INHIBITORY EFFECT OF ESSENTIAL OIL OF *OCIMUM SANCTUM* L. AGAINST PESTICIDE INDUCED GENOTOXICITY

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BY

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September, 2012

CERTIFICATE

I declare that the dissertation entitled “Inhibitory effect of essential oil of Ocimum Sanctum L. against pesticide induced genotoxicity” has been prepared by me under the guidance of Dr. Sunil Mittal, Assistant Professor, Centre for Environmental Science and Technology, School of Environment and Earth Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Inhibitory Effect of Essential Oil of Ocimum Sanctum L. against Pesticide Induced Genotoxicity

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The increase in pesticide contamination in the environment is of great concern due to its strong association with genetic material. The plants are considered as natural drugs to decline such toxic effects. In present study, the genotoxicity of monocrotophos has been evaluated in lung cancer cell line i.e. A549 and H1299. The further step is to evaluate the prevention of monocrotophos induced genotoxic effect by pre and post-treatment of O. sanctum essential oil. The results of present study suggest that eugenol (46%) and caryophyllene (28%) was the major components of essential oil of O. sanctum apart from other minor components. The essential oil caused decrease in cell proliferation rate with increase in essential oil treatment after 20 µg/ml concentration. But, the cell viability was not affected at 10 µg/ml concentration in both the cell lines. On the other hand, monocrotophos cause increase cell proliferation rate at lower concentration (6.25 µM-200µM) for 6 hrs. in A549 cells and significant decrease in cell proliferation rate at higher concentration (1mM–50mM) in both the cell line. The genotoxic study revealed that the monocrotophos caused increase in comet length, % DNA in tail, tail length, tail intensity and decrease in Head intensity but pre-treat and post-treatment show protective action with respect to various Monocrotophos concentrations. It has been observed that post-treatment was better in comparison to pretreatment of O. sanctum essential oil in all studied parameters. The results suggest that the H1299 cells, lacking p53 expression, was more sensitive to essential oil and monocrotophos concentrations. It can be concluded that the essential oil helps the cells to compensate the oxidative stress generated inside the cell and hence can protect them from pesticide induced genotoxic effect.

(Gurpreet Kaur) (Dr. Sunil Mittal)

Dedicated

To

"Akaal Purakh"

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(Gurpreet Kaur)

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviation
1.	Chinese Hamster Ovary cells	CHO cells
2.	Deoxyribonucleic Acid	DNA
3.	Dimethyl sulfoxide	DMSO
4.	Environmental Protection Agency	EPA
5.	European Union	EU
6.	Gas chromatography–mass spectrometry	GC-MS
7.	Inhibitory Concentration	Ic
8.	Intraperitoneally	i.p.
9.	Lactate Dehydrogenase	LDH
10.	Lethal Dose	LD
11.	Micronucleus test	MNT
12.	Monocrotophos	MCP
13.	Organophosphates	Ops
14.	Pesticide Action Network	PAN
15.	Prior Informed Consent	PIC
16.	Reactive Oxygen Species	ROSs
17.	World Health Organization	WHO
18.	Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH

19.	Ethylene diaminetetra acetic acid	EDTA
20.	Fetal Bovine Serum	FBS
21.	Dulbecco’s Modified Eagle’s Serum	DMEM
22.	Phosphate Buffer Solution	PBS

CHAPTER-1

INTRODUCTION

Pesticides are unique contaminants in a manner that they are purposely released into the environment to improve food production by abolition of harmful pest species. India is primarily an agriculture-based country with more than 60-70% of its population dependent on agriculture (Kale et al., 2012). India's fast growing population is projected to cross 1.3 billion by 2020 (Bhalla and Hazell, 1997). To feed this increasing population, from exhausted arable land, is of great concern to the Indian planners (Rosenzweig et al., 2001). In India, it has been estimated that 15–20% of all production is destroyed by pests (Bhalerao and Puranik, 2009). Therefore, the pesticides had become indispensable to modern agriculture to increase the crop production and fulfill the requirements of the increasing population.

Currently, among the various pesticides being used world over, organophosphates form a major and most widely used group of pesticides. The wide spread use of these pesticides over the years has resulted in various environmental and health problems which have been highlighted by different researchers. The long time exposure may lead to health problems like reproductive (Luccio-Camelo and Prins, 2011), mental retardation (Garry et al., 2004) and mutagenesis disorders (Mahboob et al., 2002).

Pesticide under investigation

Among the organophosphates, monocrotophos (Dimethyl (E)-1-methyl-2-(methylcarbomoyl) vinyl phosphate) is one of the most widely used broad spectrum systemic insecticide throughout the world including India. The commercially available monocrotophos is a reddish brown to dark brown in color, clear viscous liquid having mild ester odour. The half-life of MCP is 131 and 26 days at pH of 3 and 9 respectively at 25^{o}C (Lee et al., 1990).

Why Monocrotophos pesticide

Monocrotophos can be absorbed into the body by inhalation, through skin and by ingestion (Pain et al., 2004), and is highly toxic by all routes of exposure. The metabolic products of monocrotophos are dimethylphosphate, o-desmethyl monocrotophos and n-desmethyl monocrotophos which are more toxic than monocrotophos. The acute oral lethal dose (LD50) of monocrotophos for rats is 14 mg/kg (IPCS/World Health Organization, 1993). The classification of monocrotophos (Cas no. 6923-22-4) has been done by various international agencies based on its hazardousness (Table 1.1).

Table 1.1 Classification of monocrotophos as per various international agencies:

Sr. No.	**International agencies**	**Classification**
1	WHO classification*	Ib class (Highly hazardous class)
2	Rotterdam convention**	Banned or severely restricted for health or environmental reasons [listed in annexure iii of prior informed consent (PIC)]
3	Pesticide action network (PAN) international***	Highly hazardous
4	European Union (EU)****	Very toxic by inhalation

(http://www.inchem.org/documents/pds/pdsother/class_2009.pdf*;http://www.pic.int/Default.aspx?tabid=1132**;http://www.pan-germany.org/download/PAN_HHPList_1101.pdf***)

Monocrotophos is readily soluble in water and hence mobile in the soil (Bhalerao and Puranik, 2009), therefore can reach underground water by leaching from agricultural lands. On the other hand, it is weakly sorbed by soil particles because of its hydrophilic nature, so it is detected in ground, surface and spring runoff (Waite et al., 1992). Kumari et al. (2007) reported the presence of monocrotophos residues (1.000–4.000 $\mu g l^{-1}$) in rain water in 2002 from Hisar, India.

Protective role of herbal plants

Natural plant products are known to be an important source for biologically active drugs (Qaddouri et al., 2011). It has been well known that chemical constituents of medicinal plants have high antioxidant property which plays an important role in the prevention of various diseases (Bjelakovic et al., 2012). The concept to use plants as a source of medicine is very old. Chinese were the first to use plants as therapeutics before 4000-5000 B.C. In India, use of plants as a medicine appeared in Rigveda which has been documented in 3500-1600 B.C. (Renugadevi, 2012). The bioactive phytochemical constituents of plants responsible for curing disease include alkaloids, flavonoids, phenolics, essential oils, tannins and saponins (Surveswaran et al., 2007).

Essential oils, also known as ethereal oils, are defined as odiferous bodies, obtained almost exclusively from vegetative organs such as flowers, leaves, roots, rhizomes, barks, woods, fruits, and seeds (Skocibusic et al., 2006; Celiktas et al., 2007) which get accumulated in secretary cells, cavities, channels, and epidermic cells (Hussain et al., 2008; Anwar et al., 2009). The technique for obtaining essential oil from naturally occurring organic material was first time developed in Arab in the middle ages (Bakkali et al., 2008). Currently, about 300 essential oils have been reported to have commercial importance in various sectors like pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Sivropoulou et al., 1996; Burt, 2004; Longaray et al., 2007).

Essential oil as antigenotoxic

The plants become a reliable remedy to treat antimutagenicity and antigenotoxicity (Bakkali et al., 2008), as they have capacity to scavenge free radicals (Ikken et al., 1999). Beric et al., 2008 reported that basil oil have protective role, as some its volatile monoterpenes proven to be efficient against oxidative stress.

Plant selected for study

Plants of Lamiaceae family are widely used in traditional systems of medicine (Prakash and Gupta, 2005). Most of the aromatic plants and essential oil

commodities belong to this family in terms of world trade. It comprises about 236 genera and approximately 7000 species (http://www.britannica.com/EBchecked/topic/328710/Lamiaceae). The genus Ocimum L., consist of more than 150 species which grow commonly all over the world (Mondal et al., 2009).

Ocimum is also known as “Queen of plants” or “The mother medicine of nature”. The word "Tulsi" is sanskrit word means "the incomparable one". Ocimum sanctum, commonly known as holy basil, is frequently cultivated in several countries of America, Australia, East Asia, and Europe for the production of essential oils (De Vasconcelos Silva et al., 2004; Zheljazkov et al., 2007). It is grown in various tropical and sub-tropical regions including India (Banerjee et al., 1996). In India, two forms of Tulsi are more common - dark or Shyama (Krishna) Tulsi and light or Rama Tulsi.

Cell line selected for study

The lung cells represent the principal target site for inhalation of toxic substances. The two cell lines i.e. A459 and H1299 have been selected for study, which are epithelial in nature. The human lung, A549 cell line, is one of important cancer cell line which is frequently used as in vitro model for various environment genotoxic studies. The H1299 cell line lack p53 expression which is tumour suppressor protein plays important role to response for DNA damage in mammalian cells.

CHAPTER-2
REVIEW OF LITERATURE

Essential oils have gained much interest due to their amazing antioxidant, antitumor, antibacterial, antifungal and insecticidal properties (Bozin et al., 2006; Politeo et al., 2007). Among the plants, known for therapeutic potential, Ocimum sanctum is known for its wide medicinal properties. The therapeutic use of O. sanctum (Tulsi) has been well documented in Ayurveda and the whole plant can be used as a source of remedy for various diseases (Rajurkar et al., 1997). Major uses of O. sanctum are antioxidant (Anwar et al., 2009), anti-tumor (Adhvaryu et al., 2008, Karthikeyan et al., 1999; Sukumaran et al., 1994), anti-microbial (Rao and Nigam, 1970; Dey and choudhury, 1984; Phadke and Kulkurni, 1989; Singh et al., 2005) anti-lipidperoxidative (Kedlaya and Vasudevan et al., 2004) anti-diabetic (Chattopadhyay, 1993; Vats et al., 2004; Gupta et al., 2006; Chandra et al., 2008; Reddy et al., 2008; Agrawal et al., 1996), hepato-protective (Mondal et al., 2009; Chattopadhyay et al., 1992), anti-inflammatory (Godhwani et al., 1987; Singh and Agrawal, 1991; Singh et al., 1996), anti-carcinogenic (Aruna and Sivaramakrishnan, 1992; Banerjee et al., 1996), immuno modulatory effect (Godhwani et al., 1988; Mediratta et al., 1988; Mediratta et al., 2002; Mukherjee et al., 2005; Mondal et al., 2009), food and perfumery industries (Telci et al., 2006).

The oil of O. sanctum has revealed the presence of linoleic, stearic, oleic, linolenic and palmitic acid (Padalia and Verma, 2011; Kothari et al., 2005). But the chemical composition of the O. sanctum essential oil varied depending upon the origins and cultivars (Zheljazkov et al., 2007). Variability in essential oil composition is basically due to variation in extraction, conservation of the oils, environmental factors, cultivation practices and vegetative cycle. So, the complexity of the essential oils is a real challenge for determining their reliable and accurate compositional data.

Cyototoxicity of Essential oil

The cytotoxicity of essential oil of different plants of lamiaceae familiy has been reported in various human cancer cell lines i.e. PC-3, A549 and MCF-7 (Zu et al., 2010). There are some minor components in the essential oils that contribute to

cytotoxic activity of oils (Duh et al., 1999; Legault et al., 2003). It has been reported that mainly sesquiterpenes are responsible for the cytotoxicity of essential oils (Sylvestre et al., 2005). The components like caryophyllene oxide, *β*-elemene, *γ*-elemene, that varied with respect to geographical regions, might play a key role in the cytotoxicity profile of essential oils (Sibanda et al., 2004). It has been reported that the Ic_{50} of essential oil of O. Sanctum is 0.0848 and 0.0951 mg/ml in P388 and KB cell line respectively (Manosroi et al., 2006). Generally, the low concentration of eugenol is known to act as an antioxidant, whereas its high concentration acts as a pro-oxidant resulting from the enhanced generation of tissue-damaging free radicals.

Protective effect of *Ocimum sanctum*

Numerous studies have been carried out to explore the compounds present in plants that protect cells against direct DNA damage. The continuous efforts have been carried out all over the world to identify the different antioxidant groups such as phenolics, flavonoids, phytosterols, tannins and phytoestrogens etc**.**

The lipid peroxidation has been shown through investigations to be caused by pesticides (Abdollahi et al., 2004) which make stable free radicals chain of oxidation (Jamesdaniel and Samson, 2004). Antioxidants have wide role in protecting the human body against such damage caused by reactive oxygen species (Hussain et al., 2001). The antioxidant activity of O. sanctum is evident from significant reduction in the generation of hydroxyl radical (Ganasoundari et al., 1998). Eugenol, a major component of the O. sanctum essential oil (Kelm et al., 2000; Lee et al., 2005), has shown to posses significant antioxidant property and efficiency to inhibit lipid peroxidation (Ganasoundari et al., 1998; Gupta et al., 2002).

