EFFECT OF OCIMUM SANCTUM LEAF EXTRACT ON THE
MICRONUCLEUS FORMATION INDUCED BY
CYCLOPHOSPHAMIDE IN BONE MARROW CELLS OF
AS-30D HEPATOMA TRANSPLANTED RATS

EKACHAI KHUMPHANT

With compliments of

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Ekachai Khumphant
Holy basil (Ocimum sanctum) is an herbal plant commonly used as an ingredient for cooking and as a medicinal plant. This plant is widely used in all Thailand regions for many food recipes. Previous studies have shown that ethanol extract of Ocimum sanctum leaf (OE) possesses chemopreventive property against carcinogenesis and induction of the hepatic biotransformation enzyme profiles that are involved in detoxification of chemical mutagens.

This thesis attempted to investigate the effect of an oral administration of OE on induction of toxicity, mutagenicity, and anti-mutagenicity using rat bone marrow micronucleus test. Cyclophosphamide was used to elucidate the anti-mutagenic property of OE. With an effort to minimize the number of laboratory animals used in toxicity testing. This study has developed AS-30D hepatoma transplanted Sprague-Dawley rats that can be tested for multi modes of toxicity. The study demonstrated the use of AS-30D hepatoma transplanted Sprague-Dawley rats (in comparison with normal Sprague-Dawley rats) in an investigation of mutagenicity or anti-mutagenicity together with effects on the growth of AS-30D hepatoma of ethanolic Ocimum sanctum leaf extract (OE). OE was given daily via oral administration to Sprague-Dawley rats with or without AS-30D hepatoma transplantation to determine; (1) acute toxicity, (2) mutagenicity, and (3) anti-mutagenicity against cyclophosphamide. OE was not toxic up to the dose of 15 g/kg bw in 5-week-old rats. The mutagenicity, represented as micronucleus induction, was assessed by bone marrow using i.p. 80 mg/kg bw of cyclophosphamide 30 hours prior to bone marrow collection.

Administration of OE at a dose 5 g/kg bw did not cause mutagenicity in the rats. The AS-30D hepatoma transplanted rats yielded similar results as the normal rats on the significant inhibition of micronucleus formation against cyclophosphamide after 7 and 21-day repeat oral administration of 5 g/kg bw of OE. The anti-mutagenicity of OE suggested an enhancement of detoxification enzymes against cyclophosphamide which were reduction of micronucleus formation. It is possible that some compounds in OE may play an important role in such enhancement of detoxification mechanism. This thesis, therefore, confirmed health benefits of holy basil (Ocimum sanctum) as and ingredient of food and as a medicinal plant in reducing mutagenicity. However, the results did not see changes in the number of hepatoma harvested from the transplanted rats. Nonetheless, the rats that were transplanted with the AS-30D hepatoma cell line had been shown to be utilized successfully in short-term mutagenicity or anti-mutagenicity together with the effect on the cell growth testing. Thus, it would be of beneficial to an investigator who wants to minimize the number of rats used in genotoxic and oncologic studies, all in the same time.
EFFECT OF OCIMUM SANCTUM LEAF EXTRACT ON THE MICRONUCLEUS FORMATION INDUCED BY CYCLOPHOSPHAMIDE IN BONE MARROW CELLS OF AS-30D HEPATOMA TRANSPLANTED RATS.

E. DOCTORAL THESIS

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413674NUFT/M:  สารวิชาพยาบาลศาสตร์และโภชนาการ
วป.ม.(พยาบาลศาสตร์และโภชนาการ)

เอ.ก็ ธารผู้พัฒนา: การดำเนินการเกิด MICRONUCLEUS เนื่องจาก CYCLOPHOSPHAMIDE ด้วยสารหลักไป

กะพริบด้วยสารหลัก (70%) ในเซลล์ในกระดูกของหน่วยสัตว์พันธุ์ SPRAGUE-DAWLEY ที่ได้รับการปลูกย่อยเซลล์

มะเร็งลาง AS-30D (EFFECT OF OCIMUM SANCTUM LEAF EXTRACT ON THE

MICRONUCLEUS FORMATION INDUCED BY CYCLOPHOSPHAMIDE IN BONE

MARROW CELLS OF AS-30D HEPATOMA TRANSPLANTED RATS).

คณะนิสิตการ

ควบคุมวิทยานิยมพันธุ์: ครูนิมิ บุญกิจ ordovaลี, Ph.D., แก้ว กิตติศาสตร์ ไพศาล, Ph.D., ปัญญา เดือนธัญ, DVM, M.Sc. 75 หน้า.

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LIST OF ABBREVIATIONS

ANOVA  Analysis of variance
BW     Body weight
°C     Degree celcius
CHO    Chinese hamster ovary cell
CP     Cyclophosphamide
DNA    Deoxy nucleic acid
ED₅₀   Medial effective dose
g      Gram
GSH    Glutathione S-transferase
hr     Hour
Inc    Inches
i.p.   Intraperitoneal
kg     Kilogram
l      Litter
LD₅₀   Medial lethal dose
mg     Milligram
mm     Millimeter
MN     Micronucleus
MNPCE  Micronucleated polychromatic erythrocyte
nm     Nanometer
LIST OF ABBREVIATIONS (CONT.)

n
NCE
NLAC
NSS
OE
PBS
P450
PCE
pH
rpm
SCE
SD
SD-rat
UDS
ul
x
%

Number of animal
Normal chromatic erythrocyte
National Laboratory Animal centre
Normal saline solution
Ocimum sanctum leaves extract
Phosphate buffer saline
Phytochrome 450
Polychromatic erythrocyte
Log concentration of H⁺
round per minute
sister chromatid exchange
standard deviation
Sprague Dawley rat
Unscheduled DNA synthesis
Microlitre
Mean
percent
CHAPTER I

INTRODUCTION

Constituents of the human diet that inhibit mutagenesis and carcinogenesis are of particular importance because they may be useful in the prevention of human cancer. *Ocimum sanctum* L. (Labiatae) popularly known as “Kaprao” in Thailand, “Tulsi” in Hindi and “Holy basil” in England has been extensively used as an ingredient for cooking and as a medicinal plant. This plant was widely used in all Thailand regions for many food recipes and claimed to be valuable against a wide variety of diseases. Preliminary evaluation of this plant was first reported by Aruna and Sivaramakrishnan (1990, 1992) demonstrated the effect of *Ocimum sanctum* leaf extract (OE) against chromosomal aberrations, neoplasia of stomach and hepatomas. The plant is documented to protect adriamycin induced free radical damage (Balanchru and Nagarajan, 1992) and against radiation (Devi and Ganasoundari, 1995). It is also reported to have chemopreventive property against skin papillomagenesis (Prashar and Kumar, 1995). The mechanism of influence of *Ocimum sanctum* on the incidence of papillomas and carcinomas are yet to be known and the possible mechanism may be due to enhancement of the detoxifying enzyme system in animals (Karthikeyan *et al.*, 1999).

Earlier studies have shown that induction of drug metabolizing and conjugative enzymes are a critical determinant of carcinogenicity of chemicals (Banerjee *et al.*, 1996). Therefore, it is postulated that compounds that can modulate the activities of these
enzymes could reduce the risk of tumor development in human. Several plant components including those present in our diets have been found to decrease the incidence and mortality of certain cancers in high-risk human populations. Previous study has reported the modulatory influence of alcoholic extract of the leaves of Ocimum sanctum on the activities of cytochrome P450, cytochrom b5 and aryl hydrocarbon hydroxylase in liver. It is also reported the extract enhanced glutathione – S – transferase (GST) and a reduced glutathione level in the liver, lung and stomach of mice. All of these enzymes and cofactors play an important role in the detoxification of carcinogens and mutagens (Banerjee et al., 1996), but which component(s) of the Ocimum sanctum leaves affects the enzyme activity is not clear. A principal constituent is eugenol, which is known to induce UDP – glucuronyl transferase and GST (Yokota, 1988). Other compounds present in the leaf extract that may play an additional and/or synergistic chemoprotective role by inducing detoxification enzymes include sterols, triterpenes, alkaloids, glycosides, saponin, tannins, various sequiterpenes in essential oil of the leaves, urosolic acid and hydroxy chavicol (Prashar et al., 1998).

AS-30D hepatoma cell line was developed in vitro and in vivo by National Laboratory Animal Center Mahidol University (NLAC). The AS-30D tumor growth primarily as clusters or island of cells in the ascites fluid with single cells, doublets, and triplets making up only a minor component of the tumorous ascites. The single cell measures 11 to 15u diameter, although cells as large as 23u. It grows as single cell in suspension. Medium is 90% Dulbecco's MEM +10% Fetal bovine serum at 37 °C with 5 % CO₂ (Holstege et al., 1979; Church et al., 1988). Recently the NLAC was successful
in transplanting the AS-30D hepatoma cell into abdominal cavity of Sprague-Daley rats. This transplanted rat has been developed to evaluate the possibility of mutagenicity or antimutagenicity using bone marrow micronucleus test.

Though leaf and ethanol extract of *Ocimum sanctum* have been used for its chemopreventive activity, but the effect of ethanol extract of *Ocimum sanctum* has not been studied so far in the antimutagenicity. Thus, the present study attempts to evaluate the effect of *Ocimum sanctum* leaf extract on the micronucleus formation induced by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats.
CHAPTER II

REVIEW LITERATURE

2.1 *Ocimum sanctum*

*Ocimum sanctum* Linn. or *Ocimum tenuiflorum* Linn. (family: Labiatae), popularly known as “Kaprao” in Thai, “Tulsi” in Hindi, “Holy basil or Sacred basil” in English and “Albahaca morada” in Cuba (Luthy, 1964) was a sacred plant of the Hindus, a worshipped at home and in temples and used as spices in Thai foods (dnar: 2543). Medicinal properties had been attributed to this plant in several ancient system of medicine e.g. Ayurveda, Greek, Roman, Sidha and Unani. The leaves of the plant were used as on expectorant, diaphoretic, anti-emetic, anticancer, anthelmintic, antiseptic, and analgesic and to relieve various gastric disorder (Kirtikar and Basu, 1935; Chopra, 1958; Godhwani, 1987).

