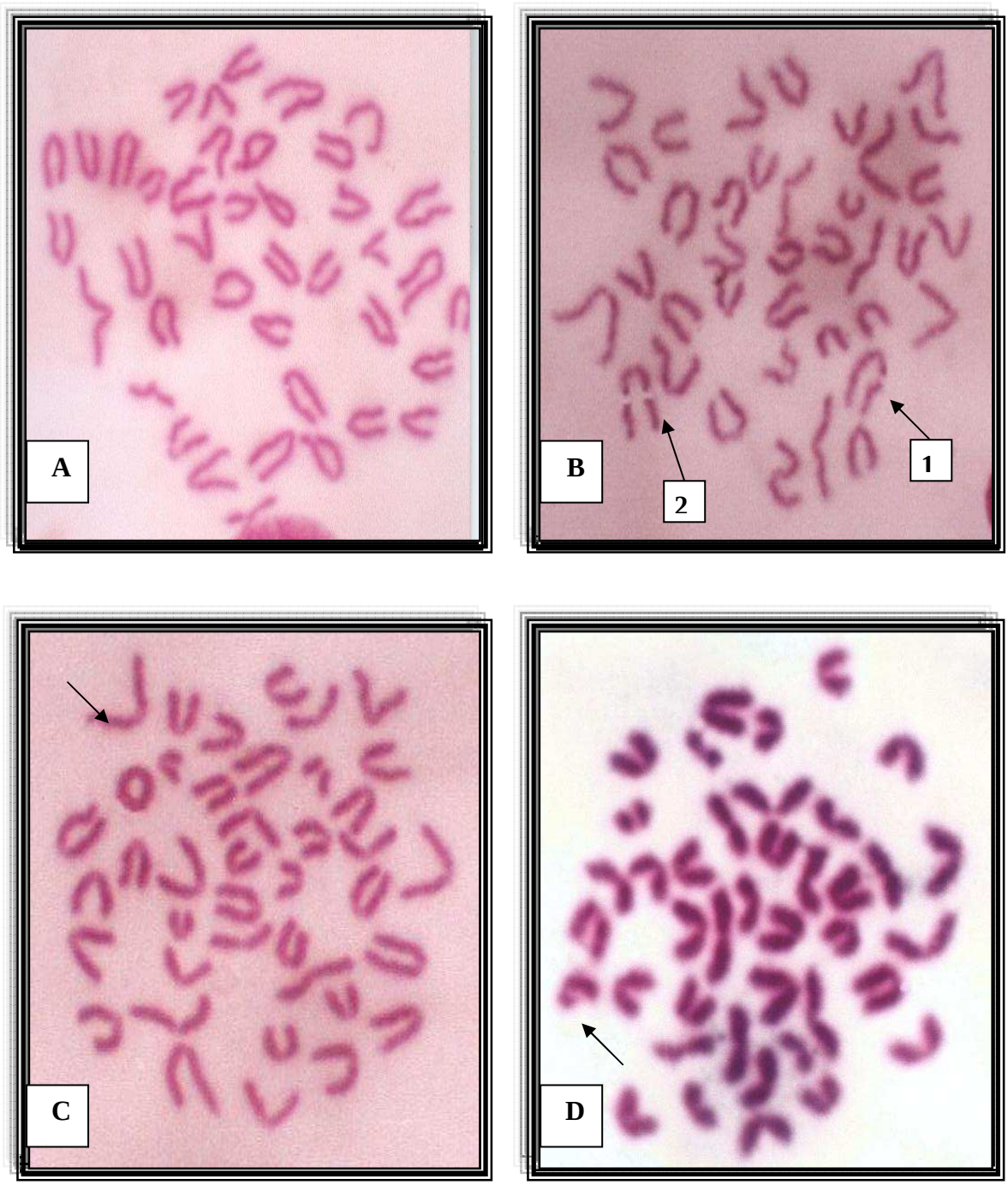


Figure (3-8): Metaphase of mouse bone marrow cells showing; normal chromosomes (A), 1.gap 2.chromosome break (B), ring (C), deletion (D). 1000X

3.6.2 Cytogenetic effect of STa on human blood lymphocytes (From normal healthy persons)

3.6.2.1 STa effect on mitotic index (MI)

The mitotic index of normal untreated human blood lymphocytes (negative control) was (3.870%) as shown in table (3-3).

However, neither the high concentration nor the low one of STa caused significant differences (P>0.05) in the MI compared with negative control. Five different concentrations were used in this study to evaluate the cytogenetic effect of STa on the MI and it was found to be (4.01, 3.850, 3.510, 3.550 and 3.630%) at concentrations of (100, 200, 400, 800 and 1600 µg/ml) respectively. Significant decrease (P<0.05) in the MI was detected after treatment with MMC (1.990%) at (5) µg/ml as compared with the negative control. This result indicated that MMC caused inhibition of mitosis which was either due to the formation of an incomplete metaphase of chromosomes or altered arrangement of spindle microtubules (Arutyunyan *et al*., 2004; Wick and Gebhart, 2005).

Significant increase in the MI was induced after treatment with some herbal plant extracts that may contain active constituents which may stimulate the mitosis process (Mowery, 1986; Hag *et al*., 1995; Hag *et al*., 1998). However, non significant differences that were indicated after treatment with STa may be because of that STa does not have any effect on the mitosis process.

3.6.2.2 STa effect on Blast index (BI)

The blast index of human blood lymphocytes of the negative control was (54.10%) as shown in table (3-3). The treatment with different concentrations of STa did not cause any significant differences (P>0.05) in the BI of human blood lymphocytes (*in vitro*) as compared with the negative control. The BI values were (53.20, 54.30, 55.10, 51.70 and 52.30%) at concentrations of (100, 200, 400, 800 and 1600 µg/ml) respectively.

A Significant decrease in the BI was noticed after treatment with (5) µg/ml of MMC which reached to (32.50%) compared to the negative control. Such reduction in both MI and BI indicated that MMC has a cytotoxic activity on human blood lymphocytes as had been indicated by some investigators (Filoni *et al*., 1995; AL-Obaidy, 2001; Wick and Gebhart, 2005). Increased percentage of BI was seen after treatment of human blood lymphocytes with many herbal plants due to the presence of active compounds in their extracts that stimulate blastogenesis or blast transformation of lymphocytes (Mowery, 1986, AL-Kayat, 1999). STa did not show to inhibit blastogenesis like MMC or to stimulate blast transformation as many herbal plants, so no significant differences in BI were seen after treatment of human blood lymphocytes with STa.

3.6.2.3 STa effect on micronucleus (MN) induction

The spontaneous frequency of MN after treatment with STa did not differ significantly ($P>0.05$) as compared with negative control (1.390%), it was (1.420, 1.170, 1.440, 1.270 and 1.390%) for STa concentration of (100, 200, 400, 800 and 1600 µg/ml) respectively (table 3-3). Significant increase ($P<0.05$) in the MN frequency was occurred after treatment with MMC (3.060%) at (5) µg/ml. Similar results were also obtained by (Fauth *et al*., 2000; Zhang *et al*., 2002; Undeger *et al*., 2004; Okamoto, 2005), they had proved that MMC is a powerful mutagenic anticancer drug.

While significant decrease ($P<0.05$) in the MN frequency was detected after treatment with many plant extracts, especially that contain quercetin as their active constituent and which in fact was used to reduce the DNA damage caused by MMC by showing antimutagenic activity (Sahelian, 2004)..

STa neither showed mutagenic nor antimutagenic activities, so no significant differences were seen after treatment with it.

Table 3-3 normal person cyto

3.6.2.4 STa effect on chromosomal aberrations (CAs)

In this study the types of CAs which have been observed were; ring, gap, acentric, dicentric, chromosome break, chromatid break and deletion. The percentage of total CAs in normal human blood lymphocytes culture was (0.204%). A significant increase ($P<0.05$) in total and in all types of CAs was noticed after treatment with MMC (0.857%) at (5) µg/ml, as compared with the negative control (table 3-3, figure 3-9). Similar results were obtained by (Latt, 1974; Tijima and Mrimoto, 1991; Baohong *et al*., 2005; Siddique and Afzal, 2005), in which, they found that MMC was effective in inducing high CAs percentage due to its ability to cause high DNA damage even with less concentrations. While significant decrease ($P<0.05$) in the CAs frequency was seen after treatment with many plant extracts that may contain active constituents, which showed powerful free radical scavengers or antimutagenic activities (Ghaskadbi *et al*., 1992).

However, non significant differences ($P>0.05$) in total and in all types of CAs were obtained (0.197, 0.20, 0.193, 0.208 and 0.202%) after treatment with different concentrations of STa (100, 200, 400, 800 and 1600 µg/ml) respectively, as compared with the negative control at (table 3-3).

This indicates that, STa lacks the effect of both MMC and plants extracts, by inducing or reducing the frequency of CAs.