Xing et al. (2012) observed that the pesticides have strong interaction with generation of oxidative stress and phase I/II enzyme genes which further increases the risk of cancer. It also has been investigated that O. sanctum provides protection against various chemical induced carcinogenesis by acting as an antioxidant or modulating phase I and II enzyme (Rastogi et al., 2007). Subramanian et al. (2005) investigated that O. sanctum has more potential than Tinospora malabarica to prevent oxidative damage to liposomal lipids and plasmid

DNA induced by various oxidants. The brief overview of the studies related to this genus has been given in table 2.2.

Table 2.2 Various studies highlighted the protective effect of O. Sanctum in various test models:

Sr. No.	Plant material	Results	References
1	O. sanctum leaves	Protection against the 3,4benzo pyrene and 3‘-methyl 4 dimethyl amino azobenzene induced squamous cell carcinoma and haematoma incidences in rats	Aruna and Sivaramakrishnan, 1992
2	O. sanctum leaf extract	Significant decrease in chromosomal damage by pre-treatment to γ-irradiation bone marrow cells in mouse	Ganasoundari et al., 1998
3	O. sanctum leaf extract	Significant reduction in the levels of 7,12-dimethylbenz (a) anthracene (DMBA) induced DNA adducts in rats	Prashar et al., 1998
4	O. sanctum aqueous extract and ethanolic extract	Prevent the early events of Dimethylbenz anthracene (DMBA) induced buccal pouch carcinogenesis in hamster buccal pouch carcinogenesis	Karthikeyan et al., 1999
5	O. sanctum seed oil	Antiproliferative and chemopreventive activities in mice	Prakash et al., 1999
6	Seed oil	Protection against 20-methylcholanthrene induced-fibrosarcoma tumors	Prakash and Gupta, 2000
7	O. sanctum	Anticancer activity against Ehrlich ascites carcinoma (EAC) and tumor in Swiss albino mice bearing	Somkuwar, 2003

Sr. No.	Plant material	Results	References
8	O. sanctum Ethanolic extracts	Antioxidant activity	Juntachote and Berghofer, 2005
9	Eugenol	Inhibits the proliferation in HL-60 human promyelocytic leukemia cells	Yoo et al., 2005
10	O. sanctum essential oils	Inhibition of the proliferation with Ic50 0.0848 and 0.0951 (mg/ml) in P388 and KB cell line respectively	Manosroi et al., 2006
11	Essential oil (Ocimum genus species)	Antioxidant activity	Trevisan et al., 2006
12	O. sanctum extract	Reduce experimentally induced Chromosomal aberrations, mitotic index, sister chromatid exchange in a dose dependent manner in human lymphocyte culture	Siddique et al., 2007
14	O. sanctum leaves	Inhibition of proliferation in N-methyl-N'-nitro-N-nitrosoguanidine induced gastric carcinogenesis	Manikandan et al., 2007
15	O. sanctum ethanolic leaf extract	Protects against 7,12-Dimethylbenz Anthracene-induced genotoxicity, oxidative stress in rats	Manikandan et al., 2007
16	O. sanctum	Anticancer activity in rats with uterine and ovarian carcinogenesis	Madhuri, 2008
17	O. sanctum combining with Azadirachta indica	Synergistic effect to reduce chemical-induced gastric carcinogenesis in rats	Manikandan et al., 2008

Sr. No.	Plant material	Results	References
18	O. sanctum extract	Reduces chromosomal aberrations and sister chromatid exchanges induced by chlormadinone acetate in human lymphocytes	Siddique et al., 2008
19	O. sanctum ethanol extracts	Induces apoptosis in A549 lung cancer cells and suppresses the in vivo growth of lewis lung carcinoma cells in a dose-dependent manner	Magesh et al., 2009
21	Ocimum viride essential oil	Cytotoxic and apoptotic activity in Human colorectal adenocarcinoma cells and COLO 205 cell	Sharma et al., 2010
22	Eugenol	Induces apoptosis and inhibits invasion and angiogenesis in a rat with gastric cancer	Manikandan et al., 2011
20	Eugenol	Induces apoptosis N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in rat	Manikandan et al., 2011

Monocrotophos relation with genetic material

Pesticides have been considered as probable chemical mutagens. The wide range of data suggests that the toxicity of pesticides is majorly contributed by oxygen free radical formation (Bagchi et al., 1995; Soltaninejad, 2009). Monocrotophos is a pentavalent phosphorus ester, which is capable of causing mutation due to its phosphorylation and methylation property (Bhunya and Jena, 1993). Thus, higher reactivity of monocrotophos may be explained by the methylation reaction of the phosphate group. If the chemical reacts with nuclear DNA, it is usually mutagenic and carcinogenic to the exposed organisms (Anwar, 1997). It has been reported that monocrotophos is more reactive than phosphorothionate esters such as chlorpyrifos or Malathion (Yaduvanshi et al., 2010). Exposure to pesticides generally doubled the level of genetic damage as measured by increased

chromosomal aberrations (Sanborn et al., 2007). There are several cytogenetic assays that have been used to evaluate the potential genotoxicity of pesticide (Bolognesi, 2003). The increased oxidative stress will causes imbalance in enzymatic and non-enzymatic antioxidant systems of cell due to monocrotophos (Siddiqui et al., 1990; Rao, 2006; Singh et al., 2006; Agrahari et al., 2009; Vanisthasree et al., 2011). Various studies supporting the genotoxicity of monocrotophos have been given in table 2.3.

Table 2.3 Various studies highlighted the genotoxic effect of monocrotophos in various test models

Sr. No.	Test system	Result	References
1.	Sister chromatid exchange assay in human lymphoid cells	Positive	Sobti et al., 1982
2.	Micronucleus test in bone marrow of Swiss mouse	Positive	Vaidya et al., 1982
3.	Reversion assay in S. typhimurium	Positive	Moriya et al., 1983
4.	Clastogenicity by chromosomal aberration test in chinese hamster ovary cell (CHO cells)	Positive	Lin et al., 1987
5.	Nucleus anomaly test in bone marrow of CHO cells	Negative	Strasser et al., 1986
6.	Sister chromatid exchange in tracheal epithelial cells and CHO cells	Positive	Wang et al., 1987
7.	Somatic chromosome aberration, micronucleus test and sperm shape abnormalities in mice	Positive	Bhunya and Behera, 1988
8.	Sister chromatid exchange assay in human lymphocytes	Positive	Rupa et al., 1988
9.	Frequency of induction of sex linked recessive lethal mutations in somatic and germ line cells of drosophila	Positive	Tripathy and Patnaik, 1992

Sr. No.	Test system	Result	References
10.	Micronuclei in erythrocytes of bone marrow and peripheral blood of chick	Positive	Jena et al., 1992
11.	Chromosome aberration (CA) assay and the micronucleus test (MNT) in bone marrow and peripheral blood erythrocytes of chick	Positive	Bhunya and Jena, 1993
12.	Genotoxicity test in Allium cepa	Positive	Gulati, 1994
13.	Micronucleus test in bone marrow erythrocytes and CHO	Positive	Peitl et al., 1996
14.	Comet assay in T. Mossambica	Positive	Saleha Banu et al., 2001
15.	Genotoxicity in spraying workers	Positive	Giri et al., 2002
16.	Comet assay in mouse peripheral blood leukocytes	Positive	Mahboob et al., 2002
17.	Comet assay in human lymphocytes	Positive	Jamil et al., 2004
18.	Sister chromatid exchange and chromosomal abnormalities in mouse somatic and germ cells	Positive	Ibrahim, 2005
19.	Numerical and structural chromosomal aberrations in males and embryos of pregnant mice	Positive	Zahran et al, 2005
20.	DNA damages in mice	Positive	Prabhavathy Das et al., 2006
21.	Comet assay in mouse and human peripheral blood leucocytes	Positive	Das et al., 2007
22.	DNA damages in liver and brain of rats	Positive	Mehta et al., 2008
23.	Comet assay in erythrocytes and tissues of Channa punctatus	Positive	Ali et al., 2008

Sr. No.	Test system	Result	References
24.	Cytotoxicity (IC50) of monocrotophos in HepG2	0.576 mM	Rahman et al., 2008
25.	Micronucleus test in Meretrix ovum	Positive	Revankar and Shyama, 2009
26.	Genotoxicity and cytotoxicity in human lymphocytes	Positive	Chakravarthi et al., 2010
27.	Enhances lipid peroxidation and micronuclei in rats	Positive	Yaduvanshi et al., 2010
28.	Induction in ROS and decrease in glutathione in PC12 cells	Positive	Kashyap et al., 2011
29.	Genotoxicity in occupational workers	Positive	Jonnalagadda et al., 2011

The previous literature suggest that there is no any studies that high lightened the effect of monocrotophos to cause genotoxicity in lung epithelial cells which is a direct site of pesticide inhalation.

CHAPTER-3

OBJECTIVE

Objectives

To achieve aim of research goal, the following specific objectives are formulated:

1. To study the composition of Ocimum sanctum essential oil.
2. Assessing the dose and time dependent genotoxic effect of monocrotophos.
3. Protective effect of essential oil of Ocimum sanctum L. against monocrotophos-induced DNA damage.

Conceptual Framework

The rationale behind the current is to investigate oxidant attack of pesticide on DNA in lung cells. It is widely thought that continuous oxidative damage to DNA has significant contribution in development of various genetic disorders including cancer. A variety of antioxidant and medicinal herb have been known to suppress the generation of reactive oxygen species and repair the damaged DNA. Much information on the genotoxicity and carcinogenicity of monocrotophos is already available. However information on prevention of oxidative DNA damage of monocrotophos has been limited.

CHAPTER-4

MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

All culture media, trypsin, heat inactivated Foetal Bovine Serum (FBS) (Gibco) antibiotic solution etc. were purchased from Invitrogen and Himedia. Penicillin/streptomycin, ciproflexin, MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, phosphate buffer solution, DMSO, low melting agarose, agarose, NaOH, NaCl, Na_2EDTA, Sodium dodecyl sulphate, Ethidium bromide, EDTA, Dulbecco's phosphate buffered saline were purchased from Loba, Sigma Eldrich, MP Biomedicals, Invitrogen and Himedia.

4.1.2 Instruments

Various Instruments being used in entire course of research work have been listed below in the table 4.1.

Table 4.1 List of instruments and their manufacturing company

Sr. No.	Name of the Instrument	Manufacturing Company
1.	Analytical Balance TE214,S	Sartorius
2.	Autoclave (vertical) NSW-227	Calton Company
3.	CO_2 Incubator	New Brunswick, UK
4.	Comet Assay Electrophoresis Unit	Sci. Plas, UK
5.	ELISA Reader 642	Systronics
6.	Fluorescent Microscope aided with computer	Olympus Magnus
7.	Hot Plate	Tarsons
8.	Inverted Microscope with live Imaging Facility	Olympus Magnus

Sr. No.	Name of the Instrument	Manufacturing Company
9.	Light Binocular Microscope with Digital Camera Attachment	Olympus Magnus
10.	Mini Centrifuge MC-02	Spinwin Daikan Scientific Co. Ltd.
11.	pH Meter	Mettler Toledo
12.	Rectangular Water Bath	New India
13.	Refrigerated Centrifuge 5430R	Eppendorf, Germany
14.	Refrigerated Circular Water Bath	Julabo, Germany
15.	UV-VIS double beam 2202 Spectrophotometer	Systronics

4.1.3 Essential oil of *O. sanctum*

Essential oil of Ocimum sanctum was purchased from Crystal aromatic company, Delhi, India.

Physical properties- Essential oil of O. sanctum was light yellow, volatile in nature with specific gravity 0.902.

4.1.4 Cell lines under study

Human epithelial lung cancer A549 and H1299 cell lines were procured from National Centre for Cell Culture, Pune.

A549 and H1299 cell lines are human alveolar basal epithelial cells, epithelial in nature. They can grow as monolayer under in vivo conditions. These cell lines are responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs. These cells contain a high percentage of unsaturated fatty acids which are required for the maintenance of membrane phospholipids in the cells.

