CHAPTER I
INTRODUCTION

Estrogen is a hormone produced by ovaries and testes responsible for the development of sexual characteristics, preservation of normal brain functions, and the formation of nerve cells (Diamanti - Kandarakis et al., 2009). If estrogen is beneficial to the health, there are synthetic substances that mimic the function of estrogen but have adverse effects. Xenoestrogens are a group of many different synthetic and naturally occurring chemicals that mimic the behavior of natural hormones (Pelekanou et al., 2011) and attach to estrogen receptor sites of humans and animals (Chen, 2001). These chemicals enter the body mainly through absorption and ingestion. Unlike natural estrogen, xenoestrogens cause negative effects on the body, because the chemicals do not fit properly into the receptor sites of cells, thus the body cannot metabolize the foreign chemicals properly and may interfere with proper hormonal signalling (Soto et al., 1995).

Xenoestrogens are considered endocrine disrupting chemicals (EDCs). Xenoestrogens, such as phthalates, can be extremely persistent in the environment. Some EDCs, for example, polychlorinated biphenyls (PCBs), are able to bioaccumulate in the food chain or in several biological matrices (as fats) and often exhibit effects at weak concentrations or in combinations (Kortenkamp et al., 1998). Among the numerous sources of exposure, the ingestion of water or contaminated food, cosmetics, pharmaceuticals, industrial exposure, and contact via professional activities (e.g., pesticides) are the most common (Singleton et al., 2003) (Figure 1.1). Many xenoestrogens are synthetic estrogens stemming from human activity, which, due to their use, can enter in contact with living organisms or be released into the
environment. For instance, workers in the production of contraceptive pills were exposed to the potent estrogen ethinyl-estradiol (EE2) which is capable of being absorbed by the skin (Harrington et al., 1978). A correlation was also established between the massive exposure to pesticide DDT (dichlorodiphenyltrichloroethane) by farm labourers and the risks of oligospermia (Degen et al., 2000).

Infertility, decreased sperm count, breast cancer, endometriosis, prostate cancer, ovarian cancer, asthma, allergies and obesity are the health consequences of estrogens dominance symptoms.

**Commonly found xenoestrogens:**

Studies showed that organochlorides are complete carcinogen by nature which can initiate and promote tumor growth. Bisphenol A widely used in polychlorinated plastic items was found to exert estrogenic activity in various animal models (Hewitt and Korach, 2011). Research revealed that 2-5 parts per billion of bisphenol A is enough to cause breast cancer cell proliferation. Parabens are constituents class of commonly used preservatives. For many years, parabens were considered to have low systemic toxicity and safe for usage in various products due to its antimicrobial activity. In contrast to the earlier reports, recent research is emerging showing its endocrine disrupting ability in human and experimental animals. Phthalates, chemicals used in cosmetics and plastic commodities, were also found to possess estrogenic activity and cause cancer, developmental and sex-hormonal abnormalities (including decreased testosterone and sperm levels and malformed sex organs) in infants, and can affect fertility (Legler et al., 2002; Fisher, 2004; Barrett, 2005; Swan et al., 2005).
Phthalates are a class of compounds made from alcohols and phthalic anhydride. They are oily, colorless, odourless liquids that do not evaporate readily. Often called plasticizers, phthalates prolongs the lifespan or durability and increases the flexibility of plastics used in the manufacture of polyvinyl chloride plastics. Phthalates are found in hundreds of products such as toys, vinyl flooring, herbal pill coatings, plastic inks, nail polishes, varnishes, shampoos, sunscreens, skin emollients, insect repellent, garden hoses and building materials (Heudoef et al., 2007). The phthalates, as additives in polymeric substances, are physically bound and consequently readily leach out from polymeric products during disposal. Various cosmetics and personal hygienic products contain low molecular weight phthalates (ATSDR 1995; ATSDR 2001). In the United States of America (USA), total phthalates consumption was 640,000 tons in indoor plastic material during 1994 and some phthalic acid esters were incorporated in house plastic supplies such as floor decorates, wall papers, floor covering, synthetic leathers and wire coatings. With respect to health effects, phthalates are often classified as endocrine disruptor or hormonally active agents (HAAs) because of their ability to interfere with the endocrine system in the body (NTP, 2007).

Phthalates, also called benzene dicarboxylic acid with formula \(\text{C}_6\text{H}_4(\text{COOH})_2\), is the name of any of three isomers. The ortho form (1,2-benzene carboxylic acid; Figure 1.2) is called simply phthalate. It is a white crystal decomposing at 191°C and slightly soluble in water and ether. This compound is mainly produced and marketed in the form of its anhydride produced by the oxidation of orthoxylene and naphthalene. Its wide application is based on the ortho-related carboxylic acid groups as their dehydration is highly reactive with broad processing conditions to produce various downstream products. It is used to make simple esters widely used as
plasticizers. It is used for making unsaturated polyester resins, alkyl resins, polyester polyols, dyes and pigments, halogenated anhydrides, polyetherimide resins, isatoic anhydride and insect repellents.

