CHAPTER 1

INTRODUCTION

This chapter emphasize the significance and characterization of curcumin and its therapeutic effects, the toxicity effect of Dimethyl Hydrazine and Chlorpyrifos were also briefly reviewed pertaining to the present work.

1.1 CURCUMIN

Curcumin (diferuloylmethane), a yellow polyphenolic pigment derived from the rhizome of turmeric plant (*Curcuma longa*) shown in Figure 1.1. Turmeric has been used as traditional medicine in India and other Asian countries for more than thousands of years (Aggarwal et al 2007). The yellow pigment of turmeric consists of about 5% curcuminoids, which contains three principle compounds: curcumin (curcumin I, 77%), demethoxycurcumin (curcumin II, 17%), and bis-demethoxycurcumin (curcumin III, 3%) (Strimpakos & Sharma 2008, Goel et al 2008) Figure 1.2. The chemical name and properties of the curcuminoids are shown in Table 1.1 (Litwinienko & Ingold 2004, Scotter 2009). The presence of two methoxy groups in curcumin appears reddish orange color, with one methoxy group demethoxycurcumin appears orange-yellow in color and without methoxy group bis-demethoxycurcumin is yellow in color. These curcuminoids are hydrophobic, insoluble in water, and soluble in organic solvents like methanol, ethanol, dimethylsulfoxide, and acetone. Curcumin constitutes up to 5% of turmeric and gives it yellow color. The maximum absorption (\(\lambda_{\text{max}}\))
of curcumin in methanol is at 430 nm and 415-420 nm in acetone (Goel et al 2008).

Figure 1.1 Turmeric Plant (Curcuma longa)
Table 1.1 Chemical name and physical properties of curcuminoids

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Curcuminoids</th>
<th>Chemical name and formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Curcumin I</td>
<td>1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione, C$<em>{21}$H$</em>{20}$O$_{6}$</td>
<td>368 g/mol. pKa= 8.54</td>
</tr>
<tr>
<td>2</td>
<td>Curcumin II</td>
<td>1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-hepta-1,6-diene-3,5,-dione, C$<em>{20}$H$</em>{18}$O$_{5}$</td>
<td>338 g/mol. pKa= 9.30</td>
</tr>
<tr>
<td>3</td>
<td>Curcumin III</td>
<td>1,7-bis-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione, C$<em>{19}$H$</em>{16}$O$_{4}$</td>
<td>308 g/mol. pKa= 10.69</td>
</tr>
</tbody>
</table>
Curcumin is an active compound among curcuminoids which exists in two tautomeric forms, keto and enol shown in Figure 1.3. The molecular configuration of curcumin may exists in different forms as the pH of the solution varies (Figure 1.4), practically curcumin exists in enolic form in solution at alkaline pH, in neutral and acidic pH curcumin shows keto forms (Strimpakos & Shrama 2008). In keto form, curcumin act as a potent H-atom donor, because of the heptadienone linkage between the two methoxyphenol rings possess highly activated carbon atom and the carbon bonds (C-H) present on this carbon are weak due to delocalization of the unpaired electron on the adjacent oxygen atom. Whereas, at alkaline pH the enol form of the heptadienone linkage dominates and makes curcumin as an electron donor (Jovanovic et al 2001). Typically, curcumin is stable at acidic pH but unstable at neutral and basic pH. In contrast, tetrahydrocurcumin or THC, one of curcumin’s major metabolites is quite stable at neutral or basic pH and still retains antioxidant activities.

![Figure 1.3 The equilibrium of keto-enol formations of curcumin](image)

1.1.1 Applications of Curcumin

Curcumin exhibit several potential pharmaceutical properties like antioxidant, anti-inflammatory, antiviral, anticancer, anti-diabetic, anti-infectious, anti-proliferative, anti-angiogenic, antitoxic and wound healing properties (Ishita et al 2004). Studies also shows that curcumin exerts protective effect against hepato, nephro, rheumatoid arthritis, myocardial infarction and thrombosis suppression (Aggarwal & Harikumar 2009). The huge rationale of curcumin is due to its non-toxic nature even at a high dose of 12 g/day (Lao et al 2006). The unique structure of curcumin allows it to
interact with many protein/enzymes like albumin, glycoprotein amyloid protein, and chelates metal ions such as iron, copper and zinc. The physiological and biochemical actions of curcumin is shown in Figure 1.5.