4.1.4.1 Physical and morphological characteristics

Table 4.2 Physical and morphological characteristics of A549 and H1299 cell lines

Physical and morphological characteristics of A549 and H1299 cell lines			
S. No.	**Property**	**A549 Cell line**	**H1299 cell line**
1.	**Growth Properties**	Adherent	Adherent
2.	**Source organism**	Homo sapiens	Homo sapiens
3.	**Morphology**	Epithelial	Epithelial
4.	**Organ**	Lung	Lung
5.	**Disease**	Carcinoma; non-small cell lung cancer	Carcinoma; non-small cell lung cancer
6.	**Derived from metastatic site**	Lymph node	Lymph node
7.	**Doubling Time**	About 22 hrs.	About 15-16 hrs.
8.	**Cellular Products**	Keratin, lecithin	Neuromedin B
9.	**Special Feature**	Express P53 expression	Partial deletion of the p53 protein, and lack expression of p53 protein

4.1.4.2 Visualization of A549 and H1299 cell line

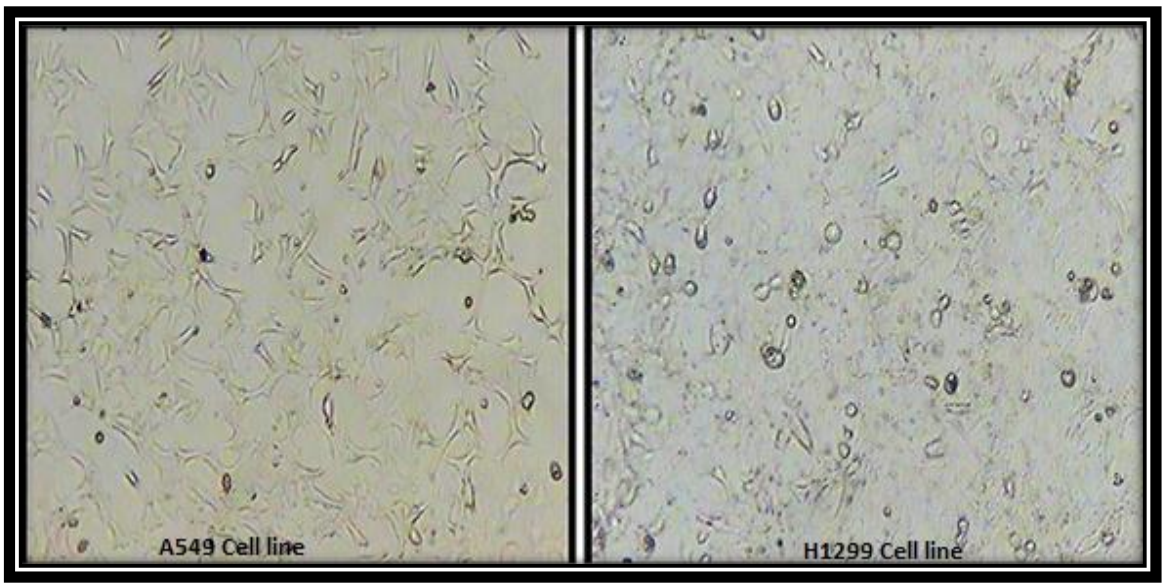


Figure 4.2 Microscopic appearance (at 20X) A549 and H1299 cell line

Methods

4.2 Experimental design and Protocols used in whole experimentation

The brief plan of work has been given below:

Experiment 1: Composition of essential oil of O. sanctum by GC-MS technique.

Experiment 2: Selection of essential oil concentration having approximately 95% cell survival rate (IC_{05}) for 24hrs. treatment in A549 and H1299 cells lines.

Experiment 3: Selection of monocrotophos concentration with approximately 80% cell viability (IC_{20}) for 6 hrs. and 24hrs. treatment in A549 and H1299 cells lines.

Experiment 4: Assessment of DNA damages in A549 and H1299 cell lines with following treatments:

4.1 Monocrotophos treatment (concentration selected from experiment 3) for 6 hrs. and 24hrs.

4.2 Pre-treatment of essential oil of O. sanctum for 24 hrs. (Concentration selected from experiment 2) to monocrotophos treated cells (4.1).

4.3 Post-treatment of essential oil of O. Sanctum for 24 hrs. (Concentration selected from experiment 2) to pre-treated monocrotophos treated cells (4.1).

4.2.1 Analysis of the oil

4.2.1.1 Gas chromatography/mass spectrometry (GC-MS) analysis

Composition of essential oil of Ocimum sanctum was studied using gas chromatography-mass spectroscopy, separated on DB-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25µm). Mass scanning range was 50 –800 m/z. About 30 µl sample in Dichloromethane was injected using split mode (50) with injector, temperature set at 250^{o}C. Column oven temperature was programmed from 40^{o}C to 220^{o}C at the rate of 4^{o}C min^{-1}, with initial and final temperatures were held for 4 and 15 minutes, respectively.

4.2.1.2 Identification of the compounds

The volatile components of oil were identified by mass spectra, calculation of Kovat index and comparing with the standard of NIST, Willey library databases.

4.2.2 Routine assay in cell culture lab

4.2.2.1 Culturing of the cell lines

The cell lines were passaged after confluency. The monolayer of cells was detached by trypsinization for 4 minutes. The trypsin was inactivated by adding 1ml of medium containing serum after detachment. Cells were harvested by centrifugation at 1200g for 10 minutes. The supernatant was discarded and resuspended the cell pellet in 2ml of medium. The cell number was counted using a haemocytometer. The cells were cultured by giving fresh medium for every three days.

4.2.2.2 Maintenance and sub-culturing of cell lines

A549 and H1299 were cultured and maintained in 25cm^2 or 75cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal Bovine serum (FBS), 1X penicillin/streptomycin and incubated at 37^{0}C in a humidified atmosphere containing 5% CO_2 and 95% humidity.

Sub culturing was done in the 25cm^2 culturing flasks after 70% confluency in the flasks. Trypsin and DMEM media containing 10% FBS, 1µg/ml penicillin /streptomycin and ciproflexin was kept at 37^{0}C in the water bath for about 10-15 minutes. The monolayer cells were detached by adding 1 ml trypsin for 5 minutes. The trypsin was inactivated by adding 1 ml of medium containing serum after cell detachment. Cells were harvested in 15ml centrifuge tube by centrifugation at 1200g for 10 minutes. The supernatant was discarded and resuspend the cell pellet in 2ml of medium and transferred to 75 cm^2 flasks containing 8ml of the media. The cell number was counted using a haemocytometer. The cells were cultured by giving fresh medium for every three days. Cells were counted in the haemocytometer and diluted the suspension to obtain the desired dilution for the experiments.

4.2.2.3 Cryopreservation and thawing of the cell lines

Cell lines were preserved in cell freezing media with 10% DMSO in the cryovials. Revival of the cell lines was done by rapid thawing at 37^{o}C and then resuspended in the 15ml centrifuge tube containing 4ml media. After centrifugation at 1200g for 10 minutes, cell pellet was resuspended in 1ml media which was reseeded in the 25 cm^{2} culturing flasks and incubated at 37^{o}C. Then, the media was changed after 24 hrs.

4.2.3 Selection of Non cytotoxic dose

4.2.3.1 MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay (Mosmann, 1983)

It is a colorimetric assay, used to determine the viability of the cells which are metabolically active by measuring the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT gets reduced to an insoluble, coloured (dark purple) formazan product after entering in the cells and passes into the mitochondria which get solubilised with an organic solvent like DMSO and can be measured spectrophotometrically.

Material: MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), Phosphate buffer solution**,** DMSO

Procedure: The 96 well plate was seeded with 100 µl of 1 x 10^{5} cells per ml and incubated for 24 hrs. Then, the cells were treated with 200µl per well of the monocrotophos (at concentrations range 1.625 µM-500 µM and 1 mm-50 mM for 6 hrs. and 24 hrs. incubation) and essential oil of O. sanctum L. (10 µg/ml-100µg/ml for 24 hrs.) as per experimental design. After treatment, the culture medium was removed from the wells. Subsequently, the cells were washed with phosphate buffer solution (PBS) and then added 50µl of 5mg/ml with MTT in each well. Then, plates were incubated for approximately 2-4 hrs. at 37°C and viewed the cells periodically for the appearance of intracellular precipitate using an inverted microscope. Discarded the MTT solution from each well and dissolved the intracellular precipitate in DMSO solution. After 20 min, the absorbance of the

samples was measured at 570nm. The data has been expressed as Mean ± SE (n) and analysed by One way ANOVA followed by Dunnett's test between different concentration with respect to control with ($p<0.005$)

4.2.4 Evaluation of DNA damage

4.2.4.1 Comet assay or Single cell gel electrophoresis technique (modified method of Olive and Banath, 2006)

Material: 1% low melting agarose, 1% normal melting agrose

a. 0.5M Na_2EDTA (pH 8.0): Add 55.8g Na_2EDTA and 6.4g NaOH to 270ml distilled water (stir for approximately 2 hrs. and adjust pH to 8.0 with additional NaOH)

b. Alkaline Lysis solution: 1.2M NaCl, 100mM Na_2EDTA, 0.1% sodium dodecyl sulphate, 0.26M NaOH (pH>13). Prepare fresh on day of experiment.

c. Rinsing solution and electrophoresis solution: 0.03M NaOH, 2mM Na_2EDTA (pH~12.3)

d. Ethidium bromide: 10mg/ml stock Etbr with working concentration 0.5µg/ml.

Procedure

1) **Slide pre-coating**: Labelled the slides using a diamond-tipped pen. Prepared agarose-precoated slides by pouring 1ml molten 1% agarose on the slides covered with coverslip. Allow agarose to air dry to a thin film and then removed the coverslip gently.

2) Sample preparation: Removed the media and washed the treated cells using phosphate saline buffer. Added trypsin to the cells and kept the cells at 37^0C for 5 minutes for cell detachment. Kept detached cells in ice-cold medium to inhibit DNA repair. Made single cell suspension and adjusted cell density to about 25,000 cells/ml in phosphate buffer using a haemocytometer. A sample of untreated cells was taken as control to confirm that background damage is low. Then, pipetted 1ml of cells into 1ml of low-gelling-temperature agarose (1%) at 40°C. Mixed well and pipetted 1ml of cell suspension onto the pre-coated slide and avoid producing bubbles. Prepared slides in triplicates for each treatment and control. Allowed agarose to gel for about 5min.

3) Alkaline lysis: After agarose got gelled, submerged slides in a covered dish containing alkaline lysis solution. Lysed samples overnight (18−20 hrs.) at 4°C in the dark.

4) Electrophoresis: After overnight lysis, carefully removed the slides and submerged the slides in rinse solution for 20 min under room temperature (18−25 °C). Repeated the same procedure two times to ensure removal of salt and detergent. Then, submerged the slides in fresh rinse solution in an electrophoresis chamber which was filled with a consistent volume of rinse solution about 1–2 mm above the top of the slides. Conducted electrophoresis in rinse solution for 30 min at a voltage of 15V (~ 0.6 V/cm) and 40 mA current.

5) Slide staining: Removed slides from electrophoresis chamber and rinsed with distilled water two times to neutralize. Pipetted about 500µl of a 10µg/ml stock solution of ethidium bromide directly onto the slide and incubated for 20 min. Placed slides in distilled water to remove excess stain.

6) Slide analysis: Analysed cells by examining at least 50 comet images from each slide by visual scoring under fluorescent microscope.

7) Evaluation of DNA damage: For visualization of DNA damage, observations of EtBr stained DNA were made using a 40x objective on a fluorescent microscope. Image analysis system was used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the comet length, % DNA in tail, head intensity, tail intensity and tail length. Selected randomly 50 cells per sample for scoring from each slide under fluorescent microscope. Scoring of the comets obtained was done using Cometscore15 software Version 1.0.1.0. The data has been expressed as Mean ± SE (n).

CHAPTER-5

RESULTS AND DISCUSSION

Results

The results of entire research work have been divided into following sections depending upon the type of parameters.

a. **Composition of *Ocimum sanctum* essential oil**

b. **Determination of non cyotoxic concentration for cells**

c. **Evaluation of DNA damage**

Section a

Composition of *Ocimum Sanctum* L. essential oil

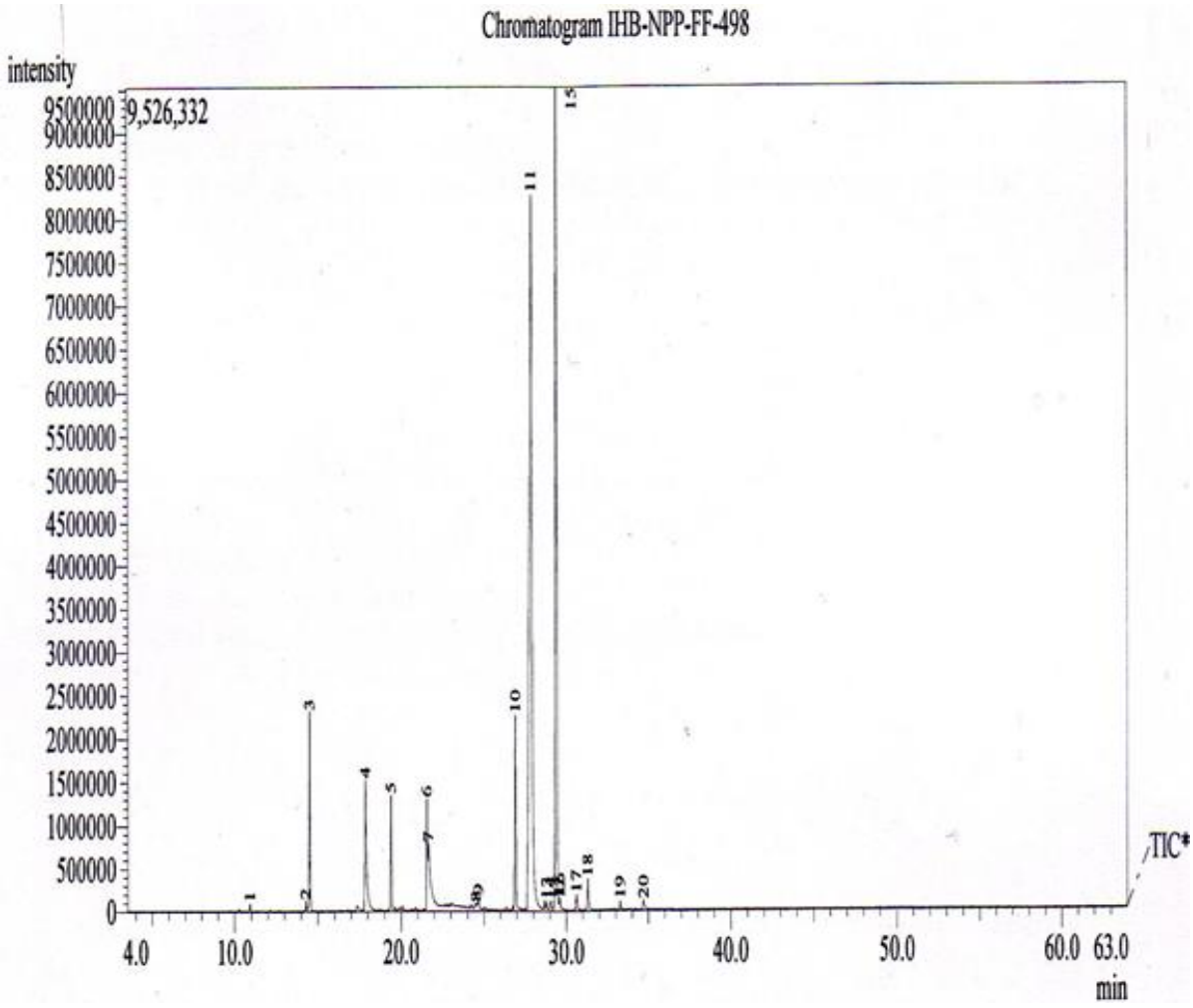


Figure 5.1 Chromatogram of mass spectra of essential oil of O. sanctum from IHBT, Palampur report (sample ID: 498)

The constituents of volatile oil had been identified and percentage composition of each constituent is listed the table (Table 5.1). From the GC-MS report, the identification of total 20 compounds had been done which constitute 99.82% of total percent area. Out of these, 74.19% of total essential oil composed of eugenol and caryophyllene with percentage area 45.5% and 28.96% respectively. The major constituents were 1,8-Cineole, *β* Linalol, Camphor, p allyl anisole and *α* terpineol which have percent areas 4.25%, 4.69%, 2.51% 2.62% and 3.32% respectively and others are Camphene (0.14%), Cinnamyl acetate (0.38%), Sabinene hydrate acetate (0.2%) and Isobornyl acetate (0.23%). Other component are present in lessen quantity given in table 5.1. Interestingly, it has been estimated that the most of compound belongs to class monoterpene (66%).

