Investigation of *in-vitro* antioxidant potential in *Crotalaria verrucosa* along with Identification and Quantification of its polyphenolic compounds

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

Certification Statement

This is to certify that this project titled "Investigation of *in-vitro* antioxidant potential in *Crotalaria verrucosa* along with Identification and Quantification of its polyphenolic compounds" 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,

_____

Countersigned by the supervisor

_____

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Abstract

Crotalaria verrucosa (Fabaceae) is a local medicinal plant, native to Chittagong, Khulna, Rajshahi and Sylhet, in Bangladesh. Traditionally, *C. verrucosa* is used for the treatment of folkloric remedies namely, scabies, heart complaints as well as in the treatment of throat and oral diseases which may be extrapolated to its antioxidant potential as deduced from this study. In this study, the preliminary phytochemical screening of methanolic leaf extract of *C. verrucosa* showed the presence of flavonoids, phenolic compounds, alkaloids, tannin, steroids and glycosides. Various *in-vitro* antioxidant studies were performed to determine its antioxidant potential. The extract showed moderate antioxidant activity in DPPH free radical scavenging assay where ascorbic acid was used as the reference standard. Also, using the DPPH free radical scavenging assay, the IC_{50} values of *Crotalaria verrucosa* and ascorbic acid was found to be 533.738ug/mL and 154.916ug/mL, respectively. Whilst the total phenolic content was found to be 152.180mg/g of gallic acid equivalent (GAE), the total flavonoid content was found to be much higher, 184.510mg/g of quercetin equivalent (QE). The total antioxidant capacity was determined to be 32.342mg of ascorbic acid equivalent (AAE) for a concentration of 1200ug/mL of methanolic extract of *C. verrucosa*. In addition to the determination of *in-vitro* antioxidant potential, HPLC coupled with a diode-array-detector was used to identify and quantify the polyphenolic compounds present in the methanolic crude extract of *C. verrucosa*. Amongst the polyphenolic compounds, the most notable of them were identified as gallic acid, (+)-catechin hydrate, vanillic acid, caffeic acid, syringic acid, (–)-epicatechin and vanillin were identified present in concentrations of 19.53, 5.08, 5.97, 6.22, 1.09, 7.16 and 8.05mg/100g of dried extract. Amongst the polyphenolic compounds quantified, gallic acid was most predominantly present (19.53mg/100g of dried extract). Although vanillic acid is a catechin-metabolite; (+)-catechin hydrate and (–)-epicatechin are flavonoids and gallic acid, syringic acid, caffeic acid and vanillin are polyphenolic compounds, all of these compounds possess established antioxidant activities.

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List of abbreviations

AA:	Ascorbic Acid
AAE:	Ascorbic Acid Equivalent
Abs:	Absorbance
AR:	Arbutin
b.w.	Body Weight
CA:	Caffeic Acid
CAT:	Catalase
CH:	(+)-Catechin Hydrate
COX	Cyclooxygenase
CV:	*Crotalaria verrucosa*
DPPH:	1, 1-Diphenyl-2-Picryl Hydrazyl
EA:	Ellagic Acid
ECA:	(-)-Epicatechin
FA:	Trans-Ferulic Acid
FCR:	Folin-Ciocalteu Reagent
FRS:	Free Radical Scavengers/Scavenging
GA:	Gallic Acid
GAE:	Gallic Acid Equivalent
GPx:	Glutathione Peroxidase
GSH:	Glutathione
GSSG:	Glutathione Disulfide
H^+:	Hydrogen Ion (Electron)
H_2O_2:	Hydrogen Peroxide
HPLC-DAD:	High Performance Liquid Chromatography-Diode Array Detection
HQ:	Hydroquinone
KF:	Kaempferol
LAF:	Laminar Air Flow
mg	Milligram
MS:	Myricetin
NADP:	Nicotine Adenine Dinucleotide Phosphate
NADPH:	Nicotine Adenine Dinucleotide Phosphate (Reduced)
ND:	Not indicated
n.d.	No date
NHB:	National Herbarium Bangladesh
NSAID	Non-Steroidal Anti-inflammatory Drug
PCA:	*p*-Coumaric Acid
pK:	Dissociation constant (of an acid or a base)
QE:	Quercetin Equivalent
QU:	Quercetin
R^2:	Regression Coefficient
RA:	Rosmarinic Acid
RNS:	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
RSD:	Relative Standard Deviation
SA:	Syringic Acid
SDV:	Standard Deviation
SOD:	Super Oxide Dismutase
TAC:	Total Antioxidant Capacity
TFC:	Total Flavonoid Content
TPC:	Total Phenolic Content
VA:	Vanillic Acid
Vit-E:	Vitamin-E
VL:	Vanillin

CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

1.1. Phytotherapy and its relationship to medicinal plants

Phytotherapy, in other words, the treatment of diseases using plant, dates back to the beginning of human's existence on Earth. Exploring the nature in search of food for survival, man discovered through trial and error, plants and herbs with medicinal uses. With the entire spectrum of human life on Earth displayed, medicinal plant preparations which were used to heal scars and injuries on the primitive stages of human life has now evolved to set the foundation of pharmacotherapy i.e. the treatment of diseases by means of drugs.

According to (Ghani, 2003d, p. 17, ¶ 1), medicinal plants can be defined as "a group of plants that possess some special properties or virtues that qualify them as items of drugs and therapeutic agents, and are useful as articles of medical management and treatment of diseases" and the World Health Organization (W.H.O.) defines medicinal plants as "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 synthesis of useful drugs" (Sofowora, 1982, p. 6, 10, 11, 74 & 114).

Thus, a medicinal plant can be claimed to possess a drug or a complex mixture of compounds that allows it to be used as a therapeutic agent either in traditional medicine preparation or its active ingredient isolated to be utilized in allopathic medicine. As in case of traditional medicine, the entire plant was not used in the remedial preparation as it was observed (through trial and error) that using entire plant reduces the potency or the indicated therapeutic effect. Thus, only the particular anatomical part of the plant that provided useful therapeutic benefits were used in preparing infusions, decoctions, paste, juice or extracts. The plant parts were intentionally used add bulk to the remedial preparation, thus acting as "excipients" in traditional medicine preparations. On the other hand, in allopathic medicine, it is the active ingredient of medicinal plants that sparked a huge interest in the area of pharmaceutical technology which in turn lead to drug discovery through isolation and purification.

Medicinal plants have always been used in the ancient times as well as now, especially in the rural areas, as a remedial treatment for various superficial diseases.

1.2. Significance of medicinal plants in drug discovery

In ancient times, healing was ensued by concoctions, extracts or brews prepared by the herbalists who would seem to always know the remedial treatment for most illness. Would healing used to always be a matter of concern right from the time of Sushrata in Ancient India to ancient Chinese, Korean, Egyptian and Africans because of the raging battles. Sushruta Samhita devoted two entire chapters on wound healing and mentions over 100 plants that can be used alone and in combination for healing wounds as well as the drugs required to obtain a clean wound (Idris, Singh, B. & Singh, G., 1994, p. 37–41; Deshpande, Pathak & Gode, 1970, p. 260-303). Medicinal plants such as *Ficus bengalensis, Ficus racemosa, Symplocos racemosa, Rubia cordifolia, Pterocarpus santalinus, Glycyrrhiza glabra, Berberis aristata, Curcuma longa, Centella asiatica, Euphorbia nerifolia*, and *Aloe vera* were used for the treatment of wounds (Biswas & Mukherjee, 2003).

Medicinal plants were used for the treatment of common ailments (Table 1.1). There used to be a remedy almost ready for a disease which is why old drugs like quinine (Kremsner, Winkler, Brandtz, Neifer, Bienzle & Graninger, 1994) and morphine were used as an anti-malarial and analgesic agent (as cited in Maridass & de Britto, 2008, ¶ 6).

Table 1.1: Some of the medicinal plants used for treating common ailments

Scientific Name (Family)	Part used	Traditional Ailment	References
Acanthus ilicifolius L.	Roots	Leucorrhoea	Hossan *et al.* (2010).
Aloe vera L.	Leaf gel	Skin diseases including eczema, irritation, wounds, abrasions, psoriasis, cold sores, sun burns, microbial skin diseases, cosmetic application and hair loss	Zari, S. T., & Zari, T.A. (2015).
Curcuma longa L.	Rhizome paste, powder	Skin diseases like eczema, wounds, irritation, inflammation, erythema, burns, itching, sunburn	Zari, S. T., & Zari, T.A. (2015).
Holarrhena antidysenterica Wall.	Bark	Used in the treatment of dysentery, dropsy, fever, diarrhea and intestinal worm infections	Chopra, R.N., Nayer, & Chopra, I.C. (1992).
Plumbago zeylanica L.	Root	Used in paralysis, secondary syphilis, leprosy and ophthalmics	Chopra, R.N., Nayer, & Chopra, I.C. (1992).

Near the beginning of 19th century, there was an increase in the knowledge and application of medicinal plants which has led to drug discovery and isolation of alkaloids from poppy (1806), ipecacuanha and strychnos (1817), quinine (1820) followed by the isolation of glycosides. The active phytocompounds such as tannins, saponosides, etheric oils, vitamins, hormones, etc. were soon discovered and isolated from medicinal plants as more and more technological advances were made towards the second half of the century (Dervendzi, 1992, p. 5–43). Next, the use of therapeutics, alkaloids, and glycosides isolated in pure form were soon increasingly replacing the drugs originally isolated from natural plant sources. It was deduced that unlike other phytocompounds, alkaloids were not only faster but the duration of drug action was long-lasting and the activity was observed to its full potential. As a result, "stabilization methods for fresh medicinal plants" were then proposed as mentioned by Lukic (1985), especially for those with labile medicinal components (as cited in Petrovska, 2012).

The Chinese, Indian and Arabic herbal medicines also made significant contribution to the health care of the present day over-populated and ageing societies. Their contribution to the healthcare was so tremendous that shortly afterwards indigenous medicinal plants were used by many people around the world for meeting their primary healthcare needs (Pan *et al.*, 2014).

Whilst the World Health Organization (WHO) has predicted that about 80% of the populations in developing countries depend enormously on traditional medicine for their primary health needs, more than half of the world's population depends exclusively on medicinal plants for their remedies (Duraipandiyan, Ayyanar & Ignacimuthu, 2006; Kumar & Navaratnam, 2013). This has led a keen eye to assess the use of medicinal plants as traditional medicine for the treatment of different diseases.

1.3. Natural products derived from medicinal plants

Liu *et al.* (2000) mentioned that approximately 50% of all marketed drugs were obtained from natural products, and their derivatives. Due to the prominence of secondary metabolites harnessing unknown medicinal potential, there is an immense interest in the field of studying medicinal plants to produce natural products that has a potential of mitigating a disease.

Phyto-constituents possessing anti-oxidant properties are believed to prevent or slow down the occurrence of disease such as cancer (Lee, Koo & Min, 2004).

Table 1.2: Some drugs isolated from natural sources

Drug	Chemical class	Source	Medical use	Mechanism of action
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis* (1820)	Anti-malarial agent (Meshnick & Dobson, 2001, p. 396).	Protein synthesis inhibitor
Artemisinin	Sesquiterpene lactone with an endoperoxide group	Chinese medicinal herb, *Artemisia annua* (Sweet wormwood)	Anti-malarial drug	Heme-mediated decomposition of the endoperoxide bridge to produce Carbon-centered free radicals
Penicillin	Penicillin	Fungus, *Penicillium niger*	Antibiotic	Inhibition of Peptidoglycan synthesis
Morphine	Alkaloid	Dried latex of Opium poppy, *Papaver somniferum* (1804)	Potent Painkiller/Analgesic, Sertürner (1805).	Opioid agonist by binding to opiate receptors (mu, delta, and kappa)
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Atrial fibrillation and Congestive heart failure	Inhibition of the Na^+/K^+-ATPase membrane pump
Colchicine	Alkaloid	Autumn crocus flowering plant, *Colchicum autumnale*	Anti-inflammatory agent in acute gout flares and as an alternative to NSAID	Tubulin polymerization inhibitor, Hartung (1954)
Paclitaxel (Taxol™)	Terpenoid	Pacific yew tree, *Taxus brevifolia*	Tubulin polymerization stabilizer, Sneader (2005).	Tubulin polymerization stabilizer

Table 1.2: Some drugs isolated from natural sources *(Continued)*

Drug	Chemical class	Source	Medical use	Mechanism of action
Salicylic acid[1]	Beta-hydroxy acid	Barks of willow tree	Aspirin is used as an analgesic, anti-inflammatory and antipyretic (NSAID)	Inhibition of COX enzymes in the COX pathway
Mevastatin[2]	Polyketide	Fungus, *Penicillum citrinum*	Cholesterol lowering drug, (Li, 2009, p. 71-96).	Inhibition of the HMG-coenzyme-A
Forskosin	Labdane diterpenoid	Tubers of *Coleus forskohlii* Briq. (Labiatae).	Anti-hypertensive agent, mentioned in the Ancient Hindu Ayurvedic scripts (Bhat, Bajqwa, Dornauer, do Scusa, & Fehlhaber, 1977).	Activation of enzyme adenylyl cyclase

1 = Later chemically modified to Aspirin; 2 = Later chemically modified to Atorvastatin
NSAID = Non-Steroidal Anti-Inflammatory Drug
COX = Cyclooxygenase

In the year of 1978, a study conducted by Ayensu & DeFilipps (cited in Prance, 1978) estimated that around 250,000 different higher plant species namely angiosperms and gymnosperms are present on this planet with a lower level at 215,000 (Cronquist, 1981). Another study conducted by Tippo & Stern (1977) estimated around 500,000 upper level higher plant species as present. However, of these, only about 6% have been screened for biological activity and 15% screened for pharmacological activities. Efficient detection and rapid characterization of these components on a molecular basis by using sophisticated instruments will offer better understanding of the pharmacological application of herbal medicines.