![Diagram of curcumin's pharmacological properties](image)

**Figure 1.5 Pharmacological properties of curcumin (Saha et al 2012)**

### 1.1.2 Limitations of Curcumin

Although curcumin has diverse medicinal properties, it lacks in bioavailability (Singh 2007), such as low serum levels, limited tissue distribution, apparent rapid metabolism and short half-life (Anand et al 2007), which significantly limits its therapeutic activities in biological systems.
Practically oral administration of drug is considered to be potential and effective way. However, administration of drug in oral form must be completely dissolved in order to be absorbed easily by epithelial layer of the intestine. But curcumin being hydrophobic in nature shows very low solubility in water, which was measured to be 0.6 μg/ml (Kurien et al, 2007; Patel et al, 2009). The solubility of drugs below 1 μg/ml are inappropriate for conventional oral administration (Pouton, 2006). Studies on oral administration of curcumin showed that, it undergoes rapid metabolism in liver but the metabolites formed shows less or no pharmacological activity (Aggarwal et al, 2007; Aggarwal & Harikumar, 2009). Being therapeutically active agent, it is appropriate to develop new formulations methods for curcumin in order to increase its oral absorption which in turn enhances its therapeutic activity (Corson & Crews, 2007).

1.2 NANOFORMULATION OF CURCUMIN - A NEW APPROACH IN DRUG DELIVERY SYSTEM FOR POTENTIAL THERAPEUTIC APPLICATIONS

Nanoparticles (NPs), size ranging from 1 to 100 nm, which possess specific physical and chemical properties that can be accomplished for drug delivery (Malam et al, 2009). It is emerging with a lot of attention on development of nanoscale techniques and tools in multidisciplinary field, especially in medicine. Nanotechnology also opens up new development in drug delivery system in terms of diagnosis, treatment and prevention. Biodegradable polymers play a vital role in the fabrication of drug delivery systems. Due to potential application of biodegradable polymers, significant research is being carried out in stabilizing and controlling drug release, also encapsulated drug NPs improves the solubility, drug release and pharmacokinetics of drugs. The mechanism in uptake of drug nanoparticles by our body is depicted in the Figure 1.6.
In general, nanoparticles carrier materials are divided into synthetic biodegradable high molecular polymers (polyvinylalcohol, polylactic acid, etc.) and natural polymers (proteins, polysaccharides, etc.) (Wang & Hanou 2010). Nanoparticles in pharmaceutical applications have gained plenty of research attention during recent decades. Nanoparticulate delivery systems for curcumin are designed to enhance the absorption, cellular uptake, bioavailability and ultimate efficiency. Although, formulations of curcumin in nanoparticles, liposome, micelles and phospholipids complexes are being explored to decrease its hydrophobicity, increase solubility to enhance blood circulation time, and its permeability through membrane barriers. Among these options nanoformulations of curcumin (Nanocrystals, Nanoemulsions and polymeric nanoparticle encapsulated curcumin) found tremendous interest and growth in the recent past.

**Figure 1.6 Mechanism of Uptake of Nanoparticles**
The main drawback of these formulation techniques is that they produce particle size in a wide-range of distribution and only a few particles are produced in the nanometer range (Reverchon & Porta 1999). Particles in nanoscale performs more effective in therapeutic applications. Unlike the other formulations process used for nanosizing the drug, liquid antisolvent precipitation process delivers a wide range of advantages in controlling the size of poorly water soluble drugs.

1.2.1 Antisolvent Precipitation Process

Anti-solvent crystallization is the separation and purification method which is used as an effective way to prepare micro to nano-size drug particles. This technique produces crystals from solutions and controls the crystalline properties such as particle size and their morphology. The use of the antisolvent in crystallization reduces the solubility of a solute in the solution and to induce rapid crystallization. The physical and chemical properties of the antisolvent can alter the rate of mixing with the solutions and thereby affect the rate of nucleation and crystal growth of the crystallizing compounds. Additionally, parameters of crystallization experiments strongly influence the mechanism of particle formation and direct the formation of crystal size and its distribution. Generally, the antisolvent contains hydrophilic stabilizer (i.e. Surfactants) which is absorbed on the crystal surface to inhibit crystal growth. Hydroxypropyl methylcellulose (HPMC) is non-toxic and has hydrophilic property which is widely used as thickening, emulsifying and stabilizing agent in food and pharmaceutical formulations. However, this technique involves some basic problems, i.e. Trouble in maintaining the size of the particles produced after precipitation, usually with a rapid growth rate which leads to a broad particle size distribution (PSD). The technique implicates dissolution, followed by precipitation and then drying. Thus, the machine-driven energy input is minimized but the resulting
nanoparticles might be crystalline or amorphous and also dependent on the process conditions. Even if the particles are crystalline, the crystal growth rate must be controlled to limit the particle size. Also, poor mixing during antisolvent process leads to accidental zones of local supersaturation and, therefore, aggregation of particles occurs. In contrast, ultrasound proves to be a feasible mixing method to provide uniform conditions throughout the container during antisolvent process (Figure 1.7).