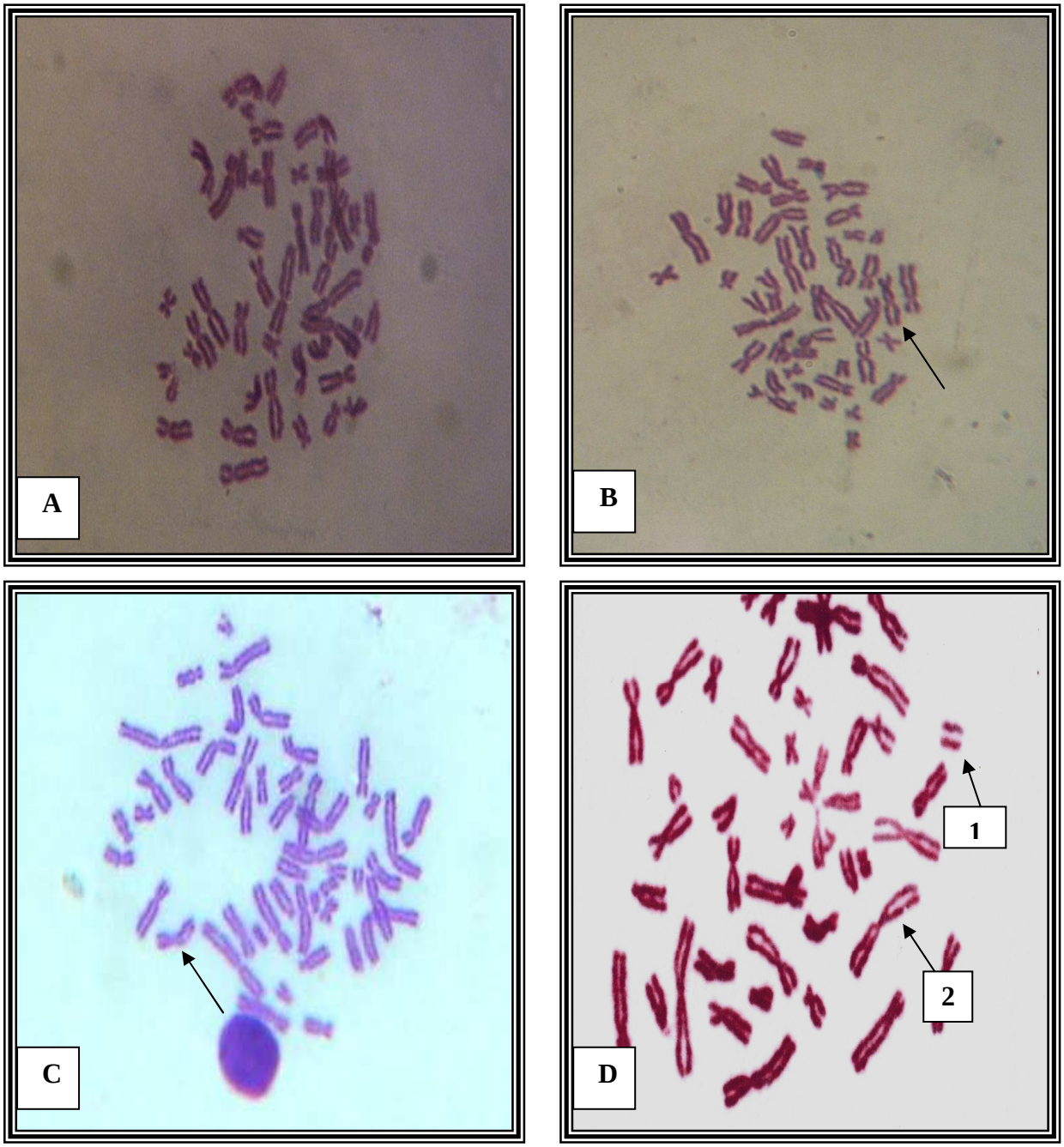


Figure (3-9): Metaphase of human blood lymphocytes showing; normal chromosomes (A), dicentric (B), deletion (C), 1. acentric, 2. gap (D). 1000X

3.6.3 Cytogenetic effect of STa on human blood lymphocytes (From colorectal cancer patients)

3.6.3 STa effect on mitotic index (MI)

Upon normal conditions and without any kind of treatments, cancer cells have a reduced MI percentage as compared with normal cells. This may be because cancer cells themselves secret some substances that cause certain chromosomal damage or inhibit the protein kinase (that play an important role in the cell cycle), thus affecting the metabolic processes that causing the cells to transfer from G_2 phase to M phase, beside most of the DNA damage can not be repaired (Clifford *et al*., 1981; Vernole *et al*., 1988; Udayakumar and Bhargava, 1994; Jennifer, 2000). As a result, cancer cells have abnormal cell cycle due to the large amount of damage in their DNA which in turn, affects the regulation of both mitosis and apoptosis processes leading to a very low number of cells that have the ability to cross the G_2 phase and thus they had reduced MI (Mitelman *et al.*, 1976; Anthony *et al*., 1999).

In this study, the MI of the negative control (untreated) human blood lymphocyte culture from colorectal cancer patients was (1.665%), as shown in table (3-4).

A gradual inhibition in MI values was noticed after using different concentrations of STa, and such inhibition was found to be concentration dependent. Although there was a gradual decrease in MI which reached to (1.630, 1.560, 1.470 and 1.305%) upon using the concentrations of (100, 200, 400 and 800µg/ml) respectively, but this inhibition was not significantly different ($P>0.05$) compared with negative control as shown in table (3-4). However, significant inhibition ($P<0.05$) in MI was achieved after treatment with STa at a concentration of (1600) µg/ml which reached to (1.105%), and this inhibition was appeared to be near to that caused by MMC (1.050%) at a concentration of (5) µg/ml. Similar results for the reduction in MI after MMC

treatment of blood lymphocytes culture from colorectal cancer patients were indicated by (Barlogie and Drewinko, 1980). It was shown that MMC had less inhibitory effect on cancer cells compared with normal cells, and one of the major reasons for that is; cancer cells have certain malformations in their cytoplasmic membrane which prevent entry of MMC into the cells leading to the inhibition of cell proliferation and this in turn explain the resistance of some cancer patients to chemotherapy drugs (Roger *et al*., 1993).

However, even the significant reduction in MI was resulted only after treatment with the highest concentration of STa, but a gradual inhibition in MI was occurred and it was concentration dependent, and may be if much higher concentrations were used, more inhibition in MI may occurred. One explanation for that is the presence of circulating colorectal cancer cells which migrated from the primary cancer site to another sites in the body by the bloodstream (Fehm *et al*., 2002). Since STa bind only to specific receptors GC-C and these receptors are expressed only by colorectal cells, and with the presence of circulating colorectal cancer cells in the blood, STa can bind to these cells and inhibits their proliferation, which was expressed by the reduction in MI percentage.

3.6.3.2 STa effect on blast index (BI)

Cancer patients usually have reduced BI when compared with normal healthy persons. The process of blastogenesis starts by the binding of certain mitogen (PHA) with certain surface receptors on lymphatic cells, then a chain of biochemical events occurs (Daniel *et al*., 1987). The reduction in the BI may be due to several reasons such as; malformations in the surface receptors to which mitogen binds or a defect in the biochemical chain which work after the binding between receptors and mitogens, and this may have occurred because of the secretion of certain hormones with abnormal biochemical activity, or

certain other substances that interfere with the cellular metabolic process by cancer cells (Anderson, 1985; Peter and Kolasek, 1997; AL-Amiry, 1999).

The BI of untreated blood lymphocytes culture from colorectal cancer patients was (30.10%) which was considered as a negative control as shown in table (3-4). A gradual inhibition in BI took place after treatment with different concentrations of STa, and this inhibition was concentration dependent. The BI when using concentrations of (100, 200 and 400µg/ml) reached to (29.90, 29.40, and 28.250%) respectively, was not significantly different ($P>0.05$) from the negative control. While the significant reduction ($P<0.05$) in BI was started from concentration (800) µg/ml, which reached to (25.80 and 22.0%) at concentrations of (800 and 1600µg/ml) respectively, as shown in table (3-4). One explanation for this gradual inhibition, as was discussed previously is the presence of circulating cancer cells, to which STa bind through the GC-C receptors and the resulting subsequent inhibition of the proliferation of these cells which was indicated by reduction in BI.

A significant decrease ($P<0.05$) was also shown after treatment with MMC (21.10%) at concentration of (5) µg/ml. Similar results were obtained by (Barlogie and Drewinko, 1980). Many anticancer drugs may act by making certain defects in the receptors to which mitogens bind, and these defects may prevent or reduce the occurrence of this binding (McCowage *et al*., 1996), and as a result reducing the BI.

This reduction in both MI and BI indicate that STa has a cytotoxic effect on human blood lymphocytes from colorectal cancer patients.

Table 3-4 cancer table

3.5.3.3 STa effect on micronucleus (MN) induction

The human blood lymphocytes obtained from cancer patients showed much higher MN frequency compared with those from normal healthy persons. This may be due to higher DNA lesions that associated with cancer development. However, unrepaired and aberrantly repaired DNA lesions that results from DNA damage, can lead to genomic instability and malignant progression (Skorski, 2002; Huebner and Croce, 2003).

No significant differences ($P>0.05$) were recorded in the frequency of MN after treatment with different STa concentrations as compared to the negative control (3.350%). As shown in table (3-4), the recorded frequencies were (3.310, 3.290, 3.435, 3.220 and 3.460%) at concentrations of (100, 200, 400, 800 and 1600µg/ml) respectively. A significant increase ($P<0.05$) in MN frequency was occurred after treatment with MMC (4.510%) at (5) µg/ml. Similar results were obtained by Nesti *et al*., (2000) who showed that MN induction increased after MMC treatment even at (0.1) µg/ml, and it was concentration dependent.

3.6.3.4 STa effect on chromosomal aberrations (CAs)

Chromosomal aberrations may act as a fundamental pathophysiological event in human carcinogenesis (Bonassi *et al*., 1995; Hagmar, 1998; Platzer *et al*., 2002), a common example includes inactivation of tumor suppressor genes by chromosomal deletions (LeaBeau, 1997). Thus, cancer cells show a higher CAs percentage compared with the normal cells (Gebhart, 1993; Hagmar *et al*., 1994; Liou *et al*., 1999; Tsafrir *et al*., 2006). Virtually all cancers reveal a tumor-specific distribution of genomic imbalance (Mertens *et al*., 1997; Korn *et al*., 1999), and individual tumor-specific chromosomal imbalances are preserved even after years of cell culture (Macville *et al*., 1999).

The percentage of total CAs in human blood lymphocytes obtained from colorectal cancer patients without any treatment was (0.863%) as shown in table (3-4). The types of CAs that were looked for in this study were; ring, gap, acentric, dicentric, chromosome break, chromatid break and deletion. Non significant differences (P>0.05) in total and in all types of CAs were observed after treatment with different concentrations of STa (0.877, 0.857, 0.848, 0.849 and 0.867%) at concentrations of (100, 200, 400, 800 and 1600µg/ml) respectively, as compared with the negative control (table 3-4).

