Investigation of *in-vitro* antioxidant and cytotoxic potential of *Crotalaria pallida* leaves

A project submitted

by

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This work is dedicated to my parents for their love and constant support…

Certification Statement

This is to certify that this project titled “Investigation of *in-vitro* antioxidant and cytotoxic potential of *Crotalaria pallida* leaves” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the supervisor,

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Acknowledgement

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Abstract

Crotalaria pallida (C. pallida) is a locally known as Kudug Jhunjhuni, and it is traditionally used for treating urinary problems, swelling of joints, the extract of the leaves is used as vermifuge. Since the antioxidant potential using methanolic leaf extract and cytotoxic activity on HeLa cell line have not been investigated before with *C. pallida* leaves, this study was aimed to screen these activities. In this study, the phytochemical screening of methanolic leaf extract of *C. pallida* showed the presence of flavonoids, phenolic compounds, alkaloids, tannins, phytosterols and carbohydrates. Various *in-vitro* antioxidant studies were performed to determine its antioxidant potential. In DPPH free radical scavenging assay, the IC_{50} value for *C. pallida* and reference standard ascorbic acid was found to be 157.01µg/mL and 276.6µg/mL respectively, which infers that, *C. pallida* has higher free radical scavenging power than the standard ascorbic acid. Whilst the total phenolic content was found to be 141.00mg of gallic acid equivalent (GAE)/g, the total flavonoid content was found to be 144.00mg of quercetin equivalent (QE)/g and the total antioxidant capacity was determined to be 90.7mg of ascorbic acid equivalent (AAE)/g of crude extract for a concentration of 1200µg/mL of methanolic extract of *C. pallida*. Other antioxidant studies conducted previously on the leaf extract with ethanol, ethyl acetate, chloroform, petroleum ether solvent showed that free radical scavenging activity of *C. pallida* reached above 80% with ethanolic extract and 60% with other extracts, whereas the study conducted with methanolic leaf extract showed 96.55% scavenging activity. The cytotoxicity of leaf extract was examined on HeLa cell line that was performed by MTT assay. The study indicated that, with the highest 2.0mg/mL concentration of leaf extract the IC_{50} value was found to be 1.25mg/mL and that inferred about 90% cancerous cell population was dead; which was the highest percentage of mortality of HeLa cells compared to 1.5, 1.0 and 0.5 mg/mL concentrations of leaf extract. Thus the study inferred that *C. pallida* leaf extract has strong antioxidant and cytotoxic potential. The presence of flavonoid provides a notion to the possibility of antioxidant activity, whereas the strong cytotoxic property attributes to the presence of tannins. The findings of this study pave the way for using this medicinal plant as antioxidant agent and possible anticancer agent. Further research and tests may confirm its' possibilities and effectiveness for treating oxidative stress degenerative diseases like Alzheimer's disease, Parkinson's disease etc. and in the treatment of cervical cancer in future.

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CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

1.1. Phytotherapy and its relation to medicinal plants

From the beginning of the human race, people were dependent on various plants and herbs in order to survive on this planet. Various diseases have emerged that challenged them to their life. The basic instinct of human being is to find the solution to the problem they face. Thus, to face those very challenges and to survive in this planet people reached to the abundant creation called plants and found the way to their wellness. In this modern age, the plants still do not fail to amaze with their limitless properties and boundless possibilities that aid to not only human but also every creature of earth.

Medicinal plants can be defined in many ways. According to the WHO, "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." When a plant is referred as 'medicinal', it is indicated that the plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation.(Sofowora, Ogunbodede, & Onayade, 2013)

Usually phytotherapy refers to the treatment in which natural plant-derived medicines are used. These plant-based medicine systems have emerged, generally depending on the geographical abundance of the plants, developed the renowned traditional medicine systems, the Ayurvedic and Unani of the Indian subcontinent, the Chinese and Tibetan of other parts of Asia, the Native American of North America, the Amazonian of South America and several local systems within Africa. (Mamedov, 2012)

In present day, plants and plant-derived medicines are one of the major sources of modern pharmaceuticals that are used to treat many diseases. There are some certain chemical constituents present in the plants those exert distinct pharmacological action on human body. These chemical constituents are also termed as bioactive compounds; among them alkaloids, flavonoids and phenolic compounds are considered to be the most significant. Researches and studies based on ethno-pharmacological information are usually considered as very significant approaches in the discovery of new drugs. (Devendra, Srinivas & Solmon, 2012)

1.2. Medicinal plants in drug discovery

Medicinal plants and their derivatives have been used as therapeutic agents for a long period of time. The various bioactive compounds are solely responsible for exerting different types of pharmacological actions inside human body and that paved the way for discovering new lead compounds to new medicines. Not only their chemical structure diversity but also their biodiversity serve as two major perspectives to screening and developing new drugs.

According to Lahlou (2013), "the 20th century revolutionized the thinking in the use f drugs as the receptor theory of drug action." At that time scientists began to believe the theory that, solely the plant extracts were not any mysterious potions rather the certain chemical compounds present in the extracts are responsible for the ailing. This conclusion has brought about a major change in the treatment and medicines where isolated bioactive compounds became the standard medicine instead of crude extracts of medicinal plants.

Medicinal plants possess numerous bioactive compounds and naturally they contain large-scale structurally diverse natural products than the synthetic compounds. Apart from this point, there is another important aspect of using medicinal plant in drug discovery; natural products most of the times express advanced binding characteristics when compared to synthetic compounds. That infers, bioactive compounds has higher capability to interact with other molecules – this is somewhat a significant characteristic for an effective drug. (Lahlou, 2013)

Some of the medicinal plants used as common medicaments are listed below with their traditionally use and pharmacological activity in **Table 1.1**. Depending on the medicinal properties the plant parts are used, because sometimes a single part of the plant can be more biologically active than other parts of the plant. Whereas the whole plant may possess different types of pharmacological activities.

Table 1.1: Some medicinal plants used for treating common diseases

Source	**Used Plant Parts**	**Local Name**	**Pharmacological activity**	**Traditional use**	**Reference**
Abroma augustac Linn (Sterculiaceae)	Leaf,	Ulatkambal	Male antifertility	Amenorrhoea (Nilufar et al., 1991)	Ranhman, Hasnat, Hasan & Ilias, (2001)
Achyrathus asperae Linn. (Sterculiaceae	Aerial part	Apang	Antidiabetic	Diuresis (Begum, 1985)	Ranhman, Hasnat, Hasan & Ilias, (2001)
Eclipta albaa Linn. (Compositae)	Tuber	Keshraj	Antihypertensive	Stomachic (Ahmed et al., 1994)	Ranhman, Hasnat, Hasan & Ilias, (2001)
Momordica charanteaf Linn. (Cucurbitaceae)	Fruit,	Corolla	Antidiabetic	Diabetes (Awal et al., 1995)	Ranhman, Hasnat, Hasan & Ilias, (2001)
Persicarie astagninag Linn. (Polygonaceae)	Whole plant.	Biscatali	Analgesic	Pain (Ahmed et al., 1997)	Ranhman, Hasnat, Hasan & Ilias, (2001)

1.3. Natural products as remedies

Today at least 120 different chemical substances are derived from the diverse plants those are believed to be have the medicinal properties, thus considered to be used as drugs in many parts of the world. Chemical substances like alkaloid, tannin, flavonoid etc. hold a great significance to express medicinal properties and when they are found to be present in certain plants in considerable amounts, it is considered that those particular plants should contain significant as well as distinct properties to heal diseases. Phyto-constituents possessing anti-oxidant properties are believed to prevent or slow down the occurrence of disease such as cancer (Lee, Koo & Min, 2004).

It is recorded that more than 4900 phytochemicals have been catalogued and they are classified by the functions and chemical characteristics. These compounds are known as secondary plant metabolites. They have various pharmacological activities including antioxidant effect, antimicrobial effect, detoxification of enzymes, stimulation of immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. The various natural products are generally accumulated in different parts of plants, such as roots, stems, leaves etc.

According to the role in plant metabolism natural bioactive compounds are very diverse. Among them alkaloids, tannins, flavonoids, phytosterols, phenols and phenolic compounds, steroid, saponin and glycosides are considered to be directly linked to various healing properties. (Saxena, Nema, Singh and Gupta, 2013)

Other bioactive compounds also contribute to various pharmacological actions in body. **Table 1.2** represents some of the important bioactive compounds are their pharmacological actions mentioning the plants they are primarily derived from. Especially digitalin, emetine, a-lobeline are very important therapeutic agents to present date which were mainly derived from different natural plants.

Table 1.2: Some drugs isolated from natural sources

Drug/chemical constituent	**Pharmacological action**	**Plant source**
Aescin	Anti-inflammatory	*Aesculushippocastanum*
Bromelain	Anti-inflammatory, proteolytic	*Ananascomosus*
Convallatoxin	Cardiotonic	*Convallariamajalis*
Demecolcine	Antitumor agent	*Colchicum autumnale*
Digitalin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephaelisipecacuanha*
a-Lobeline	Smoking deterrant, respiratory stimulant	*Lobelia inflate*

1.4. Free radical and reactive oxygen species

Free radicals are produced in the biological systems as a result of normal cellular aerobic metabolism. But in some cases they are also generated from unusual chemical reactions that are stimulated by certain diseases and xenobiotics. Whereas reactive oxygen species (ROS) term is generally used to express these reactive species together – Singlet oxygen (O_2), Superoxide anion radical ($O_2^{\cdot -}$), Hydroxyl radical ($^{\cdot}OH$), Alkoxyl radical ($RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}$), Hydrogen peroxide (H_2O_2) and Lipid hydroperoxide (LOOH). Reactive nitrogen species (RNS) are also responsible for some free radical reactions which are respectively $^{\cdot}NO$ and $ONOO^{-}$.

Many radicals are highly reactive and can either donate an electron to or extract an electron from other molecules, therefore behaving as oxidants or reductants. (Young & Woodside, 2001)

Figure 1.1 shows the major sources of free radicals in body which can be divided into two major sources: endogenous sources and environmental sources. These are the primary reasons of the production of free radicals in body that in terms causes lipid peroxidation, DNA base modification or damage of proteins in cell. These incidents ultimately lead to cellular and tissue damage.

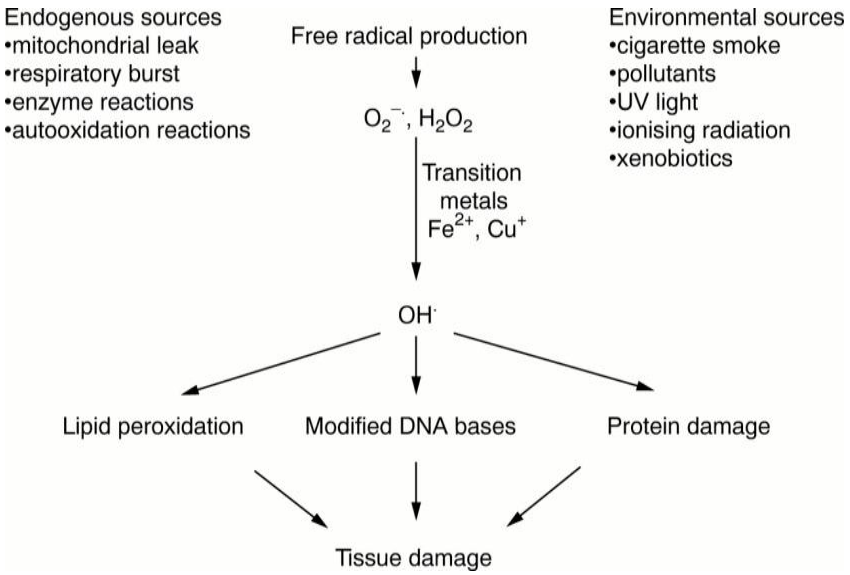


Figure 1.1: Major sources of free radicals in the body and the consequences of free radical damage (Kehrer, 2015)

1.4.1. Mechanism of tissue damage by free radicals

The mechanism of the free radical chain reaction depends on their interaction with lipid, protein or DNA base. When the free radicals or ROS/RNS interact with lipid, protein and DNA there are numerous changes and chemical reactions that take place inside the cells and ultimately leads to cell and tissue injury.

In the **Figure 1.2**, the pathway to cell and tissue injury by free radical reaction is explained. If free radicals interact with lipid molecules, then this results in lipid peroxidation reaction and it finally leads to membrane damage and injury. On the other hand, ROS/RNS's reaction with protein molecules results in activation of enzymes that will harm the cell in the final stage. Lastly, the free radicals interact with DNA, it will cause definite DNA damage or mutation and alteration of genetic expression. However, antioxidants can act on the preliminary stage of ROS/RNS interaction to alter or minimize the harmful changes. (Kehrer, 2015)

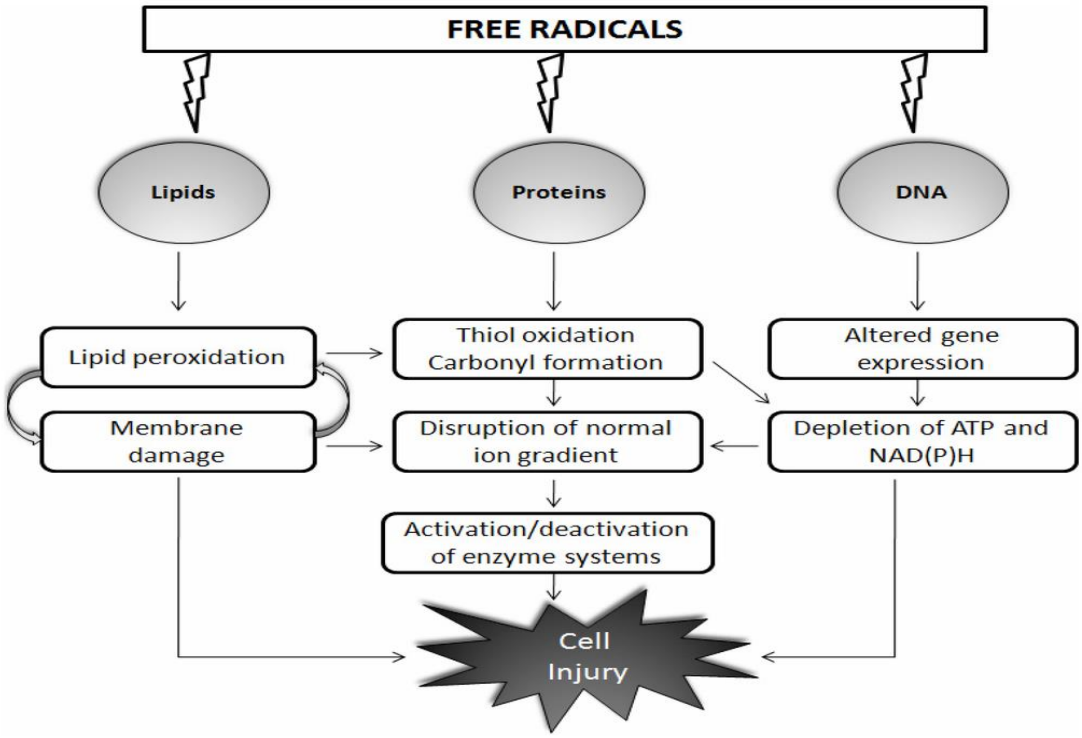


Figure 1.2: Mechanism of cell injury by free radicals

1.4.2. Antioxidant defense system against free radicals

Radicals can react in a way that can lead to damage to any cellular component. Due to this reason, both endogenous and exogenous types of antioxidant defense systems are present to protect cellular components from free radical induced damage. The defense system participating species can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins.

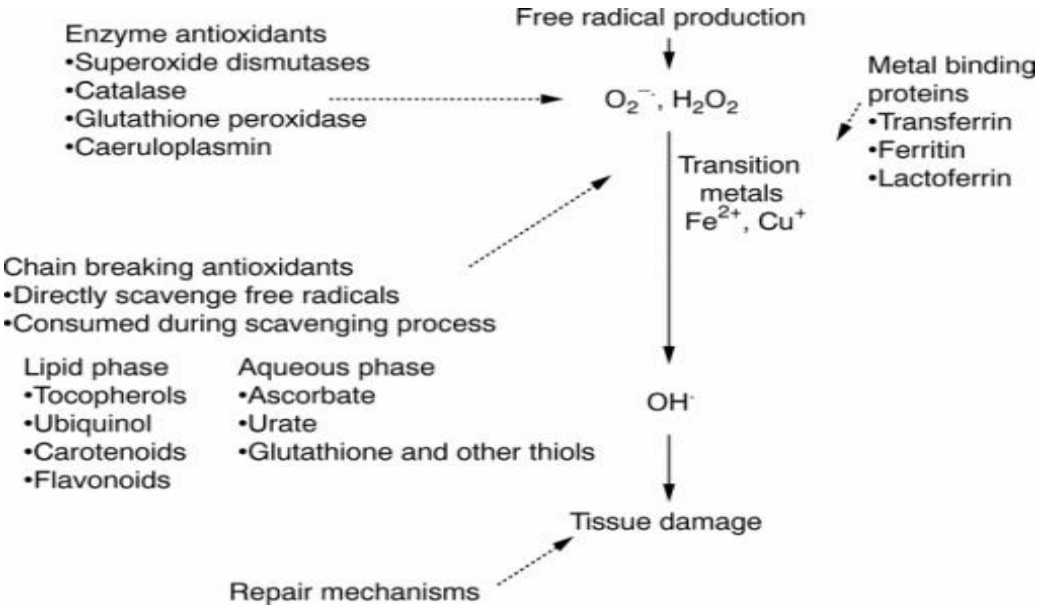


Figure 1.3: Antioxidant defense against free radical attack

In the **Figure 1.3** the mechanism of antioxidant defense is explained which occurs due to attack of free radicals. Antioxidant defenses against free radical attack. Antioxidant enzymes catalyse the breakdown of free radical species. Transition metal binding proteins prevent the interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide and thus they produce highly reactive hydroxyl radicals. Chain breaking antioxidants are very powerful electron donors and react with free radicals before important target molecules are damaged. As a result, the antioxidant is oxidized and must be regenerated or replaced.

