CHAPTER ONE

INTRODUCTION

Oxygen comprises 20% of the air we breathe and both animals and plants use oxygen in several ways. Thus, oxygen is significant for cell viability and furthermore for our life. On the other hand, it also has a potentially damage effect. Oxidation reactions occur frequently in our bodies to produce energy within the mitochondria and in the enzymatic reactions that detoxify drugs and phytochemicals. These reactions usually involve unpaired electrons catalyzed by specific enzymes Implicated in the production and maintenance of complex structures that make life achievable. An unbalanced oxidation will cause destruction of all the essential cellular elements that make life achievable, including cellular lipids, proteins, carbohydrates and DNA. As it's known oxidation is the loss of electrons, and reduction is the achievement of electrons. Oxidation and reduction reactions must occur in pairs, if one atom or molecule is oxidized, another is reduced to defend against the potentially injuring effects of free radicals. Highly reactive molecules are able to oxidize molecules that were formerly stable and possibly will cause them to become unstable species, like free radicals (Davis *et al*., 2006).

Throughout the past 30 years, the field of free radical chemistry and biology have grown from relative obscurity to become major element of biomedical research and pharmaceutical development. The first direct evidence which linked between oxygen free radicals and oxygen toxicity was shown by Gerschman *et al*. (1954) they noted that the pattern of x-radiation injure to lung tissue was similar to that induced by high oxygen concentrations. The similarity of these two pathologic phenomena was

recognized in their work by both citation of earlier observations and by the presentation of new data showing the cumulative effects of hyperoxia and x-irradiation. They also reported that many chemical agents which have the ability of protection against the toxic effect of radiation also protected against oxygen poisoning. The basic concept of their theory was that both oxygen poisoning and the biological effects of x-irradiation share a common mechanism of action, particularly the reactions of free radicals (Gerschman *et al*., 1954; Castro & Freeman, 2001). McCord and Fridovich (1969) kept going on to improve the concept of free radical mediation of oxygen toxicity by recognizing a catalytic function for the enzyme superoxide dismutase (SOD). Their work provided a direct proof of the in vivo formation of the superoxide radical (McCord & Fridovich, 1969; Castro & Freeman, 2001).

1.1 Free Radicals.

A Free radical can be defined as any element or compound that contains one or two unpaired electrons that can be neutral, positively charged, or negatively charged (Bendich *et al*., 1989). In an attempt to stabilize itself, the free radical abstracts an electron from a stable compound which in turn is converted into a new free radical. In this chain reaction the radical product of one reaction becomes the starter for another one. This series reaction will continue until the free radical containing the lone electron pairs up with another molecule containing unpaired electron, or is deactivated by an antioxidant (Grishman & McCord, 1986).

Oxygen containing reactive molecules (some of them are free radicals) are most vital for biological systems. When oxygen is exposed to a source of high energy, the energy is transferred to shape singlet oxygen (Reaction A).

$$O_2 + \text{energy} \longrightarrow O_2^{\bullet} \text{ (singlet oxygen)}$$

(Reaction A)

This singlet oxygen contains a pair of electron and thus it cannot be considered as free radical. All the same, these two electrons exist in unstable conformation in the molecule and thus the potential exists for this molecule is to contribute in the reactions that generate free radicals (Halliwell & Gutteridge, 1999).

Normally, over 95% of the oxygen utilized by aerobic organisms is enzymatically reduced to H_2O by the electron transport system in the mitochondria (Reaction B).

$$O_2^{\bullet} + 4e^- + 4\,H \longrightarrow 2H_2O$$

(Reaction B)

When molecular oxygen is reduced by one electron, the product is superoxide radical ($O_2^{\bullet -}$) (Reaction C1).

$$O_2 + e^- \longrightarrow O_2^{\bullet -} \quad \text{(Superoxide radical anion)}$$

(Reaction C1)

When a second electron is added to (Superoxide radical anion) at physiologic pH this will result in the formation of hydrogen peroxide (H_2O_2), an oxidizing species that has no unpaired electrons and thus is not a free radical (Reaction C2).

$$2\ O_2^{\bullet -} + 2\ H^+ \longrightarrow O_2 + H_2O_2 \text{ (Hydrogen peroxide)}$$

(Reaction C2)

These two products (superoxide radical anion and hydrogen peroxide) can react together to form water, however, in this reaction the highly reactive hydroxyl radical is generated which consider to be the strongest oxidant in biological systems (Reaction C3).

$$O_2^{\bullet -} + H_2O_2 + 4H^+ \longrightarrow O_2 + H_2O + HO^{\bullet} \text{ (Hydroxyl radical)}$$

(Reaction C3)

This process is known as Fenton reaction. The formation of $HO^{\bullet}$ from H_2O_2 is catalyzed by transition metals, mainly iron and copper. Together, $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$ are known as reactive oxygen species (ROS) and are continuously produced by aerobically growing cells (Castro & Freeman, 2001).

In addition to the continuous generation of reactive oxygen species (ROS) during cellular metabolism, the free radicals are also being utilized by the inflammatory cells such as neutrophils, macrophages and eosinophils in the process of killing bacteria and other pathogens that invade the body. The main cell type associated with this first line of defence against infection is neutrophil. These cells respond to chemical signals that released at the site of infection, and move to the signal (chemotaxis). Neutrophil cells have the ability to take up molecular oxygen and produce reactive oxygen containing molecules when motivated. This process is commonly called the oxidative or respiratory burst. Free radicals and other reactive molecules can destroy bacterial pathogens directly or by the reaction with granulocyte – specific enzymes to form highly toxic compounds. Neutrophils are able to produce toxic halogenated molecules

when the myeloperoxidase halide enzyme (MPO) system is activated during the oxidative burst. These halogenated species can also kill pathogens. The killing process is generally limited to intracellular vacuoles which enclose the phagocytised pathogen. During the oxidative burst neutrophiles consume vitamin C, and following activation the vitamin C concentration is reduced (Bendich *et al*., 1989).

Macrophages are other cells in the immune system that use free radicals to kill pathogens that invade the human body. These phagocytic cells have the cell-membrane associated NADPH-oxidase system, which consider being the primary (ROS) generating enzyme, including hydrogen peroxide, hydroxyl radicals and superoxide radicals (Rahman & MacNee, 2002). The oxidant load produced by eosinophils is believed to be large because these cells have several times greater capability to produce $O_2^{\bullet-}$ and H_2O_2 than do neutrophils, and the content of eosinophil peroxidise (EPO) in eosinophils is 3-10 times higher than the amount of myeloperoxidase (MPO) present in neutrophils (Walsh, 1999).

Xanthine oxidase pathway is another endogenous source of reactive oxygen species. Together xanthine dehydrogenase and xanthine oxidase are implicated in the metabolism of xanthine to uric acid. Xanthine dehydrogenase is the form of the enzyme present under physiologic conditions, but during ischemic conditions this form is changed to xanthine oxidase. Xanthine oxidase is able to reacts with molecular oxygen, and releasing superoxide free radical (Nijveldt *et al* 2001).

Beside ROS there is another group of free radicals known as nitrogen reactive species (NRS). One member of this group nitric oxide ($NO^{\bullet}$) is generated in the mammalian cells through enzymatic oxidation of the amino acid L-arginine to citrulline

and ($NO^{\bullet}$) by the action of constitutive and inducible nitric oxide synthases (Zhang & Snyder, 1995). Nitric oxide is an important mediator of varied physiologic process including neurotransmission, regulation of blood pressure, inhibition of platelet aggregation, and as an effector of immune responses. Excess production of ($NO^{\bullet}$) can be cytotoxic, reaction between ($NO^{\bullet}$) and (ROS) can yield secondary highly reactive species. For instance, the reaction of ($NO^{\bullet}$) with $O_2^{\bullet -}$ can yields peroxynitrite ($ONOO^-$) an effective oxidizing species (Koppenol *et al*., 1992). Figure 1.1 demonstrates the several intercellular sources of free radicals.

Environmental pollutants are additional sources for free radicals generation. These exogenous sources included nitrogen dioxide, ozone, cigarette smoke, radiation, halogenated hydrocarbons, heavy metals, and certain pesticides (Lykkesfeldt & Svendsen, 2007). Alcohol consumption can provoke oxidative reactions in the liver. Certain chemotherapeutic agents including doxorubicin, cyclophosphamide, 5-fluorouracil, methotrexate, and vincristine can generate oxygen radicals at doses used in cancer patients. Increased physical activity also can produce free radicals as a consequence of increased oxygen consumption during exercise (Davis *et al*., 2006). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) along with other reactive species are called oxidants.

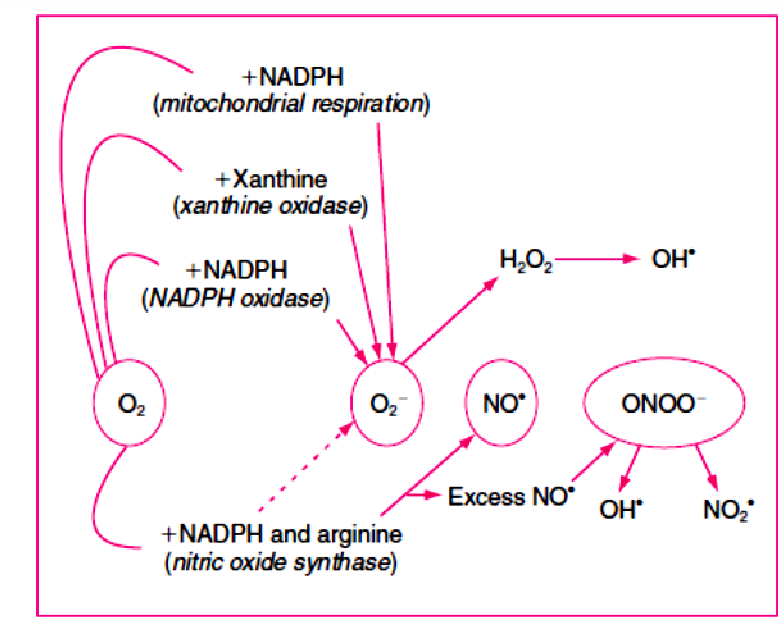

Figure 1.1. Sources of intercellular reactive oxygen species and reactive nitrogen species. (Rahman & MacNee, 2002)

As discussed previously, these oxidants or reactive species in small amount can play an important role as part of the immune defense mechanism, as growth regulator, and signal transducers. But presence of excess amount of these compounds can lead to cell damage and cause state known as oxidative stress (de Oliveira *et al*., 2009).

1.2 Oxidative Stress.

Oxidative stress can be defined as an imbalance between the oxidants and antioxidant at the cellular or individual level (Cornelli, 2009). Various factors such as drugs, diseases, pollution, can cause imbalance between oxidants production and antioxidants repair capacity, which will lead to the oxidative stress. Generally, oxidative stress is temporary condition under strict control by the antioxidant defense network and once it becomes constant oxidative stress can cause disease. Oxidative stress can seriously damage cellular macromolecules (such as lipid, proteins, and nucleic acids), cell death by apoptosis or necrosis, as well as structural tissue damage (Lykkesfeldt & Svendsen, 2007).

