Chapter 4
Application of Animal model system
**Hematotoxicity – a major public health problem**

Hematological malignancies are cancers of the blood, bone marrow, and lymph nodes. These three locations are intimately connected through the lymphatic system; therefore a disease affecting one of these three organs will often affect the others as well. Hematological malignancies are generally categorized as lymphomas, myelomas, or leukemias. **Lymphomas** are cancers that typically originate in lymph organs (lymph node, thymus, or spleen) and/or lymphatic tissue and involve an abnormal, rapid proliferation of T- or B-cells (lymphocytes). **Myelomas** are cancers involving mature B-cells (plasma cells). These neoplastic plasma cells are usually formed in lymph nodes, but migrate to and collect in the bone marrow where they can cause permanent damage. **Leukemias** are cancers originating in blood or bone marrow and are generally characterized by an abnormal proliferation of white blood cells.

The term “leukemia” includes a variety of neoplasms that are broadly divided into two large categories based on the rapidity of blood cell proliferation (acute or chronic) and the specific hematopoietic lineage involved (lymphoblastic or myelogenous). Acute leukemias display a very rapid increase in the number of immature blood cells in the bone marrow. As a result, the bone marrow becomes crowded and the production of other healthy blood cells is hindered. As the bone marrow becomes crowded, the malignant cells can overflow into the blood stream and spread to other organs. Because of the quick progression of the disease, acute leukemias must be treated immediately. Chronic leukemias are characterized by an over abundance of abnormal, more mature leukocytes. Chronic leukemias develop more slowly than acute leukemias, typically taking months or years to progress to a stage where treatment is necessary. Lymphoblastic leukemias involve abnormal lymphocyte progenitor cells and are usually of the B-cell lineage. In myelogenous leukemias, the cancerous cell is most often a myeloid progenitor such as CFU-GM, CFU-G, or CFU-M. By combining these two classification criteria, most leukemias can be categorized into four main groups: acute lymphoblastic leukemic (ALL), or chronic lymphoblastic leukemia, acute myelogenous leukemia (AML), or chronic myelogenous leukemia (Kumar et al. 2009).

**Acute lymphoblastic leukemic (ALL):**

ALL is a type of leukemia that starts from white blood cells in the bone marrow, the soft inner part of bones. It develops from cells called lymphocytes, a type of white blood cell central to the immune system, or from lymphoblasts, an immature type of lymphocyte. Acute lymphoblastic leukemia invades the blood and can spread throughout the body to other organs, such as the liver, spleen, and lymph nodes. But it does not normally produce tumors as
do many types of cancer. It is an acute type of leukemia, which means it can progress quickly. Without treatment, it can be fatal within a few months.

**Acute Myeloid Leukemia (AML):**

Acute myeloid leukemia (AML) is a type of blood cancer. AML usually develops from cells that would turn into white blood cells (other than lymphocytes). Sometimes, though, it can develop from other types of blood-forming cells.

Acute myeloid leukemia starts in the bone marrow. This is the soft inner parts of bones. With acute types of leukemia such as AML, bone marrow cells don't mature the way they're supposed to. These immature cells, often called blast cells, just keep building up.

**The Cell Cycle:**

In the development of cancer the disruption of the fine tuned regulation of cell cycle progression and division is an essential step. Lots of different regulatory factors and signals dictate the cell to proliferate or, in case of DNA damage, to die. As mammalian DNA is under constant attack by different agents, cells have developed several defensive mechanisms. Although these repair mechanisms are extremely powerful, they are not perfect, and damage of DNA can result in the development of cancer or Leukemogenesis. DNA breakdown leads to halting of the cell cycle progression via activation of different cell cycle checkpoints until elimination of the damage, or if the cell is not able to repair this defect, to programmed cell death (Hartwell & Weinert 1989).

The cell cycle is a well regulated series of events in order to duplicate DNA and subsequent cell division and in eukaryotic cell it consists of four distinct phases (Norbury & Nurse, 1992):

- **G1-phase** (gap phase 1): cellular growth and preparation for DNA synthesis
- **S-phase**: duplication of the genome
- **G2-phase** (gap phase 2): preparation for Mitosis
- **M-phase**: mitosis (cell division)

![Fig. 4.1.](image)

**Fig. 4.1.** The cell cycle and its checkpoints. The human cell cycle can be divided into four phases - G1-phase, S-phase, G2-phase and M-phase (mitosis). Cells must proceed through the cell cycle in a unidirectional manner and cell cycle progression is restricted to cells that have fulfilled specific
requirements to enter the next phase of the cell cycle. Whether requirements for cell cycle progression are met is supervised by checkpoints which hold back cells at cell cycle transitions. (Schnerch et al. 2012)

G1-, S- and G2-phase together form the interphase, while the M-phase could be divided in the metaphase (chromosomal alignment), the anaphase (segregation of sister chromosome) and the telophase (decondensation of chromosomes and formation of nuclear membranes) (McDonald & El-Deiry 2000). Besides these four phases, cells in G1-phase may temporarily or permanently leave the cell cycle in dependence on developmental or environmental signals entering a quiescent phase termed G0. Both, cell external and cell intrinsic signals together decide whether cells should enter a division cycle, but after achievement of a restriction point, progression through the cell cycle is controlled only by the intrinsic cell cycle machinery (van den Heuvel et al. 2005).

**Regulation or Control of the cell cycle:**

Regulation of the progression from one phase to another is mediated by different cyclin dependant kinases (CDKs) that are activated after binding to regulatory proteins (cyclins) (Hartwell & Kastan 1994; Michalides 1999). These CDK/Cyclin complexes do not only trigger cell cycle progression, but in case of DNA damage activated checkpoints arrest cells in either G1-, S-, or G2-phase allowing to repair the genetic material (Mailand et al. 2000).

![Fig. 4.2.: The stages of the cell cycle. The site of activity of regulatory CDK/cyclin complexes is also indicated.](image)

Until now, nine CDKs have been identified and these, five are active during the cell cycle i.e. G1 (CDK4, CDK6 and CDK2), S (CDK2), G2 and M (CDK1) (Fig. 1) (Vermeulen et al., 2003). Although CDK protein levels are constant during the cell cycle, they are only functional during distinct intervals (Meeran
While CDK4/6 regulate the entry into S-phase, CDK2 remains active through the S-phase and decrease in its activity leads to exit from S-phase. In contrast, CDK1 becomes active in G2-phase and during mitosis (Sherr 1996).

As mentioned above, association of CDKs with cyclins is essential for their activation. Two types of cyclins have been identified, the cell cycle related cyclins (Cyclins A, B, D and E) and the non cell cycle related cyclins that share structural homology (Cyclins H and C) (Sherr 1996). Cyclin D1 and Cyclin E were shown to be frequently deregulated in human cancers (Robles et al. 1998; Porter et al. 2001). Together with its catalytic subunit, cyclin E has a pivotal role in the regulation of G1-S transition and in the initiation of DNA replication (Krude et al. 1997) and its constitutive over expression at all phases of the cell cycle was observed in different cancers like breast cancer (Bortner & Rosenberg 1997) or ovarian cancer (Sui et al. 2001).

Besides the activating cyclins, there are two families of CDK inhibitors (CDKIs) that can repress CDK function, the Cip1/p21 family and the INK4 family. While members of the Cip1/p21 family (p21Cip1, p27Kip1 and p57Kip2) represent universal cyclin/CDK inhibitors, that bind both cyclin and CDK molecules simultaneously, members of the INK4 family (p15INK4, p16INK4 and p19INK4) exhibit specificity for cyclin D/CDK4/6 complexes (Meeran & Katiyar 2008). Inhibition of growth stimulatory signaling pathways has been shown to stimulate CDKI expression associated with cell growth arrest (Grana & Reddy 1995).

![Fig. 4.3.](image-url) A schematic overview of some essential steps in cell cycle regulation. P, Phosphorylated site (→: activation; -: inhibition) (Vermeulen et al. 2003).
In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. Full activation of CDK1 requires phosphorylation of threonine 161 (threonine 172 in CDK4 and threonine 160 in CDK2), brought about by the CDK7-cyclin H complex, also called CAK (Fig. 2). These phosphorylations induce conformational changes and enhance the binding of cyclins (Jeffrey et al. 1995; Paulovich & Hartwell 1995). The Wee1 and Myt1 kinases phosphorylate CDK1 at tyrosine-15 and/or threonine-14, thereby inactivating the kinase (Fig. 2). Dephosphorylation at these sites by the enzyme Cdc25 is necessary for activation of CDK1 and further progression through the cell cycle (Fig. 2) (Lew & Kornbluth 1996).