1.5. Antioxidants

Both natural and synthetic antioxidants are used to treat various diseases and to prevent the previously mentioned cellular damages that caused by free radicals or reactive oxygen species. The medicinal plants those possess antioxidant properties usually contain polyphenols and a polyphenolic chemical named Flavonoid.

According to the biochemical regulation theory, a polyphenol antioxidant's ability to scavenge free radicals happened by some metal chelation reactions. Various reactive oxygen species, such as singlet oxygen, peroxynitrite and hydrogen peroxide must be continually removed from cells to maintain healthy metabolic function.

The “deactivation” of oxidant species by polyphenolic antioxidants (POH) is based, with regard to food systems that are deteriorated by peroxyl radicals (R•), on the donation of hydrogen, which interrupts chain reactions:

R•+PhOH→R-H+PhO•

Phenoxyl radicals (PO•) generated according to this reaction may be stabilized through resonance and/or intramolecular hydrogen bonding, as proposed for quercetin or combine to yield dimerisation products, thus terminating the chain reaction:

PhO•+PhO•→PhO-OPh

1.5.1. Classification of antioxidants

According to Guttering and Halliwell, antioxidants can be classified into three classes according to their function: primary, secondary and tertiary antioxidants. Primary antioxidants prevent the oxidant formation, secondary antioxidants can scavenge ROS/RNS and tertiary antioxidants are considered to repair the oxidized molecules through some certain sources. Other than this classification antioxidants are categorized by some other modes which are discussed accordingly.

Antioxidants have vast mode of actions according to their chemical structures and functional groups present on them. They can act on different steps of tissue damage caused by free radicals. Antioxidants can be classified into six categories based on their mode of actions mentioned by Flora (2009). However, their actions are discussed in the **Table 1.3** along with relevant examples.

Table 1.3: Classification of antioxidants based on mode of actions

Mode of action	**Examples of antioxidants**
Antioxidant that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds.	Phenol, Naphthol
Antioxidants that break chains by reacting with alkyl radicals	Quinones, Nitrones, Iminoquinones
Antioxidants that decompose hydroperoxide	Sulphide, Phosphide, Thiophosphate

Antioxidants that deactivate metals	Diamine, Hydroxyl acids, Bifunctional compounds
Antioxidants that terminates the cyclic chain	Aromatic amines, Nitroxyl radical, Variable valence metal compounds
Antioxidants that act synergistically with other antioxidants	Phenol sulphide in which the phenolic group reacts with peroxyl radical and sulfide with hydroperoxide

The table indicates that phenols and naphthols act by reacting with peroxyl radicals to terminate the free radical action. On the other hand, quinones, nitrones and iminoquinones react with alkyl radicals to exert the same effect. The other antioxidants act by decomposing hydroperoxide, deactivating metals, terminating the cyclic chain and giving synergistic effect by working with several other antioxidants.

1.5.2. Enzymatic and non-enzymatic antioxidants

Antioxidants are broadly classified to enzymatic and non-enzymatic antioxidants. The antioxidant enzymatic system directly or indirectly contributes to defense against the ROS. The main enzymes that contribute to this class are: Catalase, superoxide dismutase (SOD), glutathione peroxidase andglutathione reductase. On the other hand, Non-enzymatic antioxidants are mainly: vitamins (A, C, E, and K), enzyme cofactors (Q10), minerals (Zn, Se, etc.), organosulfur compounds (allium and allium sulfur), nitrogen compounds(uric acid), peptides (glutathione), and polyphenols (flavonoids and phenolic acid). (Mehta & Gowder, 2015)

Figure 1.4 provides the broad classification of enzymatic and non-enzymatic antioxidants are further narrowed down to many more antioxidants contributes to inhibit free radical action through diverse mechanism of actions.

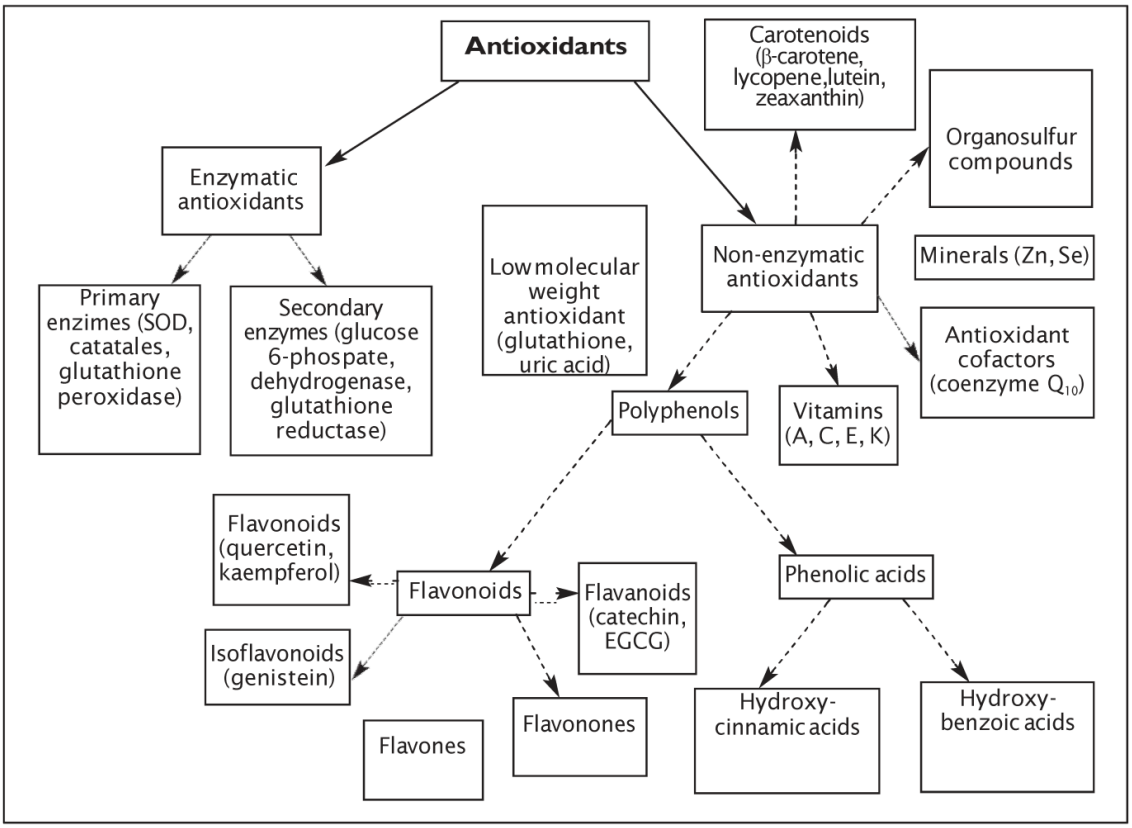


Figure 1.4: Enzymatic and non-enzymatic antioxidants (Mehta &Gowder, 2015)

The **Figure 1.4** explains that enzymatic antioxidants are classified to primary and secondary enzymes that act on various enzymatic reactions. Non-enzymatic antioxidants are further classified to polyphenols, vitamins, minerals, organosulfur compounds, cofactors and carotenoids.

Various enzymatic and non-enzymatic antioxidants differ by the locations as well as their properties. The **Table 1.4** describes the specific locations of those antioxidants and the specific properties that they possess. Enzymatic antioxidants are present in the mitochondria and cytosol of the cells. Especially enzymatic antioxidants names themselves represent their actions and properties inside the cells. Non-enzymatic antioxidants are more diversely located and their properties are different as well.

Table 1.4: Major enzymatic and non-enzymatic antioxidants along with their location and properties

Enzymatic antioxidants	**Location**	**Properties**
Superoxide dismutase (SOD)	Mitochondria and cytosol	Dismutation of superoxide radicals
Catalase (CAT)	Mitochondria and cytosol	Remove hydrogen peroxide
Glutathione Peroxidase (GSH)	Mitochondria and cytosol	Remove hydrogen peroxide and organic hyperoxide
Non-enzymatic antioxidants	Location	Properties
Vitamin C	Aqueous phase of cell	Acts as a free radical scavenger and recycles vitamin E
Vitamin E	Cell membrane	Major chain breaking antioxidant in cell membrane
Uric acid	Product of purine metabolism	Scavenger of OH radical
Carotenoids	Membrane tissue	Scavenger of ROS and singlet oxygen quencher
Glutathione	Non-protein thiol in cell	Serves multiple role in cellular antioxidant defense
Lipoic acid	Endogenous thiol	Effectual in recycling vitamin C, and also functional glutathione substitute

1.5.2.1. Mechanism of action of enzymatic and non-enzymatic antioxidants

Superoxide dismutase (SOD) is one of the most effective intracellular enzymatic antioxidants that catalyzes the dismutation of $O_2^{\bullet-}$ to O_2, resulting into the production of

less-reactive species, Hydrogen Peroxide, H_2O_2. Catalase is another enzyme present inside a cellular organelle, peroxisome that rapidly converts Hydrogen Peroxide to water and molecular oxygen, thus, preventing in the buildup of H_2O_2 within a cell. Glutathione metabolism, which involves the GP_X enzymes that in the presence of tripeptide glutathione (GSH). Glutathione metabolism is known as one of the most essential antioxidative defense mechanisms. It is the antioxidative properties of these enzymes that allow them to eliminate peroxides as potential substrates for the Fenton reaction (Mates, Perez-Gomes & de Castro, 1999).

Vitamin C, also known as ascorbic acid is a powerful antioxidant and it acts by participating in energetically favored oxidation reaction by scavenging the aqueous ROS in our body. They do not cause cellular damage (Kasparova *et al.*, 2005; Cuzzorcrea, Thiermann, & Salvemini, 2004). Vitamin C also, scavenges the ROS via rapid electron transfer that inhibits peroxidation (Jones, Kagan, Aust, Reed, & Omaye, 1995; Halliwell, Wasil & Grootveld, 1987). Vitamin E exists as eight different isomers, the two most common being- α-tocopherol& α-tocotrienol. The α-tocopherol is the most active form of Vit-E in the human body and is a powerful biological antioxidant – its major antioxidant function being protection of the cell membrane against lipid peroxidation.

1.5.3. Phytochemicals exerting antioxidant activity

Many of the natural bioactive compounds present in the dietary supplements are known to possess antioxidant properties. Some researchers suggested that, in replacement of synthetic antioxidant food additives natural antioxidant compounds should be searched and used preferably. Though there are several types of antioxidants present in food like vitamin C, vitamin E etc. major antioxidant capacity is considered to be present in phenolic acids and flavonoids.

Polyphenols are a groups of chemical substances present abundantly in plants are characterized by aromatic ring(s) and one or more hydroxyl moieties. According to Lee, Lin & Yu (2016), polyphenols are divided into four major classes including flavonoids, stilbenes, and phenolic acids (**Figure 1.5**).

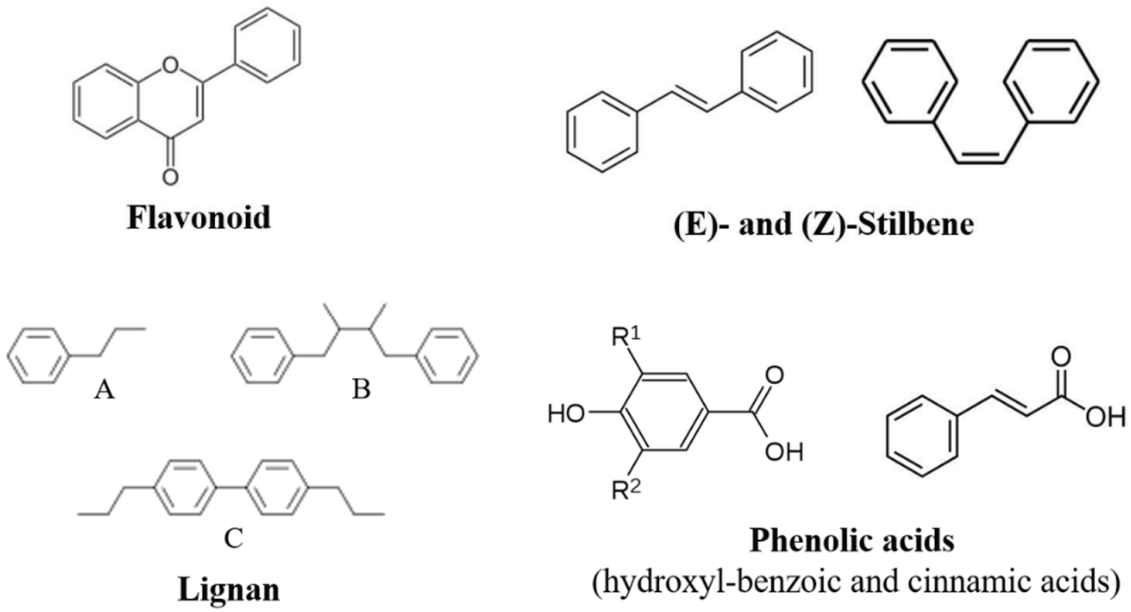


Figure 1.5: Chemical structures of flavonoid, stilbene, lignan and phenolic acids

When these compounds react with free radicals (**Figure 1.6**), the gained electron is delocalized over phenolic antioxidant. This stabilizes the resonance effect of aromatic nucleus and inhibits the continuity of the free radical chain reaction. Thus, the free radical scavenging activity is taken place. The mechanism by the resonance effect is shown on the **Figure 1.6**.

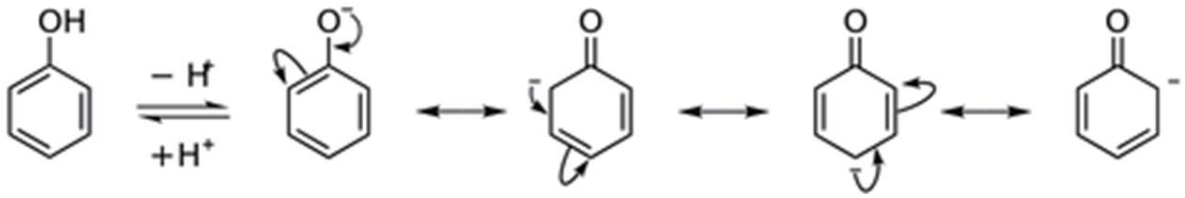


Figure 1.6: Resonance of aromatic nucleus of antioxidants when reacting with radicals

1.6. Phytochemicals

Medicinal plants possess various different chemical constituents, these chemicals presence and quantity can be varied from plant to plant. Moreover, certain plant parts such as leaves, stems etc. may carry different degrees of phytochemicals. It is important

to isolate and identify the phytochemicals in a plant extract in order to understand and obtain specific information about its pharmacological actions.

A large number of phytochemicals present in various medical plants are responsible for exerting pharmacological actions on human body that are used extensively in treating diseases. Their modes of actions are as diverse as their chemical structures. They are reported to neutralize free radicals, activate enzymes, and detoxify carcinogens and so on.

The major groups of phytochemicals are considered to be alkaloid, flavonoid, phenolic compound, carbohydrate, phytosterol, tannin, glygoside and saponin. These are present with various biological functions are discussed briefly in the **Table 1.5**.