Although STa reduced both the MI and BI, but it did not show to have the ability to cause DNA damage and thus non significant differences were seen with MN and CAs after treatment with STa. Indeed, STa is structurally and functionally homologous to the endogenous peptides (guanylin and uroguanylin) which mediate autocrine / paracrine control of intestinal fluid and electrolyte homeostasis (Forte, 1999). Under normal conditions, these two peptides bind to GC-C receptors, which in turn have been implicated in the regulation of the balance of proliferation and differentiation along the crypt-to-villus axis in the intestine (Qian *et al*., 2000; Pitari *et al*., 2001), but the mechanism by which this regulation occur still unknown. In this study, results showed that GC-C, at least, work without causing any damage in the DNA, because if the work of GC-C is combined with DNA damage; this will be a real disaster. However, significant differences (P<0.05) in total and all types of CAs were seen after treatment with MMC (1.474%) at (5) µg/ml, as compared with the negative control. Although MMC was used effectively in treating of different types of cancers, including colorectal cancer (Sato *et al*., 1990; Chong *et al*., 2005), but certain CAs were found to be associated with MMC treatment (Heim, 1985; Murty *et al*., 1987; Nakamori *et al*., 2003).

3.7 Enzymatic Assays: Effect of STa on Liver Functional Enzymes (GOT, GPT, ALP) Levels in Mouse

In this study the effect of STa on GOT (glutamate oxaloacetate transaminase), GPT (glutamate pyruvate transaminase) and ALP (alkaline phosphatase) levels have been investigated. Results in table (3-5) showed that non significant differences ($P>0.05$) in GOT level was seen after treatment with STa when compared to that of the negative control (190.33) U/L, and the recorded levels were (186.33, 196.0, 194.0, 188.67 and 192.67U/L) at doses of (100, 200, 400, 800 and 1600µg/Kg) respectively. While significant increases ($P<0.05$) were seen after treatment with MMC which reached up to (231.33 and 270.0 U/L) at doses of (2 and 5µg/Kg) respectively, as compared with the negative control.

Regarding GPT level, STa treatment did not cause any significant changes ($P>0.05$) as compared with that of the negative control (62.33) U/L. The GPT levels were (63.67, 57.0, 59.0, 58.33 and 62.67U/L) at STa doses of (100, 200, 400, 800 and 1600µg/ml) respectively. On other hand significant increases ($P<0.05$) were seen after MMC treatment which reached to (83.33 and 98.0U/L) at doses of (2 and 5µg/Kg) respectively in comparison with negative control.

At the same time, no significant differences ($P>0.05$) in ALP level were indicated after STa treatment, as compared with the negative control (65.33) U/L. They were (66.33, 68.33, 63.0, 63.33 and 62.0U/L) at doses of (100, 200, 400, 800 and 1600µg/Kg) respectively. Significant increases ($P<0.05$) were also seen after MMC treatment which reached to (87.67 and 105.33U/L) at doses of (2 and 5µg/Kg) respectively, as compared with the negative control. The levels of GOT, GPT and ALP enzymes in the serum were increased after treatment with several chemotherapeutic drugs including the MMC. This increase may be due to that, these drugs have cytotoxic effect on liver cells and this leads to increase the permeability of liver cell membrane or to cause damages in liver

tissue and thus causing the release of high quantity of these enzymes into blood (Bonnefoi *et al*., 1989; D'Mello *et al*., 1999; AL-Obaidy, 2001).

Table (3-5): STa effect on liver functional enzymes (GOT, GPT, ALP)

Treatment	GOT m±SE	GPT m±SE	ALP m±SE
PBS	a 190.33±7.51	a 62.33±6.03	a 65.33±4.51
STa 100 µg/ml	a 186.33±7.09	a 63.67±5.03	a 66.33±3.51
STa 200 µg/ml	a 196.00±7.55	a 57.00±2.00	a 68.33±3.21
STa400 µg/ml	a 194.00±6.00	a 59.00±4.51	a 63.00±1.53
STa800 µg/ml	a 188.67±5.13	a 58.33±5.51	a 63.00±2.00
STa1600 µg/ml	a 192.67±4.04	a 62.67±6.11	a 62.00±5.00
MMC2 µg/ml	b 231.33±6.51	b 83.33±5.51	b 87.67±5.69
MMC5 µg/ml	c 270.00±6.24	c 98.00±6.08	c 105.33±4.51

Differences a, b are significant (P<0.05) to compression rows

Banerjee *et al*., (1994) pointed that most of the chemical compounds work by inhibiting the activity of detoxification enzymes such as superoxide dismutase (SOD) and glutathione-S-transferase (GST) that scavenging free radicals from the cell. On other hand, some plants such as black seed, were shown to decrease the level of these enzymes after certain infection or treatment with certain drug, as a result of having antioxidant and anticytotoxic activities, (Mahmoud *et al*., 2001; Hassan, 2002).

Thus, non significant changes in the level of GOT, GPT and ALP after STa treatment indicate that STa neither causes liver damage or increases liver cell membrane permeability nor has antioxidant activity. However, this result was proved the previous result that obtained when studying the clinicopathological

effects of STa on mouse, which indicated that STa also did not cause any pathological changes in liver, because if it has, a significant changes were expected to seen in the level of these enzymes.

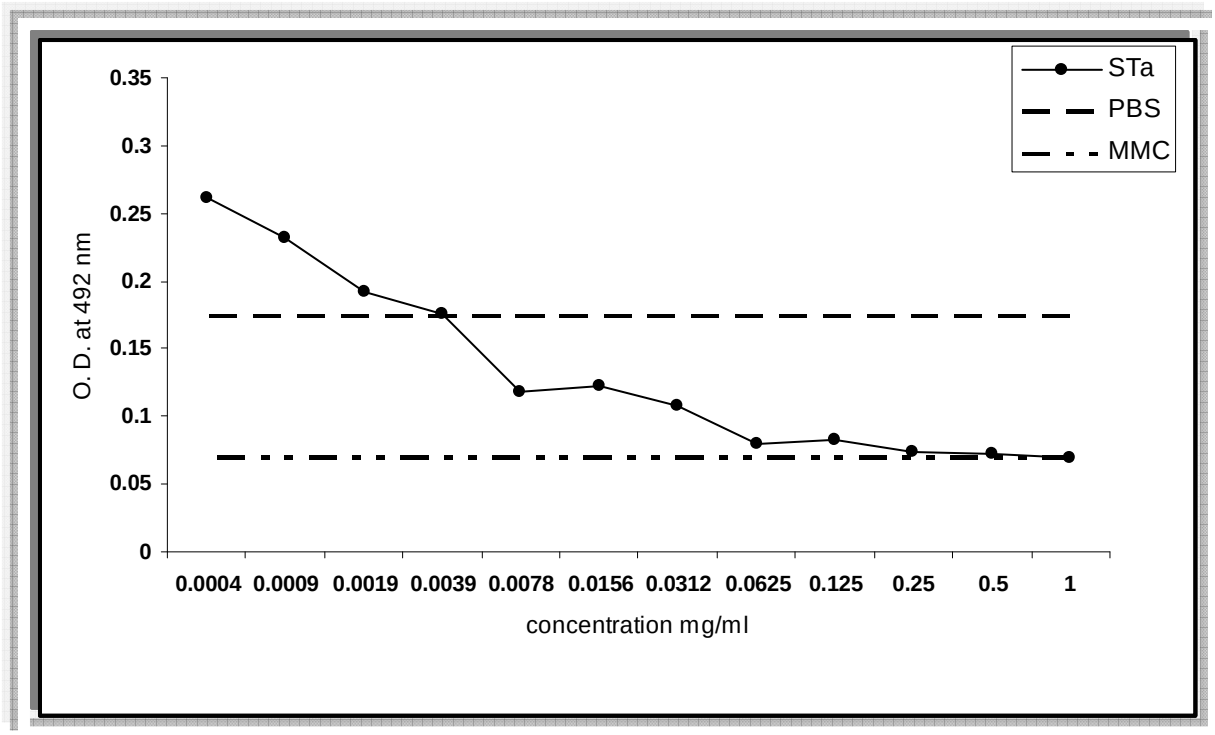
3.8 Cytotoxicity of STa on Primary Cancer Cell Cultures and Normal Cell Line

3.8.1 Cytotoxic effect of STa on primary cancer cell cultures

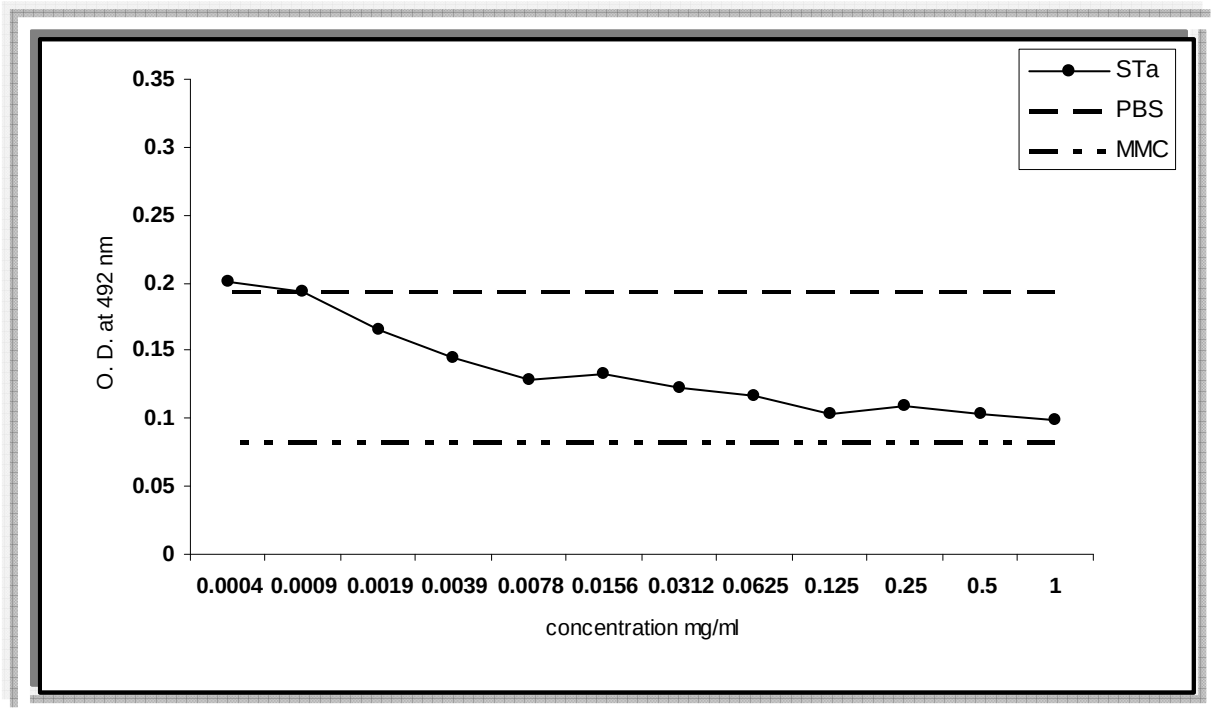
The cytotoxic effect of STa on primary cancer cell cultures was determined by evaluating its effect on: five tumor tissues obtained from patients with colon cancer, two tumor tissues obtained from patients with bladder cancer, two tumor tissues obtained from patients with breast cancer, two tumor tissues obtained from patients with stomach cancer and two tumor tissues obtained from patients with lung cancer.

