Introduction

A drug is a chemical substance used in the treatment, cure, prevention or diagnosis of disease and also used to enhance physical or mental well-being. Drugs are classified in various groups, on the basis of pharmacological properties, mode of action, chemical properties, route of administration and therapeutic effect. According to the World Health Organization (WHO) essential medicines are classified into following categories:

A sampling of classes of medicine includes: antipyretics, analgesics, antimalarial drugs, antibiotics, anticancer, antiseptics, mood stabilizers, hormone replacements, oral contraceptives, stimulants etc.

Anticancer drugs are used to control the growth of cancerous cells. Cancer is one of the most dreadful diseases and is the second leading cause of death in the United States (Jemal et al., 2006). It is a disease characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). Cancer can be treated by surgery, radiation and chemotherapies. However, the choice of treatment depends on the disease state. Localized tumors may be treated by surgery, but advanced cancers need radiation or chemotherapy. In spite of the availability of a wide range of therapies, achieving tumor selectivity is still a challenge.

1.1. Chemotherapy

Chemotherapy is the treatment of cancer with one or more anti-neoplastic drugs. The anti-neoplastic agents can be used along with other treatments, such as surgery or radiation therapy. Chemotherapeutic drugs act by killing cells that divide rapidly as the main properties of cancerous cells. Furthermore, chemotherapy can harm the cells that divide rapidly under normal conditions. The first anti-cancer agent (arsphenamine) discovered in 1909 and used to treat syphilis (Nichols and Walker, 1923).
During the world-war I, mustard gas was discovered to be a potent agent for suppressor of haematopoiesis (Krumbhaar and Krumbhaar, 1919). It was reasoned that an agent damaged the white blood cells might have a similar effect on cancerous cells. Therefore, in December 1942, several patients with advanced lymphomas were treated (Gilman, 1963). However, the first chemotherapy drug developed from this line of research was mustine. Since then, many other drugs have been developed to treat cancer, although the principles of chemotherapy discovered by the researchers still applying (Hodgson, 2008).

1.2. Alkylating Agents

The decisions on the use of chemotherapeutic agents depend on the type of cancer and also stage of malignancy. However, chemotherapeutic agents can be grouped by mechanism of action into the mitotic inhibitors, alkylating agents, antineoplastic, antibiotics, antimetabolites and hormonal agents (Cheng et al., 2013). Among these agents, alkylating agents are the oldest group of chemotherapeutics, used today. The covalent bonds between alkylating agents and biological molecules, in particular DNA, could disturb the protein synthesis process and also cellular division.

The primary cause, for the anti-cancer properties of alkylating agents is the ability of them to have the covalent binds to DNA. The alkylating agents are able to either bind twice to one strand (intra-strand crosslink) or may bind once to both strands (inter-strand crosslink) of DNA. This leads to a form of programmed cell death called apoptosis. In the Anatomical Therapeutic Chemical Classification System, alkylating agents are classified under L01A. Many of the agents are known as "Classical alkylating agents". These include true alkyl groups and have been known for a longer time than some of the other alkylating agents, e.g. cyclophosphamide, melphalan, chlorambucil etc. (McClean et al., 1999). They destroy proliferating cancer cells by adding an alkyl group to DNA molecule and preventing its replication.
1.3. Cyclophosphamide

Cyclophosphamide (trade names: Endoxan, Cytoxan, Neosar, Procytox, Revimmune) also known as cytophosphane, is a nitrogen mustard alkylating agent (Figure 1.1), from the oxazaphosphinans group (Takimoto and Calvo, 2008). An alkylating agent adds an alkyl group (C\textsubscript{n}H\textsubscript{2n+1}) to DNA. It attaches the alkyl group to the guanine base of DNA, at 7 nitrogen atom of the imidazole ring. This interferes with DNA replication by forming intrastrand and interstrand DNA crosslinks. Cyclophosphamide (CPA) is frequently used as an antineoplastic drug. The anticancer drug CPA is widely used for different forms of cancer. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas and rheumatic arthritis and in preparation for bone marrow transplantation (Goldberg\textit{ et al.}, 1986; Dollery, 1999).