*Ocimum sanctum* was found throughout Thailand and India ascending up to 1,800 m in the Himalayas, in the Andaman and Nicobar Island. At least two types of *Ocimum sanctum* were encountered with in cultivation; green type (Sri tulasi or Kaprao-khao) was the most common leaves and branch green. The red type (Krishna tulasi or Kaprao-dang) was all purple leaves and branch. Besides, there is a hybrid type with green leaves and purple branch. The plant was an erect, herbaceous, much branched, softly hairy annual. The leaves were elliptic-oblong, acute or obtuse, entire or serrate, pubescent on both sides and minutely gland-dotted; the flowers were in close whorled racemes, purplish or crimson. The nutlets were sub-globes or broadly
ellipsoid, slightly compressed, nearly smooth, pale brown or reddish and shown small black markings.

Preliminary pharmacological and clinical studies of *O. sanctum* showed that it possessed anabolic, hypotensive, cardiac depressant, smooth muscle relaxant, hypoglycemic (Chattopadhyay, 1993). It also showed the properties of sedative, antispermatogenic, antifungal, antifertility (Kasinathan, 1972), antistress (Bhargava, 1981), antioxidation, antiinflammatory (Surender, 1977; 1996) and adaptogenic action (Godhwani, 1987; Sen, 1992; Maulik, 1997).

### 2.1.1 Chemical compounds

The constituents of *Ocimum sanctum* leaves were alocinnamate, apigenin, apigenin-7-0-glucoronide, β-carotene, methyl charvical, camphor, carvacrol, caryophyllene, chavibitol, cirsimaritin, methyl cinnamate, citric acid, D-camphor, decyldehyde, 2,5-dimethyl benzoic acid, D-glucose, gratissimin, isothymusin, isothymonin, linalool, linolenic acid, luteolin, luteolin-7-0-glucoronide, malic acid, molludistin, nerol, ocimol, orientin, oxalic acid, phenols, α-pinene, γ-selinene, rosmarinic acid, succinic acid, tartaric acid, terpinene-4-ol, n-tetraatriacontane, thymol, eugenol, ursolic acid and vicenin (śwētu, 2530; Devi, 1998; Luthy, 1964; Kelm, 2000; Prasad, 1986; Singh, 1997).

### 2.1.2 Toxicity Effect of *Ocimum sanctum*

#### 2.1.2.1 Acute toxicity

The data showed the variable of toxicity due to the animal species and the route of administration. The LD50 of this plant was reported in mice given orally of 4,500 mg/kg bw and 3,240 mg/kg bw by intraperitoneal injection of 70% alcohol...
Ocimum sanctum leaf extract. It was reported that treatment with aqueous extract did not produce any acute toxic symptoms at doses up to 5 g/kg bw in mice. The LD50 values for aqueous extract was found to be 6,200 mg/kg bw and LD50 of aqueous alcohol extract in mice was 4,600 mg/kg bw (Devi and Ganasoundari, 1995). Chattopadhyay (1999) also found LD50 of 76 % alcohol extract in mice equal to 4,850 mg/kg bw.

2.1.2.2 Subchronic and chronic toxicity

The previous study demonstrated no toxicity effect of 50% alcohol Ocimum sanctum leaf extract in mice injected 1g/kg bw intraperitoneally (Dhar et al., 1998). Moreover, the high dosage up to 10 g/kg bw did not show toxicity to mice treated by intradermal injection (Mokkhasmit et al., 1971). However, there is no work reporting of the long- term administration of Ocimum sanctum leaf extract that produced any toxicity.

2.1.2.3 Reproductive and teratogenic toxicity

Kasinathan et al. (1972) reported that 10 % fresh leaves of Ocimum sanctum mixed in food inhibited spermatogenesis of mice. Benzene leaf extract of this plant could also kill sperm and inhibit spermatogenesis as well (Seth et al., 1981).

2.1.3 Mutagenicity, anti-carcinogenesis and inducing detoxification enzymes

The aqueous leaf extract of Ocimum sanctum at concentration 0.5 ml/disc did not cause mutagenicity to Bacillus subtilis both H-17 and M-45 strains (Ungsurungsi, 1982). The previous studies provided evidence for the first time that an ethanolic leaf extract of O. sanctum exhibited a chemopreventive action on DMBA- induced skin papillomagenesis in mice treated topically or through gastric intubation with the
ethanolic leaf extract (Prashar, 1994, 1995). The modulatory influence of an ethanolic leaf extract also enhances on the activities of cytochrome P450, cytochrome b5 and aryl hydrocarbon hydroxylase in liver and glutathion -s- transferase (GST) and a reduced glutathion level in the liver, lung and stomach of mice. All of these enzymes and cofactors play an important role in the detoxification of carcinogens and mutagens (Banergee et al, 1996).

The plant is also reported to protect adriamycin induced free radical damage (Balanchru and Nagarajan, 1992) and against radiation (Devi and Ganasoundari, 1995). Preliminary evaluation of this plant was also reported by Aruna and Sivaramakrishnan (1990, 1992) demonstrated its capacity against chromosomal aberrations, neoplasia of stomach and hepatomas. It is also reported to have chemopreventive property against skin papillomagenesis (Prashar and Kumar, 1995).

Immunoregulated activity, modulated activity of humoral immune responses in animals and enhancing cell mediated immunity in man have been reported on Ocimum sanctum treatment suggested that the plant may also influence the immune system to inhibit hamster buccal pouch carcinogenesis (Levij, 1968).

2.1.4 Anticancer effect

Prashar and Kumar (1995), demonstrated the aqueous and alcoholic extracts from the leaves of Ocimum sanctum shown an activity against cancer. The ethanol and aqueous extracts of this plant show significant reduction in tumor volume of Sacroma -180 transplanted in mice (Karthikeyan et al., 1999). However, the 50 % leaves extract not shows cytotoxic effect to CA-9KB cell line (Dhar et al., 1968).
2.1.5 Pharmacological effect

2.1.5.1 Antifungi and antimicrobial effect

Pandey et al. (1983) tested 50% ethanolic extract of *O. sanctum* against *Aspergillus niger* and *Absidia ramosa*. They found that 50% ethanolic extract of *O. sanctum* could inhibit growth of *Aspergillus niger* and *Absidia ramosa* at 57% and 100%. Prasad et al. (1986) reported that the essential oils from leaves of *O. sanctum* could inhibit growth of *Aspergillus fumigatus*, *A. parasiticus*, *Cryptococcus neoformans*, *Microsporium conis*, *M. gypseum*, *Sporotrichum schenckii*, *Trychophyton mentagrophyte*, *T. rubrum* and *T. verrucosum*. Damayanti et al. (1996) reported that the juice from fresh leaves of *O. sanctum* showed antifungal property against *Ceratocystist paradoxa*. Devi et al. (1998) reported that vicenin the flavonoids from the leaves of *O. sanctum* could protect mouse bone marrow cell from gamma radiation. Prasad et al. (1986) also reported that the essential oils from leaves of *O. sanctum* could inhibit gram positive bacteria as *Bacillus anthracis*, *B. sacharolyticus*, *B. stearothermophilus*, *B. subtilis*, *B. thurengiensis*. It also inhibited *Micrococcus glutamicus*, *Staphylococcus aureus*, *Staphylococcus species*, *Sarana lutea*, *Lactobacillus casei*, *L. plantalum* and gram negative bacteria as *Citrobacter sp.*, *Enterobacter sp.*, and *Salmonella weltevreden*.

2.1.5.2 Anti-inflammatory and antioxidant activity

Surender et al. (1996) reported that fixed oil from *O. sanctum* had antiinflammatory activity against PGE2, leukotriene, arachidonic acid and carragenan-induced paw edema. Besides, Kelm et al. (2000) reported that eugenol demonstrated 97% anti-inflammatory activity or cyclooxygenase inhibitory activity.
Kelm et al. (2000) reported that cirsimaritin, isothymonin and eugenol demonstrated good antioxidant activity.

2.1.5.3 Hypouricemic, uricosuric effect and antistress activity

Sarkar et al. (1990) tried to fed normal male Swiss-albino rabbits with rabbit fed mixed with 1 and 2 % of O. sanctum fresh leaf or dried seed for 4 weeks. They found that there were significantly decreased in the serum uric level with a corresponding increased in the urinary uric acid and a significantly increased occurred in the blood, as well as urinary urea level and increased in urinary volume was also observed. Previous study also reported that methanol extract from the roots of O. sanctum increased swimming time in mice suggesting a central nervous system stimulant and/or antistress activity.

2.2 AS-30D hepatoma cell line

2.2.1 Original

AS-30D hepatoma cells were established from a 3’ methyl-4-dimethylaminoazobenzine induced Sprague Dawley rat to primary tumor. Fluid from the air pouch of the 1st transplant was injecting i.p. into female SD rats and designed ascites hepatoma AS-30D in 1998. The AS-30D tumor growth primarily as clusters or island of cells in the ascites fluid with single cells, doublets, and triplets making up only a minor component of the tumorous ascites. The single cell measures 11 to 15μ diameter, although cells as large as 23μ. It grows as single cell in suspension. Medium is 90% Dulbecco’s MEM +10% Fetal bovine serum at 37 °C with 5 % CO2 (Holstege et al., 1979; Church et al., 1988).
2.2 Experimental study and developing model of AS-30D hepatoma cell line in toxicity test

Wipawee et al. (2000), study an in vitro growth of AS-30D hepatoma cells cultured. The result showed that, in the first three days the cell concentration was not change, as in the log phase. A log phase dramatically increasing of the cell number in day 3-6. Thereafter, cell viability began to decline and reach to stationary phase in day 6-7, as the day 8-9 the increasing of dead cell was observed. Wipawee et al. (1999) performed the first transplanted of this hepatoma cell line in to the abdominal cavity of the female Sprague-Dawley rat by intraperitoneal injection. The transplanted cells were fully proliferated lasted 8-10 days, as the body weigh changed to 20 g compared with the control (Wipawee et al., 1999).

2.3 Micronucleus study

At present, the micronucleus test is well established as a standard clastogenic assay in the regulatory schemes. (Sofuni, 1993; Auletta, 1993). The mammalian bone marrow micronucleus assay has become prominent among genotoxicity assay and perhaps the most commonly used of the in vivo genotoxicity test (MacGregor et al., 1987).