Table 5.1 Compounds identified in O. sanctum essential oil using GC-MS technique

Sr. No.	R. Time	% Area	Cal. RI	Compound	Chemical class
1	27.916	45.5	1379	Eugenol	Monoterpene
2	26.915	4.78	1350	*α* terpinyl acetate	Monoterpenoids
3	17.874	4.69	1110	*β* Linalol	Monoterpene
4	14.515	4.25	1029	1,8-Cineole	Monoterpene
5	21.654	3.32	1206	*α* terpineol	Monoterpene
6	19.405	2.51	1149	R-Camphor	Monoterpene
7	24.673	0.23	1287	Isobornyl acetate	Monoterpene
8	24.525	0.2	1283	Sabinene hydrate acetate	Monoterpene
9	14.285	0.19	1024	p cymene	Monoterpene
10	29.044	0.18	1412	Methyl eugenol	Monoterpene

11	10.933	0.14	951	Camphene	Monoterpene
12	29.431	28.96	1424	Caryophyllene	Sesquiterpene
13	28.8	0.18	1405	Isocaryophyllene	Sesquiterpene
14	29.604	0.28	1429	Trans-Caryophyllene	Sesquiterpene
15	34.703	0.27	1588	Caryophyllene oxide	Sesquiterpene
16	30.613	0.38	1459	Cinnamyl acetate	Volatile ethyl ester
17	31.3	0.95	1480	Ethyl trans-cinnamate	Volatile ethyl ester
18	28.664	0.19	1400	Methyl cinnamate	Volatile ethyl ester
19	21.569	2.62	1204	p allyl anisole	Terpineols

Section b

Determination of non cytotoxic concentration for cells

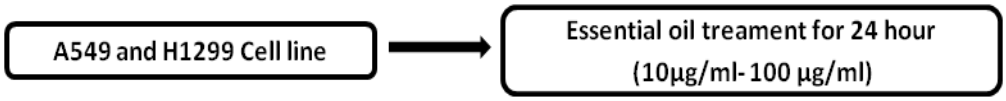
Essential oil of O. sanctum has been known to have anti-oxidant property. The aim of present study is to evaluate the genotoxicity of monocrotophos and protective effect of essential oil of O. sanctum against monocrotophos induced genotoxicity. Hence, there was need to find out the concentration at which cells must remain viable.

5.3 Cell viability in response to monocrotophos and essential oil of *Ocimum sanctum* treatment

The viability of both cell lines i.e. A549 and H1299 in response to different concentrations of essential oil of O. sanctum and monocrotophos was evaluated using MTT assay.

5.3.1 Viability of cells in response to essential oil of *Ocimum sanctum*

The aim of present study was to evaluate the protective action of essential oil, on lung cancer cell lines i.e. A549 and H1299. The essential oil of O. sanctum has been reported to cause cytotoxic response at higher concentration. So, the first step to meet this objective is to find out the concentration at which approximately 95% cells (IC_{05}) remain viable. The doubling time of both the cell line is in between 16 to 23 hrs. So, the cells had been treated with different concentration of essential oil of O. sanctum for 24 hrs. time period in order to check the maximum response of essential oil treatment. The treatment Plan has been shown below:



The two cell lines, A549 and H1299 were treated in 96 well plate with different concentrations of essential oil (range 10µg/ml - 100µg/ml) for 24 hrs. The results of the effect of essential oil of O. sanctum on cell viability in both the cell line has been shown in Fig 5.2.

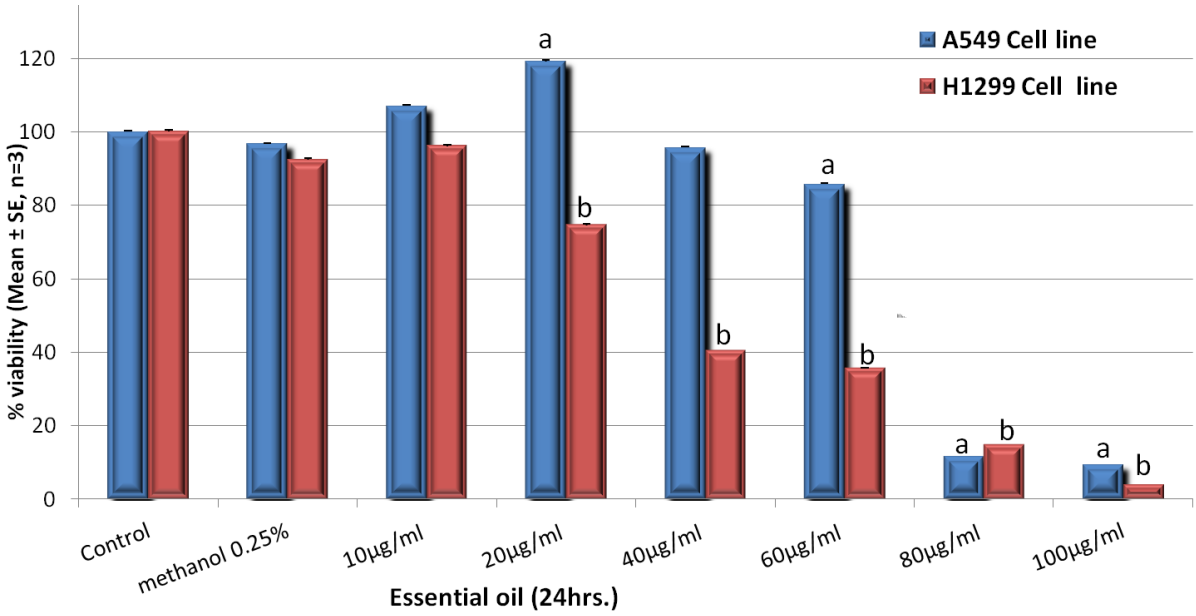


Figure 5.2 Percent cell viability of A549 and H1299 cells in response to O. sanctum essential oil treatment for 24 hrs. time period. Data is expressed as mean values ± S.E. (n=3) and had been analyzed by one way Anova followed by Dunnett's test between different concentrations of O. sanctum essential oil and compared with respect to control. Statistically significant results were indicated by "a" and "b" with ($p<0.05$) for A549 and H1299 cells respectively.

The results demonstrate that the cell viability decreased in dose dependent manner in both the cell lines. The cell proliferation of A549 cells increased significantly at 20µg/ml essential oil. At 80 µg/ml essential oil concentration, there is significant abrupt decrease in cell viability i.e. 90% inhibition in A549 cell line (figure 5.2). The H1299 had not showed any proliferation rate at any concentration of essential oil. In this cell line, the cell viability reduced from 24% to 96% at 20µg/ml to 100µg/ml essential oil concentration. The 0.25% methanol solution, as vehicle control, has not showed any significant cytotoxicity in both the cell lines.

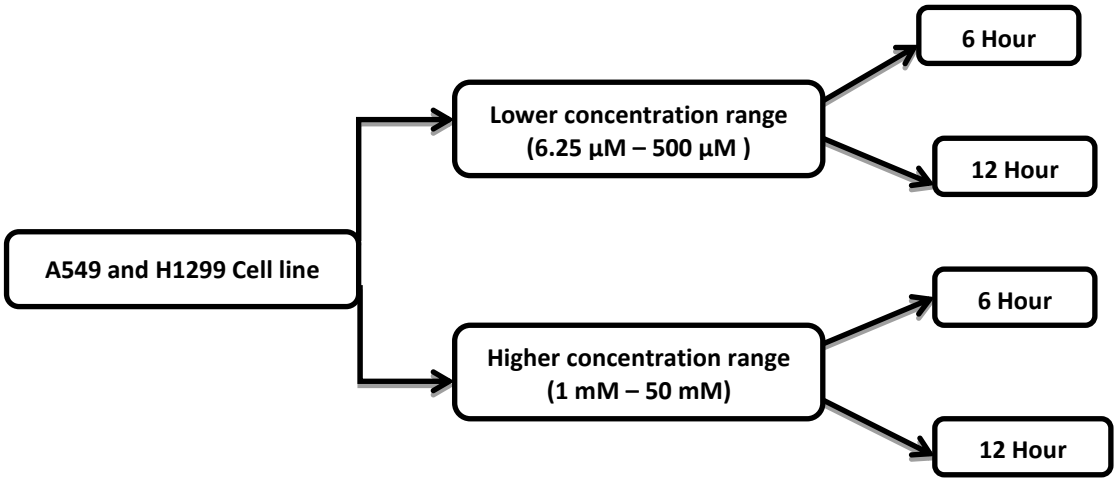
The results of present study suggest that the 10µg/ml is the concentration of O. Sanctum essential oil (figure 5.2) at which both the cell lines remain viable without

any significant cytotoxic effect. Also, the result suggests that essential oil is highly cytotoxic at higher concentrations. The H1299 cells are more sensitive to essential oil treatment as compared to A549 cells

5.3.2 Cell viability in response to monocrotophos treatment

The aim of present study was to evaluate the genotoxicity of monocrotophos in A549 and H1299 Cell lines in dose and time dependent manner. The concentration at which approximately 80% cells remain viable was selected to evaluate the genotoxicity of monocrotophos.

The two lung cancer cell lines, A549 and H1299, were treated with two concentration ranges i.e. lower concentration range 6.25µM - 500µM and higher concentration range 1 mM - 100 mM for 6 hrs. and 24 hrs. to predict the dose-time dependent response of pesticide. The treatment plan has been given below:



The lung cancer cell lines were treated with 6.25µM-500µM concentration range of monocrotophos for 6 hrs. and 24 hrs. as per above experiment plan. The response of A549 and H1299 cell line at lower concentration range i.e. 6.25µM-500µM has been shown in Fig 5.3.

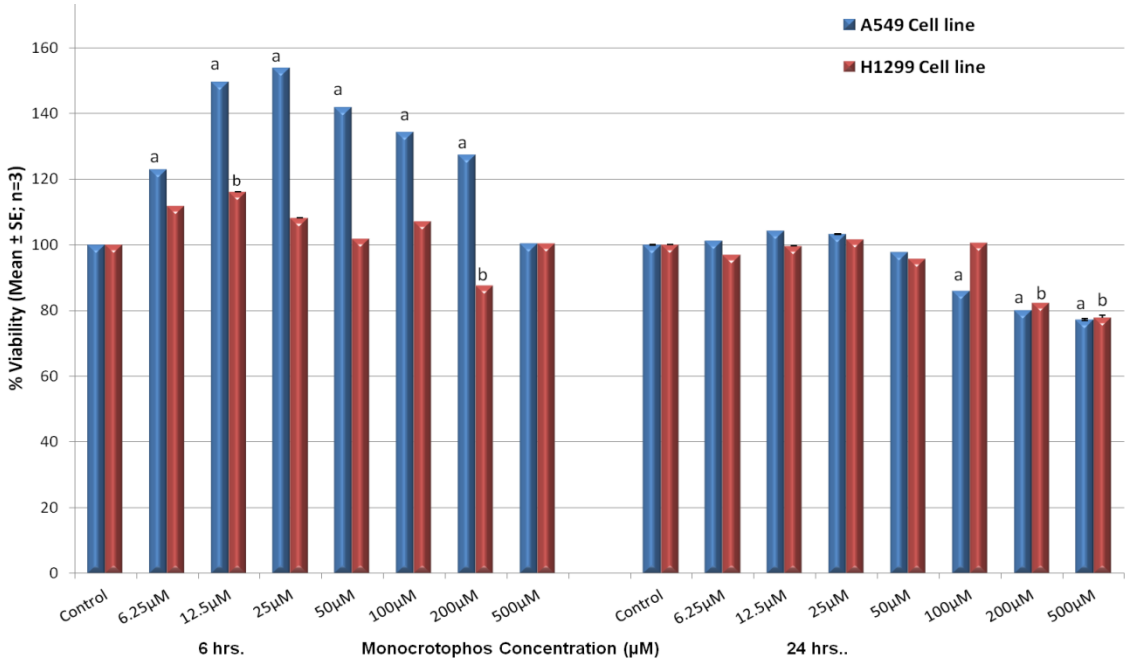


Figure 5.3 Percent cell viability of A549 and H1299 cells in response to monocrotophos (lower range: 6.25µM- 500µM) concentration for 6 and 24hrs. Data is expressed as mean values ± SE (n=3) and had been analyzed by one way Anova followed by Dunnett's test between different concentrations of monocrotophos and compared with respect to control. Statistically significant results were indicated by "a" and "b" with ($p<0.05$) for A549 and H1299 cells respectively.