**Benzene and Toxicity:**

Benzene is a ubiquitous environmental chemical that cause different types of hematotoxicity. It is known to be a very volatile liquid, with several organic and inorganic constituents. It’s exposure resulting in progressive degeneration of the bone marrow, aplastic anaemia, and leukemia, and in dysfunction of the immune system (Snyder et al. 1981). The WHO estimates a 4 in 1 million risk of leukemia on exposure to benzene to a concentration of 1 g/m³. These vapors constitute various components of pollutants in the air, which are of great environmental and human health concern. Exposures to these pollutants are common in the refineries, oil fields, refueling stations, petrochemical industries, and motor mechanical workshops. A good percent of human populace is directly or indirectly exposed to these pollutants in the course of their day-to-day activities. Generally, those occupationally exposed constitute the population at greater risk of frequent exposure (Carbolla et al. 1994; Raabe & Wong 1996). The potential health hazards associated with chronic or subchronic exposure to these ubiquitous pollutants in the environment has attracted the attention of the general public and scientific community in particular.

**Benzene-initiated Hematotoxicity and Leukemia**

**Occupational and environmental exposure to benzene:**

Benzene is a common, widely used industrial and petrochemical solvent, and, therefore large populations can be exposed to benzene either occupationally or environmentally. Chronic exposure to benzene occurs worldwide through gasoline, car exhaust, and cigarette smoke. High exposure levels of benzene are particularly prominent in workers employed in the oil, shipping, auto repair, and petro-chemical industries, as well as shoe- and rubber manufacturing plants (Fishbein 1988; Gist & Burg 1997). Benzene has a high vapor pressure at ambient temperatures; therefore exposure occurs mainly through inhalation, although contamination of drinking water (Vodela et al. 1997; Nyman et al. 2008) or dermal (Wester & Maibach 2000) exposure can also occur. Therefore, current concern is centered on the effects of exposure to long-term, low concentrations of benzene both occupationally and environmentally, as some studies have suggested that these types of exposures are associated with an increased incidence of hematological disturbances and malignancies (Lan et al. 2004). Environmental, ambient air
levels of benzene in India have been measured yearly across the country at rural and urban sites by the Analysis and Air Quality Division of the Central Pollution Control Board (CPCB), India. Although place of residence (urban or rural) impacts the amount of benzene residents are exposed to, the largest source of non-occupational exposure to benzene is through cigarette smoking. The inhaled dose of benzene from cigarettes has been reported to be in the range of 16-75 $\mu$g/cigarette (Brunnemann et al. 1989). The mean blood benzene level of nonsmokers is approximately 176 ng/l, of non-active smokers 211 ng/l, and of active smokers 365 ng/l (Angerer 1991). Blood benzene levels are directly proportional to the number of cigarettes smoked as each cigarette can increase blood benzene levels by 12 ng/l. Blood concentrations of benzene in urban smokers have been reported to be 435 ng/l, compared to 221 ng/l in urban non-smokers (Brugnone et al. 1992).

**Benzene-induced toxicity and leukemia in adult humans:**

Acute toxic effects of benzene exposure have usually been related to poor working conditions, accidents, or misuse and abuse. Inhalation of benzene produces acute toxic effects on the central nervous system in humans, although these effects are temporary and generally subside rapidly once exposure ends. Inhalation of 250-500 ppm benzene may cause symptoms such as vertigo, drowsiness, headache, and nausea, whereas higher concentrations of 1500 ppm may cause euphoria followed by giddiness, headache, nausea, staggered gait, and with continued exposure, unconsciousness. Exposure to 3000 ppm can cause death within 0.5-1.0 h, whereas exposure to massive concentrations of 20,000 ppm or higher can be fatal within 5-10 min (Duarte-Davidson et al. 2001). At these concentrations, death by benzene exposure is caused by respiratory arrest and/or cardiac arrhythmias.

Benzene has long been known to be a human carcinogen and has been classified as such by the International Agency for Research on Cancer (IARC 1987). There is strong evidence linking chronic benzene exposure with blood cancer and other hematological disorders, particularly aplastic anemia (Aksoy & Erdem 1978), myeloproliferative disorders (Irons et al. 2005), and acute myelogenous leukemia (Aksoy et al. 1974), multiple myeloma (Infante, 2005), and lymphomas (Vianna & Polan 1979; Hayes et al. 1997). Most of this evidence comes from studies evaluating workers occupationally exposed to benzene in industries such as shoemaking, petrochemical, coke production, and rubber manufacturing (Aksoy 1980; Infante et al. 1977; Infante & White 1983; Rinsky et al. 1981; Utterback & Rinsky 1995; Paxton 1996; Paustenbach et al. 1936-1976; Yin et al. 1989; Yin et al. 1987; Aksoy 1989; Glass et al. 2005). The incidence of aplastic anemia at high levels of benzene exposure (>100 ppm) has been reported to be 1/100, which drops to around 1/10000 at exposure levels of 10-20 ppm (Liang et al. 2005). Studies by Aksoy (1989) showed that there is an increased number of leukemia cases (26 cases in 28,500 workers over 6 years) compared to leukemia incidence in the general population (6 in 100, 000).
in Turkish shoe workers who were exposed to an average daily dose of benzene between 210 and 650 ppm (Aksoy 1989). Furthermore, Yin et al. (1989) indentified 30 leukemia cases among 28,460 benzene exposed Chinese workers whose average daily exposure was estimated to be at least 100ppm (Yin et al. 1989). Although the workers in these studies were exposed to benzene concentrations that were extremely high compared with concentrations experienced in these industries today, safe levels of benzene exposure have not been established, and elevated risk for hematological disorders has been shown after exposure to 10-20 ppm (Infante et al. 1997), less than 10 ppm (Hayes et al. 1997) and 1 ppm (Rinsky 1989) over a period of up to 40 years. Even at exposures lower than the current occupational limit (0.5 ppm), hematotoxicity has been observed, with lower peripheral blood cell counts and altered CFU-GEMM numbers in Chinese shoe workers (Lan et al. 2004).

Environmental exposures to sources of benzene have also been linked to a higher incidence of leukemia. In an ecological study comparing automotive gasoline consumption to data on leukemia incidence and mortality in 19 European countries, there was a positive association between the incidence of myeloid leukemia and the gasoline consumption per square kilometer (Swaen & Slangen 1995). In addition, a study in Sweden showed that there is a correlation between AML and traffic density in young adults (Nordlinder & Jarvholm 1997), whereas another study found evidence of an association between automobile ownership and the development of AML (Wolff 1992). Moreover, an increased incidence of non-Hodgkin’s lymphoma in men was associated with living within 8 km of industrial factories (Linos et al. 1991). This study also identified a greater incidence of leukemia in men living in close proximity to chemical and petroleum plants; however the trend was not statistically significant. Finally, since the late 1980s and early 1990’s, cigarette smoking has been associated with a higher incidence of AML (Doll et al. 1994; Kane et al. 1999; Brownson et al. 1991; Brownson et al. 1993; McLaughlin et al. 1989). In general, the risk of AML in smokers is 2-3 times higher than in non-smokers (Brownson et al. 1991).

Although the evidence showing a relationship between environmental benzene exposure and the increased incidence of leukemia is compelling, it is important to note that the conclusions from these epidemiological studies should be interpreted with caution because there are other chemicals and pollutants in automobile exhaust, industrial emissions and cigarettes.

**Benzene-induced toxicity and leukemia in human children:**

There are only a few known risk factors of childhood leukemia (e.g. sex, age, race, exposure to ionizing radiation and certain pharmaceuticals, and certain congenital diseases such as Down syndrome and neurofibromatosis); however these risk factors account for merely 10% of childhood leukemia cases (Belson et al. 2007). Therefore, the precise etiology leading to the development of childhood leukemia is largely unknown. It has been hypothesized that the
development of childhood leukemia may be initiated in utero after maternal exposure to benzene and/or other environmental pollutants (Gale et al. 1997; Maia et al. 2007). Studies have shown that benzene crosses the placenta as it has been detected in cord blood in amounts equal to, or greater than, those detected in the maternal blood (Dowty et al. 1976). A study in 1977 was one of the first to suggest that in utero exposure to benzene may be associated with leukemic events in the offspring (Funes-Cravioto et al. 1977). This study examined genetic outcomes in children of mothers who were occupationally exposed to benzene during pregnancy and found an increased frequency of chromatid breaks and sister chromatid exchange in lymphocytes of these children. Since then, a number of epidemiological studies have identified an increased risk for childhood ALL associated with maternal occupational exposure to benzene (Shu et al. 1970; Shu et al. 1999; Kishi et al. 1993; Feingold et al. 1992; van Steensel-Moll et al. 1985). However, other studies did not detect a relationship between benzene exposure and the development of ALL, thus this association remains controversial (Infante-Rivard et al. 2005; Kwa et al. 1980).