The micronucleus (MN) test has been used to detect the damage of chromosomes or the mitotic apparatus induced by chemical and revealed by the presence of micronucleated cells (Countryman and Heddle, 1976; Schroeder, 1966). Micronuclei consist of acentric fragments or entire anaphase lagging chromosomes as a result of spindle disturbances and has not traveled to the appropriated pole of spindle to be include in the main nucleus of the daughter cells (Schmid, 1975). It may from one or
more separate small nuclei, that is, micronuclei. Several laboratories performed the bone marrow micronucleus examination to detect the in vivo damage from chemical mutagens. Schroeder (1970), which demonstrated the occurrence of micronuclei in bone marrow cells in connection with cytogenic damage. MacGregor et al. (1987) concluded that the measurement of the frequency of newly formed micronucleated erythrocytes in bone marrow provided a convenient index of chromosomal damage in nucleated erythrocyte precursor cells. Staining the residual RNA that remains in these cells after enucleation identified the newly formed erythrocytes. Cells stained uniformly positive for RNA were referred to as polychromatic erythrocytes (PCEs) whereas cells that were not stain positively for RNA referred to as normochromatic erythrocytes (NCEs). In addition, Heddle et al. (1983) reported that PCEs in bone marrow were certainly useful for enumeration MN because of their uniform population, abundance and easy recognition.

The principle of micronucleus test was revealed in anaphase, acentric chromatid and chromosomes fragments lag behind when the centric elements move towards the spindle poles. After telophase, the undamaged chromosomes as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too. A considerable proportion is transformed into one or several secondary nuclei, which are much smaller than the principal nucleus and are therefore called micronuclei (Schmid, 1975). Micronuclei are small chromatin-containing bodies arising from chromosome fragments or hole chromosomes that were not incorporated into daughter nuclei following mitosis (Mavournin et al., 1990). Micronuclei form only in dividing cells thus they were found in any cell type of proliferation tissue: myeloblasts, myelocytes, erythroblasts etc. However, they are
most easily recognized in cell lacking main nucleus, namely erythrocytes (Venitt and Parry, 1984).

2.3.1 General procedure

Animals expose to a test substance by appropriated route at predetermined times after or during exposure by acutely or chronically. They were sacrificed at specific times and the bone marrow content was extracted, smeared and stained. Polychromatic erythrocytes from bone marrow were scored for micronuclei comparing to control groups under the light microscope (MacGregor et al., 1987).

Criteria for identifying micronuclei were based on those given by Schmid (1975) and Countryman et al. (1976) with some addition following: micronuclei were consisted of the nuclear material, fully separated from the parent nucleus, round or oval shape and area < 1/5 of the parent nucleus.

2.3.1.1 Number of animals

For routine screening, male and female animals should be test using at least 5 animals/sex/group for each sampling time (MacGregor et al, 1987). The use of a single sex or a different number of animals should be justified (Brusick, 1989).

2.3.1.2 Dose selection

Lethal dose 50 (LD50) from acute toxicity information can be used to determine dose levels. The high, intermediate and low doses are 1/2, 1/6 and 1/20 of the LD50, respectively (Brusick, 1989).
2.3.1.3 Controls

A substance known to produce micronuclei in vivo is employed as a positive control, and a negative (solvent) control group is also included in the design of each experiment. The aberration frequency induced by the test compound can be tested against the solvent and positive control values to determine whether or not it is clastogenic (MacGregor et al., 1987).

2.3.1.4 Route of administration

The animal is desirable to choose a route of administration, which maximize the dose delivered to the target tissue. The most commonly routes of application are oral intubation (p.o) and intraperitoneal (i.p) injection, although other routes may also be appropriated. However, the most appropriated route of administration should be determined by the route of human exposure to the compound (Committee on Toxicology, 1977).

2.3.1.5 Sampling times

The appearance of micronucleated PCEs induced by chemicals depends on a particular time interval during treatment. The micronuclei can not appear earlier after treatment than the interval between completion of the final erythroblast mitosis and enucleation because micronuclei are formed during division of the nucleated erythropoietic cells but scored in the anucleated young erythrocyte. Visualization of micronuclei is facilitated in these cells because they lack a nucleus. In mouse bone marrow, expulsion of the erythroblast nucleus occurs about 6 hours after the final mitosis and an increase of micronucleus frequency which was induced by the most chemicals has not been found earlier than 9 – 12 hours after treatment. Any micronucleated PCEs formed will remain in the bone marrow for at least 10–12 hours
because the life span of the PCEs within bone marrow has been reported to be between 10 and 30 hours in the mouse and rat, respectively. It is therefore not necessary to sample earlier than 19 – 24 hours after the first treatment (Salamone and Heddle, 1983).

Due to differences between test agent in the time after treatment at which the peak frequency of micronuclei occurs. It is important that two or more samples be taken if only one or two treatment is given. Available data indicate that this peak frequency usually occurs between 24 and 48 hours after treatment, but that in certain cases it may occur as late as 72 hours after treatment (Salamone and Heddle, 1983). Hence, it was recommended to take samples at 24,48 and 72 hours after treatment (MacGregor et al., 1987).

2.3.1.6 Cell population to be scored

The frequency of micronuclei can be most easily evaluated in young erythrocytes shortly after the main nucleus is expelled. These young erythrocytes are young polychromatic (PCEs) and are distinguished from the mature normochromatic (NCEs) ones by their different staining properties. With a combination of MayGrunwald and Giemsa staining, the PCEs stain bluish to purple-gray because of the high content of RNA in cytoplasm whereas the NCEs stain reddish to yellow. The PCEs are also slightly larger than the NCEs (Venitt and Parry, 1984).

2.3.1.7 Scoring

Prior to scoring, slides should be randomly coded so that the scorer is unaware of the treatment group from which each slide originated. A few slides should
be randomly chosen and examined to appraise the quality and uniformity of the stain (MacGregor et al., 1987).

In scoring for micronucleated PCEs, an area for optimal cell morphology, spacing and staining should be chosen. The great majority of real micronuclei are round, on rare occasion oval or half-moon shaped, always with a shaped contour and evenly stained. The diameters of micronuclei usually range in different cells from 1/20 to 1/5 of an erythrocyte. Even after treatment with high dose of chromosome breaking agents, most of the micronucleated cells contain just one micronucleus. However, there are always some cells with two or more of these bodies.

For each sample, the number of micronucleated PCEs among a predetermined number of PCEs is determined and at least 1,000 PCEs should be scored per animal. In order to quantity the proliferative state of the bone marrow, the ratio of PCE TO NCE should be determined by counting the number of PCEs among 100 – 200 total erythrocytes. The normal PCE:NCE ratio for bone marrow is approximately 0.4-1.0. A marked reduction in this ratio (< 0.1) indicated a cytotoxic effect, such a reduced proportion of PCE arise from either a cessation of PCE production (division and maturation of the nucleated erythroid cells have been inhibited) or massive invasion of the marrow be peripheral blood as a result of marrow depletion (Heddie et al., 1982).

2.4 Cyclophosphamide

Cyclophosphamide was known as clastogen and used widely for positive control in micronucleus test (Krishna et al., 1995). This elastogen is the respectively positive control for micronucleus assay due to it needs metabolic activation, which is typical
for most carcinogens, consistently induced well countable and even induced very high numbers of micronucleated polychromatic erythrocytes (MNPCEs) in rat bone marrow (Edenharder et al., 1998).

Cyclophosphamide is an alkylating agent (Gouyette, 1994). It is transformed via hepatic and intracellular enzymes to active alkylating metabolites, 4-hydroxycyclophosphamide, aldophosphamide, acrolein and phosphoramid mustard (Ludeman, 1999). These species cause prevention of cell division primarily by cross-linking DNA strands that result in inhibition of DNA synthesis (Fleming, 1997). Because genetic damage that results in chromosome break or structurally abnormal chromosome leads to micronucleus formation (Red book of USFDA, 2000), the incidence of micronuclei serves as an index of this type of damage caused from clastogenic property of Cyclophosphamide.

Cyclophosphamide cytotoxicity depends on activation by microsomal enzyme system. Hepatic microsomal P450 mixed-function oxidase catalyzes conversion of cyclophosphamide to the active forms: 4-hydroxycyclophosphamide and aldophosphamide (Baddy and Yule, 2000), which exert their cytotoxic effects through the covalent linkage of alkyl groups to DNA. (Hall and Tilby, 1992).

Cyclophosphamide, which is electrophilic specie prone to react readily with the tripeptide glutathione through glutathione-S-tranferase (Gouyette, 1664). Therefore, glutathione appears to be involved directly in the detoxification of cyclophosphamide and metabolites and may play a more indirect role in other processes (Gamesik, 1999). Both elevate glutathione levels and increase activity of the enzyme glutathione-S-tranferase has been associated with the resistance of cells to alkylating agent such as cyclophosphamide (Colvin et al., 1993; Tanner et al., 1997). The one mechanism of
this detoxification is the inactivation of the alkylation agent by conjugation with glutathione and catalyzed by glutathione-S-transferase (Colvin et al., 1993).

Figure 1. The structure of Cyclophosphamide
STATEMENT OF THE THESIS PROBLEM

*Ocimum sanctum* is a herbal plant that grown in every area of Thailand. This plant was normally used in both ingredients for cooking as spice and for medicinal for a long time. Several studies reported in many activities of this plant for example; antistress, antiinflammation, antifungal, antimicrobial, antioxidance, antipyretic, anticarcinogenic, non mutagenicity in Rec assay etc. In the field of mutagenicity, the previous study reported that *Ocimum sanctum* leaf extract could inhibit chromosomes aberration induced by radiation. But however, in the study of micronucleus of mutagenesis and antimutagenesis of this herb is lack in report. It was thus of interest to elucidate the effect of *Ocimum sanctum* on the properties of mutagenicity and antimutagenicity induced the micronucleus formation by cyclophosphamide.
EXPERIMENTAL OBJECTIVES

To study effect of *Ocimum sanctum* leaf extract on the micronucleus formation induced by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats.

The following aspects were studied:

1. Acute toxicity of *Ocimum sanctum* leaf extract up to dose 15 g/kg bw.
2. Mutagenicity of *Ocimum sanctum* leaf extract at dose 5 g/kg bw.
3. Anti-mutagenicity of *Ocimum sanctum* leaf extract against cyclophosphamide at dose 5 g/kg bw.
CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of *Ocimum sanctum* leaf extract (OE) from fresh basil leaves.