The results indicate that the rate of cell proliferation in A549 cell line increased as compared to control in response to monocrotophos treatment for 6 hrs. Also, the percent viability was not reduced at any concentration of monocrotophos in A549 cells for 6 hrs. treatment (figure 5.3). But, the percent viability decreased from 3% to 22.7% from 50µM to 500µM monocrotophos concentration in case of 24 hrs. treatment. On the other hand, the cell viability in H1299 was decreased from 12.45% to 19.4% for 6 hrs. treatment and 17.7% to 22.2% for 24 hrs. as compared to control (figure 5.3).

The results of concentration range from 6.25µM - 500µM did not shown any significant effect on cell viability for 6 hrs. for both the cell lines (figure 5.2). So,

there is need to test for cell viability at higher concentration range of monocrotophos to determine IC_{80}.

The cells were treated with higher monocrotophos concentration range i.e. 1mM-50mM to calculate percent cell viability for 6 hrs. and 24 hrs. time period. The response of A549 and H1299 cell lines at higher concentration range i.e. 1mM-50mM has been shown in Figure 5.4.

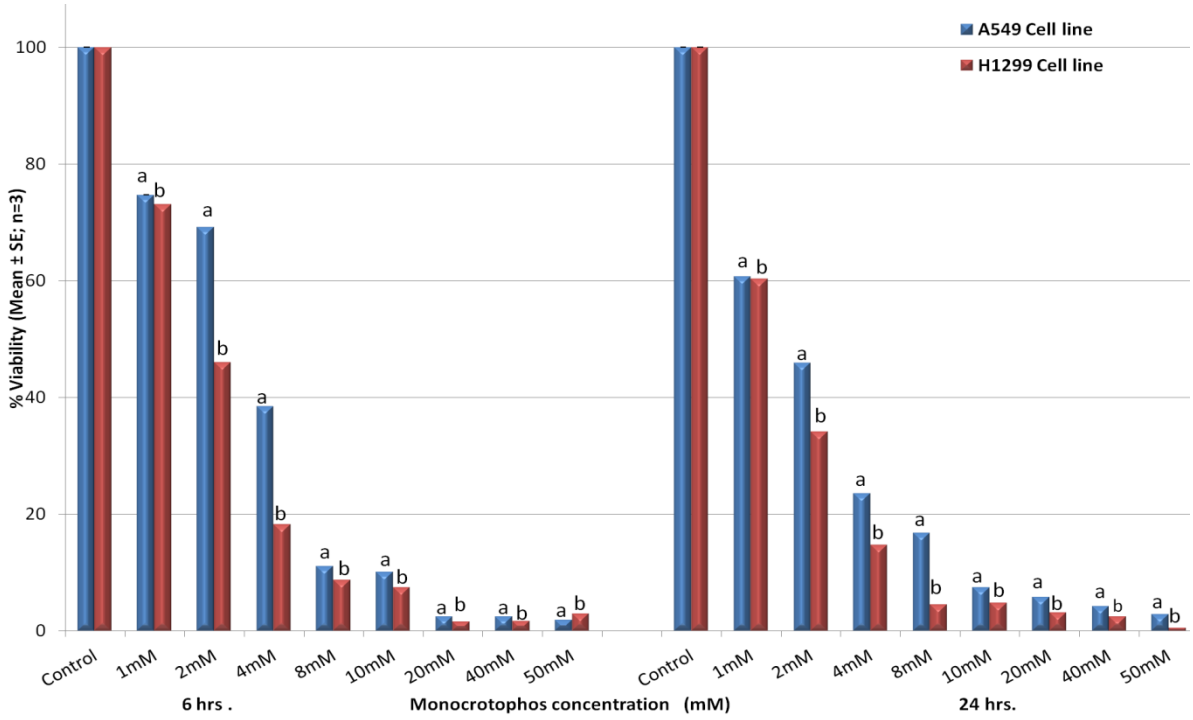


Figure 5.4 Percent cell viability of A549 and H1299 cells in response to monocrotophos (higher range: 1mM - 50mM) concentration for 6 and 24 hrs. time period. Data is expressed as mean values ± S.E. (n=3) and has been analyzed by one way Anova followed by Dunnett's test between different concentrations of monocrotophos and compared with respect to control. Statistically significant results were indicated by "a" and "b" with ($p<0.05$) for A549 and H1299 cells respectively.

It was observed that there was significant decrease in cell viability with increase in monocrotophos concentrations in case of both the cell lines. The results suggest that cell viability reduced significantly by 25.3% to 98.1% for 6 hrs. treatment and 39.2% to 97.1% for 24 hrs treatment at 1mM to 50mM monocrotophos concentration as compared to control in A549 cell line. Similarly, the H1299 cells

also showed a significant decrease in cell viability, from 27% to 97.1% for 6 hrs. treatment and 39.7% to 99.4% for 24 hrs. treatment with 1mM to 50mM concentrations of monocrotophos as compared to control. The results suggest significant decrease in cell survival with increase in monocrotophos concentration. Also, H1299 cells seem to be more sensitive as compared to A549 cells for monocrotophos treatment.

So, the essential oil showed cytotoxic nature at higher concentration of essential oil of O. sanctum. But, the 10 µg/ml is the concentration, having 95% cell viable, chosen to study its protective role against monocrotophos induced genotoxicity.

The monocrotophos has been investigated to be more cytotoxic after 1mM concentrations in both the cell lines. Interestingly, the results demonstrated that there was increase in rate of cell proliferation at lower concentration of monocrotophos (6.25µM – 200µM) for 6 hrs. in case of A549 cell lines. So, the 500µM was considered as the concentration having 80% cell viability.

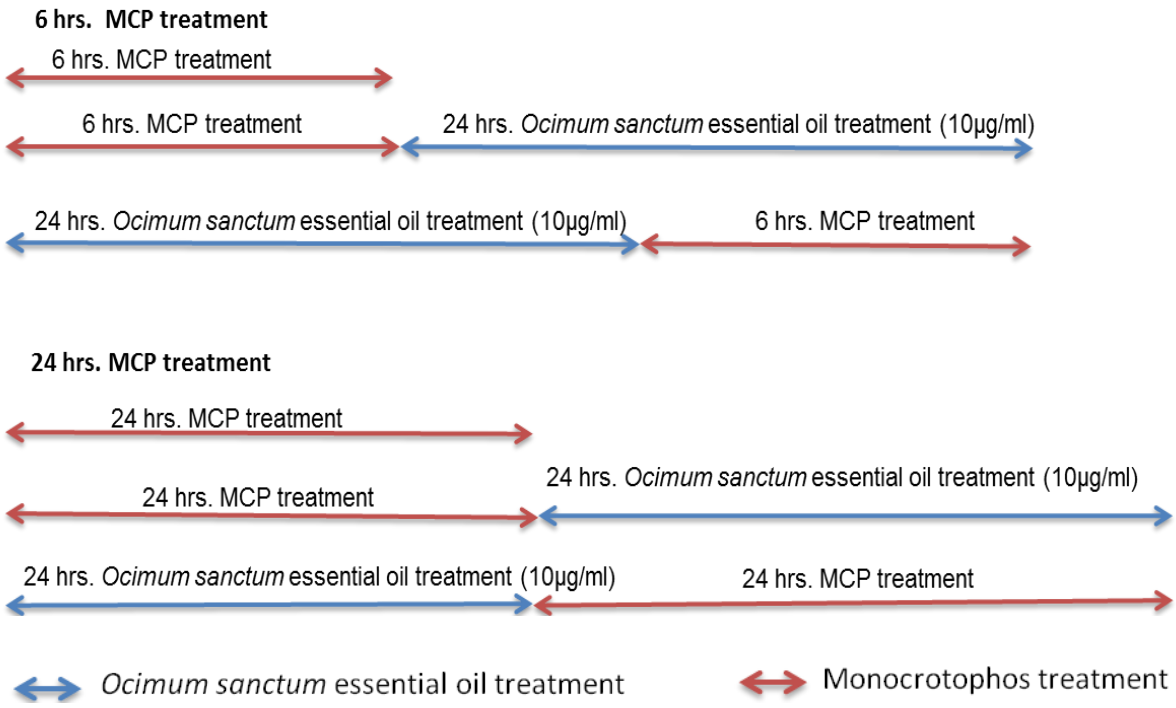
Section c

Evaluation of DNA damage

5.4 Response of *O. sanctum* essential oil against monocrotophos induced genotoxicity

The aim of present study is to evaluate the DNA damage in dose and time dependent manner for monocrotophos as well as effect of pre-treatment and post-treatment of essential oil of O. sanctum on monocrotophos treated cells of A549 and H1299 cell lines.

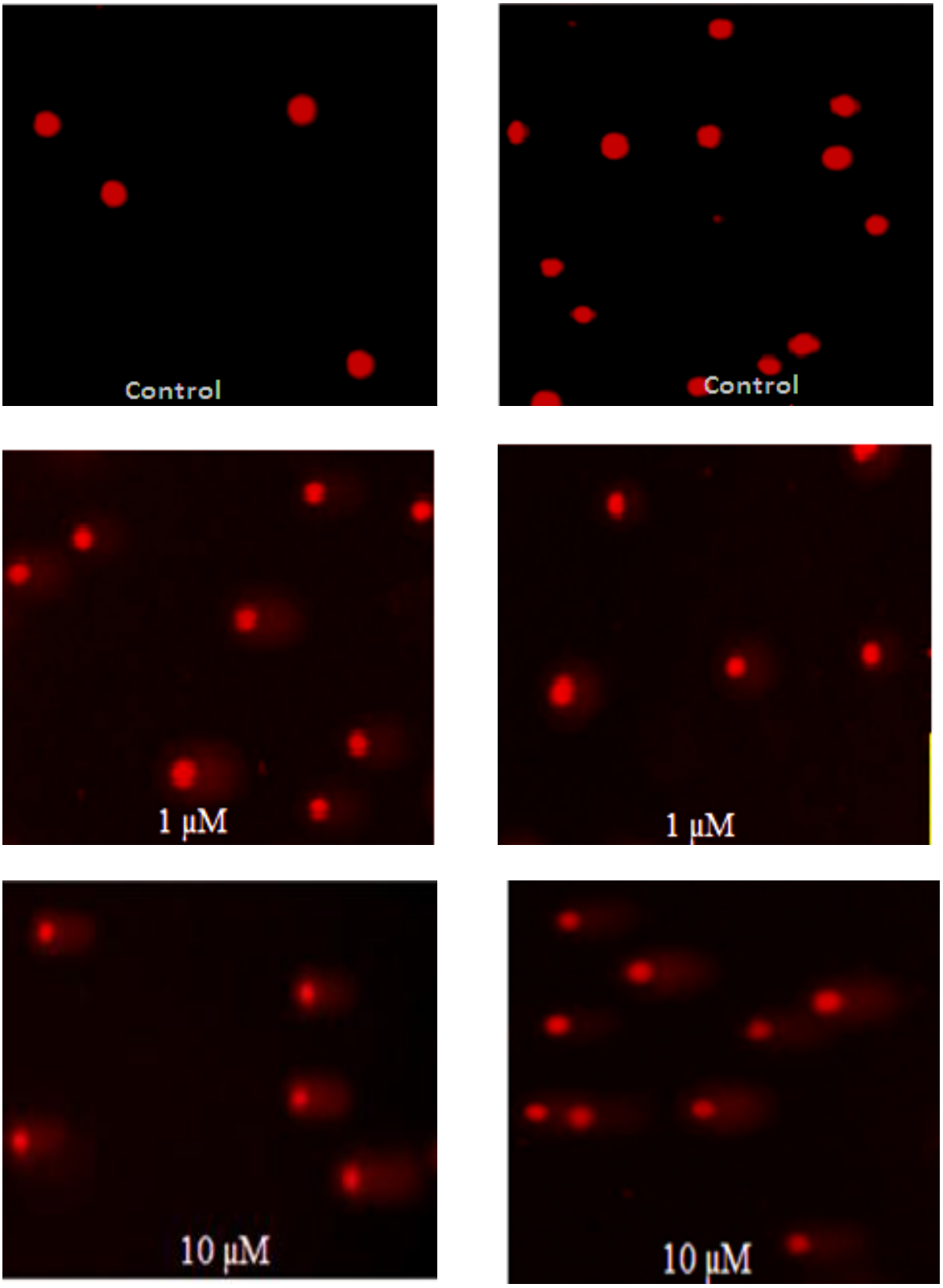
The previous results showed that the concentration at which 95% cells remain viable is 10µg/ml for essential oil of O. sanctum and concentration at which 80% cells remain viable is 500µM for monocrotophos. Based on this observation, the further experiment concentration for monocrotophos were decided as 1µM, 10µM, 100µM, 500µM and concentration for essential oil of O. sanctum as 10µg/ml. The evaluation of DNA damage in response to monocrotophos treatment and O. sanctum essential oil treatment was done using following treatment plan:



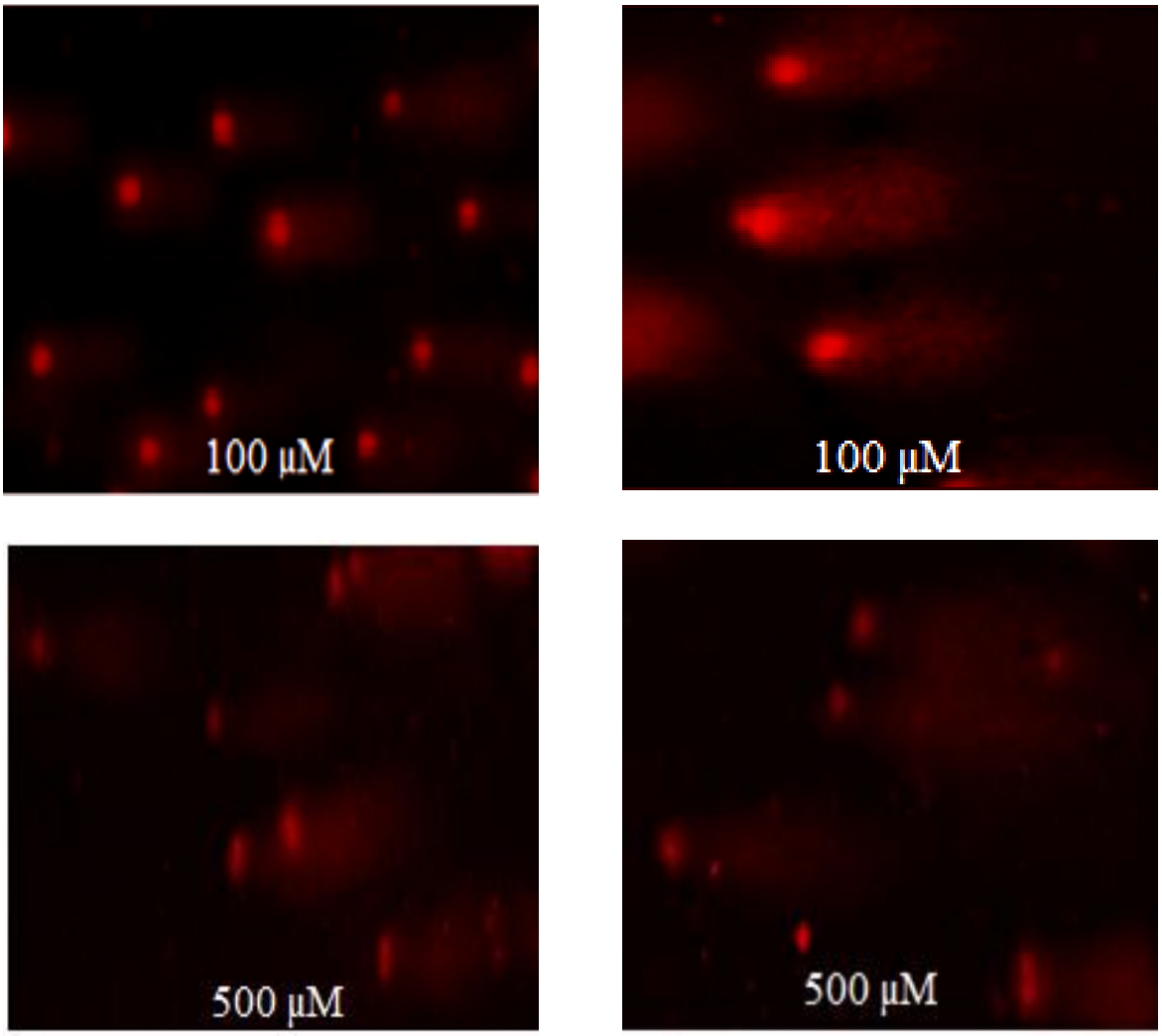
The evaluation of DNA damage was done by comet assay and scoring of the comets was done using Cometscore15 software Version 1.0.1.0.

Fig 5.5 Appearance of fluorescently stained comet slides showing DNA damage in A549 and H1299 cells in response to monocrotophos treatment (1µM-500µM)

A. A549 Cell line **B. H1299 Cell line**



A. A559 Cell line **B. H1299 Cell line**



The results of parameters scored using Cometscore15 software showing the effect of essential oil of O. sanctum against monocrotophos induced genotoxicity have been given in Figure 5.6 to 5.10.

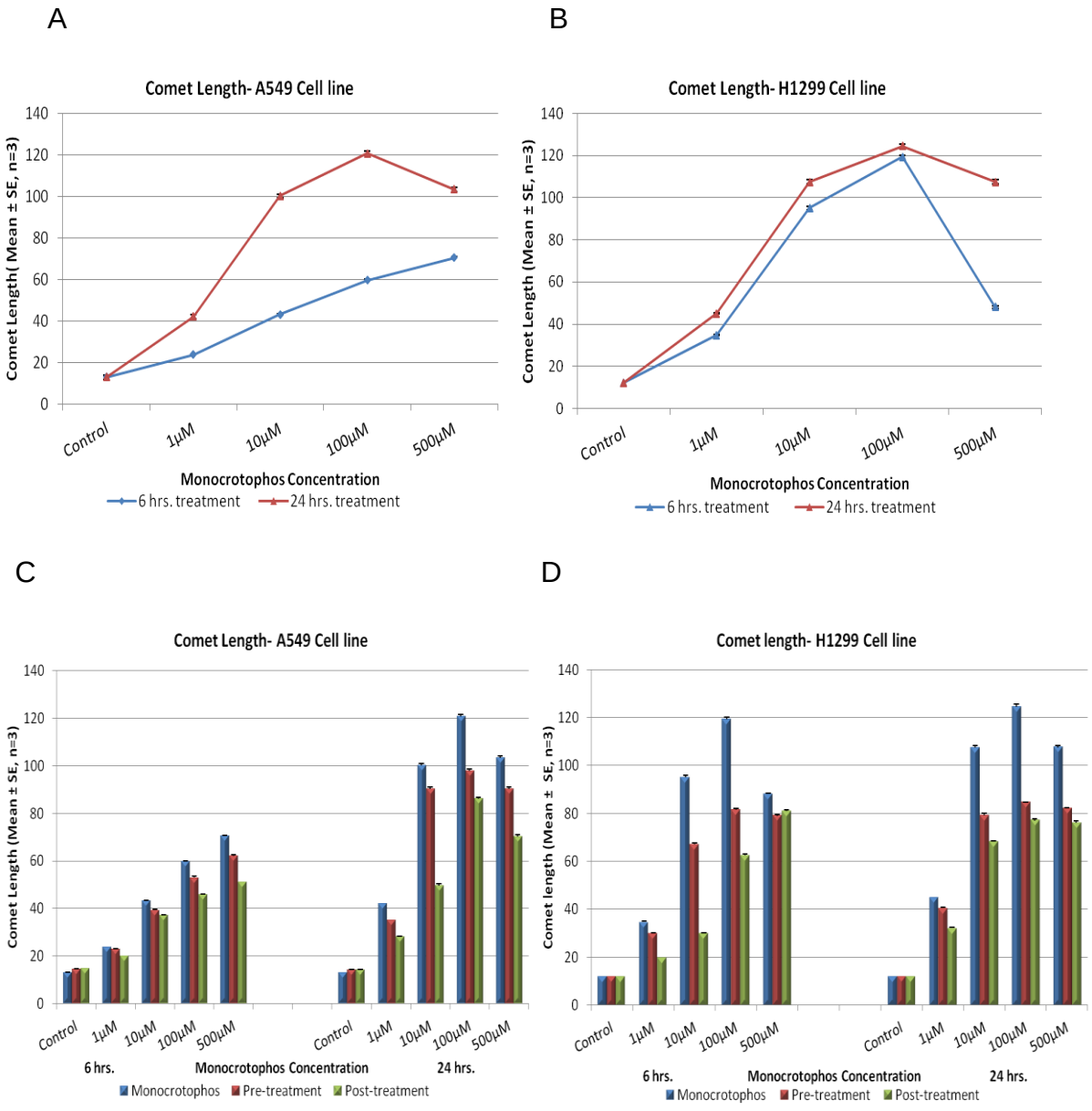


Figure 5.6 Comet length of A549 and H1299 cells in response to monocrotophos and O. sanctum essential oil treatment. Effect of monocrotophos on A549 cells (figure 5.6 A) and H1299 cells (figure 5.6 B) in dose and time dependent manner. Effect of pre-treatment and post-treatment of O. sanctum essential oil on monocrotophos treated cells of A549 (figure 5.6 C) and H1299 cells (Figure 5.6 D). Here, ■ Monocrotophos treatment concentration (1µM - 500µM), ■ Pre-treatment

of O. sanctum essential oil (10µg/ml for 24 hrs.) before monocrotophos treatment (1µM - 500µM), Post-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) after monocrotophos treatment (1µM - 500µM). All the data has been expressed as Mean ± SE (n=3).

The comet length is a diameter of comet nucleus plus migrated DNA from comet which increases with Increase of DNA damage. The results of present study suggest that comet length of A549 cells (figure 5.5 A) increased in dose dependent manner for 6 hrs. which further significantly increased after 24 hrs. treatment. It suggests the dose and time dependent increase in comet length of A549 cells. Interestingly, the comet length decreased at 500µM monocrotophos concentration in case of 24 hrs. treatment which might be due to severe damage at this concentration.

In case of H1299 cells, the comet length was to maximum at 100µM for both the time periods i.e. 6 and 24 hrs. and then start decreasing at 500µM (figure 5.5 B) which might be due to generation of more fragment of DNA which move out form the comet. Also, the tail length of H1299 cells was longer than A549 cells, which suggest that H1299 cells were more sensitive to DNA damage as compared to A549 cells in response to respective concentrations of monocrotophos.

The pre-treatment and post-treatment of essential oil resulted in decrease the comet length in A549 (figure 5.5 C) as well as H1299 cells (figure 5.5 D) with respect to monocrotophos concentrations. The post-treatment of essential oil of O. sanctum showed more response to protect against monocrotophos genotoxicity in both the cell lines.

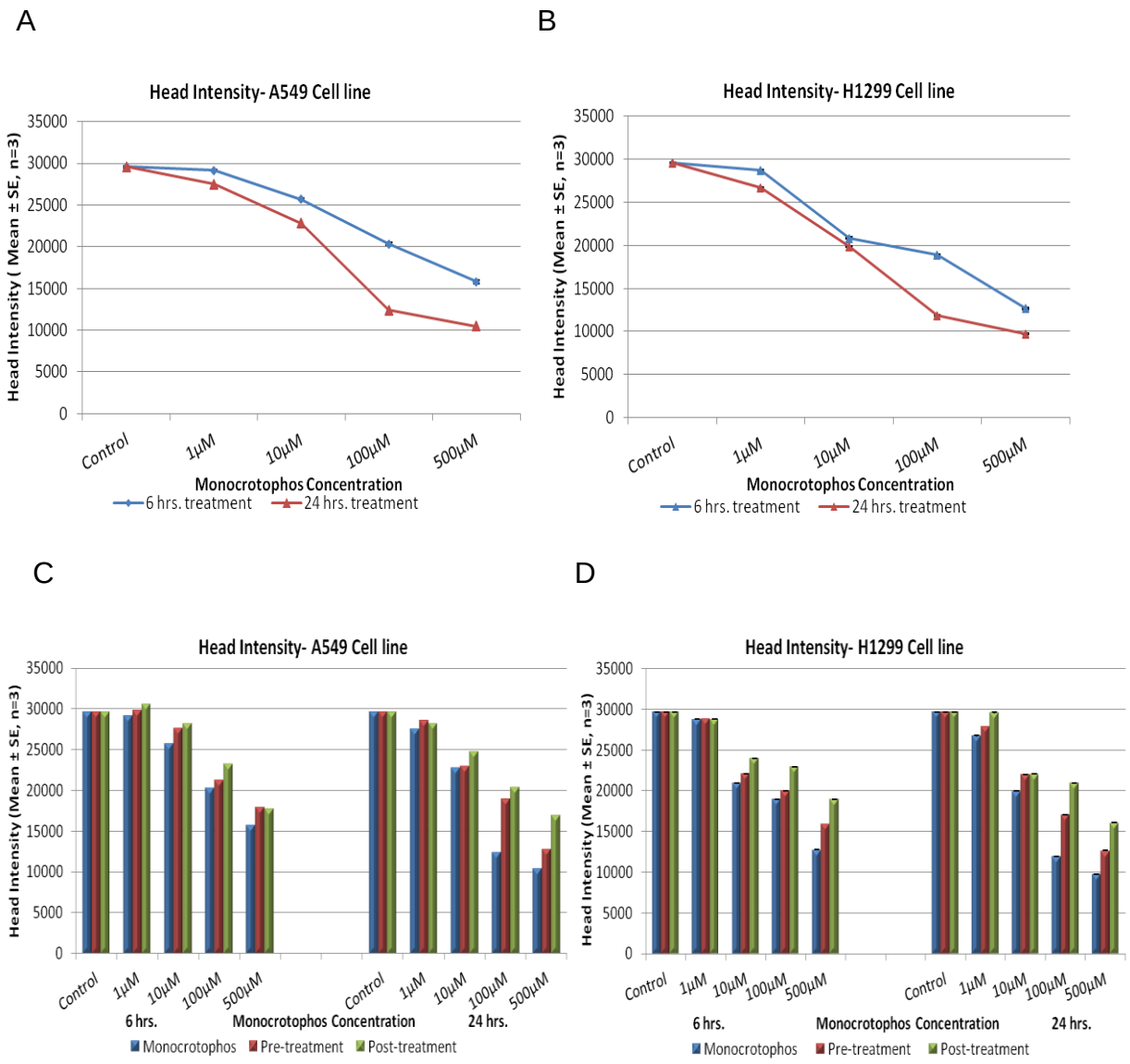
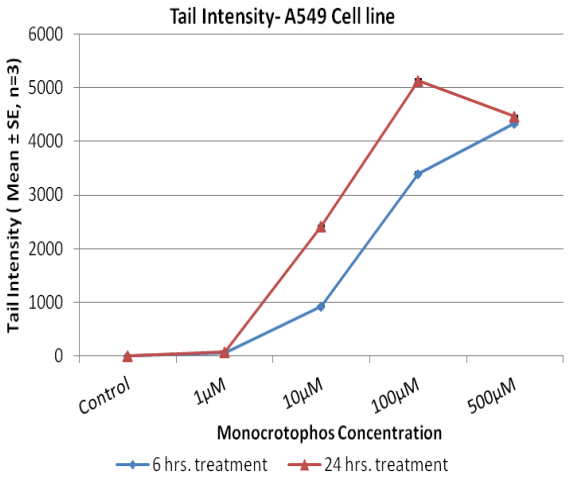