1.4. Free radicals and reactive oxygen species

1.4.1. Prevalence of free radical reaction

Most of the medicinal plants so far identified in the literature possess large quantities of antioxidants that play a key role in the adsorption and neutralization of free radicals, quenching singlet and triplet oxygen or decompose peroxides in the living systems. Within the living system, Reactive Oxygen Species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are produced as a result of normal metabolic processes (Endogenous sources) and from exogenous sources (Halliwell & Gutteridge, 1986).

Based on the types of free radicals produced as a result of metabolic reactions, free radicals can be classified as shown in Figure 1.1.

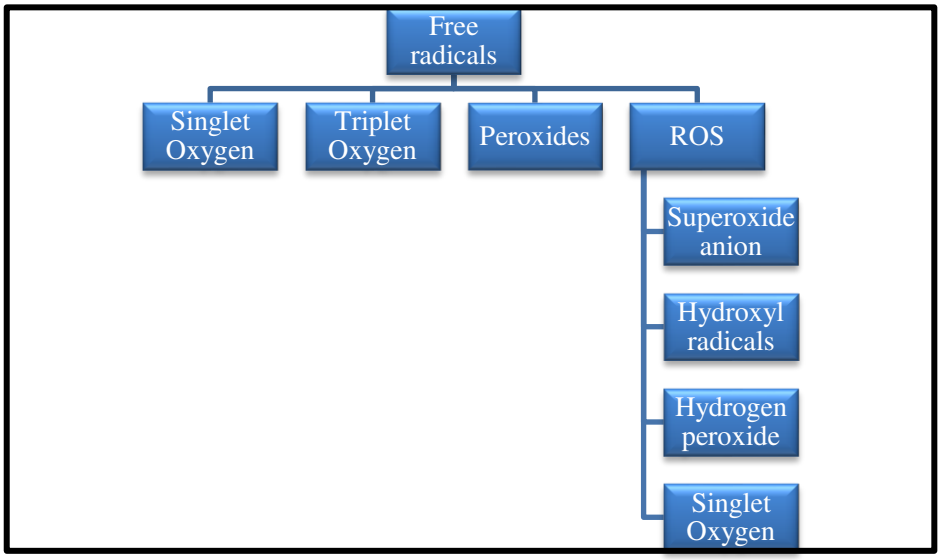


Figure 1.1: Classification of free radicals based on their type

These free radicals can be further divided based on its sources:

a) Endogenous sources: Endogenous sources are those free radicals that are produced within the animal and human bodies which pass through mitochondria, peroxisomes, NADPH oxidase, cytochrome P450 and xanthine oxidoreductase system.

b) Exogenous sources: Exogenous sources are those free radicals that are produced outside the animal and human bodies.

The diseases caused by both endogenous and exogenous sources are listed on Table 1.3.

Table 1.3: Common diseases caused by the ROS and free radicals

Endogenous Sources	Exogenous Sources
Alzheimer	Cancer
Parkinson	Dementia
Cellular oxidative stress	Atherosclerosis
Cancer risks	Vascular diseases
Chronic inflammatory diseases	Hypercholesterolemia

ROS and sometimes pathologic conditions or overproduction of oxidants in our metabolic system may lead to oxidative stress. Oxidative stress as defined by Ahmad (1995) is a condition that occurs due to "the imbalance between oxidants and antioxidants in favor of oxidants potentially leading to damage" recently has been suggested as one of the causes of ageing (Hyun, Hernandez, Mattson & de Cabo, 2006) and other diseases such as cancer (Kinnula & Crapo, 2004), atherosclerosis (Upston, Kritharides & Stocker, 2003), cardiovascular diseases (Singh & Jialal, 2006), neurodegenerative diseases (Sas, Robotka, Toldi & Vecsei 2007), Alzheimer's disease (Smith, Rottkam, Nunomura, Raina & Perry, 2000), Parkinson's disease (Bolton, Trush, Penning, Dryhurst & Monks, 2000), alcohol induced hepatic disease (Arteel, 2003) and ulcerative colitis (Ramakrishna, Varghese, Jayakumar, Mathan, & Balasubramanian, 1997).

However, if the free radicals and other ROS are not eliminated nor neutralized within the body at an early stage, it can then target the various cellular elements such as lipid membrane, proteins, DNA and RNA. And this oxidation-induced by ROS can cause cell membrane disintegration, membrane protein damage and DNA mutation which can eventually cause cancer.

1.4.2. Pathophysiology of oxidative stress

The mechanism of action of oxidative stress in a human being occurs primarily by triggering inflammation. During oxidative stress, the mitochondria produces large quantities of Reactive Oxygen and Reactive Nitrogen Species (ROS/RNS) which is primarily identified as one of the essential steps in the pathophysiology of atherosclerosis that finally manifest to different types of cardiovascular diseases. Oxidative stress, furthermore, initiates lipid metabolism, plaque rupture, thrombosis, myocardial injury, apoptosis, fibrosis and finally cardiac failure.

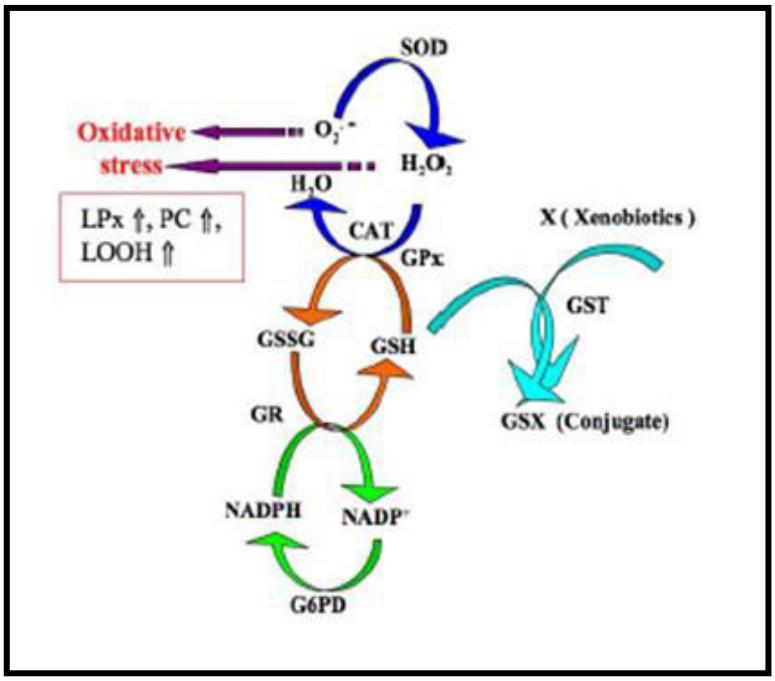


Figure 1.2: Oxidative stress caused by free radicals such as ROS and RNS

According to Salvemini, Doyle & Cuzzocrea (2006), upon exposure to chemokines, immune complexes or microbes, the leukocytes tend to extracellularly release oxygen-derived free radicals, a process in which the production of Reactive Oxygen Species (ROS) is dependent on the activation of NADPH-oxidase-system. The NADPH is thus, oxidized whilst, the oxygen is reduced to superoxide anions (O_2^-) (Figure 1.2). However, in case of neutrophils, activating signals accompanying phagocytosis triggers rapid oxidative reactions within neutrophils. The radicals then combine with Nitric oxide (NO) to produce Reactive Nitrogen Species (RNS).

It is the nature of the ROS that reacts with biomolecules like lipids protein and DNA that determine the extent of pathophysiological damage. Mostly, these radicals convert rapidly to peroxyl free radical in the presence of oxygen which then induces a chain reaction of lipid peroxidation. This in turn can manifest to physical damage such as "cross linking of protein", "change in membrane fluidity", and "formation of lipid-protein, lipid-DNA adduct" which are all injurious to proper cellular functioning.

1.5. Antioxidants

A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases. Flavonoids and polyphenolic compounds are potent antioxidants and have aroused considerable interest recently because of their potential beneficial effects on human health in fighting diseases. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity (Sannigrahi, Mazumder, Pal & Mishra, 2009).

1.5.1. Classification of antioxidants:

Antioxidants can be classified alphabetically or based on their mode of action/kinetics.

1.5.1.1. Alphabetical classification of antioxidants:

Table 1.4: Antioxidants alphabetically classified based on their structure, occurrence and mode of action, solubility and kinetics

Alphabetical name	Categories of antioxidants	Examples
Antioxidant C	Carotenoids	β-carotene, Lycopene, Lutein
Antioxidant E	Enzymes	SOD, Catalase, GPx
Antioxidant G	Glutathione	Glutathione
Antioxidant H	Hormones	Melatonin, Oestrogen
Antioxidant L	Lipid associated chemicals	Ubiquinol-10, M-acetyl cysteine, lipoic acid
Antioxidant M	Minerals	Zinc, Selenium, Copper
Antioxidant P	Phenolics	Quercetin, Catechin
Antioxidant S	Saponin, Steroids	Cortisone, Estradiol, Estriol
Antioxidant V	Vitamins	α-tocopherol, Ascorbic Acid

(Source: Flora, 2009)

1.5.1.2. Classification of antioxidants based on mode of action/kinetics

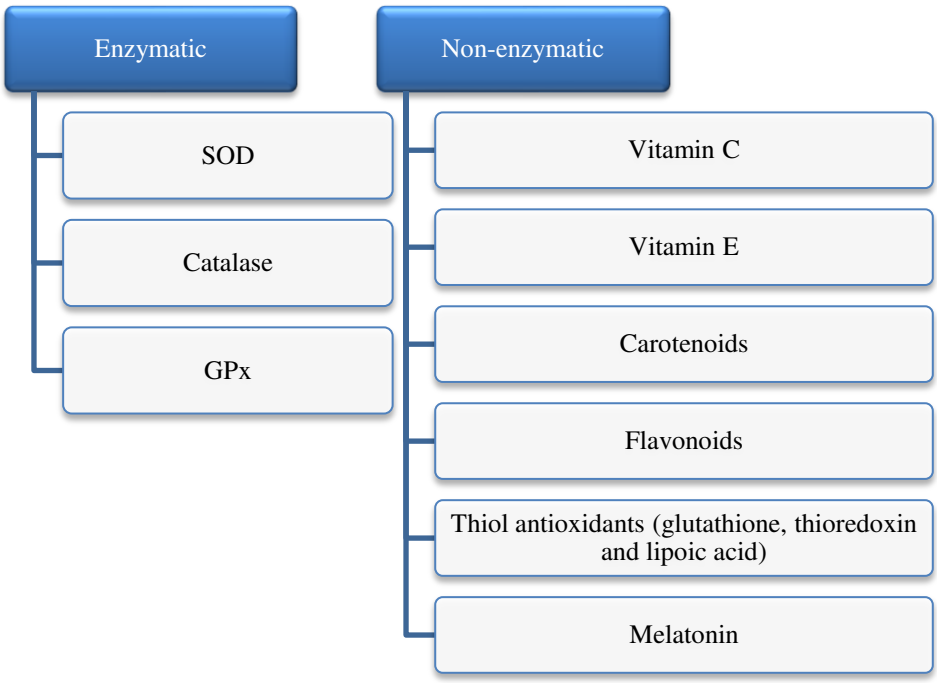
Antioxidants can be classified into six categorized based on their kinetics as mentioned by Flora (2009).

Table 1.5: Kinetic classification of antioxidants along with examples

#	Class	Examples
1.	Antioxidants that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds	Phenol, Naphthol
2.	Antioxidants that break chains by reacting with alkyl radicals	Quinones, Nitrones, Iminoquinones
3.	Hydro peroxide decomposing antioxidants	Sulfide, Phosphide, Thiophosphate
4.	Metal deactivating antioxidants	Diamines, Hydroxyl acids, Bifunctional compounds
5.	Cyclic chain termination by antioxidants	Aromatic amines, Nitroxyl radical, Variable valence metal compounds
6.	Synergism of action of several antioxidants	Phenol sulfide in which the phenolic group reacts with peroxyl radical and sulfide group with hydro peroxide

1.5.2. Mechanism of action of enzymatic and non-enzymatic antioxidants:

Below is a list of the different types of enzymatic and non-enzymatic antioxidants



1.5.2.1. Mechanism of action of Enzymatic antioxidants:

a) 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.

b) 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.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

c) Glutathione metabolism, which involves the GP_x enzymes that in the presence of tripeptide glutathione (GSH), adds two electrons to reduce peroxides. It simultaneously oxidizes GSH and decomposes peroxides. That is why, Glutathione metabolism is known as one of the most essential antioxidative defense mechanisms.

$$2\,GSH + H_2O_2 \xrightarrow{GP_x} GSSG + 2H_2O$$

$$2\,GSH + ROOH \xrightarrow{GP_x} GSSG + ROH + H_2O$$

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).

1.5.2.2. Mechanism of action of Non-enzymatic antioxidants:

a) 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. ROS oxidizes ascorbate by taking electrons from it to produce monodehydroascorbate and then dehydroascorbate. This causes the ROS to be reduced to water molecule whilst the different oxidized forms of ascorbate are relatively stable (Figure 1.3); thus, 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).