1.2.1 Lipid Peroxidation.

Lipids are important components of the lipid bilayer of the cellular membrane, and unsaturated fatty acids specially are easily oxidised and may start chain reactions resulting in oxidative damage. This process in which polyunsaturated fatty acids in the cell membrane are oxidized is known as lipid peroxidation (Oldham & Bowen, 1998). Lipid peroxidation is initiated by a hydroxyl radical OH when this species captures a hydrogen atom from a methylene carbon in the polyalkyl chain of the fatty acid. The unpaired fatty acids undergoes rearrangement by reaction with O_2 and formation of peroxyl radical ($LOO^{\bullet}$). At this step, the propagation reaction starts because ($LOO^{\bullet}$) take out a hydrogen atom from the closest fatty acid. The result is the formation of a hydroperoxide (LOOH) and an unpaired fatty acid ($L^{\bullet}$). Therefore, the peroxidation of unsaturated fatty acids can provoke the conversion of several fatty acids, which in turn leads to the generation of a chain reaction (Gate *et al*., 1999). Lipid peroxidation can

cause serious injury to cell membrane by altering their fluidity, permeability, and integrity (Schiller *et al*., 1993).

1.2.2 DNA Oxidation.

Oxidants are responsible for many types of DNA damages. They can attack DNA at the deoxyribose molecule or at any of the purine or pyrimidine bases. Attack at the deoxyribose can lead to sugar fragmentation, base loss, and strand breaks, while attack at a base can lead to modification of the nucleotide bases (Dizdaroglu, 1991). The oxidation of guanine by the hydroxyl radical (OH) to 8-hydroxy-2-deoxyguanosine (8-OHdG), modifies DNA and leads to mutagenesis and carcinogenesis (Gate *et al*., 1999). Although, several mechanisms exist to repair the DNA damage, but the mechanisms are not error proof and may not be able to repair all of the damage. DNA damage has mutagenic effect in bacteria as well as other mammalian cells, and may even cause cell death. Other effects of DNA damage include decreased cell proliferation caused by unpaired DNA replication and decreased protein production from transcription of modified DNA (Oldham & Bowen, 1998).

1.2.3 Protein Oxidation.

Proteins are also targets for oxidative stress. Protein oxidation leads to inactivating enzymes unable of achieving their cellular task. The main amino acid target for oxidative alteration in proteins is the reactive sulfhydryl of cysteine residues. For instance, oxidation of a critical cysteine in glyceraldehyde-3-phosphate dehydrogenase leads to enzyme malfunction and subsequent impairment of glycolisis in cells (Souza & Radi, 1998). Other amino acids that are susceptible to oxidative modification include methionine, phenylalanine, histidine, proline, arginine, tyrosine, and tryptophan (Castro & Freeman, 2001). Protein oxidation is associated with alteration of signal transduction mechanisms, transport system, and enzymes activities. It may also be associated with aging (Gate *et al*., 1999).

It has been demonstrated that oxidative stress is contributed to various diseases including inflammation, cancer, atherosclerosis, asthma, rheumatoid arthritis, heart disease, cellular aging (Niederlander *et al*., 2008), as well as neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (Dasgupta & De, 2007). Fortunately, our cells equipped with a comprehensive antioxidant defence network, which protect our cell against the damage caused by the oxidative stress.

1.3 Antioxidants.

According to Halliwell and Gutteridge (1999) antioxidants can be defined as "any substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate". The formation of reactive species includes three steps initiation, propagation, and termination. Antioxidant defence network can affect the formation of these reactive species during any of these three steps (Davis *et al*., 2006).

The antioxidant defence network can be categorized into four groups, the first defence line in this network is a group of enzymes function to destroy or detoxify oxidants. For instance, hydrogen peroxide one of the oxidant family, there are several enzymes capable of detoxifying it. The enzyme catalaze can reacts with hydrogen peroxide to form oxygen and water. Another group of enzymes, called glutathione peroxidases, reduce hydrogen peroxide to water (Myers & Theodorescu, 2003). Furthermore, Superoxide dismutases enzymes (SOD) catalyse the one-electron alteration of superoxide into hydrogen peroxide and oxygen (Mates, 1999). SOD and catalaze are dismutases, and thus they do not consume cofactors. The main reactions catalysed by such enzymes are not associated with any energetic cost. On the other hand, glutathione peroxidases are reductases whose reducing coenzymes are regenerated by NAD(PH) equivalents formed in metabolic pathways (Chaudieare & Ferrari-Iliou, 1999). The second group of the antioxidant network are vitamins that act as antioxidants. Vitamin C (ascorbic acid) and vitamin E (α tocopherol) are the essential members of this group. Vitamin C is water soluble and it has the ability to quench free radicals as well as singlet oxygen. Vitamin E is lipid soluble which present in all cellular membranes, and acts as antioxidant for body lipids. The third group are proteins that sequester iron and copper. This is significant because free iron and copper can

stimulate the alteration of peroxides into free radicals that rapidly react with and destroy normal tissues. Under normal conditions, this system is very successful that free iron and copper do not exist in body fluids or tissues, but when sequestration of iron fails, it causes hemochromatosis and hemosiderosis. On the other hand, failure sequestration of copper will cause Wilson's disease. The final group in the antioxidant defence network is group of dietary antioxidants. It's now apparent that most of the fruits, vegetables, and grains contain antioxidants as well as numerous medicinal plants (Myers & Theodorescu, 2003).

In addition to their medical important, naturally occurring antioxidant compounds play an important role in the preservation of food (products) and minimizing of the formation of harmful substances (Oliveira *et al*., 2009). Furthermore, natural antioxidants derived from plants can replace the synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which are now doubted due to their potential toxicity and carcinogenicity (Han *et al*., 2008).

1.4 Plant Secondary Metabolites as Natural Antioxidants.

Plant compounds can be categorized into primary or secondary compounds depending on whether or not they have a significant role in plant metabolism and are commonly present in plants. As a result of the progress in the biochemical techniques and molecular biology, nowadays, it has been clearly demonstrated that secondary products play a key role in the adaptation of plants to their environment or controlling plant growth. Secondary compounds are largely related to plant wellness, it has been demonstrated that these compounds have antibiotic, antifungal and antiviral properties, and thus they have the ability to protect plants against pathogens, ultraviolet radiation, and herbivores (Heim *et al*., 2002). In addition, secondary metabolites (phenolic compounds) are responsible for the pollination in plants by providing colours attractive to the pollinating insects (Cook & Samman, 1996), beside these functions they also involve in the processes of photosensitisation, energy transfer, the actions of plant growth hormones and growth regulators, control of respiration and photosynthesis, morphogenesis and sex determination as well as other important properties (Bravo, 1998).

Due to their enormous biological properties, plant secondary compounds have been used for long time in traditional medicine. At this time, they considered to be an important source of valuable compounds such as pharmaceutics, cosmetics, fine chemicals, and recently nutraceutics. Nowadays, in western countries 25% of the compounds used in the pharmaceutical industry are of natural plant origin. According to their biosynthetic pathways, plant secondary metabolites can be classified into three general families: phenolic compounds, alkaloids, and terpenoids (Bourgaud *et al*., 2001).

1.4.1 Phenolic Compounds.

Phenolic compounds are the most numerous and largest group of the secondary metabolites and the most widely occurring group in the plant kingdom, with more than 8000 phenolic phytochemicals have been identified (Soobratee *et al*., 2005). Structurally, phenolic compounds consist of an aromatic ring, with one or more hydroxyl substituents. Phenolics can range from simple molecules to highly polymerised compounds. Generally, phenolic compounds can be classified into several subgroups (Harborne, 1999). The main subgroups of these are flavonoid, phenolic acids, and phenolic polymers (King & Young, 1999).

Flavonoids are the largest group of phenolics with low molecular weight based on the 2-phenyl-benzo [α] pyrane or flavane nucleus, which comprises two benzene rings (A and B), linked together via a heterocyclic pyrane ring (C). They are widely distributed in fruit and vegetables and represent a common ingredient of the human diet (Cushnie & Lamb, 2005).

Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids (Bondia-Pons *et al*., 2009). Hydroxybenzoic acids mainly gallic and ellagic acid are commonly occur as hydrolysable tannins and are found basically in berries and nuts. The combination of caffeic acid and quinic acid will form chlorogenic acid which is commonly distributed in fruits and vegetables (King & Young, 1999).

Polyphenols generally known as tannins are compounds with high molecular weight. Tannins can be divided into condensed tannins which are polymers of catechins or epicatechins and hydrolysable tannins which are polymers of gallic and ellagic acid (King & Young, 1999).

The potential medicinal and pharmacological benefits of phenolic compounds are mostly related to their antioxidant activities (Heim *et al*., 2002). The antioxidant property of phenolics is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations. The structure of phenolic compounds is responsible of their antioxidant activity (Rice-Evans *et al*., 1996). Because of their diversity and extensive distribution, phenolic compound considered to be the most significant group of natural antioxidants (Robards *et al*., 1999).

1.4.2 Alkaloids.

Alkaloids are group of nitrogen-containing compounds and present 20% in plant species. Alkaloids have been used as pharmaceuticals, stimulants, and poisons (Provenza & Villalba, 2010). According to their carbon skeleton, alkaloids can be classified into many subclasses such as indole, quinoline, isoquinoline, steroidal, etc (Harborne, 1999). Many researchers have reported that alkaloids have antioxidant activities. Rackova *et al*. (2004) have revealed that alkaloids berberine and jatrorrhizine have antiradical and antioxidant activities. According to Martinez *et al*. (1992) canadine and anonaine showed interesting antioxidant properties.

1.4.3 Terpenoids.

Terpenoids are very large and diverse classes of unsaturated hydrocarbons with more than 30,000 identified compounds (Provenza & Villalba, 2010). According to the number of five-carbon (C_5) units that are present, terpenoids can be subdivided from monoterpenoids to the tetraterpenoids (Harborne, 1999). Terpenoids have antioxidant activities especially against lipid peroxidation as a result of their great lipophilicity (Grassmann *et al*., 2002). It's well known that terpenoids represent the basis of several drugs used in the treatment of many diseases correlated with ROS (Harborne, 1999).

1.5 Medicinal Plants - *Ardisia crispa*.

Since ancient times, medicinal plants have been used in almost all cultures as source of medicine (Hoareau & DaSilva, 1999). More than three-quarters of the world population depends chiefly on plants and plant extracts for health care. More than 30% of the entire plant species were used for medicinal reasons. In developed countries like United States, plant drugs represent as much as 25% of the total drugs, whereas in fast developing countries like China and India, the contribution increased to 80% (joy *et al*., 2001). In Malaysia, more than 3,200 species of higher plants are stated to have medicinal worth and have been used for generations in many traditional health care systems (Philip *et al*., 2009). Nowadays, The Malaysian herbal product market is undergoing a remarkable growth. Malaysia consumes RM 1.2 billion worth of imported herbal products every year. In response to the increased usage of medicinal plants by the Malaysian people, the government identified several plans to improve the progress of the local herbal industry (Jantan, 2004). Recently, People are returning to the natural drugs derived from medicinal plants with hope of safety and security.

A. Description of *Ardisia crispa*.

Ardisia crispa, which is also known as village ardisia or *mata ayam* (Malaysia) is a shrub up to (3 m). *Ardisia crispa* can be found in India, Japan, Malaysia and it is common in vacant plots, villages, seashores and secondary rain forest throughout Southeast Asia (Christophe, 2002).