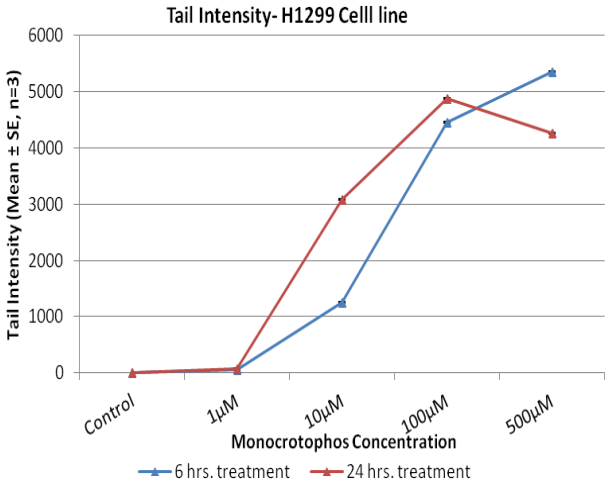
Figure 5.7 Head intensity of A549 and H1299 cells in response to monocrotophos and O. sanctum essential oil treatment. Effect of monocrotophos on A549 cells (figure 5.7 A) and H1299 cells (figure 5.7 B) in dose and time dependent manner. Effect of pre-treatment and post-treatment of O. sanctum essential oil on monocrotophos treated cells of A549 (figure 5.7 C) and H1299 cells (figure 5.7 D). Here, Monocrotophos treatment concentration (1µM-500µM) for 6 hrs. and 24 hrs. Pre-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) before monocrotophos treatment (1µM-500µM) Post-treatment of O. sanctum essential oil (10µg/ml for 24 hrs) after monocrotophos treatment (1µM - 500µM). All the data has been expressed as Mean ± SE (n=3).

The results suggest that the head intensity decreased in dose and time dependent manner in both the cell lines (figure A, B) due to increase in DNA damage with increasing monocrotophos concentration. But, the pre and post treatment (figure C, D) enhanced the Head intensity in both the cell lines. The post treatment of O. sanctum seems to be more efficient to modulate the comet intensity.

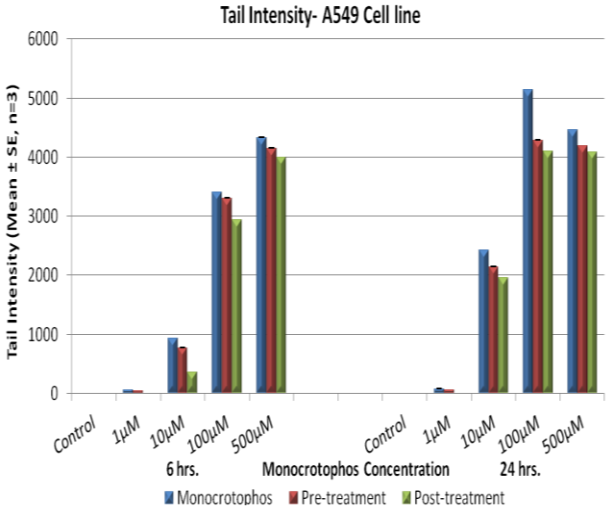
A



B



C



D

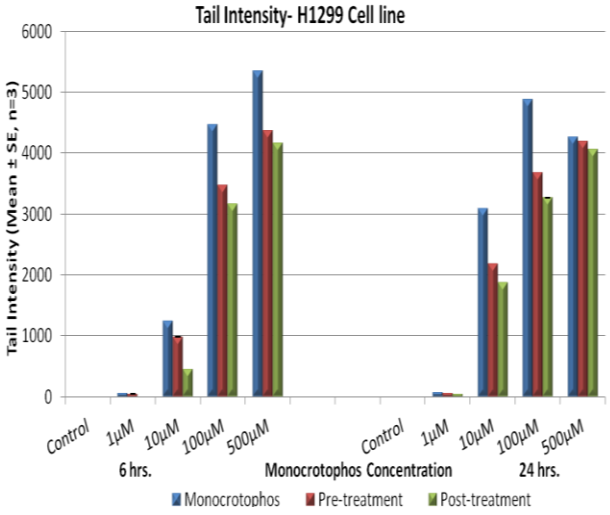
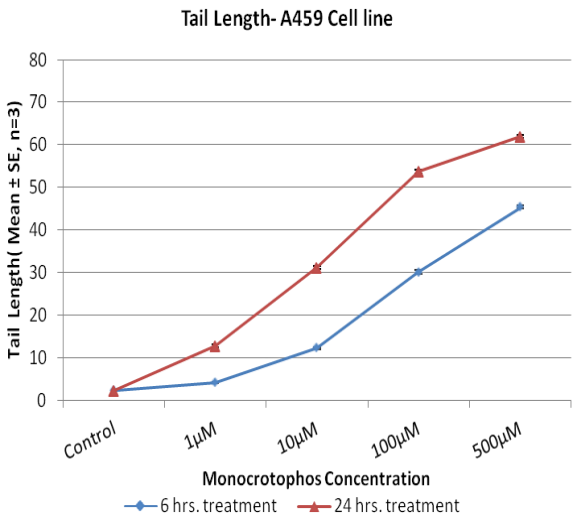


Figure 5.8 Tail intensity of A549 and H1299 cells in response to monocrotophos and O. sanctum essential oil treatment. Effect of monocrotophos on A549 cells (figure 5.8 A) and H1299 cells (figure 5.8 B) in dose and time dependent manner. Effect of pre-treatment and post-treatment of O. sanctum essential oil on monocrotophos treated cells of A549 (figure 5.8 C) and H1299 cells (figure 5.8 D). ■ Monocrotophos treatment concentration (1µM - 500µM) ■ Pre-treatment of O.

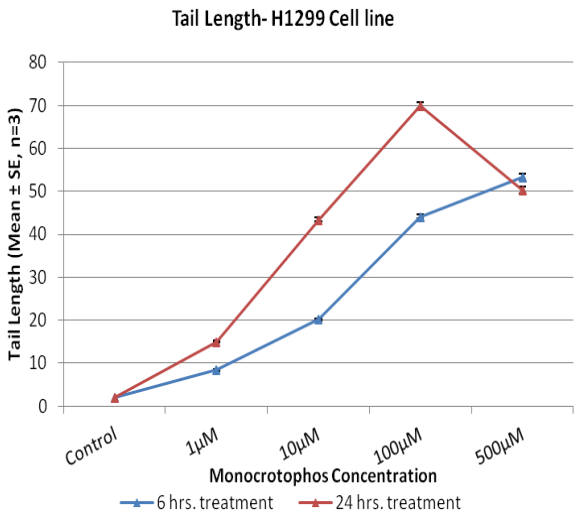
sanctum essential oil (10µg/ml for 24 hrs.) before monocrotophos treatment (1µM-500µM) Post-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) after monocrotophos treatment (1µM - 500µM). All the data has been expressed as Mean ± SE (n=3).

It was observed that tail intensity in A549 cells (figure 5.8 A) and H1299 cells (figure 5.8 B) increased in dose dependent manner for 6 hrs. treatment, but decreased after 100µM for 24 hrs. time period (figure 5.8 A, B). It may be due to more time exposure of pesticide to H1299 cell which cause even more DNA damage in the cells. Also, it has been observed that the tail intensity was more in case of H1299 cells which might be due to more migration of damaged DNA which resulted in intense tail formation. The pre and post-treatment of essential oil of O. sanctum caused decrease in tail intensity in effective manner with respect to monocrotophos concentration in A549 (figure 5.8 C) and H1299 cell line (figure 5.8 D).

A



B



C D

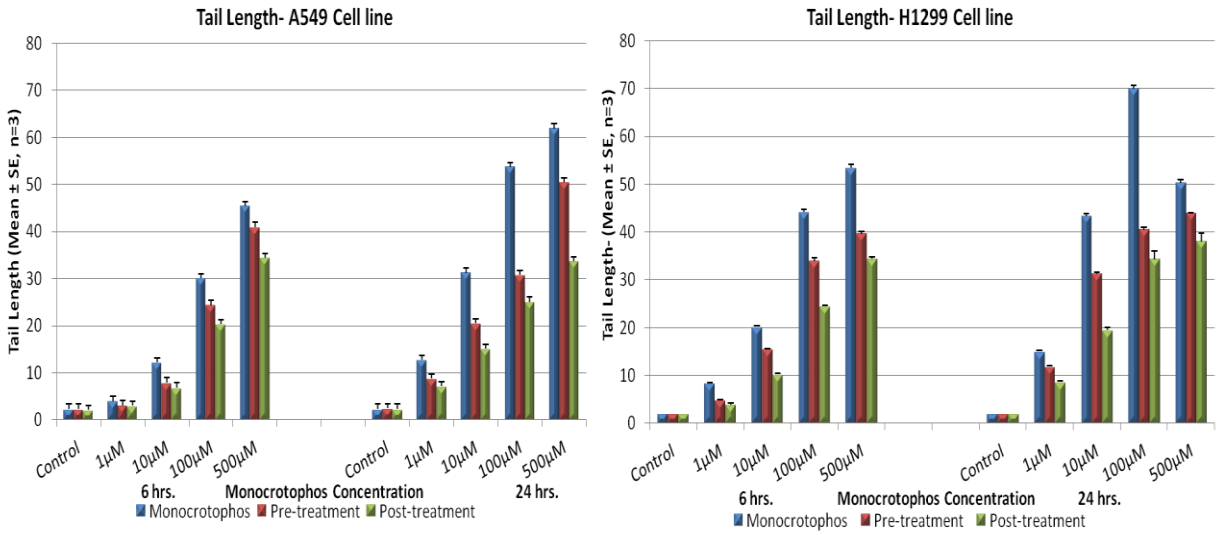


Figure 5.9 Tail length of A549 and H1299 cells in response to monocrotophos and O. sanctum essential oil treatment. Effect of monocrotophos on A549 cells (figure 5.9 A) and H1299 cells (figure 5.9 B) in dose and time dependent manner. Effect of pre-treatment and post-treatment of O. sanctum essential oil on monocrotophos treated cells of A549 (figure 5.9 C) and H1299 cells (figure 5.9 D). ■ Monocrotophos treatment concentration (1 µM-500 µM) ■ Pre-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) before monocrotophos treatment (1µM-500µM) ■ Post-treatment of O. sanctum essential oil (10 µg/ml for 24 hrs.) after monocrotophos treatment (1µM-500µM). All the data has been expressed as Mean ± SE (n=3).

The tail length has direct relation to tail intensity of comet. The results suggest that the tail length also increased in dose and time dependent manner in A549 cell line (figure 5.9 A) for 6 hrs. and in H1299 cells for 24 hrs. treatment (figure 5.8 B). In case of H1299 cell line, there was decrease in tail length at 500 µM concentration for 24 hrs. which may be due to more severe DNA damage at this concentration. Also, the pre-treatment and post-treatment of essential oil of O. sanctum resulted in decrease of tail length in effective manner (figure 5.8 C, D) in case of both the cell lines. The post-treatment of essential oil of O. sanctum is more effective to reduce the tail length in both the cell line.