Insecticide free *Ocimum sanctum* plants were grown for 60 days. Botanist of Sarerukkachart herb garden of pharmacology division Mahidol University confirmed the plant classification. The fresh leaves (40kg) were cut and washed with tap water and shed dried at room temperature (28°C) for one night. The air-dried leaves were kept in 60°C oven for 5 hours when constant dried weight was achieved. The dried leaves were ground into powder form by the grinding machine. Total amount of 5.721kg of the basil leaf powder was soaked in 4 litter of 70% ethanol for 24 hours at room temperature of 28 °C. The extract was filtered through filter paper no.3. After filtration the solid part was soaked again and filtered 24 hour later. The amount of 13.5 litter extract from three petition of extraction using 70% ethanol as previously mentioned were pooled and evaporated with the evaporator (EYELA model NE) at 60°C until the extract was turned into dark brown of paste. The paste was dried in desiccator for 2 weeks. The yield was 978.15 g.

3.2 Preparation of OE for oral gavage.

The extract was dissolved in 0.9% of sterile normal saline and kept in cold room at temperature 8°C. This method carried out according to Chattopadhyay (1999). The
dissolved extract would be gavage to Sprague-Dawley rats using five-ml syringe with ball tip needle no.16.

3.3 Animals

Prior to being assigned to the study, 5 weeks old of female Sprague-Dawley rats weighing 120±10 g were obtained from breeding colony of National Laboratory Animal Center and maintained in Strict Hygienic Conventional system. Each rat was housed in stainless still cage with dimensions of 7.5-in wide, 11.5-in long and 5.0-inch high, containing 2-cm wood shavings. Animals were maintained under standard of NLAC laboratory conditions on a 12- hr light/dark cycle at 25 ± 2°C and a relative humidity of 60±15%. Animals received commercial laboratory animal feed pellets (Chalernpokkapun Co., Ltd. No. 082), and acidified filtered water pH in range 2.3-2.5 ad libitum. In house quality assurance and vender health surveillance date confirmed that rat would be free of specified pathogenic bacteria, viruses, mycoplasmal agents, endoparasites, and ectoparasites.

3.3.1 The procedures of animals care

The AS-30D hepatoma transplanted rats were housed and maintained following the NLAC programmed. The animals were treated with the strict hygiene according to the standard procedure of NLAC. Before passing through the housing room, the researcher has to shower and wear the autoclave uniform and then walks through the clean area to the housing room. Hands clean are hygienic routine before reaching the lock rooms which setting the ultraviolet lights. The researcher must change the shoes placing in front of the AS-30D transplanted rat’s room. In the housing room, the researcher has to clean the hands and all of equipments with 70% alcohol.
The temperature and humidity were recorded following with the animal observation. Food and water in addition to the body weight of each animal were collected and recorded daily in manuscript. The new food pellets and bottles of water would be put to each cage of animals. All of procedures must operate followed the hygienic method using 70% alcohol and laboratory groove.

The animals would be treated and handled with gentle technique to reduce the pain and stress. It is widely recognized that the production of ascites fluid in rat raises important concerns regarding the potential for pain and/or distress in the animals from intraperitoneal injection of the priming agent, to the accumulation of ascites tumor fluid, and to collection of the ascites fluid, the animals may experience pain and/or distress. Therefore, close monitoring of the condition of the animals are required to minimize the potential for distress.

The routine cares of animals are including daily observations by appropriately trained researcher. Any observations of unusual behavior or symptoms during experiment must be addressed in a timely fashion. Once the ascites fluid accumulation has resulted in obvious abdominal swelling, the condition of the animal must be assessed at least once every 24 hours at regularly spaced intervals.

All animals receive special attention not only to relieve their pain, but also with respect to their husbandry and housing. A great deal would be done, beyond the routine care given to normal laboratory animals, to make these special animal models comfortable (e.g., gentle handling, improved access to food and water, housing in solid bottom cages with deep, soft bedding). The expertise of the laboratory animal veterinarian and the animal health technicians would be consulted throughout the experiment.
3.4 Chemicals

All chemicals used throughout this study were analytical grade. Giemsa and cyclophosphamide (CP) were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. through Theera Trading Co., Ltd. The sterile normal saline solution was purchased from Thai Otsuka Pharmaceutical Co., Ltd.

3.5 AS-30D hepatoma cell line

The National Laboratory Animal Center kindly supplied AS-30D hepatoma cell line, firstly provided from the Institute research of DSMZ, Germany. Cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified and 5% CO₂ incubator.

3.5.1 Transplantation AS-30D hepatoma cell line into female Sprague-Dawley rat

The ascitic fluid cell of AS-30D hepatoma was harvested from the abdominal cavity of each rat. Ten ml of the fluid cell was collected into a 50 ml conical tube containing 40 ml of washing media and then two times centrifuged at 1700 g for 5 min. at temperature 4 °C. The AS-30D hepatoma cells were adjusted for concentration of 1.0-1.5×10⁶ cells/0.5ml/animal. Finally, these cells were injected intraperitoneally as volume 0.5 ml per SD rat, weighing 120±10 g at age 5 weeks old.

3.5.2 Cell Harvesting

The AS-30D hepatoma transplanted rats were anesthetized by ether and the abdominal skin was soaked with 70% alcohol and then cut through the abdominal
cavity. All the ascitic fluid was collected by 10-ml syringe and the volumes were recorded. Then divided 2 ml of fluid volume to the appendopt tube for viability and cell concentration measurement.

3.5.3 Cell counting

The concentration of cell was determined by haemacytometer under the light microscope. Amount 100ul of ascitic fluid was collected by Pasteur pipettes for dilution in 9.9ml washing media and mixed the sample homogeneously. The 100 ul of the mixture was added in 200ul of 2% trypan blue contain in appendopt tube and shake well, then transferred the cell suspension to the edge of the haematocytometer chamber. The cell counting was performed under the light microscope selected 10X objective. Both live and dead cells were counted in five areas (Trypan blue is a stain that is actively excluded from viable cell. Therefore the cells, which are blue, are dead.). The data was calculated for percentile of cell viability and cell concentration.

3.6 Experimental Method

3.6.1 Micronucleus study

The micronucleus test is the method commonly tests by in vivo using rats or mice to evaluate the chromosome damage from clastogenic substance (Countryman and Heddle, 1976; Schroeder, 1966). The preliminary study was performed to determine the possibility in using this transplanted rat in toxicity testing using micronucleus test. Micronucleus was induced by an intraperitoneal injection of 80 mg/kg bw of CP, 30-h prior to bone marrow harvesting. The result in this preliminary indicated no significant difference in the number of micronucleus induced in both female Sprague-Dawley rats with or without the AS-30D transplantation (Figure 2.). Thus, the rats that were
transplanted with the hepatoma cell line could also be used in micronucleus testing. Thus in the present study, this transplanted rat was used to determine the effect of the OE on micronucleus formation induced by cyclophosphamide in bone marrow cell.

At predetermined times after exposure (30 h), animals were sacrificed by using phosphate buffer solution (PBS) as suspending medium. Then the bone marrow content was centrifuged, spread on slides and stained. The incidence of micronucleated (MN) cells per 1000 PCEs (polychromatic erythrocytes) were determined for each animal. The ratio of polychromatic erythrocyte (PCE) to normochromatic erythrocyte (NCE) was also evaluated the toxic effect of OE to bone marrow cells for indicating that the test compound had cytotoxic effect or not (Heddle et al., 1983).

3.6.1.1 Preparation of bone marrow from femur

Sprague Dawley rats were sacrificed at the appropriated time using ether anesthesia. A femur removed by cutting through pelvis and tibia, and muscular tissue was cleaned away by using gauze and fingers. The proximal end of the femur cut to expose the marrow and a 25- G needle mounted on a 1 ml disposable plastic syringe containing 0.5 ml of PBS, was inserted into the bone canal which was still closed at the distal end. Then the bone marrow content was flushed out from the femur by forcing out through the opening with contents of syringe. After several gentle aspiration and flushing, the bone should be visibly empty of marrow content. If not, the other end of bone was clipped off and the flushing process was repeated. (Hayashi et al., 1990)
Figure 2. The frequency of MNPCES in bone marrow cells induced by cyclophosphamide in rat with or without A-S-30D hepatoma transplantation. There is not significantly different in the frequency of MNPCES among Sprage Dawley rats with or without AS-30D hepatoma transplantation.
3.6.1.2 Bone marrow smear

After the bone marrow content of femur from each animal was collected into an individual centrifuge tube at 1,000 rpm for 5 min. The supernatant was discarded with a Pasteur pipette until a small volume remained above the pellet. Then the cells in sediment were carefully mixed for homogeneous by the Pasteur pipette. A small drop of the viscous suspension was put on the end of dry slide that was cleaned with 95% alcohol in the whole slide before used. Spread the cells evenly by pulling the droplet of cells behind a polished cover glass held at an angle of 45 degrees. The bone marrow smears were air-dried overnight at room temperature and then the slides were fixed by immersing in a jar of absolute methanol for 5 minutes (Schmid, 1975).

3.6.1.3 Staining

The staining procedure in this study was Giemsa- staining. The slides were fixed with an absolute methanol for 5 minutes, dried and then stained with Giemsa’s stain (freshly dilute with nine volume of buffered water, pH 7.0) for 20 min and rapidly washed in two changes of 0.5 M phosphate buffer pH 6.4. Finally, slides were dried in air.

3.6.1.4 Microscopic analysis of bone marrow micronuclei

The staining must allow clear discrimination between PCE and NCE. In the well-stained slides, NCE should be red and PCE should be purple-gray. For each animal, the number of micronucleated PCEs (MNPCe) among 1,000 PCEs was determined and the ratio of PCE: NCE was determined by counting the number of PCEs among 300 erythrocytes. The normal PCE: NCE ratio for bone marrow is approximately 0.4-1.0. If this ratio is ≤ 0.1 it indicates that the test substance has
cytotoxic effect. For control of bias, all slides were coded prior scoring and scored blind (Heddle et al., 1982).

3.7 Experimental Protocols

Study I: The effect of OE on the micronucleus formation in bone marrow cells of AS-30D hepatoma transplanted rats.

