INFLUENCE OF *Couroupita guianensis* FLOWER EXTRACT, ITS ALKALOID FRACTION AND ISATIN, ON OXIDATIVE BIOMOLECULAR DAMAGE AND CELL DEATH

By

Abirami.R.D

(14PBT001)

A Thesis submitted to

Avinashilingam Institute for Home Science and
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In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

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1.0 INTRODUCTION

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) that result from the cellular redox process. Free radicals are the class of compounds characterized by high reactivity due to unpaired electrons in their molecules and are generated in a variety of chemical and biological systems including the human body. Production of the free radicals in the cells is a constant process as a part of normal cellular function. However, excess free radical production from endogenous or exogenous source might play a role in various diseases (Azeemi *et al.,* 2016).

The reactive oxygen species (ROS) form as products under normal physiological conditions due to the partial reduction of molecular oxygen. ROS, are superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). Superoxide is generated directly from the reduction of oxygen and then dismutated to hydrogen peroxide. Hydrogen peroxide is a molecule with low reactivity, but it can readily penetrate cell's membranes and generate the most reactive form of oxygen, the hydroxyl radical, via Fenton's reaction (Nita and Grzybowski, 2016).

The effects of free radicals on human beings are closely related to toxicity, disease and aging. Most living species have an efficient defense system to protect themselves against the oxidative

stress induced by Reactive Oxygen Species (ROS) (Shareef *et al*., 2014).

These species play a dual role as both toxic and beneficial compounds. Thus, the delicate balance between these two antagonistic effects is clearly an important aspect of life. At low or moderate levels, ROS exert beneficial effects on cellular responses and immune function; whereas at high concentrations, they generate oxidative stress, a deleterious process, that can damage all cell structures (Gupta *et al*., 2014).

Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage. It depends upon the balance between reactive species generation and the antioxidant defense (Biswas, 2016).

Antioxidant in cells is to prevent the damage caused by the action of reactive oxygen species. Several synthetic antioxidants, such as Butylated hydroxyl anisol (BHA), Butylated hydroxytoluene (BHT) and Tetra butyl hydroquinone (TBHQ) are commercially available. Because of carcinogenicity of synthetic antioxidants, there is death for antioxidants from natural origin. Natural antioxidants play a vital role in antioxidant defense mechanism in the biological system and acts as free radical scavenger (Jayapragash *et al.,* 2016).

The antioxidant process can function in one of two ways: chain-breaking or prevention. For the chain-breaking process, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking

antioxidant (vitamin C, E, carotenoids, etc) or it simply disintegrates into an inoffensive product (Gupta *et al.,* 2014).

Endogenous antioxidant defenses include a network of compartmentalized antioxidant enzymic and nonenzymic molecules that are usually distributed within the cytoplasm and various cell organelles. In eukaryotic organisms, several ubiquitous primary antioxidant enzymes, such as SOD, catalase, and several peroxidases catalyze a complex cascade of reactions to convert ROS to more stable molecules, such as water and O_2 (Rahal *et al.,* 2014).

The synthetic antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, tertiary butyl hydroxyquinone and propyl gallate) being used in food products to prevent oxidative damages in terms of food spoilage and rancidity in fatty foods had been banned in advanced countries and being discouraged even in developing countries due to their perceived carcinogenic attributes and other toxic properties (Shahid *et al*., 2014). Most widely used synthetic antioxidants in food (butylated hydroxytoluene BHT, butylated hydroxyanisole BHA, etc.) have reported to cause side effects or negative health effects. There is need to replace the synthetic antioxidants with natural antioxidants due to their potential health risks and toxicity (Madhura *et al*., 2015).

Nature acts as a nice source of salvation for human being by providing different remedies from its plants, animals and other sources to treat almost all ailments of mankind. Among the natural sources, medicinal plants are important contributors to the medicinal preparations as raw plant materials, refined crude extracts and

mixtures etc. Several thousands of plants containing medicinal values have been identified for treating different ailments. Even in this recent time, people are still depending on the traditional medicine for their primary health care. New drugs of plant derivation are so much crucial because they are cheap and have little side effects. So medicinal plants are essential. Report shows that more than 80% of the world's population still uses plants to maintain their health and to cure their ailments (Moghal *et al.,* 2014).

It is now established and fully believed that phytoconstituents obtained from the medicinal plants serve as major and active molecules in the modern medicines. Medicinal plants are the backbone of traditional medicine. Medicinal plants used as raw materials for extraction of active ingredients are used in the synthesis of different drugs (Singh, 2015).

Medicinal plants have played an essential role in the development of human culture. Medicinal plants are resources of traditional medicines and many of the modern medicines are produced indirectly from plants. This study illustrates the importance of traditional and modern medicines in the treatment and management of human diseases and ailments (Hosseinzadeh *et al.,* 2015).

Bioactive principles present in the medicinal plants attribute to the therapeutic efficacy and it can be incorporated into modern medicine system for the development of newer drug formulation for therapeutic ailments (Santhoshkumar and Brindha, 2015).

Phytochemicals are bio-active chemicals of plant origin. Chemicals present universally in all the plants can be classified as primary and secondary metabolites. Primary metabolites include proteins, amino acids, sugars, purines and pyrimidines of nucleic acids, chlorophylls etc., while secondary phytochemicals as alkaloids to terpenoids and acetogenins to different phenols. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. They are naturally synthesized in all parts of the plant body; bark, leaves stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components (Rashid *et al.,* 2016).

Plants produce secondary metabolites as a response to adverse environmental conditions or developmental stages. The function or importance of these compounds is usually of an ecological nature as they are used as defenses against predators, parasites and diseases for interspecies competition, and to facilitate the reproductive processes (coloring agents, attractive smells, etc). From medicinal point of view, the important secondary metabolites are alkaloids, flavonoids, tannins, terpenes and phenolic compounds. These active constituents possess effective pharmacological activity (Raja and Srinivasalu, 2015).

Couroupita guianensis Aubl. is an important medicinal tree species with valuable socio-economic heritage and commonly known as Cannon ball or nagalingam tree . It grows up to the height of 30-35 m with peculiar cauliflorous inflorescence. These phytochemicals have antioxidant, antifungal, antibacterial, anticancer, antioxidant and

antimalarial activities. The whole plant is used to diagnose cold, stomach ache, tooth ache, throat infection, tumor etc (Manokari and Shekhawat, 2016). Knowing the phytochemical profile of different parts and different plants is desirable so that one can decide the part to be explored for any particular activity and it can also help one to decide the part(s) to be chosen for any synergistic evaluation. Knowing the phytochemical profile in the beginning of any experiment is desirable than random selection of the plants. The phytochemical screening of *C. guianensis* revealed the presence of alkaloids, phenolics, flavonoids, saponins, rutin, quercetin, kaempherol, lutolin, ursolic acid, hopanes, indirubin, isatin, sterols, fernesol in this plant parts (Kavitha and Padma, 2010).

Earlier studies in our laboratory have reported the important role of alkaloid fractions present in *Couroupita guianensis.* Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. Many of the earliest isolated pure compounds with biological activity were alkaloids. Naturally occurring alkaloids are nitrogenous compounds that constitute the pharmacogenically active basic principles of flowering plants. Alkaloids, comprising a large group of nitrogenous compounds are widely used as cancer chemotherapeutic agents. Alkaloids also interfere with cell division; hence the presence of alkaloids in the plant makes it a possible remedy in the treatment of cancer (Elekwa *et al*., 2009).

One such important alkaloid present in *Couroupita guianensis* is isatin. Isatin (1-*H* indole 2-3 Dione) is synthetically multipurpose compound that is use for the production of different heterocyclic

compounds. The isatin derivatives indicated variety of biological activities such as anticonvulsants, antitubercular, antibacterial, antiviral activities. Because of various biological activities of isatin derivatives our attention has focused to synthesis series of derivatives of isatin which showed remarkable BChE inhibitory and AChE inhibitory activities and used for the cure of Alzheimer's disease (AD) (Noshi *et al.,* 2016).

Isatin was found to exhibit interesting applications as research tools in physiological studies. Similarly, many other isatin-derived compounds possess a wide spectrum of medicinal properties and have thus been studied for activity against tuberculosis, leprosy, fungal, viral and bacterial infections, trypanosomiasis and as anticonvulsants (Sekularac *et al*., 2014).

The present study was aimed at the following objectives:

- To investigate the effect of the flower of *Couroupita guianensis*, its alkaloids and isatin against oxidative stress induced lipid molecules.
- To evaluate the influence of *Couroupita guianensis*, its alkaloids and isatin on the proteins subjected to oxidative stress *in vitro.*
- To analyze the DNA damage by inducing oxidative stress.
- To determine the cytotoxic effect of *Couroupita guianensis,* its alkaloids and isatin compound on oxidative induced normal goat liver cells.

2.0 REVIEW OF LITERATURE

2.1 OXIDATIVE STRESS

Oxidative stress occurs in the presence of oxygen and reactive oxidative compounds in all aerobic organisms. Nitrotyrosine, in turn, reflects nitrosative stress from peroxynitrite and is a protein adduct that can lead to altered protein activity or function. As stable footprints of oxidative stress, both 8-OHdG and nitrotyrosine are widely used as oxidative stress markers (Hinstala *et al*., 2016).

It can be considered as a mother of many human life threatening diseases. OS is a condition in which oxidation exceeds the anti-oxidant reactions, causing an imbalance between oxidative and anti-oxidant systems, with prevalence of reactive oxygen species ROS (Cacciapuoti *et al*., 2016).

Due to the oxidative stress, the cells respond by activating pathways of cell survival or programmed cell death. The initial response of the cell to a stressful stimulus is activated for helping the cell to defend itself and respond positively to the insult (Battistelli *et al.,* 2016).

Generally, if ROS steady-state levels return to the initial value within minutes/hours after stress induction, if organisms are capable and have enough resources for the corresponding response, the stress is called acute oxidative stress. However, sometimes ROS levels do not return to the initial range but stabilize at a somewhat higher level or just extend the steady-state ROS range existing under normal conditions and in this case the stress does not last for prolonged time period. This scenario is called chronic oxidative stress

and frequently occurs under pathological conditions (Volodymyr and Lushchak, 2014).

2.2 BIOMOLECULAR DAMAGE

Free radicals are reactive oxygen species (ROS) or reactive nitrogen species (RNS) which are generated in the body during normal metabolic activities or by environmental conditions. At high concentrations, free radicals are dangerous and can attack biological molecules such as lipids, proteins, enzymes and DNA (Pandey *et al*., 2016).

2.2.1 LIPIDS

Lipid peroxidation is initiated by the hydroxyl radical, while its major final products are MDA and 4-hydroxyl-2-nonenal (HNE). Therefore, MDA is one of the most used biomarkers to evaluate oxidative damage in both local and systemic disorders (Greabu *et al*., 2016).

2.2.2 PROTEINS

Oxidative damage to proteins may result in chemical modification of amino acids, aggregation, or cross-linking of proteins or protein fragmentation. Supplementing the diets of rats with 1% EGCG significantly inhibited increases in muscle protein carbonyl content induced by electrical muscle stimulation. Protein glycation results from the reactions between primary amino groups of proteins and reducing sugars, such as glucose. Oxidation and structural rearrangement of glycated proteins results in the formation of advanced glycation end products, such as NЄ-(carboxymethyl) lysine and pentosidine (Ibrahim *et al*., 2016).