**Benzene-induced toxicity and carcinogenicity in laboratory animals:**

Multiple studies have demonstrated that benzene is a multisite carcinogen in rodents when administered by inhalation or ingestion. Cancers detected in mice and rats after benzene exposure include the nasal and oral cavity, lung, stomach, liver, skin, zymbal gland, mammary, ovarian and uterine, lymphoma, and leukemia (Maltoni et al. 1989; Mehlman 2002; Huff et al. 1989; Maltoni & Scarnato 1979; Cronkite et al. 1989; Cronkite et al. 1984; Farris et al. 1993; Snyder et al. 1980). Animal studies have also shown that mice are more sensitive to benzene toxicity than rats (Zhu et al. 1995; Henderson 1996). For example, mice exposed to 300 ppm exhibit decreased survival and weight gain, as well as peripheral blood lymphocytopenia, anemia and neutrophilia. Although rats exposed to 300 ppm benzene exhibit decreased survival and lymphocytopenia, other blood disorders are not evident and the effects that are evident are not as severe as those observed in mice (Snyder et al. 1980; Zhu et al. 1995; Henderson 1996; Snyder et al. 1980). Exposing mice to 100-200 ppm benzene for 1 week produces similar hematotoxic effects as those that are observed in humans after chronic exposure to 1-10 ppm benzene, such as decreased peripheral blood cell counts and anemia (Lan et al. 2004, Green et al. 1982). Therefore, due to similarities in the nature of the blood disorders that are observed between mice and humans after benzene exposure, mice are the preferred animal model when studying benzene toxicity. Interestingly, reports in the literature indicate that mice exhibit different blood disorders after chronic exposure to benzene depending on the strain. For example, C57Bl/6, and B6C3F1 mice exposed to 300 ppm benzene 6 hr/day for at least 2 years have a significant increase in lymphomas compared to control animals (Huff et al. 1989; Snyder et al. 1980) whereas CBA/Ca mice exposed to 300 ppm benzene 6 hr/day for 16 weeks have a higher incidence of lymphomas and myelogenous neoplasms (Krajinovic et al. 2000; Farris et al. 1993). Furthermore, AKR/J and
CD-1 mice chronically exposed to 300 ppm benzene for 6 h daily do not display a significant increase in the incidence of lymphomas or leukemias, although the CD-1 mice do display increased incidences of myeloproliferative disease (Snyder et al. 1980; Goldstein et al. 1982; Snyder et al. 1982; Snyder et al. 1988). Yoon et al. (2001) investigated effect of benzene on the cell cycle of hemopoietic stem cells. In their experiment, bromodeoxyuridine UV (BUUV) suicide assay was performed in normal C57BL/6 and p53 knockout (KO) C57BL/6 mice during and after exposure to 300 ppm benzene for 2 weeks. They finding benzene suppresses the cell cycle by p53 mediated over expression of p21, a cyclin-dependent kinase inhibitor, resulting not simply in suppression of hemopoiesis but rather in a dynamic change of hemopoiesis during and after benzene exposure.

Toxicogenomic studies have also been conducted on hematopoietic cells of mice exposed to high levels of benzene. Microarray analysis revealed altered mRNA expression of various apoptosis, cell cycle and growth control genes in HSCs from mice exposed to 100 ppm. inhaled benzene for 6 h/day, 5 days/week for 2 weeks, compared with control mice (Faiola et al. 2004). In a study of wild-type and p53-knockout mice exposed to 300 ppm. benzene for 6 h/ day, 5 days/week for 2 weeks (Yoon et al. 2003), the p53 tumor suppressor gene was shown to be central to the mechanism of benzene action in bone marrow cells, by strictly regulating specific genes involved in the pathways of cell cycle arrest, apoptosis and DNA repair. It was proposed that dysfunction of the p53 gene, possibly through effects of repeated benzene exposure, could lead to problems in these critical functions and lead to hematopoietic malignancies. In a more recent study, heterozygous p53- deficient mouse models (C3H/He and C57BL/6 strains) were found to produce a higher incidence of hematopoietic neoplasms and a higher than threshold incidence of hematopoietic neoplasms at lower doses, compared with the corresponding wild-type strains, and AML was induced in the p53-deficient C3H/He mice exposed to benzene 300 ppm. (Kawasaki et al. 2009).

**Fig. 4.4.** Benzene metabolism and possible mechanism of benzene-induced leukemogenesis (Hirabayashi et al. 2004)
**Current Treatment Strategies: Promises and Pitfalls**

**Conventional therapy and Systemic Toxicity:**

Conventional therapeutic strategies such as chemotherapy, radiation therapy, bone marrow transplantation, biological therapy, blood transfusion and medications have been developed based upon the type of malignancies. For example, ionising radiation induces DNA damage that, upon multiple cell divisions, may lead to errors in transcription and translation resulting in cell death (Ryдberg 2001). Similarly, cytotoxic chemotherapy may interrupt microtubule formation that is essential for mitotic events that ultimately affect cell survival (Marchetti et al. 2002). This is true for many haematopoietic malignancies, however, as little as 5% of some solid tumours actually consist of rapidly proliferating, and therefore, susceptible cells (Rang et al. 1999). As a result, only a small subset of cancers such as Hodgkin’s lymphoma, testicular cancer, acute lymphoid leukemia and non-Hodgkin’s lymphoma are routinely cured using these agents (Abeloff & Armitage 2004). This is primarily because therapies that are directed against rapidly proliferating cells result in the death of normal tissues that also show enhanced proliferation rates, such as the bone marrow, gastrointestinal (GI) tract and hair follicles (Kaelin 2005). Side effects such as nausea, vomiting, hair loss, alopecia, liver and kidney damage and occasionally more serious affects including neutropenia and cardiotoxicity means that anticancer chemotherapeutics are often administered at sub-optimal doses, which eventually leads to the failure of therapy (DeVita 1997; Foote 1998).

**The Emergence of Multi-Drug Resistance (MDR):**

The development of drug resistance is also a major obstacle in patients receiving prolonged chemotherapeutic treatment. Clinical resistance to anticancer agents can occur at the time of presentation, as well as during the course of treatment and after relapse (Quesada et al. 1996). Although a number of different resistance mechanisms have been described, such as insufficient activation of the drug, utilization of alternate metabolic pathways, mutations in the p53 gene and over expression of the Bcl-2 gene family, the most intensely studied has been the decreased accumulation of drugs in cells, which is the leading cause of multi-drug resistance (Gottesman et al. 2002). Such resistance is characterized by a failure to respond to a variety of chemotherapeutic agents, many of which are structurally dissimilar and do not share a common intracellular target (Rang et al. 1999). The mechanism responsible for MDR in mammalian cells involves the overexpression of a 170 kDa cell surface, energy dependant plasma membrane glycoprotein (P-gp) encoded on the MDR1 gene (Bellamy 1996). The physiological role of P-gp is thought to be in the protection of cells against environmental toxins and works by exporting drugs out of mammalian cells, lowering the intracellular drug concentration below the toxic threshold (Gottesman & Pastan 1993; Patel & Rothenberg 1994) is therefore to find ways of overcoming drug resistance due to the expression of P-gp, which involves a search for clinically
novel drugs that retain relatively good activity on MDR cells. However, the chemotherapy of cancer, as compared with that of bacterial disease, poses a difficult problem. Microorganisms are both quantitatively and qualitatively different from human cells, while, cancer cells and normal cells are so similar that it has proved difficult to find general, exploitable biochemical differences between them (Rang et al. 1999). This is illustrated by the number of drugs selected for preclinical or clinical testing, based on their activity in experimental animal systems, that do not become clinically useful agents due to their severe or unpredictable toxicity towards normal cells, or because they lack any therapeutic advantage. The prevalence of MDR and systemic toxicity in association with currently administered cancer chemotherapies therefore suggests that alternative avenues need to be explored in the hope of finding new and effective therapeutic agents.

**Revival of Natural Product Research:**

Natural products have been investigated for promising new leads in pharmaceutical development (McChesney et al. 2007; Bailly 2009) as well as promising new source of therapeutic against in plant secondary metabolites that characterize certain plants or plant group (Seigler 1998). The use of natural products in the discovery of new medicines has been the single most successful strategy, primarily because the chemical diversity of natural products is greater than any other source. Between 1983 and 1995 as many as 60% of the approved drugs and drug application candidates for anti-infective and anticancer treatments were of natural product origin. In 1997, of the 42 new chemical entities that were submitted for approval by the FDA, 32 (76%) were natural products or derivatives thereof (Grabley & Thiericke 1999) and in 2000, 57% of all drugs in clinical trials for cancer were natural products or were based on natural product leads (Demain & Zhang, 2005).