The meta form (Figure 1.2) is isophthalic acid (IPA) (1,3-benzene carboxylic acid). It is a white crystal subliming at 345°C, and slightly soluble in water, alcohol and acetic acid (insoluble in benzene). It is produced by oxidizing meta-xylene with chromic acid, or by fusing potassium meta-sulphobenzoate or meta-brombenzoate with potassium formate. Isophthalic acid has excellent performance characteristics for coatings including excellent hardness, corrosion and stain resistance, hydrolytic stability of coatings and gel coats, excellent thermal stability and low resin color. It is a key ingredient for such products as marine, automotive and corrosion resistant pipes and tanks. Polyesters containing IPA are also used extensively in industrial coating applications for home appliances, automobiles, aluminium sliding and metal office furniture. It is used as an intermediate for polyesters, polyurethane resins and plasticizers.

The para form (Figure 1.2) known as terephthalic acid (1,4-benzenecarboxylic acid) (TPA) is a combustible white powder that is insoluble in water, alcohol and ether (soluble in alkalies), and sublimes at 300°C. It can be produced by oxidizing caraway oil, a mixture of cymene and cuminol or by oxidizing para-diderivatives of benzene with chromic acid. Terephthalic acid has been used mainly as a raw material for polyester fiber but lately it has been exploited for various uses in the non-fiber field for PET (polyethylene terephthalate)- bottles, PET-films, engineering of plastics and as poultry feed additives. Phthalate derivatives are also widely used to make dyes, medicines, synthetic perfumes, pesticides and other chemical compounds.
Phthalate esters (PEs) are a diverse group of chemicals having a vast range of applications (Stanley et al., 2003). The higher molecular weight phthalates are added to vinyl resin to improve its flexibility; di-2-ethylhexyl phthalate (DEHP), di-isononyl phthalate (DiNP), and di-isodecyl phthalate (DiDP) are the predominant PEs used as vinyl plasticizers. The lower molecular weight phthalates have a considerable range of applications. Dimethyl phthalate (DMP) is used as a stabilizing diluent for the shipping and storage of organic peroxides. Diethyl phthalate (DEP) is used as a fixative or carrier for perfumes and fragrances and also in time-released pharmaceuticals. Dibutyl phthalate (DBP) and diisobutyl phthalate (DiBP) are used in vinyl acetate emulsion adhesives and in cellulose lacquers. Butyl benzyl phthalate (BBP) is normally used with other general-purpose plasticizers in chloride applications (Table 1.1).

**EXPOSURE OF PHTHALATE**

Phthalates have been measured in numerous media, including: surface water, groundwater, landfill leachate, drinking water, sediment, suspended particulate matter, soil, air (outdoor and indoor), dust, precipitation, waste-water, sewage sludge, food, vegetation, and wildlife (Clark et al., 2003). The source of some of these environmental concentrations is migration from materials/media in the environment, e.g., landfills or injected sewage sludge (Bauer, 1997). Weathering of plastics and other phthalate esters-containing articles results in the release of phthalate to the environment, including air and water (Bauer, 1997; Michael et al., 1984; Tabor and Loper, 1985). The source of phthalates in indoor air, dust, or soil may be from weathering of products containing phthalates or directly from household products containing phthalates.
Humans may be exposed to phthalates simultaneously through a variety of exposure pathways, including ingestion of food, drinking water, dust, and soil; and inhalation of air (outdoors and indoors). The use of the lower molecular weight phthalates in consumer products such as cosmetics and pharmaceuticals may result in their direct release to air or direct absorption through the skin or gastrointestinal tract. Phthalates have been measured in human milk, blood, and urine (Koo and Lee, 2005; Zhu et al. 2006; Hogberg et al., 2008), and their metabolites have been measured in human urine, blood, amniotic fluid, and milk (Barr et al., 2003; Calafat et al., 2004, 2006; Teitelbaum et al., 2008).

**Diethyl phthalate**

Diethyl phthalate (DEP) is a phthalate ester, namely the diethyl ester of phthalic acid. It is a clear substance that is liquid at room temperature and is only slightly more dense than water. DEP is a man-made colorless liquid with a slight aromatic odor and a bitter, disagreeable taste (Table 1.2). Worldwide annual production and/or import volumes of DEP were reported around 700 tonnes in Japan and 10 000 tonnes in EU countries based on 1999 data, and between 4500 to < 23 000 tonnes in US in the calendar year 2005 (IPCS, 2003; US EPA, 2010).

International sources report that diethyl phthalate is used in a diverse range of consumer products and applications. The European Council for Plasticisers and Intermediates lists diethyl phthalate as a plasticiser widely used in tools, automotive parts, toothbrushes, food packaging, cosmetics and insecticide (ECPI, 2010). According to IPCS (2003), a common use of diethyl phthalate as a plasticiser is for cellulose ester plastic films and sheets (photographic, blister packaging, and tape applications) and moulded and extruded articles (toothbrushes, automotive
components, tool handles, and toys). For cosmetic applications, DEP is frequently found in skin care preparations, eye shadows, hair sprays, perfumes and other fragrance preparations, in toiletries, soaps, bath preparations, nail polish and enamel removers, and nail extenders. More specifically, DEP is used in perfumes as a fixative and solvent, in toiletries as an alcohol denaturant, in nail polish as a solvent for nitrocellulose and cellulose acetate, and in fingernail elongators as a plasticiser. It is also used in detergents, insecticide sprays and mosquito repellents, dye applications, adhesives, sealants, surface lubricants for food and pharmaceutical packaging, and medical tubing devices (IPCS, 2003; US EPA, 2010).