Thirty-two females of AS-30D hepatoma transplanted rat at age 5-wk. old weighing 120±10 g were devised into 4 groups (8 rats per group). OE was dissolved in 0.9% Normal saline (NSS) to a concentration of 5 g/kg bw. Treated animals were given orally with OE at a dose 5 g/kg bw for 7 days. Negative control was treated with solvent only (NSS). For positive controls were treated with 80 mg/kg bw cyclophosphamide (dissolve in 0.9 % NSS) by intraperitoneal (i.p.). Untreated animal was also used to compare the treated animals. All animals were killed at 30 hours post-treatment for bone marrow and cell line collection. The cells were harvested for fluid volume and then measured for cell viability and cell concentration. Contents of bone marrow from a femur were smeared on the slides and then stained with Giemsa staining as previously mentioned. The frequency MNPCEs in 1,000 PCEs per animal and the ratio of PCE: NCE in 300 erythrocytes were counted under light microscope.

Study II: The effect of OE on the micronucleus formation induced by cyclophosphamide in bone marrow cells of Sprague-Dawley rats with or without AS-30D hepatoma transplantation

This study was divided to 2 experiments. First experiment was performed to study the effect of 7 and 21-day repeat oral administration of 5 g/kg bw of OE on
micronucleus formation induced by CP using AS-30D hepatoma transplanted rat. For 7-day experiment, twenty-four female AS-30D hepatoma transplanted Sprague-Dawley rats at 5 weeks old weighing 120±10 g were divided to 3 groups, 8 animals per group. Group I was given orally with 5 g/kg bw of OE for 7 day and following intraperitoneally injected with 80 mg/kg bw of cyclophosphamide (dissolve in 0.9 % NSS) 30-h prior to bone marrow harvesting. Group II was 7-day repeat oral administration of 0.5 ml/kg bw of NSS and Group III was intraperitoneally injected with 80 mg/kg bw of cyclophosphamide, 30-h prior to bone marrow harvesting, which is the positive control. For 21-day experiment, Group I and II of female Sprague-Dawley rats weighing 50±60g were given orally with 5 g/kg bw of OE or 0.5 ml/kg bw of NSS for 14 days prior to AS-30D hepatoma cell line transplantation. After cell line transplantation rats were continually given of OE or NSS until the end of experiment (day 21). Cyclophosphamide was intraperitoneally injected to induce micronucleus 30-h prior bone marrow harvesting of OE treated group (Group I). Cyclophosphamide was also used as positive control group (Group III).

The second experiment was performed to study the effect of 7 and 21-day repeat oral administration of 5 g/kg bw of OE on micronucleus formation induced by CP using Sprague-Dawley rat. Forty-eight SD rats were divided to 3 groups. Group I was 7-day or 21-day given orally with 5g/kg bw of OE for 7 or 21 day and following intraperitoneally injected with 80 mg/kg bw of cyclophosphamide (dissolve in 0.9 % NSS) 30-h prior to bone marrow harvesting. Group II was 7-day or 21-day repeated oral administration of 0.5 ml/kg bw of NSS and Group III was intraperitoneally injected with 80 mg/kg bw of cyclophosphamide, 30-h prior to bone marrow harvesting, which is the positive control. All animals were killed at 30 hours post-
treatment for bone marrow and cell line collection. The cells were harvested for fluid volume and then measured for cell viability and cell concentration. Contents of bone marrow from a femur were smeared on the slides and then stained with Giemsa staining as previously mentioned. The frequency MNPCes in 1,000 PCEs per animal and the ratio of PCE: NCE in 300 erythrocytes were counted under light microscope.

**Study III : Acute toxicity test**

Males and females Sprague-Dawley rats weighing 120±10 g were divided into 4 groups (two control and two treatment groups). Each group consisted of 10 animals. On day 3, 15 g/kg bw of OE dissolved in NSS was given orally to the animals. The amount of OE extract was equally separated feeding in three periods, namely: 8 a.m., 12 p.m. and 5 p.m. All rats were checked and theirs general and the occurrence of any abnormal sign were recorded after 12, 24, 36, 48, 72, and 96 hours of 15 g/kg bw of OE administration. On day 1 until day 17 all of animals were recorded the body weight and food consumption. Finally, at the end of experiment, (day 17) the animals were killed and autopsied to determine the toxicity effect. The toxicity of OE would be shown in number of dead animals, abnormal behavior and organ sign observation.

**3.8 Statistical Method**

In acute toxicity, rat bone marrow micronucleus test, and effect of OE to the growth of AS-30D hepatoma cell were analyzed as the means and the standard deviation (X±SD). The differences of MNPCes and PCE: NCE ratio among the control and treatment group and between the rat with or without AS-30D transplantation in micronucleus study were compared by Man Whitney u-test (p<0.01). In acute toxicity
test, the body weight and food consumption among control and treatment were compared according to Student t-test. ANOVA also used to compare the fluid volume, cell concentration and cell viability as well. Statistical significant of difference between groups were taken at values of less than 0.01 (p<0.01).
CHAPTER IV

RESULTS

4.1 Acute toxicity test

In the present study, this experiment was performed to evaluate the toxicity of OE. The growth curves (Figure 3) showed that, there was no significantly different in body weight between control and OE-treated groups of both sexes recorded throughout the experiment. The cumulative food consumption of rats showed in Figure 4, demonstrated no significantly different of food consumption in OE treated group when compared to the control group. Averages daily food consumption throughout the experiment of female rats were 15.26±0.24 g in OE-treated group and 15.18±0.24 g in control group, slightly consumed by male SD rats. The male-SD rats showed the increasing of body weight compared to the female SD rat (Table 1.) and also increasing in the amount of food consumption as well (Table 2). The averages daily food consumption of male were 16.98±0.6 g and 17.21±0.73 g in OE treated rat and control, respectively.

4.1.1 Survival and body weight

The numbers of dead animal in both sexes of rats were not show in this study. Growth rate for the control and OE-treated rats showed in Figure 3 in both male and female rats. The result demonstrated no significant differences in body weight in rats of either male or female when compared to the control.
Figure 3. Body weight of male and female SD-rat after receiving one time of 15 g/kg bw of OE compared with control groups. There is not significantly different among OE-treated group and control in both sexes of rat.
Figure 4. Food consumption of male and female SD-rat after receiving one time of 15 g/kg bw of OE compared with control groups. There is not significantly different among OE-treated group and control in both sexes of rat.
Table 1. Mean body weight of Sprague-Dawley rat after oral administration of 15 g/kg bw of *Ocimum sanctum* leaf extract.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Dose (/kg BW)</th>
<th>Mean of Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>OE</td>
<td>15 g</td>
<td>193.34±9.55</td>
</tr>
<tr>
<td></td>
<td>Normal saline</td>
<td>15 ml</td>
<td>191.73±10.39</td>
</tr>
<tr>
<td>Female</td>
<td>OE</td>
<td>15 mg</td>
<td>163.87±4.52</td>
</tr>
<tr>
<td></td>
<td>Normal saline</td>
<td>15 ml</td>
<td>162.77±4.28</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 15 g/kg bw
Normal saline 15 ml/kg bw

1Value is means ± SD of 10 rats/group
By student *t*-test, no statistical differences were found between OE treated rat and control in each sex at p<0.01
By variance analysis (ANOVA), there is no significantly difference between both sexes at p<0.01
Table 2. Food consumption of Sprague-Dawley rat after oral administration of 15 g/kg bw of Ocimum sanctum leaf extract

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Mean of cumulative food consumption (g)</th>
<th>Mean food consumption (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>OE</td>
<td>288.6</td>
<td>16.98±0.63</td>
</tr>
<tr>
<td></td>
<td>Normal saline</td>
<td>292.5</td>
<td>17.21±0.73</td>
</tr>
<tr>
<td>Female</td>
<td>OE</td>
<td>259.2</td>
<td>15.26±0.24</td>
</tr>
<tr>
<td></td>
<td>Normal saline</td>
<td>258.0</td>
<td>15.18±0.24</td>
</tr>
</tbody>
</table>

OE = Ocimum sanctum leaf extract at a dose of 15 g/kg bw  
Normal saline at a dose of 15 ml/kg bw  
1Values are means ± SD of food consumption, n=10 rats/group  
2Values are means of cumulative food consumption, n= 10 rats/group  
By student t-test, no statistical differences were found between OE treated rat and control in each sex at p< 0.01  
By variance analysis (ANOVA), There is no significantly different between both sexes at p< 0.01
4.1.2 General appearance

No specific sign of toxicity showed in the rats feeding with 15g/kg bw of OE at any time point observation and until the end of experiment.

4.1.3 Organ autopsy reported

The rats were killed at the end of experiment. The animals were determined to observe the sign of organs for toxicity effect of OE. The first observation was performed on skin and also subcutaneous and then following with the important organs namely, liver, heart, kidney, lung, spleen, pancreas, stomach and ovary (testis). The result showed no abnormal sign of organs were observed in this study, therefore it suggested that the ethanol leaf extract of Ocimum sanctum at a concentration of 15 g/kg bw did not exert any toxicity effect to the rats.

4.2 The effect of OE on the micronucleus formation in bone marrow cells of AS-30D hepatoma transplanted rats.

The frequencies of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of PCE to NCE induced by cyclophosphamide after exposure to OE (5g/kg bw) in A-S-30D hepatoma transplanted rats were summarized in Table 3. No effect of OE (5g/kg bw) has been found to induce any significant increasing the frequency of MNPCEs in AS-30D hepatoma transplanted rats compared with that found in the negative control and untreated transplanted Sprague-Dawley groups. The frequencies of micronuclei in negative control group and untreated Sprague-Dawley rats were 1.81±0.27 and 1.25±0.28 MNPCEs/1,000 PCEs, respectively. The frequencies of micronuclei in AS-30D hepatoma transplanted rats after OE treatment was 1.88±0.16
Table 3. Frequencies of micronucleated polychromatic erythrocytes (MNPCES) in rat bone marrow after 7-day oral administration 5 g/kg bw of Ocimum sanctum leaf extract in AS-30D hepatoma transplanted rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN PCEs/1,000 PCEs&lt;sup&gt;1&lt;/sup&gt;</th>
<th>PCE: NCE ratio&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OE</td>
<td>1.88 ± 0.16*</td>
<td>0.53 ± 0.02*</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>1.81 ± 0.27*</td>
<td>0.51 ± 0.02*</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>39.13 ± 2.01</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.25 ± 0.28*</td>
<td>0.50 ± 0.02*</td>
</tr>
</tbody>
</table>

OE = Ocimum sanctum leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw, Cyclophosphamide 80 mg/kg bw i.p.= intraperitoneal, MNPCES = Micronucleated Polychromatic Erythrocytes PCE = Polychromatic Erythrocyte, NCE = Normochromatic Erythrocyte

<sup>1</sup>Mean ± SD, n = 8, 1000 PCEs scored per animal; <sup>2</sup>Mean ± SD, n = 8, 300 erythrocytes (PCE/NCE) scored per animal.