Free radicals produced during mitochondrial electron transport chain stimulate protein degradation. Oxidative protein damage may be brought about by metabolic processes that degrade a damaged protein to promote synthesis of a new protein. In the process of cataractogenesis, oxidative modifications play a significant role in cross-linking of protein, leading to high molecular weight aggregates, loss of solubility and opacity. The consequence of these events may include loss of enzyme activity, cytolysis and even cell death (Bhattacharya and Chakraborty, 2015).

2.2.3 DNA

ROS may promote tumor onset and progression by increasing DNA damage and genome instability, as well as inducing pro-tumorigenic signaling. At the same time, extensive oxidative DNA damage caused by elevated ROS may abrogate the cellular repair capacity to trigger DNA damage response (DDR), which then lead to cell cycle arrest, premature cellular senescence, or programmed cell death, therefore blocking cancer development and progression (Ding *et al*., 2016).

2.3 DISEASE CONDITION

ROS including superoxide free radical, hydroxyl free radical, hydrogen peroxide and singlet oxygen play a key role in the oxidative damage of various diseases such as cardiovascular disease, cancer, diabetes mellitus, atherosclerosis and Alzheimer's disease (Umesh *et al*., 2016).

2.3.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the principal cause of death and disability in the developed countries and the most important cause of premature death worldwide, as outlined by the World Health Organization. Although the ability of oxidative stress biomarkers to predict CVD has yet to be established, the cardioprotective effect of food antioxidants has long been known. In humans, a diet based on antioxidant-rich foods results in an increase in serum antioxidant capacity and a decrease in oxidative stress, and protects from cancer and heart disease (Pellegrino *et al*., 2016).

2.3.2 CANCER

Cancer cells experience an increase in oxidative stress. The pentose phosphate pathway (PPP) is a major biochemical pathway that generates antioxidant NADPH. Here, we show that transketolase (TKT), an enzyme in the PPP, is required for cancer growth because of its ability to affect the production of NAPDH to counteract oxidative stress. It shows that TKT expression is tightly regulated by the Nuclear Factor, Erythroid Protein and CNC Homolog oxidative stress sensor pathway in cancers (Xua *et al.,* 2016).

2.3.3 DIABETES MELLITUS

Diabetes mellitus is characterized by raised level of oxidative stress with associated increased generation of glycoxidation products, notably, HbA above the benchmark plasma value <7%. The presence of hyperglycemia promotes increase in intracellular levels of advanced glycation end products (AGEs). Furthermore, auto-oxidation of glucose generates ROS, such as O_2•−, H_2O_2 and •−OH

which in turn, accelerate lipid peroxidation with corresponding accumulation of advanced lipoxidation end products (ALEs) and more free radicals . Increased levels of ROS in T2DM also contribute to a hypercoagulable state and evidence suggests that accumulation of oxidation products occur prior to the development of Diabetes mellitus (Chikezie *et al*., 2015).

2.3.4 ATHEROSCLEROSIS

Atherosclerosis is responsible for most cardiovascular disease (CVD) and is caused by several factors including hypertension, hypercholesterolemia, and chronic inflammation. Oxidants and electrophiles have roles in the pathophysiology of atherosclerosis and the concentrations of these reactive molecules are an important factor in disease initiation and progression. Overactive NADPH oxidase (Nox) produces excess superoxide resulting in oxidized macromolecules, which is an important factor in atherogenesis. Although superoxide and reactive oxygen species (ROS) have obvious toxic properties, they also have fundamental roles in signaling pathways that enable cells to adapt to stress. In addition to inflammation and ROS, the endocannabinoid system (eCB) is also important in atherogenesis (Ross *et al*., 2015).

2.3.5 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and its incidence increases with age. AD is characterized by the presence of several pathological hallmarks including neuronal loss, formation of senile plaques composed by extracellular deposits of amyloid beta (Aβ) caused by an abnormal

processing of amyloid-beta precursor protein (APP), intracellular neurofibrillary tangles (NFT) composed of aggregated hyperphosphorylated tau proteins in brain, proliferation of astrocytes, and activation of microglial. These features are accompanied bymitochondrial dysfunction and alterations in neuronal synapses (Barbagallo *et al*., 2015).

2.4 ANTIOXIDANTS

Antioxidants are act as free radical scavengers, reducing agents and quenchers of singlet oxygen molecule, and activators for antioxidant enzymes to suppress the damage induced by free radicals in biological system. Human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally or externally supplied through foods and/or supplements (Nithiya and Kumar, 2016).

It also major plant products that play a role as anticancer agents by acting as reducing agents, hydrogen donators, and singlet oxygen quenchers that suppress the naturally produced free radicals and delaying oxidative reactions such as lipid oxidation (Elansary, 2014).

There are highly complex antioxidant systems (enzymatic and non-enzymatic) in human cells, working in collaboration in other to protect the body against free radical damage (Al-Dalaen and Al-Qtaitat, 2014).

2.5 PLANT AS A SOURCE OF ANTIOXIDANTS

Plants are the basic source of knowledge of modern medicine. Almost all the parts of the plant, namely leaves, flowers, fruits, roots, stem and seeds are known to have various medicinal properties (Gousia *et al.,* 2013).

The alcoholic extract of *Couroupita guianensis* significantly stimulated the wound concentration this is due to the presence of stigma sterol and flavonoids in the extract. The results indicated that *Couroupita guianensis* accelerates the wound healing process by decreasing the surface area of the wound and increasing the tensile strength (Swapnalatha and Rajeswari, 2014).

2.6 MEDICINAL PLANT

The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents. Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases (Waddod *et al.,* 2013).

Many hundreds of plants worldwide are used for medicinal purposes as well as for treatments of bacterial infections. Medicinal plants are used as a source to a variety of drugs in developed countries. According to World Health Organization (WHO) more than 80% of the population requires medicinal plants for their primary health care needs due to their fewer side effects. It is important source for pharmacological properties and can act as a new agent against pathogen which exhibit antibacterial activity (Debnath *et al*., 2014).

The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines particularly plant drugs, for their primary health care needs. Herbs are safe but many unsafe and fatal side effects have recently been reported. Hence, there is a urgent need to study the screening of antimicrobial properties of herbs, which will be helpful in the treatment of several diseases caused by microorganisms (Abdul-Hafeez *et al.,* 2014).

Medicinal plants are used to find natural antimicrobial compounds with minimum side effects on health. They are important due to the increasing number of antibiotic-resistant strains. The rise of antibiotic resistant microorganisms is one of the severe problems in health care system of the world and infectious diseases are the second most serious cause of death (Mouafi *et al*., 2014).

Complementary and alternative forms of medicine (CAM) have been developed throughout the world for centuries. Majority of these medicines developed from the use of crude plant extract or purified products from different parts of plants and is dependent on the biodiversity available within the regions, thereby forming a local heritage. In India, CAM is practiced for more than 5000 years in different forms such as Ayurvedha, Siddha, Homeopathy and Unani and these are all licensed by the Government (Saritha *et al.,* 2015).

Plants produce hundreds to thousands of chemical compounds with different biological activities. Mostly, the plants have been used in the treatment of various human diseases for thousands of years all over the world (Manonmani *et al.,* 2015).

2.7 BIOACTIVE COMPOUNDS

Plants are the major source of medicine having nutritive values. The medicinal value of the plants is more important in 1developing countries because of the relative unavailability of medicines and the emergence of widespread drug resistance (Sutha *et al.,* 2014).

Plants are good sources of pharmacological compounds. Natural products could be potential drugs for humans or livestock species. It also acts as intermediate for the synthesis of useful drugs. These bioactivities of the plants produce definite physiological actions on human body (Shehadi e*t al.,* 2014).

Medicinal plants produce an array of secondary metabolites, many of which have shown promising microbiostatic and microbiocidal activities against some pathogenic microbionta. Some of the biologically active compounds elicit chemotherapeutic and chemo prophylactic properties against infectious diseases. These phytochemicals include the alkaloids, flavonoids, tannins, terpenoids, glycosides, saponins, and anthraquinones among others (Godstime *et al*., 2014).

Since ancient times, different varieties of plants have been used as a source of medicines. About 80% of individuals especially developing countries are used in their traditional medicines for primary health care needs. Mostly, the plants produce a wide variety of bioactive molecules that are secondary metabolites such as alkaloids, flavonoids, steroids, resins, fatty acids, tannins and phenol compounds, etc., Those are used in the treatment of diarrhea, dysentery, cough, cold, fever, bronchitis, cholera, etc., These plant

derived products are more effective, less side effects, reduced cost, and easy availability (Shinde and Mulay, 2015).

Plants produce secondary metabolites and play an important role in protecting plants against predators and microbial pathogens due to the biocidal properties against microbes or repellence to herbivores. Some metabolites are involved in defence mechanisms against biotic stress (e.g., UV –B exposure). In general, 100,000 secondary metabolites are involved in plant chemical defence system. Among these metabolites, it is estimated that over 3000 essential oils are known, of which 300 are commercially important for flavor and fragrance industries. Most of the essential oil possesses higher antimicrobial activity which is found in the oxygenated terpenoids, while some hydrocarbons also exhibit antimicrobial effects (Bassole and Juliani, 2012).

Plants exhibit protective mechanism against pathogens. Plant derived compounds have general antimicrobial activity. Plants produce bioactive molecules having a rich source of different types of medicines. These are effective against microorganisms' inturn to protect humans against infectious diseases (Packialakshmi and Naziya, 2014).

A number of plants have been used in traditional medicine for many years, due to their antimicrobial properties. Specifically, some chemical substances perform physiological function of the human or animal body. Medicinal plants contain a number of compounds which may be a potential antibacterial treatment for common bacterial infections. Plants derived medicines are safer than synthetic medicines (Anyanwu and Nwosu, 2014).

Medicinal plants contain some organic compound which produces several actions on the human body. Phytochemicals deal with organic substances present in plants. Chemical compounds such as carbohydrates, proteins, lipids and compounds like glycoside, alkaloids and flavonoids are used as food and medicines by various ways (Dhawale and Pawar, 2015).

2.7 *Couroupita guianensis*

Couroupita guianensis is widely cultivated for its large showy flowers and reddish-brown woody capsular fruits upto 20 cm in diameter. It is grown in Indian gardens as an ornamental tree. It is native to south India and Malaysia and is commonly known as Nagalinga pushpam in Tamil. C.*guianensis* has shown that plant contains several chemical constituents with novel structures and possesses bio-active moieties.

PLANT DESCRIPTION

Kingdom	: Planate
Subkingdom	: Tracheobionta
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Lecythidales
Family	: Lecythidaceae
Genus	: Couroupita
Species	: *Couroupita guianensis* Aubl.

The flowers, which are borne only on special stems on the main trunk, are orange, scarlet or pink, forming racemes up to 3m long (Morankar, 2013).