The leaves of *Ardisia crispa* are large (10cm x 4cm), simple, alternate, and without stipules. Petiole slender. Blade narrowly elliptical, tapered to apex and base, and margin laxly crenate. Inflorescence terminal. Flowers are small and pink. Calyx consists of 5 free sepals. Corolla tube short and consisting of 5 spreading petals. Stamens are 5 with short pedicels and large anthers forming a conspicuous cone around the style. The fruit is a bright, red, and small berry. *Ardisia crispa* is widely distributed in the region with 2000 meters of altitude and widely distributed in Asia stretching from Japan and the Himalayas to java and Philippines (Christophe, 2002).

Figure 1.2. Leaves and fruits *of Ardisia crispa*

B. Medicinal Uses of *Ardisia crispa*.

The root and leaves are commonly used in folklore medicine. The root is used as one of the traditional ingredient in 'meroyan' where the root is boiled and used to treat pain in the throat and chest as well as to treat rheumatism. The mixture of its leaves and root is used as skin liniment (Muhamad & Mustafa, 1994). In Thailand, the root is mixed with other plants and used to wash 'dirty blood' or in woman with dysmenorhea. In China the roots of *Ardisia crispa* are used to reduce fever and to stop excessive salivation. In Malaysia the juice expressed from the leaves is used to treat scurvy. In Taiwan this plant is used to promote urination (Christophe, 2002).

C. Chemical Constituents of *Ardisia crispa*:

The reports about the phytochemistry of *Ardisia crispa* are very limited. (Jansakul *et al*., 1987) reported that roots of *Ardisia crispa* contain two triterpenoid saponins namely ardisiacrispins A and B. Hofstra *et al*. (1970) have stated the presence of anthocyanin in the leaves of *Ardisia crispa*. Yoshida *et al*. (1987) have isolated n-peptide compound from the whole plant of Ardisia crispa and the compound has shown antihypertensive and antiplatelet aggregating activities. AC7-1 was isolated from *Ardisia crispa* and identified as 2-methoxy-6-tridecyl-1,4-benzoquinone, this compound was reported to have antimetastatic and antitumor effects (Kang *et al*., 2001).

1.7 The Research Objectives:

1. To extract the chemical compounds from the leaves and fruits of *Ardisia crispa*.

2. To separate the chemical compounds using Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC).

3. To evaluate the antioxidant activities of crude extracts from *Ardisia crispa* using three different assays
   a. DPPH free radical scavenging assay
   b. Reducing power assay
   c. Metal chelating assay

4. To determine the total phenol and flavonoid content.

5. To determine the toxicity of crude extracts of *Ardisia crispa* using Brine Shrimp Lethality Assay (BSLA).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Preparation of Plant sample.

Fresh leaves and fruits of *Ardisia crispa* were obtained from area inside the University of Malaya campus. The leaves and fruits were separated and dried at room temperature for approximately 2 weeks. After drying the leaves and the fruits were ground into fine powder using grinder.

2.2 Preparation of Crude Extracts.

Four solvents namely hexane, chloroform, methanol, and water were used for plant extraction. 20 g of each dried sample were soaked in 200 ml of Hexane for 3 days at 42 °C using water bath. The solvent containing – extracts were then filtered through Whatman No. 1 and concentrated to 10 ml using rotary evaporator. The residues were further extracted with 200 ml of chloroform for 3 days and filtered. The same step was repeated with methanol. For water extraction, 10 g of each dried sample were mixed with 100 ml of distilled water and put in water bath at 42 °C for 5 hr. The extracts were filtered through Whatman No. 1 and followed by concentration to approximately 10 ml using rotary evaporator.

Types of crude extracts have been prepared.

LH**:** Leaves Hexane extract of *Ardisia crispa*

LCh**:** Leaves Chloroform extract of *Ardisia crispa*

LM**:** Leaves Methanol extract of *Ardisia crispa*

LW**:** Leaves Water extract of *Ardisia crispa*

FH**:** fruits Hexane extract of *Ardisia crispa*

FCh**:** fruits Chloroform extract of *Ardisia crispa*

FM**:** fruits Methanol extract of *Ardisia crispa*

FW**:** fruits Water extract of *Ardisia crispa*

2.3 Separation of Bioactive Compounds from *Ardisia crispa*.

A. Thin layer chromatography (TLC).

Thin layer chromatography (TLC) was used in this study to detect the presence of bioactive compounds in the leaves and the fruits of *Ardisia crispa*. Aluminum supported silica gel 60 F_{254} TLC plates, size 20X20 cm with thickness of 0.25 mm were prepared. The samples were applied on the TLC plates as spots using fine glass capillary tube and then developed in saturated chromatographic tanks with various appropriate solvent systems at room temperature. The spotted TLC plates were examined under UV light and later sprayed with specific reagents.

Detection of Chemical Compounds Using Specific Reagents.

1. Dragendroff's Reagent.

Preparation:

Solution A: 1.7g of bismuth nitrate in 100ml distilled water : acetic acid (80 : 20).

Solution B: 40g of Potassium iodide in 100ml distilled water.

Solution C: 20ml acetic acid in 70ml distilled water.

5ml of solution A was mixed with 5ml of solution B then added to solution C.

Application of the reagent:

Orange spot indicates the presence of alkaloid compounds.

2. Vanillin-sulphuric acid Reagent.

Preparation:

1ml of concentrated sulphuric acid was added to 1g of vanillin, and then this solution was mixed with 100ml of ethanol and stirred. The TLC plates which sprayed with this reagent were heated at 120 °C.

Application of the reagent:

Purple spot indicates presence of terpeniod compounds

Red spot and other indicates presence of phenolic compounds

3. Anisaldehyde-Sulphuric Acid Reagent.

Preparation:

0.5ml of anisaldehyde was mixed with 80ml of methanol, 10ml of glacial acetic acid, and 5ml of sulphuric acid. TLC plates sprayed with reagent were heated at 100 °C.

Application of the reagent:

Red spot indicates the presence of flavonoids

Purple spot indicates the presence of terpenoid compounds

Blue and black spot indicates the presence of saponins

B. High Performance Liquid Chromatography (HPLC).

The HPLC analysis was performed as described by (Chen *et al*., 2001). The HPLC system from model Shimadzu consisted of LC-10AT pump system (Shimadzu Co. Japan) and an SPD-M10A DIODE array detector. The bioactive compounds were detected at both 280 and 360 nm. The column used was a Thermo scientific Hypersil BDS-C_{18} (250 x 4.6 mm, 5µm). A gradient solvent system consisting of solvent A (water-acetic acid, 97:3, v/v) and solvent B (MeOH) was used. The solvent gradient is presented in table 2.1.

Table 2.1. HPLC solvent gradient elution program

Time (min)	Solvent B (%)	Flow-rate (ml/min)
0	0	0.9
10	10	1.0
40	70	1.0
44	0	0.9
47	0	0.9

2.4 Antioxidant Activity of Crude Extracts.

A. DPPH Radical Scavenging Activity Assay.

The scavenging activity of plant extracts on 2,2 diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to the method described by (Sanchez-Moreno *et al.,* 1999) with slightly modification.

Preparation of Extracts.

A stock solution of each extract was prepared by dissolving the extract in methanol at concentration of 20mg/ml and wrapped with aluminum foil.

Preparation of Ascorbic Acid.

A stock of ascorbic acid was prepared by dissolving in methanol at concentration of 400µg/ml. The stock solution was kept in flask wrapped with aluminum foil.

Preparation of DPPH.

A stock of DPPH was prepared by dissolving in methanol at concentration of 8mg/ml. The stock was kept in flask wrapped with aluminum foil.

Scavenging Activity of Ascorbic Acid as Positive Reference.

Ascorbic acid was used as positive standard in DPPH radical scavenging activity assay. The reaction mixture of ascorbic acid, DPPH, and methanol was prepared as shown in table 2.2a.

Table 2.2a. Preparation of reaction mixture of ascorbic acid, DPPH, and methanol for DPPH assay.

Concentration of ascorbic acid (mg/ml)	**Volume of methanol (µl)**	**Volume of ascorbic acid (µl)**	**Volume of DPPH solution (µl)**
200.00	475.00	500.00	25.00
100.00	725.00	250.00	25.00
50.00	850.00	125.00	25.00
25.00	912.00	62.50	25.00
12.50	943.75	31.25	25.00
6.25	959.38	15.63	25.00
3.12	967.19	7.81	25.00
1.56	971.09	3.91	25.00
Control	975.00	-	25.00
Blank	1000.00	-	-

The reaction mixtures were allowed to stand at room temperature for 30 minutes. The reduction of DPPH radical was measured using spectrophotometer at 517 nm. The degree of discoloration indicates the free radical scavenging efficiency of ascorbic acid.

Scavenging Activity of Crude Extracts on DPPH.

The scavenging activity of each crude extract was tested at different concentrations (5mg/ml, 4mg/ml, 3mg/ml, 2mg/ml, and 1mg/ml). Reaction mixtures containing crude extracts, DPPH, and methanol were prepared according to table 2.2b. All tests were carried out in duplicates and the mean values were used to calculate the IC_{50}.

Table 2.2b. Preparation of reaction mixtures of crude extracts, DPPH, and methanol for DPPH assay.

Concentration of crude extracts (mg/ml)	**Volume of methanol (µl)**	**Volume of ascorbic acid (µl)**	**Volume of DPPH solution (µl)**
5	725.00	250.00	25.00
4	775.00	200.00	25.00
3	825.00	150.00	25.00
2	875.00	100.00	25.00
1	925.00	50.00	25.00
Control	975.00	-	25.00
Blank	1000.00	-	-

Determination of Percentage of Inhibition.

DPPH radical scavenging activity of each concentration of crude extracts was expressed as percentage of inhibition which was calculated according to the following formula:

% inhibition = $[(OD_{control} - OD_{sample}) / OD_{control}] \times 100$

Where, $OD_{control}$ = absorbance value of control

OD_{sample} = absorbance value of sample

A graph of percentage of inhibition against concentration was plotted to determine the IC_{50} value for each crude extract.

B. Ferric Reducing Antioxidant Power Assay (FRAP).

The reducing power of crude extracts was determined using the method described by (Oyaizu, 1986) with some modifications. The purpose of this assay is to evaluate the ability of each crude extract to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}).

Preparation of 1% Potassium Ferricyanide $K_3[Fe(CN)_6]$ Solution.

A stock of potassium ferricyanide was prepared at the concentration of 10 mg/ml. 600 mg of potassium ferricyanide stock was dissolved in 60 ml distilled water. The stock solution was kept in flask wrapped with aluminum foil.

Preparation of 10% Trichloroacetic Acid (TCA).

A stock of trichloroacetic acid was prepared at the concentration of 100 mg/ml. 6 g of trichloroacetic acid was dissolved in 60 ml distilled water. The stock was kept in flask wrapped with aluminum foil.

Preparation of 0.1% Ferric Chloride Solution ($FeCl_3$).

A stock of ferric chloride (sigma) was prepared at the concentration of 1 mg/ml. 0.01 g of ferric chloride stock was dissolved in 10 ml distilled water. The stock was kept in flask wrapped with aluminum foil.

Preparation of 0.4 M Monobasic Stock Solution.

13.9 g of sodium phosphate monobasic (Na_2HPO_4) was dissolved in 500 ml distilled water. The stock was kept in flask prior to preparing 0.2 M phosphate buffer.

Preparation of 0.4 M Dibasic Stock Solution.

28.4 g of sodium phosphate dibasic (NaH_2PO_4) was dissolved in 500 ml distilled water. The stock was kept in flask prior to preparing 0.2 M phosphate buffer.

Preparation of 0.2 M Sodium Phosphate Buffer Solution.