Based on a survey of fragrance manufacturers conducted in 1995-1996 by the Research Institute for Fragrance Materials, approximately 4000 tonnes were used in the preparation of fragrance mixtures worldwide (Api, 2001; IPCS, 2003). Concentrations of diethyl phthalate in cosmetics and fragrance preparations ranged from < 0.1% to 28.6% (97.5th percentile of use based on data from the International Fragrance Association), although < 1% was found in most products. A 2001 survey of fragrance manufacturers in the US indicated maximum concentrations of 1%-11% diethyl phthalate in perfume and up to 1% in deodorants and other personal care products (Api, 2001; IPCS, 2003). Also, a Greenpeace International report (2005) identified diethyl phthalate as the most prevalent phthalate found in 34 out of the 36 perfumes tested and with concentrations ranging from below detection to 2.2%. Similarly, DEP was found in 12 out of 17 popular perfumes, colognes and body sprays at concentrations ranging from 0.0098% to 3.2% (The Campaign for Safe Cosmetics, 2010).
Environmental contamination:

Based on 1994 Toxics Release Inventory data, US EPA (1995) estimated that 72 tonnes and 341 kg of diethyl phthalate would be released annually to the air and water, respectively, as a result of manufacturing, use, or disposal, and 364 kg of diethyl phthalate would be released annually to the environment as a result of landfilling activities. Total off-site releases were 1.26 tonnes annually.

Approximately 75% of the total environmental release of phthalate plasticizers from dump sites result from low-temperature burning, with subsequent vaporization. Diethyl phthalate may also be released directly to the atmosphere as a result of volatilization/evaporation from consumer items such as cosmetics and toiletries, insect repellents, and insecticides (Peakall, 1975).

Diethyl phthalate was detected in 4.96% of the groundwater samples and 1.42% of the surface water samples taken at NPL sites included in the Contract Laboratories Program Statistical Database (CLPSD) at mean concentrations of 12.50 and 12.10 µg/L, respectively, in the positive samples (CLPSD, 1989). It has been estimated that the phthalate esters released to the environment may be approximately 1% of the phthalate content of plastic materials in direct contact with water or other liquids (Peakall, 1975). EPA (1981) estimated that 300 metric tons of diethyl phthalate would be released annually to surface water as a result of manufacturing, use, or disposal, based on 1977 production data.

Based on 1977 production data, EPA (1981) estimated that 6,800 metric tons of diethyl phthalate would be released annually to the environment as a result of landfilling activities. Diethyl phthalate has been detected in 4.26% of the soil samples.
taken from the NPL sites included in the CLPSD at a mean concentration of 39.06 µg/kg in the positive samples (CLPSD, 1989).

**General population and occupational exposure:**

Human exposure to diethyl phthalate can result from eating food into which diethyl phthalate has leached from packaging material, eating contaminated seafood, drinking contaminated water, or breathing contaminated air, or as a result of medical treatment involving the use of PVC tubing (e.g. dialysis patients). The use of diethyl phthalate in consumer products, and intake from contaminated foods, however, are likely to be the primary sources of human exposure. Diethyl phthalate has been detected in adipose tissue samples taken from people in the USA. Occupational exposure may occur in industrial facilities where diethyl phthalate is used in the manufacture of plastics or consumer products.

Diethyl phthalate was quantified from food at concentrations of 0-0.51 mg/kg (Giam and Wong, 1987). Baked food packaged in card cord board boxes with cellulose acetate windows [containing 16-17 % diethyl phthalate] had diethyl phthalate concentrations of 1.7 – 4.5 mg/kg. It was suggested that diethyl phthalate may volatilize from the plastic window to the food without direct contact or be adsorbed in condensate on the window, which then fall back onto the food (Castle et al., 1988). Diethyl phthalate was found in food by Castle et al. (1988). Kamrin and Mayer, (1991) estimated a total daily dietary exposure to diethyl phthalate of 4 mg based on a daily ingestion of 1 kg of cellulose acetate wrapped food containing 4 mg/kg diethyl phthalate.

Diethyl phthalate is an important solvent and vehicle for fragrance and cosmetic ingredients. A survey of over 2000 perfume compounds intended for
hydroalcoholic cosmetic products reported a 97.5 percentile of use for diethyl phthalate of 28.6%. Diethyl phthalate is also approved for use as a component of food manufacturing equipment and packaging at unlimited concentrations (Anonymas 1985) and drug product containers (Kamrin and Mayor, 1991).

Diethyl phthalate concentrations ranging from 0.01 µg/litre (in 6 of 10 US cities) to 1.0 µg/litre (in Miami, Florida) were found in drinking-water samples from water treatment plants in the USA (Keith et al., 1976). US EPA (1989) summarized various studies in which diethyl phthalate was detected in the ground water of 33% of 39 public water wells in New York State. Based on an average concentration of diethyl phthalate in Toronto, Canada, drinking-water of 0.0107 µg/litre, the mean drinking – water exposure for the years 1978-1984 was estimated to be approximately 6 µg/year, assuming an average consumption of 1.5 litres of water per day (Davies, 1990).