![Figure 1.7 Antisolvent Precipitation method](image)

1.2.1.1 Ultrasound Assisted Antisolvent Crystallization

Ultrasound has widely been used in crystallization. The sonocrystallization (crystallization using ultrasound) offers unique features which distinguish it from precipitation without ultrasound. Main features of sonocrystallization are,

(a) Fast primary nucleation; fairly constant throughout the sonicated volume,

(b) Early initiation of nucleation for materials which are difficult to nucleate,
(c) Initiation of secondary nucleation,

(d) Uniform, pure and smaller size crystal production.

It has been also reported that in ultrasound assisted precipitation of nanoparticles, ultrasound mainly changes mixing efficiency, nucleation, crystal growth, particle size, agglomeration and to certain extent supersaturation. It has been reported that ultrasound converts the less ordered form to ordered form of lattice structure such as amorphous to crystal form. Moreover, ultrasound has been found to assist in reordering of surfactant molecules at solid–liquid interface or increasing adsorption of polymer and decreasing surface energy leading to reduced particle size. Despite of a wide use of ultrasound in cooling crystallization, it has been rarely used in liquid anti-solvent (LAS) precipitation process.

The use of ultrasonic waves in crystallization has been increased at laboratory scale for;

(i) Rapid and uniform nucleation throughout the sonicated volume leads to smaller and uniform-sized particles, and

(ii) Reduction of agglomeration of particles and controlling the number of nuclei.

The mean particle size and its distribution can be effectively controlled by adjusting ultrasound variables such as the power intensity and ultrasonic time during crystallization. When ultrasound waves propagate through a liquid medium, its influence will initiate an important phenomenon known as cavitation. The formations of cavitational bubbles are occurring during the negative pressure period of the sound wave. These bubbles will grow up to their resonance size and then they implode, generating a localized hot spot with a high temperature and pressure including the release of powerful shock waves. The power of ultrasound and cavitation phenomena
will initiate the nucleation and thereby forming crystal growth in a crystallization process. The use of ultrasound may also influence the solubility and thereby the super solubility. It can also alter the crystal habit as the ultrasound can increase or decrease the growth rate of certain crystal faces. Restricted hot spots may influence the crystal lattice and have some effect on the crystal habit change due to abrasion.

Thus, Liquid antisolvent process result in producing various size and morphology of particle (Lonare & Patel 2013). Bhawana et al 2011, studied that the free nanocurcumin prepared by wet-milling technique exhibit high stability and also disrupts cell organelles of microorganisms by anchoring the cell wall. It was also reported that the activity of curcumin was enhanced by high solubility rate and reduction in size. Since ultrasound helps in forming controllable crystal particles it can be implemented in preparing nanoparticles, thereby reducing its size and shape. Therefore, by combining ultrasound process with LAS may extend the study on physicochemical characterization, potential effect and evaluation of nanoparticle-behavior during different stages of preparation, surface modification and stabilization in different environments. Increasing knowledge about these kinds of systems extends the understanding and facilitates the prediction of the behavior of similar systems in man.