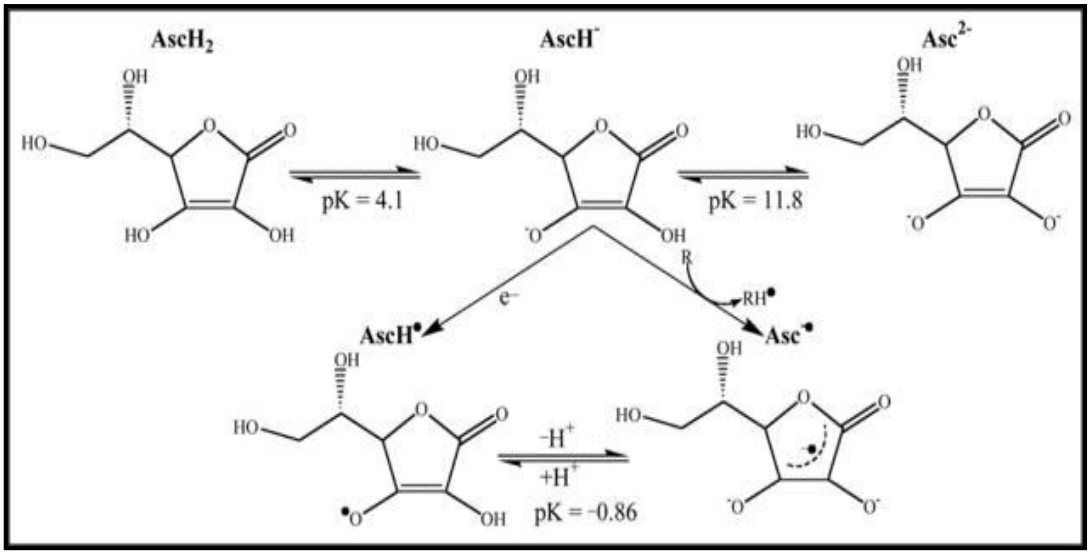


Figure 1.3: Conversion of ascorbic acid ($AscH^-$) to its reduced forms at various pH when interacting with Radicals ($R^\bullet$), indicating the possible binding sites and free electrons responsible for antioxidant and chelating property

b) Vitamin E exists as eight different isomers, the two most common being- α-tocopherol & α-tocotrienol (Figure 1.4). 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.

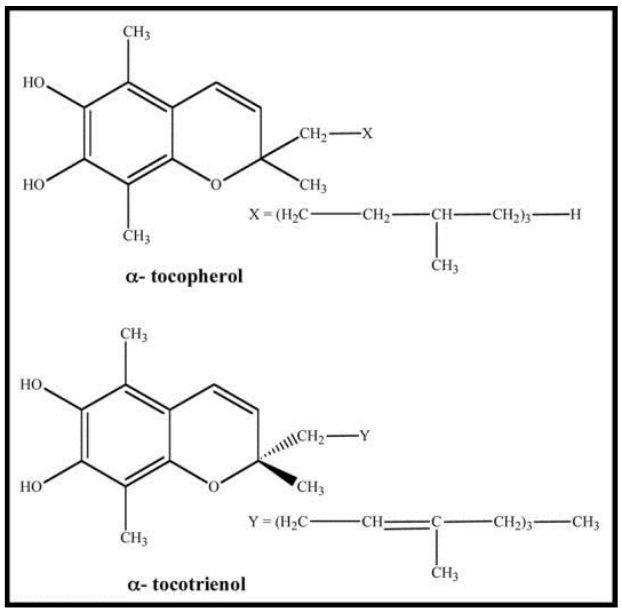


Figure 1.4: Two isomers of Vit-E namely, α-tocopherol & α-tocotrienol

α-tocopherol is converted to α-tocopherol radical during an antioxidant reaction, which can easily be reduced to the original, α-tocopherol, by Ascorbic acid (Kojo, 2004).

Furthermore, Vit-E also functions by interrupting the free radical chain reactions via "capturing the free radical". It is the free hydroxyl group on its aromatic ring which is responsible for antioxidant properties. Furthermore, as a micronutrient, Vit-E participates in maintaining both the structural and functional integrity of immune cells, thus, enhancing immunity. It also has the ability to prevent cellular injury by quenching free radicals and maintaining the sulfhydryl groups of the membrane proteins (Basu & Dickerson, 1996, p. 214-227).

c) Flavonoids are polyphenolic compounds possessing a structural backbone of diphenylpropane. While phenolic compounds may function as terminators of free radical chain reaction, it most actively participates in the protection against "oxidative stress". Due to their favorable reduction potential relative to alkyl peroxyl radicals, flavonoids are ideal scavengers of peroxy radicals and in this manner; they effectively inhibit lipid peroxidation (Rice-Evans, 2001). Another structural feature worth taking notice is the presence of 2,3-unsaturation in conjugation with a 4-oxo group in C-ring. In addition to this, the presence of dihydroxylated ring-B, exposes the molecule to donate hydrogen/electron that subsequently scavenges reactive radical species (Polovka, Brezova & Stasko, 2003).

d) Carotenoids are pigments (red or yellow) found in plants, and vegetables (carrot). The antioxidant activity of carotenoids primarily arises due to their ability to delocalize unpaired electrons. Thus, α-carotene would be able to physically quench singlet oxygen species without degradation (Mortensen, Skibsted, & Truscott, 2001). Moreover, according to Burton and Ingold (1984), a very high concentration of carotenoids can protect against lipid peroxidation. As for β-carotene, a study conducted by Karas, *et al.* (2000) has shown that it can help in reducing the growth of cancer cells by inhibiting the anti-apoptotic protein Bcl-2 expression in cancel cells.

1.6. Phytochemical evaluation of crude extract of different medicinal plants

As mentioned by Ghani (2003d, p. 18), the medicinally active compounds from the plants are generally by-products of normal synthesis and metabolism of the primary metabolites known as the secondary metabolites that may be distributed throughout the whole plant or accumulated in certain parts of the plant e.g. leaves.

Bioactive compounds of plants include substances like alkaloids, flavonoids, tannins and phenolic compounds. Polyphenolic compounds are a structural class of compounds that consists of many phenolic units in one structure and comprise of various classes of compounds namely flavonoids, phenolic compounds/phenols, tannins and sometimes, glycosides. These are chemical substances that possess medicinal value and are of huge interest in pharmacology and phytochemical research. When these molecules are isolated from the plants, they exhibit various pharmacological actions including, antibacterial, antiviral, antifungal, anti-inflammatory, analgesic, anti-pyretic, anti-cancer, antioxidant and many more. Nonetheless, studies have recently shown that compounds obtained from natural sources are better than their synthetic analogues because of their environmental safety and biodegradability. However, for reasons of unavailability as well as the limited number of medicinal plants to treat the human population regionally and world-wide, synthetic analogues of drugs are used for the management of disease, although, the concern of toxicity from the medicinal plant remain (Khan *et al*., 2011).

Phytochemical evaluation provides us both qualitative and quantitative information pertaining to a particular class of compounds. Furthermore, Bowman, Krishnaraju and Nigam (1970) mentions that recently, with the use of sophisticated methods in scientific approach, scientists have identified secondary plant metabolites as possessing potential pharmacological properties, yet to be identified. On this note, the WHO admit that the best possible source of herbal drugs are the medicinal plants, which is why the importance of studying medicinal plant and their role in pharmacology is very important.

1.6.1. Alkaloids

Alkaloids are the most abundant chemical constituent found in the plants, both medicinally beneficial and poisonous (Ghani, 2003d, p. 22-23). Alkaloids possess a bitter taste and they represent one of the largest classes of compounds that are physiologically active and many are important drugs. As shown in table 1.6, they constitute a large class of compounds and they are classified based on the type of group attached to them.

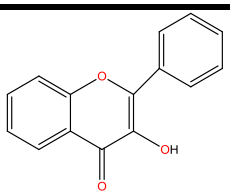
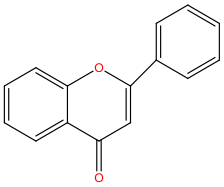
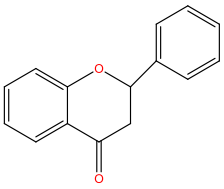
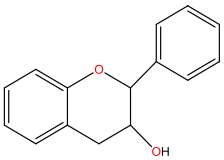
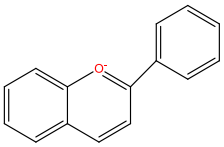
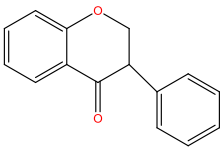
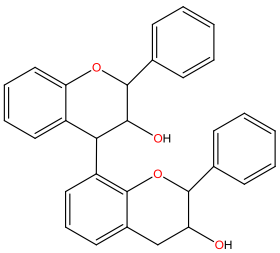
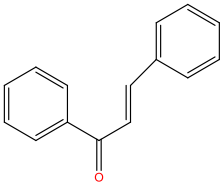
Table 1.6: Classification of alkaloids along with examples

SL #	Class of Alkaloid	Example
1.	Pyridine group	Arecoline (*Areca catechu*
2.	Tropane group	Atropine (*Atropa belladonna)*
3.	Isoquinoline group	Morphine (*Papaver somniferum)*
4.	Quinoline group	Quinine (*Cinchona* species)
5.	Indole group	Reserpine (*Rauvolfia serpentina)*
6.	Steroidal group	Digitoxin (Foxglove digitalis)
7.	Imidazole group	Pilocarpine *(Pilocarpus* species)
8.	Phenylethylamine group	Ephedrine (*Ephedra* species)
9.	Alkaloidal amines	Colchicine (*Colchicum autumnale)*

1.6.2. Flavonoids

Flavonoids are one of the largest class of polyphenolic compounds. One of the best described properties of flavonoids is its antioxidant activity. The presence of a hydroxyl group within its B-ring structure plays a major role in scavenging ROS during “oxidative stress” (Rice-Evans, 2001). Besides its antioxidant potential, flavonoids are also known for anti-inflammatory, antibacterial, anti-cancer and hepatoprotective activity. Rutin is a flavonoid present in the lemons that helps in “strengthening the walls of blood capillaries” (Ghani, 2003d, p. 22). Based on their chemical structure, Flavonoids can be classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones, and the positioning of the hydroxyl group in their structure, determines the nature of the pharmacological activity.

Table 1.7: Classification of flavonoids based on their structural backbone along with examples

S.L. #	Flavonoid Sub-class	Schematic Structure	Examples	Food source
1.	Flavonol		Quercetin, Kaemferol, Galangin, Myricetin	Onion, kale, broccoli, apple, cherries, berries, tea
2.	Flavone		Apigenin, Luteolin, Tangeritin	Parsley, thyme, citrus fruits
3.	Flavanone		Naringenin, Hesperetim	Citrus fruits
4.	Flavan-3-ol		Catechin, Epicatechin (EC), Epicatechin gallate, Epigallo-catechin (EGC) and Epigallocatechin gallate (EGCG)	Apple, tea, grapes
5.	Anthocyanin		Cyanidin, Pelagonidin, Mavidin, Peonidin, Rosinidine, Aurantinidin, Luteolinidin	Cherries, grapes, strawberries, raspberries
6.	Isoflavone (not a flavonoid)		Daidzen, Genistein	Soybeans, legumes
7.	Procyanidin (dimers, oligomers and polymers)		Epicatechin-(4-β-8)-Epicatechin	Apples, grapes, cocoa and pine bark
8.	Chalcone (not a flavonoid)		Phloretin, Phloridzin	Apples

(Source: Barrington, R. 2011)

1.6.3. Phenols

Phenols such as thymol possess particular antimicrobial and anti-inflammatory properties which make it common to be used in various antiseptic and anti-inflammatory medicinal preparations. Thymol is particularly found in the fruits of umbelliferous plants (Ghani, 2003d, p. 20). Thymol and salicylic acid are found most commonly in a number of plants.

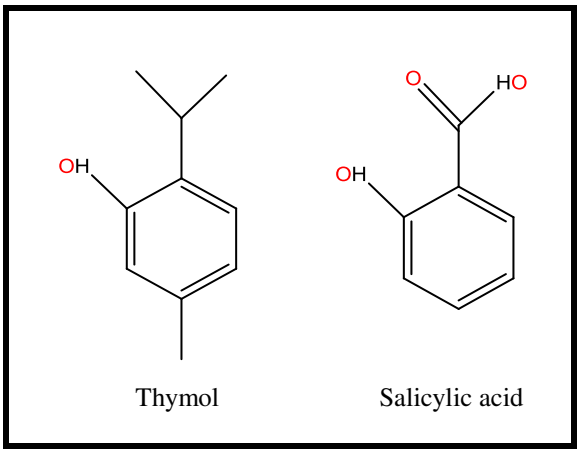


Figure 1.5: Structures of Thymol and Salicylic acid

1.6.4. Tannins

Chemically, tannins are polyhydroxyphenolic substances. Tannins are generally indicated for gastrointestinal problems such as diarrhea, dysentery, ulcer and some skin diseases (Ghani, 2003d, p. 20). However, antiseptic and antibiotic activity is also demonstrated by tannins whereby, they coagulate the microbial protoplasm. Examples of medicinal plants containing tannin include *Eucalyptus* species (Eucalyptus) and *Cinnamomum cassia* (Cassia bark).