In the past decade, research into natural products in the pharmaceutical industry has declined, owing to issues such as the development of high-throughput screening (HTS) against defined molecular targets and the development of combinatorial chemistry (Koehn & Carter 2005). Consequently, many pharmaceutical companies have placed greater emphasis on high-throughput screening of mass-produced combinatorial libraries over natural product research. One problem however, is that the percentage of isolated or synthesised compounds that are showing biological activity is low, and the expected surge in productivity has not been as great as projected. For example, the number of new active substances (NASs), also known as new chemical entities (NCEs), has hit a 20 year low of 37 in 2001 and is still declining (Newman et al. 2003). This is because combinatorial chemistry is limited to the rearrangement of known chemical structures, whereas completely novel chemical skeletons can only be found in nature. For this reason, many researchers are now turning back to natural products and investigating organisms from different niches that were previously inaccessible or unconsidered.
**Ocimum as a source of novel anti-hematotoxicity agents:**

*Ocimum basilicum* L. popularly known as Tulsi in Hindi and “Sweet Basil” in English in one of the sacred herbs for Hindus in the Indian subcontinent. And India has one of the oldest, richest, and most diverse cultural living traditions associated with the use of medicinal plants (Brown, 1980). The entire plant of *Ocimum basilicum* L. has medicinal value although mostly the leaves, and sometimes the seeds, are used. It has a versatile role in traditional medicine. Earlier studies in particular, the leaves of *Ocimum basilicum* L. offer promise to biologically active constituents such as insect repellent, nematocidal, antibacterial, antifungal and antioxidants properties (Lee et al. 2005; Deshpande & Tipnis 1977; Simon et al. 1999; Juliani & Simon 2002; Saha et al. 2013). The study was prompted by the reported use of the fresh juice of this plant to treat a maggots-infested nasal disease in India (Chopra et al. 1941; Deshpande & Tipnis 1977; Karawa et al. 1974). Antitubercular and antimalarial action of oil is also reported (Ramawamy & Sirsi 1967; Spencer et al. 1947; Wome 1982). Estragole (methyl chavicol), a major component in some sweet basil oils, has been shown to produce hepatocellular carcinomas in mice (Drinkwater et al. 1976; Hartwell 1969; Narayana et al. 1975). The plant is also documented to have anti-ulcer activity against animal model (Sing et al. 1999). Chiang et al. (2005) reported antiviral activities of extracts and selected pure constituents of *Ocimum basilicum*. In their experiment, results showed that crude aqueous and ethanolic extracts of OB and selected purified components, namely apigenin, linalool and ursolic acid, exhibit a broad spectrum of antiviral activity. Antiproliferative activity against p388 cell lines has also been reported (Manosroi et al. 2006). In addition, Kehkashan et al. (2010) reported anticancer activity of *Ocimum basilicum* L. extract and its fractions was also evaluated using human cancer cell lines. Recently Saha et al. (2012) anti-hematotoxicity activity against benzene induced mice has been reported.

**Hypotheses and Objectives:**

There is evidence in the literature indicating that benzene as a carcinogen, a genotoxicant and a hematotoxicant, and exposure of this chemical to humans and animals are associated with the development of a number of hematological pathologies including leukemia and aplastic anemia. Chromosomal abnormalities, gene mutations and gene expression abnormalities all affect critical protein levels and functions in primary human AML cells and may exert profound effects on chemosensitivity. Pharmacological targeting of these protein abnormalities has emerged as a possible therapeutic strategy in human malignancies, including AML. However, given the heterogeneity of AML one would expect differences between patients in their responses to such targeted therapies.

The increased incidence of multidrug resistance (MDR) and systemic toxicity to conventional chemotherapeutic agents suggests that alternative avenues need to be explored in the hope of finding new and effective treatments for metastatic disease. Natural products have made
enormous contributions to many of the anticancer agents used clinically today and as a result, the
cytotoxic molluscan metabolite tyrindoleninone as well its oxidative artifact 6-bromoisatin, were
initially used as templates for drug design. Both small molecule inhibitors and LDPs have also
shown much therapeutic potential in the treatment of a variety of malignancies, yet their
development still requires further optimisation, as very few drugs that enter preclinical trials are
ever approved for clinical use.

In the light of the above, the objectives of present study were

- To develop a mice model of benzene induced hematotoxicity.
- To study sub-acute toxicity of leaf methanolic extract of *Ocimum basilicum* L.
- To investigate the protective role of methanol leaf extract of *Ocimum basilicum* L.
  against benzene induced hematotoxicity in Swiss Albino mice.
Pharmacological experiments involving animals described in the present work were carried out and get approved by Animal Ethical Committee, Department of Zoology, University of Kalyani, Kalyani (Under Committee for the Purpose of Control and Supervision of Experiments on Animals, India).

**Plant material collection:**
The plant of *Ocimum basilicum* L. were collected from medicinal and aromatic plant garden (Fig. 4.6.), Department of Botany, University of Kalyani, Kalyani, India during the months of August 2011.

**Authentication of Plant material:**
The plants were identified by Dr. G. G. Maity, Professor of Taxonomy, Taxonomy and Plant systematic Unit, Department of Botany, University of Kalyani and Botanical Survey of India, Howrah, West Bengal, India. The voucher specimens (PDG/Bot. 136) was deposited and preserved in the Department of Botany, University of Kalyani, Kalyani, India, for reference.

**Preparation of crude methanolic extract from plant:**
The air-dried plants powdered. And the preparation of the extract was according to the procedure referred in materials and methods section *(Chapter 2).*

![Figure: Schematic representation for the isolation of methanol extract from plant parts of *Ocimum basilicum* L.](image)

**Drugs and Chemicals:**
The primary antibodies specific for cyclin D1, cyclin E, CDK2, CDK4, CDK6, Cip1/p21, p53 and β-actin and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate...
pyruvate transaminase (SGPT), Serum alkaline phosphatase (ALP), Bilirubin, Serum total cholesterol, Total protein, Urea, Uric acid, and Creatinine kits were purchased from Span Diagnostics, Surat, India. All other chemicals used in our experiments were of analytical grade.

**Experimental Animals:**
Swiss albino mice (20-28 gm, 6 to 8 weeks old) were purchased from a reputed supplier at a regular basis from maintained strain and were housed at ambient temperature (25±1°C), in relative humidity (55±5%) in a stainless steel wire cages (Tarson, India) and maintained on a 12/12 light-dark cycle. Pellet diet (West Bengal Diary and Poultry Development Corp. Ltd., Kalyani Industrial Area, Kalyani) was provided *ad libitum* except during the 6-hour daily period of benzene inhalation. Water was supplied *ad libitum* automatically through the tubing throughout the study.

**Benzene exposure:**
Benzene (MERCK, India) vapour was generated by heating liquid benzene to 16°C and channeled into the inhalation chamber. The groups of experimental mice were exposed 300 ppm, benzene for 6 hr/day, 5 days/week for 2 weeks in 1.3m³ inhalation chambers (S.B. Equipments, WB, India) and one control group was exposed to ambient air for the same time duration, interval, and periods. Benzene concentration in the chambers was monitored at half-hours intervals during daily exposures. The temperature and humidity in the chambers were automatically maintained at 24 ± 1°C and 55 ± 10%, respectively. This animal can act as a model for benzene-exposed hematotoxicity and leukemogenesis leading to secondary acute myelocytic leukemia.

**Sub acute oral toxicity study of the plant extract:**
Toxicity study was performed according to the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD 2001). In order to study acute toxic effect of the plant extract 30 mice was randomly divided into three groups of ten animals (5 males and 5 females) were used. Before oral administration of a dose, the mice were fasted for one to two hours (Kasolo et al. 2011) and body weights of each of them were recorded. Then the extract was dissolved in distilled water and administered daily by oral gavage to the mice, groups II and III and the control group (group I) receiving respective volume of distilled water (vehicle), for 29 days. The mice in group II were given orally the extract of *O. basilicum* L. at a dose of 50 mg/kg, and the mice in group III were given the extract at dose of 1500 mg/kg of body weight. Then, the mice were observed continuously for one hour after the treatment; intermittently for four hours, and thereafter over a period of 29 days. The mice were observed for gross behavioral changes, coat conditions, discharge, movement, body weight changes, serum biochemical parameters, hematological parameters, and mortality for 30 days to test subacute toxicity.
**Measurement of Body weight:**
The change of body weight in grams of each animal was recorded using ACCULAB digital balance, (Model No. ALC-310.3, Sartorius Mechatronics India Pvt. Ltd., Bangalore, India).

**Biochemical analysis**

**Blood sample collection and analysis for biochemical parameters:**
After the administration of the last dose, the animals were given rest overnight and then on the next day, they were sacrificed on the 30th day by decapitation and blood was collected. Blood samples collected from sub-acute treatment group were used for the estimation of serum biochemical parameters. The following experiments were done according to the instructions of manufacturer.