Distribution

- As ornamental trees along highways and in parks.
- Native to tropical northern South America, especially the Amazon rainforest, and the southern Carribean.
- Also occurs in India, where it is probably native, and Thailand.

Constituents

- Flowers yield an alipathic hydrocarbon and stigmasterol.
- Flowers yielded alkaloids, phenolics and flavonoids.
- Yielded active principles isatin and indirubin (vital to its antimicrobial activity).
- Phytochemical screening yielded flavonoids: 2',4'-dihydroxy-6'-methoxy - 3', 5' - dimethylchalcone, 7 - hydroxy 5 methoxy 6, 8 dimethyl flavanone and the phenolic acid 4-hydroxybenzoic acid.

*Couroupita guianens*is has showed various biological and pharmacological activities such as antioxidant, antibacterial, anti-inflammatory, anti-ulcer, anti-depressant, anti-fertility, anti-mycobacterial, anti-biofilm activities. This plant is grown as an ornamental tree around the temples due to its pleasant aroma, and the magnificent Nagalingam flowers have attracted the people religiously in India because the flower resembles the Linga of Lord Shiva (reduced stigma) protected by the holy King cobra (staminal sheath) (Raja and Koduru , 2014).

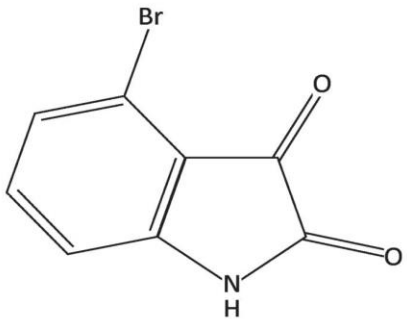
2.8 ISATIN

The derivatives of isatin (indole-2,3-dione), as well as its Schiff and Mannich bases, have already been reported to show a variety of biological activities, such as antibacterial, antifungal and anti-HIV activities (Sekularac *et al.,* 2014).

Isatin (1H-indole-2,3-dione) is an endogenous compound, identified in humans and possesses a wide range of biological activities such as anxiogenic, sedative, anticonvulsant activities and acts as a potent antagonist on atrial natriuretic peptide receptors *in vitro*. Derivatives of isatin have been reported to have cytotoxicity against human carcinoma cell lines derived from breast, prostate, human acute lymphoblastic leukemia (MOLT-4), colon, and lung (Premanathan *et al.,* 2013).

Isatin is one of the most promising new classes of heterocyclic molecules having many interesting activity profiles and well-tolerated in human. Since Isatins have active area for medicinal chemistry there

is a great need to create novel Isatin derivatives for emerging drug targets (Souza and Chattree, 2015).



Isatin

Isatin and its derivatives accomplish various reactions for the synthesis of organic compounds; Schiff bases of isatin are used for their pharmaceutical properties and spirooxindoles have significant biological activities. In recent years isatin-related review articles have been published on enantioselective reactions with isatin, biochemical and pharmacological characterization of isatin and its derivatives, isatins as privileged molecules in design and synthesis of spiro-fused cyclic frameworks, recent progress on routes to spirooxindole systems derived from isatin, synthesis of spiro and multi spiro heterocyclic compounds from isatin, the use of water in the synthesis of isatin based spirocyclic compounds, and synthesis of heterocyclic compounds based on isatin through 1, 3-dipolar cycloaddition reactions. The importance of isatin as a building block in organic synthesis, and since there is a wide range of reactions that include isatin in the synthesis of heterocyclic compounds, this review gives an overview of the synthesis of different types of spiro-fused heterocyclic scaffolds through multicomponent reactions from the year 2012 to

2015 in which one of the starting materials is isatin (Ziarani *et al.,* 2016).

A spiro compound N,N'-(2-oxo-3'H-spiro [indoline-3,2'-thiadiazole] 3'5'-diyl) diacetamide were synthesise by refluxing Isatin and thiosemicarbazone in ethanol and then on heating with acetic anhydride underwent to cyclization into intermediate that on acetylation of the NH and NH_2 groups afforded the spiro compound (Tripathi and Sonawane, 2014).

Hence, the present study was designed to investigate the effects of *Couroupita guianensis*, its alkaloids and isatin compound to reduce the oxidative stress in the lipid, protein, DNA and Cytotoxic effects.

3.0 EXPERIMENTAL PROCEDURE

Couroupita guianensis Aubl. (cannon-ball tree), originating in tropical north-eastern South America, especially the Amazon rainforest, belongs to the Lecythidaceae family and is widely planted as a botanical curiosity in tropical and sub-tropical regions of the world . It is one of the most spectacular plants because of its showy scanty flowers and peculiar spherical woody fruit. *Couroupita guianensis* has been frequently found in wet areas of lowlands and river banks subject to periodic flooding, and it grows well in moist soils but thrives under dry conditions because of its hygrophyte and heliophyte nature. It is a fast-growing deciduous tree, and almost all of the parts of this species, namely leaves, fruit, flowers, stems, roots, and seeds, have been reported to contain chemical constituents, such as triterpenes, phenolics, couroupitine, indirubin, isatin, and oils (Khan and Noguchi, 2016).

The present study focused on the effect of methanolic extracts of *Couroupita guianensis*, its alkaloids and isatin and on the biomolecules subjected to oxidative stress also their Cytotoxic effects.

3.1 COLLECTION OF PLANT SAMPLE

The fresh flowers of *Couroupita guianensis* plant were collected from Coimbatore, and the specimen was identified (voucher number – 2430).

3.1.1 SAMPLE PREPARATION

3.1.1.1 PREPARATION OF METHANOLIC EXTRACT

10g of fresh flowers were homogenized in 100ml of methanol. The plant extract was then left overnight in the shaker at room temperature and then centrifuged at 4000rpm for 20mins. The supernatant containing the plant extract was then transferred to a preweighed beaker and the extract was concentrated by evaporating the solvent at 60°C. The crude extract was weighed and dissolved in a known volume of dimethyl sulphoxide.

3.1.1.2 PREPARATION OF ALKALOID FRACTIONS

Flowers (5g) of *Couroupita guianensis* were crushed in a mortar and pestle with 10% acetic acid in ethanol (200ml) and incubated for 4hours in the dark. After incubation, the extract was filtered and the solution was concentrated to $1/4^{th}$ volume in a boiling waterbath. To the extract, 25% ammonium hydroxide was added until a precipitate was formed and then centrifuged at 2500rpm for 5mins. The residue obtained was washed with 1% ammonium hydroxide and filtered. The residue that contained alkaloids was then weighed, dissolved in ethanol at 4°C.

3.2 TREATMENT GROUPS

The treatment groups set up to study the effect of *Couroupita guianensis* flower extract, its alkaloids and isatin on oxidative induced damage to biomolecules are as follows,

1. Untreated control
2. Treated control

3. *Couroupita guianensis* flower extract
4. Methanolic extract + H_2O_2
5. Alkaloid fractions of *Couroupita guianensis*
6. Alkaloid fraction + H_2O_2
7. Isatin alone
8. Isatin + H_2O_2

3.3 PARAMETER ANALYSED

The methods adopted for the estimation of various parameters involved in the present study are given below:

3.3.1 LPO IN RBC GHOSTS

The extent of lipid peroxidation in RBC ghost was estimated by the method of Dodge *et al.* (1963) as explained in Appendix I.

3.3.2 LPO IN LIVER HOMOGENATE

The lipid peroxidation in goat liver homogenate was assessed by the method of Okhawa *et al.* (1979) and the detailed procedure is given in Appendix II.

3.3.3 LPO IN LIVER SLICES

Lipid peroxidation in goat liver slices was assessed by the method of Okhawa *et al*. (1979) and the stepwise procedure has been explained in Appendix III.

3.3.4 PROTEIN OXIDATION IN BOVINE SERUM ALBUMIN

The protein carbonyl group as a measure of protein oxidation at 375nm was estimated by the method of Jean *et al*. (2010) .The procedure is described in Appendix IV.

3.3.5 PROTEIN OXIDATION IN SDS PAGE

In the presence and absence of H_2O_2, with or without the flower extracts of *Couroupita guianensis*, its alkaloid fractions and isatin were done to separate the proteins by SDS PAGE. The procedure is explained in Appendix V.

3.3.6 DNA STRAND BREAKS IN λ DNA AND pUC 18 DNA

The damage to deoxyribose sugar unit that make up the backbone of DNA was quantified according to *Chang et al.* (2002) in λ DNA AND P UC 18 DNA. The procedure is explained in Appendix VI.

3.3.7 DNA STRAND BREAKS IN HERRING SPERM DNA

The extent of DNA damage was measured by an increase in absorbance at 532nm according to Aseschlach *et al*. (1994) as explained in Appendix VII.

3.3.8 MTT ASSAY

The viability of the cells in the presence and absence of H_2O_2 with or without flower extract of *Couroupita guianensis*, its alkaloid fractions and isatin was estimated by MTT assay as proposed by Igarashi and Miyazawa (2001) and explained in Appendix VIII.

3.3.9 FLOW CYTOMETRY

The dead cells were measured by using Flow Cytometry as proposed by Krishan *et al.*, (1973) and explained in Appendix IX.

4.0 RESULTS AND DISCUSSION

Oxidative stress is the balance between the environmental production of reactive oxygen species (ROS), including hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2), and the ability of biological systems to readily detect and detoxify them, or repair the resulting damage are oxidative stress. It is a common condition caused by biological systems in aerobic conditions such that antioxidants cannot scavenge the free radicals. This causes an excessive generation of ROS, which damages cells, tissues and organs. Highly reactive radicals cause the oxidative damage of different macromolecules proteins, DNA, and lipids leading to loss of function, an increased rate of mutagenesis, and ultimately cell death (Kashmiri and Mankar, 2014).

The controlled generation of RONS in the extracellular space by these enzymes was developed evolutionarily as part of the innate immune system to kill bacteria. However, an overwhelming release of RONS may also induce deleterious effects, causing damage to host biological structures. Another group of enzymes release RONS intracellularly as a byproduct of metabolic processes (Weidinger and Kozlov, 2015).

Antioxidants are chemical or biological agents able to neutralize the potentially damaging action of free radicals such unstable molecules as peroxyl radical, hydroxyl radical, and singlet oxygen and peroxynitrate radicals. The oxidation process of other macromolecules is avoided or slows down by antioxidants. The destructive effect of free radicals in cells is minimized or terminated by

antioxidants. The tissues or cell damage by toxic metabolites is minimized by antioxidants (Khan *et al*., 2015).

In the present study, the influence of methanolic extracts of *Couroupita guianensis* flower, its alkaloids and isatin on the biomolecules subjected to oxidative stress and their cytotoxic effects have been analysed.

4.1 BIOMOLECULAR DAMAGE

Free radicals attacks the biological molecules in the human body. When the ROS reacts with the biomolecules such as lipids, proteins and DNA and causes biomolecular damage that leads to lipid peroxidation, Protein oxidation and DNA damage and finally result in cell death. Due to that it is essential to study the influence of flower extract of *Couroupita guianensis*, its alkaloids and isatin on oxidative damage to lipids, proteins and DNA (Sharma *et al.,* 2014).