1.6.5. Glycosides

Glycosides are also another class of compounds much wider in occurrence than the alkaloids. Most, but not all, are non-toxic (Ghani, 2003d, p. 21-22). Glycosides comprise a class of very important drugs for the modern medicine and can be classified into five major classes namely, anthraquinones, cardiac, saponin, thiocyanates and miscellaneous glycosides (Table 1.8).

Table 1.8: Classification of Glycosides

S.L.#	Class	Properties	Example
1.	Anthraquinone glycosides	Laxatives, purgatives and cathartics	Sennosides (Senna or *C. angusifolia*)
2.	Cardiac glycosides	Treatment of Cardiac diseases	Digitoxin and Digoxin (*Digitalis purpurea* and *D. lanata*)
3.	Saponin glycosides	Demulcent, expectorant, laxative and as an aphrodisiac	*Panax* species
4.	Thiocyanate glycosides	Local irritants and emetics	Sinigrin (Black mustard), Sinalbin (white mustard)
5.	Miscellaneous glycosides	Anti-rheumatic, anhelmintic, diuretic and astringent	Santonin (*Artemisia cina)*, Arbutin (*Uva ursi)*

1.7. *In-vitro* antioxidant activity of *C. verrucosa*

Four *in-vitro* antioxidant activities are performed for the purpose of this study:

1.7.1. DPPH free radical scavenging assay

The DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radical scavenging assay of methanolic leaf extract *C. verrucosa*, was determined using the method described by Braca *et al.* (2001). In this method, DPPH 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 percentage free radical scavenging activity or percentage inhibition of free radical scavengers is then calculated, using the equation as follows:

$$\% \text{ Inhibition of free radical scavengers} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, $\mathbf{A_0}$ = The absorbance of the control

$\mathbf{A_1}$ = The absorbance of the sample/standard

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)

The total phenolic content (TPC) of the plant extract of *C. verrucosa* was determined by the modified folin-ciocalteu method as mentioned by Wolfe, Wu and Liu (2003).

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:

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

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) 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)

1.7.3. Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) of the extracts of *C. verrucosa* was determined according to the method as described by Kumaran and Karunakaran (2007) with slight modification. In this method a stable acidic complex 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.

The total flavonoid content (C) of each of the fractions was then, calculated and 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 quercetin obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

1.7.4. Determination of total antioxidant capacity (TAC)

Also known as the phosphomolybdenum method, the total Antioxidant capacity of plant extract of *C. verrucosa* was determined using the method described by Prieto, Pineda & Aguilar (1999). 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.

The total antioxidant capacity (A) of each of the fractions was then expressed as ascorbic acid equivalent (AAE) using the following equation:

$$A = \frac{(c \times V)}{m}$$

Where, **A** = 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)

1.8. Analysis of Polyphenolic compounds in *C. verrucosa* using HPLC

HPLC (High Performance Liquid Chromatography or High Pressure Liquid Chromatography) is most commonly used now-a-days for the identification and quantification of compounds in pharmaceutical industries, chemical industries, food industries, for environmental analysis, and many more. However, it is most widely used method of analysis in research–no field has more use of HPLC than in research. HPLC is so powerful that it can separate compounds dissolved in solution and is primarily used as a "quantitative method of analysis for non-volatile organic compounds" (Summerfield, 2010).

As a highly sophisticated instrument with multiple functionalities i.e. one can choose between a gradient elution or isocratic elution; a reversed-phase HPLC or a normal-phased HPLC; different types and sizes of column, etc. It's a highly compact feature; it also demonstrates very reliable results with more robustness, linearity, accuracy, optimal limit of detection and optimal limit of quantification. Just as the "input" process of injecting sample from sample injector to the column is performed sophisticatedly, the "output" in the form of a chromatogram is performed sophisticatedly.

1.8.1. Instrumentation

The HPLC instrument consists of mainly six functioning units (Figure 1.6) namely the mobile phase reservoir, the pump, a sample injector/autosampler/sample manager, the HPLC column housing the stationary phase, the detector and finally, the recorder.

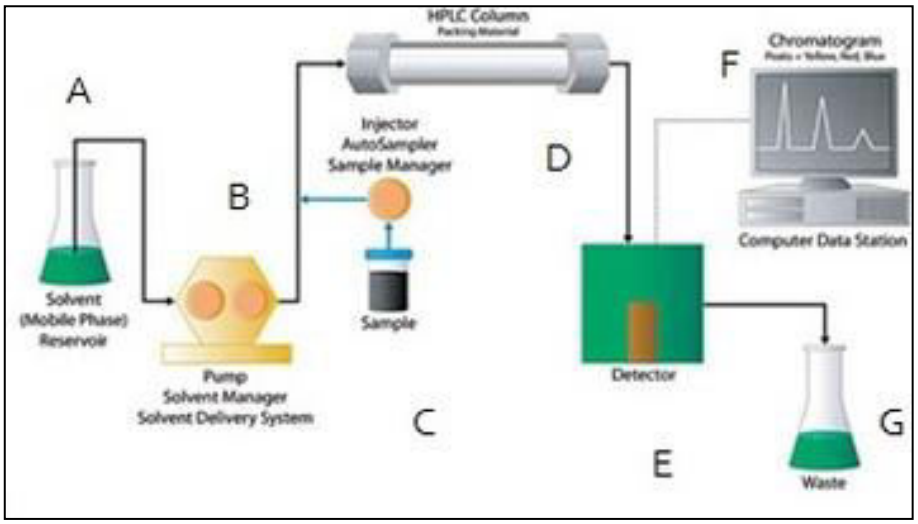


Figure 1.6: Schematic diagram of an HPLC system

After placing the sample in the sample compartment, the sample is injected into the HPLC column by the aid of sample injector. The sample injector withdraws a predefined volume of the sample mixture (5-25uL) from the sample compartment and then injects it into the column. Once present in the stationary phase, different compounds move across the stationary phase at different rates, based on the "partitioning behavior" of the compound between the mobile liquid phase and the stationary phase. The detector then measures the retention times of the compounds undergoing analysis and once complete, produces an output –which is known as a chromatogram.

The constituents of a sample can be identified by comparing its retention time with those of known standards because one chemical constituent will comprise only one peak (Summerfield, 2010). By using a Diode Array Detector (DAD), the UV/Visible spectra of separated compounds can be recorded which aids in quantification. Besides identification, HPLC can also be used for quantification of compounds by comparing the peak area of the sample with that of the known standard concentration.

Degassing of solvents is very important to allow proper functioning of the HPLC system. It is done so as to remove air bubbles present in the solvent. Pumps provide a steady high pressure with no pulsating and can be programmed to vary the composition of the mobile phase by allowing either gradient elution or isocratic elution or both (Summerfield, 2010). After use, the eluent is disposed off in a waste bin.

1.8.2. HPLC Column

The columns are typically 10-30cm in length, 3-5mm in diameter and are made up of stainless steel tubing which is packed with 3-10um silica particles as stationary phase (Summerfield, 2010). There are mainly 2 types of HPLC column based on the composition of stationary and mobile phase, namely the normal phased HPLC and the reversed-phase HPLC (RP-HPLC).

A RP-HPLC has been utilized for the purpose of this study. It utilizes a relatively non-polar stationary phase of "surface modified silica" support e.g. octadecyl carbon chain (C18)-bonded silica (USP classification L1) and a polar mobile phase such as binary or tertiary mixtures of water, methanol or acetonitrile. In RP-HPLC, the polar compounds are eluted first.

1.8.3. HPLC Detector

There are many different types of detector which can be used with HPLC. The selection of the type of detector is based on the nature of analyte to be separated. Typically, it includes diode-array-detector (HPLC-DAD), UV-Visible detector (HPLC-UV-Vis), florescence detector, refractometer, mass spectrometry (HPLC-MS), amperometric detector and conductivity detector.

The present study focuses on the identification and quantification of polyphenolic compounds present in the crude extract of *C. verrucosa* for which the most appropriate type of detector chosen was the diode-array-detector (known as HPLC-DAD).

The Diode-Array-Detector is sensitive at up to 10^{-9} to 10^{-4}g/mL and it allows the detection of compounds at multiple wavelengths, which can be used for identification of compounds using the chromatogram of the sample comparable to that of the standard.

HPLC-DAD was used in the present study as the choice of detector to simultaneously analyze nineteen polyphenolic antioxidant compounds because of its rapidity (analysis time was 40min), accuracy, precision, linearity and stability of the system to excellently identify and quantify polyphenolic compounds present in *C. verrucosa*.

1.8.4. Significance of HPLC over other types of chromatographic technique

The main reasons why RP-HPLC was chosen over other types of chromatographic analysis are:

a) Results obtained are precise, accurate, and reliable

b) Results obtained are reproducible

c) Economic because smaller volume of organic solvent is used in the entire analytical procedure

d) Use of hydrophobic stationary phase allows for using a combination of most of the organic solvents, including water.

1.9. Bangladeshi medicinal plant

1.9.1. Overview of Bangladeshi medicinal plant

In the *material medica* of traditional medicine it has been noted that in the Asian subcontinent alone, approximately 2000 plants with medicinal properties have been enlisted (Chopra, R.N. Chopra, I.C. Handa & Kapur, 1958) out of which more than 500 medicinal plants are currently listed as amongst those growing in Bangladesh (Yusuf, Chowdhury, Wahab & Begum, 1994). From amongst these plants are herbs or plants with definite medicinal virtues which are thereafter used as principle therapeutic agents or as an excipient in traditional medicinal preparation to enhance the therapeutic effect or confer stability to the final preparation. Some of the medicinal plants are those which have been introduced rather empirically by handing over through generations of use or based on the individual experience or maybe are isolated cases of beneficial effects. Some of the plants are recommended as providing efficacious therapeutic effect by practicing physicians after being clinically tried.

Currently in Bangladesh, more than 250 medicinal plants are commonly used in the preparation of traditional medicine (Ghani, 2003b, p.7-8), some of which as listed below (Table 1.9).

Table 1.9: Medicinal uses of certain medicinal plants in Bangladesh in traditional medicine preparation

Name of plant	Medicinal uses
Abroma augusta	Curing urogenital and female-related diseases
Allium sativum	Reducing blood cholesterol
Andrographis paniculata	Curing fever and hepatic diseases
Bacopa monniera	Increasing the longevity of life and as a brain tonic
Catharanthus roseus	Treatment of diabetes and cancer
Centella asiatica	Treatment of diarrhea and dysentery
Coccinea indica	Management of diabetes
Rauvolfia serpentina	Treatment of high blood pressure, insanity and insomnia
Terminalia arjuna	Treatment of heart diseases

1.9.2. Selection of *Crotalaria verrucosa* L. for the present study

After doing extensive literature review of the various bioactivity studies performed on medicinal plants in Bangladesh, it was found that amongst the publications of *Crotalaria verrucosa*, its antioxidant potential was not studied. After thorough research online, it was sufficiently concluded that neither *in-vivo* nor *in-vitro* antioxidant potential was determined. Thus was taken the initiative to determine the antioxidant potential of *C. verrucosa*.

Figure 1.7: *Crotalaria verrucosa*

1.9.3. Introduction to *Crotalaria* taxon

The *C. verrucosa* belongs to the Leguminosae family also known as Fabaceae. Leguminosae is known as the legume, pea or bean family and currently are the largest most economically important family of the flowering plants and is the third largest land plant family in terms of number of species, with the Orchidaceae and Asteraceae being the top two (MDidea.com, n.d).

Of the 630 legume genera known (over 18,860 species), one of the largest genera is Crotalaria which approximately has 700 species. Other largest genera include Astagalus (over 2,000 species), Acacia (over 1,000 species), Indigofera (around 700 species) and Mimosa (around 500 species). These top 5 largest legume genera constitutes one-fourth of all the legume species. The economic value of all *Crotalaria* species is illustrated in table 1.10.

Table 1.10: Economic value of *Crotalaria* species

***Crotalaria* taxon**	**Habitat**	**Occurrence (%) in nature**	**Economic value**
Crotalaria nana	Herb	ND	Cover crop and green manure
Crotalaria pallida	Herb	ND	Cover crop and green manure
Crotalaria retusa	Shrub	12.5	Cover crop, green manure and pesticide
Crotalaria striata	Herb	9.7	Cover crop, green manure and medicinal
Crotalaria verrucosa	Herb	18.1	Medicinal

KEY: ND = Not Indicated *(Source: Arun et al., 1999; Rao & Sherief, 2002)*

1.9.4. Plant description

Crotalaria verrucosa L. is also known as "Blueflower", "Rattlepod", or "Blueflower" and it belongs to a Fabaceae (Leguminosae) family. It was first recorded in *Species Plantarum* in 1753 by Linnaeus, C. von. In Bengali, *Crotalaria verrucosa* is called "Jhanjhania" or "Bansan" (Ghani, 2003c, p. 190). As shown in table 1.10, *Crotalaria verrucosa* is the only perennial shrub that is described as solely possessing medicinal value compared to other *Crotalaria* species. It can be best identified by their copiously branched stems that possess curved spines holding on to ovate-elliptic-shaped leaves. Ghani further describes that the flowers are pea-shape contained within "racemose spikes and hirsute leguminous pods". When small, the plant simply produces flowers, and once it is between 1-1.5m in height, it starts to bear fruits in addition to flowers (Australian Tropical Rainforest Plants, n.d.).