<sup>*</sup>Significant difference from cyclophosphamide at p< 0.01 by Mann-Whitney u- test
Table 4. The amounts of cell volume, viability, cell concentration and total cell of AS-30D hepatoma cell line on 7-day oral administration of *Ocimum sanctum* leaf extract.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume (ml)</th>
<th>Viability (%)</th>
<th>Cell conc. ($10^8$/ml$^a$)</th>
<th>Cell No. ($10^8$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>18.38±1.72</td>
<td>97.16±0.27</td>
<td>1.70±0.16</td>
<td>31.0±4.6</td>
</tr>
<tr>
<td>Normal saline</td>
<td>23.38±0.96</td>
<td>97.04±0.34</td>
<td>1.80±0.18</td>
<td>41.0±4.4</td>
</tr>
<tr>
<td>OE</td>
<td>23.50±1.15</td>
<td>97.25±0.40</td>
<td>1.54±0.11</td>
<td>36.0±3.9</td>
</tr>
<tr>
<td>Untreated</td>
<td>21.63±1.02</td>
<td>97.41±0.24</td>
<td>1.56±0.11</td>
<td>33.2±2.1</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw  
CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal  
$^a$ The concentration of cell line per 1 ml of volume  
$^b$ The total number of cell line derived from cell concentration multiply volumes  
$^1$ Values are MEAN ± SD of 8 rats/group  

By variance analysis (ANOVA), no significant differences at p<0.01 were found between each group in fluid volume, cell concentration, viability and number of cell.
MNPCEs/1,000 PCEs. For the positive control the result showed the high frequencies of MNPCEs (39.13±2.01) induced by cyclophosphamide, which is significantly different from the others groups.

The ratio of PCE to NCE in OE treated group was not significantly different from that observed in the negative control and untreated SD groups. The ratio of PCE to NCE in control and untreated SD groups were 0.51±0.02 and 0.50±0.02, respectively as the ratio of PCE to NCE after OE treatment was 0.53±0.02. The ratio of all groups were significantly increased from the positive control group that presented 0.38±0.09.

There was a significant increase (p<0.01) in the frequencies of MNPCEs in A-S-30D hepatoma transplantation rats treated with cyclophosphamide, a positive control substance. Therefore, cyclophosphamide 80 mg/kg bw produced a very high frequency of MNPCEs, that was 39.13±2.01MNPCEs/1,000 PCEs.

The amount of cell volume, cell viability, cell concentration and total cell of AS-30D hepatoma cell line in this experiment were summarized in Table 4. There was no significantly different in each group. For the OE treated group, there was no significantly different in cell volume, cell viability, cell concentration and total cell compared with normal saline and Sprague-Dawley groups.

The bodyweight and food consumption curves of AS-30D collected throughout experimental period showed in Figures 5 and 6. There was no significantly different of bodyweight in each group. The food consumption of all groups demonstrated that there was no significant difference between OE treated rat and of control group.
Figure 5. Body weight of A-S-30D hepatoma transplanted Sprague-Dawley rats after 7-day repeat oral administration of 5 g/kg bw Ocimum sanctum leaf extract compared with control groups. There is not significantly different among OE-treated group and controls.
Figure 6. Food consumption of A-S-30D hepatoma transplanted Sprague-Dawley rats after 7-day repeat oral administration of 5 g/kg bw Ocimum sanctum leaf extract compared with control groups. There is not significantly different among OE-treated group and controls.
This data suggested that no significant increase in the MN frequency of PCEs and no toxic effect to red blood cell in AS-30D hepatoma transplanted rats after fed with 5g/kg bw of Ocimum sanctum leaf extract.

4.3 The effect of OE on the micronucleus formation induced by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats

The effect of OE on frequency of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of PCE to NCE of OE induced by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats summarized in Tables 5 and 6.

The fluid volume, cell concentration, cell viability and total cell of A-S-30D hepatoma cell line were showed in Table 7.

OE (5g/kg bw) was significantly decreased in the number of micronuclei in A-S-30D hepatoma transplanted rat induced by cyclophosphamide in both 7 and 21 days experiments compared with that found in positive control group. In the 7 days experiment the frequencies of micronuclei in the positive control group was 39.13±2.01 MNPCEs/1,000PCEs as the frequencies of micronuclei in OE treated group was 20.25±1.80 MNPCEs/1,000PCEs after 30 hours of cyclophosphamide injection. For negative control the frequencies of micronuclei was 1.81±0.27 MNPCEs/1,000 PCEs. For the 21 days experiment the frequencies of micronuclei in the positive control group was 38.94±1.72 MNPCEs/1,000PCEs as the frequencies of micronuclei in OE treated group was 25.18±1.60 MNPCEs/1,000PCEs after 30 hours.
Table 5. Inhibitory effect of 7-day oral administration of *Ocimum sanctum* leaf extract on the induction of micronucleated polychromatic erythrocytes (MNPCES) by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN PCEs/1,000 PCEs(^1)</th>
<th>PCE: NCE ratio(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>1.81 ± 0.27</td>
<td>0.51 ± 0.02*</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>39.13 ± 2.01</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td>OE interact with CP (i.p)</td>
<td>20.25 ± 1.80*</td>
<td>0.41 ± 0.02</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal, MNPCES = Micronucleated Polychromatic Erythrocytes, PCE = Polychromatic Erythrocyte, NCE = Normochromatic Erythrocyte.

\(^1\) Mean ± SD, n = 8, 1000 PCEs scored per animal; \(^2\) Mean ± SD, n = 8, 300 erythrocytes (PCE/NCE) scored per animal.

* Significant difference from cyclophosphamide at p< 0.01 by Mann-Whitney u-test.
Table 6. Inhibitory effect of 21-day oral administration of *Ocimum sanctum* leaf extract on the induction of micronucleated polychromatic erythrocytes (MNPCES) by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN PCEs/1,000 PCEs(^1)</th>
<th>PCE: NCE ratio(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>1.31 ± 0.27</td>
<td>0.51 ± 0.03*</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>38.94 ± 1.72</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>25.18 ± 1.60 *</td>
<td>0.40 ± 0.01</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal, MNPCEs = Micronucleated Polychromatic Erythrocytes
PCE = Polychromatic Erythrocyte, NCE = Normochromatic Erythrocyte
\(^1\)Mean ± SD, n = 8, 1000 PCEs scored per animal; \(^2\)Mean ± SD, n = 8, 300 erythrocytes (PCE/NCE) scored per animal.
*Significant difference from cyclophosphamide at p< 0.01 by Mann-Whitney u-test.
cyclophosphamide injection. For negative control the frequencies of micronuclei was 1.31±0.27 MNPCEs/1,000 PCEs.

The ratios of PCE to NCE in OE treated group of both experiments were increased from that observed in positive control group. In the 7 days experiment the ratio in positive control group was 0.38±0.09 and in the OE treated group was 0.41±0.02. The PCE: NCE ratio of negative control was significantly increased when compared to another groups. The ratio in positive control group of the 21 days experiment was 0.36±0.01 and in the OE treated group was 0.40±0.01. The negative control showed the high in PCE: NCE ratio (0.51±0.03) and significantly different from the other groups.

For cell line observation, Both 7 and 21 days experiments did not significantly different in fluid volume, cell concentration, cell viability and total cell in each group. OE (5 g/kg bw) did not show significantly different in fluid volume, cell concentration, cell viability and total cell compared with negative control group (NSS). The means of fluid volume, cell concentration, cell viability and total cell in control group of the 7 days experiment were 23.38±0.96, 97.04±0.34, 1.80±0.18(10^5) and 41.0±4.4(10^5), respectively. As the means of fluid volume, cell concentration, cell viability and total cell in 5g/kg bw of OE treated group were 23.13±0.81, 96.91±0.63, 1.40±0.10(10^5) and 37±6.3(10^5), respectively. For 21 days experiment the means of fluid volume, cell concentration, cell viability and total cell in control group were 23.25±1.00, 97.44±0.85, 1.40±0.10(10^5) and 31.0±1.8(10^5), respectively. For 5 g/kg bw of OE treated group were 23.00±1.12, 97.10±0.54, 1.40±0.13(10^5) and 31.0±3.4 (10^5), respectively.
Table 7. The amounts of cell volume, viability, cell concentration and total cell of A-S-30D hepatoma cell line after oral administration of Ocimum sanctum leaf extract.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of treatment (day)</th>
<th>Volume (ml)</th>
<th>Viability (%)</th>
<th>Cell conc. (10^8)/ml</th>
<th>Cell No. (10^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>7</td>
<td>18.38±1.72</td>
<td>97.16± 0.27</td>
<td>1.70±0.16</td>
<td>31.0±4.6</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>21.75±1.06</td>
<td>97.24± 0.45</td>
<td>1.50±0.13</td>
<td>32.0±2.9</td>
</tr>
<tr>
<td>Normal saline</td>
<td>7</td>
<td>23.38±0.96</td>
<td>97.04± 0.34</td>
<td>1.80±0.18</td>
<td>41.0±4.4</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>23.25±1.00</td>
<td>97.44± 0.85</td>
<td>1.40±0.05</td>
<td>31.0±1.8</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>7</td>
<td>23.13±0.81</td>
<td>96.91± 0.63</td>
<td>1.40±0.10</td>
<td>37.0±6.3</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>23.00±1.12</td>
<td>97.10 ±0.54</td>
<td>1.40±0.13</td>
<td>31.0±3.4</td>
</tr>
</tbody>
</table>

OE = Ocimum sanctum leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal

a The concentration of cell line per 1 ml of volume

b The total number of cell line derived from cell concentration multiply volumes

1 Values are MEAN ± SD of 8 rats/group

By variance analysis (ANOVA), no significant differences at p<0.01 were found between each group in fluid volume, cell concentration, viability and number of cell.
Figure 7. Body weight of A-S-30D hepatoma transplanted Sprague-Dawley rats after 7-day repeat oral administration of 5 g/kg bw *Ocimum sanctum* leaf extract compared with control groups. There is not significantly different among treated group and controls.
Figure 8. Body weight of A-S-30D hepatoma transplanted Sprague-Dawley rats after 21-day repeat oral administration of 5 g/kg bw Ocimum sanctum leaf extract compared with control groups. There is not significantly different among OE-treated group and controls.
Figure 9. Food consumption of A-S-30D hepatoma transplanted Sprague-Dawley rats after 7-day repeat oral administration of 5 g/kg bw *Ocimum sanctum* leaf extract compared with control groups. There is not significantly different among OE-treated group and controls.
Figure 10: Food consumption of A-S-30D hepatoma transplanted Sprague-Dawley rats after 21-day repeat oral administration of 5 g/kg bw Ocimum sanctum leaf extract compared with control groups. There is no significantly different among OE-treated group and controls.
This result indicated that OE at a dose 5g/kg bw was significantly decreased in the number of micronuclei formation in AS-30D transplanted rat induced by cyclophosphamide. On the other hand, there was no significantly different in fluid volume, cell concentration, cell viability and total cell in AS-30D hepatoma cell line at the same time.