**Estimation of serum glutamate oxaloacetate transaminase (SGOT): (IFCC method):**
(Span Diagnostics Ltd., Surat, India)

**Principle:**
SGOT catalyses the transfer of amino group from L-aspartate to 2-oxoglutarate forming oxaloacetate and L-glutamate. The rate of this reaction is monitored by an indicator reaction coupled with malate-dehydrogenase (MDH) in which the oxaloacetate formed is converted to malate in the presence of reduced nicotinamide adenine dinucleotide (NADH). The oxidation of NADH in this reaction is measured as a decrease in absorbance of NADH at 340 nm, which is proportional to SGOT activity.

**Reaction:**
L-Aspartate + 2-Oxoglutarate $\xrightarrow{\text{AST}}$ L-Glutamate + Oxaloacetate

Oxaloacetate + NADH $\xrightarrow{\text{MDH}}$ Malate + NAD

Sample pyruvate + NADH $\xrightarrow{\text{LDH}}$ L-Lactate + NAD

AST: Aspartate aminotransferase.

MDH: Malate dehydrogenase

LDH: Lactate dehydrogenase

**Reaction Temperature:** 37°C

**Procedure:**

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>50 µl</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
**Estimation of serum glutamate pyruvate transaminase (SGPT):** *(UV-Kinetic method):*

(Span Diagnostics Ltd., Surat, India)

**Principle:**

SGPT catalyses the transfer of amino group from L-alanine to 2-oxoglutarate with the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to react with NADH to produce L-lactate. The rate of this reaction is monitored by an indicator reaction coupled with LDH in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a decrease in the absorbance of NADH at 340 nm, which is proportional to SGPT activity.

**Reaction:**

\[
\begin{align*}
\text{L-Alanine} + \text{2-Oxoglutarate} & \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate} \\
\text{Pyruvate} + \text{NADH} & \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}
\end{align*}
\]

ALT: Alanine aminotransferase  
LDH: Lactate dehydrogenase

**Reaction Temperature:** 37°C

**Procedure:**

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>50 µl</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

**Estimation of serum alkaline phosphate (ALP):** *(p-Nitrophenyl Phosphate (pNPP)-AMP) – IFCC method):*

(Span Diagnostics Ltd., Surat, India)

**Principle:**

Serum alkaline phosphatase hydrolyses p-nitrophenyl phosphate into p-nitrophenol and phosphate in the presence of oxidizing agent Mg\(^{2+}\). This reaction is measured as absorbance is proportional to the ALP activity.

**Reaction:**

\[
\begin{align*}
\text{P-Nitrophenyl phosphate} + \text{H}_2\text{O} & \xrightarrow{\text{Mg}^{2+} \text{ ALP}} \text{P-Nitrophenol} + \text{Phosphate}
\end{align*}
\]

**Wavelength:** 405 nm  
**Reaction Temperature:** 37°C

**Procedure:**

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>20 µl</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
**Estimation of Bilirubin: (Diazo method, end point):**

(Span Diagnostics Ltd., Surat, India)

**Principle:**

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form pink colored azobilirubin with absorbance directly proportional to Bilirubin concentration. Direct Bilirubin, being water soluble directly reacts in acidic medium. However indirect or unconjugated Bilirubin is solubilised using a surfactant and then it reacts similar to Direct Bilirubin.

**Reaction:**

\[
\text{Sulfanilic acid} + \text{NaNO}_2 \xrightarrow{\text{HCl}} \text{Diazotized sulfanilic acid} \\
\text{Bilirubin} + \text{Diazotized sulfanilic acid} \xrightarrow{\text{pH 1.4}} \text{Azobilirubin}
\]

**Reaction Temperature:** 37°C

**Procedure:**

Prepare Total Bilirubin reagent by adding 0.2ml of Sodium Nitrite Reagent to 10ml of Total Bilirubin. Direct Bilirubin reagent is prepared by adding 0.1ml of Sodium Nitrite Reagent to 10ml of Direct Bilirubin.

**Blank Solution:** To 500 μl of above working reagent add 25μl of distilled water.

**Standard solution:** To 500μl of above working reagent add 25μl of standard solution.

**Test Solution:** To 500 μl of above working reagent add 25μl of test solution of serum.

Mix well; incubate for 5 minutes at 37°C. Read the absorbance at 546 nm for Total Bilirubin and 630 nm for Direct Bilirubin against reagent blank.

**Calculation:**

Total Bilirubin (mg/dl): Abs. of Test × 23 (Factor)
Direct Bilirubin (mg/dl): Abs. of Test × 17 (Factor)
Indirect Bilirubin (mg/dl): TB – DB.

**Estimation of Total Cholesterol (CHOD-PAP method):**

(Span Diagnostics Ltd., Surat, India)

**Principle:**

Cholesterol esterase (CHE) hydrolyses cholesterol ester. Free cholesterol is oxidized by the cholesterol oxidize (CHO) to choloest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce pink colored quinoneimine dye. The intensity of color produced is proportional to cholesterol concentration.

**Reaction:**

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{CHOD}} \text{Cholesterol} + \text{fatty acids} \\
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{CE}} \text{Cholest-4-ene-3-one} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\text{ H}_2\text{O}
\]
**Procedure:**

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Total Cholesterol (Tc) as shown below:

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Serum sample</td>
<td></td>
<td></td>
<td>10 µl</td>
</tr>
<tr>
<td>Reagent-1</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mix, incubate for 5 min. at 37°C and Read the absorbance of standard, total cholesterol against Blank at 505 nm.

**Calculations:** Total Cholesterol (mg/dl) = Abs. of Test / Abs. of Std X 200

**Estimation of Total Protein (Modified Biuret method, End point assay):**
(Span Diagnostics Ltd., Surat, India)

**Principle:**
Peptide bonds in protein react with cupric ion in alkaline solutions to form a colored chelate, the absorbance of which is measured at 578 nm. The Biuret reagent contains sodium-potassium tartrate to complex cupric ions and maintains their solubility at alkaline pH. The absorbance of final colour is proportional to protein concentration of total protein in the sample.

**Reaction:**

\[ \text{Protein} + \text{Cu}^+ \xrightarrow{\text{Alk pH}} \text{cu-protein complex} \]

**Procedure:**

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret Reagent</td>
<td>1000 µL</td>
<td>1000 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C or at RT for 5 minutes. Read the absorbance at 578 nm against reagent blank.

**Protein Standard:** 6.5 mg/dl

**Calculations:** Total Protein (gm/dl) = Abs. of Test/ Abs. of Std X 6.5

**Estimation of Urea (Urease-Glutamate dehydrogenase: enzymatic UV test):**

**Principle:**
Urea is hydrolysed in presence of urease to produced ammonia and CO₂. The ammonia produced combines with 2-oxoglutarate and NADH in presence of GLDH to yield glutamate and NAD.

**Reaction:**

\[ \text{Urea} + \text{H}_2\text{O} + 2\text{H}^+ \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{CO}_2 \]
2NH₄⁺ + 2- Oxoglutarate + 2NADH → \( \text{GLDH} \) → H₂O + 2NAH⁺ + Glutamate

**Procedure:**
- Mix 4 parts of reagent 1 with 1 part of reagent 2 (Mono reagent). Leave the mono reagent for at least 30 min. at 15-25°C before use and protected from light.

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea standard</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
<tr>
<td>Monoreagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

- Mix and start stopwatch at the same time. After 60 sec. read absorbance (A₁) at 340 nm and exactly after another 60 sec. read absorbance (A₂). A\(_{\text{sample}}\) as well as A\(_{\text{std}}\) = A₂ - A₁

**Calculation:** Urea (mg/dL) = A\(_{\text{sample}}\) / A\(_{\text{std}}\) X conc. of Std

**Estimation of Uric acid: (Uricase – Peroxidase/ Uricase - POD):**
(Span Diagnostics Ltd., India)

**Principle:**
Uric Acid is converted by uricase into allantoin and hydrogen peroxide. The hydrogen peroxide oxidizes the reaction product of 4- aminoantipyrine (4-AAP) with 3, 5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) in presence of a peroxidase to form a red colored dye complex. The increasing in absorbance correlates with (is proportional to) the uric acid concentration of the sample.

**Reaction:**

\[
\text{Uric Acid} + 2\text{HO}_2 + \text{O}_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-AAP} + \text{DHBS} \xrightarrow{\text{POD}} \text{Quinoeimine Dye} \quad \text{(Red Dye)}
\]

**Procedure:**

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 μl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>20 μl</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>20μl</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C or at RT for 5 minutes. Read the absorbance at 505 nm against reagent blank.
**Calculation:**

Uric Acid concentration in the sample can be calculated using the following formula:

\[
\text{Uric Acid (mg/dl)} = \frac{\text{Absorbance of sample} \times \text{Concentration of std. (mg/dl)}}{\text{Absorbance of standard}}
\]

**Estimation of serum creatinine (Picrate method):**

(Span Diagnostics Ltd., India)

**Principle:**

Creatinine in a protein free solution reacts with alkaline picrate and produces a red colored complex, which is measured colorimetrically at 520nm.