1.9.5. Plant taxonomy

Table 1.11: Taxonomy of *C. verrucosa*

Rank	**Scientific name (Common name)**
Kingdom	Plantae (Plants)
Subkingdom	Tracheobionta (Vascular plants)
Superdivision	Speratophyta (Seed plants)
Division	Magnoliophyta (Flowering plants)
Class	Magnoliophyta (Dicotyledons)
Subclass	Rosidae
Order	Fabales
Family	Fabaceae/Leguminosae (Pea family)
Genus	*Crotalaria* L. (Rattlebox)
Species	*Crotalaria verrucosa* L. (Blue rattlesnake)

1.10. Rationale of the project:

Previous study on this legume plant included investigation into its *in-vivo* anti-diabetic, anti-fertility, antipyretic, CNS depressant potential, wound-healing and hepatoprotective activity; and *in-vitro* anticoagulant activity, thrombolytic and antibacterial activity. No previous bioactivity study has yet been conducted on the anti-oxidant potential of *C. verrucosa.* In addition to that, it has been found in a study conducted by Kumar, Asha and Babu (2014), that *C. verrucosa* L. is composed of flavonoids, steroids and steroidal nucleus. Since plants rich in polyphenolic compounds like flavonoids have demonstrated to possess anti-inflammatory, antiallergenic, antiviral, antiageing and anticarcinogenic activities, these can be attributed to their antioxidant properties and thus, this study will focus mainly on determining the anti-oxidant potential of crude extract of *C. verrucosa.*

1.11. Aim of the project:

The aim of the study is the investigation of *in-vitro* antioxidant potential in *Crotalaria verrucosa* along with the identification and quantification of its polyphenolic compounds.

1.12. Objectives of the project:

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

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) Identification and quantification of the polyphenolic compounds using HPLC.
e) Revealing the presence or absence of accessory bioactive properties to justify its use in folkloric remedies.

CHAPTER TWO

LITERATURE REVIEW

Chapter 2: Literature Review

2.1. Previously studied pharmacological properties of *C. verrucosa*

Literature reviews *C. verrucosa* have been carried out and it is reported to possess various pharmacological activities including antipyretic, thrombolytic, anti-diabetic, CNS depressant, antimicrobial, anti-fertility and would healing activity (Table 2.1).

Table 2.1: Previous studies on pharmacological activity of *C. verrucosa*

Pharmacological activity	Plant part	Type of extract	References
Antipyretic	Leaves	Ethanol	Nawrin *et al.* (2015)
Thrombolytic	Leaves	Ethanol	Nawrin *et al.* (2015)
Anti-diabetic	Leaves	Ethanol	Nawrin *et al.* (2015)
CNS depressant	Leaves	Ethanol	Nawrin *et al.* (2015)
Antibacterial	Leaves	n-butanolic	Riazunnisa, Prasad, Sudha, & Khadri (2015)
Anti-fertility	Leaves	70% ethanol, 95% ethanol and aqueous	Singh *et al.* (2011)
Wound-healing	Leaves	Aqueous	Kumari *et al.* (2010)
Hepatoprotective activity	Leaves	Ethanol	Lekharani *et al.* (2013)

2.1.1. Antipyretic activity: The study conducted by Nawrin *et al.* (2015) primarily induces pyrexia in Wistar rats through subcutaneous injection of brewer's yeast (20% w/v in distilled water at 10mL/kg b.w.). After a period of 19hrs, the initial rectal temperature was recorded and the *Crotalaria verrucosa* extracts of different concentrations (100, 250 and 500 mg/kg b.w.) were orally administered to different rats, the results of which were comparable to administration of standard drug, Paracetamol 150mg/kg. The reduction in temperature was recorded at 1hr, 2hr and 3hrs following the treatment. It was observed that the sample dose of 500mg/kg b.w. produced a significant reduction in temperature (from 40.33°C to 37.48°C) comparable to that of Paracetamol 150mg/kg (from 40.42°C to 37.51°C). And it was sufficiently concluded that the 500mg/kg b.w. possesses strong antipyretic activity greater than 250mg/kg (temperature reduction from 40.67°C to 38.55°C) whilst 100mg/kg b.w. does not decrease hyperthermia significantly (from 40.27°C to 39.63°C).

2.1.2. Thrombolytic activity: From the study conducted by Nawrin *et al.* (2015), it was successively concluded that the leaf extract of *C. verrucosa* failed to produce a strong clot lysis activity in comparison to the standard drug, Streptokinase. The percentage of thrombolytic activity of *Crotalaria verrucosa* leaf extract against HRBC clot denaturation was observed to be 26.81% in comparison to that of Streptokinase (80.65%). In this study, 100uL of *C. verrucosa* extract was taken for each alpine tube containing thrombus to which the 500uL of blood withdrawn from healthy volunteers was added and weighed. Similar was done for the standard drug, Streptokinase. The tubes were then incubated at 37°C for 90min after which the supernatant was removed from the tubes, which was then reweighed to observe clot disruption and the % thrombolytic activity was then calculated.

2.1.3. Anti-diabetic activity: The anti-diabetic activity of *C. verrucosa* has been investigated by Nawrin *et al.* (2015) by artificially developing type-II diabetes on Wistar rats via intraperitoneal injection of alloxan monohydrate. At the 72nd hour the blood glucose level was confirmed to be above 140mg/dL, thus, confirming diabetic condition. Thereafter, 3 different doses of *C. verrucosa* were orally administered to the Wistar rats: 100mg/kg, 250mg/kg, 500mg/kg b.w., the results of which were compared with that of Standard drug, Glibenclamide 2.5mg/kg b.w. during a time interval of 0, 7, 14 and 21 days using commercially available glucose kits. The blood glucose level of the Wistar rat induced with *C. verrucosa* extract of 500mg/kg b.w. on Day-0 and Day-21 was observed to be 284.54mg/dL and 190.33mg/kg b.w. respectively, whereas that of Glibenclamide showed a significant decrease in blood glucose level from 281.49mg/dL to 177.12mg/dL. The author thus, concludes that the ethanolic extract of *C. verrucosa* possesses significant anti-diabetic activity, less than that of Glibenclamide (2.5mg/kg).

2.1.4. CNS depressant: The CNS depressant activity of *C. verrucosa* was observed on Swiss albino mice via "Hole cross test" and "Open field test". The test described by Khatoon *et al.* (2014) was followed for the "Hole cross test" and the method described by Al-Mahmud, Bachar, Qais, & Shams-Ud-Doha, (2013) was followed for "Open field test" with selective modifications. For both the tests, Diazepam 1mg/kg b.w. was selected as the reference standard which was orally administered to the animals 20min before the start of experiment. The reduction in "Hole cross activity" reflected the CNS depressant activity of the sample on the Swiss albino mice, whereas for the "Open field

test", the decrease in the number of squares crossed by the animal in an open field in 20min is an indication of CNS depressant activity. The result of the experiment conducted by Nawrin *et al.* (2015), sufficiently concluded that the maximum decrease in motor function was exhibited by the *Crotalaria verrucosa* extract of 500mg/kg b.w. which was slightly less than that of Diazepam (1mg/kg b.w.).

2.1.5. Antibacterial activity: In a study conducted by Riazunnisa, Prasad, Sudha, and Khadri (2015), the antibacterial activity was examined for n-butanol extracts of *C. verrucosa* (100ug/mL) by measuring the diameter (mm) of the zone of inhibition using the Agar well diffusion method against the bacterial strains namely, *Bacillus subtilis* (G +ve), *Klebsiella pneumonia* (G –ve), *Escherichia coli* (G –ve), *Proteus vulgaris* (G –ve) and *Pseudomonas aeruginosa* (G +ve). In this study, Gentamycin was used as the standard drug. It was observed that the zone of inhibition in *C. verrucosa* extract for *B. subtilis* and *K. pneumonia* was 15mm each, 13mm for *E. coli*, 14mm for *P. vulgaris* and 12mm for *P. aeruginosa*. On the other hand, for Gentamycin, the zone of inhibition for the above strains were 20mm, 16mm, 18mm, 15mm and 20mm, respectively. Thus, the n-butanol extract of *C. verrucosa* possesses a "broad spectrum" of antibacterial activity against a panel of bacteria responsible for most of the common diseases.

2.1.6. Anti-fertility activity: The data obtained in the study conducted by Singh *et al.* (2011) indicated that 70% ethanolic, aqueous and 95% ethanolic extract of aerial part of *C. verrucosa* exhibited significant anti-implantation and early abortifacient activity in dose dependent manner in female Wistar albino rats compared to 95% ethanolic extract. The 95% ethanolic, 70% ethanolic and aqueous extracts of *C.* verrucosa at dose of 500 mg/kg b.w., 250 mg/kg b.w. were found to possess highly significant estrogenic activity as indicated by increase in uterine weight, vaginal cornification and uterotropic responses. Other biochemical changes such as the concentration of glucose, cholesterol and alkaline phosphatase was observed to be higher in the treated group (*C. verrucosa*) in comparison to the control group and significantly less than standard group.

2.1.7. Wound-healing activity: The aqueous extract of *C. verrucosa* was found to possess significant wound-healing potential on Wistar albino rats when it was tested on three wound models including incision, excision and dead space wounds. It was observed that coadministration of *C. verrucosa* with dexamethasone significantly increased the breaking strength in incision-type of model in comparison with the

dexamethasone treated group. As for the excision wound model, the percentage of wound contraction was significantly increased by two doses of test extract (all except 12^{th} and 16^{th} day of drug treatment) and it also reversed dexamethasone suppressed wound contraction. Furthermore, it reduced the time required for epithelialization and reversed the epithelialization delaying effect of dexamethasone. This successfully represents the significant wound-healing activity of *C. verrucosa* which was evident by the decrease in the period of epithelialization, increase in rate of wound contraction, skin breaking strength and granulation tissue dry weight content (Kumari *et al.*, 2010).

2.1.8. Hepatoprotective activity: In a study investigated by Lekharani *et al.* (2013), *C. verrucosa* confirmed the hepatoprotective action against paracetamol-induced-hepatotoxicity in Wistar rats. The study concludes that the treatment of ethanolic extract of *C. verrucosa* causes significant protection against both paracetamol induced liver damage and protects against any increase in serum enzyme levels and bilirubin in a dose responsive manner. Other parameters such as LP (Lipid peroxidation), SOD (Superoxide dismutase), CAT (Catalase), GSH (Glutathione) and glycogen contents were also measured from the liver. Furthermore, upon performing the histopathological studies, it was observed that the Wistar rats treated with ethanolic extract of *C. verrucosa* showed absence of centrilobular necrosis and absence of vacuolization of cytoplasm of the hepatic cells, thus proving the protective action against hepatic damage by *C. verrucosa*.

2.2. Phytocompounds previously isolated from *C. verrucosa*

Table 2.2: Phytocompounds isolated from *C. verrucosa*

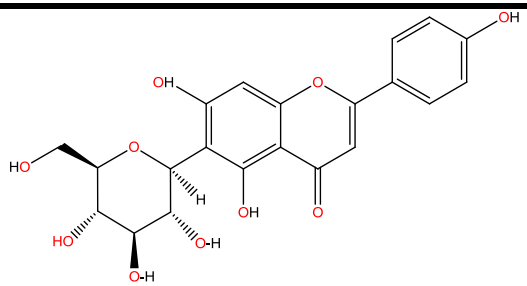
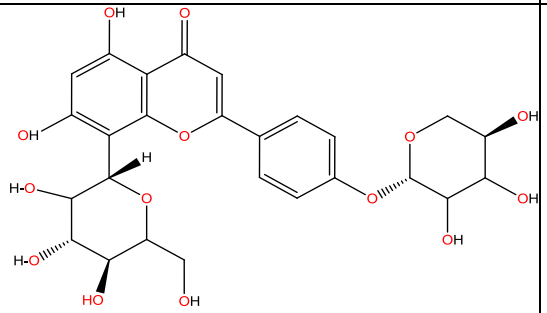
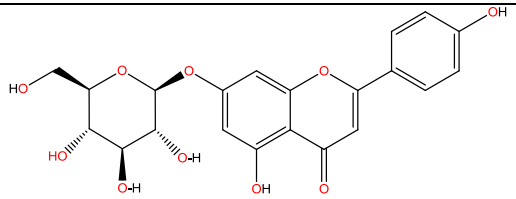
Chemical class	Phytocompounds	Plant part	References
Flavonoids	Isovitexin 	Seeds	Indian Journal of Pharmacy (1967 & 1972); Phytochem (1976)
Flavonoids	Vitexin-4'-O-glucoside 	Seeds	Rastogi & Mehrotra (1993, p. 2)
Flavonoids	Vitexin (Apigenin-8C-glucoside) 	Seeds	Rastogi & Mehrotra (1993, p. 2)