Table 1.5: Major Phytochemicals and their biological functions

Class of Phytochemicals	**Mechanism of action**	**Example**
Alkaloid	Alkaloids act as topoisomerase (I) inhibitor and interacts with tubulin. Some of them target the mitochondria of a cancer cell, they show cytotoxicity effect in different stages of cell proliferation.	Arecoline (*Areca catechu*) Atropine (*Atropa belladonna*) Digitoxin (*Digitalis purpurea*)
Flavonoids	They can inhibit or minimize the cellular damage caused by free radical reactions. These compounds possess antimicrobial, estrogenic, anti-allergic and, cytotoxic antitumor activity.	Flavones Flavonols Flavanones
Phenol and Phenolic compounds	They contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways.	Stilbenes Lignins Phenolic acids
Carbohydrate	Carbohydrates serve as injectable anticoagulant, trigger the creation of antibodies, provide hormones etc.	Heparin Amylopectin
Phytosterol	Phytosterols have been found to inhibit proliferation, induce apoptosis, and reduce invasiveness of cancer cells in culture.	Campesterol Stigmasterol
Tannin	Tannins are capable of forming reversible and irreversible complexes with proteins, alkaloids, nucleic acids and minerals.	Gallotannin Ellagitannin
Glycoside	These compounds are potent inhibitors of cellular Na^+/K^+-ATPase.	*Digitalis purpurea*

1.7. *In-vitro* antioxidant activity study methods

Natural antioxidants like flavonoids, ascorbic acid etc. present in plants are capable of scavenging free radicals that are responsible for many diseases. Bioactive compounds present in plants showed significant role in preventing several neurodegenerative diseases caused by cell and tissue injury. To identify the antioxidant compounds and activities, a number of quantitative *in-vitro* tests are performed.

According to a review study by Alagumanivasagam, Pasupathy, Kottaimuthu & Manavalan (2012), there are 27 different types of *in-vitro* antioxidant activity study methods are used.Different types of tests require diffrent oxidizing agents and reagents to conduct the tests *in-vitro*. The **Table 1.6** describes the *in-vitro* tests that are conducted to measure the antioxidant capacity.

Table 1.6: Various *in-vitro* antioxidant tests (Alagumanivasagam, Pasupathy, Kottaimuthu & Manavalan, 2012)

Serial No.	**Name of the *in-vitro* antioxidant test**
1	DPPH(1,1-diphenyl-2-picryl-hydrazyl)free radical scavenging assay
2	Ferric reducing antioxidant power (FRAP) assay
3	Hydrogen peroxide radical scavenging (H_2O_2) assay
4	Hydroxyl radical scavenging (HO)assay
5	Metal chelating activity
6	Nitric oxide radical scavenging (NO) assay
7	Oxygen radical absorbance capacity (ORAC) assay
8	Reducing power (RP)
9	Superoxide anion radical scavenging (SO) assay
10	Total phenolic content (TPC)
11	Total flavonoid content (TFC)
12	Total antiosidant capacity (TAC)

13	Trolox equivalent antioxidant capacity (TEAC) assay
14	Cupric ion reducing antioxidant capacity (CUPRAC)
15	Enhanced chemiluminiscence (ECL)
16	Total radical trapping antioxidant parameter (TRAP)
17	Phospho molybdenum method
18	DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) method
19	Microsomal lipid peroxidation or thiobarbituris acid (TBA) assay

Generally more than one *in-vitro* antioxidant assays are used to calculate antioxidant activity because "both in food and *in-vitro*, oxidation reactions are complex", mentioned Badarinath, RAo, Chetty, Ramkanth, Rajan & Gnanaprakash (2010). There may be certain difference between the liberaton of antioxidant in food digestion and liberation for any *in-vitro* tests. Moreover, the mechanism of action inside the human body and in the laboratory test tubes may differ as there might be presence of absence of many factors. So quite a few *in-vitro* antioxidant assays are used to measure antioxidant capacity.

1.7.1. DPPH free radical scavenging assay

DPPH (1, 1-diphenyl-2-picryl-hydrazyl)is used as a free radical scavenger that helps in determining the free radical scavenging activity of the antioxidant present in the sample plant extract. In methanol or aqueous solution, DPPH generates stable free radicals by the delocalization of the free electrons which in turn produces a deep purple colored solution that is characteristic of absorption at 517nm. In the presence of antioxidants/hydrogen donors, DPPH is reduced to hydrazine that produces a characteristic change in the color of the solution from purple to yellow. Thus, at any given time, a decrease in the absorbance of DPPH at 517nm is directly proportional to the scavenging activity which can be observed visually as well by the degree of intensity of color changing from purple to yellow.

The rationales behind using DPPH over other free radical scavengers are as follows:

(a) During the delocalization of free electron from the DPPH molecule, the entire molecule does not dimerize as does other free radical scavengers.
(b) Less time consuming as it involves use of no additional reagent nor any temperature equilibration. Thus, it is easy and rapid (Alam, Bristi, & Rafiquzzaman, 2012).

1.7.2. Determination of total phenolic content (TPC)

Polyphenols can act as hydrogen or electron donors, thus their antioxidant property lies in the capability ro stabilize and delocalize the unpaired electron that serves as chain breaking function to inhibit the free radical reaction. Moreover, they potentially form chelate metal ions that terminates the Fenton reaction. (Rice-Evans et all, 1997)

This method intends to measure the reducing capacity of the sample by using folin-ciocalteu reagent (FCR). Whilst the exact chemical nature of FCR is still unknown, it is believed that the compound is a heteropolyphosphotunstates-molybdate that undergoes a sequence of reduction reactions involving transfer of one or two electrons that result into the formation of a blue-colored complex, possibly $(PmoW_{11}O_{40})^{-4}$. In this form of complex, it is believed that molybdenum is easier to be reduced and electron-transfer reactions occur between the reducing agent and Mo (VI) as follows:

$$Mo\ (VI) + e^{-} Mo\ (V)$$

The absorbance of the blue-colored complex at 765nm is measured against blank (methanol and reagents) using the UV-Visible Spectrophotometer and the total phenolic content (C) of each of the fractions were expressed as gallic acid equivalents (GAE).

1.7.3. Determination of total flavonoid content (TFC)

Flavonoids have various machanism of action including scavenging of free radicals, chelation of metal ions like iron and copper, and inhibition of enzymes those act to generate free radical reaction. Depending on their chemical structure, flavonoids can scavenge free radicals in different mechanisms.(Benavente-Garcia, 1997)

This method intends to determine the total flavonoid content by a stable acidic complex that is formed between 3 substrates – the C-4 keto-group with either the C-3 or C-5 hydroxyl group of the flavones and flavanols present in the sample with that of

aluminium chloride (Pal, Sannigrahi & Mazumder, 2009). Furthermore, aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of the flavonoids. The intensity complex formation is measured spectrophotometrically at 415nm.

1.7.4. Determination of total antioxidant capacity (TAC)

This method is Also known as the phosphomolybdenum method. In this method, the total antioxidant capacity can be quantitatively determined by following spectrophotometric analysis at 695nm. The assay is basically based on the reduction of Mo (VI) to Mo (V) by the sample analyte which subsequently results in the formation of a green-colored phosphate-molybdenum (V) complex at an acidic pH.

1.8. Cervical cancer

Cervical cancer is one of the major causes of cancer deaths in women. According to a research conducted by Nour(2009), about 260,000 women die of cervical cancer and among them 85% deaths occur in deveoping countries. Whereas WHO estimates that among 500,000 new cases of cervical cancer anually, 80% women are affected by it whose age is between 15 and 45 years residing in developing countries. HPV(Human Papilloma Virus) is responsible for the cervical cancer occured in cervix, as well as cancer of vagina, vulva, penis and anus, along with some head and neck cancers, angeogenital warts and recurrent respiratory papillomatosis.

1.8.1. Prevalence of cervical cancer globally and in Bangladesh

According to WHO cervical cancer is the fourth most common cancer in women worldwide. In the global report of incidence of mortality due to cervical cancer (2012), it was found out that about 528,000 new cases of cervical cancer are reported globally. The majority of these cases are found to be from the less developed regions of many parts of the world. The **Table 1.7** shows the incidence, mortality and prevalence of cervical cancer according to the WHO report from the year 2012.

Table 1.7: Estimated incidence, mortality and prevalence of cervical cancer worldwide (WHO, 2012)

Estimated numbers (thousands)	Cases	Deaths	5-year preview
World	528	266	1547
More developed regions	83	36	289
Less developed regions	445	230	1258
WHO Africa region (AFRO)	92	57	236
WHO Americas region (PAHO)	83	36	279
WHO East Mediterranean region (EMRO)	15	08	42
WHO Europe region (EURO)	67	28	225
WHO South-East Asia region (SEARO)	175	94	465
WHO Western Pacific region (WPRO)	94	43	299
IARC membership (24 countries)	206	103	595
United States of America	13	07	47
China	62	30	190
India	123	67	309
European Union (EU-28)	34	13	115

According to the estimate number of incidence, mortality and prevalence of last 5 years (WHO, 2012), it is evident that among the 528,000 new cases of cervical cancer 266,000 deaths were recorded worldwide. Whereas, among all the new cases recorded throughout the world, it is estimated that 445,000 new cases were from less developed region, which apparently the lion's share of the total cervical cancer cases recorded worldwide. In the less developed region about 230,000 deaths have been recorded due to cervical cancer. It is estimated from the records of WHO that, apparently few cases of cervical cancer was recorded in United States of America, East Mediterranean region and European Union and less deaths occurred compared to other regions of the world. It is also evident from that chart that more developed regions prone to have less new cases of cervical cancer and thus they face less mortality cases due to cervical cancer compared to less developed countries.

According to ICO HPV information center's annual report of 2017, annually 11,956 new cases of cervical cancer have been reported which makes this cancer as the second leading cause of female deaths in Bangladesh. Cervical cancer also stands as the second most common cancer in women whose cage is between 15-44 years.

Table 1.8: Cervical cancer incidence in Bangladesh (estimates for 2012)

Indicator	Bangladesh	Southern Asia	World
Annual number of new cancer cases	11,956	145,946	527,624
Crude incidence rate	15.9	17.1	15.1
Age-standardized incidence rate	19.2	19.3	14.0
Cumulative risk (%) at 75 years old	2.1	2.1	1.4

The **Table 1.8** indicates the annual number of new cases recorded in Bangladesh compared to Southern Asia and the world. It appears in total 527,624 new cases are reported annually whereas 145,946 new cases are reported to be from Southern Asia's other countries including Bangladesh. Whereas, alone in Bangladesh 11,956 new cases are recorded each year, which is definitely a huge number. Compared to the other countries Bangladesh has high incidence rate of cervical cancer which is 15.9. On the other hand, the cumulative risk (%) of cervical cancer is 2.1 which are much higher than the % risk (1.4) occurred in throughout the world. It appears that Bangladesh has high incidence rate for cervical cancer compared to Southern Asia and other countries of the world.

The statistics of ICO HPV annual report of Bangladesh of 2017 also infers that 6582 female deaths are recorded from cervical cancer. After the breast cancer, female deaths are more prominent in cases of cervical cancer. The **Table 1.9** shows the mortality report of female in Bangladesh due to cervical cancer.

Table 1.9: Cervical cancer mortality in Bangladesh (estimates for 2012)

Indicator	Bangladesh	Southern Asia	World
Annual number of deaths	6582	79,958	265,672
Crude mortality rate	8.7	9.4	7.6
Age-standardized mortality rate	11.5	11.0	6.8
Cumulative risk (%) at 75 years old	1.3	1.2	0.8

About 265,672 female deaths have been reported throughout the world, whereas in Southern Asia 79,958 female deaths have been reported according the estimation. The crude mortality rate is definitely high (8.7) compared to world (7.6) and the cumulative

risk (%) for the death due to cervical cancer is also high in Bangladesh compared to other countries of the world except Southern Asia region.

1.8.2. Human papilloma virus and its' mechanism to cause cervical cancer

For the first time in 1970s Haral zur Hausen identified HPV in warts and cervical cancer. Later he isolated and cloned various types of strains of HPV. HPV is a double-stranded DNA vius with a non-enveloped structure. A capsid shell made of L1 and L2 designated major and minor structural protein protects the genome of the virus. To present date, more than 100 subtypes of HPV are discovered but HPV type 16 and 18 are mainly responsible for causing cancer in over 70% of cervical cancer cases. Moreover, these subtypes of HPV have been found in 99.7% of women suffering from cervical cancer. The predominant cause of spreading the virus is through sexual contact. (Nour, 2009)

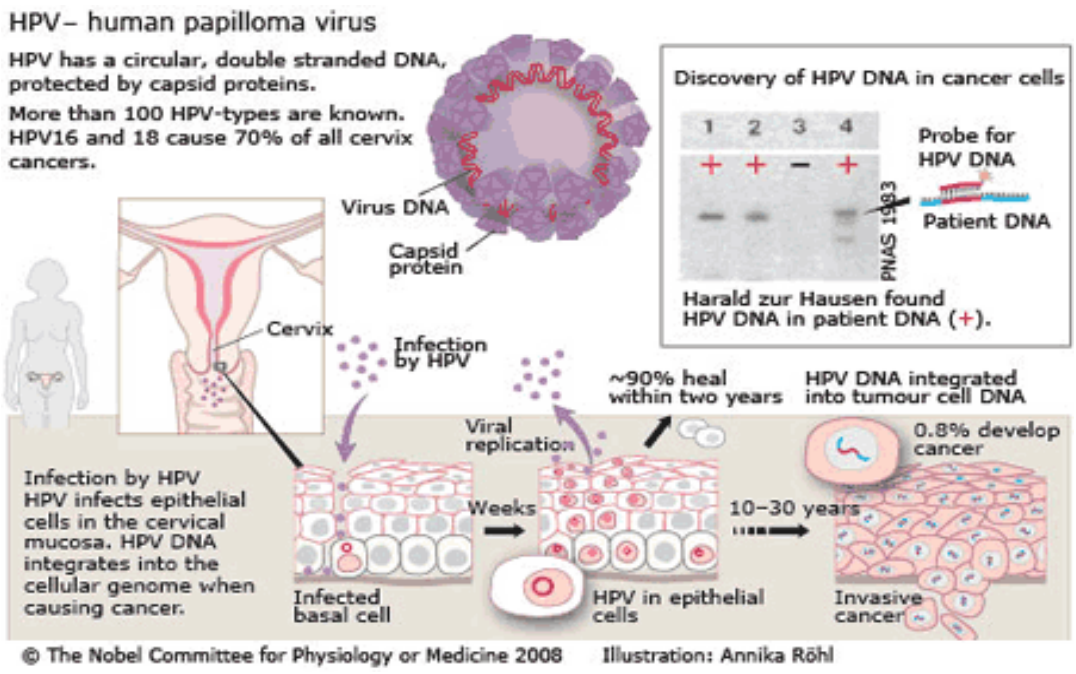


Figure 1.7: The mechanism of HPV attacking human cell and causing cervical cancer (Nour, 2009)

The **Figure 1.7** describes the whole mechanism by which HPV attack the human cell and causes cervical cancer gradualy. When HPV enters the gost body first it effects the intraepithelial layer of cervical mucosa. It is found that 50% of the patients in this stage

develops serum antibiotics, although these are no effective enough until they target the L1 protein. After approximately 8 to 12 months the seroconversion takes place. Seroconveriosn is actually the definite time period in which an antibody develops in body and becomes detectable in blood stream. However, after the infection the infected cells develop pre-cancerous properties that results in cervical intraepithelial neoplasia(CIN) or adenocarcinoma in-situ (AIS). If the patient is not treated, then these situations lead to squamous cell carcinoma or adenocarcinoma in respective organs or parts of body.

1.8.3. Risk factors for cervical cancer

The virus responsible for causing cervical cancer is itself a sexually transmitted virus. So the risk factors may as well be similar to other sexually transmitted diseases. The major risk factors are listed below (Nour, 2009):

(a) Early age at first sexual activity
(b) Multiple sex partner
(c) Early age at first delivery
(d) Increased number of pregnencies
(e) Smoking
(f) Immunosuppression (HIV)
(g) Long term use of oral contraceptives

Most of these factors are easily relatable to the scenario of the women living developing or less developed countries. That perspective provides a clear picture about the high occurrence and mortality rates of women living in these regions.

1.8.4. Treatment and prevention of cervical cancer

Cervical cancer is the third most common malignancy among women (Ordikhani, Arslan, Marcelo, Sahin, Grigsby, Schwarz & Azab, 2016). The treatment strategies and selection of drugs depend on th stage of cancer, thus it is a “stage-specific” treatment. To treat the metastatic cancers several chemotherapeutic agents have shown attractive activity and performance in healing this cancer. These agents are: cisplatin, carboplatin, paclitaxel, ifosfamide and topotecan. The chemical structure of these anticancer agents

are given in **Figure 1.8** (Ordikhani, Arslan, Marcelo, Sahin, Grigsby, Schwarz & Azab, 2016).