**Pharmacokinetics:**

Diethyl phthalate is hydrolyzed to monoester, monoethyl phthalate and ethanol after oral administration in the lumen of the gastrointestinal tract or in the intestinal mucosal cells. Hydrolysis of diethyl phthalate also takes place at the kidney and liver after systemic absorption. After tissue distribution throughout the body, DEP accumulates in the liver and kidney. The metabolites are excreted in the urine (Api, 2001). Diethyl phthalate is metabolized by carboxyl esterase, which is synthesized in the human liver. *In vitro* studies show that DEP reduces the glucuronyl transferase activity. It was also observed that the activity of peroxisomal enzyme carnitine acetyl transferase is increased in cultures of rat liver cells (Api, 2001).
Once formed, the monoester derivative can be further hydrolysed \textit{in vivo} to phthalic acid and excreted or conjugated to glucuronide; the terminal or next-to-last carbon atom in the monoester can be oxidized to an alcohol; or the alcohol can be successively oxidized to an aldehyde, ketone, or carboxylic acid (Albro \textit{et al.}, 1973; Albro and Moore, 1974; Kluwe, 1982; US EPA, 1989). Absorption of diethyl phthalate and three other phthalates were measured using rat dorsal epidermal skin \textit{in vitro} (Scott \textit{et al.}, 1987). Lag time for absorption was 1.1 h, and the steady-state absorption rate was 414 µg/cm$^2$ per hour. The different percutaneous absorption rates between human and rat would suggest differences in bioavailability and subsequent differences in toxicity following dermal exposure. Percutaneous absorption of diethyl phthalate was evaluated \textit{in vitro} in flow-through diffusion cells using full-thickness male rat skin (Mint \textit{et al.}, 1994).

\textbf{Toxicological evaluation:}

A variety of \textit{in vitro} and \textit{in vivo} studies have focused on the biological and toxicological effects of diethyl phthalate. Multiple studies in rabbits, guinea pigs and rat of skin irritation following single or repeated dermal applications of undiluted DEP to intact or abraded skin under open or occluded conditions for 4-24 hours produced slight to moderate irritation (Api 2001; SCCNFP, 2001). The National Toxicology Program (NTP, 1995) found that long-term repeated dermal application of diethyl phthalate at 100 or 300 µl produced mild dermal acanthosis in rats. Significantly depressed body weight (15-25% less than controls) was noted in rat administered diethyl phthalate in the diet for 16 weeks at a concentration of 5% (Brown \textit{et al.}, 1978). Lamb \textit{et al.} (1987) and NTP (1984) reported significantly depressed terminal body weight (8-12% lower than controls) in F1 parental CD-1
mice administered diethyl phthalate in the diet during premating, mating and gestation at a concentration of 2.5%.

Studies by Pereira and co – workers (Pereira et al., 2007; Pereira and Rao 2007) reported a host of changes in liver endpoints, including serum and liver chemistry, liver weight, and histopathology in rats exposed to doses as low as 0.57 mg/kg bw/day for 5 months. Mapuskar et al. (2007) reported that three different doses (10, 25 and 50 mg/kg of the diet/day) of diethyl phthalate caused significant dose-dependent increase in liver aspartate and alanine aminotransferase (AST and ALT) activities and liver glycogen, cholesterol contents as well as acid phosphatase (ACP), lactate dehydrogenase (LDH) activities and triglycerides level in serum. Liver histology by light microscopy showed intracellular vacuolations in all the diethyl phthalate -treated groups which was more evident in 25 and 50 ppm diethyl phthalate – treated mice while hepatocellular degeneration and hypertrophy of hepatocytes was evident in 50 ppm diethyl phthalate- treated mice (Mapuskar et al., 2007). Mapuskar (2007) also reported proliferation of mitochondria and peroxisomes evident in the electron micrographs of the 10 ppm diethyl phthalate – treated mice while increase in lipid droplets and severe mitochondrial proliferation were seen in 25 and 50 ppm diethyl phthalate – treated mice. Pereira et al. (2006) studied chronic toxicity of diethyl phthalate in male Wistar rats and reported a significant increase in serum ACP, LDH and ALT enzyme activities as well as liver triglycerides, glycogen, total cholesterol and lipid peroxidation. Total glutathione (GSH) also decreased in liver of diethyl phthalate – treated mice. He also showed severe vacuolations, fatty degeneration and loss of hepatic architecture in diethyl phthalate – treated liver histology. Simultaneous administration of diethyl phthalate and ethyl alcohol leads to severe impairment of lipid metabolism coupled with toxic injury to the liver as
evident from significantly altered lipid and enzyme levels in liver and serum (Sonde et al., 2000). Sonde et al. (2000) also reported that long-term simultaneous exposure to diethyl phthalate and ethyl alcohol may have severe implication for humans who are occupationally exposed to these two xenobiotics. Combined administration of diethyl phthalate and polychlorinated biphenyls caused increase liver weight as well as liver function tests and severe vacuolations, loss of hepatic architecture and granular deposits in the liver (Pereira et al. (2006). Burse et al. (2007) showed increase liver weight bioaccumulations of diethyl phthalate in liver, testis, brain, gills and in muscle tissue of fish which increased significantly with increase of dose from 1 to 5 ppm. Liver and muscle AST, ALT, SDH, ACP, ALP were significantly increased as well as brain acetylcholinesterase (AchE) activity was significantly decreased by diethyl phthalate in Cirrhina mrigala fish (Ghorpade et al., 2002). Increases in kidney weight were reported in several oral studies. Fujii et al. (2005) reported 7-9% increase in kidney weight in F1 parental female rats receiving diethyl phthalate from the diet at 1375 mg/kg bw/day for up to 17 weeks.