178.5 ml of monobasic solution was mixed with 112.5 ml dibasic solution, followed by 600 ml distilled water. The solution was adjusted to pH 6.6.

Preparation of FRAP Assay.

0.5 mL of each crude extract at different doses (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml) was mixed with phosphate buffer (0.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (0.5 ml). The mixture was incubated at 50 °C for 20 min. After incubation 0.5 ml of 10% trichloroacetic acid was added and then mixture was centrifuged for 10 min at 3000 rpm. The upper layer (0.5 mL) was mixed with 0.5 mL of distilled water and 0.1 ml of 1% $FeCl_3$ for 10 min, and the absorbance was measured at 700 nm using spectrophotometer with higher absorbance indicating greater reducing power.

In this method butylated hydroxyyanisole (BHA) was used as positive reference standard using the same procedure mentioned above. All tests were carried in duplicates.

C. Metal Chelating Activity Assay.

The chelating activity of crude extracts was determined according to the method of (Dinis *et al*., 1994). The purpose of this assay is to evaluate the ability of crude extracts to chelate ferrous ion and preventing the formation of ferrozine-Fe^{2+} complex.

Preparation of 5mM Ferrozine.

0.0246 g of ferrozine stock was dissolved in 10 ml deionized water. The stock was kept in flask wrapped with aluminum foil.

Preparation of 2mM Ferrous Chloride ($FeCl_2$).

0.004 g of $FeCl_2$ stock was dissolved in 10 ml deionized water. The stock was kept in flask wrapped with aluminum foil.

Preparation of Metal Chelating Activity Assay for EDTA as Positive Reference.

Ethylenediaminetetraacetic acid (EDTA) was used as a positive reference standard. 0.1 mg/ml of EDTA was prepared by dissolving 0.01 g of EDTA in 4 ml of deionized water. The pH was adjusted while stirring with NaOH solution until most of EDTA dissolved. Once the EDTA dissolved the total volume was adjusted with deionized water until 10 ml. volumes of EDTA at different concentrations was mixed with deionized water followed by ferrous chloride ($FeCl_2$) and ferrozine respectively according to Table 2.3a.

Table 2.3a. Preparation of reaction mixture of EDTA, ddH_2O, $FeCl_2$, and ferrozine.

Concentration of EDTA (mg/ml)	**Volume of dd H_2O (µl)**	**Volume of EDTA (µl)**	**Volume of $FeCl_2$ 2mM (µl)**	**Volume of ferrozine 5mM (µl)**
1	-	1000	50	200
0.5	500	500	50	200
0.25	750	250	50	200
0.125	875	125	50	200
0.0625	937.5	62.5	50	200
Control	1000	-	50	200

The reaction mixtures were shaken vigorously, and the absorbance of each sample was measured at 562 nm using spectrophotometer. Deionized water was used as blank. All samples were tested in duplicates. The percentage of inhibition of ferrozine-Fe^{2+} complex of each sample was calculated according to the following formula:

% inhibition = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

Where, $Abs_{control}$ = absorbance reading of control

Abs_{sample} = absorbance reading of sample

Preparation of Metal Chelating Activity Assay for Crude Extracts.

The crude extracts at different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml) were mixed with deionized water, followed by $FeCl_2$ and ferrozine respectively as shown in Table 2.3b. The reaction mixtures were shaken vigorously, and the absorbance of each sample was measured at 562 nm using spectrophotometer. Deionized water was used as blank. All samples were tested in duplicates and the mean values were used to calculate the IC_{50}. The percentage of inhibition of ferrozine-Fe2+ complex of each sample was calculated according to the following formula:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Where, $Abs_{control}$ = absorbance reading of control

Abs_{sample} = absorbance reading of sample

Table 2.3b. Preparation of reaction mixtures of crude extracts, deionized water, $FeCl_2$, and ferrozine.

Concentration of crude extract (mg/ml)	**Volume of methanol (µl)**	**Volume of crude extract (µl)**	**Volume of $FeCl_2$ 2mM (µl)**	**Volume of ferrozine 5mM (µl)**
1	-	1000	50	200
0.5	500	500	50	200
0.25	750	250	50	200
0.125	875	125	50	200
0.0625	937.5	62.5	50	200
Control	1000	-	50	200

2.5 Determination Total Flavonoid Content.

Total flavonoid content was measured according to the method described by (Zhishen *et al*., 1999).

Preparation of 10% Aluminum Chloride ($AlCl_3$) Solution.

Aluminum chloride was prepared by dissolving 10 g of $AlCl_3$ in 90 ml distilled water.

Preparation of 5% Sodium Nitrate ($NaNO_3$) Solution.

Sodium nitrate was prepared by dissolving 5 g of $NaNO_3$ in 95 ml distilled water.

Preparation of 1M Sodium Hydroxide (NaOH) Solution.

Sodium hydroxide was prepared by dissolving 20 g of NaOH in 500 ml distilled water

Preparation of Quercetin Standard Solution.

Quercetin was used as positive standard in this assay. 0.025 g of quarcetin stock was dissolved in 10 ml of methanol.

Preparation of Total Flavonoid Assay.

1 mL of each extract was added to 0.3 mL of 5% $NaNO_3$. After 5 min. incubation in water bath at 37 °C, 0.3 ml of 10 % $AlCl_3$ was added and the mixture was incubated at 37 °C for 6 min. 2ml of 1M NaOH were added to the mixture followed by 10 ml of distilled water. The solution was mixed well and absorbance was measured

using spectrophotometer at 510 nm, all samples were tested in duplicates. Methanol was used as blank. A calibration curve, using quercetin with concentrations range from 0.5 – 2.5 mg/ml was prepared. The results were mean values and were expressed as mg of QE (Quercetin equivalents)/ml.

2.6 Determination Total Phenolic Content.

Total phenolic content was measured according to the method described by (Velioglu *et al*., 1998)

Preparation of Folin-Ciocalteau Reagent.

Folin-Ciocalteau reagent was prepared by mixing 20 ml of the reagent with 200 ml of distilled water.

Preparation of 1M Sodium Bicarbonate Solution.

Na_2CO_3 was prepared by dissolving 15.8985 g of Na_2CO_3 stock in 150 ml distilled water.

Preparation of Gallic Acid 0.001 g/ml Standard Solution.

Gallic acid was prepared by dissolving 0.01 g of gallic acid stock in 10 ml distilled water.

Preparation of Total Phenolic Assay.

0.5 mL of each extract was added to 5 mL of Folin-Ciocalteau reagent followed by addition of 4 ml of 1M Na_2CO_3 and allowed to stand in water bath at 45 °C for 15 min. The solution was mixed well and absorbance was measured using spectrophotometer at 765 nm, all samples were tested in duplicates. Methanol 50% was used as blank. A calibration curve, using gallic acid with concentrations range from 0.5 – 2.5 mg/ml was prepared. The results were mean values and were expressed as mg of GAE (gallic acid equivalents)/ml.

2.7 Brine Shrimp Lethality Assay (BSLA).

This test was carried out according to the method of (Rahman *et al*., 2001) with slight modifications. In this test the eggs of the brine shrimp or *Artemia salina* nauplii were used. Artificial sea water was prepared by dissolving 38g sea salt in 1L of distilled water using magnetic stirrer, and then filtered out to remove any insoluble salt. The sea water was poured in small tank and shrimp eggs were added to the larger compartment of the tank which is darkened by covering it with aluminum foil. The brine eggs were hatched by incubation for a period of 48hr with continues presence of intense light.

Plant extracts were prepared at 20mg/ml from this stock solution 3 different concentration were obtained 1000 µg/ml, 100 µg/ml, and 10 µg/ml respectively. After hatching the *Artemia salina* nauplii were transferred into vials at different concentrations 1000µg/ml, 100µg/ml, and 10µg/ml. A triplicate was done for each concentration. After 24hr the number of surviving *Artemia salina* nauplii was counted and percentage of mortality was calculated. Finney's Probit analysis was used to determine the LC50 of each extract.

CHAPTER THREE

RESULTS

3.1 Separation of Bioactive Compounds from *Ardisia crispa*

A. Thin Layer Chromatography (TLC):

The extracts of both leaves and fruits of *Ardisia crispa* were subjected to TLC using TLC aluminum silica gel 60 F_{254} sheets. The presence of various secondary metabolites was detected by spraying sheets with specific visualization reagents. The results as shown below:

Table 3.1. Thin Layer Chromatography of leaves hexane extract from *Ardisia crispa*

Label compounds	**Rf value x 100 Solvent system $CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
LH1	94	Brown (++)	-ve	-ve	-ve	Black (++)	Saponin
LH2	88	Brown (++)	-ve	Orange (++)	-ve	-ve	Alkaloid

Indication for intensity of colour:
+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.2. Thin Layer Chromatography of leaves chloroform extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system $CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
LCH1	95	Brown (+++	Brown (+++)	-ve	-ve	Black (++)	Saponin
LCH2	93	Yellow (++)	-ve	Orange (++)	-ve	-ve	Alkaloid
LCH3	90	-ve	-ve	-ve	-ve	Purple (+)	terpenoid
LCH4	75	Green (++)	-ve	-ve	-ve	-ve	Chlorophyll
LCH5	62	Green (+)	-ve	-ve	Red (++)	Red (++)	Flavonoid
LCH 6	22	-ve	-ve	-ve	Red (+)	Red (+)	Flavonoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.3. Thin Layer Chromatography of leaves methanol extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
LM1	97	Green (++)	-ve	-ve	-ve	Black (++)	Saponin
LM2	95	Yellow (++)	-ve	-ve	-ve	Purple (++)	Terpenoid
LM3	87	-ve	-ve	Orange (++)	-ve		Alkaloid
LM4	77	-ve	-ve	-ve	Red (++)	Red (++)	Phenolic
LM5	70	-ve	-ve	-ve	Red (+)	Red (+)	Phenolic
LM6	54	-ve	-ve	-ve	-ve	Purple (++)	Terpenoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.4. Thin Layer Chromatography of leaves water extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-** **H_2SO_4**	**Anisaldehyde-** **H_2SO_4**	
LW1	94	Brown (+)	-ve	-ve	Purple (++)	Purple (+)	Terpenoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.5. Thin Layer Chromatography of fruits hexane extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
FH1	97	Brown (++)	-ve	-ve	-ve	Black (++)	Saponin
FH2	94	Yellow (++)	-ve	-ve	-ve	Purple (+++)	Terpenoid
FH3	85	Green (+)	-ve	-ve	-ve	-ve	Chlorophyll
FH4	77	Red (+)	-ve	-ve	Red (+++)	Red (+++)	Phenolic
FH5	58	-ve	-ve	-ve	-ve	Purple (+)	Terpenoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.6. Thin Layer Chromatography of fruits chloroform extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
FCH1	97	Brown (++)	-ve	-ve	-ve	Black (++)	Saponin
FCH2	94	Yellow (++)	-ve	-ve	-ve	Purple (++)	Terpenoid
FCH3	84	Green (+)	-ve	-ve	-ve	-ve	Chlorophyll
FCH4	77	Red (+)	-ve	-ve	Purple (+++)	Purple (+++)	Terpenoid
FCH5	68	Green (+)	-ve	-ve	-ve	-ve	Chlorophyll
FCH6	60	-ve	-ve	-ve	-ve	Purple (+)	Terpenoid
FCH7	25	-ve	-ve	-ve	Red (+)	Red (+)	Phenolic