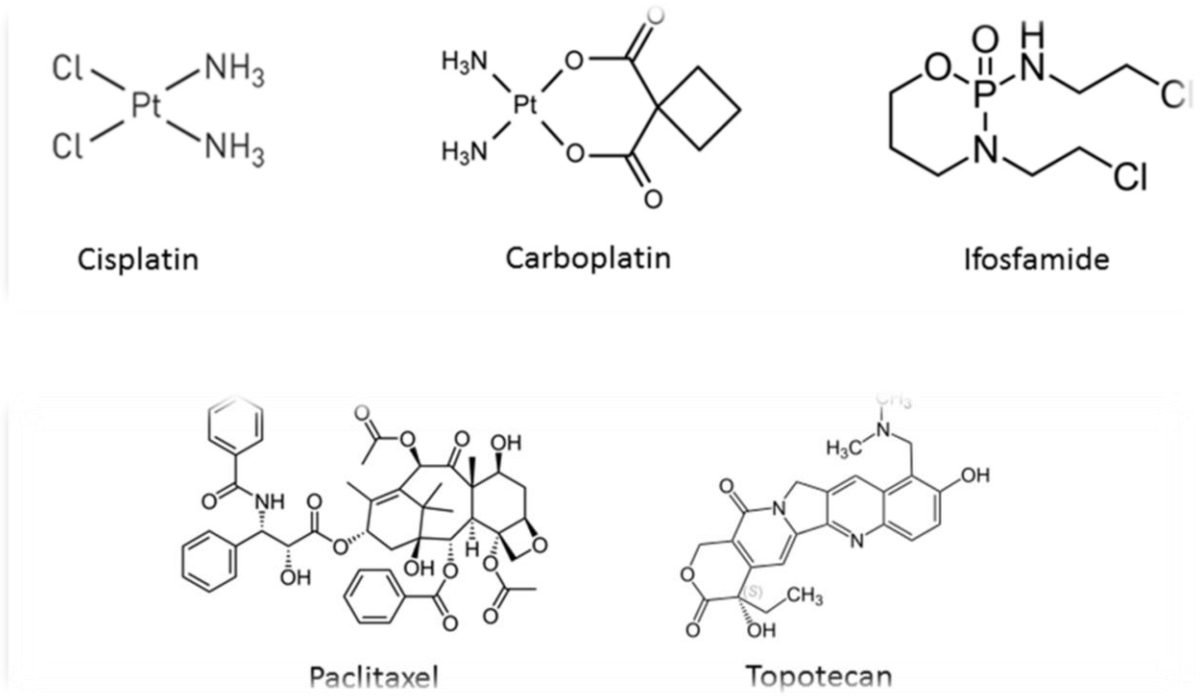


Figure 1.8: Chemical structure of anticancer drugs used to treat cervical cancer

Among all these agents, cisplatin is recorded to to be the most effective chemotherapeutic agent for treating advanced cervical cancer. Cisplatin can interact with DNA forming adducts and force to stop gene transcription that induces oxidative stress and appoptosis in tumor cells.

Preventive HPV vaccines have been produced to take a preventive action against cervical cancer. These vaccines are mainly non-infectious protein based, virus-like particles (VLPs) that induce high levels of nutralizing antibodies when directed against L1 protein epitopes. (Lowy, Solomon, Hildersheim, Schiller & Schiffman, 2008)

Currently there are commercial versions of vaccines available approved by FDA. The composition and respective manufacturers are listed in the **Table 1.10**. These three cervical cancer vaccines are commercially available, among these Cervarix was first synthesized and marketed which is composed of VLPs from HPV-16 and HPV-18. However, these two subtypes are known to be majorly responsible for cervical cancer.

Later Gardasil and Gardasil 9 vaccines were synthesized which contain many other VLPs for other subtypes including HPV-16 and HPV-18.

Table 1.10: HPV vaccines along with the composition and manufacturer name

Brand name	**Manufacturer**	**Composition**
Cervarix	GlaxoSmithKline (GSK)	Bivalent vaccine Composed of L1 VLPs from HPV-16 and HPV-18
Gardasil	Merck & Co.	Quadrivalent vaccine Composed of L1 VLPs from HPV-16, HPV-18 and HPV-6, HPV-11
Gardasil 9	Merck & Co.	Nonavalent vaccine Composed of L1 VLPs from HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52, HPV-58 and HPV-6, HPV-11

1.9. Cytotoxic activity

Cytotoxicity is a property of certain chemical substances that leads to cell death. When a cytotoxic agent is applied to a cancerous cell or normal cell, it may undergo cell necrosis. However, cytotoxic study is a very important step to determine the potential toxicity of substance, where it helps to determine and understand the efficacy and capability to inhibit cancerous cell growth and provide significant perspective in cancer treatment. If a potential agent is confirmed to kill both normal and cancerous cells, that agent is considerably called cytotoxic agent. Whereas, if an agent can potentially kill only cancerous cells but do not harm the normal cells instead, that agent can be considered as an anticancer agent and further clinical trials and tests are conducted before using it as anticancer agent. Therefore, it is an important step to identify the cell viability or cytotoxic assay to find out its' capability to harm normal cell or cancerous cells or both.

1.9.1. Methods of screening cytotoxicity

Cytotoxic screening for a substance can be carried out in two major ways: *in-vitro* and *in-vivo*. Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests for chemicals. These studies are based on various cell functions, such as enzyme activity, cell membrane permeability, cell adherence, ATP production, coenzyme production and nucleotide uptake activity. There are many methods which are established to count the number of live cells, such as Colony formation method, Crystal Violet method, MTT, WST method etc.

1.9.1.1. *In-vivo* cytotoxicity screening

In the *in-vivo* screening of cytotoxic activity, generally mice models are used and cytotoxic T lymphocytes (CTL) are used to target certain cells. The cytotoxic activity is determined by measuring degradation of radioactively labeled DNA in target cells that have undergone apoptosis. The CTL effector cells are generated in particular mice which are virally infected or peptide-primed. Then these amice are injected with target cells labeled with carboxyfluoresceindiacetatesuccinimidyl ester (CFSE). The CTL activity is then quantified by flow cytometry, using spleen or lymph node cells from these model mice by calculating the loss of CFSE bright cells. (Wonderlich, Shearer, Livingstone & Brooks, 2006)

1.9.1.2. *In-vitro* cytotoxicity screening

When a number of test molecules or compounds are screened to find out if they have any role in cell proliferation or show direct cytotoxic effects that may result in cell death, cell-based assays are often used. Moreover, these assays are used to measure receptor binding and studying signal transduction events that can play important role in genetic reporters, trafficking cellular components or monitoring organelle functions. To know the quantity of viable eukaryotic cells, different types of cell-based assays are widely used. The most used *in-vitro* cytotoxicity test methods include: tetrazolium reduction, resazurin reduction, protease markers, and ATP detection method. (Riss, Moravec, Niles, Duellman, Benink, Worzella & Minor, 2016)

In the tetrazolium reduction, resazurin reduction, and protease activity assays, the aspect of general metabolism or enzymatic activity is measured as the marker of viable eukaryotic cells. In these methods, first the reagents are incubated along with a

population of viable cells so that the substrate is converted to a fluorescent product and later it is detected by a plate reader or hemocytometer. Usually this incubation period will result in a signal that is proportional to the number of viable cells present in that medium. Along with the cell death, their ability to covert the substrate to product also decreases. Based on this theory generally the cell-based assays are done. The ATP assay has different approach than these; no incubation period is needed here as the reagent immediately ruptures the cells in this method. (Riss, Moravec, Niles, Duellman, Benink, Worzella & Minor, 2016)

1.9.2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is a type of tetrazolium reduction assay which was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening. It is a widely used assay to detect cell viability. First the MTT substrate is prepared in a solution, added to cells in culture and then incubated for 1 to 4 hours. The theory depicts the quantity of formazan produced is directly proportional to the number of viable cells. Then the measurement is taken by recording the changes in absorbance at 570 nm using plate reading spectrophotometer.

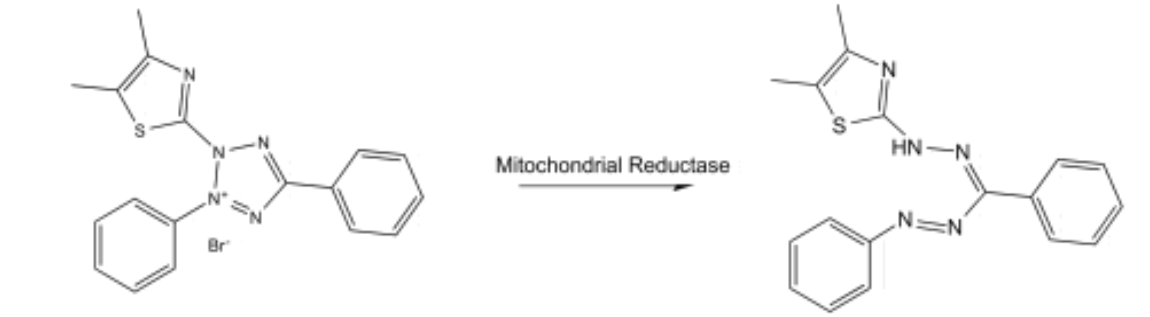


Figure 1.9: Structure of MTT and colored formazan product

The viable cells in culture convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm (**Figure 1.9**). When the cell's active metabolism decreases or cell death occurs, they ability to convert MTT to formazan also reduces. It is estimated that the conversion from MTT to formazan involves reaction with NADH or

similar reducing molecules that transfer electrons to MTT. As only the active metabolism-able viable cells can convert MTT to formazan, this color formation serves as useful marker only for the viable eukaryotic cells. (Riss, Moravec, Niles, Duellman, Benink, Worzella & Minor, 2016) However, formazan forms as purple needle shaped crystals in the cells which are insoluble in water. Thus, organic solvents (DMSO, dimethylformamide, SDS etc.) are used to solubilize the crystals, this is an important step in MTT cell viability assay.

Among all the other enzyme-based cytotoxicity screening assays, MTT is widely used because it is an easy-to-use method; it has high reproducibility and safe method. It is also the best method to determine the mitrochondrial dehydrogenase activity in the cells.

1.10 Overview of Bangladeshi medicinal plant

It is estimated that about 87% of drugs source comes from nature to treat various types of diseases. About 25% prescribed drugs were originated from plants. (Khatun, Rahman[1], Haque, Rahman[2], Akter[1], Akter[2]&Jhumur, 2014).Uddin et al. mentioned that about 80% people in developing countries are depended on plant-based or herbal medicines for their primary health care. The medicinal plants possess many pharmacological activities such as, antibacterial, antifungal, anticancer, antioxidant, anti-inflammatory etc. properties. Traditionally people are dependent on these herbs and plants to treat their primary ailment with the ancient knowledge of the plants, especially in rural areas.

Bangladesh possesses a rich source of various medicinal plants among all other South Asian countries. The traditional resources and diverse ecology is the key reason for the natural resources. It is estimated that almost 250 species of medicinal plants are used to prepare traditional medicines in many parts of Bangladesh (Khatun, Haque, Rahman, Akter & Jhumur, 2014).

Some medicinal plants and their traditional uses are discussed in the **Table 1.14** below. Usually these medicinal plants are found throughout Bangladesh and used traditionally for mild illness. For an instance, *Adhatoda vasica* plant leaves are randomly used in treating cough and minor cold. *Helitropium indicum* plant is not very hard to find, it grows in almost every part of the country and the leaves are widely used to treat pyresis.

These are just mere examples of medicinal plants of Bangladesh; the tropical regions of the country possess a wide variety of medicinal plants.

Table 1.11: Some medicinal plants with traditional uses

Plant name	**Local name**	**Traditional use**
Adhatoda vasica	Basak	Cough
Cinnamomum zeylanicum	Dalchini	Vomiting
Eugenia jambolana	Jam	Dysentery
Helitropium indicum	Hatissur	Pyresis
Psidium guajava	Piyara	Diarrhoea

1.10.1. Selection of *C. pallida* for present study

After doing extensive literature review on medicinal plants of Bangladesh, it was found that a number of *Crotalaria* genus plants were previously studied for cytotoxity using different cell lines. But no study for the cytotoxic activity was found to be carried out using *C. pallida* in HeLa cell which is human cervical carcinoma cell line. Moreover, the study of antioxidant activity was not carried out in Methanolic extract of plant previously. That is why, the study of antioxidant activities with Methanolic extract of the plant and cytoxocity activity on HeLa cell line was planned for the present study.

1.10.2 Introduction to *C. pallida*

C. pallida belongs from the Fabaceae family which is also known as Leguminosae. Leguminosae is the largest economically important family. It is also known as the pea, legume or bean family.

Among the 690 species of *Crotalaria* genus, this species is widely distributed in tropical Africa and Madagaskar. This species is also found in Bangladesh, India, China, Sri Lanka and USA (Kabir et al, 2015). **Figure 1.10** shows the picture of *C. pallida* plant.

Figure 1.10: *Crotalaria pallida* plant

1.10.3. Plant taxonomy

The plant taxonomy of *C. pallida* plant is shown in the **Table 1.15** below.

Table 1.12: Plant taxonomy of *C. pallida*

Rank	**Scientific name (Common name)**
Kingdom	*Plantae* (Plants)
Subkingdom	*Viridiplantae*
Infrakingdom	*Streptophyta*(land plants)
Superdivision	*Embryophyta*
Division	*Tracheophyta*(vascular plants)
Subdivision	*Spermatophytina*(spermatophytes, seed plants)
Class	*Magnoliopsida*

Superorder	*Rosanae*
Order	*Fabales*
Family	*Fabaceae*(peas, legumes)
Genus	*Crotalaria L.* (rattlebox)
Species	*Crotalaria pallida*(smooth rattlebox)

1.10.4. Plant description

C. pallida is also known as "smooth rattlebox" and it belongs to a Fabaceae (Leguminosae) family. In tribal regions of Bangladesh it is also known as KudugJhunjhuni (Chakma); Easygaas (Tanchunga); ThaSimNoi, RatiAapa (Marma); Roa Bay (Murang).The plant is an erect low shrub up to 1.5 m high. The leaves are 3-foliolate, leaflets 7-10 cm long, obovate-oblong, obtuse or subacute, glabrous above and obscurely silky beneath. Usually racemes are 20-50-flowered, reaching 15-30 cm long. Corolla yellow striped with red. The pods are turgid, deflexed, rather recurved, 4-5 cm long, 20-30-seeded.

1.10.5. Habitat and traditional use of *C. pallida*

C. pallida plant is usually grown in fallow lands all over the tropical areas of the world. In Bangladesh, it can be found on hilly tracts of Chittagong and Sylhet. Apart from Bangladesh, this plant is grown in a large amount in Sri Lanka, India and native in tropical Africa.

Traditionally this plant is used to treat urinary problems, reducing fever, skin problems like eczema and the leaf extract is taken as vermifuge. A poultice made of the roots is applied to painful swelling of joints. Apart from medicinal use, various parts of this plant are taken as food in several parts of the world. In Indo-China, a kind of coffee is prepared with the roasted seeds of the plant. In west Java, a fermented product was formerly made from the seeds. The seeds were boiled for two hours, wrapped in banana leaves and left to ferment for several days to remove poisonous components. In Cambodia the flowers are taken as a vegetable. (Hamem & Macsen, 1997)

1.11. Rationale of the project

Previous study on this legume plant included investigation into its *in vivo* anti-diabetic, *in vitro* anticoagulant activity, *in vivo* anti-fertility, antipyretic, thrombolytic and CNS depressant potential has been investigated. No previous study has yet been conducted on cytotoxicity of *C. pallida* on using any cell line. Previously estrogenic and mutagenic activity has been found in this plant but this study aims to find cytotoxic effect on cancerous cells. Moreover, although antioxidant properties have been found in leaf extracts using ethanol, ethyl acetate, petroleum ether and water as solvents but previously *in-vitro* antioxidant study was not carried out using methanolic leaf extract. So, this study will provide a scope to conduct comparison between antioxidant activity between methanolic leaf extract and other alcoholic leaf extracts.

1.12. Aim of the project

Investigation of *in-vitro* antioxidant and cytotoxic potential of *C. pallida* leaves.

1.13. Objectives of the project

After studying the literature review pertaining to the previous findings of *C. pallida*, the objectives of the project were made as follows with regards to using methanolic leaf extract of *C. pallida*:

(a) Carrying out phytochemical screening in order to qualitatively determine the presence of chemical constituents.

(b) Determination of its antioxidant potential using various *in vitro* methods.

(c) Assessment of its free radical scavenging potential comparable to that of standard ascorbic acid using DPPH free radical scavenging method.

(d) Scope to compare antioxidant activity between methanolic leaf extract and other alcoholic leaf extracts.

(e) Investigation of cytotoxicity of methanolic leaf extract on HeLa cell line.

CHAPTER TWO
LITERATURE REVIEW

Chapter 2: Literature Review

2.1. Previously studied pharmacological activities of *C. pallida*

According to previous researches carried out and from their findings, *C. pallida* plant possesses various pharmacological activities such as anti-inflammatory, antioxidant, antibacterial etc. The pharmacological activities and their used plant parts are listed below in **Table 2.1**.