![2D structure](image1.png)

![3D view](image2.png)

\textbf{Figure 1.1:} The anticancer drug: Cyclophosphamide (a) 2D structure; (b) 3D view (Black- Carbon; Red- Oxygen; Blue- Nitrogen; White- Hydrogen, Orange- Phosphorus and Green- Chlorine)
1.4. Physical and Chemical Properties of Cyclophosphamide

The properties of CPA are listed in Table 1.1.

Table 1.1: Properties of CPA

<table>
<thead>
<tr>
<th>Properties</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systematic (IUPAC) name</td>
<td>(RS)-N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide</td>
</tr>
<tr>
<td>Appearance</td>
<td>Fine white crystalline powder. Odorless with a slightly bitter taste.</td>
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<tr>
<td>Bioavailability</td>
<td>&gt;75% (oral)</td>
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<tr>
<td>Protein binding</td>
<td>&gt; 60S</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Half-life</td>
<td>3-12 hours</td>
</tr>
<tr>
<td>Excretion</td>
<td>Renal</td>
</tr>
<tr>
<td>Formula</td>
<td>C_7H_{15}Cl_2N_2O_2P</td>
</tr>
<tr>
<td>Melting point</td>
<td>2 ºC (36 ºF)</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>261.09</td>
</tr>
<tr>
<td>Density</td>
<td>1.479 g/cm³</td>
</tr>
<tr>
<td>Boiling point</td>
<td>336 ºC</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in chloroform, dioxane and glycols; slightly soluble in benzene, carbon tetrachloride, water, very slightly soluble in ether and acetone.</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td>0.63</td>
</tr>
<tr>
<td>PKa</td>
<td>4.5-6.5</td>
</tr>
<tr>
<td>Stability</td>
<td>Hydrolysis occurs at temperatures above 30 ºC, with removal of chlorine atoms. Sensitive to oxidation, moisture and light.</td>
</tr>
</tbody>
</table>
1.5. Cyclophosphamide: Mechanism of Action and Side Effects

CPA requires metabolic activation by hepatic microsomal cytochrome P450 mixed function oxidase system for both its therapeutic and its toxicological actions (Smith and Kehrer, 1991; Lindley et al., 2002). Metabolic activation through the predominant pathway (4-hydroxylation), yields 4-hydroxycyclophosphamide that exists in equilibrium with aldophosphamide, which degrades by β-elimination to form the DNA cross-linking agent, phosphoramidate mustard (PM) and an equimolar amount of the toxic byproduct, acrolein (Lindley et al., 2002; Pass et al., 2005). PM brings about interstrand cross-links between opposite DNA strands and hampers the replication and transcription process that characterizes the clinical activity of CPA represented in Figure 1.2 (Paolo et al., 2004).
Figure 1.2: Metabolism of cyclophosphamide.
Hence, the therapeutic effect of CPA is attributed to PM, while acrolein is associated with unwanted side effects (Colvin, 1999). Bioconversion of CPA to these metabolites leads to the formation of high levels of reactive oxygen species (ROS), which result in decreased antioxidative capacity (Stankiewicz et al., 2002). It is well known that excessive production of ROS could culminate in oxidative stress (Scherz-Shouval and Elazar, 2007). Mounting evidences suggest that oxidative stress plays a predominant etiological role in CPA-induced toxicity (Stankiewicz et al., 2002; Manda and Bhatia, 2003; Selvakumar et al., 2005).

Upon the administration of CPA, drug distributed throughout the body and interacts with both cancerous and healthy tissues. Along with inducing apoptosis in cancerous cells, CPA undergoes many non-selective reactions with a variety of bio-molecules, such as proteins and phospholipids. Despite the success of the drug, CPA has several severe side effects such as hepatotoxicity, nephrotoxicity and reproductive toxicity (Shulman et al., 1980; Bacon and Rosenberg, 1982; Snover et al., 1989).