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.7. Thin Layer Chromatography of fruits methanol extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-H_2SO_4**	**Anisaldehyde-H_2SO_4**	
FM1	97	Brown (++)	-ve	-ve	-ve	Black (++)	Saponin
FM2	94	-ve	-ve	-ve	-ve	Purple (++)	Terpenoid
FM3	81	Green (+)	-ve	-ve	-ve	-ve	Chlorophyll
FM4	71	-ve	-ve	-ve	Red (+++)	red (+++)	Phenolic
FM5	55	-ve	-ve	Orange (+)		-ve	Alkaloid
FM7	32	-ve	-ve	-ve	-ve	Purple (+)	Terpenoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

Table 3.8. Thin Layer Chromatography of fruits water extract from *Ardisia crispa*

Label compounds	**Rf x 100** **Solvent system** **$CHCL_3$: Me0H: H_2O (40: 9: 1)**	**Visible light**	**UV light**	**Spraying Reagents**			**Remarks**
				Dragendorff's	**Vanillin-** **H_2SO_4**	**Anisaldehyde-** **H_2SO_4**	
FW1	94	Brown (+)	-ve	-ve	Purple (++)	Purple (++)	Terpenoid

Indication for intensity of colour:

+++ = Strong
++ = Medium
+ = Weak
-ve = no colour observed

B. High Performance Liquid Chromatography (HPLC).

In order to identify the compounds present in the leaves and fruits extracts from *Ardisia crispa*, HPLC was done to 4 crude extracts namely Fruits methanol extract, leaves methanol extract, fruits water extract, and leaves methanol extract. The standard used for validation was gallic acid, the retention time for gallic acid as shown in Figure 3.1. From the HPLC analysis of the crude extracts two major peaks A and B on the chromatogram were screened. Peak A was identified as gallic acid when compared to the standard, approximately both gallic acid standard and peak A have the same retention time. Since we used only one standard in our study, peak B was not identified. Chromatograms for crude extracts are shown below.

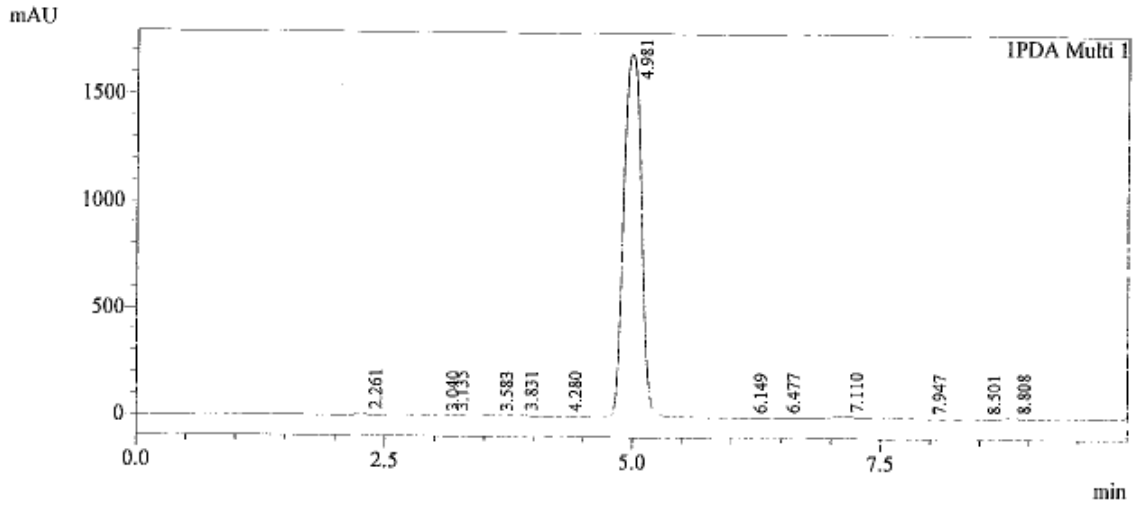

Figure 3.1. HPLC chromatogram of gallic acid standard

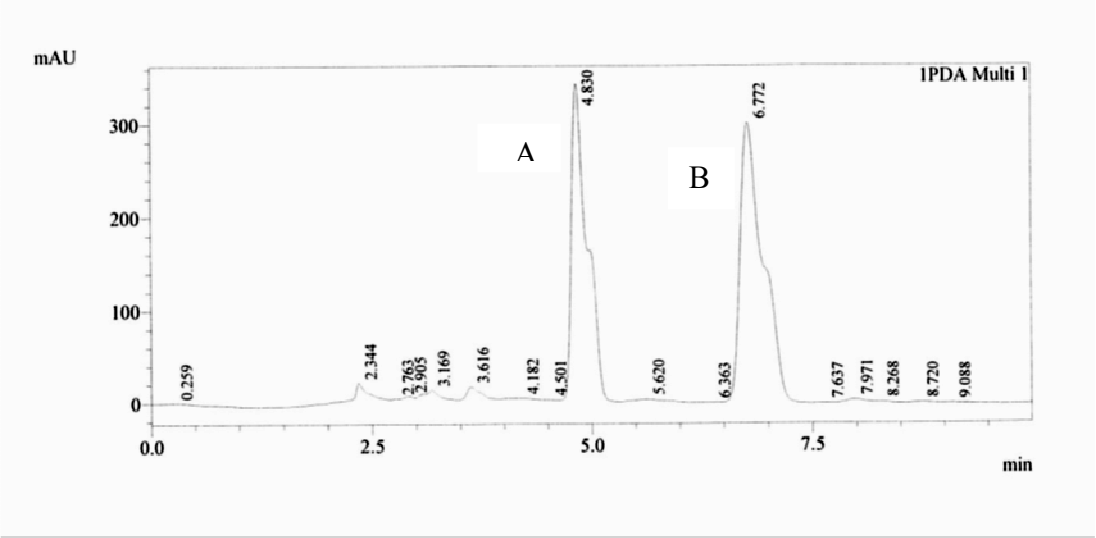

Figure 3.2. HPLC chromatogram of fruits methanol extracts from *Ardisia crispa*

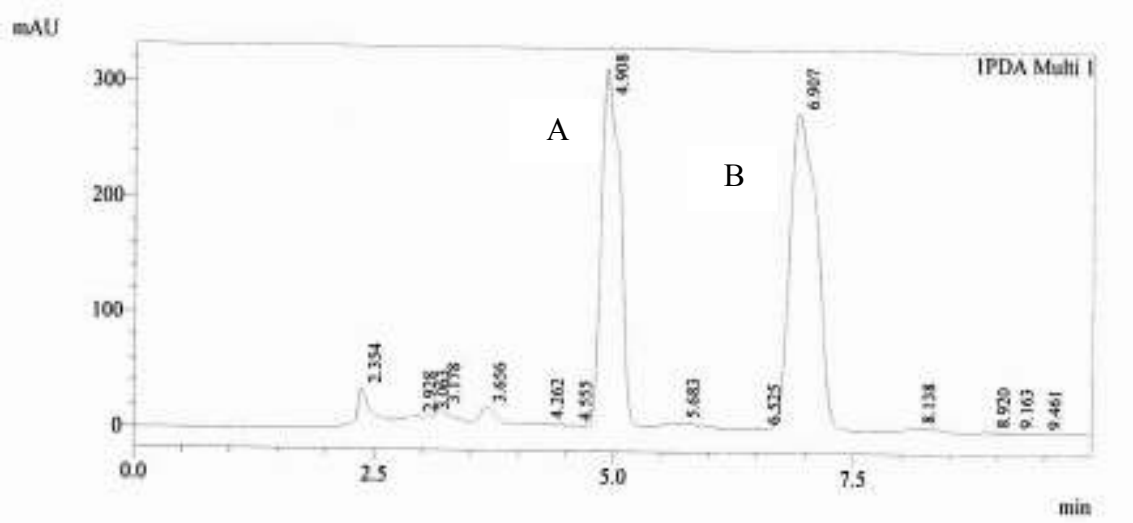

Figure 3.3. HPLC chromatogram of leaves methanol extracts from *Ardisia crispa*

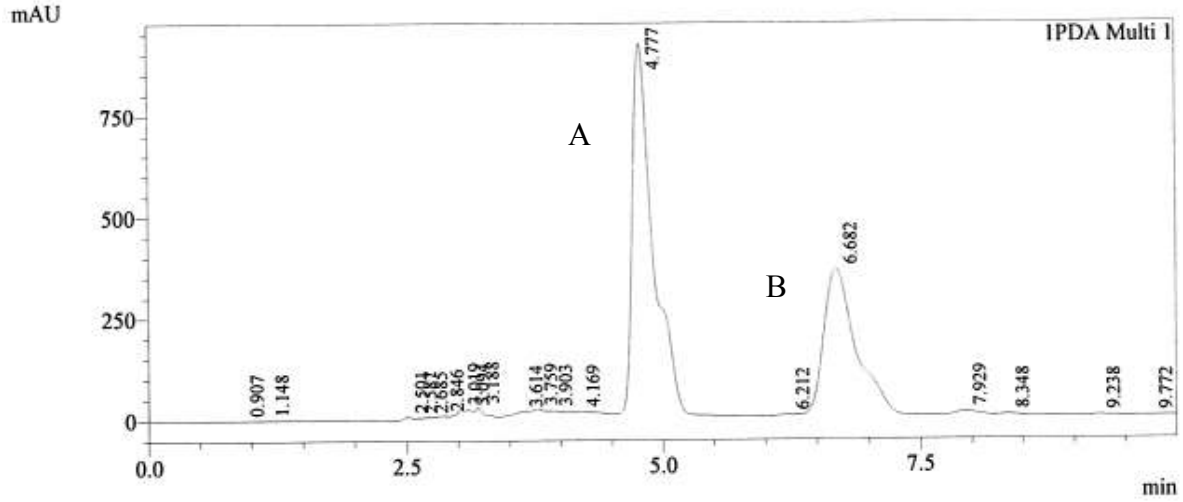

Figure 3.4. HPLC chromatogram of leaves water extracts from *Ardisia crispa*

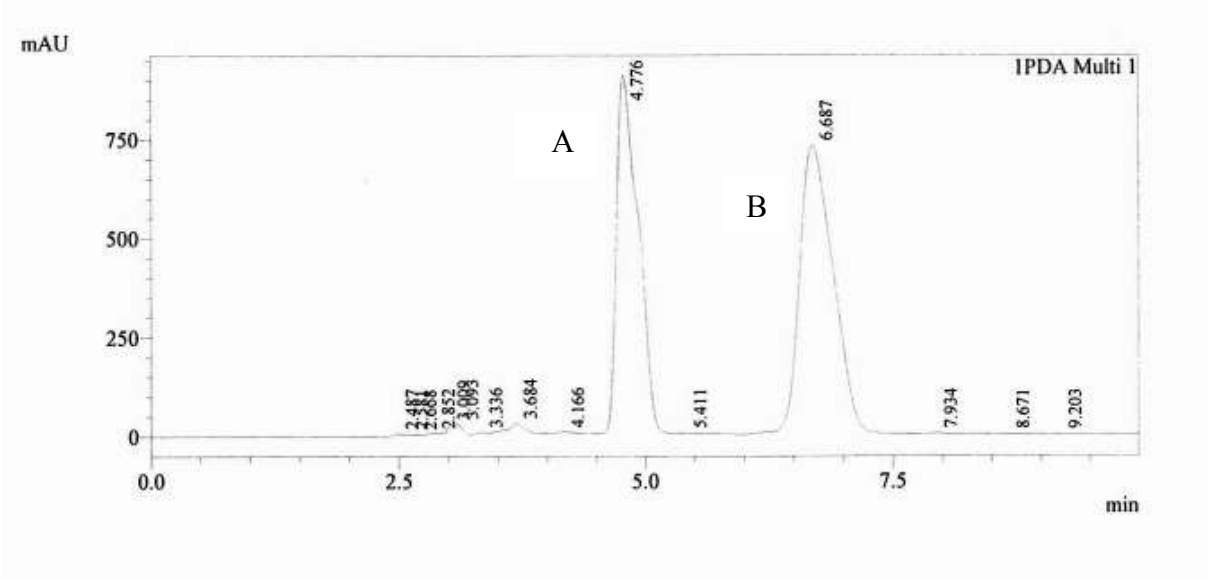

Figure 3.5. HPLC chromatogram of fruits water extracts from *Ardisia crispa*

3.2 Screening for Antioxidant Activity of *Ardisia crispa*.

The crude extracts of leaves and fruits of *Ardisia crispa* were tested for their antioxidant activity using three different assays:

A. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

B. Reducing power assay

C. Metal chelating assay

A. DPPH Radical Scavenging Assay.

The potential antioxidant activity of leaves and fruits extracts of *Ardisia crispa* was evaluated depending on their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH is stable radical with an unpaired electron at one atom of nitrogen bridge, and maximum absorbance range between 515-520 nm with deep purple colour. Presence of a hydrogen/electron donor (antioxidant) will decrease the absorption intensity, and the radical solution is discoloured according to the number of electrons captured from deep purple to yellow colour.