There is some evidence that exposure to DEP may result in alterations within selected biomarkers of male reproductive functions in human. Jönsson et al. (2005) assessed urine, serum, and semen samples from 234 young Swedish men and found that subjects within the highest quartile for urinary MEP had 8.8% (95% confidence interval=0.8–17) fewer sperm, 8.9% (0.3–18) more immotile sperm, and lower serum luteinizing hormone values (0.7 IU/L; 0.1–1.2) compared to those subjects in the lowest quartile for urinary MEP. A dose-response relationship between sperm deoxyribonucleic acid (DNA) damage (as assessed by the comet assay) and urinary MEP levels was reported for two groups of men who presented at a health facility for semen analysis as part of an infertility investigation (Duty et al., 2003; Hauser et al.,
Pant et al. (2008) reported a significant (p < 0.05) inverse relationship between sperm concentration and level of DEP in the semen of a group of 300 males between the ages of 20 and 40 years. Decreased testicular weight in diethyl phthalate-treated groups may be due to impaired synthesis or enhanced metabolism resulting in down regulation of testicular androgenesis (Andric et al., 2000) which correlate well with reduced serum testosterone and androstenedione level. Fujii and co-workers (2005) also showed a significant decrease in serum testosterone levels in rats fed with diethyl phthalate at high concentration. Jones et al. (1993) reported ultrastructural changes in Leyding cells of rats treated with diethyl phthalate showing mitochondrial swelling, focal dilation of smooth endoplasmic reticulum and vesiculation. Pereira et al. (2008) reported diethyl phthalate enhanced ROS production, lead to mitochondrial swelling of the Leyding cells (Jones et al., 1993) might inhibit the StAR protein (Steroidogenic acute regulatory protein) expression and function. StAR protein results in reduction in testosterone levels (Diemer et al., 2003).

Toxic effects of DEP on thyroid glands have also been observed from another three-generation study in Wistar rats (F0 dosed at 0.57, 1.43, or 2.85 mg/kg bw/d whereas F1 and F2 at a single dose of 1.43 and 0.57 mg/kg bw/d respectively for 150 d each generation). In F2, thyroid glands showed follicular shrinkage, loss of thyroglobulin and fibrosis of the interfollicular epithelium with a lesser intensity seen in F0 and F1 rats (Pereira et al., 2008).

In one study of pregnant rats, administration of DEP in the diet at a concentration producing a dose level of 3,210 mg/kg bw/day during GDs (gestation days) 6–15 resulted in increased incidence of extra ribs, percent of litters with malformed fetuses, and percent of litters with variations (Field et al., 1993). In a two-
generation reproductive toxicity study of rats, depressed pup weight at weaning, delayed pinna detachment in male pups, and delayed onset of vaginal opening were noted in offspring of rats receiving DEP from the diet at 1,016 mg/kg bw/day (males) and 1,375 mg/kg bw/day (females); these effects were not seen at doses of 197 mg/kg-day (males) and 267 mg/kg bw/day (females) (Fujii et al., 2005). Administration of DEP to rats and mice has led to the increase in skeletal defects or rib number alteration in offspring (Singh et al., 1972; Kamrin, 1991; Field et al., 1993; WHO 2003).

At culture concentrations of 0.05, 0.167, and 0.5 μg/L, DEP produced a concentration-related increase in the number of relative sister chromatid exchanges per chromosome. This effect occurred only in the presence of the S9 fraction from rat liver homogenates (NTP, 1993). Diethyl phthalate has been shown to be mutagenic for Salmonella typhimurium strains TA100 and TA1535 only without metabolic activation (Kozumbo et al., 1982; Agarwal et al., 1985). Hokanson et al. (2009) reported failure to develop of a normal brain/central nervous system due to in utero, and perhaps to early post-natal exposure to DEP and its mono ethyl phthalate. Monoethyl phthalate, a main metabolite of DEP, is reported to cause increased DNA damage and also significantly associated with increased incidence of breast cancer in premenopausal women (El Tahir et al., 1993).

Additional evidence supporting this mode of toxin action is provided by the observation that DEP failed up-regulate expression of m-RNA of the enzymes SOD, GST or CAT while are induced as counter measures to adverse effects of oxidative stress (Mankidy et al., 2013). This data corroborate recent reports in the literature (Kang et al., 2010; Erkekoglu et al., 2014). The results of in vitro experiments suggested that apoptosis was occurring and a plausible mechanism for observed
toxicity of DEP to fathead minnow embryos. These observations suggested a link between oxidative stress and programmed cell death (Carvour et al., 2008) and places apoptosis downstream of events generating oxidative stress in cells. Diethyl phthalate affects the apoptotic system in PC12 cells and may enhance oxidative stress such as that induced by reactive oxygen species (Sun et al., 2012). Increased liver lipid peroxidation levels correlates well with the increased number of peroxisomes observed in the electron micrographs in the DEP - treated rats (Pereira et al., 2006) which indicates high levels of ROS (reactive oxygen species) production due to continuous liver insult by DEP. This correlates with the increase liver damage and reduced GSH levels observed. Hence exposure to DEP for a longer period increased ROS production leading to depletion of GSH reserves, which is involved in antioxidant cellular defence due to oxidative stress.

Oxidative stress is among major apoptotic stimuli in ischemic heart disease. During ischemia the respiratory cytochromes become redox-reduced, allowing them to directly transfer electrons to oxygen. Reactive oxygen species (ROS) are therefore excessively generated from a likely mitochondria source and then precipitate DNA damage, protein oxidation, lipid peroxidation, and other direct cellular injuries, consequently launching apoptosis in cells.