Table 2.1: Previously studied pharmacological activities of *C. pallida*

Pharmacological activity	**Plant parts**	**Extraction solvent**	**Reference**
Antibacterial	Leaves	Methanol, ethanol, ethyl acetate, petroleum ether, chloroform, water	Kiruthiga, Rakkimuthu & Aravinthan (2014) Govindappa, Bharat, Shruthi, Sadananda&Sharanappa (2011)
Anti-inflammatory	Leaves	Ethanol, ethyl acetate, petroleum ether, chloroform, water	Govindappa, Bharat, Shruthi, Sadananda & Sharanappa (2011)
Antioxidant	Leaves	Ethanol, ethyl acetate, petroleum ether, chloroform, water	Govindappa, Bharat, Shruthi, Sadananda & Sharanappa (2011)
Estrogenic activity	Leaves	Ethanol	Boldrin, Resende, Hohne, Camargo, Espanha, Nogueira, Melo, Vilegas & Varanda (2013)
Mutagenic activity	Leaves	Ethanol	Boldrin, Resende, Hohne, Camargo,

			Espanha, Nogueira, Melo, Vilegas & Varanda (2013)
Thrombolytic	Leaves	Methanol	Kabir, Murad, Hasanat, Hamid, Islam, Chowdhury, Hasan, Hossain, Masum, Uddin (2015)
HIV-protease inhibitor	Leaves, flowers and stems	Methanol and ethanol	Govindappa, Kumar & Santoyo (2011)

According to previous researches, it is evident that not only the leaves of *C. pallida* but also other plant parts also possess pharmacological activities of various types. It is also found out that methanol and ethanol solvents were mostly used for the extraction of plant parts to further analyze its' pharmacological activities. To the present date, *C. pallida* is reported to have antibacterial, anti-inflammatory, antioxidant, estrogenic, mutagenic, thrombolytic activities and it is found to act as HIV-protease inhibitor.

2.1.1. Antibacterial activity

The study conducted by Kiruthiga, Rakkimuthu & Aravinthan (2014) on the methanolic leaf extract of *C. pallida* provided adequate information about prominent antibacterial activity of the plant. In this study only methanolic extract was used as the solvent and the antibacterial activity was tested against *Escherichia coli, Klebsiella pneumoneae, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus.* The activity was observed by agar diffusion method where Nutrient Agar plates were swabbed with 12-hours old broth culture of respective bacteria and then different concentrations of methanolic leaf extract was added to observe the zone of inhibition. The plates were incubated at 37℃ for 24-48 hours. The result of this study showed that, *Escherichia coli* and *Klebsiella pneumoneae* showed maximum susceptibility at 25mg/mL concentration compared to other three bacteria.

Another study of antibacterial activity of this plant extract was conduct using *Escherichia. Coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas fluorescens, Clavibacter michiganensis, Michiganensis, Xanthomonas oryzae, Xanthomonas axanopodis* bacteria by Govindappa, Bharat, Shruthi, Sadananda & Sharanappa (2011). In that study paper-disk method was used to determine zone of inhibition. Moreover, the solvents which were used here respectively ethyl acetate, ethanol, petroleum ether, water and chloroform. The result expressed that, the highest activity of ethanol extract observedon *X. axanopodis, E. coli* and *C.michiganensis* spp. *michiganensis*. The ethanolic extract was more active against all bacterial strains.

2.1.2. Anti-inflammatory activity

According to a study by Govindappa, Bharat, Shruthi, Sadananda & Sharanappa (2011), the leaf extract of the plant showed five different methods to determine the anti-inflammatory activity.

(a) Inhibition of Albumin denaturation: In this test, sample extract and 1% aqueous solution of bovine albumin fraction was mixed along with small amount of HCl. The mixture was incubated for 20 minutes in 37℃, and then followed by heating and cooling the turbidity was measured at 660 nm. As a result, maximum inhibition was observed from ethanol extract followed by petroleum ether, ethyl acetate, water and lastly chloroform.

(b) Membrane stabilization test: For this test, at first 10mL of fresh whole human blood was collected and centrifuged. The then volume of blood was measured and reconstituted as 10% v/v suspension with normal saline. Then 10% RBCs suspension was mixed with test sample solution and incubated at water bath at 56℃ for 30 minutes. Again the mixture was centrifuged and the absorbance of supernatant was measured at 560 nm. Percentage of Membrane stabilization activity was calculated by an established formula. As a result, ethanolic extract has shown highest membrane stabilization activity followed by petroleum ether and ethyl acetate.

(c) Protein Inhibitory Action**:** In this test, test sample was mixed with trypsin and TrisHCl buffer and then incubated for 37℃ for 5 minutes. Then 0.8% w/v casein was added. 70% perchloric acid was added at last to terminate the reaction. The cloudy

suspension was centrifuged and the supernatant was measured for absorbance. As a result, ethanol extract has shown highest protein inhibitory activity.

(d) Xanthine Oxidase Assay: In this assay, two types of buffer solution were prepared using xanthine and Nitro Blue Tetrazolium Chloride and another consisted of xanthine oxidase. Adding the solvent extract the mixture was incubated for 20 minutes and then the activity was observed. As a result, maximum inhibition of xanthine oxidase was observed from ethanol extract.

(e) Acetylcholinesterase Inhibitory Activity: The assay mixture for this test contained Tris HCl, BSA buffer and Acetylcholinesterase along with the sample extract. The mixture was incubated before adding DTNB and Acetylcholinesterase iodide. The developing yellow color was measured and the percentage of acetylcholinesterase inhibitory activity was measured using the formula. As a result, ethanol and petroleum ether showed best activity.

2.1.3. Antioxidant activity

According to a study by Govindappa, Bharat, Shruthi, Sadananda & Sharanappa (2011), to determine the antioxidant activity of plant extract Ferrous reducing antioxidant property (FRAP) assay and DDPH radical assay tests were conducted. The result from these studies shows that ethanol extract of *C. pallida* had higher activity than that of other solvent extracts. At a concentration of 0.1 mg/mL, the scavenging activity of ethanol extract of *C. pallida* reached above 80% while petroleum ether, ethyl acetate, chloroform and water extracts also reached 60%.

2.1.4. Estrogenic and mutagenic activity

The recombinant yeast assay with the strain BY4741 of *Saccharomyces cerevisiae*, was performed with the ethanolic extract, dichloromethane fraction and stigmasterol isolated from the leaves of *C. pallida*. Mutagenic activity was evaluated by the Salmonella/microsome assay (Ames test), using the Salmonella typhimurium tester strains TA100, TA98, TA97 and TA102, with (+S9) and without (−S9) metabolization, by the preincubation method. Results express that all samples showed estrogenic activity, mainly stigmasterol. The ethanolic extract from *C. pallida* leaves showed mutagenic

activity in the TA98 strain (−S9), whereas dichloromethane fraction and stigmasterol were found devoid of activity.

Considering the excellent estrogenic activity performed by stigmasterol in the RYA associated with the absence of mutagenic activity when evaluated by the Ames test, stigmasterol becomes a strong candidate to be used in hormone replacement therapy during menopause. (Boldrin, Resende, Hohne, Camargo, Espanha, Nogueira, Melo, Vilegas & Varanda, 2013).

2.1.5. Thrombolytic activity

According to a study by Kabir, et al (2015), the test was performed using this particular method where lyophilized streptokinase was mixed with sterile water. The venous blood was collected from the volunteers and distributed in the mixture. The mixture was incubated at 37℃ for 45 minutes to form the clot. After the incubation and clot formation, the serum was removed carefully to weigh the clot weight. Then the ethanol extract was added there and incubated for 90 minutes to observe clot lysis. After incubation, the tubes were again weighed to measure the difference in weight after clot disruption. In the *in-vitro* thrombolytic activity study *C. pallida* showed highest clot lysis effect.

2.1.6. HIV-protease inhibitor activity

To conduct the test Govindappa, Kumar and Santoyo (2011) used pepsin enzyme which closely resembles the proteolytic activity of HIV-1 Protease. Pepsin, hemoglobin and different parts of *C. pallida* plant extract were mixed and incubated for 20 minutes. Then 5% TCA was added to stop the reaction. The then mixture was centrifuged and the supernatant was taken for measuring the absorbance. The ethanol and methanol stem and flower extracts exhibited strong inhibition of pepsin enzyme. Strong inhibition was noticed in methanol stem extract followed by ethanol stem extract, methanol flower extract and ethanol flower extract while other extracts with negligible toxic effect.

CHAPTER THREE
METHODOLOGY

Chapter 3: Methodology

3.1. Collection and authentication of plant material

C. pallida was as the plant for this investigation since no previous study was conducted on its cytotoxicity and several antioxidant potential tests. With the aid of comprehensive literature study of this plant and its availability, it was decided to be chosen for investigation of *in-vitro* antioxidant and cytotoxic potential of *Crotalaria pallida* leaves. *C. pallida* plant belongs to the Fabaceae family and in this study only the leaves were taken for the investigation.

The whole plant *C. pallida* was collected in the month of August 2017 from Chittagong, Bangladesh. Thereafter, it was submitted to the National Herbarium of Bangladesh (NHB), Mirpur, Dhaka for its authentication. A week later, its voucher specimen was collected and the plant was identified (Accession No.: DACB-45302) and authenticated by the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka.

3.2. Extraction procedure

The entire extraction procedure can be virtually divided into 2 parts:

(a) Plant material preparation and drying (2-steps)

(b) Extraction process (5-step)

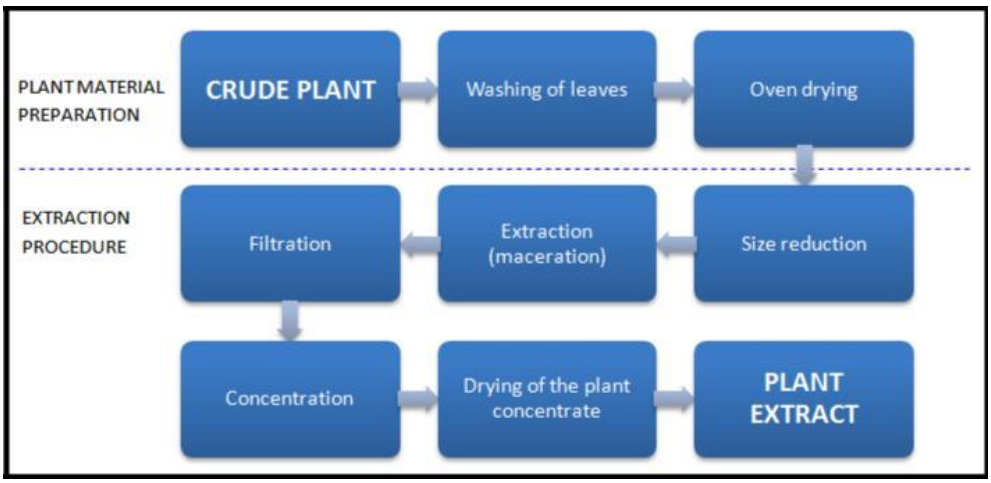


Figure 3.1: Flowchart showing a step-by-step procedure for the extraction

The **Figure 3.1** shows the steps involved in the extraction of ingredients from the crude medicinal plant. First the leaves of the crude plant are separated and then washed and

dried. This is the plant material preparation, the first step of extraction process. Then the other procedures take place which are shown through the flow chart (Figure 3.1).

3.2.1. Plant material preparation and drying

The leaves were plucked off from the plant stem and washed thoroughly with clean water to remove plant debris and dust particles. The clean leaves were then shade-dried for several days and the dried leaves were then prepared for the next step.

3.2.2. Extraction process

3.2.2.1. Size reduction and weighing

The dried, crispy leaves were then grounded to coarse powder using a high capacity grinding machine. This was followed by packaging into air-tight plastic containers with necessary labeling which were finally left in a cool, dry and dark place until further investigation. During the grinding process, necessary measures were taken to avoid cross-contamination.

The powdered leaves were taken in a beaker and then the total weight was weighed in a weight machine.

3.2.2.2. Extraction

For the purpose of this study, the maceration process of extraction was used for extraction of plant materials and methanol was utilized as the organic solvent. The beaker containing powdered plant material of *C. pallida* was soaked in 900mL of methanol for a period of 7 days at normal room temperature (22-25°C) with occasional agitation.

The outcome of maceration process was a 3-layer phase: the lower-most phase is the sediment and the upper-most is a methanolic solution of the extract which is separated by a dirty suspension of plant parts.

3.2.2.3. Filtration

After seven days of maceration, the contents of the beaker were decanted first before filtering them using Whatman filter (pore size: 110mm).

3.2.2.4. Concentration

The collected filtrate was concentrated using rotary evaporator (Heidolph) at 100rpm at 30°C, until concentrated methanolic extract is produced. Thereafter, the mixture was transferred into a beaker for drying under LAF.

3.2.2.5. Drying

Finally, the beaker was placed under Laminar Air Flow (LAF) to evaporate the solvent from the extract, leaving behind dry and semi-solid extract. LAF was used as a preventive measure so as to avoid any chances of microbial growth on the extract while it's drying. After successful drying of the extract, the beaker was covered in Aluminum foil and refrigerated for further use.

3.3. Phytochemical screening

Phytochemical screening was performed on the crude extracts of *C. pallida* in order to access its qualitative chemical compositions namely, alkaloids, carbohydrates, tannin, flavonoids, glycosides, etc.

3.3.1. Detection of alkaloid

For the qualitative determination of alkaloids, three tests were performed. 0.5g of methanolic extract of *C. pallida* was dissolved in 5mL of 1% Hydrochloric acid, boiled in a water bath followed by filtration. Using the filtrate obtained the following tests were performed:

3.3.1.1. Hager's test

To 2mL of the filtrate, a few drops of Hager's reagent (1% picric acid solution) was added and the presence of alkaloids were confirmed by the formation of yellow precipitate (Waldi, 1965).

3.3.1.2. Mayer's test

According to Evans (1997), a 10mL Mayer's Reagent is prepared by dissolving 0.1358g of Mercuric (II) Chloride and 0.5g of Potassium Iodide in 10mL distilled water.

Then, to a 2mL of the filtrate, a few drops of Mayer's reagent were added along the sides of the test tube. The formation of a white or creamy precipitate indicates the presence of alkaloids.

3.3.1.3. Wagner's test

According to Wagner (1993), a 10mL Wagner's Reagent is prepared by dissolving 0.2g of Iodine crystals and 0.6g of Potassium Iodide in 10mL distilled water.

To a 2mL of the filtrate, a few drops of Wagner's reagent were added. Formation of a brownish-black precipitate confirms the presence of alkaloids in the sample.

3.3.2. Detection of carbohydrate

According to Ramkrishnan, Prasannan and Rajan (1994), carbohydrate can by qualitatively detected by weighing out 0.5g of methanolic extract of *C. pallida* and dissolving it in 5mL of distilled water and then filtering the mixture. To the filtrate obtained, the following two tests were performed:

3.3.2.1. Molisch's test

2mL of the filtrate obtained was treated with 2 drops of Molisch's Reagent i.e. alcoholic solution of α-napthol to which 2mL of concentrated sulfuric acid was pipetted along thesides of the test tube and was allowed to stand for a while. The formation of a violet ring indicates the presence of carbohydrates.

3.3.2.2. Fehling's test

To 2mL of the filtrate, 1mL of each of the Fehling's solution A and B were added in a 1:1 ratio and then boiled for a few minutes. Formation of a brick-red precipitate indicates the presence of reducing sugar.

3.3.3. Detection of flavonoid

3.3.3.1. Lead acetate test

The methanolic extract was treated with a few drops of lead acetate solution and the formation of yellow colored precipitate signifies the presence of flavonoids.

3.3.3.2. Zinc ribbon test

According to Sindhu, Uma and Manorama (2013), the presence of flavonoids can be confirmed by another method. To a test tube containing 0.5mL of alcoholic extract, 5-10 drops of concentrated Hydrochloric acid and a small piece of Zinc was added. The solution was then boiled for a few minutes and then left to stand. The formation of a red to crimson color solution indicates the presence of flavonoids.

3.3.4. Detection of phenols/phenolic content

3.3.4.1. Ferric chloride test

According to Soni and Sosa (2013), this test is performed by measuring 2mL of extract in a test tube followed by adding 3-4drops of 15% (w/v) Ferric chloride solution. The formation of a bluish-black precipitate signifies the presence of phenols

3.3.5. Detection of phytosterols

3.3.5.1. LibermannBurchard's test

To a small amount of extract, 1 L of chloroform was added and filtered. The filtrate was then treated with a 2mL of acetic anhydride, boiled and cooled. Finally, 1mL of concentrated sulfuric acid was added to the solution. Formation of a brown ring at the junction indicates the presence of phytosterols (Soni & Sosa, 2013).

3.3.6. Detection of steroids

3.3.6.1. Salkowski test

To 1mL of extract, 2mL of chloroform, 1mL of sulfuric acid were added. The appearance of red color indicates the presence of steroids (Ghani, 2003).

3.3.7. Detection of tannins

3.3.7.1. Lead acetate test

To 1mL of the extract, a few drops of 1% Lead acetate solution were added and the formation of a yellow-colored precipitate indicates the presence of tannins (Tiwari and BimLesh, 2011).