Table 2.2: Phytocompounds isolated from *C. verrucosa (Continued)*

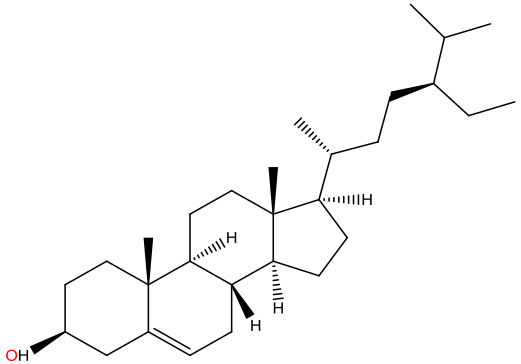
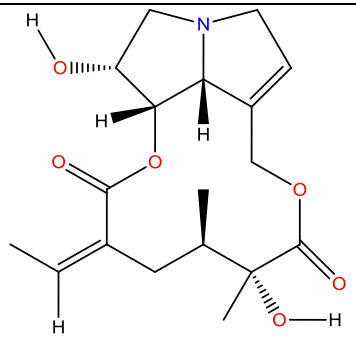
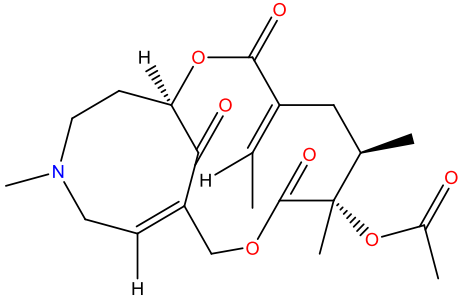
Chemical class	Phytocompounds	Plant part	References
Phytosterols	β-sitosterol 	Seeds and stems	Indian Journal of Pharmacy (1967 & 1972); Phytochem (1976)
Pyrollizidine alkaloids	Crotalaburnine (also known as Anacrotine) 	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976), Roeder & Wiedenfield (2013)
Pyrollizidine alkaloids	Crotaverrine acetate (O-acetylcrotaverrine or Ligularidine) 	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976), Roeder & Wiedenfield (2013)

Table 2.2: Phytocompounds isolated from *C. verrucosa (Continued)*

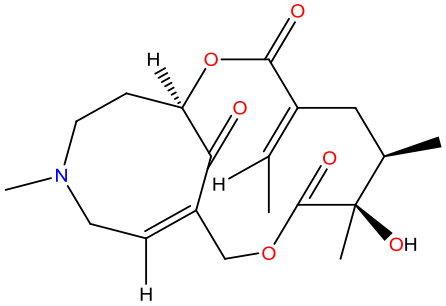
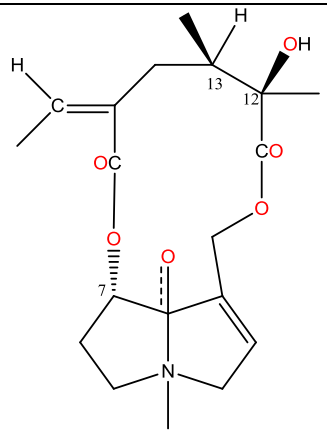
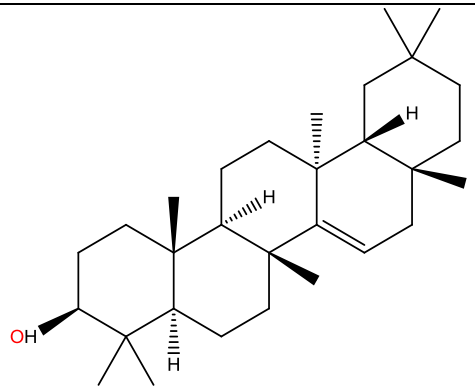
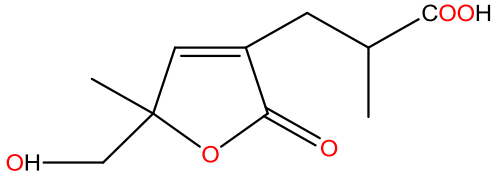
Chemical class	Phytocompounds	Plant part	References
Pyrollizidine alkaloids	Crotaverrine (O-crotaverrine) 	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976)
Pyrollizidine alkaloids	Isosenkirkine 	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976)
Pyrollizidine alkaloids	Isosenkirkine acetate (Structure not found)	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976)

Table 2.2: Phytocompounds isolated from *C. verrucosa (Continued)*

Chemical class	Phytocompounds	Plant part	References
Saturated fatty acid	Not found	Stem	Yadava & Mattews (1993)
Tri-terpenoid	Taraxerol (Alnulin) 	Stem	Yadava & Mattews (1993)
Necic lactone	2-methyl-3-(2-oxo-[5H]-5-hydroxymethyl-5-methylfuran-3-yl)-propanoic acid 	Leaves	Suri, O.P., Suri, K.A., and Dhar, K.L. (1989)
Tropane alkaloids	Not found	Leaves	Indian Journal of Pharmaceutical Sciences (1979)
Unsaturated fatty acid	Not found	Stem	Yadava & Mattews (1993)

2.3. Folklore remedies/medicinal uses

In the native, Bangladesh, the plant possesses a multitude of medicinal functions as mentioned by Ghani (2003c, p. 190). The ethanolic extract of *C. verrucosa* acts as a CNS depressant and diuretic (Indian Journal of Experimental Biology, 1977). The leaves obtained from this plant have been used both internally and externally in the treatment of scabies and impetigo. Further literature reviews suggest that they are also used as an expectorant and as an emetic. They are considered efficacious in diminishing salivation (Kumari *et al.*, 2010) probably due to the presence of tropane alkaloids and are used in the treatment of dyspepsia, biliousness, in reducing fever, treating impurities of blood, in relieving heart complaints and in the treatment of throat and oral diseases. Amongst the Chakma and Marma tribes in Bangladesh, the leaves of *C. verrucosa* were used in treating skin allergies (Yusuf, Begum, Hoque & Chowdhury, 2009). In Nigeria it is used for the treatment of colic, flatulence and various skin diseases (Nadkarni, A.K. & Nadkarni K. M., 2000, p. 596-598).

2.4. Habitat and Distribution

The perennial, non-climbing shrub is native to tropical Asia (Polhill, 1990, p. 80). In Bangladesh, it is distributed across Chittagong, Khulna, Rajshahi and Sylhet (about 5%) (Ghani, 2003c, p. 190). It is widely spread in tropics and is found in waste places of native India (Nadkarni, A.K. & Nadkarni K. M., 2000).

CHAPTER THREE

METHODOLOGY

Chapter 3: Methodology

3.1. Collection and authentication of plant material

Crotalaria verrucosa was as the plant for this investigation (Figure 3.1) since no previous study was conducted on its antioxidant potential. With the aid of comprehensive literature study of this plant and its availability, it was decided to be chosen for investigation.

Table 3.1: Chemical investigation of *Crotalaria verrucosa*

NAME OF THE PLANT	FAMILY	PLANT PART
Crotalaria verrucosa	Fabaceae	Leaves

The whole plant *Crotalaria verrucosa* was collected in the month of August 2015 from Sylhet, 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-41865) and authenticated by the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka.

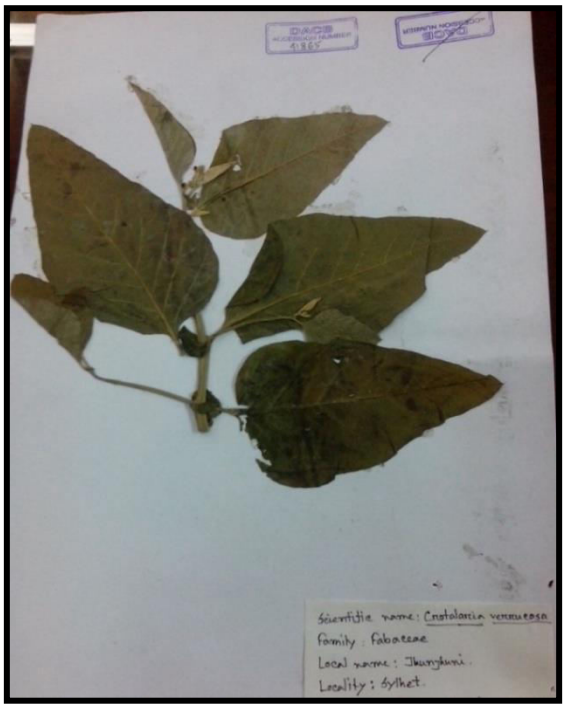


Figure 3.1: *Crotalaria verrucosa* leaves obtained from the National Herbarium of Bangladesh, Mirpur, Dhaka

3.2. Extraction procedure

Steps involved in the extraction of Medicinal plant:

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

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

b. Extraction process (5-step)

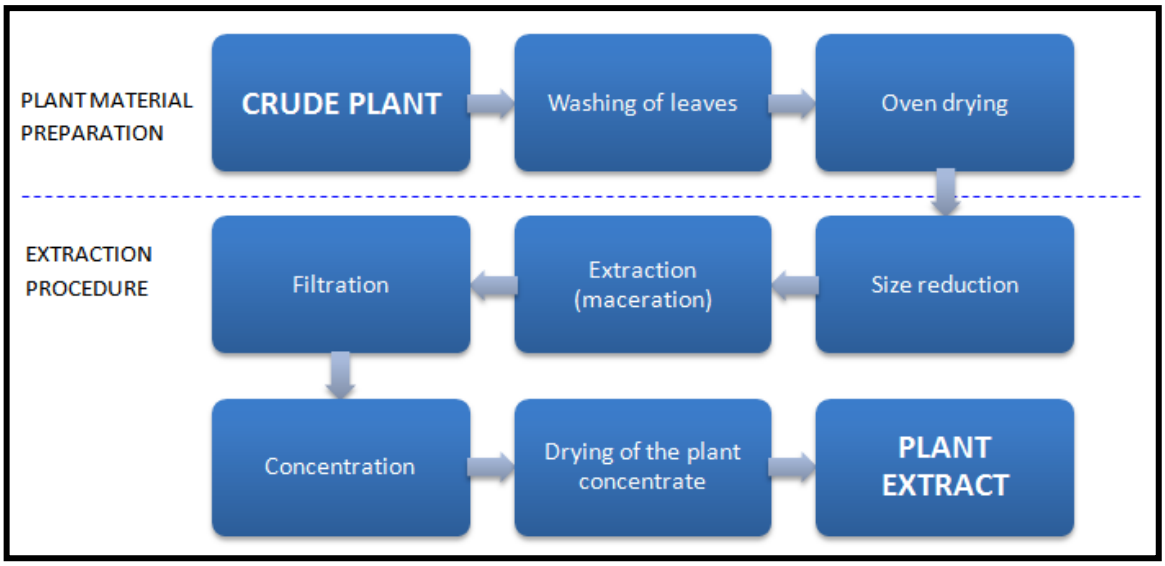


Figure 3.2: Flowchart showing a step-by-step procedure for the extraction of ingredients from the crude medicinal plant

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 total weight of the powdered plant material was roughly divided into 3 parts and weighed separately in a beaker, the results of which were recorded (Chapter 4: Results, Table 4.1).

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. Each of the three beakers containing powdered plant material of *Crotalaria verrucosa* was soaked in 900mL of methanol for a period of 2 days at normal room temperature (22-25°C) with occasional agitation.

Table 3.2: Indicates the weight of powdered plant material along with the volume of methanol used for the maceration process

	Beaker-1	**Beaker-2**	**Beaker-3**	**Total**
Weight of Plant Powder/g	98.59	90.86	88.85	278.30
Volume of methanol/mL	900	900	900	2700

Figure 3.3: The outcome of maceration process

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

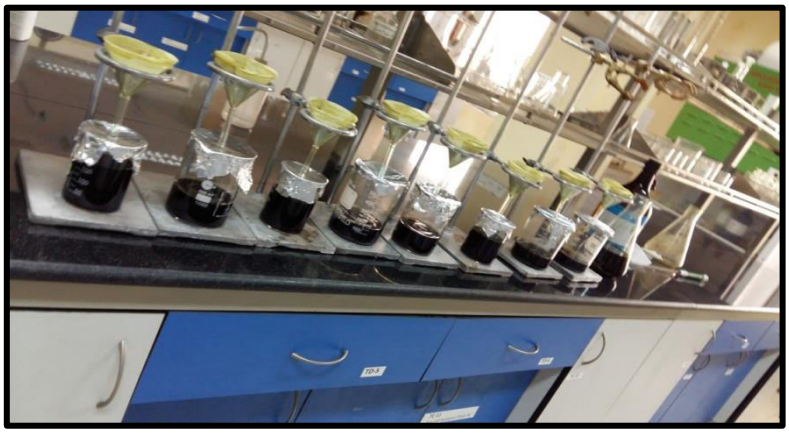


Figure 3.4: Demonstration of the filtration process

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 onto petri-dishes for drying under LAF.

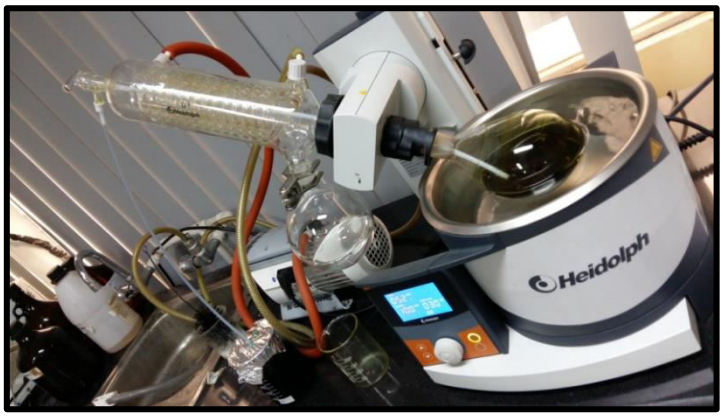


Figure 3.5: Concentrating the filtrate of *C. verrucosa* plant using a Rotary evaporator

3.2.2.5. Drying

Finally, the petri-dishes were 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 its drying. After successful drying of the extract, the petri-dishes were covered in Aluminum foil and refrigerated for further use.