ROS are highly reactive oxygen moieties that arise from electrons from the mitochondrial respiratory chain or through the activity of intracellular oxidases, including NADPH oxidase (NOX) and xanthine oxidase. A number of antioxidants are in charge to remove excessive ROS and to maintain a physiological redox balance (Halliwell et al., 1985). Various remodelling stimuli (e.g., neurohormones, growth factors, and cytokines) that are released in response to cardiac injury enhance ROS production by activating ROS-generating enzymes and/or decreasing the antioxidant
defense capacities, which results in a net increase of ROS (oxidative stress) (Dhalla et al., 1994, 1996, Hill et al., 1996). Excessive ROS may directly induce cellular injury via oxidation of DNA, lipids, and proteins associated with cell death, different diseases and premature aging. ROS also participate in cell signalling through activation of redox-sensitive signalling cascades. Thereby, they initiate both protective (adaptive) and damaging (maladaptive) cellular events.

In principle, oxidative stress can result from:

i) Diminished antioxidants, e.g. mutations affecting antioxidant defence enzymes. Depletions of dietary antioxidants and other essential dietary constituents can also lead to oxidative stress.

ii) Increase production of ROS/RNS, e.g. by exposure to elevated O₂ or the presence of toxins that are metabolised to produce ROS/RNS or are themselves reactive species, e.g. NO₂⁻ or excessive activation of natural ROS/RNS-producing systems, e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases.

According to the redox-homeostasis model, the following three strategies to modify oxidative stress-associated processes can be delineated:

(i) Scavenging or neutralization of ROS by enhancing antioxidant capacities,

(ii) Inhibition of sources of ROS, and

(iii) Protection of potential targets from oxidation.

**Antioxidants:**

Antioxidants may be defined as any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or
prevents oxidation of that substrate in a chain reaction (Leong and Shui, 2002; Halliwell and Whitemann, 2004). Humans have evolved a highly complicated antioxidant protection system, which involves a variety of endogenous and exogenous compounds that are able to function interactively and synergistically to neutralize free radicals. These include antioxidant enzymes that catalyze free radical quenching reactions, metal binding proteins that sequester free iron and copper ions that are capable of catalyzing oxidative reactions, diet-derived antioxidants, and other low molecular weight compounds such as \( \alpha \)-lipoic acid (Kaliora et al., 2005). Antioxidants have become a popular research topic because they cannot be generated by the human body and hence have to be consumed in the diet. Many fruit and vegetables have been found to be rich sources of antioxidants. Since a large portion of the human diet is based on fruit and vegetables, it is important to understand the biological and biochemical interactions between these dietary antioxidants and living systems.

**Nigella sativa**

Medicinal plants are used in the preparation of herbal medicines as they are considered to be safe as compared to modern allopathic medicines. Many researchers are focusing on medicinal plants since only a few plant species have been thoroughly investigated for their medicinal properties, potential mechanism of action, safety evaluation and toxicological studies. *Nigella sativa* is an annual flowering plant, native to south and southwest Asia. It grows to 20-30 cm (7.9-12 inch) tall, with finely divided, linear leaves. The flowers are delicate, and usually coloured pale blue and white, with five to ten petals. The fruit is a large and inflated capsule composed of three to seven united follicles, each containing numerous seed (Figure 1.3). The seed is used as a spice.
**Plant profile:**

Kingdom : Plantae  
Unranked : Angiosperms  
Unranked : Eudicots  
Division : Magnoliophyta  
Order : Ranunculales  
Family : Ranunculaceae  
Genus : Nigella  
Species : sativa  
Synonyms : *Nigella cretica* Mill.

**Traditional uses:**

Traditionally the seeds are used in several diseases. The seeds are considered as bitter, pungent, aromatic, appetizer, stimulant, diuretic, deodorant, digestive, constipating. They are used in ascites, cough, jaundice, hydrophobia, fever, paralysis, conjunctivitis, piles, skin diseases, anorexia, dyspepsia, flatulence, abdominal disorders, diarrhoea, dysentery, intrinsic haemorrhage and amenorrhea.

**Nutritional facts:**

*Nigella sativa* constitute a good alternative source of essential fatty acids compared with common vegetable oils and could contribute to the overall dietary intake. On the other hand, in terms of both quantity and quality, these seeds are potentially attractive source of protein, lipid and some common minerals that appear to have a very positive effect on human health (Ali et al., 2012).
**Phytochemical constituents:**

Millions of people in the Mediterranean region and on the Indian subcontinent use the oil from the seed of *Nigella sativa* daily as a natural protective and curative remedy. The seeds are very rich and diverse in chemical composition. They contain amino acids, proteins, carbohydrates, fixed and volatile oils (Khan, 1999). *Nigella sativa* seeds contain other ingredients, including nutritional components such as carbohydrates, fats, vitamins, mineral elements, and proteins, including eight of the nine essential amino acids (Bhatia *et al.*, 1972; Correa *et al.*, 1986; Jassir *et al.*, 1992; Omar *et al.*, 1999; Chun *et al.*, 2002).