The bodyweight curves showed in Figures 7 and 8. There was no significantly different of bodyweight in each group of both 7 and 21 days experiments. The food consumption of all groups demonstrated that there were no significantly different of food consumption. However, the OE treated group was a bit decreasing in food consumption compared with negative control group (Figures 9 and 10).

The effect of OE on frequency of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of PCE to NCE of OE induced by cyclophosphamide in bone marrow cells of normal Sprague-Dawley rats summarized in Tables 8 and 9.

In both 7 and 21 days experiments, OE (5g/kg bw) significantly decreased in the number of micronuclei formation in Sprague-Dawley rats induced by cyclophosphamide compared with that found in positive control group. The frequencies of micronuclei in the positive control group in 7 days experiment was 39.31±2.10 MNPCEs/1,000PCEs as the frequencies of micronuclei in OE treated group was 25.38±0.94 MNPCEs/1,000 PCEs after 30 hours cyclophosphamide injection. For negative control group the frequencies of micronuclei was 1.18±0.36 MNPCEs/1,000 PCEs. This results similarly observed in 21 days experiment demonstrated the frequencies of micronuclei in the positive control group was 39.13±1.89 MNPCEs/1,000PCEs as the frequencies of micronuclei in OE treated group was
Table 8. Inhibitory effect of 7-day oral administration of *Ocimum sanctum* leaf extract on the induction of micronucleated polychromatic erythrocytes (MNPCEs) by cyclophosphamide in bone marrow cells of Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN PCEs/1,000 PCEs</th>
<th>PCE: NCE ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>1.18 ± 0.36</td>
<td>0.50 ± 0.02*</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>39.31 ± 2.10</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>25.38 ± 0.94*</td>
<td>0.41 ± 0.02</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal, MNPCEs = Micronucleated Polychromatic Erythrocytes

PCE = Polychromatic Erythrocyte, NCE = Normochromatric Erythrocyte

1Mean ± SD, n = 8, 1000 PCEs scored per animal; 2Mean ± SD, n = 8, 300 erythrocytes (PCE/NCE) scored per animal.

*Significant difference from cyclophosphamide at p< 0.01 by Mann-Whitney u-test.
### Table 9  Inhibitory effect of 21-day oral administration of *Ocimum sanctum* leaf extract on the induction of micronucleated polychromatic erythrocytes (MNPCEs) by cyclophosphamide in bone marrow cells of Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN PCEs/1,000 PCEs(^1)</th>
<th>PCE: NCE ratio(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>1.88 ± 0.30</td>
<td>0.48 ± 0.10*</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>39.13 ± 1.89</td>
<td>0.37 ± 0.09</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>25.19 ± 1.63 *</td>
<td>0.41 ± 0.02</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal, MNPCEs = Micronucleated Polychromatic Erythrocytes  
PCE = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocyte  
\(^1\)Mean ± SD, n = 8, 1000 PCEs scored per animal;  
\(^2\)Mean ± SD, n = 8, 300 erythrocytes (PCE/NCE) scored per animal.  
\(^\ast\)Significant difference from cyclophosphamide at p< 0.01 by Mann-Whitney u- test
25.19±1.63 MNPCEs/1,000 PCEs after 30 hours cyclophosphamide injection. For negative control, the frequencies of micronuclei was 1.88±0.30 MNPCEs/1,000 PCEs.

The ratio of PCE to NCE in OE treatment group were increased from that observed in positive control group of both 7 and 21 days experiments. In 7 day experiment the ratio in positive control group was 0.36±0.09 and in the OE treated group was 0.41±0.02. The negative control showed the high in PCE: NCE ratio (0.50±0.02) that significant difference from the other groups. The ratio of PCE to NCE in 21 days experiment was significantly increasing in NSS group, that was 0.48±0.10, higher than that found in OE and CP treated rat. OE treated group was increased from that observed in positive control group. The ratio in positive control group was 0.37±0.09 and in the OE treated group was 0.41±0.02.

This result indicated that OE at a dose 5 g/kg bw given orally for 7 and 21 days were significantly decreased in the number of micronuclei formation in Sprague-Dawley rats induced by cyclophosphamide.

There was no significantly different of the frequency of micronuclei formation between rats with or without A-S-30D transplantation on 7 or 21-day experiment of 5g/kg bw ethanol leaf extract of Ocimum sanctum induced micronucleus by CP (Tables 10 and 11). The amount MNPCEs of OE treated rat in AS-30D transplanted rat was 20.25±1.80 and in normal SD-rat was 25.38±0.64, as the positive control were 39.13±2.01 and 39.31±2.10 in transplanted rat and normal rat, respectively, after 7-day treatment. The frequency of MNPCEs in OE treated rat in 21-day experiment of transplanted rat was 25.18±1.60 and in control was 38.94±1.72, as the MNPCEs found in normal rats were 25.19±1.63 and 39.13±1.89 in OE treated rat and control.
**Table 10.** The comparative frequency of MNPCEs and PCE:NCE ratio in rat bone marrow micronucleus test between A-S-30D transplanted rat and normal SD-rat after 7 -day oral administration of *Ocimum sanctum* leaf extract.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat</th>
<th>No. of MN PCEs/1,000 PCEs</th>
<th>PCE: NCE ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>Transplanted rat</td>
<td>1.81 ± 0.27</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>1.18 ± 0.36</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>Transplanted rat</td>
<td>39.13±2.01</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>39.31±2.10</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>Transplanted rat</td>
<td>20.25±1.80</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>25.38±0.94</td>
<td>0.41±0.02</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal

MNPEs = Micronucleated Polychromatic Erythrocytes

PCE = Polychromatic Erythrocyte, NCE = Normochromatic Erythrocyte

1Mean ± SEM, n = 8, 1000 PCEs scored per animal; 2Mean ± SEM, n = 8, 300 erythrocytes (PCE/NCE) scored per animal. There is no significantly different between both rats at p< 0.01 by Man –Whitney u- test.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat</th>
<th>No. of MN PCEs/1,000 PCEs (^1)</th>
<th>PCE: NCE ratio (^2)</th>
</tr>
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<tbody>
<tr>
<td>Normal Saline</td>
<td>Transplanted rat</td>
<td>1.31± 0.27</td>
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<td></td>
<td>Normal</td>
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</tr>
<tr>
<td>Cyclophosphamide (i.p.)</td>
<td>Transplanted rat</td>
<td>38.94±1.72</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>39.13±1.89</td>
<td>0.37 ± 0.09</td>
</tr>
<tr>
<td>OE interact with CP (i.p.)</td>
<td>Transplanted rat</td>
<td>25.18±1.60</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>25.19±1.63</td>
<td>0.41 ± 0.02</td>
</tr>
</tbody>
</table>

OE = *Ocimum sanctum* leaf extract 5 g/kg bw, Normal saline 0.5 ml/kg bw, CP = Cyclophosphamide 80 mg/kg bw, i.p. = Intraperitoneal MNPCEs = Micronucleated Polychromatic Erythrocytes PCE = Polychromatic Erythrocyte, NCE = Normochromatic Erythrocyte

\(^1\) Mean ± SEM, \(n = 8\), 1000 PCEs scored per animal; \(^2\) Mean ± SEM, \(n = 8\), 300 erythrocytes (PCE/NCE) scored per animal. There is no significantly different between both rats at \(p < 0.01\) by Man-Whitney \(u\)-test.
respectively. The negative control of both rats showed similar yield of MNPCEs of all 7 and 21-day experiment.

The PCE: NCE ratios between both rats did not show any significantly different (Tables 10,11). These results indicated there were no significantly different of the frequency of micronuclei formation and PCE: NCE ratio between both rats after feeding of 5g/kg bw of OE in rat bone marrow micronucleus test.
CHAPTER V

DISCUSSION

5.1 Acute toxicity test of ethanol Ocimum sanctum leaf extract

In this study used the dose at a concentration of 15 g/kg bw of OE given orally to the male and female Sprague-Dawley rats. The result demonstrated, there is no toxic effect of the orally given of 15 g/kg bw of OE. Observations were determined by survival of animals, body weight change, general behavior and as well as autopsy investigated reports. The number of animals died were not show in this study and no significantly different in body weight were observed in OE-treated rats of both sexes compared to the control groups. Furthermore, the food consumption between OE-treated rats and control did not significantly different in this study. The generally behavior observation showed no specific sign of toxicity in any animals as similar result derived from an autopsy technique that demonstrated no abnormal sign occurring in observed organs.