Results of the cytotoxic effect of STa on tumor tissues obtained from five different colon cancer patients showed that STa caused an obvious inhibition in the growth of cancer cells compared with the negative control and this inhibition was close to the inhibition that was seen with the positive control (MMC, at concentration of 10µg/ml), as shown in figure (3-10/ A, B, C, D and E). These results that associated with growth inhibition of primary colon cancer cell culture after STa treatment were similar to those obtained by many authors (Pitari *et al*., 2001; Ghanekar *et al*., 2003; Morgan, 2003; Pitari *et al*., 2003; Carrithers *et al*., 2003; Pitari *et al*., 2005), and in fact some of them were able to reduce the growth of cancer cell lines with less concentrations of STa. They showed that STa induce colorectal cancer cytostasis without cell death by targeting guanylyl cyclase-C (GC-C) signaling and the anticancer action of this toxin is mediated by cGMP that dependent influx of Ca^{+2} through the cyclic nucleotide-gated channels. Thus, GC-C is the specific receptor for STa, and without it, STa will be unable to exhibit its cytostasis effect (Mann *et al*., 1997; Charney *et al*., 2001; Ghanekar *et al*., 2004; Albano *et al*., 2005). In fact some

chemotherapeutic drugs show their cytotoxic effect by activation of cGMP (Pitari *et al*., 2006).

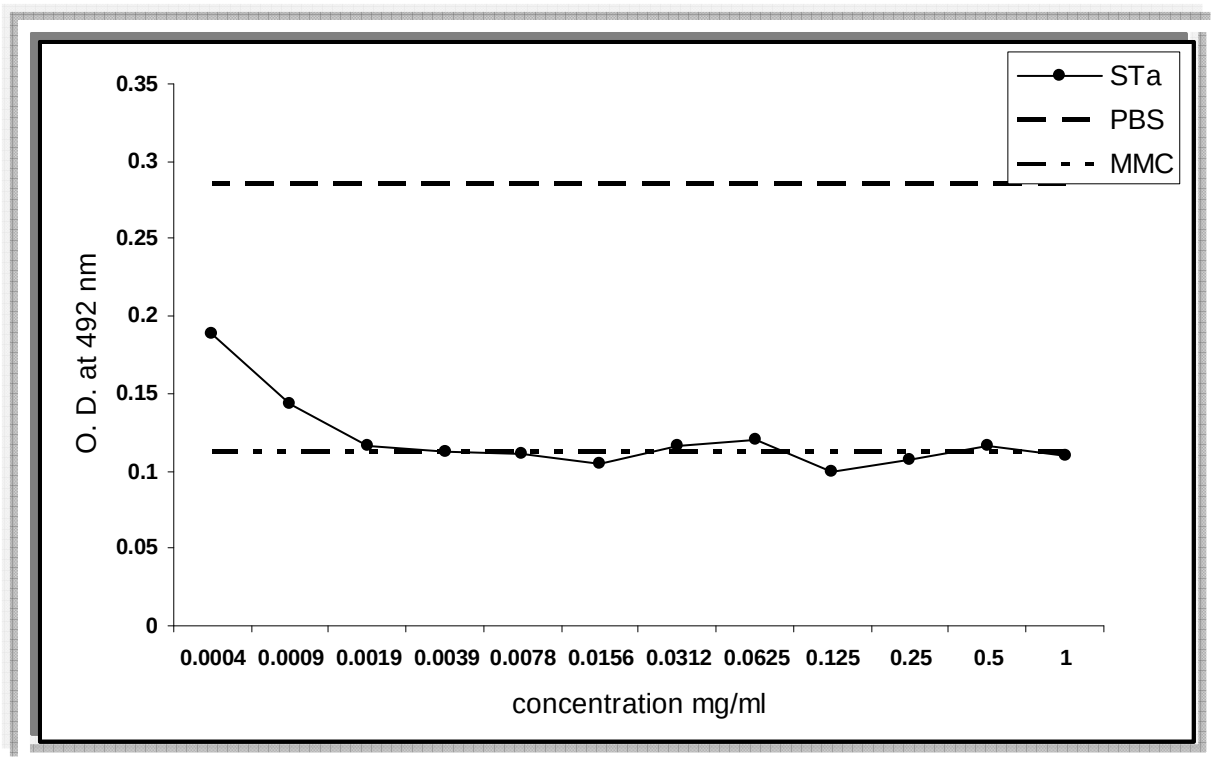


(A)

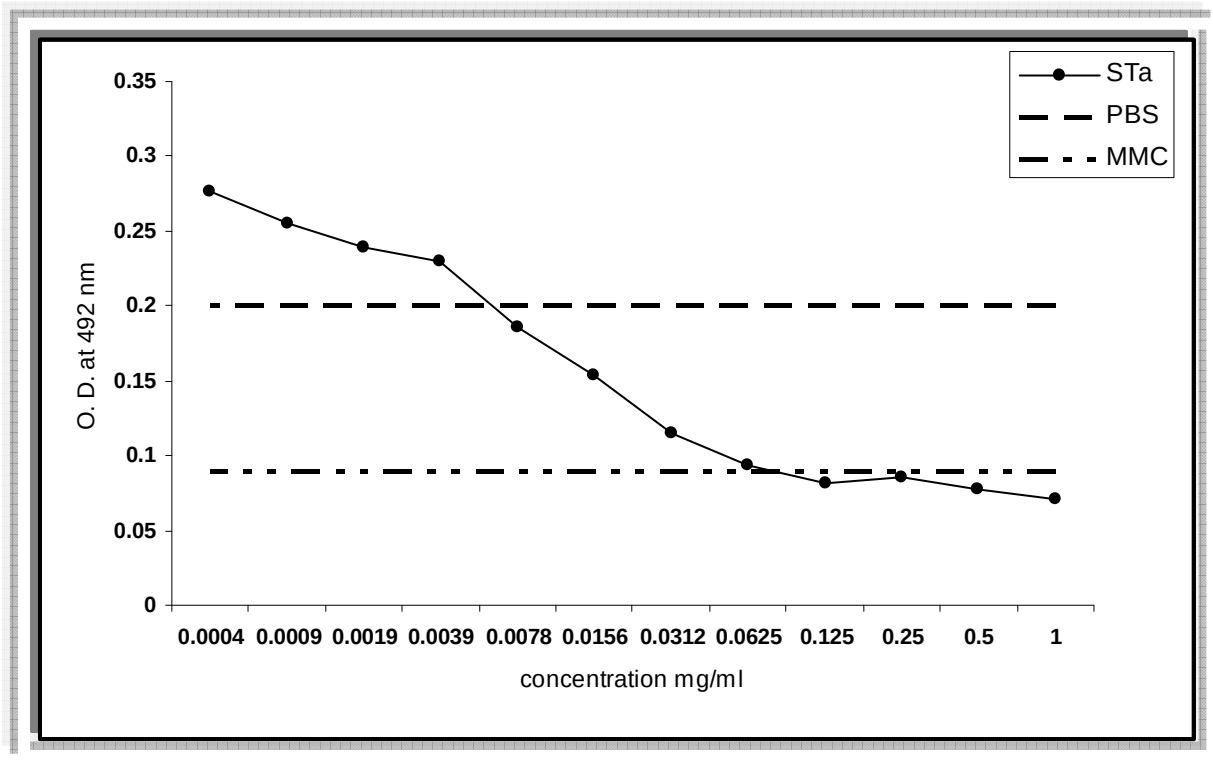


(B)

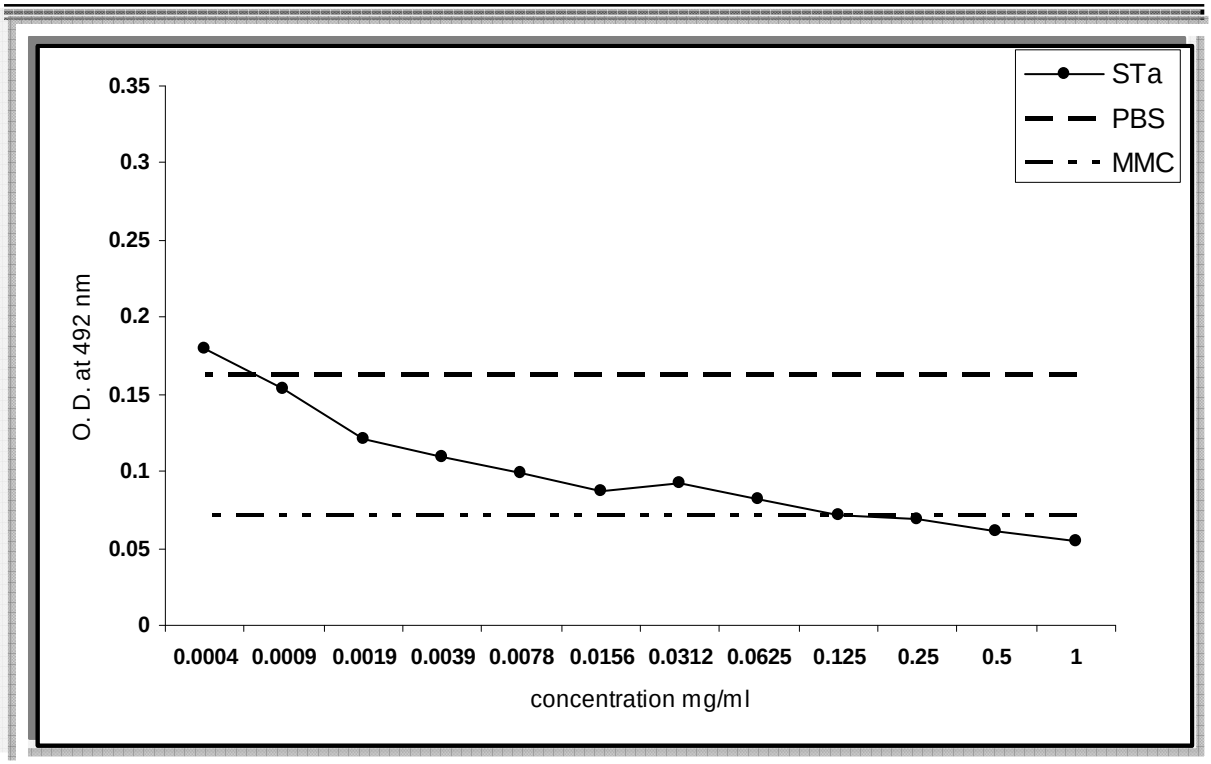
Figure (3-10): Cytotoxic effect of STa on primary cancer cells cultures obtained from five patients with colon cancer.