1.3 **OXIDATIVE STRESS AND ANTIOXIDANTS PROPERTIES**

Oxidative stress is a state in which the level of free radicals or toxic reactive oxygen intermediates (ROI) overcomes the antioxidant defense mechanism of the host. These free radicals or toxic reactive oxygen intermediates are mainly released by phagocytic cells during cell injury and inflammation of tissues. The oxidative stress may cause secondary damage to cells by hindering cellular death and inflammation. In general, increase in the formation of hydrogen peroxide \( \text{H}_2\text{O}_2 \) and oxygen-derived free radicals is a
sign of oxidative stress (Van Houten 2006). Many diseases like diabetes, hypertension, heart failure, Parkinson’s, Alzheimer’s, lung, liver and neurodegenerative diseases are related with Oxidative stress. Oxidative homeostasis is the process that maintains our body’s free radical (oxidants) and antioxidants production. Thus, partially reduced form of O₂ including both the radical and non-radical are termed as Reactive oxygen species (ROS) or ROI (Imlay 2003, Semenza 2007). An imbalance in normal physiological stability increases the level of production of ROS that overcomes the endogenous antioxidants present in the cell which may affect the integrity of the cell and cellular structure by inducing the oxidative degradation in cell molecules like DNA, proteins and lipids (Imlay 2003, Evans et al 2003). The most important reactive oxygen and nitrogen species are H₂O₂, superoxide radical (O₂⁻), hydroxyl radical (HO⁻) and nitric acid radical (NO), peroxynitrite (ONOO⁻) (Jenner 2003). The concept of oxidative stress is shown in the Figure 1.8.

Figure 1.8 Concept of Oxidative stress (Fujii et al 2003)
In general, cells have a variety of defense mechanisms that seize free radicals to prevent or reduce the level of intracellular damage and ameliorate the harmful effects of ROS (Deavall et al 2012). Antioxidants are of three major types, the first type of defense against free radicals consists of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), non-enzymatic antioxidants like glutathione (GSH), protein-SH, ascorbic acid, dietary antioxidants and minerals like Se, Mn, Cu and Zn. The second type of antioxidants are polyphenolic substances majorly derived from plants. The third type involves the enzymes that repair DNA damage and proteins. The activity of first type of antioxidant enzymes alters immediately against the prevention of free radicals. SOD primarily acts as quencher of superoxide, CAT catalyses decomposition of H$_2$O$_2$ to water and oxygen. GPx contains selenium that catalyses the reduction of H$_2$O$_2$ and lipid hydro peroxide to water using reduced glutathione as substrate. Glutathione (GSH), vitamin C (ascorbic acid), uric acid, thiols, albumin, vitamin E ($\alpha$-tocopherol), carotenoids, flavonoids are second line of defense antioxidants. $\beta$-carotene and Vitamin E are an excellent scavenger of singlet oxygen ($1O^2$) and peroxyl radical intermediates in lipid peroxidation respectively. Flavonoids, phenolic compounds in plants, which inhibit lipid peroxidation. The third type of antioxidants is complex form of enzymes like lipase, protease and reductase. These antioxidants repair DNA and protein damage and also stop proliferation of peroxyl lipid radical. Minerals like Cu and Zn exerts its antioxidant activity through the cytosolic superoxide dismutase and induces normal growth. Thus, it is clearly indicates that antioxidants intermediates free radicals which induces oxidative stress (Singh et al 2009, Halliwell 2006). So, in the present study, we evaluated the level of serum lipid peroxidation (LPO) and antioxidant defense enzymes like SOD, CAT, GPx and GSH to observe the alteration in oxidant during induced toxicity both \textit{in vitro} and \textit{in vivo}. 
1.4 OXIDATIVE STRESS AND INFLAMMATION

Inflammation is a physiological process that repairs tissues in response to endogenous or exogenous antagonisms. Inflammation leads to oxidative stress and vice versa. However, a chronic state of inflammation may have detrimental consequences. High levels of interleukin (IL)-6, IL-1, tumor necrosis factor-α (TNF-α), and C-reactive protein are associated with increased risk of morbidity and mortality. In particular, studies have indicated TNF-α and IL-6 levels as markers of susceptibility (Michaud et al 2013). The inflammatory caused by environment pollution leads to the generation of oxygen and nitrogen free radicals as well as pro-inflammatory cytokines. The oxidative stress results in the release of pro-inflammatory mediators or cytokines, intercellular chemicals that alerts the immune system when an infection is present (Brown et al 2004, Long et al 2004). Cytokines are intracellular signalling molecules that mediate many protective physiological functions such as increasing the blood circulation and engaging leukocytes (white blood cells) at the site of an infection. Cytokines can also induce potentially harmful responses such as prolonged tissue inflammation and development of fibrosis in response to toxicants (Nelson & Martin 2000, Thèze 1999). Cytokines are regulators of host responses to infection, immune responses and inflammation; some cytokines act to make disease worse (pro-inflammatory), whereas others serve to reduce inflammation and promote healing effects (anti-inflammatory) (Dinarello 2000).