**Reaction:**

\[
\text{Creatinine} + \text{Sodium picrate} \xrightarrow{\text{NaOH} (\text{Alkaline medium})} \text{Creatinine-picrate complex (Yellow-orange)}
\]

**Procedure:**

Deproteinization of test sample

0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.

2.0 ml of supernatant from the above step was mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively. All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520nm.

**Hematological parameters:**

Hematological parameters were estimated after completion of the treatment with plant extract (29 days). After the administration of the last dose, the animals were given to rest overnight and sacrificed on the 30th day by decapitation and blood was collected. Blood samples were taken by puncturing heart, in heparinized capillary tubes and analyzed for haematological parameters.

Hemoglobin (Hb) content (gm %) was estimated by Sahli’s hemoglobinometer. When blood is added to 0.1 N hydrochloric acid, hemoglobin is converted to brown colored acid hematin. The resulting color after dilution is compared with standard brown glass reference blocks of a Sahli haemoglobinometer. By using a pasteur pipette added 0.1 N hydrochloric acid in the tube up to the lowest mark (20% mark). Blood was drawn up to 20 μl mark in the Hb-pipette. Blood column adjusted carefully without bubbles. Blood was transferred to the acid in the graduated tube; reaction mixture and allowed the tube to stand for at least 10 minutes. The solution was diluted with distilled water by adding few drops at a time carefully and by mixing the reaction mixture,
until the color matches with the glass plate in the comparator. The matching was done only against natural light. The level of the fluid was noted at its lower meniscus and the reading corresponding to this level on the scale was recorded in gm % of hemoglobin.

Hematological parameters like total white blood cells (WBC), total red blood cells (RBC) were counted using Neubauer’s hemocytometer according to Sharma and Pandey (2010). Other blood parameters including platelet count, differential count of monocyte, lymphocyte, neutrophil and differentials of RBC such as hematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were also performed.

**Bone marrow sampling:**

The femur bone of the sacrificed experimental mice was dissected and both the proximal and distal ends were removed. Phosphate buffer solution of pH 7.2 was injected gently into one end of the shaft. This process was followed to flush out the bone marrow through the opposite end into a collection vessel to prepare single cell suspension within one hour of stopping benzene inhalation.

**Western blot detection of p53, p21, CDK2, Cyclin E, CDK4, CDK6, Cyclin D1 and β-actine after benzene exposure protein extraction from bone marrow cells:**

Protein extracts of the bone marrow were prepared using 5 different animals individually by sonicating femoral bone marrow cells in a cell-lysis buffer containing 20% sodium dodecyl sulfate (SDS), 2mM phenylmethylsulphonyl fluoride, and a protease inhibitor/phosphate inhibitor (Yoon et al., 2001). The concentration of protein was quantified using Bradford method (Bradford, 1976) and measured in a Beckman spectrophotometer.

**Western blot analysis for p53, p21, CDK2, Cyclin E, CDK4, CDK6, Cyclin D1 and β-actine:**

Protein extracts from bone marrow (15 µg for p21 and 10 µg for other) were denatured, subjected to 12% (W/V) SDS-PAGE, and then transferred to Polyvinylidene difluoride (Bio-Rad) membranes. After blocking nonspecific binding sites by incubating the membranes with 5% nonfat dehydrated milk in Tris buffered saline containing 0.1% Tween 20 (TTBS, pH 7.4) for 1 hour at room temperature, the membranes were incubated overnight at 4°C in the presence of diluted primary antibodies (p53, p21, CDK2, Cyclin E, CDK4, CDK6, Cyclin D1 and β-actine; Santa Cruz Biotechnology, In., Santa Cruz, CA, USA). The membranes were then washed with TTBS and incubated with 1:2000 horseradish-peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, In., Santa Cruz, CA, USA) for 50 minutes at room temperature. To visualize the bands, the membranes were treated with a detection reagent for 1 minute. Band densities were measured using an image analyzer (image J, NIH).
**DNA Fragmentation Assay:**

DNA was extracted from bone marrow single cell preparation (as mentioned before) using reagents and protocol suggested by Bangalore Genei (PI.NO: KT23). DNA concentration was measured at 260nm using a spectrophotometer (Varian, Germany). DNA extracts were then subjected to 0.8% agarose gel electrophoresis using 1X TAE buffer as running buffer (composition: 40mM Tris-acetate, 1mM EDTA) and ethidium bromide (final concentration into gel 0.5 μg/mL) as staining agent. 5 μL of 100 bp DNA ladder was loaded into one well in each assay. Finally, the gel was visualized and photographed using a gel documentation system (Genei image system).

**Treatment of Benzene-Exposed Mice with Crude methanolic leaf extract:**

Since no sign of toxicity of the plant extract was found in both low- and high-dose of plant extract-treated group, benzene-exposed mice were treated with the extract at a dose of 100 mg/kg body weight orally from the date of commencement of benzene exposure for 4 weeks daily. Control group of mice were treated with respective volume of distilled water containing 10% DMSO following the same schedule. During treatment, mice were supplied pelleted feed and water *ad libitum*.

**Experimental design:**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>No received any treatment</td>
</tr>
<tr>
<td>Group II</td>
<td>Benzene exposed, received only vehicle, distilled water containing 10% DMSO</td>
</tr>
<tr>
<td>Group III</td>
<td>Benzene exposed, received plant extract suspended in distilled water containing 10% DMSO at a dose of 100 mg/kg body weight for 4 weeks.</td>
</tr>
</tbody>
</table>

**Statistical Analysis:**

Analysis of variance (ANOVA) followed by multiple comparison two-tail “t” test was used for statistical analysis of collected data [36]. Differences were considered significant at $P < .05$. All the values were indicated in the figures as Mean ± S.E.M (Standard Error of Mean).
Results

Oil profiles of O. basilicum L. leaves:

The results obtained by Gas chromatography (GLC) analysis of the essential oil of Ocimum basilicum L. revealed 10 compounds, which are identified and quantified in Table 4.1. The unidentified component mainly consisted of a mixture of oxygenated monoterpenes, sesquiterpenes and aromatic compounds. As shown in table 1, 63.91% (10 compounds) of the oil was identified. The GLC result profile revealed the oil was predominantly composed of Geraniol (34.89%), while the second major compound was Citral (23.51%), followed by Linalool (2.21%), Eugenol (1.33%) and Camphor (0.64%). Citronellal (0.59%), Vanillin (0.27%), α-Pinene (0.23%), β-Pinene (0.19%) and Cineole (0.05%).

Table 4.1.: Percentage composition of the essential oils of Ocimum basilicum L. cultivated in West Bengal

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention indices (RI) in min.</th>
<th>Essential oil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>4.76</td>
<td>0.23</td>
</tr>
<tr>
<td>Camphor</td>
<td>4.87</td>
<td>0.64</td>
</tr>
<tr>
<td>Citral</td>
<td>5.26</td>
<td>23.51</td>
</tr>
<tr>
<td>Geraniol</td>
<td>5.76</td>
<td>34.89</td>
</tr>
<tr>
<td>Cineole</td>
<td>5.83</td>
<td>0.05</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>5.92</td>
<td>0.19</td>
</tr>
<tr>
<td>Citronellal</td>
<td>5.96</td>
<td>0.59</td>
</tr>
<tr>
<td>Eugenol</td>
<td>6.57</td>
<td>1.33</td>
</tr>
<tr>
<td>Vanillin</td>
<td>6.72</td>
<td>0.27</td>
</tr>
<tr>
<td>Linalool</td>
<td>7.41</td>
<td>2.21</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Evaluation of Non-toxicity of plant extract:

Physicochemical observations were recorded to determine potential toxic effects of infused plant material in the animals are summarized Table 4.2. No adverse effect such as behavioral changes, coat conditions alterations, discharge and movement abnormality was seen for both group II and group III animals. No mice were found to be died during the study and non significant changes of the body weight (p<0.05) were recorded after 30 days of observation. The normal control mice exhibited in body weight 23±1.2 g, while lower and higher dose showed 23±1.5 g and 23±1.4 g, respectively. Whereas, biochemical status of animals i.e SGOT, SGPT, ALP, Bilirubin, cholesterol, total protein, urea, uric acid and creatinine content showed no significant changes (p<0.05) in the both (low and high dose) treated mice compared to the control mice. In hematological parameters (i.e. RBC, Hb, WBC and Platelet) also showed no significant changes (p<0.05) in the treated mice compared to the control mice. High dose (1500 mg/kg body weight) led no significant rice (p<0.05) in RBC (6.7±0.13×10¹²/L), Hb (12.9±0.31g/dl), WBC
(32.1±0.26×10^9/L), and Platelet (750±42.4×10^9/L) as compared to low doses and control values (p<0.05). So, the selected dose of plant extract is non-toxic.