4.1.1 EFFECTS OF *C.guianensis* AGAINST *in vitro* LIPID PEROXIDATION

In lipid peroxidation, the three different membrane models namely goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used. The percent of *in vitro* lipid peroxidation by methanolic extracts of *C.guianensis* flower, its alkaloids and isatin in all the three membrane systems is given in Figure 1.

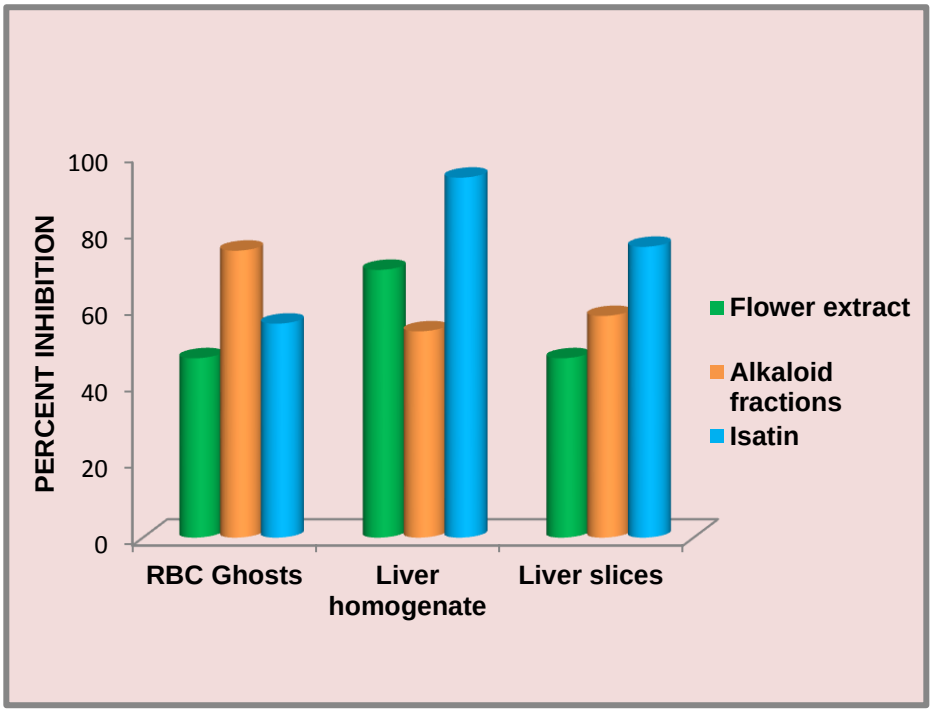


Figure 1. Effects of *C.guianensis* against *in vitro* lipid peroxidation

The results obtained showed that all the extracts namely methanolic extract of *Couroupita guianensis* flower, its alkaloids and isatin substantially decreased the extent of lipid peroxidation in all the three membrane preparations. Among the three extracts, the methanolic extracts of *Couroupita guianensis* rendered the maximum protection against lipid peroxidation in liver homogenate and liver slices.

Alkaloid fractions of *Couroupita guianensis* attains better protection to plasma membrane lipids (RBC ghosts) and equal protection to intracellular lipids (liver homogenate) than the intact cells (liver slices). In the isatin treated groups, the maximum response was observed in goat liver homogenate followed by liver slices and RBC ghosts.

There are many scientific reports available about the efficient of medicinal plants in lipid peroxidation. In recent studies, described that the comparative analysis of antioxidant and antimicrobial activity of methanolic, hexanic, and aqueous extracts from *Adiantum* leaves and showed the higher inhibition in the aqueous extracts when compared to the other extracts (Ahmed *et al*., 2015).

Similarly, the methanol extracts were used from the dried *Carissa carandas* and the plant extracts have good degree of lipid peroxidation in goat liver homogenate (Anupama and Madhumitha, 2015).

Dildar Ahamed *et al*., in 2015 stated that the aqueous extract of *Adiantum caudatum* leaves were the strong inhibitor of lipid peroxidation and the hexanic extract was more potent than aqueous extract.

Another study stated that the methanolic extract of *Carissa carandas* dried fruit showed 85% lipid peroxidation inhibition in chicken and goat liver cells (Anupama *et al.,* in 2015).

The methanolic extract of *Samedera indica* showed inhibition of lipid peroxidation in all concentrations, which showed 50% inhibition effect at 171.2µg/ml and the extract inhibition value was found to be more than the standard vitamin-E (Raja Sundarajan *et al.,* in 2015). *Antidesma bunius* fruit extract exhibited a significant activity in decreasing lipid peroxidation in egg yolk homogenate similar to L-ascorbic acid (Barcelo *et al*., 2016).

Ranjibar Nedamani *et al*., in 2014 stated that a combination of Rosemary and oak extracts showed a significant syngergistic effect in all combined combinations. An increase or decrease in concentration of one or more compounds may influence these interactions and decrease the antioxidant activity.

Sasikumar *et al*., 2015 reported that the alkaloid from *Amaranthus viridis* Linn inhibits the membrane lipid peroxidation triggered by the injurious oxygen radicals generated from hydrogen peroxide.

In the present study, among all the extracts, the methanolic extract of *Couroupita guianensis*, its alkaloids and isatin showed that the methanolic extracts obtained maximum protection against lipid peroxidation in liver homogenate and liver slices.

Alkaloid fractions rendered better protection to plasma membrane lipids (RBC ghosts) and almost equal protection to intracellular lipids (liver homogenate) and intact cells.

Thus, the results showed that the methanolic extracts of *Couroupita guianensis* flower, its alkaloids and isatin rendered a better inhibition on lipid peroxidation.

4.2 OXIDATIVE DAMAGE TO PROTEINS

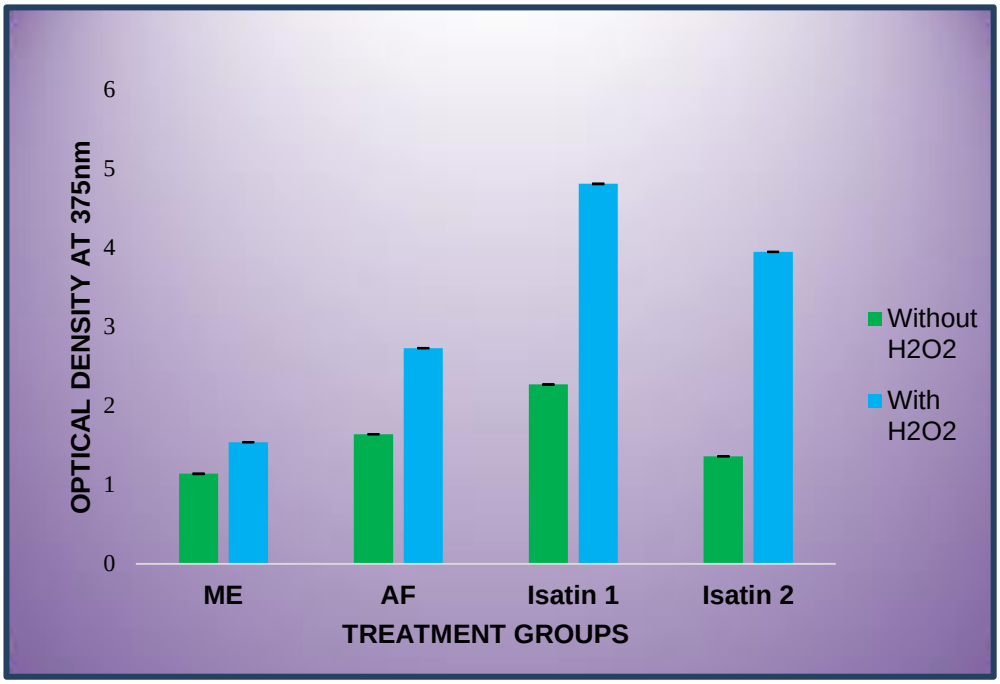
Protein oxidation is involved in regulatory physiological events as well as in damage to tissues and is thought to play a key role in the pathophysiology of diseases and in the aging process. Protein-bound carbonyls represent a marker of global protein oxidation, as they are generated by multiple different reactive oxygen species in blood, tissues and cells (Weber *et al*., 2015).

4.2.1 EFFECT OF *C.guianensis* ON PROTEIN CARBONYL FORMATION

The oxidant is increased due to the formation of protein carbonyl. The oxidation of proteins were compared in the treated and untreated groups of methanolic extracts of *C.guianensis* flower, its alkaloid and isatin and is given in Figure 2.

Among all the tested groups, the methanolic extract of the flower of *C.guianensis* rendered a lesser protection against the protein oxidation compared to alkaloid fractions and isatin. Protein oxidation on BSA was tested with two different concentration of isatin among which 0.25mg of isatin showed a better protection against protein oxidation.

There are many reports available for protein carbonyl formation.



ME – Methanolic extract AF- Alkaloid fractions Isatin 1-25mg Isatin 2-50mg

Figure 2. Effects of *C.guianensis* on protein carbonyl formation

The methanolic extract of *Piper nigrum* fruits was done for anxiolytic and antidepressant profile, and the results induced a decrease in oxidative stress due to the methanolic extracts in the protein carbonyl (Hritcu *et al.,* 2015).

Similarly, It was observed that a mixture of aqueous extract of *Allium sativum* and methanolic extract of *Lagerstroemia speciosa* decreased the hepatic protein carbonyl levels significantly in the type-II diabetic rats (Kesavanarayanan *et al.*, 2013).

Methanolic extract of *Perna viridis* showed significant inhibitory effects on protein oxidation (Jena *et al.,* in 2010).

Razack *et al*., 2015 suggested that the *Nardostachys jatamansi* has showed a good ability against protein, good source of antioxidants and could protect against oxidative insults.

The observation signifies the protective effects of the flowers of *Couroupita guianensis*, its alkaloids and isatin against the protein oxidation.

4.2.2 EFFECT OF *C.guianensis* ON ONE DIMENSIONAL GEL ELECTROPHORESIS

The effect of the flower extracts on protein oxidation *in vitro* was evaluated by one dimensional gel electrophoresis probing of oxidized proteins. The differences in the electrophoretic mobility of the protein bovine serum albumin subjected to oxidative stress *in vitro* were determined in the presence and/or absence of H_2O_2 and the flower extract of *C.guianensis,* its alkaloids and isatin. The results are depicted in plate 2.