1.4.1 Markers of inflammation

Pro-inflammatory cytokines and oxidative stress play a vital role in the early pathophysiological events of the disease. Interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) are the major mediators which initiate and propagate the early inflammatory response syndrome (Escobar et al 2009). They can stimulate the production of acute-phase proteins by the liver,
mobilize neutrophils to the site of insult, direct the hypothalamus for a fever response, and increase the adhesion molecules on the vascular epithelium. TNF is a potent cytokine with multiple immunologic and inflammatory effects, associated with rheumatoid arthritis, osteoarthritis and inflammatory bowel disease. Other cytokines seen in inflammation include interleukin-1 receptor antagonist (IL-1ra), IL-8, IL-1, IL-18 and interferon-γ (Margioris 2009, Wright et al 2006). Hence, monitoring the levels of cytokines may reflect the toxicity and the antioxidant property of any compound. Therefore, the release of pro-inflammatory and anti-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α and IL-4 were measured as a biological endpoints in this study.

1.5 OXIDATIVE STRESS IN CANCER

Cancer is a multistage process defined by three stages: initiation, promotion, and progression (Guyton & Kensler 1993). Oxidative stress interacts with all three stages of this process. During the initiation stage, ROS may produce DNA damage by introducing gene mutations and structural changes of the DNA. In the promotion stage, ROS can initiate abnormal gene expression, blockage of cell to cell communication, thus causing an increase in cell proliferation or a decrease in apoptosis of the initiated cells. Finally, oxidative stress also involves in the progression stage of the cancer process by altering DNA in the initiated cells (Klaunig et al 1998). ROS such as H$_2$O$_2$ and superoxide anion induce mitogenesis and cell proliferation has been revealed in several mammalian cell types. However, incoherently high concentrations of ROS can trigger apoptotic or necrotic cell death (Chang et al 2007, Halliwell 2007).

Since, Metabolism of toxic components such as drugs, carcinogens, mutagens and pollutants takes place in liver, the major detoxifying organ (Vyskočilová et al 2013). It is of great concern in day to day life in human
health where countries produce large amount environmental pollution. Liver cancer is being third foremost cause of death worldwide over the last decades. Since millions of individuals were diagnosed for liver cancer every year (Jemal et al 2011). In the mechanism of liver, a carcinogen causes tissue damage that (presumably acting via Toll-like receptors, TLR) activates the MyD88–NF-κB signalling pathway in Kupffer cells. These cells, a type of macrophage, produce interleukin-6 (IL-6), which in turn promotes inflammation, tissue damage, cell proliferation and tumour formation. Oestrogens interfere with NF-κB activity and IL-6 production (Figure 1.9). Subsequently, hepatocellular carcinoma has become great concern among the researchers in order to develop a potential drug to minimize its cause.