In this assay, the crude extracts of both leaves and fruits of the medicinal plant were tested for their scavenging activity against DPPH free radical at concentrations of 5mg/ml, 4mg/ml, 3mg/ml, 2mg/ml, and 1mg/ml to determine the IC_{50} value. Crude extracts possessing at least 50 % inhibition at 5mg/ml considered as positive extracts. In the present study ascorbic acid was used as a positive standard. Figure 3.6 shows the scavenging activity of ascorbic acid. The scavenging activity of ascorbic acid on DPPH radical was 94.17±0.0 at concentration of 200µg/ml and the IC_{50} value was 18µg/ml.

For crude fruits extracts, the inhibition activity range between 75.14±0.02% - 90.16±0.007% at concentration of 5mg/ml. for crude leaves extracts, the inhibition activity range between 45.77±0.0% - 82.24±0.02% at concentrations of 5mg/ml. In general, the inhibition activity by fruits extracts was higher than their equivalent of leaves extracts at concentration of 5mg/ml, Table 3.9a. All the crude extracts except leaves hexane extract showed inhibition activity more than 50% and they considered as positive extracts.

Figure 3.7 shows the inhibition activity of DPPH radical caused by different crude extracts at concentrations ranging from 1mg/ml - 5mg/ml. The inhibition effect of crude extracts increased with the increase in the extracts concentrations. Fruit methanol extract showed the highest inhibition activity (90.16±0.0%) at concentration of 5mg/ml. leave hexane extract showed The lowest effect (45.77±0.0%) at concentration of 5mg/ml. Fruit methanol extract exhibited the highest antioxidant activity with IC_{50} value of 0.9 mg/ml followed by fruit water extract with IC_{50} value of 1mg/ml, Table 3.9b.

Table 3.9a. Percentage of inhibition activity of DPPH radical by leaves and fruits extracts of *Ardisia crispa*.

Crude extracts	**Inhibition activity (%)**	
	Fruits	**Leaves**
Hexane extract	80.28 ± 0.0	45.77 ± 0.0
Chloroform extract	75.14 ± 0.02	62.38 ± 0.08
Methanol extract	90.16 ± 0.0	82.24 ± 0.02
Water extract	81.54 ± 0.0	74.18 ± 0.01

Table 3.9b The IC_{50} values of leaves and fruits extracts of *Ardisia crispa*.

Crude extracts	**IC_{50} value (mg/ml)**	
	Fruits	**Leaves**
Hexane extract	1mg/ml	> 5mg/ml
Chloroform extract	1.76mg/ml	4.1mg/ml
Methanol extract	0.9mg/ml	1.5mg/ml
Water extract	1.02mg/ml	2.74mg/ml

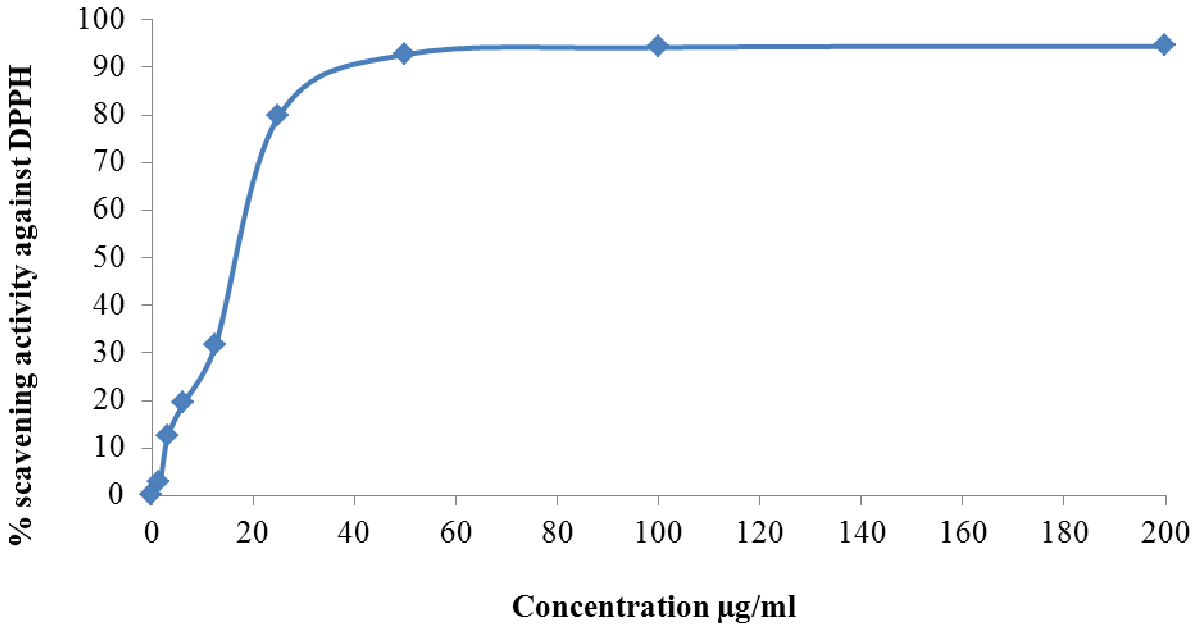

Figure 3.6. The inhibition activity of ascorbic acid on the DPPH free radical.

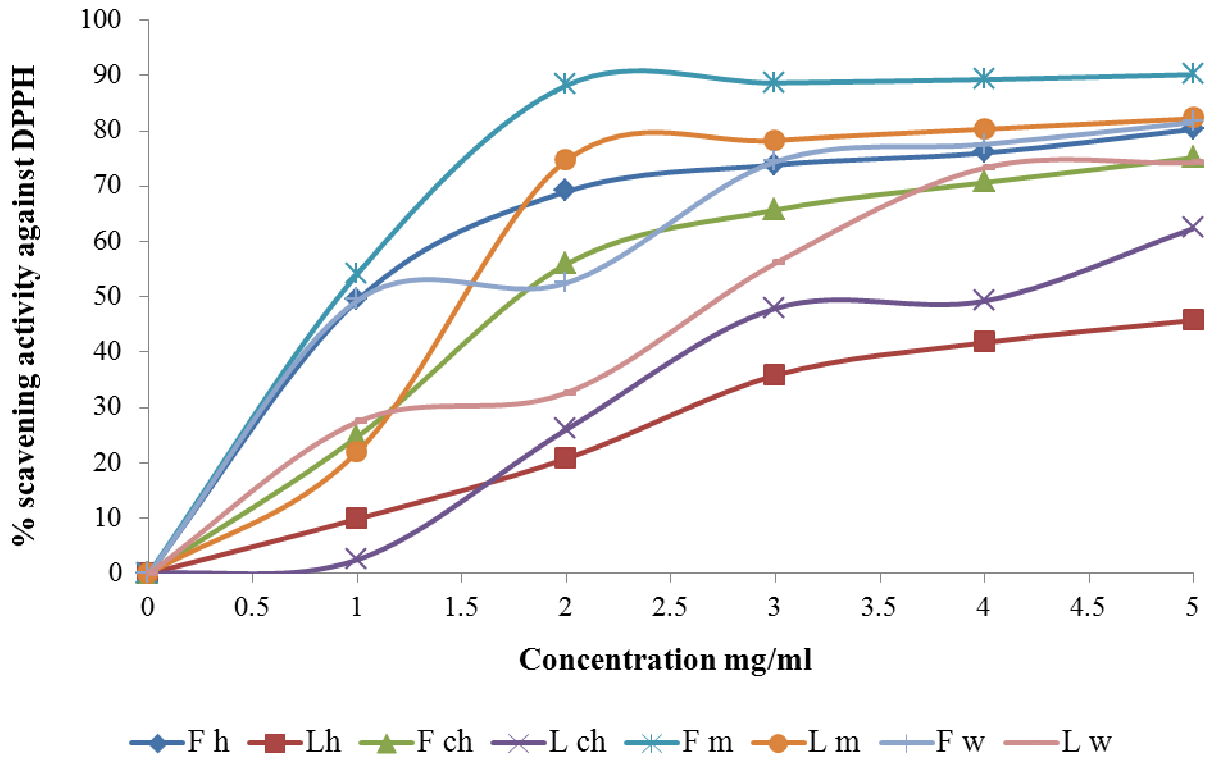

Figure 3.7. The inhibition effect of *Ardisia crispa* crude extracts on DPPH free radical.

B. Ferric Reducing power Assay.

In this assay, the yellow colour of the test solution changes to green and blue depending on the reducing power of each crude extract. The existence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). Thus, the formation of Fe^{2+} can be monitored at 700 nm.

Figure 3.8 shows the reducing power of leaves and fruits crude extracts. Fruit methanol extract exhibited the highest reducing power 2.960±0.0 at concentration of 1mg/ml followed by leave methanol extract with reducing power of 2.062±0.01 at concentration of 1mg/ml. The lowest reducing power was shown by leave hexane extract with 0.544±0.03 at concentration of 1mg/ml. In general, the fruit extracts showed higher reducing power than leave crude extracts.

In the present assay butylated hydroxyanisole (BHA) was used as standard, the reducing power for BHA was 3.101±0.05 at concentration of 1mg/ml Figure 3.8. Comparing to BHA standard the crude extract showed good reducing power specially fruit and leave methanol extracts.