1 2 3 4 5 6 7 8 9 10 11

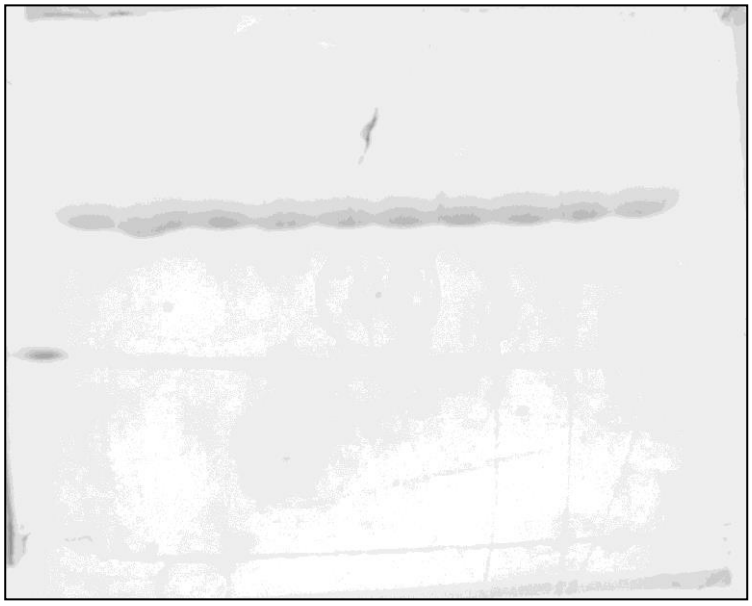


Plate 2.Effect of *Couroupita guianensis* flower, its alkaloids and Isatinon SDS PAGE subjected to oxidative stress

Lane 1 – Marker

Lane 2 - Untreated control

Lane 3 – Treated control

Lane 4 – Methanolic extract

Lane 5 – Methanolic extract + H_2O_2

Lane 6 - Alkaloid fractions

Lane 7 - Alkaloid fractions + H_2O_2

Lane 8 - Isatin 1 (25mg)

Lane 9 - Isatin 1 + H_2O_2

Lane 10 - Isatin 2 (50mg)

Lane 11 - Isatin 2 + H_2O_2

It is evident from the results of the SDS-PAGE, that the intensity of the bands in the H_2O_2-treated group (lane 2) showed a significant decrease when compared to that of the untreated control (lane 1). This effect was counteracted by the co-treatment with the flower extract of *Couroupita guianensis* (Lane 4), alkaloid fraction of *Couroupita guianensis* (Lane 6),isatin of 0.25mg (Lane 8) and isatin of 0.5mg (Lane 10).

Thus, results showed that in the presence and absence of H_2O_2 with the methanolic extracts of *Couroupita guianensis*, its alkaloids and isatin, protein damage has been counteracted to a better extent.

Makri *et al.* (2013) confirmed the protective effect *Crocus sativus* stigmas (saffron) extract against selenium-induced crystalline proteolysis of rat lens proteins using SDS-PAGE. Park *et al.* (2010) showed a decreased multimerization pattern of apolipoprotein (apo) A-I on SDS-PAGE after treatment with the purple sweet potato extract).

Inhibitory effect of epicatechin conjugates obtained from grape against oxidation of erythrocyte membrane proteins was confirmed using SDS-PAGE (Martínez *et al.*, 2012).

Gugliucci *et al.* (2009) found that chlorogenic acid and caffeic acid are the main bioactive compounds that are responsible for anti-glycation effect of yerba maté tea (*Ilex paraguariensis*) using SDS-PAGE analysis.

The anticarcinogenic activity of methanolic extracts were done by the SDS PAGE shows that the indication of the band formation and

also has better activity in the methanolic extracts of the fennel seeds (Zaahkouk *et al*., 2015).

4.3 OXIDATIVE DAMAGE TO DNA

In cells, DNA molecules are the primary targets for oxidative stress. Continuous oxidative damage of the DNA has been implicated in the pathophysiology of various diseases such as cancer, atherosclerosis and aging (Neofytou *et al*., 2012). The effect of the flowers of the *C.guianensis* on oxidant induced DNA damage was assessed *in vitro* in commercially available DNA preparations. DNA from different hierarchies of evolutionary development were selected for the analysis, which included the commercially available preparations of viral DNA (λ DNA), bacterial plasmid DNA (pUC18) and DNA of animal origin (herring sperm DNA). Hydrogen peroxide was used to induce oxidative stress in all the types of DNA both in the presence and the absence of the flower of *C.guianensis*, its alkaloids and isatin.

4.3.1 EFFECT OF *C.guianensis* on λ DNA and pUC18 DNA SUBJECTED TO OXIDATIVE STRESS

The extent of DNA damage in λ DNA and pUC18 DNA was analysed using agarose gel electrophoresis in which the migration of DNA was observed. The results are presented in Plate 3 and 4. In both λ DNA and pUC18 DNA, the absence of specific bands, wherein the DNA was treated with oxidant alone indicated the significant damage induced by H_2O_2. The treatment with the flower of *C.guianensis*, its alkaloid and isatin alone did not cause any damage to λ DNA and pUC18 DNA. The exposure to the oxidant in the

presence of the flower extracts significantly inhibited the oxidant-induced damage of both λ DNA and pUC18 DNA, which is evident from the intact DNA bands.

1 2 3 4 5 6 7 8 9 10

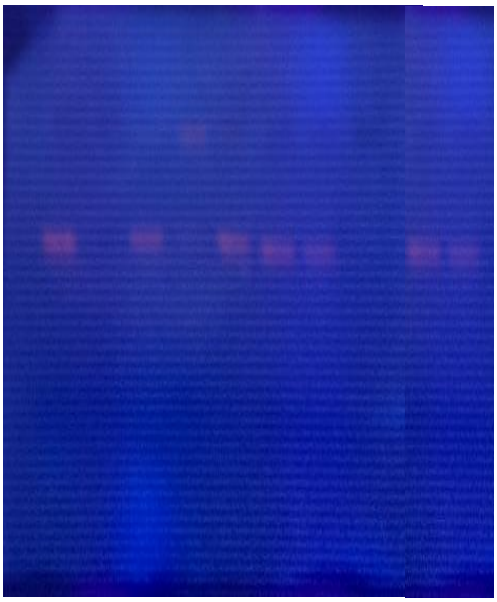


Plate 3. Effect of *Couroupita guianensis* flower, its alkaloids and isatin on λ DNA subjected to oxidative stress

Lane 1 – Untreated control

Lane 2 - Treated control

Lane 3 – Methanolic extracts

Lane 4 – Methanolic extracts + H_2O_2

Lane 5 - Alkaloid fractions

Lane 6 - Alkaloid fractions + H_2O_2

Lane 7 - Isatin 1

Lane 8 - Isatin 1 + H_2O_2

Lane 9 - Isatin 2

Lane 10 - Isatin 2 + H_2O_2

1 2 3 4 5 6 7 8 9 10

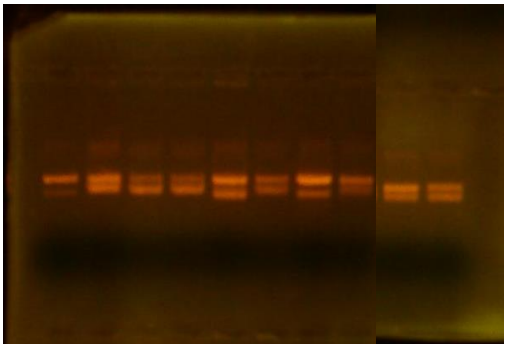


Plate 4. Effect of *Couroupita guianensis* flower, its alkaloids and isatin on pUC 18 DNA subjected to oxidative stress

Lane 1 – Untreated control

Lane 2 - Treated control

Lane 3 – Methanolic extracts

Lane 4 – Methanolic extracts + H_2O_2

Lane 5 - Alkaloid fractions

Lane 6 - Alkaloid fractions + H_2O_2

Lane 7 - Isatin 1

Lane 8 - Isatin 1 + H_2O_2

Lane 9 - Isatin 2

Lane 10 - Isatin 2 + H_2O_2

Parihar *et al*., 2015 reported that the methanolic extracts of *P.hysterophorus* contains antioxidant activity and protect the DNA. Similarly, the effect of methanolic extracts of *Phyllanthus emblica* on λ DNA showed the, ability of the extract in preventing DNA from oxidative damage (Ashadevi *et al*., 2014).

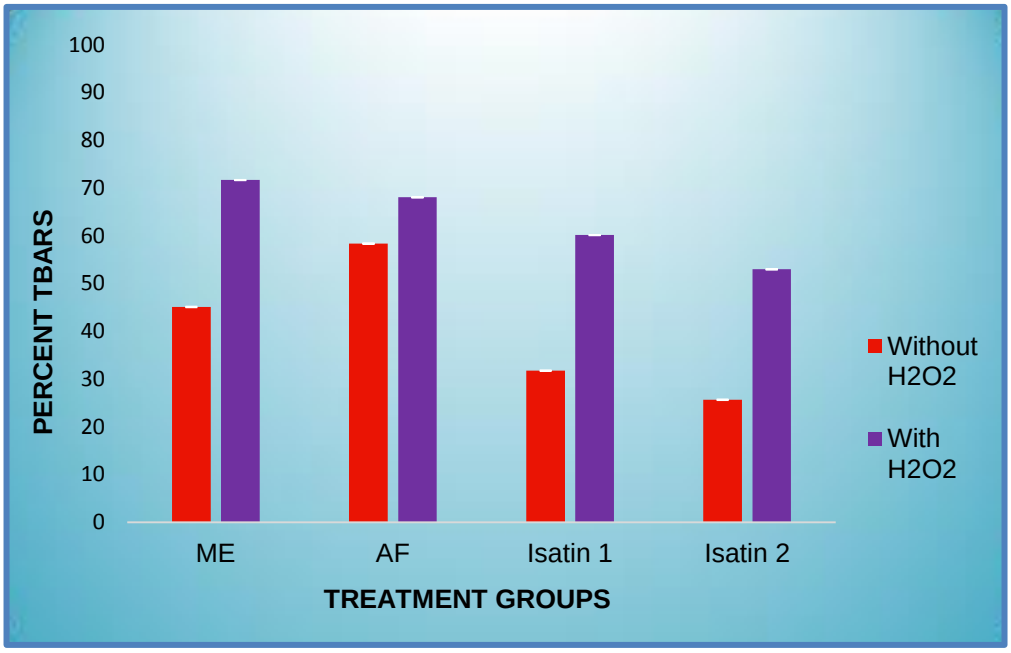
4.3.2 EFFECTS OF *C.guianensis* ON HERRING SPERM DNA

The extent of DNA damage in herring sperm DNA was measured by spectrophotometric analysis of TBARS formation and the results are depicted in Figure 3. The extent of damage to herring sperm DNA was increased markedly on exposure to H_2O_2, which was significantly decreased on co-treatment with the flower extracts, alkaloid fractions and isatin. The protection rendered by the methanolic extract of the *C.guianensis* was more pronounced than that of the alkaloid fraction and different concentrations of isatin.

The results obtained showed that all the extracts substantially decreased the extent of DNA damage. Methanolic extracts of *Couroupita guianensis* rendered better protection than the alkaloid fraction and Isatin.

Balasubramanian and Nirmala, (2014) described that the *Foliose lichens* having antioxidant properties showed the prevention of Herring sperm DNA damage.

Similarly, Radha *et al.,* (2013) reported that the *Phyllanthus niruri* leaves are an excellent source of natural antioxidants with the potential of lipid and DNA damage inhibition. The aqueous extract of the leaves exhibited maximum protection than methanol and chloroform extract. This study strongly warrants closer attention to this



ME – Methanolic extract AF- Alkaloid fractions Isatin 1-25mg Isatin 2-50mg

Figure 3. Effects of *C.guianensis* on Herring sperm DNA

traditional plant for the development of drugs to treat various complications initiated by free radicals.