3.3.7.2. Potassium dichromate test

10% Potassium Dichromate solution is prepared by dissolving 1g of Potassium Dichromate was dissolved in 10mL distilled water to prepare this solution. 5mL aqueous solution of crude extract was dissolved in 1mL of 5% ferric chloride solution and the formation of yellow precipitation indicates the presence of tannin (Ghani, 2003).

3.3.7.3. Ferric chloride test

5% Ferric chloride solution is prepared by dissolving 0.5g of ferric chloride in 10mL distilled water.5mL aqueous solution of crude extract was dissolved in 1mL of 5% ferric chloride solution and the formation of greenish black precipitation indicates the presence of tannin (Ghani, 2003).

3.3.8. Detection of resins

According to Soni and Sosa (2013), presence of resin can be identified by adding 5-10 drops of acetic anhydride to 2mL of the extract and heating the solution gently. This is then followed by addition of 0.5mL of sulfuric acid to the solution. Presence of resin is identified by the formation of a bright purple color.

3.3.9. Detection of glycosides

The methanolic extract of *C. pallida* was hydrolyzed with dilute Hydrochloric acid before subjecting it to Borntrager's Test (Mariappansenthilkumar, 2013).

3.3.9.1. Borntrager's test (modified)

To the 5 L of filtrate, 5mL of 5% Ferric (III) chloride and 5mL of dilute Hydrochloric acid were added. This was followed by heating the mixture for 5min in a boiling water-bath and cooling it down. Then, 5mL of benzene was added to the mixture and shaken thoroughly. The organic layer was then separated by using a separating funnel and an equivalent volume of dilute ammonia solution was added. The formation of a pinkish-red color in the ammonical layer signifies the presence of glycosides (Kamalakar, Prabhakar & Shailaja, 2014).

3.3.10. Detection of saponin

3.3.10.1. Froth test

The extract is diluted with distilled water and the volume was made up to 20mL and the contents of the cylinder were shaken in a graduated cylinder for about 15min. The formation of a foam layer of about 2cm in height indicates the presence of saponins (Kokate, 1999).

3.4. *In-vitro* antioxidant activities

There are various in vitro methods of determining anti-oxidant activities of a plant extracts. Of the various methods, four methods were chosen to determine the antioxidant activity in plant extract of *C. pallida*, namely DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC).

3.4.1. DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

The DPPH free radical scavenging assay of plant extract *C. pallida*, was determined using the method described by Braca *et al.* (2001).

3.4.1.1. Reagents and chemicals

The reagents and chemicals that are required to carry out the determination of DPPH scavenging activity is listed below in **Table 3.1**.

Table 3.1: Reagents and chemicals used to determine DPPH scavenging activity

Name of reagent/chemical	**Supply**
DPPH	Sigma Aldrich, U.S.A.
Methanol	Active Fine Chemicals Ltd., Bangladesh
L-ascorbic acid	Merck, Germany

3.4.1.2. Reagent preparation

0.004% (w/v) DPPH solution was prepared by dissolving 2mg of DPPH in 50mL distilled water and then stored in the refrigerator at -4°C till before use.

3.4.1.3. Sample and standard solution preparation

120mg of extract was measured and dissolved in 10mL of methanol to produce a concentration of 12mg/mL. This became the sample stock solution.

The sample concentrations were prepared by serial dilution of the sample stock solution to derive 8 serially diluted concentrations: 1200, 800, 400, 200, 100, 50, 25 and 12.5µg/mL.

The standard used was L-ascorbic acid and it was prepared in the same manner as theextract resulting in eight serially diluted concentrations, ranging from 1200-12.5µg/mL.

3.4.1.4. Experimental procedure

(a) 1mL of each of the fractions of sample and standard (L-ascorbic acid) were taken in test tubes.

(b) To each of the test tube, 2mL of 0.004% (w/v) DPPH solution was added.

(c) Then, the test tubes were incubated for 30 minutes at room temperature which was followed by measuring the absorbance of the resulting solutions and control (DPPH and

methanol) at 517nm against blank (Methanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(d) The percentage of free radical scavenging activity (% FRS) was then calculated from the equation as follows:

$$\% \text{ Free radical Scavenging (\%FRS) activity} = \frac{(A0 - A1)}{A0} \times 100$$

Where, A_0 = The absorbance of the control

A_1 = The absorbance of the sample/standard

(e) Finally, the % Scavenging activity was plotted against concentration from which IC_{50} value was calculated (IC_{50} value is defined as the concentration at which 50% of total DPPH free radical is reduced by the antioxidants which can be determined by plotting % inhibition against the corresponding concentrations).

3.4.2. Determination of total phenolic content (TPC)

The TPC of the plant extract of *C. pallida* was determined by the modified Folin-Ciocalteu method as mentioned by Wolfe, Wu and Liu (2003).

3.4.2.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total phenolic content is listed below in **Table 3.2**.

Table 3.2: Reagents and chemicals used to determine total phenolic content

Name of reagent/chemical	**Supply**
Folin-Ciocalteu reagent (FCR)	LOBA Chemie Pvt. Ltd., India
Gallic acid monohydrate (Standard)	Sigma Aldrich, USA
Sodium carbonate	Merck Specialties Pvt.Ltd., Mumbai
Mehanol	Active Fine Chemicals Ltd., Bangladesh

3.4.2.2. Reagent preparation

20mL of 10% FCR solution was prepared by taking 2mL of FCR in a volumetric flask and diluting it with distilled water.

100mL of 7.5% (w/v) Sodium carbonate was prepared by measuring out 7.5g of Sodium carbonate in a 100mL volumetric flask and then diluting it with distilled water to 100mL mark.

3.4.2.3. Sample and standard preparation

120mg of *C. pallida* extract was measured and dissolved in 10mL of methanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive four serially diluted concentrations: 1200, 800, 400 and 200µg/mL.

Gallic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400 and 200µg/mL.

3.4.2.4. Preparation of the blank

The blank solution contained the same volume of FCR solution and Sodium carbonate as was used in the experiment i.e. 5mL and 4mL, respectively, and 1mL of methanol was used to make the volume up to 10mL.

3.4.2.5. Experimental procedure

(a) 1mL of each of the fraction of sample and standard (gallic acid) concentrations was taken in test tubes.
(b) To which 2.5mL of FCR solution was added.
(c) 2.5mL of Sodium Carbonate solution was added.
(d) Each of the mixture was vortexed for 15s and then allowed to stand for 30min at 40°C in a water bath.
(e) Finally, the absorbance of standard and sample solutions was measured against blank at 765nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(f) The total phenolic content, C, for each of the fractions were expressed as Gallic Acid Equivalents using the following equation:

$$C = \frac{c \times V}{m}$$

Where, C = Total content of phenolic compounds, milligram of gallic acid per gram of dried plant extract, expressed as gallic acid equivalent (GAE)

c = Concentration of gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.4.3. Determination of total flavonoid content (TFC)

The total flavonoid content of the extracts of *C. pallida* was determined according to the method as described by Kumaran & Karunakaran (2007).

3.4.3.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total flavonoid content are listed below in **Table 3.3**.

Table 3.3: Reagents and chemicals used to determine total flavonoid content

Name of reagent/chemical	**Supply**
Potassium acetate	Merck KGaA, Germany
Quercetin (Standard)	Sigma Aldrich, Germany
Aluminium chloride	Merck Specialties Pvt.Ltd., Mumbai
Mehanol	Active Fine Chemicals Ltd., Bangladesh

3.4.3.2. Reagent preparation

100mL of 10% Aluminium chloride solution was prepared by measuring 10g of Aluminium chloride in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

100mL of 1M Potassium acetate solution was prepared by measuring 9.815g of Potassium acetate in a 100mL volumetric flask and diluting it with distilled water up to 100mL mark.

3.4.3.3. Sample and standard preparation

120mg of *C. pallida* extract was measured and dissolved in 10mL of methanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL.

Quercetin was used as the standard and the stock solution was prepared in the same manner as the extract resulting in 4 serially diluted concentrations, ranging from 1200, 800, 400 and 200µg/mL.

3.4.3.4. Preparation of the blank

The blank solution contained 200µL of 10% Aluminium chloride solution, 200µL of 1M Potassium acetate solution, 5.6mL of distilled water and 4mL of methanol, such that the final volume of the solution was 10mL.

3.4.3.5. Experimental procedure

(a) 1mL of each of the fraction of sample and standard (Quercetin) concentrations was taken in test tubes.
(b) To which 3mL of methanol was added.
(c) And 200µL of 10% Aluminium chloride solution and 200µL of 1M Potassium acetate solution were added to each of the test tubes using 1000µL micropipette.
(d) Finally, 5.6mL of distilled water was added to the test to make the final volume of the solution 10mL.
(e) The test tubes were then incubated at room temperature for 30min.
(f) Afterwards, the absorbance of each of the sample and standard solutions were measured at 415nm against blank using spectrophotometer (U-2910 UV-Vis Spectrophotometer).
(g) The total flavonoid content, C, for each of the fractions were expressed as Quercetin equivalents (QE) using the following equation:

$$C = \frac{c \times V}{m}$$

Where, C = Total content of flavonoid compounds, milligram of quercetin per gram of dried plant extract, expressed as quercetin equivalent (QE)

c = Concentration of gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.4.4. Determination of total antioxidant capacity (TAC)

The total Antioxidant capacity of plant extract of *C. pallida* was determined by using the method as described by Prieto, Pineda and Aguilar (1999).

3.4.4.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total antioxidant capacity are listed below in **Table 3.4**.

Table 3.4: Reagents and chemicals used to determine total antioxidant capacity

Name of reagent/chemical	**Supply**
Ammonium Molybdate	Active Fine Chemicals Ltd., Bangladesh
L-ascorbic acid (Standard)	Merck, Germany
Tri Sodium phosphate ($Na_3PO_4.12H_2O$)	MerckKGaA, Germany
Mehanol	Active Fine Chemicals Ltd., Bangladesh
Concentrated sulfuric acid (98%)	Merck, Germany

3.4.4.2. Reagent preparation

100mL of 0.6M Sulfuric acid was prepared by measuring 3.28mL of 98% concentrated sulfuric acid in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

100mL of 0.004M Ammonium Molybdate solution was prepared by measuring 4.494g of Ammonium Molybdate in a 100mL volumetric flask and diluting it with distilled water up to 100mL mark.

100mL of 0.028M Tri Sodium Phosphate solution was prepared by measuring 1.0645g of Tri Sodium Phosphate in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

3.4.4.3. Sample and standard preparation

120mg of *C. pallida* extract was measured and dissolved in 10mL of methanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL.

Ascorbic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400 and 200µg/mL.

3.4.4.4. Preparation of the blank

3mL of reagent solution and 300µL of methanol was used for preparing the blank solution.

3.4.4.5. Experimental procedure

(a) 300µL of each of the fraction of sample and standard (L-ascorbic acid) concentrations were taken in test tubes.

(b) 3mL of Reagent solution (0.6M sulfuric acid, 0.028M Sodium phosphate and 0.004M Ammonium molybdate) was added into the test tubes.

(c) The test tubes (sample, standard and blank) were then incubated at 95°C in a water bath for 90min.

(d) Finally, the absorbance of the sample and standard solutions were measured against blank at 695nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(e) The total antioxidant capacity, A, for each of the fractions were expressed as Ascorbic Acid Equivalents (AAE) using the equation as follows:

$$C = \frac{c \times V}{m}$$

Where, C = Total antioxidant capacity, milligram of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (AAE)

c = Concentration of ascorbic acid obtained from calibration curve (mg/ mL)

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.5. Cytotoxicity activity study by MTT assay

3.5.1. Solutions preparation

1% penicillin-streptomycin solution

Penicillin streptomycin solution usually known as pen-strep is used in MTT assay to control the bacterial contamination and maintain the sterile condition throughout the process. The solution contains a mixture of 10000 unit of penicillin per mL and 10 mg of streptomycin per mL.

The rationale of using the antibiotics is to simply prevent the growth of bacteria and fungi in the medium.

10 % fetal bovine serum

50 mL of Fetal Bovine Serum was added to the 500 mL of DMEM to prepare 10 % FBS. FBS is high in albumin that provides nutrion and adequate environment to grow the cells more quickly.

Trypsin

0.25% trypsin was used in the medium.

2% DMSO solution

2% DMSO solution was prepared by adding 60 µl in 2940 mL of distilled water for negative control.

3.5.2. Used consumables

96-well plate, 15-mL tubes, Tips, Gloves, PTFE Syringe Filter (0.45µm pore size and 25mm diameter), Culture flask, Cell culture media, 1% Penicillin-Streptomycin, Gentamycin, Serological pipette, Trypsin etc.

3.5.3. Used Instruments

Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA)**,** CO_2 Incubator (Nuaire, USA)**,** Trinocular Microscope with Camera (Olympus, Japan)**,** Hemocytometer.

3.5.4. Celltiter 96 assay kit

Celltiter 96® assay kit is an accumulation of qualified reagents that give a fast furthermore advantageous system with determination of the amount of proliferation and cytotoxicity (Ifere et al., 2010). It is a view of change of a cell division with tetrazolium salt under a formazan product that is undoubtedly distinguished utilizing a 96-well plate. A mixer of dye solution was added in 96 well plates to culture cells to perform this assay.

3.5.5. HeLa cell line

HeLa cell line was collected from Centre for Advance Research and Science (CARS). This cell line was cultured and maintained in DMEM (Dulbecco's Modified Eagles Medium) by using cell culture flask. It was stored in liquid nitrogen.

DMEM is a widely accepted medium and used in many cell lines. It is high in glucose, vitamins and amino acids to help providing the necessary nutrition to cells.

3.5.6. Preparation of the different concentrations of plant extract

The assay was be performed by using four concentration 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.5 mg/mL of the *C. pallida* leaves extract. 2.0mg/mL concentration was

made by adding 20 mg of leaves extract in 1mL DMSO solution and it was the stock solution. 1.5 mg/mL concentration was made by diluting 2.0mg/mL solution by DMSO. In this way1.0 mg/mL and 0.5 mg/mL concentrations were made by serial dilution with. Then the samples were filtered through 0.45 μm syringe filter prior to examination.

3.5.7. Cell Culture

3.5.7.1. Preparation of assay plates

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagles Medium) in addition with 1% penicillin-streptomycin, 0.2% Gentamycin, 10% fetal bovine serum.

3.5.7.2. Thawing of cells

HeLa cells were preserved in liquid nitrogen in cryovials. The cryovial was taken and rapidly defrosted by swirling the vial delicately using a waterbath at 37°C until there was a small piece of ice left in the vial. Afterward the thawed cells were transferred into a centrifuge tube which contained the DMEM medium drop wise under a laminar airflow hood. Thereafter, cells were suspended in the medium gently and transferred to the culture vessels.

3.5.7.3. Cell Passage

To get a fresh cell suspension cell passaging was done by transferring the cells into a new medium. The used cultured media was washed by FBS and followed by addition of 800μl of trypsin for detaching the cells from the top of the culture vessels. Then the cells were incubated and checked for the detachment under a microscope. After watching 90% of cells detached, 5 mL DMEM media was added to the vessels and blended using a pipette. Finally 1 mL of this solution was taken and mixed with 4 mL of DMEM in a new vessel and kept in a incubator for further use.

3.5.7.4. Harvesting of cells

The cell was harvested using trypsin in log phase growth. Then cell was counted and seeded into 96 well plates.

3.5.7.5. Counting of cells

Cell counting had been carried out by using a Hemocytometer. The Hemocytometer was prepared by cleaning and polishing the mirror like surface deliberately with ethanol and lens paper. For adding the cell suspension, the coverslip was put in the counting surface. A Pasteur pipet was used to introduce the fresh cell suspension into the hemocytometer. Enough suspension was presented so that the surface had been simply overflowed. Afterward the counting chamber was set in the microscope stage then the counting grid was focused. In a standard hemocytometers with Neubauer rulings 1 entire grid can be observed at 40X magnification. The cells of the 4 large squares were counted. Either upper and left sides touching cells or lower or right sides touching cells were counted.

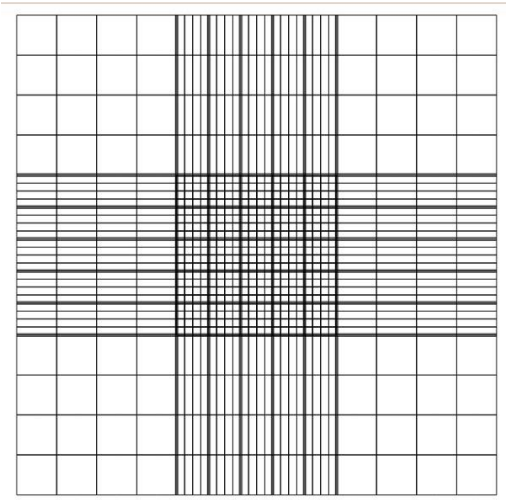


Figure 3.2: Hemocytometer

3.5.8. Procedure

Cytotoxic effect was performed in The Centre for Advanced Research in Sciences using their services. The MTT colourimetric assay was performed by using celltiter 96 non-radioactive cell proliferation assay kit (Promega,USA). Cells were seeded onto 96 well plates and incubated at 37 °C and 5% of CO_2atmosphere. After 24 hours of incubation, 10 µL of sample was added into each well. Then it was again incubated for 2 days. After 2 days of incubation, cytotoxicity was examined using celltiter 96 non-radioactive cell

proliferation assay kit. Then the absorbance was measured at 570nm using a 96-well plate reader. Same procedure had been followed for all 4 concentrations, negative control and positive control. Negative control was contained medium with 2% DMSO solution and blank was contained only medium. Duplicate wells were used for each sample. Cytotoxic activity was calculated by using a formula which is given below-

$$\%\ \text{of cytotoxic activity} = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of negative control}} \times 100$$

CHAPTER FOUR

RESULTS

Chapter 4: Results

4.1. Determination of percentage yield of the plant extract, *C. pallida*

4.1.1. The total weight of powdered plant material

The table shows the initial weight of the powder along with the beaker and the total weight of the powdered plant material.