STa
PBS
MMC
O. D. at 492 nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.0004 0.0009 0.0019 0.0039 0.0078 0.0156 0.0312 0.0625 0.125 0.25 0.5 1
concentration mg/ml

(C)


STa
PBS
MMC
O. D. at 492 nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.0004 0.0009 0.0019 0.0039 0.0078 0.0156 0.0312 0.0625 0.125 0.25 0.5 1
concentration mg/ml

(D)

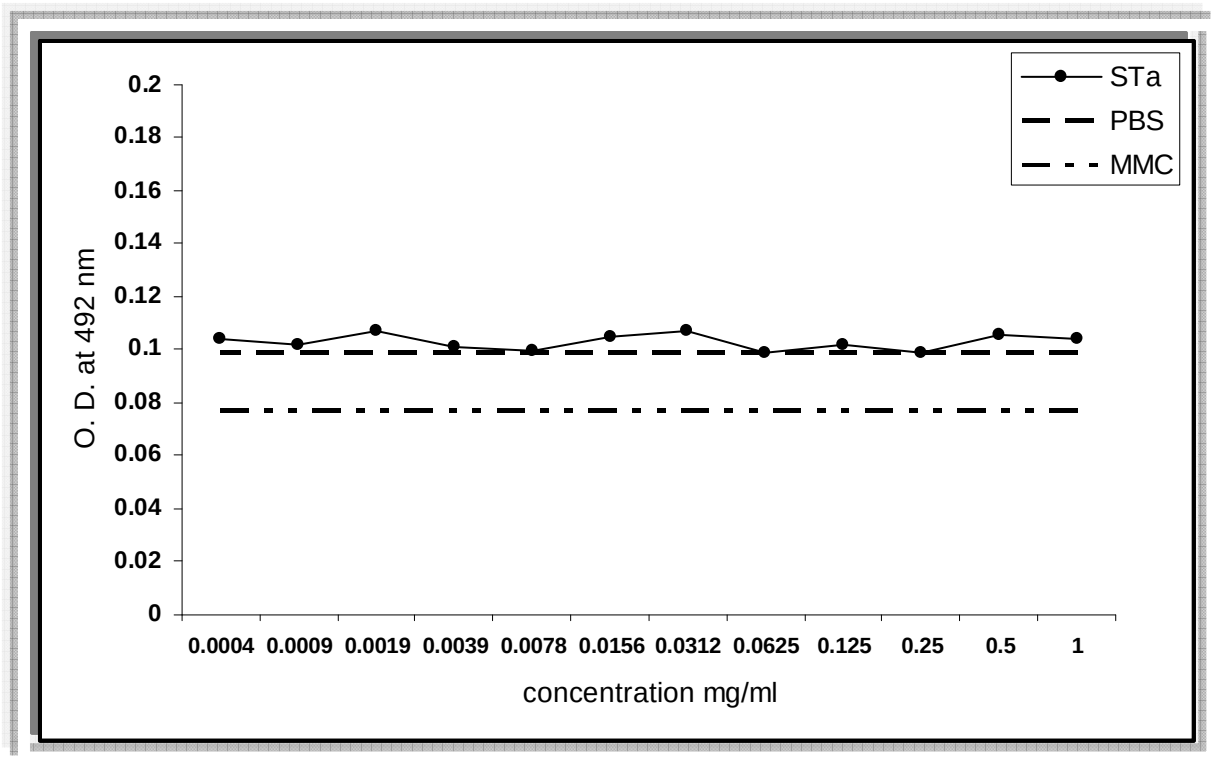


(E)

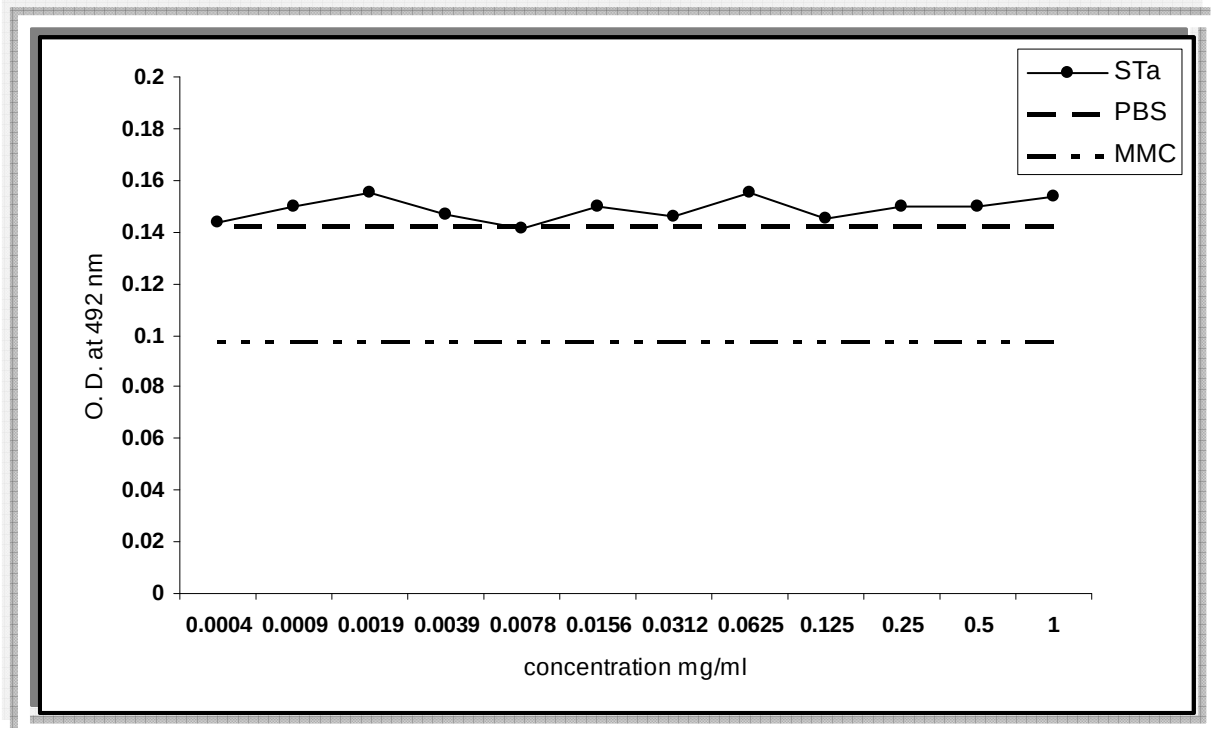
Previous studies showed that STa penchant for intestinal cells only, indicating that, as a drug, it would focus just on these cells and leaves others alone, and upon its injected into the blood it might even specifically combat colon cancer cells that had been migrated to other parts of the body. Thereby, derailing metastasis which is a serious problem in this type of cancer. Pitari and coworkers predicated that even if this toxin only slow the growth of colon cancer cells without killing them, it speculate that it may lead to possible therapy that would control colorectal cancer spread.

Thus, if the sacrifice for one is to have occasional diarrhea and prevent the tumor in the colon from ever forming or progressing, it worth's it (Carrithers *et al*., 2003).

At the same time the cytotoxic effect of STa on primary cancer cell cultures obtained from other organs have been also studied. Results showed that STa had no inhibitory effect on the growth of cancer cells obtained from (bladder, breast, stomach and lung cancer) as shown in figures (3-11, 3-12, 3-13 and 3-14, A and B) respectively.