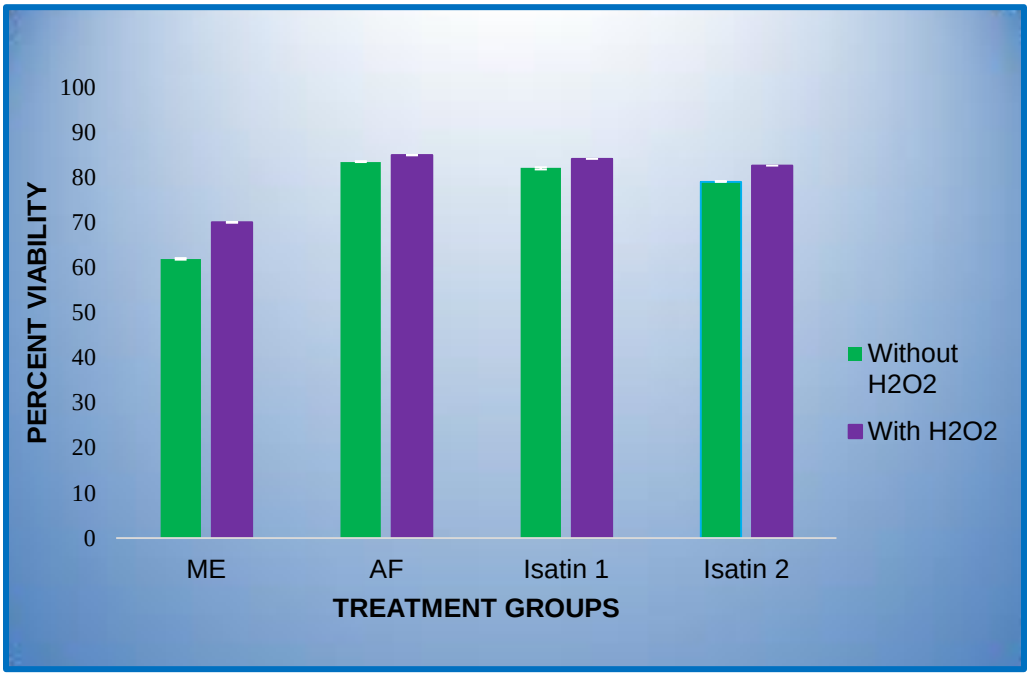
Sakina Razack *et al*., in 2015 stated that *Nardostachys jatamnsi* extract significantly inhibited the AAPH-induced DNA damage by decreasing the formation of open circular form.

Hydro–alchoholic extract of *Desmostachya bipimata* showed protective effect on DNA due to the presence of flavonoids and phenolic compounds like trycin and quercetin which can prevent the production of ROS by complexing cations such as copper and iron that participate in hydroxyl radical formation.

Thus, the results clearly demonstrate the effects of the methanolic extracts of *Couroupita guianensis* flower, its alkaloids and isatin on oxidatively damaged DNA and showed a good protection by counteracting the oxidative stress.

4.4 EFFECT OF *C. guianensis* ON THE VIABILITY OF GOAT LIVER CELLS

The influence of the flowers of *C.guianensis,* its alkaloids and isatin on the goat liver cell survival was determined by quantifying the per cent cell viability using MTT. The extent of viability in the different treatment groups are presented in Figure 4. Exposure to H_2O_2 caused a marked decrease in the viability of the goat liver cells. All the three groups increased the viability of the cells subjected to oxidative stress. The maximum cytoprotection was rendered by alkaloid extracts. When compared to the untreated control group, a minimal decrease in the cell viability of goat liver cells in groups treated with all the extracts alone was also observed.



ME – Methanolic extract AF- Alkaloid fractions Isatin 1-25mg Isatin 2-50mg

Figure 4. Effects of *C.guianensis* on MTT Assay

The methanolic extracts of *Azima tetracantha* Lam were assessed in MTT assay and the results showed non-toxic to the normal cells (Gayathri *et al*., 2015).

The synthesis and characterization of isatin derivatives for MTT assay was done by Rani *et al.,* (2015). They reported that the isatin was effective against the entire cell line.

The alkaloid from the stem of *Toddalia asiatica L* has strong anticancer activity and implies that alkaloids was safe against the normal cells than the methanolic, flavonoids, tannins etc (Praveena and Suriyavathana, 2015).

Inhibition of protein carbonyl formation in sham-operated rats after treatment with polyphenols isolated from *Euterpe oleracea* Mart. extract was reported in a study by Costa *et al.* (2012).

It is evident from our results that the methanolic extract of flowers of *Couroupita guianensis*, the alkaloid fractions and isatin are found to be non-toxic to the normal cells.

4.5 EFFECT OF *C.guianensis* ON OXIDANT EXPOSED GOAT LIVER CELLS IN CELL CYCLE

The flow cytometric analysis was performed in the goat liver cells treated with H_2O_2 and / or, the methanolic extract of flowers of *Couroupita guianensis*, the alkaloid fractions and isatin and the scattergrams and the corresponding histograms were recorded after PI staining. It is evident that all the three tested samples protects the liver cells, exposed *in vitro* to the

oxidant, against cell death. The scatterograms and histograms are depicted in Figure 5, 6 and 7.

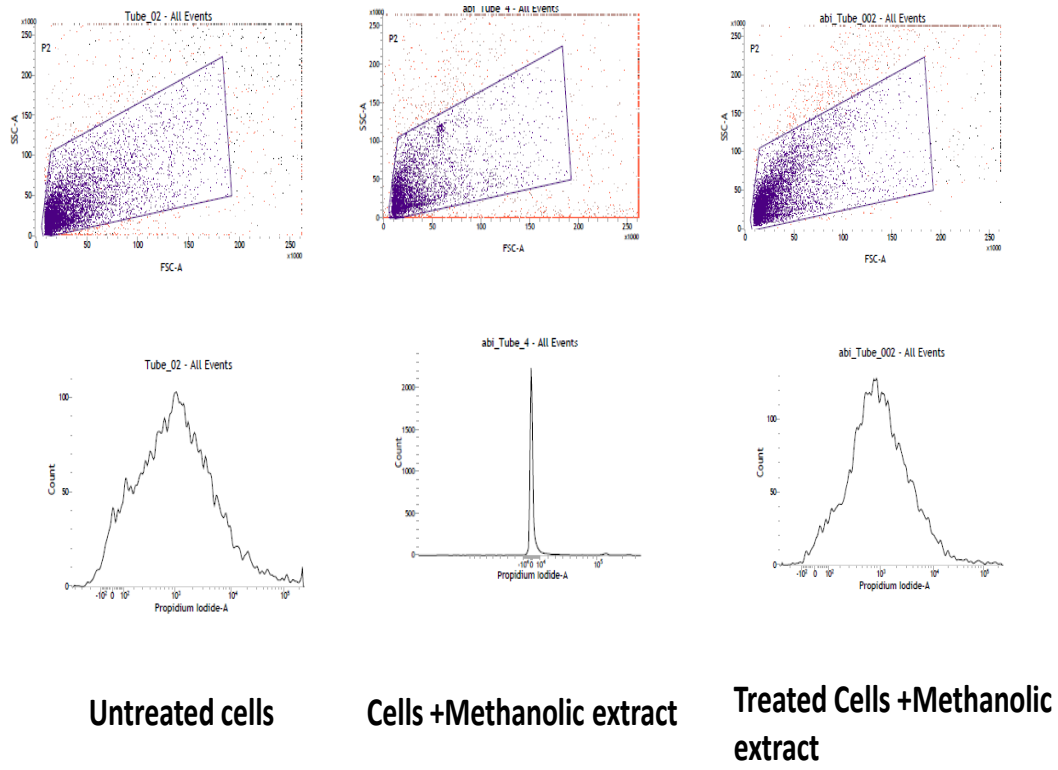
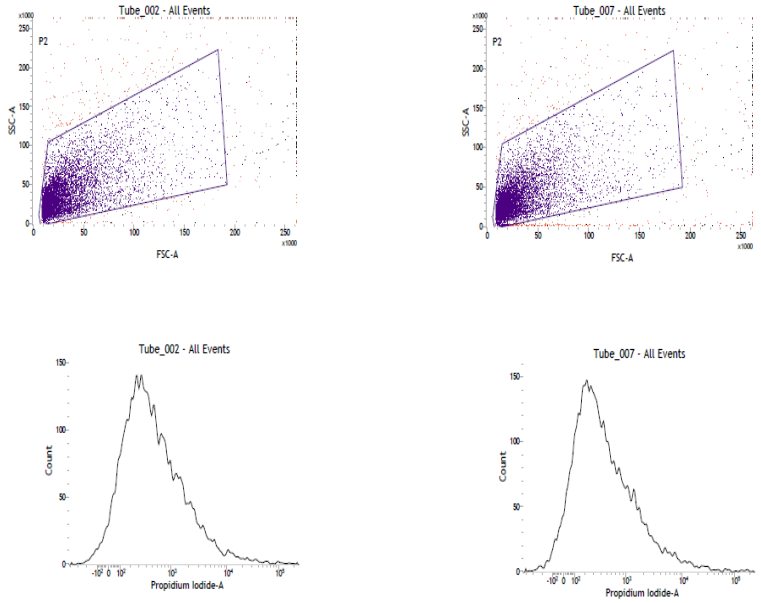


FIGURE 5
EFFECT OF *C.guianensis* ON OXIDANT EXPOSED GOAT LIVER CELLS IN CELL CYCLE



Cells + Alkaloid fractions

Treated Cells +Methanolic extract

FIGURE 6
EFFECT OF *C.guianensis* ON OXIDANT EXPOSED GOAT LIVER CELLS IN CELL CYCLE

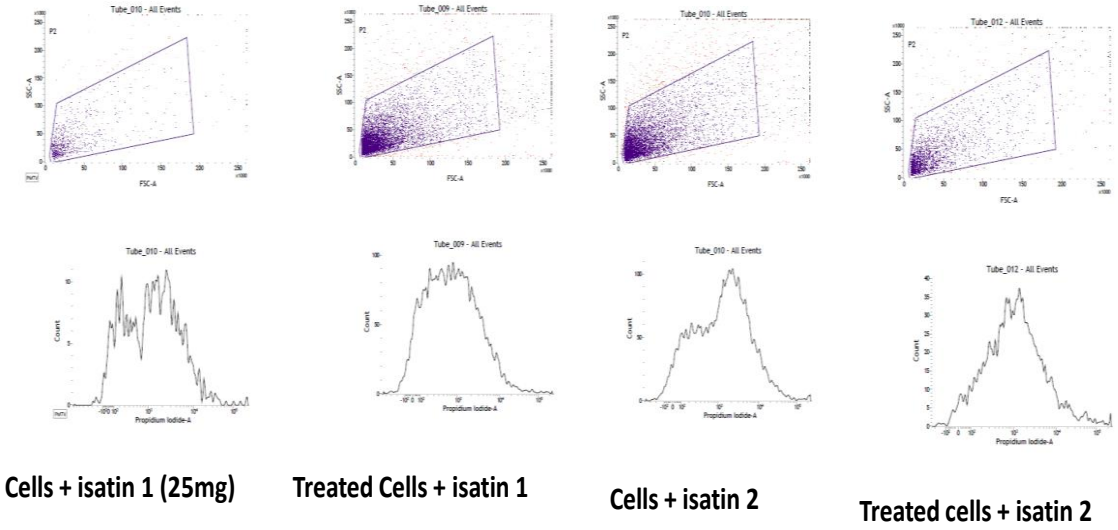


FIGURE 7
EFFECT OF *C.guianensis* ON OXIDANT EXPOSED GOAT LIVER CELLS IN CELL CYCLE

Our results showed that, in the control group (liver cells), cells are distributed throughout the phases of the cell cycle indicating that there are mixture of cells.However, most of the cells are locked in G_0-G_1 phase of the cell cycle, containing 1X DNA content, indicating resting phase. But when the cells were exposed to H_2O_2, it showed the sub-G_0 phase indicating the 1X DNA content to be very less (Krishan, 1975). In the goat liver cells exposed to H_2O_2 and the flower extract of *C.guianensis,* its alkaloids and isatin, there was a shift in the histogram peak and scattergram of DNA intensities in the S-phase and lesser sub- G_0 phase. This clearly proves that the cell death was much lower.