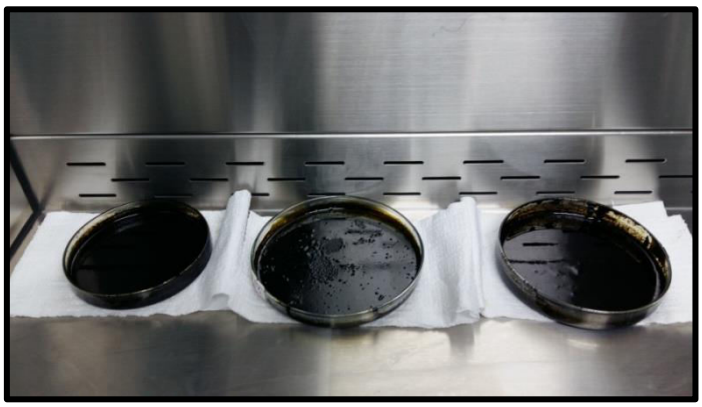


Figure 3.6: The petri-dishes containing the methanolic leaf extract was placed under LAF for drying

3.3. Phytochemical screening

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

Figure 3.7: Demonstrates the various phytochemical tests performed on the methanolic extract of *C. verrucosa*

The following qualitative tests were performed:

3.3.1. Detection of Alkaloid:

For the qualitative determination of alkaloids, three tests were performed. 0.5g of methanolic extract of *C. verrucosa* 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 dissolving0.1358g 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 Carbohydrates

According to Ramkrishnan, Prasannan and Rajan (1994), carbohydrate can by qualitatively detected by weighing out 0.5g of methanolic extract of *C. verrucosa* 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 the

sides 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 Flavonoids

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 compounds

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

Libermann Burchard's Test:

To a small amount of extract, 1mL 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

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 *Crotalaria verrucosa* was hydrolyzed with dilute Hydrochloric acid before subjecting it to Borntrager's Test (Mariappansenthilkumar, 2013).

Borntrager's Test (modified):

To the 5mL 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 Saponins

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, 4 methods were chosen to determine the antioxidant activity in plant extract of *C. verrucosa*, 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. verrucosa*, was determined using the method described by Braca *et al.* (2001).

3.4.1.1. Reagents and chemicals

Table 3.3: Reagents and chemicals required for determining DPPH scavenging activity

S.L. #	Name of Reagent/Chemical	Suppliers
1.	DPPH	Sigma Aldrich, U.S.A.
2.	Methanol	Active Fine Chemicals Ltd., Bangladesh
3.	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 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 7 serially diluted concentrations: 1200, 800, 400, 200, 100, 50 and 25ug/mL.

The standard used was L-ascorbic acid and it was prepared in the same manner as the extract resulting in 7 serially diluted concentrations, ranging from 1200-25ug/mL.

3.4.1.4. Preparation of Blank solution

The blank solution contained 3mL methanol.

3.4.1.5. 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:

$$\% \text{ Inhibition of free radical scavengers} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, $\mathbf{A_0}$ = The absorbance of the control

$\mathbf{A_1}$ = 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. verrucosa* was determined by the modified Folin-Ciocalteu method as mentioned by Wolfe, Wu and Liu (2003).

3.4.2.1. Reagents and Chemicals required

Table 3.4: Reagents and chemicals required for determining total phenolic content

S.L. #	Name of Reagent/Chemical	Suppliers
1.	Folin-Ciocalteu Reagent (FCR)	LOBA Chemie Pvt. Ltd., India
2.	Gallic acid monohydrate (Standard)	Sigma Aldrich, USA
3.	Sodium carbonate	Merck Specialities Pvt. Ltd., Mumbai
4.	Methanol	Active Fine Chemicals Ltd., Bangladesh

3.4.2.2. Reagent preparation

250mL of 10% FCR solution was prepared by taking 25mL of FCR in a 250mL volumetric flask and diluting it with distilled water to 250mL mark.

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. verrucosa* 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 200ug/mL.

Gallic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in 7 serially diluted concentrations, ranging from 1200, 800, 400, 200, 100, 50 and 25ug/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 5mL of FCR solution was added.

c) And 4mL 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 TFC of the extracts of *C. verrucosa* was determined according to the method as described by Kumaran and Karunakaran (2007).

3.4.3.1. Reagents and Chemicals required

Table 3.5: Reagents and chemicals required for determining total flavonoid content

S.L.#	Name of Reagent/Chemical	Suppliers
1.	Quercetin (Standard)	Sigma Aldrich Co., Germany
2.	Methanol	Active Fine Chemicals Ltd., Bangladesh
3.	Potassium Acetate	Merck KGaA, Germany
4.	Aluminium Chloride	Merck Specialities Pvt. Ltd., Mumbai

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. verrucosa* 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 200ug/mL.

Quercetin was used as the standard and the stock solution was prepared in the same manner as the extract resulting in 7 serially diluted concentrations, ranging from 1200, 800, 400, 200, 100, 50 and 25ug/mL.

3.4.3.4. Preparation of the blank

The blank solution contained 200uL of 10% Aluminium chloride solution, 200uL 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 200uL of 10% Aluminium chloride solution and 200uL of 1M Potassium acetate solution were added to each of the test tubes using 1000uL 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 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 quercetin 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. verrucosa* was determined by using the method as described by Prieto, Pineda and Aguilar (1999).

3.4.4.1. Reagents and Chemicals required

Table 3.6: Reagents and chemicals required for determining total antioxidant capacity

S.L. #	Name of Reagent/Chemical	Suppliers
1.	Ammonium Molybdate	Active Fine Chemicals Ltd., Bangladesh
2.	L-Ascorbic Acid (Standard)	Merck, Germany
3.	Sodium Triphosphate ($Na_3PO_4.12H_2O$)	Merck KGaA, Germany
4.	Concentrated sulfuric acid (98%)	Merck, Germany
5.	Methanol	Active Fine Chemicals Ltd., Bangladesh

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 Potassium acetate in a 100mL volumetric flask and diluting it with distilled water up to 100mL mark.

100mL of 0.028M Sodium Phosphate solution was prepared by measuring 1.0645g of Aluminium chloride 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. verrucosa* 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 200ug/mL.

Ascorbic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in 7 serially diluted concentrations, ranging from 1200, 800, 400, 200, 100, 50 and 25ug/mL.

3.4.4.4. Preparation of the blank

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

3.4.4.5. Experimental procedure

a) 300uL 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:

$$A = \frac{(c \times V)}{m}$$

Where, A = 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. Analysis of Polyphenolic compounds in *C. verrucosa* using HPLC-DAD

3.5.1. Chemicals

Arbutin (AR), gallic acid (GA), hydroquinone (HQ), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), Syringic acid (SA), (-)-epicatechin (EC), vanillin (VL), *p*-coumaric acid (PCA), *trans*-ferulic acid (FA), rutin hydrate (RH), ellagic acid (EA), benzoic acid (BA), rosmarinic acid (RA), myricetin (MC), quercetin (QU), *trans*-cinnamic acid (TCA), and kaempferol (KF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC-grade), methanol (HPLC-grade) and acetic acid (HPLC-grade) were obtained from Merck (Darmstadt, Germany).

3.5.2. HPLC detection and quantification of phenolic antioxidants

Detection and quantification of phenolic antioxidants in the methanolic leaf extract of *Crotalaria verrucosa* were determined by HPLC-DAD analysis as described by Khirul *et al.* (2014) with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS).

Separation was performed using Acclaim® C_{18} (5µm) Dionex column (4.6 x 250 mm) at 30 ºC with a flow rate of 1 ml/min and an injection volume of 20 µl. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) with the gradient elution program of 5%A/95%B (0-5 min), 10%A/90%B (6-9), 15%A/75%B/10%C (11-15), 20%A/65%B/15%C (16-19 min), 30%A/50%B/20%C (20-29 min), 40%A/30%B/30%C (30-35) and 100%A (36-40 min). The UV detector was set to 280 nm for 22.0 min, changed to 320 nm for 28.0 min, again change to 280 nm for 35 min and finally to 380 nm for 36 min and held for the rest of the analysis period while the diode array detector was set at an acquisition range from 200 nm to 700 nm. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing arbutin (AR), (–)-epicatechin (ECA) (5 µg/ml each), gallic acid (GA), hydroquinone (HQ), vanillic acid (VA), rosmarinic acid (RA), myricetin (MC) (4 µg/ml each), caffeic acid (CA), Syringic acid (SA), vanillin (VL), *trans*-ferulic acid (FA) (3 µg/ml each), *p*-coumaric acid (PCA), quercetin (QU), kaempferol (KF) (2 µg/ml each), (+)-catechin hydrate (CH), ellagic acid (EA) (10 µg/ml each), *trans*-cinnamic acid (TCA) (1 µg/ml), rutin hydrate (RH) (6 µg/ml) and benzoic

acid (BA) (8 µg/ml). A solution of the extract was prepared in methanol with a concentration of 10 mg/ml.

Prior to HPLC analysis, all the solutions (mixed standards, sample, and spiked solutions) were filtered through 0.20 µm syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min. Data acquisition, peak integration, and calibrations were calculated with Dionex Chromeleon software (Version 6.80 RS 10).

3.6. Statistical analysis

The mean and standard deviations for each of the methods, namely, total phenolic content (TPC), total flavonoids content (TFC) and total antioxidant capacity (TAC) were performed in triplicates (n=3) whereas that of DPPH was performed in duplicates (n=2). In case of HPLC analysis, however, the content of polyphenolic compounds present in the extract was presented as the mean and standard deviation of five determinations (n=5). All the statistical analysis, involving calculation of mean and standard deviation; plotting of graphs and determination of regression coefficient (R^2), were performed using Microsoft Office Excel 2007.

CHAPTER FOUR

RESULTS

Chapter 4: Results

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

4.1.1. The total weight of powdered plant material before maceration

Table 4.1: Weight distribution of powdered plant material amongst three beakers

	Beaker-1	**Beaker-2**	**Beaker-3**
Initial Weight/g (Beaker)	309.31	260.37	310.76
Final Weight/g (Beaker + plant powder)	407.90	351.23	399.61
Weight of Plant Powder/g	98.59	90.86	88.85
Total weight of plant powder/g (W_2)			**278.30**

Interpretation: The weight of the powdered plant material in each of the beaker before the maceration process were 98.59, 90.86 and 88.85g which in total is 278.30g of powdered plant material ready for maceration process.

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

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

	Petri-dish 1	**Petri-dish 2**	**Petri-dish 3**
Initial weight/g (Petri-dish)	46.75	51.32	51.72
Final weight/g (Petri-dish + extract)	56.32	52.54	63.44
Weight of extract/g	9.57	1.21	11.72
Total weight of extract/g (W_1)			**22.50**

Interpretation: A total of 22.50g of plant extracts was produced as a result of maceration and subsequent drying of the methanolic extract of *Crotalaria verrucosa*; that was used in carrying out subsequent experiments.

4.1.3. Calculation of percentage yield of the extract

Percentage 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 the extract= (22.5 x 100) / 278.30

= 8.084%

Interpretation: The total weight of the extract after maceration was found to be 22.50g whilst that before maceration was 278.30g; therefore, the % yield of *Crotalaria verrucosa* was calculated to be 8.084%.

4.2. Phytochemical screening of *C. verrucosa*

Table 4.3: Phytochemical screening of *Crotalaria verrucosa*

S.L.#	Class of compound	Result
1.	Alkaloids	+++
2.	Flavonoids	++
3.	Phenols/Phenolic compounds	+
4.	Glycosides	+
5.	Tannins	+++
6.	Carbohydrates	++
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. verrucosa* showed the presence of alkaloids, flavonoids, phenolic compounds, glycosides, tannins, carbohydrates, resins and steroids whilst showing the absence of phytosterol and saponin.

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

4.3.1. Table of DPPH free radical scavenging assay (Absorbance vs. concentration)

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

Concentration (ug/mL)	Absorbance of Standard (AA) at 517nm (Mean ± SDV)	Absorbance of Sample (CV) at 517nm (Mean ± SDV)
12.5	0.385 ± 0.007	0.631 ± 0.001
25	0.255 ± 0.001	0.614 ± 0.001
50	0.113 ± 0.001	0.597 ± 0.001
100	0.0275 ± 0.001	0.585 ± 0.001
200	0.0255 ± 0.001	0.491 ± 0.001
400	0.024 ± 0.001	0.303 ± 0.001
800	0.0235 ± 0.001	0.090 ± 0.001
1200	0.0225 ± 0.002	0.090 ± 0.001

The data was presented as mean ± standard deviation of duplicate experiments

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

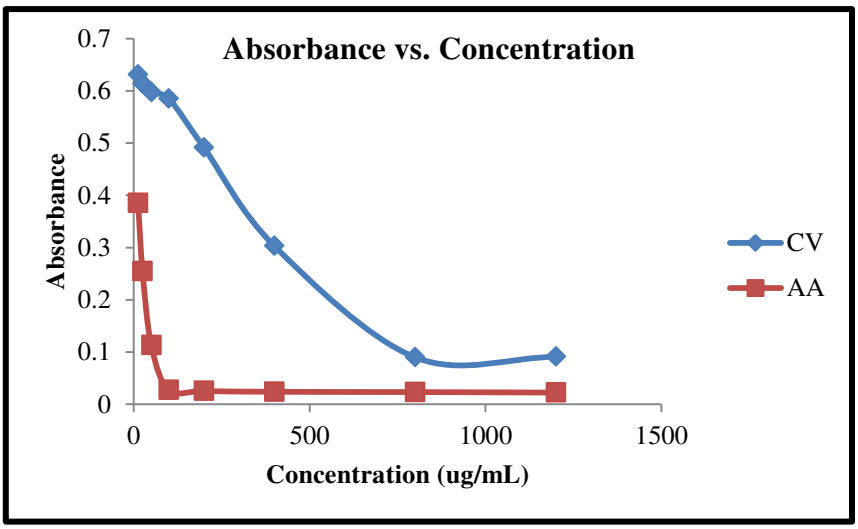


Figure 4.1: Graph of DPPH Absorbance vs. Concentration (ug/mL) for *Crotalaria verrucosa* (CV) and ascorbic acid (AA)

Interpretation**:** From the table 4.4 and figure 4.1 above, it was observed that with an increase in concentration of *C. verrucosa* from 12.5 – 1200ug/mL, the absorbance decreased slowly from 0.630 to 0.091 in comparison to that of ascorbic acid (0.385-0.0225); thus, implying that the steeper slope i.e. ascorbic acid (red) had a higher %

inhibition of DPPH free radical scavengers than the less steeper slope (blue) i.e. methanolic extract of *C. verrucosa*.