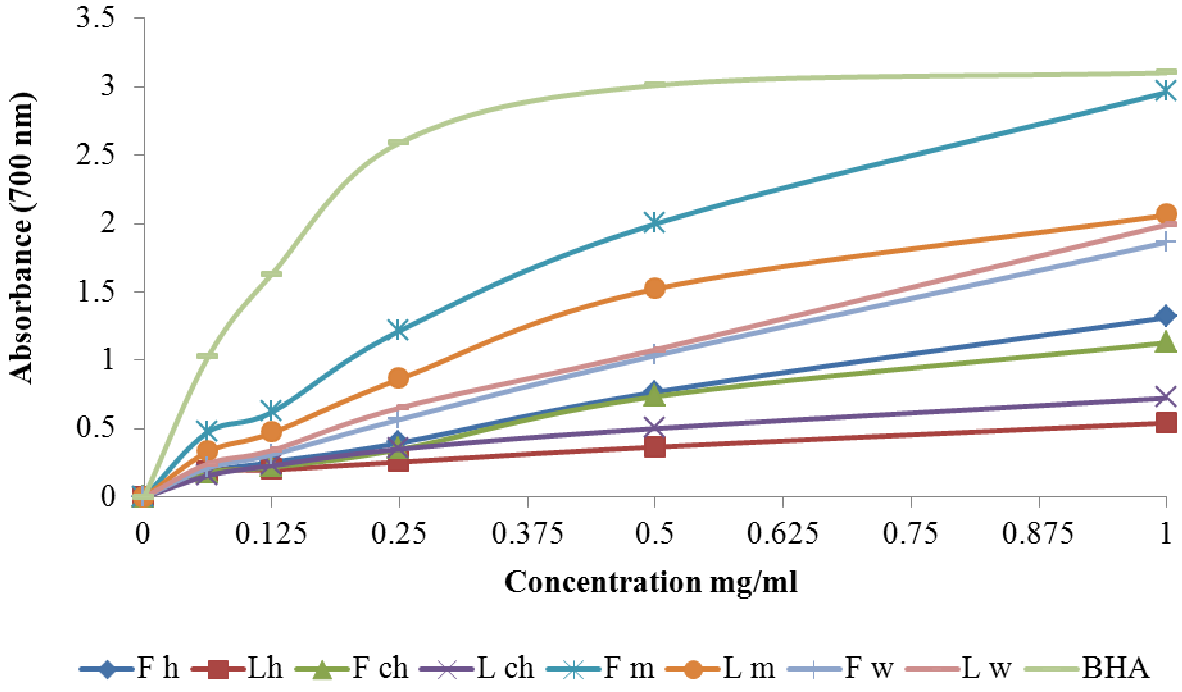

Figure 3.8. The reducing power activity of BHA standard and *Ardisia crispa* crude extract.

C. Metal Chelating Assay.

In this assay, the ability of crude extracts to chelate metal ions (Fe^{2+}) was tested at different concentrations range from 1mg/ml – 0.0625mg/ml. Ferrozine can form complexes with Fe^{2+}. Presence of chelating agents will lead to the disruption of complex formation, resulting in a decrease in the red colour.

EDTA was used as standard in this assay. EDTA was excellent chelator for ferrous ions, at 1mg/ml EDTA showed very high chelating activity 96.76±0.0%, Table 3.10. All crude extracts presented much lower chelating activity than EDTA standard, and all of them showed chelating activity less than 50%, Figure 3.9. The highest metal chelating activity (41.94±0.0%) was achieved with fruit water extract followed by leave water extract 37.85±0.11% at 1mg/ml, fruit hexane extract showed the lowest chelating activity with 4.78±0.01% at 1mg/ml, Table 3.10.

Table 3.10. Chelating activity (%) of leaves and fruits extracts at concentration of 1mg/ml.

Crude extracts	**Chelating activity (%)**	
	Fruits	**Leaves**
Hexane extract	4.78±0.01	6.69±0.0
Chloroform extract	7.06±0.0	7.34±0.0
Methanol extract	24.07±0.0	19.22±0.0
Water extract	41.94±0.0	37.85±0.11

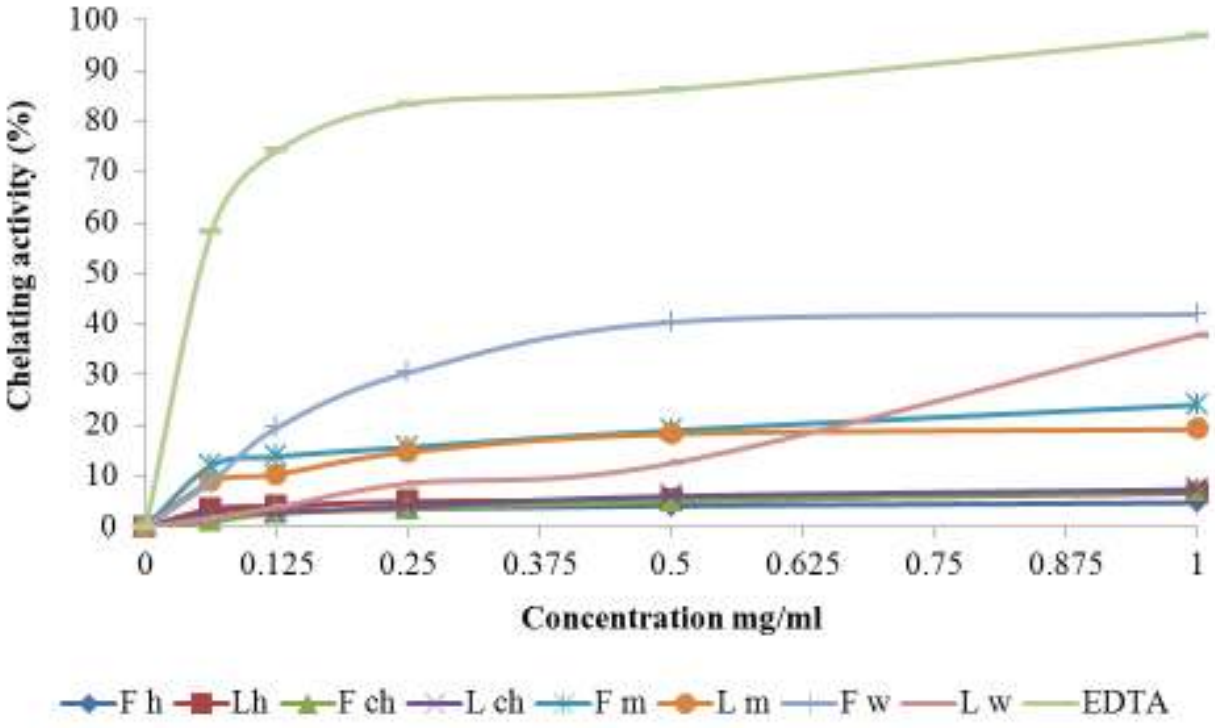

Figure 3.9. The metal chelating activity of different crude extract and EDTA standard.

3.3 Determination of Total Phenolic Content.

The amount of total phenolic compounds in the leaves and fruit extracts of *Ardisia crispa* was determined using the Folin-Ciocalteau method. A calibration curve of gallic acid was prepared, and the results were expressed as mg GAE/ml.

All the crude extracts showed high total phenolic content. Among the crude extracts, fruit methanol extract showed the highest phenolic content followed by leave methanol extract. Their phenolic contents were 3.14±0.0mgGAE/ml and 2.57±0.0mgGAE/ml, respectively. Leave hexane extract showed the lowest phenolic content, 0.67±0.0mgGAE/ml. Generally, fruit crude extracts showed higher phenolic contents comparing to their equivalent of leave crude extracts, Table 3.11.

Table 3.11. Total phenolic content of crude extracts from leaves and fruits of *Ardisia crispa*.

Crude extracts	**Total phenolic content mg GAE/ml**	
	Fruits	**Leaves**
Hexane extract	0.95±0.01	0.67±0.0
Chloroform extract	2.33±0.10	1.78±0.06
Methanol extract	3.14±0.0	2.57±0.0
Water extract	1.86±0.01	1.34±0.0

3.4 Determination of Total Flavonoid Content:

The total flavonoid content of crude extract was determined according to the method reported by (Zhishen *et al*., 1999). A calibration curve of quercetin was prepared, and the results were expressed as mg QE/ml.

All the crude extracts showed high total flavonoid content. Among the crude extracts, fruit chloroform extract had the highest flavonoid content followed by leave chloroform extract, their total flavonoid contents were 2.50±0.02 mgQE/ml, 2.00±0.0mgQE/ml. The lowest total flavonoid content was shown by leave water extract 0.47±0.009mgQE/ml. in general, fruits extracts had higher flavonoid content than leaves extract, Table 3.12.

Table 3.12. Total flavonoid content of crude extracts from leaves and fruits of *Ardisia crispa*.

Crude extracts	**Total flavonoid content mg QE/ml**	
	Fruit extracts	**Leave extracts**
Hexane extract	1.87±0.02	1.61±0.0
Chloroform extract	2.50±0.02	2.00±0.0
Methanol extract	1.54±0.0	1.12±0.0
Water extract	0.52±0.0	0.47±0.0

3.5 Determination of Toxicity of Crude Extracts Using BSLA Assay.

The brine shrimp lethal assay was used as inexpensive rapid method to determine the toxicity of *Ardisia crispa*. All the crude extracts were tested at concentration of 1000µg/ml, 100 µg/ml, and 10µg/ml, and the LC_{50} value was calculated using Finney program. All the crude extracts which tested at these concentrations showed very low levels of toxicity. In the present study, none of the crude extracts showed 100% mortality.

Fruit hexane extract caused the highest mortality 56.7%, 43.3%, and 10% at 1000µg/ml, 100µg/ml, and 10µg/ml respectively, LC_{50} value was 384.69µg/ml. All other crude extracts showed very low mortality or none at all, Table 3.13.

Table 3.13. The toxic effects of crude extracts from *Ardisia crispa* on brine shrimp larvae (ND = not detected, LC_{50} value >1000 µg/ml)

Extract	Concentration (µg/ml)	Mortality %	LC_{50} values (µg/ml)	95 % confidence interval
Leaves hexane	1000 100 10	6.7 0 0	ND	-
Fruits hexane	1000 100 10	56.7 43.3 10	384.69	66.67 – 3.06
Leaves chloroform	1000 100 10	16.7 3.3 0	9715.52	1062.43 – infinity
Fruits chloroform	1000 100 10	33.3 30 10	7141.70	297.84 – infinity
Leaves methanol	1000 100 10	10 3.3 0	7763.12	1193.89 – infinity
Fruits methanol	1000 100 10	43.3 0 0	ND	-
Leaves water	1000 100 10	13.3 0 0	ND	-
Fruits water	1000 100 10	3.3 0 0	ND	-

CHAPTER FOUR

DISCUSSION

4.1. Identification of Bioactive Compounds Present in *Ardisia crispa*.

4.1.1. Thin Layer Chromatography (TLC).

Thin Layer Chromatography (TLC) is one of the chromatographic techniques which frequently applied in phytochemical analysis. TLC is a simple, rapid, and inexpensive method widely used for qualitative analysis of organic compounds.

Results from our study showed that there were different kinds of secondary metabolites present in the crude extract of leaves and fruits of *Ardisia crispa*, the main secondary compounds were terpenoids and flavonoids. In addition, saponins and alkaloids have been identified in the crude methanol extracts of both leaves and fruits. TLC analysis of the crude extracts revealed that these extracts contain flavonoids and indicated when sprayed with anisaldehyde – sulphuric acid reagent and Vanillin-sulphuric acid Reagent.

TLC results of the present study is supported by several reports indicated the presence of terpenoids , phenolic compounds, and saponins in this plant. According to Kobayashi & de Mejía (2005) the genus *Ardisia* produces several groups of biologically active compounds including triterpenoid saponins, coumarins, and quinones. Duke (2001) stated the presence of several compounds in *Ardisia japonica* including 2-hydroxi-5-methoxy-pentadecenyl-benzoquione, ardisin, ardisinol I and II, bergenin, embelin, myricitrin, Quercetrin, and rapanone.