Table 4.1: The weight of powdered plant material

Initial weight (beaker)	309.31g
Final weight (beaker + plant powder)	388.81g
Weight of plant powder	79.50g

Interpretation: The weight of the powdered plant material in the beaker before the maceration process was 79.05g of powdered plant material and which is ready for maceration process.

4.1.2. The net weight of the plant extract obtained after maceration

After the maceration procedure the weight of the plant material is reduced and the table shows the final weight of the leaf extract.

Table 4.2: The total weight of methanolic leaf extract of *C. pallida* obtained as a result of the complete extraction procedure

Initial weight (beaker)	140.05g
Final weight (beaker + extract)	150.05g
Weight of extract	10.00g

Interpretation: A total of 10.00g of plant extracts was produced as a result of maceration and subsequent drying of the methanolic extract of *C. pallida*; that was used in carrying out subsequent experiments.

4.1.3. Calculation of the percentage yield of the extract

Extract yield (%) = (W_1 x 100) / W_2

Where, W_1 = Net weight of extract after maceration (g)

W_2 = Total weight of powder taken for extraction (g)

Percentage Yield of extract = (10.00 x 100) / 79.50

= 12.58%

Interpretation: The total weight of the extract after maceration was found to be 10.00g whilst that before maceration was 79.50g; therefore, the % yield of *C. pallida* was calculated to be 12.58%.

4.2. Phytochemical screening of *C. pallida*

The presence or absence of the phytochemical compounds in the plant extract is listed below in **Table 4.3**.

Table 4.3: Phytochemical screening of *C. pallida*

Class of compound	**Result**
1. Alkaloid	+++
2. Flavonoid	++
3. Phenols/Phenolic compound	+
4. Glycoside	-
5. Tannins	+++
6. Carbohydrate	+
7. Phytosterol	+
8. Resins	-
9. Steroids	-
10. Saponin	+
(+) means presence in a single method test, (++) means presence	

experimented in two methods, (+++) means presence experimented in three methods, and (-) means absence

Interpretation: The phytochemical screening of *C. pallida* showed the presence of alkaloids, flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol and saponin whilst showing the absence of glycoside, steroids and resins.

4.3. DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

4.3.1. Table of DPPH free radical scavenging activity of *C. pallida*(CP)

The concentration and the absorbance values of sample (CP) and standard (AA) shown in the **Table 4.4** below.

Table 4.4: DPPH free radical scavenging activity (Absorbance vs. Concentration)

Concentration (µg/mL)	**Absorbance of Standard (AA) at 517nm (Mean ± SDV)**	**Absorbance of Sample (CP) at 517nm (Mean ± SDV)**
12.5	0.102± 0.001	0.081± 0.001
25	0.111 ± 0.001	0.065 ± 0.001
50	0.113 ± 0.001	0.041 ± 0.001
100	0.043 ± 0.001	0.038 ± 0.001
200	0.025 ± 0.001	0.020 ± 0.001
400	0.019 ± 0.001	0.015 ± 0.001
800	0.012 ± 0.001	0.009 ± 0.001
1200	0.009 ± 0.001	0.003 ± 0.001

4.3.2. Graph of DPPH Absorbance vs. Concentration for CP and AA

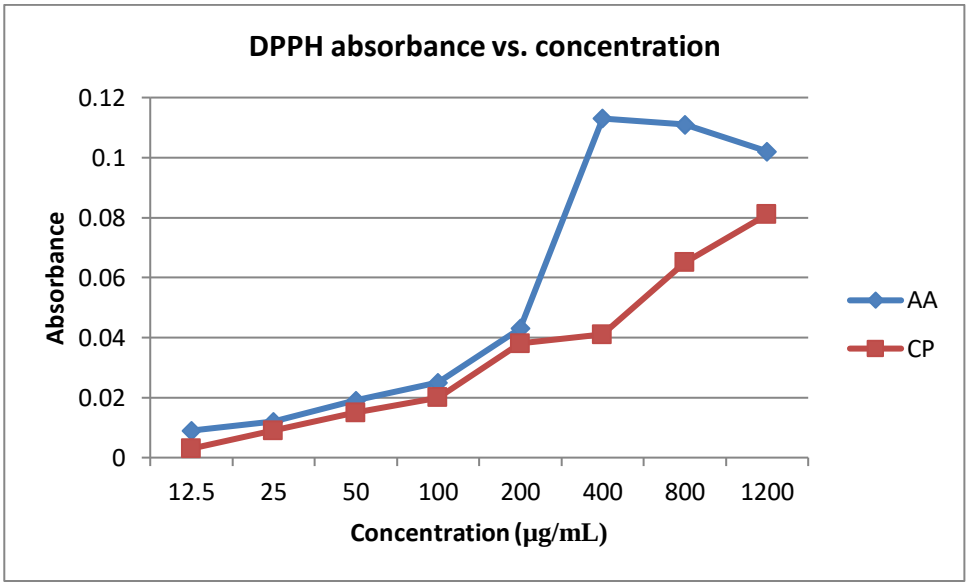


Figure 4.1: Graph of DPPH Absorbance vs. Concentration (µg/mL) for *C. pallida* (CP) and ascorbic acid (AA)

Interpretation: From the **Table 4.4** and **Figure 4.1** above, it was observed that with an increase in concentration of *C. pallida* from 12.5– 1200µg/mL, the absorbance increased gradually in comparison to that of ascorbic acid; thus, implying that the steeper slope i.e. *C. pallida* (red) had a higher inhibition of DPPH free radical scavengers than the less steeper slope (blue) of ascorbic acid standard.

4.3.3. Table of % inhibitory activity of *C. pallida*

The table shows the % inhibition of DPPH free radical scavengers by methanolic extract of *C. pallida*.

Table 4.5: % Inhibition of DPPH free-radical scavengers by dried extract of *C. pallida* (CP) with respect to standard ascorbic acid (AA)

Concentration (µg/mL)	% Inhibition (AA) (Mean ± SDV)	% Inhibition (CP) (Mean ± SDV)
12.5	1.7 ± 1.095	6.8 ± 0.219
25	27.5 ± 0.219	25.28 ± 0.219
50	29.8 ± 0.219	52.87 ± 0.219

100	50 ± 0.109	56.32 ± 0.219
200	71 ± 1.095	77.01 ± 1.095
400	78 ± 0.219	82.75 ± 0.219
800	86.20 ± 0.219	89.65 ± 0.219
1200	89.65 ± 0.109	96.55 ± 0.109

Interpretation: For any given concentration, the % inhibition of free radical scavengers' by the crude extract of *C. pallida* was observed to be more than the corresponding concentration of ascorbic acid. However, the highest free radical scavenging activity for CP was found to be 96.55% at 1200µg/mL.

4.3.4. Graph of % inhibition by *C. pallida* vs. corresponding concentration

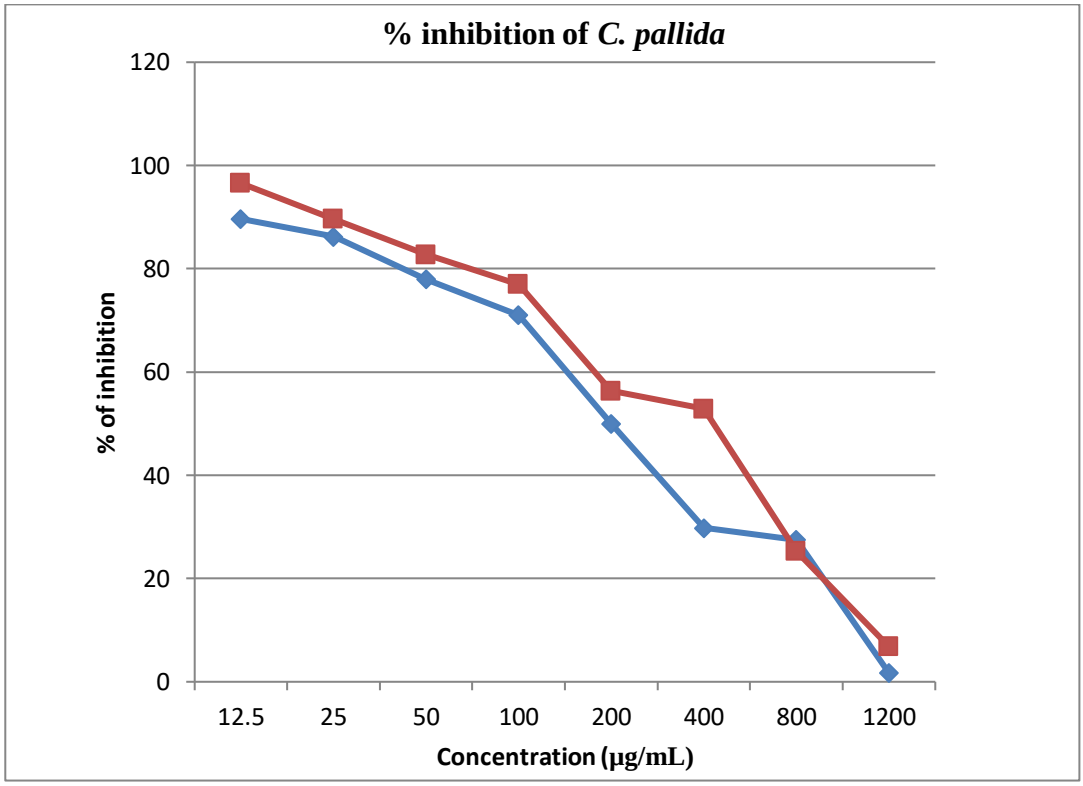


Figure 4.2: % inhibition of DPPH free radical scavengers by methanolic leaf extract of CP (red) and standard ascorbic acid (blue)

Interpretation: The less-steeper slope (blue) of *C. pallida* indicates that as its concentration was increased, the % inhibitory activity has also increased.

4.3.5. Determination of the IC_{50} of *C. pallida* (CP) and standard ascorbic acid (AA)

Table 4.6: Comparison between the IC_{50} values of *C. pallida* and standard ascorbic acid

Name of the sample	IC_{50}
C. pallida	157.01µg/mL
Ascorbic acid	276.60µg/mL

Interpretation: The IC_{50} value of dried extract of *C. pallida* signifies that a much lower concentration of 157.01µg/mL is required for inhibiting 50% of all DPPH free radical scavengers, whilst a large concentration of 276.60µg/mL is required to achieve the same inhibitory effect for ascorbic acid.

4.4. Determination of total phenolic content (TPC)

4.4.1. Calibration curve of gallic acid

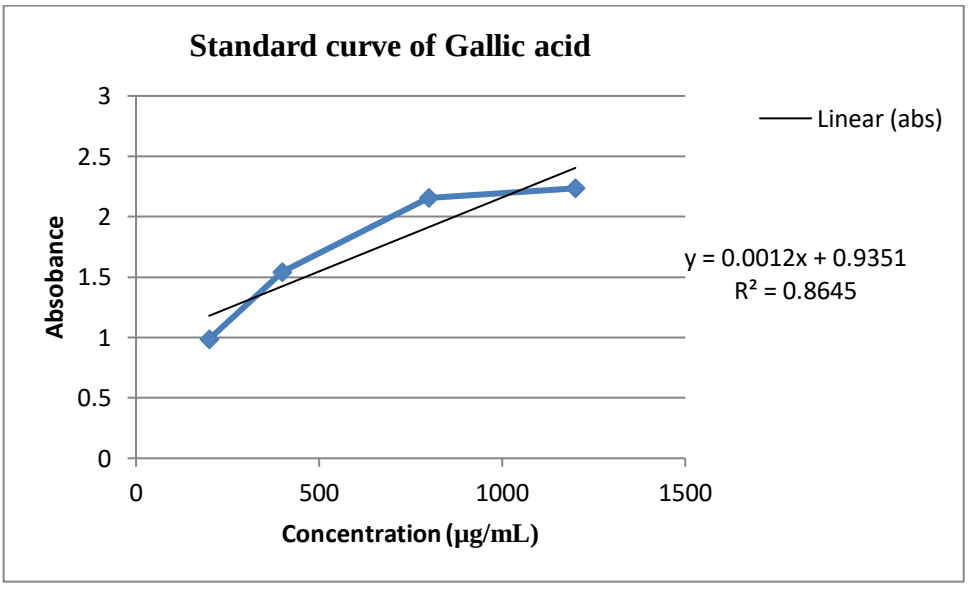


Figure 4.3: Calibration curve of standard gallic acid (GA) at 765nm for determining TPC in CP

4.4.2. Table of total phenolic content in *C. pallida* (CP)

Table 4.7: Total phenolic content of *C. pallida* (CP) represented as gallic acid equivalent (GAE)

Concentration of CP (µg/mL)	TPC (GAE) (Mean ± SDV)
200	12.00 ± 1.815
400	28.20 ± 0.900
800	100.20 ± 2.876
1200	141.00 ± 2.688
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of *C. pallida* was increased from 200–1200µg/mL, the total phenolic content also increased from 12.00 to 141mg of gallic acid per gram of dried extract. Therefore, it indicates that with increases in total phenolic content, its antioxidant activity also increases.

4.5. Determination of total flavonoid content (TFC)

The figure shows the calibration curve of standard quercetin for the determination of total flavonoid content.

4.5.1. Calibration curve of quercetin

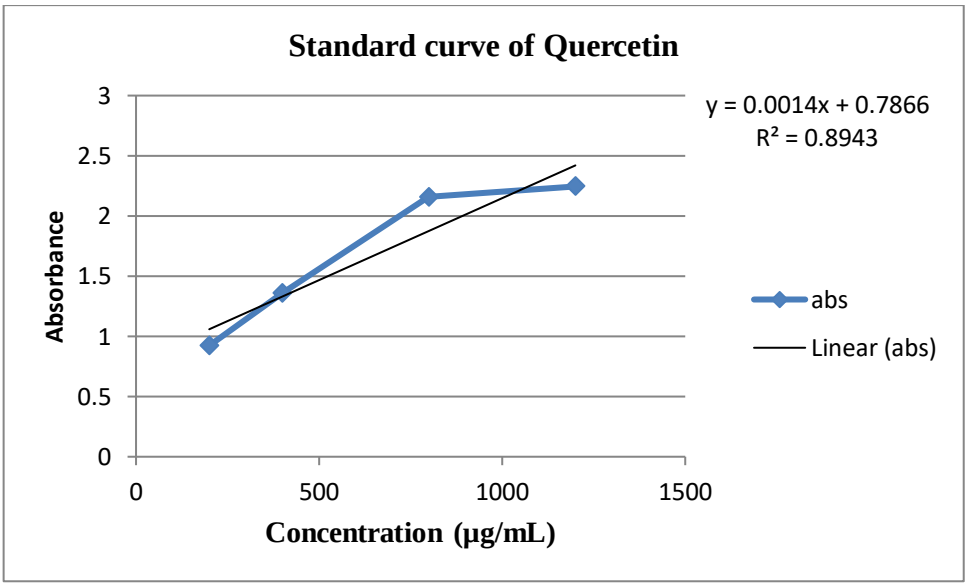


Figure 4.4: Calibration curve of standard quercetin (at 415nm) for determining TFC inCP

4.5.2. Table of total flavonoid content (TFC) in *C. pallida* (CP)

Table 4.8: Total flavonoid content of *C. pallida* (CP) represented as QE

Concentration of CP (µg/mL)	**TFC (QE) (Mean ± SDV)**
200	24.78 ± 4.167
400	54.00 ± 2.700
800	126.00 ± 2.622
1200	144.00 ± 2.778
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of *C. pallida* was increased from 200–1200ug/mL, the total flavonoid content also increased from 24.78 to 144.00mg of quercetin per gram of dried extract. Therefore, it indicates that there is a positive correlation between total flavonoid content of *C. pallida* and its antioxidant activity.

4.6. Determination of total antioxidant capacity (TAC)

The figure shows the calibration curve of ascorbic acid to determine total antioxidant capacity.