**Table 4.2.:** Evaluation of nontoxicity of the plant material. Effect of leaf methanolic extract on serum biochemical, hematological parameters, and body weight after subacute administration. Data are represented as mean±SEM.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (group 1)</th>
<th>Lower dose (50mg/Kg body weight) (group II)</th>
<th>Higher dose (1500 mg/Kg body weight) (group III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT (IU/dL)</td>
<td>40.75±1.12</td>
<td>41.08±1.19</td>
<td>40.96±1.15</td>
</tr>
<tr>
<td>SGPT (IU/dL)</td>
<td>34.22±1.19</td>
<td>34.98±1.16</td>
<td>35.11±1.13</td>
</tr>
<tr>
<td>ALP (IU/dL)</td>
<td>81.12±1.78</td>
<td>80.56±1.76</td>
<td>80.01±1.72</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.86±0.13</td>
<td>0.85±0.11</td>
<td>0.86±0.12</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>149.25±9.3</td>
<td>150.33±8.7</td>
<td>150.69±8.9</td>
</tr>
<tr>
<td>Total protein (mg/dL)</td>
<td>6.90±2.09</td>
<td>6.89±2.01</td>
<td>6.98±2.33</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>41.64±1.32</td>
<td>42.09±1.21</td>
<td>42.67±1.31</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.48±1.77</td>
<td>5.12±1.56</td>
<td>5.19±1.67</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.93±0.12</td>
<td>0.91±0.21</td>
<td>0.92±0.18</td>
</tr>
<tr>
<td>RBC (×10^12/L)</td>
<td>6.6±0.12</td>
<td>6.6±0.16</td>
<td>6.7±0.13</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>13.2±0.22</td>
<td>13.1±0.28</td>
<td>12.9±0.31</td>
</tr>
<tr>
<td>WBC (×10^9/L)</td>
<td>31.3±0.42</td>
<td>31.2±0.38</td>
<td>32.1±0.26</td>
</tr>
<tr>
<td>Platelet (×10^9/L)</td>
<td>756±41.2</td>
<td>736±46.1</td>
<td>750±42.4</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23±1.2</td>
<td>23±1.5</td>
<td>23±1.4</td>
</tr>
</tbody>
</table>

**CDK expression and plant material arrest benzene induced cell cycle deregulation:**

The results of the immunoblot analysis (Fig. 4.8.), significantly reduced expression of cell cycle regulatory proteins such as p53 and p21 has been implicated in benzene induced hematotoxicity in mice, whereas, expression levels of cyclins (cyclins D1, E), CDK2, CDK4, and CDK6 were considerably higher in benzene-induced mice compared with control mice. However, treatment of mice with the material after benzene exposure resulted in inhibition of benzene-induced expression levels of cyclins (cyclins D1, E), CDK2, CDK4 and CDK6 compared with proteins obtained from non-treated mice. Treatment of plant material resulted in the enhancement of the levels of p21 and p53 in benzene-exposed mice compared with non-treated benzene exposed mice.

**Hematological parameters:**

The results shown in Table 4 indicate that the number of red blood cells (RBC), white blood cells (WBC), and hemoglobin (Hb) (g%) has been decreased in benzene-exposed group compared to control group of animals (Table 4.3.). Lower levels were also observed for the major leukocyte cell, including neutrophil %, monocyte %, Lymphocyte % and red blood cells (RBC) including
hematocrit % (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular and hemoglobin concentration was elevated (Table 4.4.).

Hb (g %), RBC and WBC have been decreased to 9.96 ± 1.3, 2.11 ± 0.19, and 3.28 ± 0.45, respectively. After treatment of benzene-exposed mice with the plant leaf extract the above parameters increase significantly (P < 0.05) and the value reaches to 11.96 ± 1.22, 3.26 ± 0.33, and 4.67±0.31, respectively. This result is also in accordance with over expression of p21 in plant extract-treated group of animals as shown in immunoblot analysis. Among total WBC count, lymphocyte count increases significantly (P < 0.05) after treatment with the plant extract. Hct as RBC differential also increased significantly (P < 0.05) after treatment (Table 4.3.). However, after treatment with the plant material, decrease in blood parameters was found to be less as compared to control group of animals (Table 4.3.).

**Table 4.3.:** Variation of blood parameters among BE (benzene exposed) mice and treated with PE (plant extract) group. Data are expressed as mean±SEM

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (g %)</th>
<th>RBC (×10¹²/L)</th>
<th>WBC (×10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.29±2.1</td>
<td>4.08±0.38</td>
<td>8.21±1.2</td>
</tr>
<tr>
<td>BE</td>
<td>9.96±1.3</td>
<td>2.11±0.19</td>
<td>3.28±0.45</td>
</tr>
<tr>
<td>Treated with PE</td>
<td>11.96±1.22*</td>
<td>3.26±0.33</td>
<td>4.67±0.31*</td>
</tr>
</tbody>
</table>

*Significant increase (p<0.05) in parameters.

**Table 4.4.:** Variation of differential blood parameters among BE (benzene-exposed) mice and treated with PE (plant extract) group. Data are expressed as mean±SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>Neutrophil</th>
<th>Monocyte</th>
<th>Lymphocyte</th>
<th>Hct (%)</th>
<th>MCV (µm³)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
<th>Platelet count (×10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.03±1.98</td>
<td>1.92±0.06</td>
<td>68.34±5.12</td>
<td>38.09±2.11</td>
<td>52.78±3.46</td>
<td>16.22±0.12</td>
<td>26.44±0.35</td>
<td>750±38.21</td>
</tr>
<tr>
<td>BE</td>
<td>21.68±1.32</td>
<td>1.88±0.05</td>
<td>48.49±3.51</td>
<td>10.23±0.96</td>
<td>47.55±2.98</td>
<td>14.11±0.14</td>
<td>24.33±0.38</td>
<td>798±40.08</td>
</tr>
<tr>
<td>Treated with PE</td>
<td>21.92±1.41</td>
<td>1.90±0.06</td>
<td>62.12±4.98*</td>
<td>24.35±1.67*</td>
<td>48.34±2.76</td>
<td>14.56±0.13</td>
<td>24.49±0.36</td>
<td>776±39.97</td>
</tr>
</tbody>
</table>

*Significant increase (p<0.05) in parameters.

**DNA fragment analysis:**

As shown in Fig. 4.9. DNA fragmentation assay of bone marrow cells characteristic of apoptosis was clearly detected after benzene exposure to mice and it is being less fragmented in methanolic leaf extract treated group. The data were coincident with the results of the cell cycle regulating kinases.
Preparation of Plant extract

Plant extract

Composition of plant extract (GC analysis)

Sub acute toxicity study

Treatment with plant extract

Development of hematotoxicity mice model

Swiss albino mice

Benzene exposure @ 300 ppm X 6 hrs/day X 5 days/wk for 2 wks
Yoon et al., (2001)

Mice with hematotoxicity that includes disturbance in cell cycle regulation

Acute myeloid leukemia

Recovered mice to be normal

Different blood & bone marrow parameters studied including blood RBC, WBC, Hg%, DNA fragmentation & Western blotting analysis of Cell cycle regulatory proteins.

Fig. 4.5: Experimental Design
**Fig. 4.6:** *Ocimum basilicum* L. in the experimental garden, Department of Botany, University of Kalyani

**Fig. 4.7:** Chromatograms GC analysis of essential oils from *Ocimum basilicum* L.

**Fig. 4.8:** Immunoblotting of p53, p21, CDK2, cyclin E, CDK4, CDK6, cyclin D1. Panel 1: proteins from control animal; panel 2: proteins from benzene exposed animal; panel 3: proteins from animal treated with plant extract.