Many of the pharmacological activities mentioned above have been attributed to quinone constituents in the seed. As early as 1956, Chopra *et al.* (1956) found that thymoquinone (TQ) is the main active constituent of the black seed. Mahfouz and El-Dakhakhny (1960) were the first to report on the isolation of ‘nigellone’ from *Nigella sativa* seed, using Girard’s reagent. Nigellone was later found to possess antihistaminic properties in relatively low concentrations (Mahfouz *et al.*, 1965). El-Dakhakhny (1963) was able to isolate the constitutive components of *Nigella sativa* seeds from its essential oil, among which TQ was later shown to be the main constituent of the volatile oil (Houghton *et al.*, 1995). In addition, El-Dakhakhny (1963) determined that the ‘nigellone’ isolated earlier was a dimer of TQ, which was later named dithymoquinone (TQ2). The latter compound was shown to be formed via photodimerization of TQ as a consequence of exposure to sunlight during separation and extraction of the quinones from the seed. El-Fatatry (1975) reported the isolation of thymohydroquinone (THQ) from *Nigella sativa* seed volatile oil. In another study (Aboutabl *et al.*, 1986), the chemical composition of the black seed of *Nigella sativa* was found to contain a fixed oil (30%) and a volatile oil (average 0.5%,
maximum 1.5%). The volatile oil was found to contain 54% TQ and many monoterpenes such as p-cymene and a-pinene, TQ2 and THQ.

The seeds of *Nigella sativa* have been subjected to a range of phytochemical investigations. They have been shown to contain more than 30% (w/w) of a fixed oil with 85% of total unsaturated fatty acid (Houghton *et al.*, 1995). The seeds also contain alkaloids of unknown pharmacological actions, such as nigellidine, nigellimine and nigellicine, saponins and crude fiber as well as minerals such as calcium, iron, sodium and potassium. Other constituents of the volatile oil, include thymol (TOH). Recently, the presence of TQ, TQ2 and TOH in *Nigella sativa* seed was confirmed using thin layer chromatography (TLC) and normal phase high-performance liquid chromatography (HPLC) methods (Abou-Basha *et al.*, 1995; Aboul-Enein and Abou Basha, 1995). The content of TQ in *Nigella sativa* seed, obtained from different origins, was measured by gas chromatography (GC) analysis and found to be in the range of 0.13–0.17% w/v of the oil (Houghton *et al.*, 1995). The seeds are also rich in proteins; when whole *Nigella sativa* seeds were fractionated using SDS-PAGE, they were found to contain a number of protein bands ranging from 10 to 94 kDa molecular mass (Haq *et al.*, 1999) (Figure 1.4).

An HPLC method for quantifying the putative pharmacologically active constituents (TQ, TQ2, THQ and TOH) in the *Nigella sativa* seed was recently described by Ghosheh *et al.* (1999). In this procedure, the four compounds mentioned were separated and quantified in commercial *Nigella sativa* seed with good resolution, reproducibility and sensitivity. Both heat and light are known to affect the levels of the constituents in the oil. Since various storage and manufacturing conditions are expected to make a difference in the amounts of the quinone constituents of the oil,
the analytical HPLC method described by Ghosheh et al. (1999) can be used to quantify the levels of the above constituents in the oil and seed extracts of *Nigella sativa* under different manufacturing conditions. The protocol is also useful as a quality control method for the determination of pharmacologically active quinones in *Nigella sativa* seed.

Using TLC, black seed was found to contain TQ and the terpenoid components carvacrol, t-anethole and 4-terpineol (Burits and Bucar, 2000). Differences were mainly restricted to the quantitative composition (Burits and Bucar, 2000). *Nigella sativa* seeds contain fixed oils and volatile oils, which are rich sources of quinones, unsaturated fatty acids, amino acids and proteins and contain traces of alkaloids and terpenoids. Most of the studies on the biological effects of *Nigella sativa* have dealt with its crude extracts in different solvents; however, some studies used its active principles. Among the components isolated from the volatile oil of *Nigella sativa*, TQ has been shown to be the principal active ingredient (Mahfouz and El-Dakhakhny, 1960) and thus is the most studied of all.

**Pharmacological properties:**

*Nigella sativa* has been shown to exert effect on various system including the respiratory, cardiovascular, gastric and uterine smooth muscle. The effect of intravenous administration of *Nigella sativa* was investigated on the respiratory system of the guinea pig (El-Tahir et al., 1993). The fatty and petroleum extracts of *Nigella sativa* seed shortened bleeding time and inhibited fibrinolytic activity in rabbits (Ghoneim et al., 1982). In a recent study, the crude extract of *Nigella sativa* seeds were found to exhibit spasmolytic and bronchodilator activities mediated
possibly through calcium channel blockade and this activity was concentrated in the organic fraction of the extract (Gilani et al., 2001).

Traditionally *Nigella sativa* plant has been used in many Middle Eastern countries as a natural remedy for diabetes. Significant reduction in blood glucose and cholesterol levels in humans following the use was reported by Bamosa et al. (1997). The oil of this plant has a great potential in the treatment of diabetic animals because of its combined (Al-Hader et al., 1993; Zaoui et al., 2002) and immunopotentiating properties (Haq et al., 1999). The ability of *Nigella sativa* to lower blood glucose concentrations was confirmed in streptozotocin diabetic rats following 2,4 and 6 weeks of the treatment (El-Dakhakhny et al., 2002). In many Arab counties *Nigella sativa* and its derived products are consumed abusively for traditional treatment of blood homeostasis abnormalities and as a treatment for dyslipidemia (Zaoui et al., 2002). Several studies support the use of *Nigella sativa* extract for treatment of thrombosis and dyslipidemia (Labhal et al., 1997; Enomoto et al., 2001; Zaoui et al., 2002). The purified components [2-(2-methoxypropyl)-5 methyl-1,4-benzenediol, thymol and carvacrol] obtained from the methanol-soluble portion of *Nigella sativa* showed inhibitory effects on arachidonic acid-induced platelets aggregation and blood coagulation. Some aromatic compounds present in the extract were found to be more potent than aspirin, which is well known as a remedy for thrombosis (Enomoto et al., 2001). In addition, an aqueous suspension of *Nigella sativa* seeds was found to decrease the serum total lipids and body weight in *Psammommys obesus* sand rat (Labhal et al., 1997). Evidence indicates that *Nigella sativa* has a protective role against gastric ulcers (El-Dakhakhny et al., 2000). A final physiological effects of *Nigella sativa* includes its potential as an antioxytocic agent (Aqel and Shaheen 1996).
**Antimicrobial and antiparasitic effects:**