Figure 1.9 Mechanism of Liver Cancer
Currently chemotherapy is used as an agent for cancer treatment (El-Serag 2011). Though, chemotherapy cures the cancer it has some limitations causing severe side effects to normal tissues (Armstrong 2002, Markman 2008). There has been increased focus on understanding the chemopreventive and chemotherapeutic potential of curcumin against a variety of cancers. Several studies have been performed in cancerous cells as well as in animal models to show the anti-cancer effect of curcumin. As represented in Figure 1.10, curcumin arrests different phases of cell cycle in order to inhibit the proliferation of cancer cells derived from breast, prostate, colon, liver, kidney, blood and skin (Karunagaran et al 2005, Hatcher et al 2008).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Arrested cell cycle phase</th>
<th>Cell cycle-related mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60 (Human acute myeloid leukemia)</td>
<td>G2/M first, then in G0/G1 phase</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>Human multiple myeloma cells</td>
<td>G1/S</td>
<td>Down regulation of cyclin D1, inhibition of IKK and NF-κB</td>
</tr>
<tr>
<td>CA46 cells (human Burkitt's lymphoma)</td>
<td>G0/G1 or G2/M and S</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>MDA 686LN (human head and neck squamous cell carcinoma)</td>
<td>G1/S</td>
<td>Down regulation of cyclin D1, inhibition of IKK and NF-κB</td>
</tr>
<tr>
<td>HCl-116 (human colon cancer)</td>
<td>G2/M</td>
<td>P53 and p21-independent Down regulation of cyclin D and E but not B Activation of cdc2</td>
</tr>
<tr>
<td>HT-29 and HCT-15 (human colon adenocarcinoma)</td>
<td>G2/M</td>
<td>Prostaglandin independent</td>
</tr>
<tr>
<td>COLO205 (colorectal carcinoma)</td>
<td>G1</td>
<td>Inhibition of Ca2+ dependent endonuclease, reduction of p53 gene expression</td>
</tr>
<tr>
<td>Love (colon cancer)</td>
<td>S, G2/M</td>
<td>inhibition of DNA synthesis</td>
</tr>
<tr>
<td>HUVEC (human umbilical vein endothelial cells)</td>
<td>S</td>
<td>inhibition of DNA synthesis</td>
</tr>
<tr>
<td>ECV304 (immortalized human umbilical vein endothelial cells)</td>
<td>G0/G1 and/or G2/M</td>
<td>up-regulation of p21WAF1/CIP1, p27KIP1, and p53</td>
</tr>
<tr>
<td>A7r5 (Rat aortic smooth muscle cell line)</td>
<td>G0/G1 and S</td>
<td>inhibition of protein tyrosine kinase activity, protein kinase C activity, c-mye mRNA expression and bcl-2 mRNA expression</td>
</tr>
<tr>
<td>PCC4 (mouse embryonal carcinoma cells)</td>
<td>G1</td>
<td>differentiation characterized by increase of nuclear/cytoplasmic ratio</td>
</tr>
<tr>
<td>MCF-7/TH (multidrug-resistant human breast carcinoma)</td>
<td>G2M and subG0/G1</td>
<td>Reduction in the expression of Ki67, PCNA and p53 mRNAs</td>
</tr>
<tr>
<td>Human breast cancer cells</td>
<td>S and G2/M</td>
<td>inhibition of ornithine decarboxylase activity</td>
</tr>
</tbody>
</table>

Figure 1.10 Effects of Curcumin on the Cell Cycle (Hatcher et al 2008)
It has been reported to induce apoptosis in several malignancies such as leukemia, melanoma, breast, prostate, lung, colon, renal and ovarian carcinomas by interference of multiple signaling pathways and down-regulation of transcription factors such as nuclear factor kappa B (NF-κB). Recently, promising results have been seen during the clinical trials of curcumin in patients with colorectal and pancreatic cancers which shows its potential future use as a therapeutic agent (Dhillon et al 2008, Sharma et al 2004). Curcumin exhibits chemopreventive and therapeutic response against cancer by targeting a variety of pathways that are associated with tumor initiation and progression. ROS: reactive oxygen species; RNS: reactive nitrogen species; NFkB: nuclear factor kappa B; Nrf2: (NF-E2)-related factor 2; HO-1: heme oxygenase-1; GST glutathione S-transferase; GR: glutathione reductase. COX: cyclooxygenase; MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor (Figure 1.11).

Figure 1.11 Targets associated with the Anti-cancer activity of Curcumin
1.6 OXIDATIVE STRESS IN ENVIRONMENTAL POLLUTANTS

1.6.1 Dimethyl hydrazine

1,2-dimethyl hydrazine or DMH is present in the rocket fuel and also reported to be a metabolic product of RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine, an explosive widely used for military purpose. It is a toxic environmental pollutant and a procarcinogen (Saini et al 2012, Lima et al 2005). Humans are exposed to DMH and other hydrazines through environment. DMH is a potent colon carcinogen induces colorectal tumors (Newell & Heddle 2004, Saini et al 2009). Fiala (1975) has reported that active metabolite of DMH is metabolized in liver to form azoxymethane and methylazoxymethanol, which is excreted through bile to cause carcinogenic effect and the diazonium ion present elicits an oxidative stress. The induction of oxidative stress results in inflammation and tumour which leads to promutagenic effect. Therefore, DMH treated rat models can be used as a reliable tool to assess the antioxidant and antitoxic efficacy of natural and synthetic drugs (Hamiza et al 2012, Devasena & Menon 2007). The structure of DMH is shown in the Figure 1.12.