Thus, the present study strongly iterates the medicinal value of the plant, *Couroupita guianensis* by protecting cellular biomolecules and are found to be non toxic to the normal cells and scientifically validates it for use as a component of medicinal preparations, to address the myriad diseases caused by oxidative stress.

5.0 SUMMARY AND CONCLUSION

Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. They are produced either from normal cell metabolisms *in situ* or from external sources (like pollution, cigarette smoke, radiation and medication). When an overload of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and/or supplements.

Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Antioxidants can also protect the human body from free radicals and ROS effects.

Development of safer natural antioxidants from the extracts of many plant materials that can replace synthetic antioxidants is of interest today. Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, antiinflammatory, antiallergic, antithrombotic and vasodilatory activities. *Couroupita guianensis* is also called as Cannonball tree or Sal tree or Ayauma tree. The tree has spread widely all over the world. The tree has a enormous medicinal values since most of the parts like leaves, flower, fruit and stem are used as medicine to cure various diseases. It is enriched with a number of

compounds such as oils, keto steroids, glycosides, couroupitine, indirubin, isatin and phenolic substances. This plants have various activities such as anti inflammatory activity, anti ulcer activity and anti cancer activity, antioxidant activity and antimicrobial activity. Different strategies including the use of *in vitro* system, have been extensively studied to improve the production of plant chemicals.

In this study, the effect of methanolic extracts of flowers of *Couroupita guianensis*, its alkaloids and isatin on oxidatively stressed cellular biomolecules such as lipids, proteins and DNA have been analysed. Lipid Peroxidation in biological membranes increases, thereby affecting the physiological processes of the cell. Three different membranes were used, namely intracellular membrane (RBC ghosts), plasma membrane and internal membranes (liver homogenate) and intact cells (liver slices).

Among the three extracts, the methanolic extracts of *Couroupita guianensis* rendered the maximum protection against lipid peroxidation in liver homogenate and liver slices. Alkaloid fractions of *Couroupita guianensis* attains better protection to plasma membrane lipids (RBC ghosts) and equal protection to intracellular lipids (liver homogenate) than the intact cells (liver slices).

Proteins can be damaged in oxidative conditions by their reactions with LPO products, such as 4-hydroxy-2-nonenal (HNE). Oxidative changes to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity.

It is evident from the results of protein carbonyl assay that the *Couroupita guianensis* flowers, alkaloid fractions and isatin have good protection against protein oxidation in which alkaloid fraction showed a better effect than other two groups.

ROS is capable of inducing damage to almost all cellular macromolecules including DNA which includes base deletion, pyrimidine dimers, cross-links, strand breaks and base modification, such as alkylation and oxidation. DNA damage can cause damage to all components of the DNA molecule.

The DNA damage was studied by three different sources such as Herring sperm DNA, pUC 18 DNA (circular plasmid DNA) and lambda DNA (linear phage DNA). The extent of the λ DNA and pUC 18 DNA in the presence and absence of plant extracts of *Couroupita guianensis* were analysed by the migration of treated and untreated DNA on agarose gels. Methanolic extracts of *Couroupita guianensis* rendered better protection than the alkaloid fractions and Isatin. Similar results were observed with Herring sperm DNA.

The MTT assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The results clearly demonstrated that the methanolic extract of flowers of *Couroupita guianensis*, the alkaloid fractions and isatin are found to be non-toxic to the normal cells.

The flow cytometric analysis was performed in the goat liver cells treated with H_2O_2 and / or , the methanolic extract of flowers of *Couroupita guianensis*, the alkaloid fractions and isatin and the scattergrams and the corresponding histograms were recorded after PI staining. It is evident that

all the three tested samples protects the liver cells, exposed *in vitro* to the oxidant, against cell death.

Thus, the present study strongly iterates the medicinal value of the plant, *Couroupita guianensis* by protecting cellular biomolecules and are found to be non toxic to the normal cells and scientifically validates it for use as a component of medicinal preparations, to address the myriad diseases caused by oxidative stress.

Suggestions for the future study

- The phytochemical fractions may be purified further to isolate many single active component from the fractions and its structure can be elucidated.
- The active component can be subjected to clinical trials to develop into a novel drug.
- The active components can be structurally modified *in silico* and a QSAR (Quantitative Structure Activity Relationship) analysis can be done to identify the most potent derivative.

Appendix I

INHIBITION OF LIPID PEROXIDATION IN GOAT RBC GHOSTS

Dodge *et al. (*1963)

The method explained by Dodge *et al. (*1963) was employed to prepare goat RBC ghosts.

REAGENTS

1. Isotonic KCL (1.15%)
2. Hypotonic KCL (0.5%)
3. Tris Buffered Saline (TBS) – 10mM Tris, 0.15M NaCl (pH 7.4)
4. Ferrous sulphate (10µM)
5. Thiobarbituric acid (TBA) (1%)
6. Ascorbic acid (0.06mM)
7. Ethanol (70%)
8. Acetone

PREPARATION OF RBC GHOSTS

A clean sterile container containing acid- washed stones was used for the defibrination of fresh goat blood (50ml). The defibrinated blood was transferred into a fresh sterile container and diluted with sterile isotonic KCL (1:1) and was centrifuged at 3000g for 10 minutes at 4 □C. The supernatant was discarded and the pellet was washed thrice with isotonic KCL. The resulting pellet was suspended in hypotonic KCL and incubated at 37 □ C for one hour for complete lysis. The lysate was centrifuged at 5000g for 15 minutes at 4 □ C and repeatedly washed with hypotonic KCL until a pale pink pellet

containing erythrocyte ghosts was obtained. The ghosts preparation was suspended in 1.5ml of TBS and used as a source of membrane lipids.

PROCEDURE

The assay mixture contained RBC ghosts (0.1ml), test samples (0.1ml), $FeSO_4$ (0.1ml), ascorbate (0.1ml) and TBS (0.1ml). A blank was also prepared by eliminating test samples and lipid source but only $FeSO_4$, ascorbate and TBS to a final volume of 0.5ml. An assay medium corresponding to 100% oxidation was prepared by adding all other constituents except the test samples. The experimental medium corresponding to auto-oxidation contained only the RBC ghosts. All the tubes were heated in a water bath at 37^o C for one hour. The reaction was stopped by the addition of 70% alcohol (0.5ml). TBA (0.5ml) was added to all tubes and heated in a boiling water bath for 20 minutes to allow colour development. After cooling, acetone (0.5ml) was added all the tubes. The intensity of the pink colour, as the indication of TBARS produced, was measured at 535nm.

Appendix II

INHIBITION OF LIPID PEROXIDATION IN LIVER HOMOGENATE

(Okhawa *et al.,* 1979)

Goat liver was collected fresh from the slaughter house and washed with TBS to remove blood. A 20% goat liver homogenate was prepared in ice-cold TBS using Teflon homogenizer. The homogenate was centrifuged to remove the debris and the supernatant was used as a membrane source for the LPO assay (Okhawa *et al.,* 1979).

REAGENTS

1. KCL (30mM)
2. $FeSO_4$ (0.16mM)
3. TBA (1%)
4. Glacial acetic acid
5 n-propanol: pyridine (15:1 v/v)

PROCEDURE

The reaction mixture contained 0.1ml each of liver homogenate, KCL, $FeSO_4$ and ascorbate. The mixture was incubated for one hour at 37 ^{o} C both in the presence and absence of 5µl of *Couroupita guianensis* flower extract, alkaloids fractions and isatin. An aliquots (0.4ml) of the assay mixture was mixed with 1.5ml each of TBA and acetic acid. The mixture was heated in a boiling water bath for 20 minutes and allowed to cool at room temperature, followed by the addition of 1.0ml of distilled water and 5.0ml of a mixture of n- proponal and pyridine. The pink coloured chromophore obtained after centrifugation was measured at 532nm.

Appendix III

INHIBITION OF LIPID PEROXIDATION IN LIVER SLICES

Dodge *et al.,* (1963)

The extent of inhibition of LPO in goat liver slices was followed by the method of Dodge *et al.,* (1963).

REAGENTS

1. Phosphate buffered saline (PBS)
2. H_2O_2 (30%)
3. Alcohol (70%)
4. TBA (1%)
5. Acetone

PREPARATION OF GOAT LIVER SLICES

The goat liver was collected fresh from the slaughter house, plunged in ice- cold sterile PBS and maintained at 4^o C till use. Very thin slices (1mm) of the liver were cut using a sterile blade.

PROCEDURE

Thin slices of goat liver (250mg) were taken in 1.0ml of sterile PBS in flat- bottomed flasks. The slices were incubated in the presence and/or the absence of 5µl flower extracts of *Couroupita guianensis*, its alkaloids and isatin and oxidant (5µl of 30% H_20_2). Approximate controls were set up. The assay mixture was incubated at 37^o C with mild shaking for one hour followed by homogenization using a Teflon homogenizer and the homogenate was used for the assay.

An aliquot of the homogenate (0.5ml) was mixed with 0.5ml of 70% alcohol to terminate the lipid peroxidation reaction. TBA (1ml) was added to all the tubes and heated in a boiling water bath for 20 minutes. After cooling at room temperature, 500µl of acetone was added and the pink colour developed was measured spectrophotometrically at 535 nm as an indication of TBARS formed.

Appendix IV

PROTEIN CARBONYL ASSAY

Jean *et al*. (2010)

The method explained by Jean *et al*. (2010) was used to analyze the protein carbonyl with slight modifications.

PRINCIPLE

Protein carbonyls in bovine serum albumin (BSA) standards or protein samples are derivatized with 2,4-dinitrophenylhydrazine (DNPH) first. Proteins are then TCA precipitated and free DNPH is removed by washing the protein pellet. After dissolving the protein pellet in guanidine hydrochloride (GuHCl), the absorbance of protein-hydrazone is measured at 375nm, and the protein carbonyl content is calculated.