4.6.1. Calibration curve of ascorbic acid (AA)

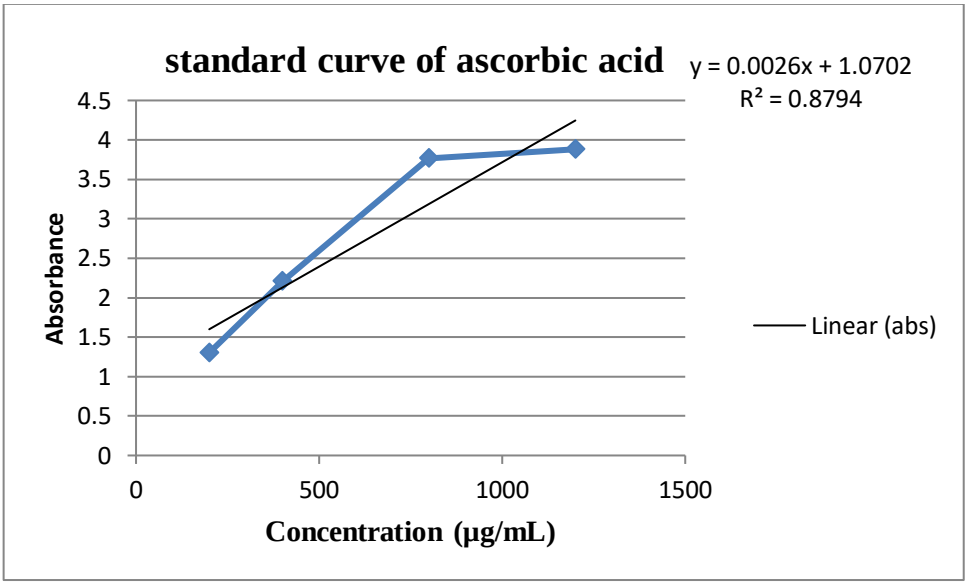


Figure 4.5: Calibration curve of ascorbic acid (AA) at 695nm for determining TAC in CP

Interpretation: The equation of the calibration curve of ascorbic acid was found to be y=-0.002x+1.070 which would be used to determine the total antioxidant capacity of *C. pallida*. A regression coefficient of 0.879 was also obtained indicating a good relationship between the concentration and the absorbance.

4.6.2. Table of total antioxidant capacity (TAC) in *C. pallida*

Table 4.9: Total antioxidant capacity of *C. Pallida* (CP) represented as AAE

Concentration of CP (µg/mL)	**TAC (AAE) (Mean ± SDV)**
200	9.50 ± 0.652
400	37.45 ± 0.245

800	67.65 ± 0.285
1200	90.70 ± 0.136
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of *C. pallida* was increased from 200µg/mL to 1200µg/mL, the total antioxidant capacity also increased of ascorbic acid per gram of dried extract. This indicates therefore, that a positive correlation exists between total antioxidant capacity of *C. pallida* and its antioxidant activity.

4.7. *In vitro* cytotoxic Activity of methanolic extract of *C. pallida* by MTT assay

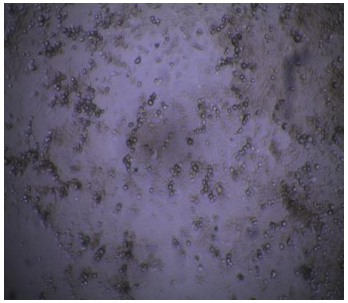
The cytotoxic activity of *C. pallida* leaves extract was performed by MTT assay on HeLa cell line. Different concentration (0.5mg/mL, 1.0mg/mL, 1.5mg/mL, 2.0mg/mL) of leaves extract were used to analyze the cytotoxic effect. 2% DMSO in DMEM medium was used as positive control. Absorbance was observed for each concentration. The results are given in the **Table 4.10** below:

Table 4.10: Survival of HeLa cells in different concentrations of plant leaf extract

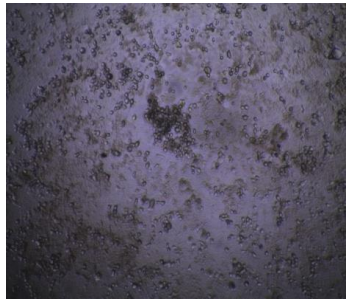
Sample	Survival of HeLa cells
Without solvent (-)	100%
With solvent (+)	95%
0.5mg/mL	80%
1.0mg/mL	70%
1.5 mg/mL	30%
2.0 mg/mL	10%

Interpretation: According to the percentage of the survival of the HeLa cells that was observed through trinocular microscope, it is evident that prominent cell cytotoxicity was observed on HeLa cell line at 2.0mg/mL and moderate cytotoxity at 1.5mg/mL concentration of leaf extract. As only 10-20% cells were alive at 2.0mg/mL

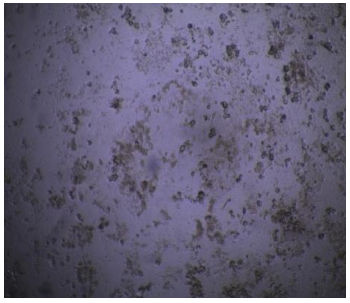
concentration, it can be seen that approximately 80% cells were dead at that concentration thus shown the cytotoxicity in well manner.



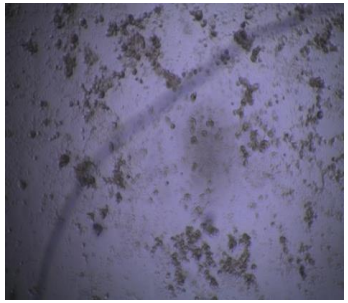
(a) 0.5mg/mL plant leaf extract



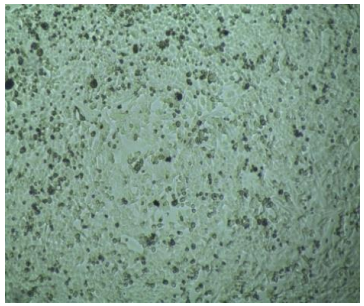
(b) 1.0mg/mL plant leaf extract



(c) 1.5mg/mL plant leaf extract



(d) 2.0mg/mL plant leaf extract



(e) Negative control (2% DMSO solution)

Figure 4.6 (a), (b), (c), (d) & (e): Cell viability of methanolic extract of *C. pallida* at different concentration and 2% DMSO as a positive control after incubating 48 hours.

Interpretation: In the **Figure 4.6** the cell viability of HeLa cells in methanolic extract of *C. pallida* at 0.5, 1.0, 1.5 and 2.0mg/mL concentrations and 2% DMSO solution as a positive control is shown. The **Figure 4.6(e)** shows the negative control (2% DMSO), when no leaf extract was added in the cell culture. In the second picture **Figure 4.6(a)**, it is seen that the population of cells had decreased due to adding leaf extract of 0.5mg/mL concentration. 30% cells are dead due to the cytotoxic effect of plant extract. The **Figure 4.6(b)** shows almost same population of HeLa cell alike second picture as leaf extract of 1.0mg/mL concentration was added. In the fourth picture **Figure 4.6(c)** 1.5mg/mL concentration of leaf extract was added and a large number of population decreases is evident.70% cells are dead due to the cytotoxic action of plant leaf extract. The last picture **Figure 4.6(d**) shows that greater number of cell population is dead when 2.0mg/mL plant leaf extract is added. This concentration shows the highest cytotoxicity on HeLa cell line as it was capable of killing 90% of cell population.

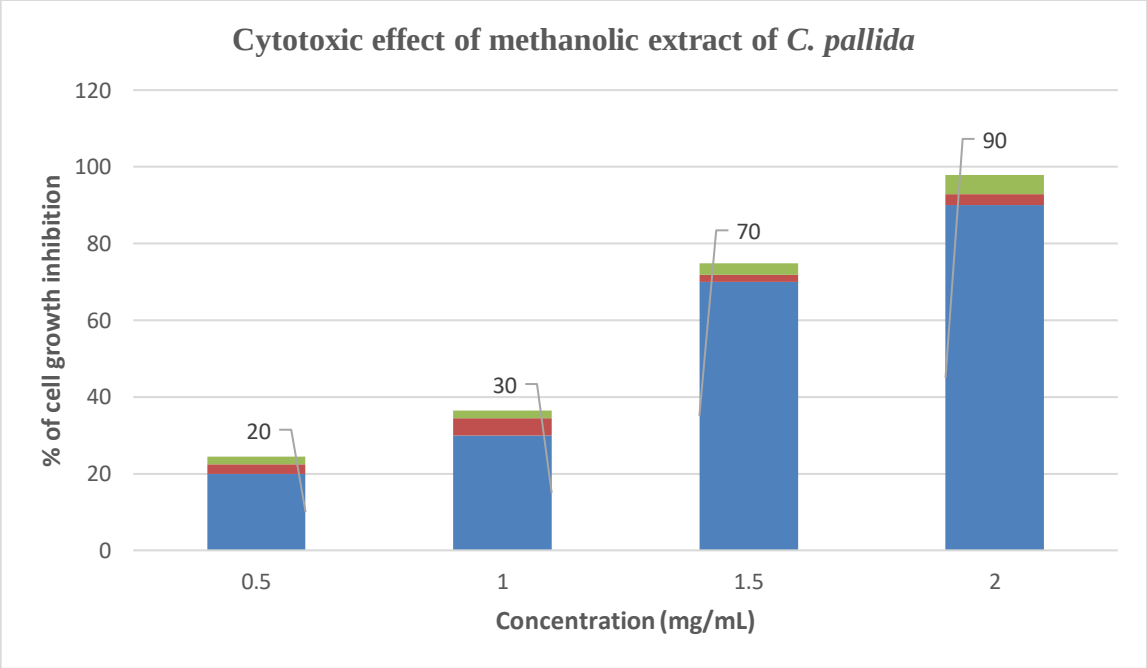


Figure 4.7: Cytotoxic effect of methanolic extract of *C. pallida*

Interpretation: The **Figure 4.7** expresses that, in the highest concentration of 2mg/mL plant extract the % of cell growth inhibition is the highest which is 90%. In 1.5mg/mL

concentration the % cell growth inhibition is slight less 70%, compared to other two concentrations respectively 1 and 0.5mg/mL.

The IC_{50} value of *C. pallida* in case of cytotoxicity is 1.25mg/mL which shows the cytotoxic characteristic for the cancerous HeLa cells

CHAPTER FIVE

DISCUSSION

Chapter 5: Discussion

This present study was started with 79.05g of plant powder of *C. pallida* plant and methanolic extract of the leaves of 10.00g was obtained by extraction process followed by evaporation and drying. After obtaining the crude extract various experiments were carried out to understand its properties.

Firstly, phytochemical screening was carried out using methanolic plant extract that evidents the presence or absence of various chemical constituents. Prior to some qualitative tests and experiments, it was found that alkaloid, carbohydrate, flavonoid, phenolic compounds, phytosterol, tannin and saponins were present in the extract. However, steroid, resin and glycosides were absent in the extract. Previously in another study ethanol, ethyl acetate, petroleum ether, chloroform and water was used as extraction solvents where glycoside was present. But in this study, methanol was used as the solvent but glycoside was not present. The presence of these components gives us an opportunity to understand and predict the activities that may or may not be found in future. For an example, tannin was present in the plant extract and so it could be predicted that anticancer activity may be present in *C. pallida* plant. Later, the cytotoxicity test on HeLa cell line proved that at least the leaf extract of this plant possess cytotoxic properties. Thus, it left us a scope to estimate the anticancer property may be present in the leaf extract.

Then to understand the antioxidant properties of the leaf extract several *in-vitro* antioxidant tests were carried out. DPPH free radical scavenging assay, Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Antioxidant Capacity (TAC) tests were carried out and the data showed that leaf extract of *C. pallida* possess antioxidant properties. Especially DPPH free radical scavenging assay shows that 96.55% of inhibition of free radicals are evident for the highest concentration of 1200µg/mL concentration of the leaf extract. The IC_{50} was found to be 157.01µg/mL for the plant extract, whereas the IC_{50} was found to be 276.60µg/mL for the standard ascorbic acid. Thus, the extract possessed higher antioxidant potential than the standard ascorbic acid. That infers that relatively small amount of plant extract is needed to inhibit the free radical reaction and thus it is more potent than ascorbic acid. The three other tests also revealed that there are antioxidant properties present in the leaf extract. L-ascorbic acid was taken as the standard for DPPH and Total Antioxidant Capacity tests.

For Total Flavonoid Content quercetin and for Total Phenolic Content gallic acid was taken as standard to compare the values of sample extract. The total phenolic content was found to be 141.00mg of gallic acid equivalent (GAE)/g, total flavonoid content was found to be 144.00mg of quercetin equivalent (QE)/g and the total antioxidant capacity was found to be 90.70mg of ascorbic acid equivalent (AAE)/g of crude extract for a concentration of 1200µg/mL.

Though cytotoxic study has been done with other species such as *Crotalaria retusa* (antiproliferative nature against Jurkat cell line), *Crotalaria verrucosa* (Human cervical cancer cell line), *Crotalaria juncea* (Human cervical cancer cell line) etc. However, there has been no cytotoxic study of *C. pallida* performed before. In the current study, *in vitro* cytotoxicity activity was examined on cervical cancer cell line (HeLa) where different concentrations of leaves extracts were used to determine the cell viability. 2% DMSO was used as the negative control and viable cells was determined. From the images it was clear that the mortality of the cells increased with the increase of plant extract in it. To compare the result, first a solvent/extract-free image was taken where abundant cell population was observed clearly. Then the population started to decrease as the concentration of the plant extract started to increase in the culture. The highest % of cell growth inhibition seen for 2.0mg/mL concentration of leaf extract. About 90% of cell population was dead in this concentration of leaf extract thus made the IC_{50} value 1.25mg/mL. So, this can be concluded that methanolic extract of *Crotalaria pallida* leaves contain cytotoxic properties and thus they are capable of killing cancerous HeLa cells.

To conclude, it can be inferred that methanolic extract of *C. pallida* plant leaves has strong cytotoxic effect as well as antioxidant property. Thus it can be used as anticancer agent to treat cervical cancer and to treat oxidative degenerative diseases like Alzheimer's disease and Parkinson's disease.

CHAPTER SIX
CONCLUSION

Chapter 6: Conclusion

C. pallida is a traditional medicinal plant mostly found in the tropical regions of Bangladesh. Though various *in-vitro* studies have been carried out using *C. pallida* plant, no study have been carried out previously to find out its' antioxidant and cytotoxic potential. In this study, the aim was to investigate about the antioxidant property and cytotoxic potential of *C. pallida* methanolic leaf extract using various *in-vitro* methods.

The phytochemical screening *C. pallida* plant extract revealed the presence of alkaloids, tannins, flavonoid, phenolic compound, phytosterol and carbohydrate. Whereas the methanolic leaf extract presented negative result for steroid, resin and glycosides.

To investigate the antioxidant potential of the plant extract four *in-vitro* antioxidant studies were carried out. In DPPH free radical scavenging assay *C. pallida* leaf extract showed higher antioxidant activity than standard ascorbic acid. The IC_{50} value was found to be 157.01µg/mL which is higher than that of ascorbic acid, 276.60µg/mL. The total phenolic content (TPC) was found to be 141.00mg/g of gallic acid equivalent (GAE), the total flavonoid content (TFC) was found to be 144.00mg/g of quercetin equivalent (QE) and the total antioxidant capacity (TAC) was found to be 90.7mg/g of ascorbic acid equivalent (AAE) for the highest concentration 1200µg/mL of methanolic leaf extract.

The investigation of cytotoxic potential was carried out using HeLa cell line (Human Cervical Carcinoma). MTT *in-vitro* cytotoxicity assay was used to find out the cytotoxicity of leaf extract of *C. pallida* plant. The highest concentration 2.0 mg/mL showed the highest mortality rate of HeLa cell population as it was able to kill 90% cell population, whereas the IC_{50} value was 1.25 mg/mL.

On the concluding note, the study revealed that *C. pallida* plant extract possess antioxidant property as well as strong cytotoxic activity. This shows a promising future to investigate more about *C. pallida* plant in the ground of new drug discovery.

The result of the present study established that methanolic extract of *C. pallida* possesses moderate cytotoxic and antioxidant activity thus it broadening up a dimension full of different scopes for further studies:

- Other cancerous cell lines can be used to carry out the cytotoxicity investigation such as, prostate cancer, breast cancer etc. cell line as no other cell lines were used previously to study cytotoxic potential.
- The phytochemical screening was carried out and that revealed the presence of a number of bioactive compounds such as, alkaloids, flavonoid, tannin etc. More detailed isolation of the bioactive compounds can be carried out to investigate the presence of definite structural bioactive compounds as well as their quantitative analysis.
- This study only focuses on the methanolic leaf extract of *C. pallida*. In future other plant parts such as, roots, stems etc. can be used to identify their antioxidant and cytotoxic potential. Moreover, other extraction solvent except methanol can also provide new scopes to reveal more about this plant's bioactivity.

CHAPTER SEVEN

REFERENCE

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