**Fig. 4.9:** DNA fragmentation analysis (lane 1: M marker; lane 2: control DNA; lane 3: DNA from benzene-exposed group; lane 4: DNA from plant extract-treated group).
Discussion

The availability of appropriate disease model system is crucial to development of effective treatment. Animal models are an approximation of reality and their use in developing anticancer drugs is controversial. There exists several types of model system in nature for development of anticancer drugs, such as human cell lines, mouse cell lines, human embryonic stem cell (hECS) derived cell lines, xenografted human primary cell lines, genetically engineered mice (GEMs) and in silico models. But in vivo models offer the most promise at present for mimicking the complexities of disease, including its multigenic nature, interactions with the host, treatment resistance and establishment of therapeutic indices. Therefore, in the present study we developed benzene induced hematotoxicity in mice model. Exposure of mice to benzene severely reduces the number of hemopoietic and lymphoid cells in the peripheral blood (Cronkite 1986; Cronkite 1989; Selling 1916; Gill et al. 1980; Snyder et al. 1980; Green et al. 1981; Farris et al. 1997) by inhibiting DNA synthesis in progenitor cells in the bone marrow (Moeschlin & Speck 1967; Lee et al. 1988). It is well established that benzene and/or its metabolites cause chromosomal aberrations in the peripheral blood lymphocytes of chronically exposed humans (Bogadi-Soare et al. 1997; Ding et al. 1983; Forni et al. 1971; Kasuba et al. 2000; Picciano 1979; Sasiadek 1992; Sasiadek et al. 1990; Sasiadek et al. 1989; Tompa et al. 1994; Tough et al. 1965; Tough et al. 1970; Yardley-Jones et al. 1990; Zhang et al. 1998; Zhang et al. 2007; Zhang et al. 2002). Benzene exposure has been associated with higher levels of chromosomal changes commonly observed in AML, including 5q-/-5 or 7q-/-7, +8 and t (Lan et al. 2004; Vermeulen et al. 2005) in the peripheral blood lymphocytes of highly exposed workers (Zhang et al. 2002; Smith et al. 1998; Zhang et al. 1999). However, the mechanisms underlying benzene-induced toxicity and leukemogenicity are not yet fully understood, they are likely to be complicated by various pathways, including those of metabolism, growth factor regulation, oxidative stress, DNA damage, cell cycle regulation and programmed cell death.

The simple quantitative analysis of the crude methanolic extract showed remarkable variations in essential oil composition of *O. basilicum* L. from West Bengal. The yield of the essential oils obtained from dry leaf of *O. basilicum* L. was 1.77% (w/v) and the dominant or major constituents in sample were geraniol and citral ranging between 34.89% and 23.51% of the total oils (Table 4.1.; Fig. 4.7.). Although methyl chavicol, linalool, methyl cinnamate, methyleugenol, eugenol, and geraniol are reported as major components of the oils of different chemotypes of *O. basilicum* L. (Grayer et al. 1996; Marotti et al. 1996; Chalchat et al. 1999). The variation in the oil composition could be attributed to differences in soil conditions and altitude. It was observed that linalool and geraniol are the main components in *Ocimum basilicum* L. from Bangladesh, and the authors concluded that the oil composition could be dependent on climatic
conditions (Mondello et al. 1996). In the present study, our results showed that geraniol and citral and the main constituents of *O. basilicum* L. in respect of West Bengal are condition. The observed variations may be due to different environmental and genetic factors and the nutritional status of the plants as well as other factors that can influence the oil composition.

Toxicity studies of herbal extract in animals are commonly used to assess potential health risk in humans, caused by intrinsic adverse effects of chemical compounds or plant extracts (Ashafa et al., 2011). The deleterious effects of these extracts may be accompanied or preceded by clinical signs of toxicity such as salivation, loss of hair, changes in animal eye color, decreased respiratory rate and motor activity. In present study, thirty days of oral administration of *Ocimum basilicum* L. (50 mg/Kg body weight and 1500 mg/Kg body weight) extracts showed no significant changes in serum biochemical, hematological and body weight as compared to control mice. In this study, serum enzymes ALP in treated groups of mice did not show any change in comparison to the control groups (Table 4.2.). Liver cell damage is characterized by a rise in serum enzymes viz. ALP (Brautbar & Williams 2002). Generally, SGPT concentrations are consistently higher than SGOT levels and this is expected as body cells generate more SGPT than SGOT (Mayne 1996). Usually, about 80% of SGPT is found in mitochondria whereas SGOT is a purely cytosolic enzyme. Therefore, SGPT is found in mitochondria whereas SGOT is a purely cytosolic enzyme. Therefore, SGPT appears in higher concentrations in a number of tissues (liver, kidneys, heart and pancreas) and is released slowly in comparison to SGOT. But since SGOT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than SGPT and within its limits can provide a quantitative assessment of the degree of damage sustained by the liver (Mammary et al. 2002). Since SGOT and SGPT activities are not showing any significant difference with the treatment of extracts, it does not indicate damage to liver cells. The markers of renal functions viz. urea, uric acid and creatinine levels were not changed in the group of mice treated with *Ocimum* extracts. This view strengthened by the fact that the relative weight of the kidneys did not show any evidence of toxicity. Also, total protein, cholesterol, and bilirubin (total and direct) level of treated mice of all dose groups shows no changes in comparison to control group mice (Table 4.3.).

The assessment of hematological parameters in mice can be used to determine the extent of deleterious effect of a plant extract on animal blood (Yakubu et al. 2007). It is inferred from this study that the methanolic extract of *O. basilicum* L. may have no toxic effect on the hematological parameters which include red blood cell count, hemoglobin concentration, white blood cell count and Platelet. This showed that the oxygen carrying capacity of the red blood cells was not compromised thus, the plant may be safe as used in traditional medicine. Some
Plants have been reported to cause destruction of the red blood cells which leads to anaemia and ultimately cell death (Blood & Radostits 1989; Adedapo 2002; Adedapo et al. 2004, 2007a). The extract of *O. basilicum* L. caused an increase in the level of the total WBC count and its differentials (neutrophil, monocyte and lymphocyte). This is an indication that the principle function of phagocytes, (which is to defend against invading microorganisms by ingesting and destroying them, thus contributing to cellular inflammatory processes), will be enhanced (Paul 1993; Swenson & Reece 1993; Jimoh et al. 2008). The observed changes in the levels of the WBC count and its differentials may provided a basis for the antibacterial, antifungal, antimalarial and antitubercular properties of *O. basilicum* (Saha et al. 2013; Afifi 1975; Ramaswamy & Sirsi 1967; Spencer et al. 1947; Wome 1982). Reduced expression of cell cycle regulatory proteins such as p53 and p21 has been implicated in benzene induced hematotoxicity in mice (Yoon et al., 2001). The level of Cip1/p21, which is a universal inhibitor of cell cycle progression, is transcriptionally activated by p53 after benzene exposure (Yoon et al. 2001; Farris et al. 1997). Mice exposed to benzene affect cell cycle regulators in bone marrow and blood. Regulation of cyclin-CDK complexes plays a key role in cell cycle progression at different phases in which CDKs are negatively regulated by a group of functionally related proteins known as CDK inhibitors, such as Kip/Cip family members (Kastan and Bartek et al., 2004; Vermeulen et al. 2003; Satyanarayana & Kaldis 2009). p21 is a universal CDK inhibitor and p21 inhibits DNA replication process (Park et al. 2004; Abukhdeir & Park 2008), whereas p53 is upregulated in response to anti-proliferative signals. Consistent with these reports, methanolic leaf extract treatment resulted in an upregulation of p53 and p21 and significantly reduced level of CDK4 and CDK6, cyclin D1, and moderately reduced level of CDK2 and cyclin E in benzene induced animal with hematotoxicity, which may have caused cell cycle arrest mainly in G2 phase in hematopoietic cells. Thus, it can be suggested that modulation in cell cycle progression and inhibition of cell proliferation could be one of the possible mechanisms through which the leaf extract inhibits benzene-induced cell cycle deregulation. Blood parameters analysis is relevant to risk evaluation as the hematological system has a higher predictive value for toxicity in humans (91%) when assay involve rodents and nonrodents (Olson et al. 2000). Blood is an important index of physiological and pathological status in man and animals and the parameters usually measured are hemoglobin, total red blood cell (RBC), and leukocyte (WBC) counts (Schalm et al. 1975). The hematotoxicity of benzene is characterized by suppression of erythromyelopoiesis, resulting in the depression of leukocyte (lymphocyte count mainly) and erythrocyte levels in peripheral blood (Farris et al. 1997; Cronkite et al. 1989). This result is also in accordance with overexpression of p21 as shown in immunoblot analysis (Fig. 4.8.). But after treatment with the plant material, decrease in blood parameters was found to be less.
In conclusion, protection by plant secondary metabolites is currently an important strategy for controlling the process of various diseases including hematotoxicity. Therefore, there is a need to explore medicinal plants or other natural agents that can work as protective agents against hematotoxicity. In the present work, our results show significant activity of methanolic leaf extract of *Ocimum basilicum* L. against benzene-induced hematotoxicity in mice. These extract may promote chemopreventive activity in human. On the basis of above evidence, it is possible that monoterpenes like geraniol, citral, and eugenol, present in the extract may be responsible for this activity. However, this claim demands extensive studies on synergistic pharmacodynamic interactions of the phytochemicals as well as the effects on signal modulation which are essential for the development of multiactive natural drugs from *Ocimum basilicum* L. for cancer chemoprevention in the near future.