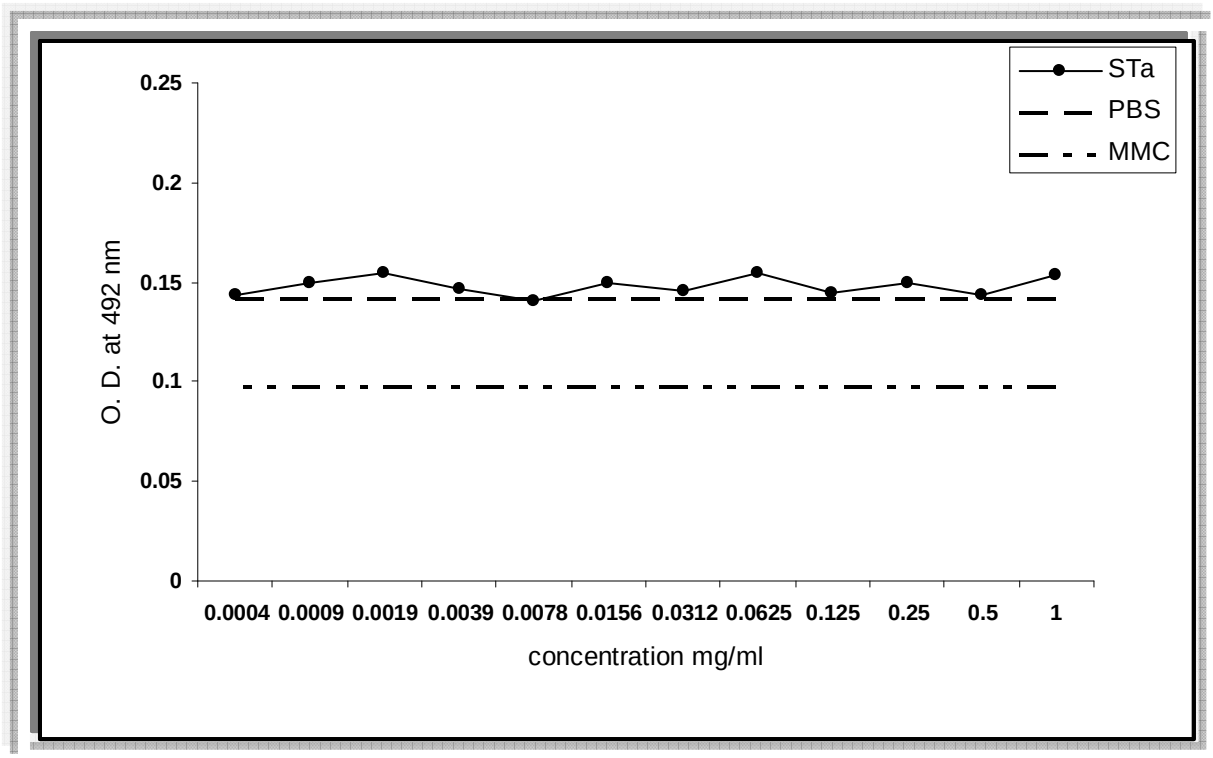


(A)

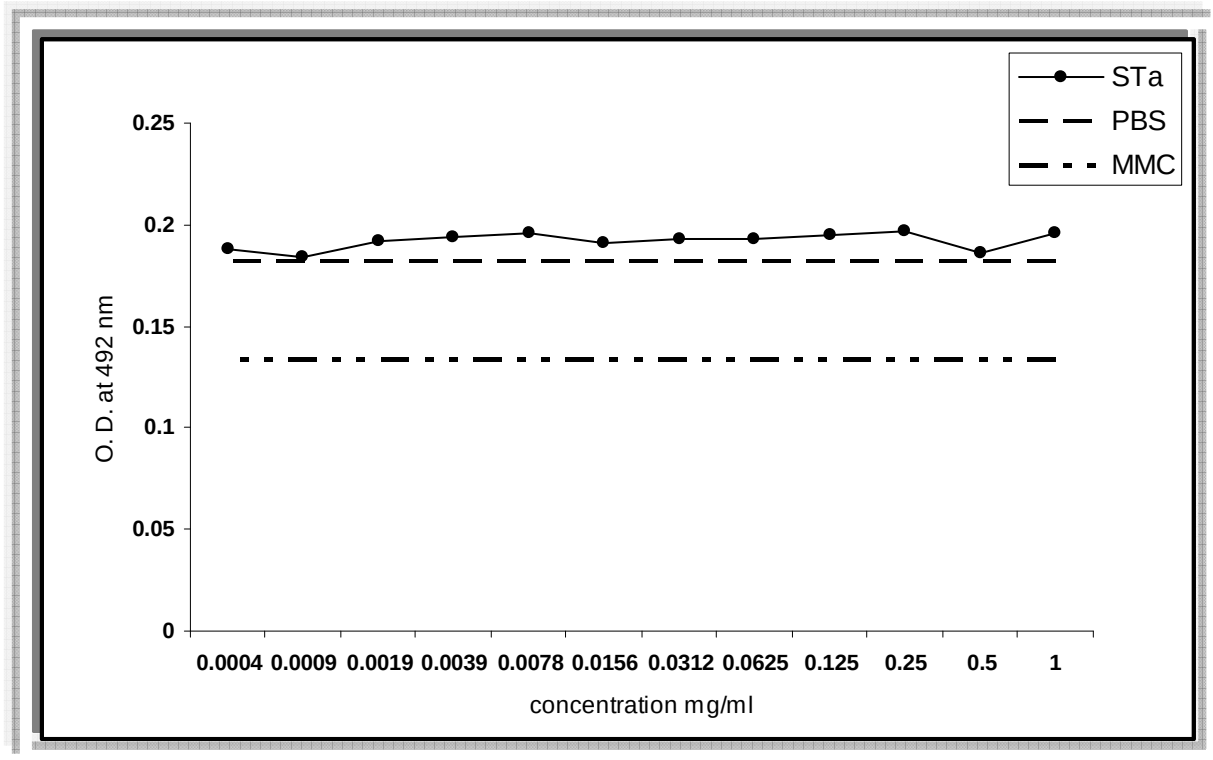


(B)

Figure (3-11): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with bladder cancer.

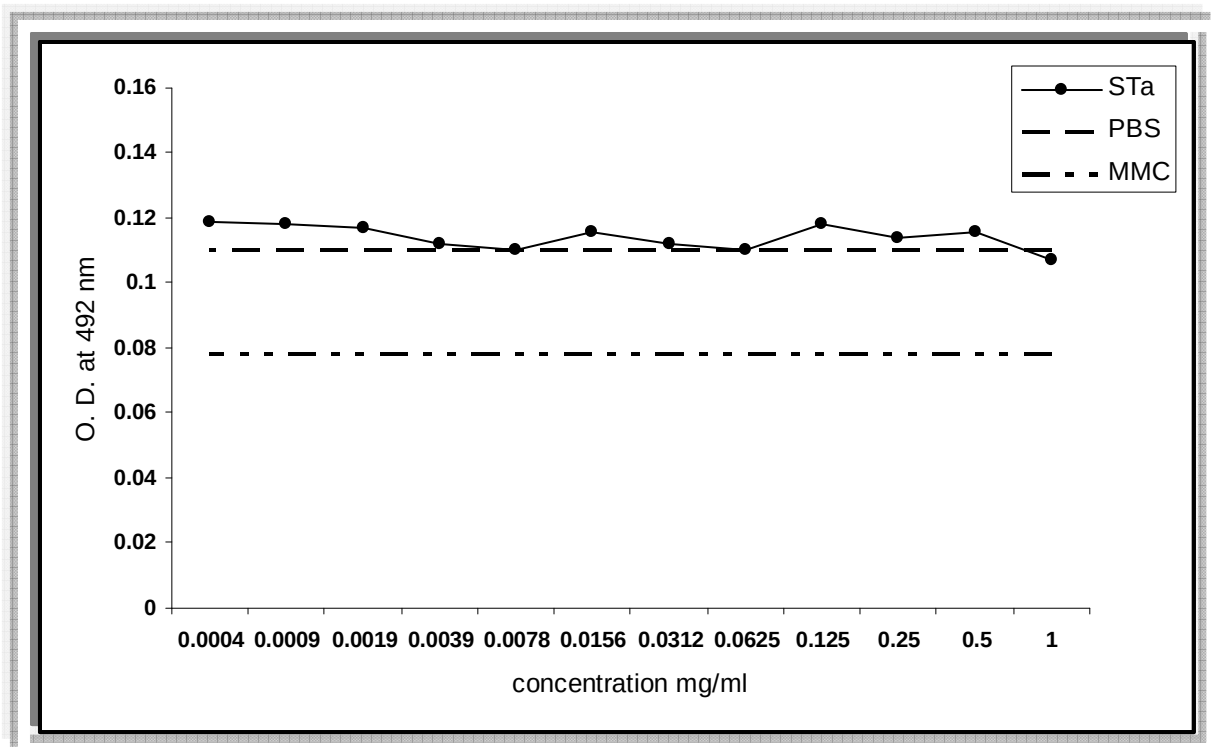


(A)

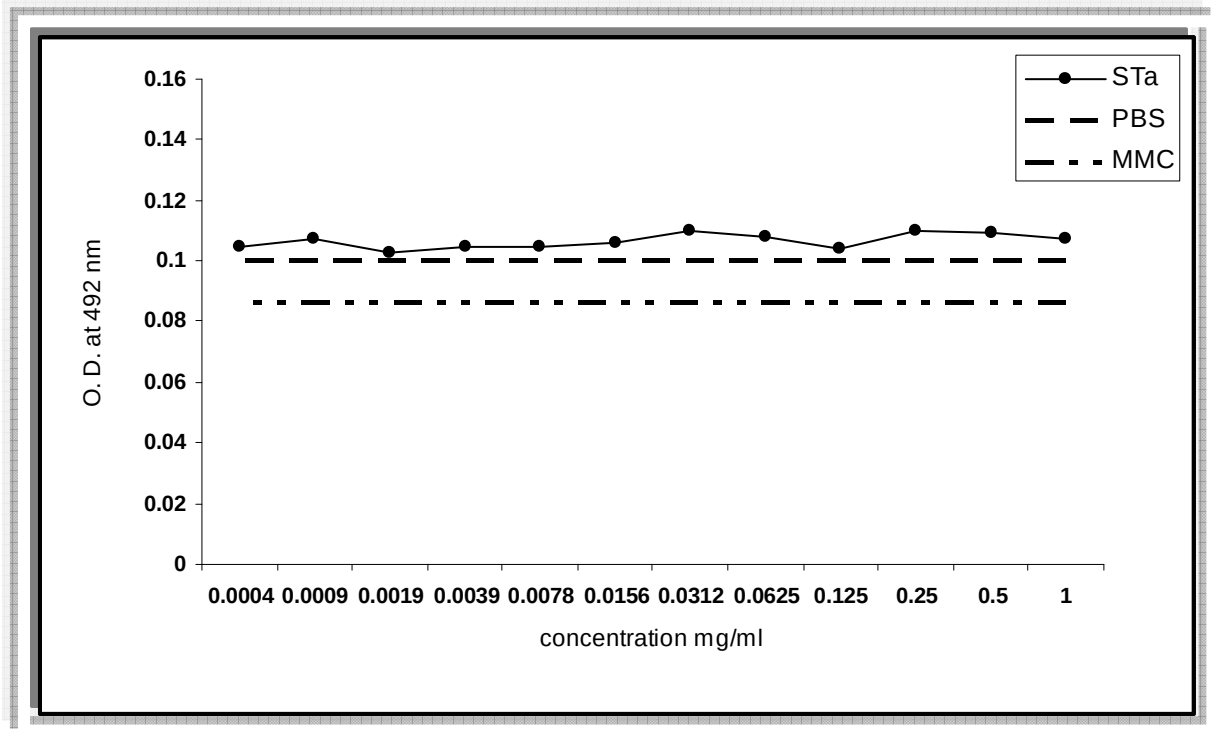


(B)

Figure (3-12): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with breast cancer.