(i) Hepatotoxicity

CPA is a widely prescribed non-cell-cycle-specific antineoplastic drug which is known to cause toxic effects including hepatotoxicity (DeLeve, 1996). The damage to the structure of liver after CPA treatment is commonly assessed by the determination of serum aminotransferases (SGOT and SGPT) activities (Ishak and Zimmerman, 1995). Oxidative stress plays an important role in CPA-induced hepatotoxicity (Fasihi et al., 2012). Ficus hispida, sequalene etc. protected oxidative damage in liver and also enzymatic antioxidants have the important protective responses against CPA toxicity in the liver of mice (Senthilkumar et al., 2006; Shanmugarajan et al., 2008).

(ii) Nephrotoxicity

Chemotherapeutic agents are capable of causing nephrotoxicity (Rossi, 1997; McDonald et al., 1991). The acute renal toxicity is the most important side effect of CPA, even following a single dose (Lavin and Koss, 1971). There is pharmacological evidence that the breakdown of CPA into biologically active alkylating compounds
takes place principally in the liver (Brock and Hohorst, 1967). Therefore, the changes observed in epithelial cells of the proximal segment of uriniferous tubules were presumably the result of the cytotoxic effect of metabolites of CPA. This cytotoxicity resulted in necrosis of tubular epithelial cells within the first few hours after the CPA was administered (Lavin and Koss, 1971).

(iii) Reproductive Toxicity

CPA, a widely used anticancer drug, is associated with testicular damage and sterility. However, CPA has toxic effects on male reproduction, causes oligospermia and azoospermia in both animal models (Elangovan et al., 2006) and in humans (Garolla et al., 2006). It can cause damage during a key point of sperm chromatin remodeling, thereby affecting the chromatin structure. In animals, this often results in embryonic loss, which can occur pre- or post-implantation (Arnon et al., 2001; Codrington et al., 2004; Elangovan et al., 2006). The cytotoxic effect of CPA targets rapidly dividing cells and testis is especially a good target for damaging effects. Decrease in weight of reproductive organ, impaired fertility, growth and development of next generation was also observed in low dose CPA treated male rats (Trasler et al., 1986).

1.6. Reactive Oxygen Species (ROS)

The first theory of ROS induced aging, was reported by Harman in 1955. According to Harman theory free radicals produced during the biological reactions are leading to oxidative stress. This theory highlights a loss of the protective mechanisms that reduce the ability to oxidative challenges. The ROS are the natural by-product of the metabolism of oxygen and have important roles in cell signaling and homeostasis. Furthermore, free radicals are the important contributor of pathological conditions including degenerative diseases. However, during the environmental stress, the levels of ROS can increase dramatically; this will result, damage to cell structures (Devasagayam et al., 2004). The ROS could affect many cellular functions by oxidizing proteins, damaging nucleic acids and lipid per-oxidation. It is important to note that whether ROS will act as damaging, signaling or protective factors depends
on the equilibrium between production and scavenging of ROS at the proper site of action (Bulua et al., 2011). Oxidative stress occurs in case of this critical balance due to depletion of antioxidants or accumulation of ROS in different body organs.

1.7. ROS and the Biological Systems

A number of defense mechanisms of body are evolved to provide a balance between production and removal of ROS in biological systems. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$).

\[
\cdot 2\text{O}_2^+ + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

In the peroxisomes, the enzyme CAT converts H$_2$O$_2$ to water and oxygen, and completes the detoxification initiated by SOD.