Extracts of *Nigella sativa* have shown promising effects against bacteria, fungi, viruses, parasites and worms. *Nigella sativa* was found to have high antimicrobial effect against gram-positive microorganisms (El-Fatatry 1975). *Nigella sativa* seed extracts also inhibit the growth of *Escherichia coli*, *Bacillus subtilis* and *Streptococcus faecalis* (Saxena and Vyas, 1986). The antimicrobial activity of *Nigella sativa* against several species of pathogenic bacteria and yeast (Topozada et al., 1965; Hanafy and Hatem 1991). Crude extracts of *Nigella sativa* showed promising antimicrobial isolates with multiple resistances against antibiotics (Morsi, 2000). The antiparasitic action of *Nigella sativa* have been well documented by several researchers (Agarwal et al., 1979; Akhtar and Riffat 1991; Abdel-Salam et al., 1993; Mahmoud et al., 2002). The antiviral effects of *Nigella ativa* seeds were more potent than the action of Chinese traditional herbal medicine hochuekki – against murine cytomegalovirus (Hossain et al., 1999).

**Anticancer effects:**

Black seed preparations have been demonstrated to have significant antineoplastic activity against various tumour cells *in vitro* (Salomi et al., 1991, 1992; Swamy and Tan, 2000). The active principles of *Nigella sativa* showed 50% cytotoxicity against Ehrlich ascites carcinoma, Dalton’s Lymphoma ascites and sarcoma-180 cells at a concentration of 1.5, 3 and 1.5 µg/mL, respectively, with little activity against lymphocytes (Salomi et al., 1991). The ethyl acetate fraction of *Nigella sativa* seeds was later found to exhibit significant growth inhibition on a variety of cancer cell lines without inhibiting the growth of normal human endothelial cells (Swamy and Tan, 2000).
**Anti-inflammatory and immunomodulatory effects:**

*Nigella sativa* and its derived products have been traditionally used as a treatment for rheumatism, liver diseases and related inflammatory disorders. The effect of black seed on the immune system has been investigated by several researchers (Houghton *et al.*, 1995; Haq *et al.*, 1995; Ek-Dakhakhny *et al.*, 2000; Al-Ghamdi 2001). The ability of thymoquinone to modulate cytokines and enhance the immune system has been implicated as the main reason for its protective effect against schistosome egg infection in the liver (Mahmoud, 2002). *Nigella sativa* enhanced the production of IL-3 by human lymphocytes and had a stimulatory effect on macrophages (Haq *et al.*, 1995).

**Antioxidant and hepatoprotective effects:**

Health food stores sell *Nigella sativa* seeds as a natural remedy for a variety of complaints including liver diseases (Boulos, 1983). *Nigella sativa* is known to possess strong antioxidant activities (Aboul-Enein *et al.*, 1999; Nagi and Mansour 2000; Meral *et al.*, 2001; El-Dakhkhny *et al.*, 2002; Mahmoud *et al.*, 2002). *Nigella sativa* seed has been shown to inhibit lipid peroxidation in Ox brain phospholipid liposomes (Houghton *et al.*, 1995). Houghton (1995) also reported that the *Nigella sativa* had antioxidant effects greater than thymoquinone, the active constituent of *Nigella sativa*. Clinical and experimental investigations have shown that *Nigella sativa* has a protective effect against oxidative damage in isolated rat hepatocytes (Daba and Abdel Rahaman, 1998; Kanter *et al.*, 2003). In addition, it was suggested that *Nigella sativa* treatment increases the antioxidants defences activity in experimentally induced diabetic rabbits (Meral *et al.*, 2001).
SCOPE OF THE THESIS:

✓ This thesis embodies the study regarding the toxicity of Diethyl phthalate in liver and kidney of mice. Activities of liver marker enzymes, the extent of lipid peroxidation, alterations in ingredients enzymatic and non-enzymatic antioxidant contents systems, changes in levels of important constituents like proteins, DNA and RNA and other relevant biochemical parameters and histopathological examinations were taken into consideration in this study.

✓ As medicinal herb *Nigella sativa* is having long history of herbal usage, is selected as an antidote to mitigate diethyl phthalate-induced toxicities. The antioxidative potential of the plant extract has been ascertained using superoxide dismutase, nitrous oxide, hydroxyl and DPPH radical scavenging assay.

✓ The mitigatory effects of the plant extracts on DEP – induced toxicity were determined biochemically and histopathologically. The liver and kidney protecting property of all the tested compounds have been compared on the basis of their hepatoprotective/renoprotective index (H/R).

✓ The detailed study methodology followed and the results of these investigations are discussed in the following pages.