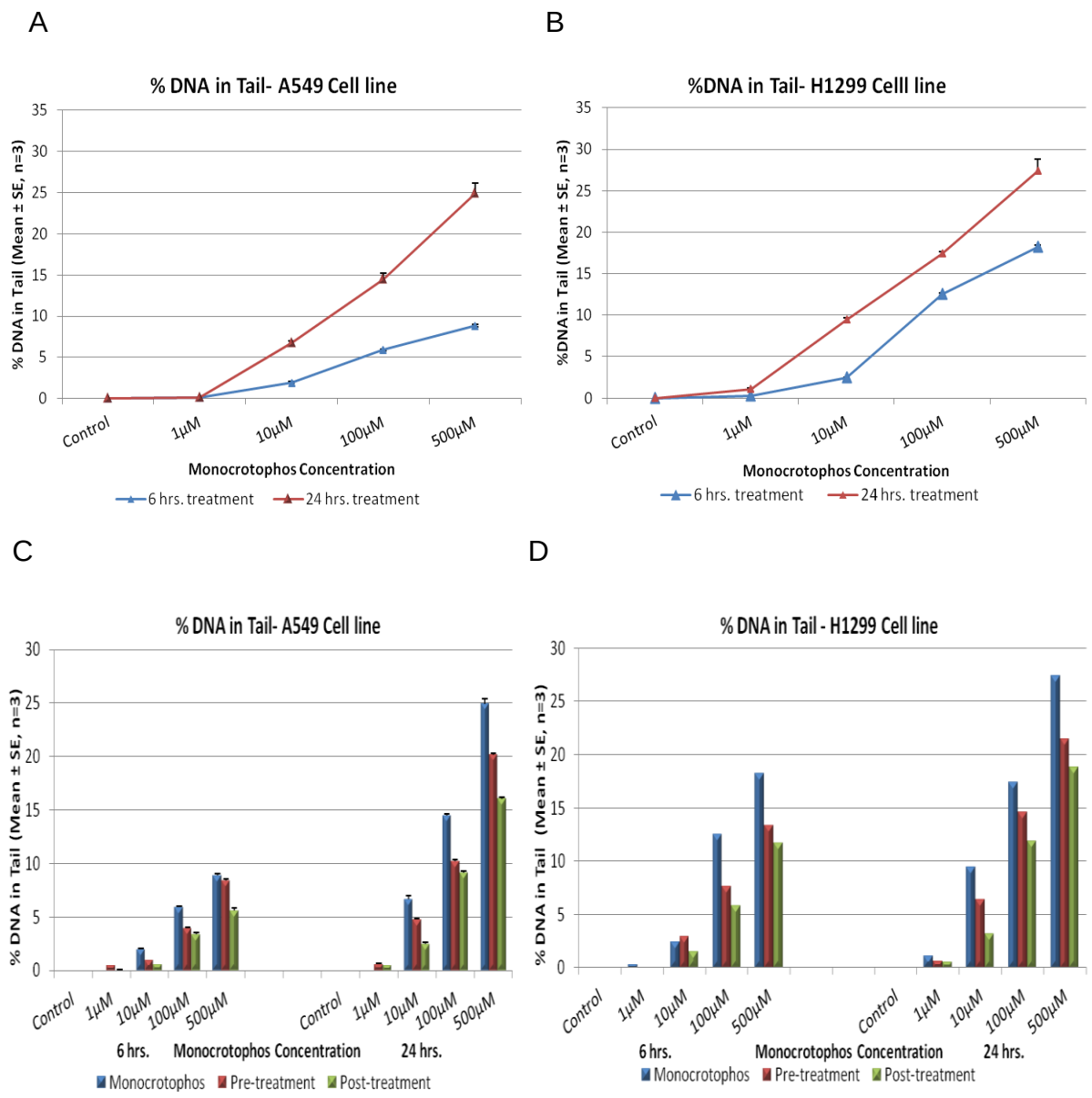


Figure 5.10 Percent DNA in tail of A549 and H1299 cells in response to monocrotophos and O. sanctum essential oil treatment. Effect of monocrotophos on A549 cells (figure 5.10 A) and H1299 cells (figure 5.10 B) in dose and time dependent manner. Effect of pre-treatment and post-treatment of O. sanctum essential oil on monocrotophos treated cells of A549 (figure 5.10 C) and H1299 cells (figure 5.10 D). ■ Monocrotophos treatment concentration (1µM - 500µM) ■ Pre-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) before monocrotophos treatment (1µM-500µM) ■ Post-treatment of O. sanctum essential oil (10µg/ml for 24 hrs.) after monocrotophos treatment (1µM-500µM). All the data has been expressed as Mean ± SE (n=3).

It has been observed that the % DNA in tail has also been increased with increase in monocrotophos concentration (figure 5.10 A, B) as well as with time of exposure of treatment in case of both the cell lines. The H1299 cells seem to be more prone to DNA damage at all respective concentrations of monocrotophos as compared to A549 cell line. The pre-treatment of O. sanctum essential oil decreased the % DNA in tails of both the cell lines (figure 5.10 C, D) which suggest the protective as well as repairing action of O. sanctum essential oil. The results suggest that the post-treatment of essential oil of O. sanctum is better than the pre-treatment.

Discussion

Concern to genotoxicity of pesticides, such as organophosphate (OP) compounds is increasing, due to increasing reports of pesticide contamination in the recent years. The increase in pesticide use is further significant in health concerns, as 70% population of Punjab is directly or indirectly associated with agriculture (Thakur et al., 2008). There are number of reports suggesting the contamination of pesticides in water, milk, fat, adipose tissues, blood and vegetables in Punjab (Kalra and Chawala, 1981; Battu et al., 1989; Aulakh et al., 2006; Thakur et al., 2008; Gill et al., 2009). So, this problem increases further because of potential of such hazardous pesticides to cause harm to a large variety of non-target organisms (Ramaneswari and Rao, 2008).

The plants have been considered to have protective role against various chemical induced toxicity in cells. But, the composition of that particular plant is necessary to know actual component responsible for its activity. The GC-MS report of O. sanctum essential oil suggests that the major constituents present in essential oil were monoterpene including monoterpenic hydrocarbon (a-camphene), monoterpenic alcohol (eugenol and a-terpineol, camphor) and monoterpenic ester (bornyl acetate). It was calculated that about 74.19% of essential oil is made of eugenol (46%) and caryophyllene (29%). The other major constituents are 1,8-cineole (4.3%, beta linalol (4.7%), camphor (2.5%), p-allyl anisole (2.6%) and alpha terpineol (3.32%). The camphene (0.14%), cinnamyl acetate (0.38%), sabinene hydrate acetate (0.2%) and isobornyl acetate (0.23%) components were in lesser quantities.

The toxicity of monocrotophos has been widely investigated on insects and animal models, but there are few reports of cytotoxicity and genotoxicity in the in vitro models. (Rupa, 1989; Amer and Aly, 1992; Chakravarthi et al., 2010). Thus, studies on genotoxicity and mutagenicity of pesticides are important to evaluate their response in living cells. In the present investigation, attempts were made to study the effect of MCP on cellular DNA due to generation of oxidative stress. The effect of essential oil and monocrotophos treatment on the cell viability was estimated. The results suggest that A549 cell line shows increased rate of proliferation at 20µg/ml O. sanctum essential oil concentration for 6hrs. treatment.

The H1299 has not showed any proliferation rate with different concentration of essential oil (10µg/ml-100µg/ml). Interestingly, it has been observed that there is sharp decrease in cell proliferation rate at 80µg/ml in A549 cell line and of 40 µg/ml in H1299 cell line which may be due to high cytotoxicity of essential oil at this concentration. The results suggest that the concentration at which approximately 95% cells remain viable is 10µg/ml for essential oil of O. sanctum. The concentration at which 80% cell remain is 500µm for monocrotophos. It has been observed that there was increase in rate of cell proliferation at lower concentration range of monocrotophos (6.25 µM - 500 µM) for in A549 cell line for 6 hrs. treatment, but there is no significant change in cell proliferation in H1299 cells at this time period. Also, there is significant decrease in rate of cell proliferation at higher concentration (1mM - 100mM) in dose dependent manner for both the cell line.

Although, free radicals and other highly reactive forms of oxygen are produced in cells during normal metabolism but exposure to pesticides results in increase of free radicals generation (Banerjee et al., 1996; Kale et al., 1999; El-Demerdash, 2012; Lu et al., 2012; Kazi and Hashem, 2012). Also in cell system, there are several antioxidant enzymes which scavenge the free radicals and eliminate the harmful by products inside the cell (Chelikani et al., 2004; Ranjbar et al., 2002; Pompella et al., 2003; Amin and Hashem, 2012; Mishra et al., 2012; Kazi and Oommen, 2012). But, the exposure to environmental genotoxic compounds results in imbalance of antioxidant system which ultimately cause the DNA damage in cell (Zahran et al., 2005; Prabhakaran, 1993). The comet assay is able to detect various types of DNA damage under in vivo and in vitro conditions in many prokaryotic and eukaryotic systems (Tice et al., 2000; Singh et al., 2000).The results of the present study clearly demonstrate that monocrotophos causes DNA damage in dose and time dependent manner in A549 and H1299 cell lines, as evidenced by the increase in comet length, % DNA in tail, tail length, tail intensity and decrease in head intensity due to increase in DNA migration resulted from more DNA damage. Interestingly, it has been observed that there was decrease in comet area, % DNA in tail, comet length and tail moment in cells at 500 µM concentration of monocrotophos treatment for 24hrs. which may be because of

more severe DNA damage at this concentration which lead to migration of DNA away from the comet. In current study, the effect of monocrotophos pesticide on all said parameters was increased in dose and time dependent. It has also been evidenced that MCP exposure increase DNA damage in the dose dependent manner (Zahran et al., 2005; Saleha et al., 2001).

The Ocimum sanctum has been reported to decrease oxidative stress by reducing free radical (Sethi et al., 2004; Yanpallewar et al., 2004; Shetty et al., 2008; Suanarunsawat et al., 2010; Sakr and Al-Amoudi, 2012) and by enhancing the antioxidant system of cell (Banerjee et al., 1996; Prakash and Gupta, 2000; Samson et al., 2007; Nair et al., 2007; Kim et al., 2010). The results of present study suggest that the essential oil of O. sanctum has property to protect the cell from oxidative stress to the DNA damage by pesticide treatment. The pre and post-treatment of essential oil resulted in decrease in the % DNA in tail, comet length, tail length, tail intensity and increase in head intensity with respect to increasing concentration of monocrotophos. The results suggest that the post-treatment of O. sanctum essential oil is more effective in protecting the cells from DNA damage with respect to various monocrotophos treatment in H1299 and A549 cells. Eugenol, major component of O. sanctum, was reported to have antioxidant activity (Yoo et al., 2005) which may be responsible for this activity. In the present study, the results indicate that both pre-treatment and post-treatment with O. sanctum oil seems to be efficacious against monocrotophos induced DNA damage. The post-treatment of O. sanctum essential oil seems to be more effective to protect the cells from free radicals generated due to oxidative stress induced by various concentrations of monocrotophos.

CONCLUSION AND FUTURE PROSPECTIVE

In the present study, the composition of essential oil of Ocimum sanctum, genotoxicity of monocrotophos (pesticide) and protective role of O. sanctum against monocrotophos induced genotoxicity has been investigated. The results of research suggest the following conclusion as:

- The major component of essential oil of O. sanctum belong to monoterpene with eugenol (46%) and caryophyllene (28%) as major components. It also contain 8-cineole, beta linalol, camphor, p-allyl anisole and alpha terpineol. Other components are present in much less quantity like cinnamyl acetate, sabinene hydrate acetate and isobornyl acetate.
- The cell proliferation rate increased at lower concentration of 20µg/ml (A549 cell line), but there is drastic decrease in cell proliferation rate with increase in essential oil concentration. The 10µg/ml has chosen as concentration with no observable cytotoxic effect in both the cell line.
- The cell proliferation rate increased at lower concentration (6.25µM-200µM) and decreases with higher concentration (1mM-50mM) of monocrotophos concentrations in both the cell line.
- Monocrotophos causes DNA damage in dose and time dependent manner. The H1299 cells are more sensitive to essential oil and monocrotophos concentrations.
- The pre and post-treatment of O. sanctum essential oil show protective action against oxidative stress generated due to monocrotophos treatment to cells. The post-treatment is more effective to comparison to pre-treatment of O. sanctum essential oil in all studied parameters.

There is requisite to further investigate the effect of each individual component of essential oil of O. sanctum against chemical induced genotoxicity. It may be helpful to find the exact component responsible for the protective action of essential oil. Also, the genotoxic evaluation of pesticides needed to be elaborate to know its harmful consequences.

SUMMARY

The long time over usage of pesticides is one of the major cause for prevalence of various genetic disorders associated with its genotoxicity. To safeguard the human life from toxic effects of pesticides, the evaluation of genotoxic extend of pesticide is vital. Also, there is current need to reduce morbidity and mortality related to pesticide genotoxicity by the use of natural herbs. The aim of present work is to study the genotoxic dose and time response assessment of monocrotophos treatment as well as anti-genotoxic effect of essential oil of O. sanctum L. against monocrotophos induced DNA damage in A549 and H1299 Cell line.

The results of present study demonstrated that monocrotophos caused significant increase in cell proliferation at lower cell concentration (6.25µM–200µM) for 6 hrs. treatment (A549 cell line) and significant inhibition of cell viability at higher concentration (1mM-50mM) for 6 hrs. as well as 24 hrs. treatment (A549 and H1299 cell line). The increase of DNA damage was observed in A549 and H1299 cell lines in dose and time dependent manner. The pre-treatment and post-treatment with O. sanctum essential oil (10µg/ml) caused attenuation in genotoxic effect of monocrotophos in A549 and H1299 cell line. The H1299 cells were sensitive to monocrotophos genotoxicity as compared to A549 cell line. The pre-treatment and post-treatment reversed subsequent effect of monocrotophos induced genotoxicity. These results indicate that oxidative damage is likely to be reduced in pre-treatment and post-treatment of essential oil of O. sanctum.

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