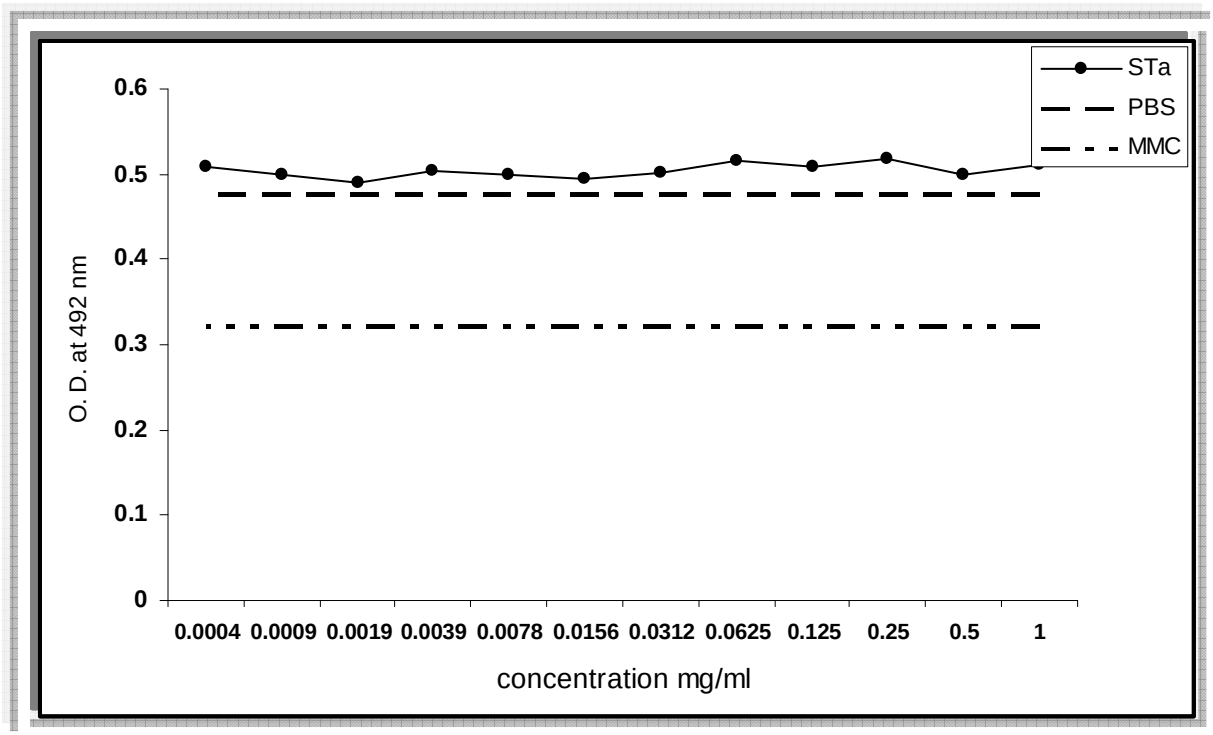


(A)

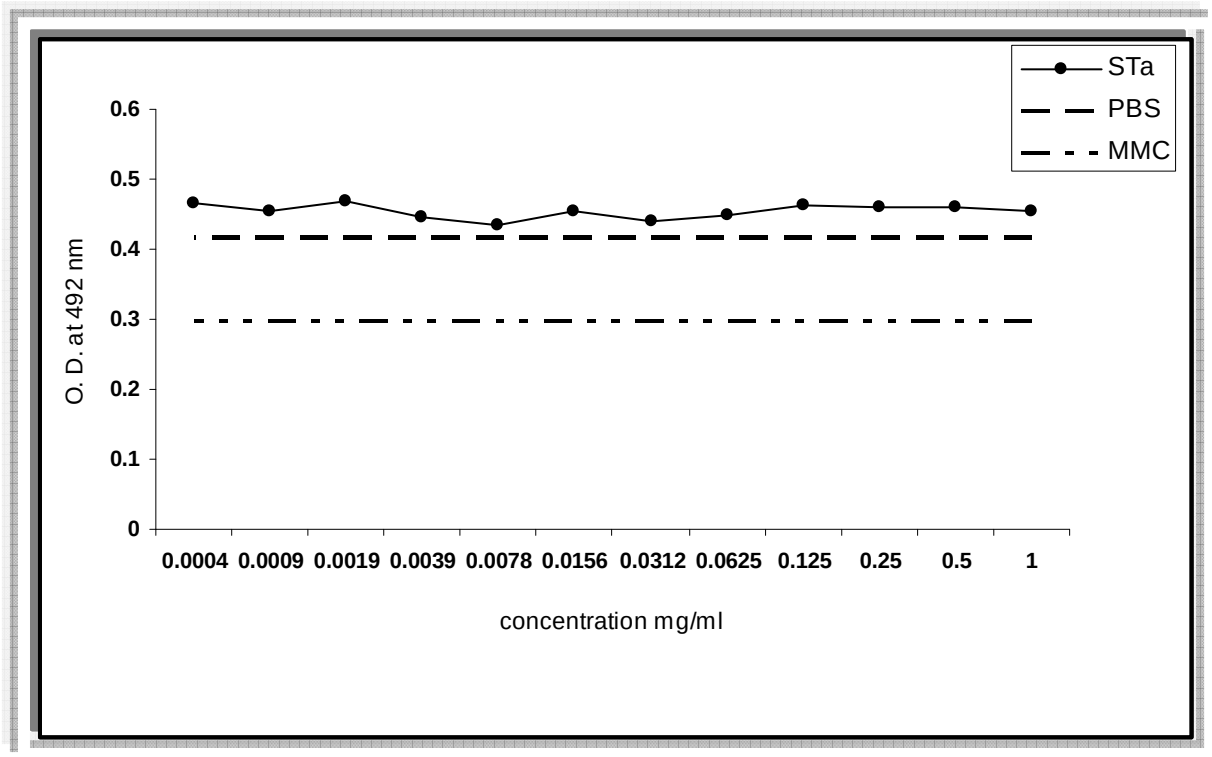


(B)

Figure (3-13): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with stomach cancer.



(A)



(B)

Figure (3-14): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with lung cancer.

The most reasonable explanation for the absence of STa effect on these primary cancer cell cultures is that STa does not bind to these cells due to the absence of the expression of GC-C receptors to which STa binds and exerts its effect (Albano *et al*., 2005).These results were in close agreement with those declared by (Krause *et al*., 1994; Carrithers *et al.*, 1996; Cagir *et al*., 1999), in which they examined the binding affinity of STa to extraintestinal tissues by using many different types of cancers and with the using of more precise techniques including reverse transcription-polymerase chain reaction (RT-PCR), they showed that STa binding were not detected in all these extraintestinal cancers, and thus no cytostasis effect for STa was detected.

3.8.2 Cytotoxic effect of STa on normal cell line (REF)

When any substance was suspected to be used in cancer therapy, it must show a selective toxicity on cancer cells but not on normal cells in order to say it is safe. So, when cancer cell lines are use in any study, normal cell lines should be also use as a control for comparison. In this study, REF (rat embryo fibroblast) cell line was used as a normal cell line. Results shown in figure (3-15) indicated that STa has no cytotoxic effect on normal line (REF) as compared with negative control, and there is an increase in the growth of cells as the STa concentration decrease. This inverse relationship may be explained by the increase in the growth medium concentration at the time of STa concentration decrease, since growth medium concentration play a significant role in the cell culture technique (Freshney, 2000). Similar results were indicated by (Carrithers *et al.*, 1995), who examined the binding and function of STa in normal extraintestinal human tissues and colorectal tumors. They found that STa was able to bind specifically to all colon and rectum tumors that were examined, while neither STa binding nor STa activation of GC-C was detected in all examined normal extraintestinal tissues. In addition, DNA of tumor cells

was found in a relaxant shape, and the DNA molecule was found in unstable figure because of the far away between the H-bonds which connect the both strands of DNA and this makes easy for compounds to interfere or associated to both DNA strands. On the other hand, DNA of normal cells has strong H-bonds that connect the both strands to each others and thus making it more stable, so by this way, the compounds can not interfere or associate with DNA strands (Belijanski, 2002). However, inhibition of normal cell line growth was detected with MMC treatment at a concentration of (10) µg/ml, as compared with the negative control, and this result came in agreement with Wang *et al*., (1999), in which they showed that MMC has toxicity to both normal and tumor cell lines and this toxicity could be increase or enhance by certain substances.