REAGENTS

1. BSA(2mg)
2. Ferric chloride(50µM)
3. Hydrogen peroxide(1mM)
4. Ascorbic acid(100mM)
5. Dinitrophenylhydrazine(DNPH,10mM)
6. Hydrochloric acid(2.5M)
7. TCA(20%w/v)
8. TCA(10%w/v)
9. Ethanol/ethylacetate(1:1,v/v)
10. Guanidine hydrochloride(6M)

PROCEDURE

BSA was oxidized by a Fenton type reaction .The reaction mixture containing 2mg BSA ,$Fecl_3$ (5µl), H_2O_2 (10µl) ,ascorbic acid (10µl) and flower extract of *Couroupita guianensis*, its alkaloids and isatin compound (5µl) was made upto 1ml with distilled water .it was incubated for 30 minutes at 37ºC.An aliquot 200µl of sample was transferred to two 2ml plastic tubes ,one tube as the sample and the other as control. To each of the sample tubes,800µl of DNPH was added whereas 800µl of 2.5M HCl was added to the control tubes. The tubes were incubated in the dark at room temperature for one hour with brief vortexing every 15 minutes. TCA 1ml of 20% was added to each tube and left for 5minutes on ice. The tubes were then centrifuged at 10,000g for 10 minutes at 4ºC .The supernatant was discarded and the pellet was resuspended in 1ml of 10% TCA for 5 minutes on ice. The centrifugation was repeated and the pellet was suspended in 1ml of (1:1)ethanol/ethylacetate mixture. The tubes were vortexed thoroughly and centrifuged at 10,000g for 10 minutes at 4ºc. The washing step was repeated twice and after the final wash, the protein pellet was resuspended in 6M guanidine hydrochloride. The tubes were again centrifuged at 10,000g for 10 minutes at 4ºC to remove any leftover debris. The supernatant was transferred to fresh tubes and the absorbance of the samples was measured at 375nm against its blank. The protein carbonyl content was calculated using the formula,

Protein carbonyl (nmol per mg) = corrected absorbance × 45.45/Protein concentration (mg)

Where

Corrected absorbance=$A_{Sample} - A_{Blank}$

The extinction coefficient for dinitrophenyl hydrazine at 370nm is 22,000 $M^{-1}Cm^{-1}$.

Appendix V

ONE DIMENSIONAL GEL ELECTROPHORESIS

Proteins are the early targets of ROS. The oxidative modification of proteins has been shown to play a major role in a number of human diseases. However, the ability to identify specific proteins that are more susceptible to oxidative modifications is difficult .Separation of proteins using polyacrylamide gel electrophoresis (PAGE) offers the analytical potential for the recovery. The ability of this method was used in the present study to visualize the *in vitro* damage to proteins.

REAGENTS

1. Tris (25mM, pH 8.3)
2. Tris (1.5mM, pH8.8)
3. Tris (1M, pH6.8) (From this other buffer concentrations like 50mM and 100mM tris of pH6.8 were prepared respectively and used)
4. DTT (100mM)
5. Glycine(250mM)
6. Glycerol
7. SDS (20%)
8. Bromophenol blue (0.1%)
9. Ammonium per sulphate(10%)
10. SDS(10%)
11. Acrylamide: Bisacrylamide(29:1)
12. composition of gels

S.No.	COMPONENTS	SEPARATING GEL (12%)	STACKING GEL (5 %)
1	Acrylamide stock	12.0ml	1.75ml
2	Tris buffer	7.5ml (pH8.8)	1.25ml (pH6.8)
3	Water	9.9ml	6.8ml
4	SDS	0.3ml	0.1ml
5	APS	0.3ml	0.1ml
6	TEMED	12µl	0µl

13. Gel loading dye: 5ml of 10mM tris(pH6.8) ,1ml of 20% SDS, 10mg of bromophenol blue (tracking dye) and 1ml of glycerol were mixed and made upto 9ml with distilled water . To this, 1ml of 1M DTT stock was added fresh just before use and mixed.

14. Staining solution: coomasie brilliant blue R250 (250mg) was dissolved in 45ml of water and 45ml of methanol ,and 10ml of glacial acetic acid was added to it

15. Destaining solution : This was prepared by freshly mixing together 45ml water,45ml methanol and 10ml glacial acetic acid.

PROCEDURE:

The glass plate were set without any leakage .The resolving gel was allowed to polymerize followed by the stacking gel with the Teflon combs in the vertical position. Samples (methanolic extracts of *Couroupita guianensis* flower, alkaloids fraction and isatin) were prepared by heating to100ºC for 3minutes in the sample loading buffer. After polymerization ,gel plates were mounted in the apparatus (Hoefer inc.,USA)

The tubes were then incubated at 37ºC with gentle shaking for one hour.

ELECTROPHORESIS

From the incubated reaction mixture ,70µl was taken in a microfuge tube and mixed with 30µl gel loading buffer containing DTT. The tubes were tightly corked and kept in a boiling water bath for exactly one minute .They were then allowed to cool to room temperature and pulse spun to bring the solution down .The samples were then loaded into the wells of the gel at a concentration of 50µg protein /well (25µl) and electrophoresed at 8V /cm(0.65mA/Cm^2) till the tracking dye reaching a distance of 1cm from the bottom of the gel (3-4hours). The leads were disconnected and the plates were dismantled and gently pried apart with a flat spatula .The gel was marked for the direction of run by clipping off the right hand corner and rinsed quickly in tris buffer to remove SDS. It was then gently shifted to the staining solution taken tn a staining box and incubated on a gel rocker overnight at room temperature. Following this ,the stain was drained off and the gel was rinsed several times in freshly prepared destaining solution and put on the gel rocker. Several

changes of destaining solution were given at a minimum interval of 30 to 45 minutes ,till the bands were clearly visible in a transparent background of the gel .the gel was then documented in a digital gel documentation system and the integrated density values of the bands were recorded.

Appendix VI

ESTIMATION OF DNA DAMAGE IN λ DNA AND pUC 18 DNA

Chang *et al* (2002)

The method proposed by Chang *et al* (2002) was used to determine DNA damage.

REAGENTS

1. λ DNA /pUC 18 DNA (2µg/5µl)
2. Tris buffer (50mM, pH 7.4)
3. H_2O_2 (30%)
4. $FeCl_3$ (500µM)
5. Agarose (1%) in 1X TAE buffer
6. TAE buffer (50X) (tris base 24.2g, glacial acetic acid 5.71ml and EDTA 18.61g, in a total volume of 100ml, pH 8.0).
7. EtBr (10mg/ml)
8. Gel loading dye (1.28g bromophenol blue, 0.25g xylene cyanol and 50ml glycerol in a total volume of 100ml.)

PROCEDURE

The reaction mixture (30µl) contained 5µl of tris buffer or λ/ pUC 18 DNA and 5µl of tris buffer or flower extract of *Couroupita guianensis*, its alkaloids and isatin, 10µl of 30% H_2O_2 and 5µl of $FeCl_3$ and incubated at 37 ^{o} C for 15minutes for pUC 18 DNA and 30 minutes for λ DNA. The assay mixture (10µl) was mixed with 5µl of gel loading dye and loaded onto 1% agarose gel with 5µg/ml EtBr. Electrophoresis was carried out at 100V for 15 minutes in a submerged gel electrophoretic apparatus. The DNA was visualized in a UV transilluminator and photographed using Alpha Digi Doc digital gel documentation system (Alpha Digi Doc, USA).

Appendix VII

ESTIMATION OF DNA DAMAGE IN HERRING SPERM DNA

Aeschlach *et al*. (1994)

The H_2O_2 induced DNA damage in herring sperm DNA was studied by the method described by Aeschlach *et al*. (1994).

REAGENTS

1. Herring sperm DNA (0.5mg/ml in 10mM tris buffer, pH 7.4)
2. H_2O_2 (30%)
3. NaCl (5mM)
4. $FeCl_3$ (50µM)
5. EDTA (0.1M)
6. TBA (1%)
7. HCl (25%)
8. Tris buffer (10mM, pH 7.4)

PROCEDURE

The assay mixture contained herring sperm DNA (50µl), H_2O_2 (0.167ml), $FeCl_3$ (0.05ml) and flower extract of *Couroupita guianensis*, its alkaloids and isatin (5µl). The volume was made up to 0.5ml with tris buffer and was incubated at 37 ^{o} C for one hour. EDTA (10µl) was added to terminate the reaction. TBA (0.5ml) and HCl (0.5ml) were added and incubated at 37^{o} C for 15 minutes for colour development. After centrifugation, the supernatant was taken and the extent of DNA damage was measured by the increase in absorption at 532nm spectrophotometrically.

Appendix VIII

CYTOTOXICITY ASSAY

Igarashi and Miyazawa (2001)

The extent of cytotoxicity in the oxidant induced cells both in the presence and the absence of the flower extract and isatin compound was determined by the MTT dye reduction assay as described by Igarashi and Miyazawa (2001).

PRINCIPLE

The 2 - (4, 4 – dimethyl – 2 – tetrazoyl) - 2, 5 – diphenyl - 2, 4-tetrazolium salt (MTT) is converted into its formazon derivative by living cells. The amount of formazon formed is a measure of the number of surviving cells. After solubilisation of the formazon in a suitable solvent, the cell viability can be measured in a microtitre plate reader.

REAGENTS

1. PBS (phosphate buffered saline)
2. MTT – 3mg/ml in PBS
3. Isopropanol in 0.04N HCl (acid-propanol)

PROCEDURE

The goat liver cells (100 µl), were incubated with 50µl of MTT at 37°C for 3 hours. After incubation, 200µl of PBS was added to all the samples(flower extract of *Couroupita guianensis*, its alkaloids and isatin). The liquid was then carefully aspirated. Then 200µl of acid-propanol was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Anthos 2020,

Austria). The optical density of the control cells were fixed to be 100% viability and the per cent viability of the cells in the other treatment groups were calculated.

Appendix IX

FLOW CYTOMETRY ANALYSIS

(Krishan, 1973)

Flow cytometric analysis was performed using propidium iodide.

REAGENTS

1. Propidium iodide staining solution: 3.8mM sodium citrate, 50 µg/ml PI in PBS.
2. RNase A stock solution: 10 µg/ml RNase A (boiled for 5 minutes, aliquoted and stored frozen at -20 ^{o}C).

PROCEDURE

The goat liver cells were harvested and single cell suspension in buffer was prepared and the cells were washed and spinnned at 300 x g for 5 minutes and resuspend at 3-6 x 10^{6} cells/ml. The 500µl cells were aliquoted in a 15 ml polypropylene, V-bottomed tube and 5 ml cold 70% ethanol was added dropwise and it was gently vortexed. If cells are not vortexed on addition to the ethanol, they are fixed to each other in clumps. The cellswere fixed for at least 1 hour at 4^{o}C. (Cells may be stored in 70 % ethanol at -20^{o} C for several weeks prior to PI staining and flow cytometric analysis). The cells were washed in PBS as described above. (It may be necessary to centrifuge cells at a slightly higher "g" to pellet after ethanol fixation). Propidium iodide staining solution (1ml) was added to cell pellet and it was mixed well. RNaseA stock solution (final concentration 0.5ug/ml) (50µl) was added and incubated overnight (or at least 4 hours) at 4ºC. The samples were stored at 4^{o} C until analyzed by flow cytometry.

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