Many reports have revealed the presence of triterpenoid saponins in *Ardisia* plants including *Ardisia crenata* (Koike *et al*., 1999), *Ardisia mamillata* Hance (Wang *et al*., 1992), and *Ardisia pusilla* D.C. (Zhang *et al*., 1993). As stated by Sumino *et al*. (2002) the fruits of *Ardisia colorata* containing myricetin, quercetin, gallic acid, as well as other biological compounds such as novel alkylphenols A-C, embelin, bergenin, norbergenin, and kaempferol.

4.1.2. High Performance Liquid Chromatography.

Nowadays, HPLC is frequently used for the separation of complex mixtures of phenolic compounds and other natural products in plant extracts (Chen *et al*., 2001). The HPLC analysis was done for the methanol and water extracts which showed the highest scavenging and reducing power activities. HPLC results showed the presence of gallic acid in methanol and water extracts from *Ardisia crispa* according to the characteristic retention time of compound A in comparison with the standard. The presence of gallic acid in *Ardisia* species has been reported previously. Sumino *et al*. (2002) has reported the presence of gallic acid in the fruits of *Ardisia colorata*, (de Mejía *et al*., 2006) has mentioned that *Ardisia compressa* contain gallic acid. Since one standard was used in this study it's difficult to identify compound B, however, from TLC results it could be predicted that this compound could be one of terpenoids since terpenoid compounds are one of the main compounds present in *Ardisia* plants. Further analyses are needed to confirm this result such as NMR.

In summary, the presence of various chemicals in *Ardisia crispa* could be responsible for the medicinal value of this plant.

4.2. Antioxidant activity of crude extracts from *Ardisia crispa*.

Plants represent a significant source of natural compounds that of valuable benefit to the human life due to their biological properties. Nowadays, it's well known that intake of fruits and vegetables has been related with reduced risk of several diseases such as cardiovascular diseases, cancer, neurodegenerative diseases, and inflammatory diseases (Kohen & Nyska, 2002). Previous studies have shown that wide range of plants have a strong antioxidant activities, the protective effect of these plants is attributed to their antioxidant compounds such as phenolic compounds, carotenoids, and vitamins C and E (Katalynic *et al*., 2006).

So far, there are no studies about the antioxidant activity of plant *Ardisia cirspa*. This led us to investigate the antioxidant capacity of extracts from this plant. A number of methods have been developed for the assessment of the antioxidant efficiency. Since many active species and reaction mechanisms are involved in oxidative stress process, thus, there is no simple universal method can be applied for accurate and quantitative measurement of antioxidant capacity (Frankel & Finley, 2008). Generally, in these methods a radical is generated and the antioxidant capability of a sample against the radical is evaluated (Erel, 2004).

In the present study, the antioxidant activity of crude extracts of *Ardisia crispa* were measured using 3 assays namely, DPPH radical scavenging assay, reducing power assay, and metal chelating assay.

4.2.1. DPPH radical scavenging activity.

The scavenging activity of crude extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used to evaluate the antioxidant activity of plant, the use of DPPH method provides rapid and easy way to examine the antiradical activities of samples (Brand-Williams *et al*., 1995), in addition, DPPH radical is unaffected by certain side reaction of polyphenols, for instance metal ion chelation and enzyme inhibition. This method has been widely used to evaluate the antioxidant activity of fruits, vegetables, and other plants (Villano *et al*., 2007). Hydrogen-donating capacity of the antioxidant is responsible for its free radical-scavenging property (Sentandreu *et al*., 2008).

DPPH radical assay results revealed that most of the crude extracts have strong DPPH radical scavenging activity. Fruit and leave methanol extracts showed the highest scavenging effects of 90.16 ± 0.0% and 82.24 ± 0.02% respectively at concentration of 5mg/ml, with IC_{50} value of 0.9mg/ml and 1.5mg/ml respectively. The results demonstrated that crude extracts of *Ardisia crispa* were free radical inhibitors, and their inhibition activity may attribute to their hydrogen donating ability. The leave hexane extract showed the lowest scavenging effect 45.77±0.0% at 5mg/ml, this indicate that the ability of this extract to donate a hydrogen electron to the free radical was not sufficient.

4.2.2. Ferric Reducing power assay.

FRAP assay was frequently used to study the antioxidant activity of plants (Li *et al*., 2006). FRAP assay measures the ferric to ferrous ion reduction in the presence of antioxidant. FRAP assay results showed that methanol extracts of fruits and leaves from *Ardisia crispa* revealed the strongest reducing power. The reducing power of a sample may serve as important pointer of its potential antioxidant capacity (Meir *et al*., 1995). The presence of antioxidant compounds cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) monitored at 700 nm (Sousa *et al*., 2008). All the crude extracts presented an activity increasing the absorbance at 700 nm with increasing concentration. These result revealed the crude extracts specifically methanol extracts of both leaves and fruits are electron donor which may help converting the free radicals into more stable products, and thus, terminating radical chain reaction.

The reducing power assay confirmed the results obtained by the DPPH assay, the fruit extracts showed higher scavenging and reducing power activities than leave extracts. Both fruit and leave methanol extracts showed the highest scavenging and reducing power capacities. The correlation between the DPPH radical scavenging activity and the reducing power activity presented by the extracts is explained by the fact that both assays depend on the mechanism of electron/hydrogen donation.

4.2.3. Metal chelating assay.

Metal chelating activity is significant as it reduces the concentration of the catalysing transition metal in lipid peroxidation through the Fenton reaction (Hseu *et al*., 2008). Metal chelating assay results revealed that all crude extract showed metal chelating activity lower than 50%, which is insufficient to consider the crude extracts as metal chelators. All the crude extract showed metal chelating capacity much lower than EDTA standard, this possibly due to the presence of phenolic compounds. Metal chelating capacity of phenolic compounds is governed by their unique phenolic structure and the number and location of the hydroxyl groups (Santoso *et al*., 2004). Many authors have reported that metal chelating potency play a minor role in the overall antioxidant activities of some polyphenols (Rice-Evans *et al*., 1996). Andjelkovic *et al*. (2006) reported that the ability of phenolic compounds to chelate iron were far lower than that of EDTA. Hseu *et al*. (2008) has stated that the metal chelating activity of gallic acid extracted from the leaves of *Toona Sinensis* was extremely low. Since the HPLC results of the present study showed the presence of gallic acid this possibly could be responsible for the low metal chelating capacity of *Ardisia crispa*.

Several reports have shown that many plants from the genus *Ardisia* possess antioxidant activities. de Mejia & Ramirez-Mares, (2002) reported that *Ardisia compressa* leaves possess a high antioxidant capacity. Sumino *et al*. (2002) stated that *Ardisia colorata* fruits showed antioxidant capacity toward DPPH free radical. Ryu *et al*. (2002) pointed out that *Ardisia japonica* possess antioxidant activity.

4.3. Total Phenolic and Total Flavonoid Contents.

Numerous studies have revealed that flavonoids and related phenolic compounds are responsible for to the total antioxidant activity of many fruits and vegetables (Luo *et al*., 2002). The concentration of phenols in the crude extracts was expressed as mg of GAE (Gallic acid equivalent)/ml. The fruit extracts showed higher phenolic content than the leave extracts, methanol extracts of fruits and leaves showed the highest phenolic content 3.14±0.0mgGAE/ml and 2.57±0.0mgGAE/ml, respectively.Total phenolic content results revealed that there is a positive correlation between the total phenolic content of crude extracts and their antioxidant activities. Extracts with highest phenolic contents showed the highest DPPH radical scavenging activity and reducing power activity, thus, phenolic compounds may play a role in the antioxidant activity of *Ardisia crispa*. This finding is agreed by several reports revealed that the antioxidant capacity of plants materials is well correlated with their phenolic content (Velioglu *et al*., 1998). Li *et al*. (2008) reported a high correlation between the antioxidant capacity and total phenols of methanol extracts from 45 medicinal plants.

For total flavonoid content the concentration expressed as mg of QE (quercetin equivalent)/ml. Fruit extracts showed higher flavonoid content than leave extracts. In this study, there is no correlation between the total flavonoid content and antioxidant activity of crude extracts. Chloroform extracts of fruit and leave showed the highest flavonoid content 2.50±0.02 mg of QE/ml and 2.00±0.0 mg of QE/ml respectively but they showed lower DPPH radical scavenging activity and reducing power activity than methanol and water extracts. (Vundac *et al*., 2007) reported that the correlation between the flavonoid content and DPPH scavenging activity of seven Croatian *Stachys* taxa was poor. Dasgupta & De (2007) stated that there is no correlation between the total flavonoid content and antioxidant actvitiy of 11 Indian medicinal plants. On the other

hand, recent reports have shown that various flavonoids and related polyphenols contribute significantly to the total antioxidant capacity of many fruits and vegetables (Dastmalchi *et al*., 2008).

It can be concluded from the above observation, that *Ardisia crispa* can be considered as good source of natural antioxidants since it extracts were found to possess high antioxidant activity toward DPPH radical and ferric ion. The results suggested that the antioxidant activity of this plant may be attributed to the chemical components present in this plant. This plant could be a good source of compounds that would help increase the overall antioxidant capacity of an organism and protect it against oxidative stress.

4.4. Toxicity of *Ardisia crispa* using brine shrimp lethality assay (BSLA).

Brine shrimp lethality assay was used in present study In order to evaluate the toxicity of *Ardisia crispa*. BSLA is an efficient, rapid, inexpensive test for initial assessment of toxicity of plant extracts (McLaughlin *et al*., 1998). The results exhibited that all crude extracts did not show high levels of toxicity or could be considered toxic as the percentage mortality was low in the *Artemia salina* nauplii. According to Meyer *et al*. (1982) the crude extracts can be categorized into nontoxic (LC_{50} value > 1000 µg/ml) and toxic (LC_{50} value < 1000 µg/ml).

The highest toxic effect was achieved by fruits hexane extract with 56% mortality at concentration of 1000µg/ml and LC_{50} value of 384.69µg/ml. All other crude extracts showed very low mortality less than 50% or none at all, and the LC_{50} values were more than 1000µg/ml. It's hard to consider the extracts to be none toxic according to our results, because several factors influence the acute toxicity of crude extracts such as temperature, composition, salinity of the medium, and the age of larvae (Hartl & Humpf, 2000); in addition, it was very difficult to completely dissolve the crude extracts in salt water.

It must be understood that one cannot extrapolate the result of this assay to toxicity of mammals (McGaw & Eloff, 2005). This test is normally used as preliminary test and additional tests should be done to obtain further reliable data (Hartl & Humpf, 2000).

CHAPTER FIVE

CONCLUSION

From this study, it can be concluded that:

1. Several compounds exist in the fruits and leaves of *Ardisia crispa*, the main compounds were terpenoids and phenolic compounds, as well as alkaloids and saponins.

2. DPPH radical scavenging assay and ferric reducing assay indicated the crude extracts specifically fruits and leaves methanol extracts had high antioxidant activities. All the crude extracts showed metal chelating activity less than 50% and considered to be insufficient as metal chelators.

3. Total phenolic content results of crude extracts showed a positive correlation between antioxidant activities of crude extracts and their phenolic content. The presence of phenolic and flavonoid compounds indicates that *Ardisia crispa* could be a good source of natural antioxidant for human health and food industry.

4. Brine shrimp lethality assay results revealed that the majority of crude extracts of *Ardisia crispa* showed low levels of toxic effects.