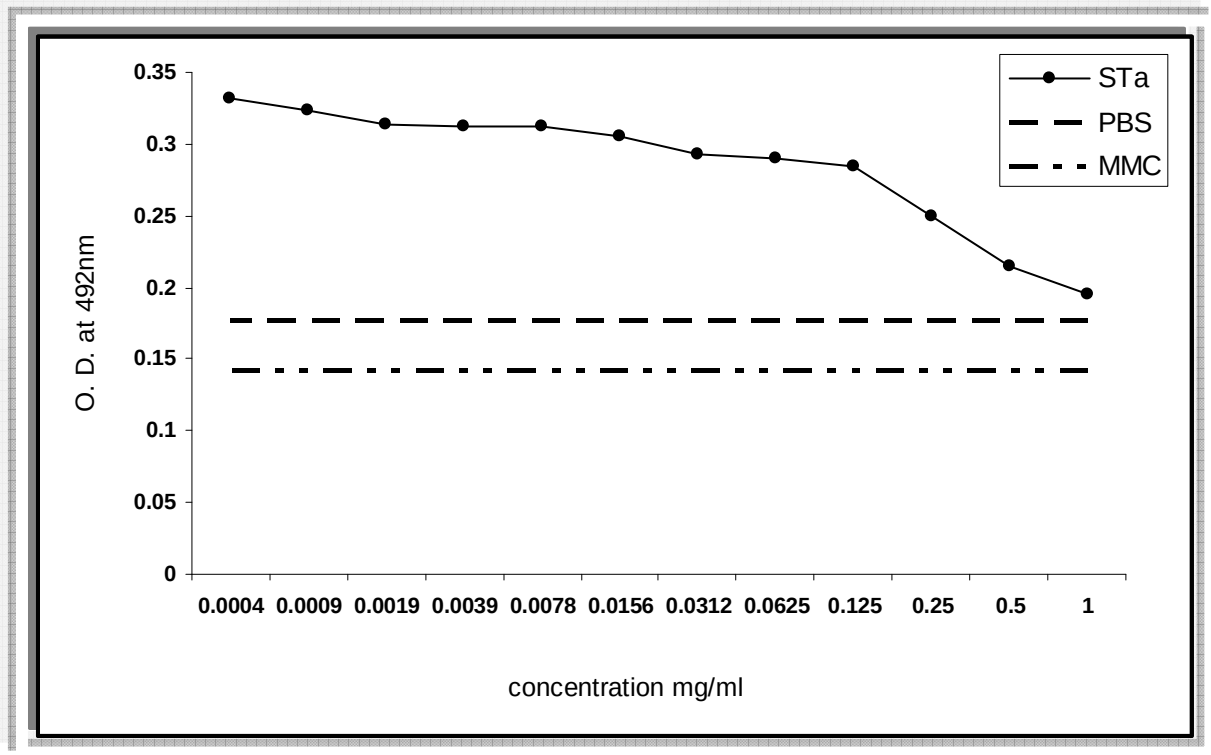


Figure (3-15): Cytotoxic effect of STa on normal cell line (rat embryo fibroblast / REF).

Chapter four: Conclusions and Recommendations

4.1 Conclusions

1. STa has no cytotoxic effect on mouse bone marrow cells (*in vivo*), it did not cause any significant decrease or increase in the spontaneous frequency of CAs, MN or MI, beside no changes in the serum level of liver functional enzyme (GOT, GPT, ALP) or clinicopathological effects were seen.

2. STa has no cytotoxic effect on human blood lymphocytes (*in vitro*) obtained from normal healthy persons, in which neither reduction nor induction was seen in the spontaneous frequency of CAs, MN, MI or BI after STa treatment, while it showed the ability to reduce only the spontaneous frequency of both MI and BI but with no significant changes in the spontaneous frequency of both CAs and MN of the human blood lymphocytes (*in vitro*) obtained from colorectal cancer patients.

3. *In vitro* study proved that STa has cytotoxic effect on primary cancer cell cultures obtained from colon cancer but not on primary cancer cell cultures obtained from other types of cancer.

4. This study showed that, STa has no cytotoxic effect on normal cell line (*in vitro*).

4.2 Recommendations

1. Performing complete purification of STa to obtain more activity and then more cytotoxic effect by using lower concentrations.

2. *In vivo* study of STa on tumor bearing animals.

3. Further studies were required for the STa effect on the inhibition of genotoxic effects produce by many chemotherapeutic drugs including; MMC (interaction study).

4. Further studies were needed for detecting the effect of STa on the immune system.

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Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Chapter Four

Conclusions and Recommendations

References

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

دراسة وراثية خلوية للسم المعوي الثابت حراريا (أ) المنتج من قبل بكتريا ايشيريشيا القولون السمية المعوية على مرضى سرطان القولون والمستقيم وتأثيره على الخلايا السرطانية

أطروحة

مقدمة الى كلية العلوم جامعة النهرين

كجزء من متطلبات نيل درجة الدكتوراه فلسفة

في التقانة الأحيائية

من قبل

مها فخري مجيد الطائي

بكالوريوس تقانة احيائية / جامعة النهرين/ ٢٠٠٠

ماجستير تقانة احيائية / جامعة النهرين/٣ ٢٠٠٢

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Table (3-4): Continued

Chromosomal aberrations % m±SE								
Treatment	**Ring**	**Gap**	**Acentric**	**Dicentric**	**Chromosome breaks**	**Chromatid breaks**	**Deletion**	**Total**
PBS	**a 0.036±0.018**	**a 0.326±0.030**	**a 0.045±0.020**	**a 0.131±0.019**	**a 0.024±0.015**	**a 0.207±0.023**	**a 0.094±0.20**	**a 0.863±0.048**
STa 100 µg/ml	**a 0.038±0.021**	**a 0.305±0.046**	**a 0.052±0.017**	**a 0.130±0.016**	**a 0.027±0.011**	**a 0.218±0.035**	**a 0.107±0.027**	**a 0.877±0.017**
STa 200 µg/ml	**a 0.032±0.022**	**a 0.310±0.032**	**a 0.043±0.018**	**a 0.140±0.022**	**a 0.020±0.014**	**a 0.210±0.025**	**a 0.102±0.030**	**a 0.857±0.060**
STa 400 µg/ml	**a 0.032±0.016**	**a 0.314±0.007**	**a 0.045±0.018**	**a 0.145±0.025**	**a 0.019±0.010**	**a 0.211±0.027**	**a 0.082±0.021**	**a 0.848±0.011**
STa 800 µg/ml	**a 0.040±0.025**	**a 0.331±0.038**	**a 0.051±0.020**	**a 0.118±0.018**	**a 0.026±0.012**	**a 0.195±0.020**	**a 0.088±0.020**	**a 0.849±0.057**
STa 1600 µg/ml	**a 0.043±0.021**	**a 0.341±0.035**	**a 0.054±0.014**	**a 0.110±0.023**	**a 0.020±0.009**	**a 0.191±0.017**	**a 0.108±0.017**	**a 0.867±0.053**
MMC 5 µg/ml	**b 0.072±0.012**	**b 0.517±0.045**	**b 0.081±0.020**	**b 0.227±0.022**	**b 0.057±0.014**	**b 0.349±0.022**	**b 0.171±0.017**	**b 1.474±0.72**

Differences a, b are significant (P<0.05) to compression rows

Table (3-4): Cytogenetic effects of STa in comparison with negative (PBS) and positive (MMC) controls on human blood lymphocytes obtained form colorectal cancer patients (*in vitro)*

Treatment	Blast Index % m±SE	Mitotic Index % m±SE	Micronucleus % m±SE
PBS	a 30.100±0.898	a 1.665±0.111	a 3.350±0.095
STa 100 µg/ml	a 29.900±1.017	a 1.630±0.128	a 3.310±0.097
STa 200 µg/ml	ab 29.400±1.015	a 1.560±0.125	a 3.290±0.085
STa 400 µg/ml	ab 28.250±1.109	ab 1.470±0.133	a 3.435±0.0116
STa 800 µg/ml	b 25.800±1.191	ab 1.305±0.142	a 3.220±0.085
STa 1600 µg/ml	c 22.000±1.211	b 1.105±0.137	a 3.460±0.091
MMC 5 µg/ml	c 21.100±0.720	b 1.050±0.85	a 4.510±0.122

Differences a, b, c are significant (P<0.05) to compression rows

Table (3-3): Cytogenetic effects of STa in comparison with negative (PBS) and positive (MMC) controls on human blood lymphocytes obtained form normal healthy persons (*in vitro)*

Treatment	Blast Index % m±SE	Mitotic Index % m±SE	Micronucleus % m±SE
PBS	a 54.10±1.214	ab 3.870±0.120	a 1.390±0.101
STa 100 µg/ml	a 53.20±1.027	a 4.010±0.098	a 1.420±0.085
STa 200 µg/ml	a 54.30±1.030	ab 3.850±0.100	a 1.170±0.085
STa 400 µg/m	a 55.10±1.353	b 3.510±0.161	a 1.440±0.113
STa 800 µg/ml	a 51.70±0.950	b 3.550±0.154	a 1.270±0.091
STa 1600 µg/ml	a 52.30±0.950	ab 3.630±0.117	a 1.390±0.103
MMC 5 µg/ml	b 32.50±1.460	c 1.990±0.120	b 3.060±0.110

Differences a, b, c are significant (P<0.05) to compression rows

Table (3-3): Continued

Chromosomal aberrations % m±SE								
Treatment	**Ring**	**Gap**	**Acentric**	**Dicentric**	**Chromosome breaks**	**Chromatid breaks**	**Deletion**	**Total**
PBS	**a 0.00±0.00**	**a 0.150±0.005**	**a 0.00±0.00**	**a 0.001±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.053±0.005**	**a 0.204±0.009**
STa 100 µg/ml	**a 0.00±0.00**	**a 0.156±0.004**	**a 0.000±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.041±0.00**	**a 0.197±0.007**
STa 200 µg/ml	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.002±0.00**	**a 0.00±0.00**	**a 0.000±0.00**	**a 0.059±0.006**	**a 0.200±0.004**
STa 400 µg/ml	**a 0.00±0.00**	**a 0.142±0.009**	**a 0.00±0.00**	**a 0.001±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.050±0.004**	**a 0.193±0.008**
STa 800 µg/ml	**a 0.00±0.00**	**a 0.149±0.003**	**a 0.00±0.00**	**a 0.001±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.058±0.007**	**a 0.208±0.007**
STa 1600 µg/ml	**a 0.00±0.00**	**a 0.155±0.008**	**a 0.00±0.00**	**a 0.000±0.00**	**a 0.00±0.00**	**a 0.00±0.00**	**a 0.047±0.003**	**a 0.202±0.008**
MMC 5 µg/ml	**b 0.005±0.001**	**b 0.468±0.025**	**b 0.009±0.002**	**b 0.073±0.002**	**b 0.006±0.001**	**b 0.009±0.002**	**b 0.287±0.018**	**b 0.857±0.029**

Differences a, b are significant (P<0.05) to compression rows

Table (3-1): Purification steps results of STa by ion-exchange chromatography (DEAE-Sephadex A-50) column (2x10 cm)

Steps	Volume (ml)	Activity (U/ml)	Protein concentration (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Supernatant	250	56	0.16	40	14,000	350	1	100
Crude STa	5	594	0.41	2.05	2,970	1448.7	4.13	21.21
DEAE-Sephadex A-50 column chromatography	27	95	0.04	1.08	2,556	2366.6	6.76	18.25