![Figure 1.12 Structure of 1, 2-Dimethylhydrazine](image)

Figure 1.12 Structure of 1, 2-Dimethylhydrazine
1.6.2 Pesticide

Pesticides have undoubtedly benefited human health sector in increasing agricultural yield by controlling pests and also by protecting from insect-borne diseases. But these have some grave drawbacks also, such as potential toxicities to humans and other non-target animals. In epidemiological studies, an increased risk of cancer has been linked with both residential and occupational exposures to pesticides. The presence of pesticide residues is a concern for consumers because pesticides are known to have potential harmful effects to other non-targeted organisms than pests and diseases. The major concerns are their toxic effects such as interfering with the reproductive systems and foetal development as well as their capacity to cause cancer and other physiological changes in human health (Gilden et al, 2010). Some of the pesticides are persistent and therefore remain in the body causing long term exposure which stimulates oxidative stress.

1.6.2.1 Chlorpyrifos

Chlorpyrifos (CPF) belongs to the class of organophosphate (OP) compounds which are widely used as pesticides. One of the key mechanisms of CPF effect is its ability to inhibit cholinesterases. Unfortunately, there is the growing amount of data showing that CPF is much more dangerous for the animal and human organism, especially for children than previously thought. Nervous system is the most vulnerable target of this toxicant. Research works conducted in recent years have shown the existence of other cholinesterase independent mechanisms of influence of CPF on a living organism. It is very actual to find the ways to explore the dermal toxicity of this compound and determine its features. Chlorpyrifos are known to inhibit acetylcholinesterase and pseudocholinesterase in target tissues resulting in
accumulation of acetylcholine in synaptic junctions (Shenouda et al 2009). This excessive accumulation of acetylcholine in synapses leads to activation of cholinergic, muscarinic and nicotinic receptors and hyperactivity in the cholinergic pathways. However, AChE inhibition does not explain all the symptoms of OP intoxication. Other systems that may be affected by OP exposure are the immune system (Galloway & Handy 2003). Hematological system (Jintana et al 2009), and reproductive system (Farag et al 2000 and Uzun et al 2009). OP pesticides have demonstrated genotoxic, alkylating and clastogenic properties; thus they are potentially mutagenic and clastogenic (Mehta et al 2008). More recently, it has been postulated that OP pesticides produce oxidative stress in different tissues through the formation of reactive oxygen species (ROS) (Akhgari et al 2003, Abdollahi et al 2004, Mehta et al 2009). The chemical structure of chlorpyrifos is shown in Figure 1.13.

![Figure 1.13 Structure of Chlorpyrifos](image)

1.6.3 Exposure of pesticides

Pesticides have been hazardous by affecting skin, liver, lung, kidney and nervous system of our body. It causes carcinogenicity, reproductive,
oncogenic, teratogenicity and mutagenic effects (Galloway & Handy 2003, Kaur et al 1997, El Dib et al 1996). Several pesticides are being used largely in agriculture, industry and domestic, especially chlorpyrifos, an organophosphate pesticide have been utilized extensively. Several reports are available proving that organophosphates are the major agent of self-poisoning, with increased fatality rates. The prolonged use of pesticides in protection of plants may lead to the significant effect on dermal exposure and causes severe impact on genotoxicity, cardiotoxicity and cholinesterase activities (Swamy et al 1992, Schulze-Rosario & Loosli 1994, Bhunya & Jena 1993). Oxidative stress has been reported as a possible mechanism of pesticide-induced toxicity in human (Banerjee et al 2001). It may lead to cell injury and malfunction through free-radical mediated damage of vital molecules (Sahnoun et al 1997). Lipid peroxidation is one of the most biologically important free radical reactions. If unopposed with an efficient local anti-oxidative defense system, peroxidative injury to plasma phospholipids may lead to severe cell damage (Nair et al 2007). It has been reported that DNA is also a major target of constant oxidative damage by endogenous oxidants (Jones 2008).

Meanwhile, a human skin may be exposed to hazardous chemicals specifically through:

- direct contact with polluted surfaces,
- aerosols deposition,
- Physical agents such as temperatures and radiation (UV radiation).
- Mechanical strain includes scrapes, cuts and bruises.
• Biological mediators include parasites, microorganisms, plants and other animal resources.

Studies have also showed that absorption of chemicals through the skin can occur without being noticed by the worker, and in some cases, may represent the most significant exposure pathway. Many commonly used chemicals in the workplace could potentially result in systemic toxicity if they penetrate through the skin (i.e. pesticides, organic solvents). These chemicals enter the blood stream and cause health problems away from the site of entry. The effect of chemical toxicity has been represented in Figure 1.14.