These results suggested that 15 g/kg bw of OE was not toxic to both sexess of Sprague-Dawley rats, supporting no toxicity of this well known flavoring plant exposure to human as food recipe or medical uses. The previous study of toxic assessment reported that 70% alcoholic extract of 4500 mg/kg bw by oral administration is the LD50 of mice and the dose of 3.24 g/kg bw intraperitoneal causing in 50% of animal death. (Bhagarva and Singh, 1981). However, there is no toxic effect of 50% alcohol extract of O. sanctum given intraperitoneally to mice at a dose 1g/kg bw (Dhar et al., 1968) or 10 g/kg bw by
subcutaneous injection (Mokkhasmit, 1971). It is also reported that treatment with aqueous extract did not produce any acute toxic symptoms at dose up to 5g/kg bw (Devi and Ganasoundari, 1995). The dose of 15 g/kg bw of OE used in this study was higher than LD50 determined in mice (4850 mg/kg bw of OE given by oral administration) and also higher than the LD50 of mice fed 6200 mg/kg bw aqueous leaf extract of Ocimum sanctum.

However, the result of this study suggested the orally administrated of 15 g/kg bw of OE did not produce any toxicity evaluated by using male and female Sprague-Dawley rats. The study, therefore, confirmed that Ocimum sanctum is the safe plant.

5.2 The effect of OE on the micronucleus formation in bone marrow cells of AS-30D hepatoma transplanted rats.

The in vivo micronucleus assay has become increasingly accepted as the model of choice for evaluation of chemistry induced cytogenetic damage in the intact animal. The earliest application of this model focused on the frequency of micronuclei in polychromatic erythrocytes (MNPCEs) in mouse bone marrow (Heddle et al., 1991). However, rat is also the most frequently used rodent species in micronucleus testing. Several recent studies have demonstrated the rat on bone marrow micronucleus testing. In this study using an in vivo rat bone marrow micronucleus assay which is the well established, reliable and moderately expensive short-term test to check the mutagenic and antimutagenic effect of the extract (Edenharder et al., 1998)

In this study, OE was tested for its potential to induce micronuclei formation in rat bone marrow cells. It showed that OE at a concentration of 5g/kg bw did not significantly
increase the frequency of micronuclei formation in rat bone marrow PCEs after 7 days oral administration to Sprague-Dawley rats (Table 3). This result was associated according to the work of Ganasoundari et al. (1997) that studied toxicity of OE by sister chromatic exchange method, the result shown the ethanolic extract of *Ocimum sanctum* leaves did not induce sister chromatic in the rats. The ethanolic extract of this plant was also reported to protect mouse bone marrow chromosome against radiation clastogenicity and did not cause mutagenicity or toxicity to the bone marrow cells (Devi et al., 1998). Given with the previously supporting data of the present study, the *Ocimum sanctum* leaf extract, therefore, did not exert the mutagenic property yielding by the rat bone marrow micronucleus assay.

5.3 The effect of OE on the micronucleus formation induced by cyclophosphamide in bone marrow cells of AS-30D hepatoma transplanted rats.

In this study, we demonstrated the used of AS-30D hepatoma transplanted SD rat in comparison with normal Sprague-Dawley rat of an investigation the effect of OE on the micronucleus formation induced by cyclophosphamide in bone marrow cells of rats.

From the result, the transplanted rats showed similarly result as normal rats on the effect of OE that significantly decreased the micronucleus formation induced by cyclophosphamide in bone marrow cells.

The result of this study showed the significant decreasing of MNPCEs of the 5 g/kg bw of OE compare with control in the rats with or without AS-30D hepatoma
transplantation. This result suggested that the ethanol leaf extract of OE at a dose 5 g/kg bw possesses the inhibiting activity against micronucleus forming in rat bone marrow cells.

The aqueous and alcoholic extracts from the leaves of this plant have been investigated extensively for various pharmacological activities including their activity against cancer (Prashar et al., 1998). In this study found clearly that an ethanol leaf extract of Ocimum sanctum inhibited the micronucleus formation in rat bone marrow cells. The mechanism of this property is still unknown. But several previous studies indicated the interestingly possible of the potential of this plant involved carcinogenesis.

The data supports the several naturally occurring dietary or non-dietary constituents, as well as parts of several species of edible plant having pharmacological activity, that may influence the hepatic biotransformation enzyme profiles that are involved in activation and detoxification of xenobiotic compounds, including chemical carcinogen (Ganasoundari et al., 1997, 1998). These supporting data are evaluated the inhibiting effect of OE against micronucleus formation in this study, may involve in the hepatic biotransformation enzyme, which is may play a role in detoxification of cyclophosphamide induced mutagenesis. Cyclophosphamide is an alkylating agent possessing the clastogenic effect which produced the formation of micronuclei in bone marrow cells (Fleming 1997; Countryman 1976). This clastogen requires activation by hepatic microsomal P450 mixed-function oxidase before being metabolized to its respective cytotoxic species, 4- hydroxycyclophosphamide, aldophosphamide, phosphoramidemustard and acroleine (Ludeman 1999; Fleming 1997). These metabolites
of cyclophosphamide alkylate DNA, forming DNA-DNA cross linking that result in inhibit of DNA synthesis (Fleming 1997; Colvin 1999). Because genetic damage that results in chromosome break or structurally abnormal chromosome leads to micronucleus formation (USFDA, 2000), the incidence of micronuclei serves as an index of this type of damage caused from clastogenic property of cyclophosphamide. This alkylating agent is electrophilic specie prone to react readily with the tripeptide glutathione through glutathione-S-tranferase (Gouyette, 1664). Therefore, glutathione appears to be involved directly in the detoxification of cyclophosphamide and its metabolites (Gamcsik, 1999). Both elevate glutathione levels and increase activity of the enzyme glutathione-S-tranferase has been associated with the resistance of cells to cyclophosphamide (Colvin et al., 1993; Tanner et al., 1997). The one mechanism of this detoxification is the inactivation of the alkylating agent by conjugation with glutathione and catalyzed by glutathione-S-tranferase (Colvin et al., 1993). Since it has been well documented that Ocimum sanctum leaf extract produces a substantial elevation in the hepatic microsomal cytochrom p450, cytochrom b5 and aryl hydrocarbon hydroxilase activites in a dose-dependent manner (Banerjee et al., 1996; Prashar et al., 1994). It could effectively elevate the glutathion -S-transferase activity and the reduced glutathion levels in the liver as well as in extra-hepatic organs of mice (Barnerjee et al., 1996; Prashar et al.,1994), that was consistent with Aruna and Sivaramakrishnan (1990, 1992) reported that OE increased glutathion and GST enzymes. Hence, from these accumulating evidences, it is possible that Ocimum sanctum leaf extract may be accelerating detoxification of
cyclophosphamide in the hepatic and extra-hapatic tissues of rat by increasing the microsomal cytochrom p450 and glutathione-S-transferase activities.

The earlier studies provide evidences that OE exhibits a chemopreventive action on DMBA-induced skin papillomagenesis in mice (Prashar and Kumar, 1995), and inhibits DNA-binding of 7,12 DMBA in rat hepatocytes in vitro. These results suggested that OE blocks or suppresses the events associated with chemical carcinogenesis by inhibiting metabolic activation of the carcinogen (Karthikeyan, 1999).

It is not possible at this stage to suggest any plausible mechanism of action, since OE used in this study contains a number of compounds and the observed effect cannot be attributed to any particular compound (Prashar et al., 1998).

Science hepatic metabolism of xenobiotic chemicals often quantitatively predominates over organ specific metabolism, induction of the above-mentioned enzyme parameters many effectively contribute in protecting against insult by toxic xenobiotic compounds, including carcinogens. In this study using an indirect carcinogen; cyclophosphamide as the positive control produced MNPCEs in rat bone marrow. Metabolizing of detoxification enzymes including phase I and phase II are effectively protecting of this clastogen to biding with DNA, resulting in adduct formation.

The OE may be suggested in this study of induction of carcinogen- metabolizing enzymes, but with component(s) of the OE affects the enzyme activity is not clear. A principle constituent is eugenol (Prashar et al., 1998) which is known to induce UDP-glucoronyl transferase and GST (Yokota et al., 1988).
Although eugenol is the active principle, but actually most derived in the volatile oil extract via steam distillation from the leaves and seed of OE (Sinha and Gulati, 1990). This essential oil exerts the flavoring in specific odor characteristic using to flavor food, dental and oral products, in fragrance, and in traditional rituals and medicines. Other constituents of the plant extract, though minor ingredients, may play major role in determining the overall biologic activity of the medical plant. The other compounds that found in the leaf extract may play and additional and/or synergistic chemoprotective role by inducing detoxification enzymes include sterols, triterpines, alkaloids, glycosides, saponins, tannins, various sequiterpene in essential oil of the leaves, and hydroxy chavicol (Prashar et al., 1998). These compounds, not particular in this study, may influence in the important role in reduction of the micronucleus formation induced by CP detected by rat bone marrow cells.

This finding of the present study, suggested that the 5 g/kg bw ethanol leaf extract of Ocimum sanctum possesses the inhibiting effect against micronucleus formation determined by the significantly decreasing in MNPCEs induced by cyclophosphamide in both Sprague-Dawley rats with or without AS-30D hepatoma transplantation. Nonetheless, the rats that were transplanted with AS-30D hepatoma cell line had been shown to be utilized successfully in short-term micronucleus testing.
CHAPTER VI
CONCLUSION

1. In acute toxicity test, any significant changes that can be considered as induced by administration of 15 g/kg bw of OE was not found by overall judgements from the result of general behavior, the number of animal death and organ autopsy observation. The growth rates of bodyweight and food consumption in rats also demonstrated no toxicity effect of this plant at a dose 15 g/kg bw given by oral administration.

2. Administration of OE at a dose 5 g/kg bw did not cause mutagenicity in the rats. The AS-30D hepatoma transplanted rats yielded similar results as the normal rats on the significant inhibition of micronucleus formation against cyclophosphamide after 7 and 21-day repeat oral administration of 5 g/kg bw of OE. The anti-mutagenicity of OE suggested an enhancement of detoxification enzymes against cyclophosphamide which were reduction of micronucleus formation. It is possible that some compounds in OE may play an important role in such enhancement of detoxification mechanism. This thesis, therefore, confirmed health benefits of holy basil (*Ocimum sanctum*) as and ingredient of food and as a medicinal plant in reducing mutagenicity. However, the results did not see changes in the number of hepatoma harvested from the transplanted rats. Nonetheless, the rats that were transplanted with the AS-30D hepatoma cell line had been shown to be utilized successfully in short-term mutagenicity or anti-mutagenicity together with the effect on the cell growth testing. Thus, it would be of beneficial to an investigator who wants to minimize the number of rats used in genotoxic and oncologic studies, all in the same time.
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