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2
\]

1.8. Oxidative Stress

Lipid peroxidation is one of the most important indicators of oxidative stress. Unsaturated fatty acids present in cellular membranes are a common target for ROS. The reaction occurs where a free radical will capture hydrogen from an unsaturated fatty acid to form water (Figure 1.3). Lipid peroxides are unstable and decompose to form a complex series of compounds, such as malondialdehyde (Wang et al., 2013). However, measurement of lipid peroxidation has relied on the detection of thiobarbituric acid reactive compounds such as malondialdehyde (MDA) generated (Pryor et al., 1976).
Superoxide detection is based on the interaction of superoxide with the other measurable compounds. The reduction of ferricytochrome c to ferrocytochrome c has been used to measure the rate of superoxide formation. Hydrogen peroxide is another member of reactive oxygen species. A number of colorimetric substrates such as tetra-methyl-benzidine and phenol red are using to measure hydrogen peroxide levels.

\[
\text{Fe}^{3+} \text{cytochrome c} + \cdot \text{O}_2 \rightarrow \text{Fe}^{2+} \text{cytochrome c} + \text{O}_2
\]

### 1.9. Natural Antioxidants

Natural antioxidants are present in all parts of plant. These components belong to the class of phytochemicals such as alkaloids, phenols, tannins and flavonoids, which are able to scavenge free radicals such as superoxide or lipid peroxides (Gutteridge and Halliwell, 2000).
The most current research on natural antioxidants has been focused on polyphenolic compounds such as flavonoids (Manach et al., 1998). Fruits, plant extracts and vegetables are rich sources of polyphenols, such as flavonoids and carotenoids, whose activities have been established in recent years. The anti-oxidative potential of plant-based antioxidants resulted from the action of lesser-known compounds or from the action of the cocktail of antioxidants present in plants (Baker et al., 1990).

The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites (Singh et al., 2007). They possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Han et al., 2007). Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds (Brown and Rice-Ewans, 1998; Krings and Berger, 2001). Natural antioxidants mainly come from plants in the form of phenolic compounds such as flavonoid, phenolic acids, tocopherols etc. (Ali et al., 2008).

Dietary polyphenols constitute one of the most numerous groups of natural products in the plant kingdom. More than 8000 phenolic structures are known, and among them over 4000 flavonoids have been identified (Harborn and Williams, 2000; Cheynier, 2005).

Flavonoids are the most common group of polyphenolic compounds in the human diet. The major sources of flavonoids include fruits, tea, wine and plants extracts (Anderson and Tolkovsky, 1999; Tsao, 2010). The main groups of flavonoids are flavonols, flavones, isoflavones, flavanones and anthocyanidins. The biological importance of flavonoids has been attributed to their reducing capacities on the intracellular conditions (Rice-Evans, 2001).

Herbs are defined as any part of a plant that is used as a drug for their protective properties. Herbs have identified as source of various phytochemicals and many of them possess powerful antioxidant activity (Dragland et al., 2003). A variety of
diseases have been reported to cure with herbs, from degenerative disease, infections to AIDS and even malaria. Nowadays, about 35% of medicines are of plant origin and their properties have been reported to work like chemical drugs (Dragland et al., 2003). The benefits of herbs are including their low price, availability and lesser side effects compared to chemical drugs (Linde et al., 2001). It was proved that some herbs contain anti-oxidative properties which are more active than those of vegetables and fruits (Valko et al., 2007).

1.10. Objectives of the Study
The aims of the present study is to focus on the evaluation of protective role of Phyllanthus fraternus Webster and Aegle marmelos L. against CPA-induced oxidative stress. In view of the foregoing discussion, the study was undertaken with the following objectives:

- To evaluate the phytoconstituents present in different plants extract by qualitative and gas chromatography - mass spectrometry (GC-MS) method.
- To evaluate the hepatoprotective activity of AEPF and AEAM against CPA-induced liver damage in mice.
- To evaluate the protective effect of AEPF and AEAM against CPA-induced renal toxicity in mice.
- To study the effect of AEPF and AEAM on CPA-induced changes in sperm characteristics and testicular oxidative damage in male mice.