Figure 1.14 Toxicity effect of chemical exposure in human organs
The rate of dermal absorption depends largely on the outer layer of the skin called the stratum corneum (SC). The SC serves an important barrier function by keeping molecules from passing into and out of the skin, thus protecting the lower layers of skin. The extent of absorption is dependent on the following factors:

- Skin integrity (damaged vs intact)
- Physical and chemical properties of the harmful substance
- Concentration of a chemical
- Duration of exposure
- The surface area of skin exposed

It has been stated that skin absorption occurs through diffusion. The mechanisms by which chemicals diffuse dermally have been proposed:

1. Intercellular lipid pathway, where chemicals penetrate through these lipid-filled intercellular spaces through diffusion (Figure 1.15a).
2. Transcellular permeation, chemicals to be absorbed through the skin by cell-to-cell, permeation (Figure 1.15b).
3. Through the appendages, chemical diffuse through the skin appendages like hair follicles (Figure 1.15c).
Figure 1.15 (a, b, c) Mechanism of absorption of chemicals through skin
Pesticides can be toxic by ingestion, dermal exposure, inhalation, or ocular exposure. Of all these, dermal exposure of pesticide causes inflammation in skin cells and it is the second major source of risk assessment. Exposure profile can be described as

(i) Time period of exposure and sources

(ii) Impact of variability

(iii) Transport of pesticides and pathways of exposure

Pesticides absorbed on the outer surface of the skin enter into inner surface or into the body, finally enters into the blood stream causes severe health problems and its major action is away from the site of entry. Feldmann & Maibach (1974) have studied the penetration of 12 radio labelled pesticides and the skin absorbed herbicides in male volunteers, by topical application maximum of 22% of pesticides caused damage to the skin cells. So, it may be essential to identify the cytotoxic effect of pesticides on cell proliferation under \textit{in vitro} conditions in human cell lines.

Despite many toxicity assessment of pesticides exposure in various animal models both \textit{in vitro} and \textit{in vivo}, a very limited research is been carried out in ameliorating the toxicity effect. Hence, there is a need, for rapid and short-term \textit{in vitro} and \textit{in vivo} bioassays to screen the toxicity of pesticides using biochemical techniques in order to identify the cellular and molecular mechanisms involved in the toxicity, to design a drug which must act precisely against the toxicity induced by pesticides. The study may give astuteness about the toxicity of pesticides and delivers extensive knowledge regarding the environmentally relevant concentration causes toxic effects on human.
1.7 RATIONALE AND NOVELTY OF THE STUDY

Since the activity of drug nanoparticles depends on size, shape and surface charge it is essential to design the drug safely and efficiently. Perhaps, liquid antisolvent process helps in preparing size controllable and shape modified drugs without any carrier molecules. These type of drugs requires further examination, as the size and shape of the drugs changes the property may also change consistently. Several in vitro and in vivo studies were conducted on the potential effect of drug carrier nanoparticles, but less or inadequate study on non-carrier drugs were shown. Thus, sufficient studies have to be perform in order to understand the activity of those structurally modified drugs. This interest encouraged us to investigate the shape modified curcumin nanorods (CNRs) prepared by ultrasound mediated liquid antisolvent precipitation process.

Hence, the present study holds novelty in the synthesis of curcumin nanoparticles using simple and less time consumption protocol, surface modification, carrier-free and analyzing its therapeutic effect in in vitro and in vivo models. The in vitro characterization of CNRs was evaluated for anticancer activity on hepatocellular carcinoma (HepG2) cells and amelioration activity on HaCaT (human keratinocyte) cells. The in vivo study of CNRs were investigated, for circulatory and hepatoprotective activity against DMH and the dermal toxicity induced by chlorpyrifos.

1.8 AIM AND OBJECTIVE OF THE THESIS

- The aim of our study is to standardize a protocol to synthesize curcumin nanoparticles (nanocurcumin) using antisolvent liquid precipitation method.
To characterize the curcumin nanoparticles for its size, morphology, molecular transformation, surface charge, thermal stability using various nanoparticles characterization methods.

To investigate the therapeutic applications of nanocurcumin by analysing the

- Anticancer activity \textit{in vitro} using HepG2 cells
- Antioxidant and antitoxic activity \textit{in vitro} using HaCaT cells.
- Circulatory and hepatotoxicity induced by Dimethyl hydrazine induced in wistar albino rats.
- Antioxidant activity against pesticide induced dermal toxicity in wistar albino rats.