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

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

Concentration (ug/mL)	% Inhibition (A.A.) (Mean ± SDV)	% Inhibition (C.V.) (Mean ± SDV)
12.5	40.402 ± 1.095	2.322 ± 0.219
25	60.526 ± 0.219	4.954 ± 0.219
50	82.508 ± 0.219	7.585 ± 0.219
100	95.743 ± 0.109	9.443 ± 0.219
200	96.053 ± 0.109	23.994 ± 0.219
400	96.285 ± 0.219	53.096 ± 0.219
800	96.362 ± 0.109	86.068 ± 0.219
1200	96.517 ± 0.328	86.068 ± 0.219
The data was presented as mean ± standard deviation of duplicate experiments		

Interpretation: For any given concentration, the % inhibition of free radical scavengers' by the crude extract of *C. verrucosa* was observed to be lower than the corresponding concentration of ascorbic acid. However, the highest free radical scavenging activity for CV was found to be 86.068% at 800ug/mL.

4.3.4. Graph of % inhibition by CV vs. corresponding concentration

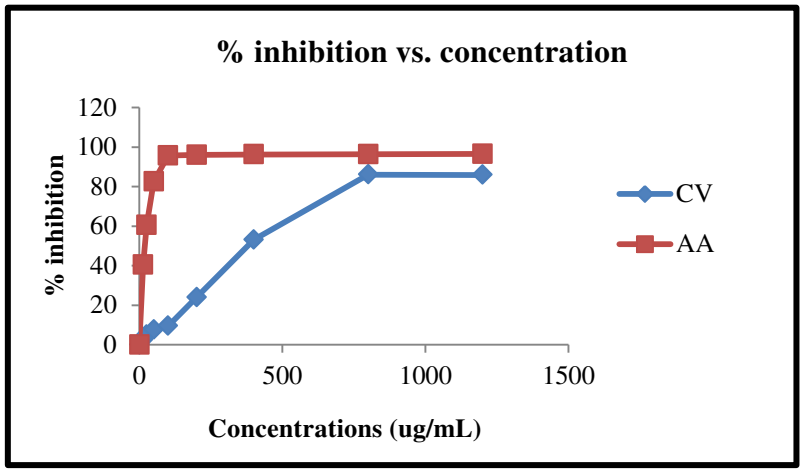


Figure 4.2: % inhibition of DPPH free radical scavengers by methanolic leaf extract of CV (blue) and standard ascorbic acid (red)

Interpretation: The less-steeper slope (blue) of *C. verrucosa* indicates that as its concentration was increased, the % inhibitory activity also increased, although lower than the corresponding concentration of ascorbic acid.

4.3.5. Determination of the IC_{50} value of *C. verrucosa* (CV) and standard ascorbic acid (AA)

Table 4.6: Comparison between the IC_{50} value of *Crotalaria verrucosa* and standard ascorbic acid.

S.L. #	Name of the sample	IC_{50}
1.	*Crotalaria verrucosa*	533.738 ug/mL
2.	Ascorbic acid	154.916 ug/mL

Interpretation: The IC_{50} value of dried extract of *C. verrucosa* signifies that a much higher concentration of 533.738ug/mL is required for inhibiting 50% of all DPPH free radical scavengers, whilst a small concentration of 154.916ug/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

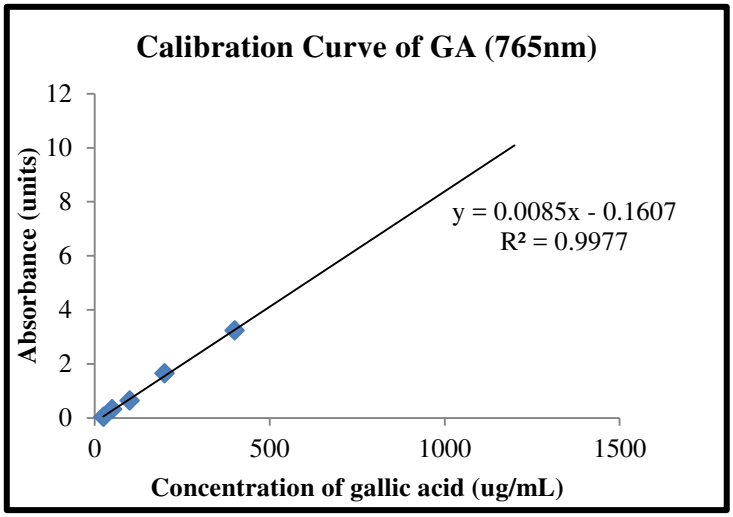


Figure 4.3: Calibration curve of gallic acid (GA) at 765nm for determining TPC in CV

4.4.2. Table of total phenolic content (TPC) in CV

Table 4.7: Total phenolic content of *Crotalaria verrucosa* (CV) represented as GAE

Concentration of CV (ug/mL)	**TPC (GAE) (Mean ± SDV)**
200	34.775 ± 1.815
400	59.721 ± 0.900
800	99.564 ± 2.876
1200	152.180 ± 2.688

The values are the average of triplicates of experiments and are represented as mean ± standard deviation

Interpretation: It is observed that as the concentration of *C. verrucosa* was increased from 200–1200ug/mL, the total phenolic content also increased from 34.775 to 152.180mg 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)

4.5.1. Calibration curve of quercetin

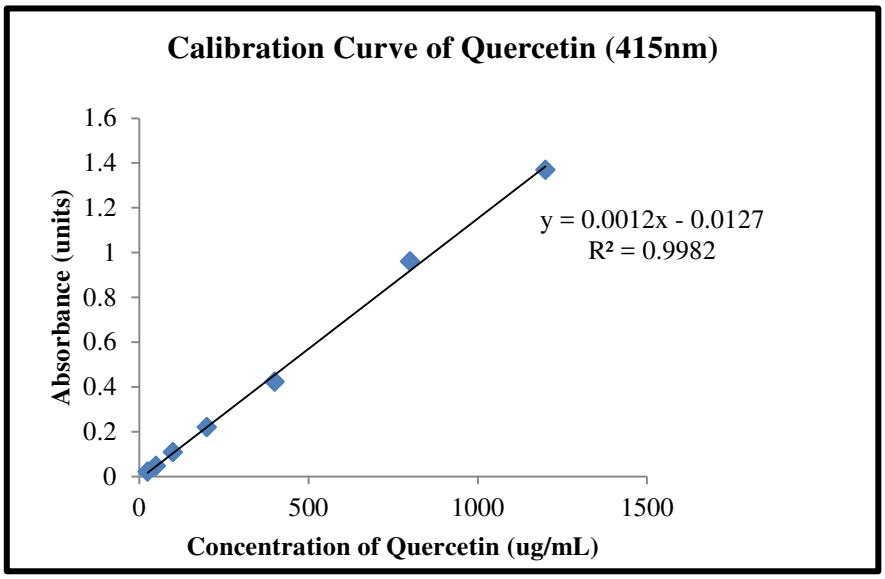


Figure 4.4: Calibration curve of quercetin at 415nm for determining TFC in CV

4.5.2. Table of total flavonoid content (TFC) in *C. verrucosa* (CV)

Table 4.8: Total flavonoid content of *Crotalaria verrucosa* (CV) represented as QE

Concentration of CV (ug/mL)	**TFC (QE) (Mean ± SDV)**
200	32.083 ± 4.167
400	78.938 ± 2.700
800	120.600 ± 2.622
1200	184.510 ± 2.778

The values are the average of triplicates of experiments and are represented as mean ± standard deviation

Interpretation: It is observed that as the concentration of *C. verrucosa* was increased from 200–1200ug/mL, the total flavonoid content also increased from 32.083 to 184.51mg of quercetin per gram of dried extract. Therefore, it indicates that there is as well, a positive correlation between total flavonoid content of *C. verrucosa* and its antioxidant activity.

4.6. Determination of Total Antioxidant Capacity (TAC)

4.6.1. Calibration curve of ascorbic acid

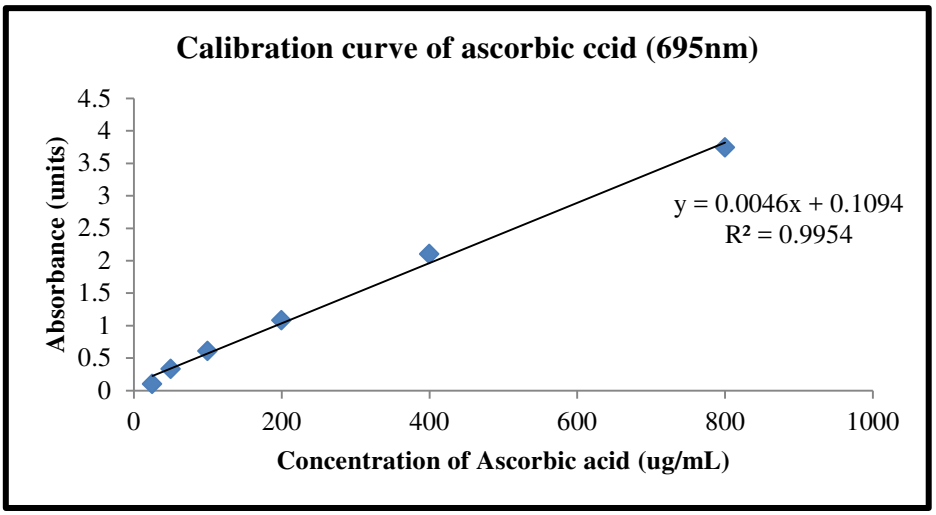


Figure 4.5: Calibration curve of ascorbic acid (AA) at 695nm for determining TAC in CV

Interpretation: The equation of the calibration curve of ascorbic acid was found to be y=0.004x-0.109 which would be used to determine the total antioxidant capacity of *Crotalaria verrucosa*. A regression coefficient of 0.995 was also obtained indicating a good relationship between the concentration and the absorbance.

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

Table 4.9: Total antioxidant capacity of *Crotalaria verrucosa* (CV) represented as AAE

Concentration of CV (ug/mL)	**TAC (AAE) (Mean ± SDV)**
200	8.348 ± 0.652
400	19.582 ± 0.245
800	24.628 ± 0.285
1200	32.342 ± 0.136

The values are the average of triplicates of experiments and are represented as mean ± standard deviation

Interpretation: It is observed that as the concentration of *C. verrucosa* was increased from 200ug/mL to 1200ug/mL, the total flavonoid content also increased from 8.348–32.342mg of ascorbic acid per gram of dried extract. This indicates therefore, that a

positive correlation exists between total antioxidant capacity of *C. verrucosa* and its antioxidant activity.

4.7. Relationship between DPPH free radical scavenging activity of *C. verrucosa* with the other three *in-vitro* antioxidant methods

Table 4.10: Summary of the DPPH free radical scavenging activity, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) of methanolic leaf extract of *C. verrucosa*

Concentration of CV (ug/mL)	TPC (GAE)	TFC (QE)	TAC (AAE)	DPPH FRS assay (% Inhibition)
200	34.775 ± 1.815	32.083 ± 4.167	8.348 ± 0.652	23.994 ± 0.219
400	59.721 ± 0.900	78.938 ± 2.700	19.582 ± 0.245	53.096 ± 0.219
800	99.564 ± 2.876	120.600 ± 2.622	24.628 ± 0.285	86.068 ± 0.219
1200	152.180 ± 2.688	184.510 ± 2.778	32.342 ± 0.136	85.913 ± 0.219

Interpretation: For higher concentrations of methanolic extract of *C. verrucosa,* it was observed that the total flavonoid content was always greater than the total phenolic content. A smaller value of total antioxidant capacity for any given concentration relative to total flavonoids content and total antioxidant content can be explained as follows. For instance, at a concentration of 1200ug/mL, the lower value of TAC indicates that lesser amount of antioxidants prefer the chemical pathway of ascorbic acid (32.342mg/g of dried extract) than that of quercetin (184.510mg/g of dried extract) or gallic acid (152.180mg/g of dried extract). However, upon comparing its total antioxidant capacity to that of *Gardenia jasminoides,* it was found that *C. verrucosa* possessed higher total antioxidant capacity (Chapter 8: Appendix, Table 8.13).

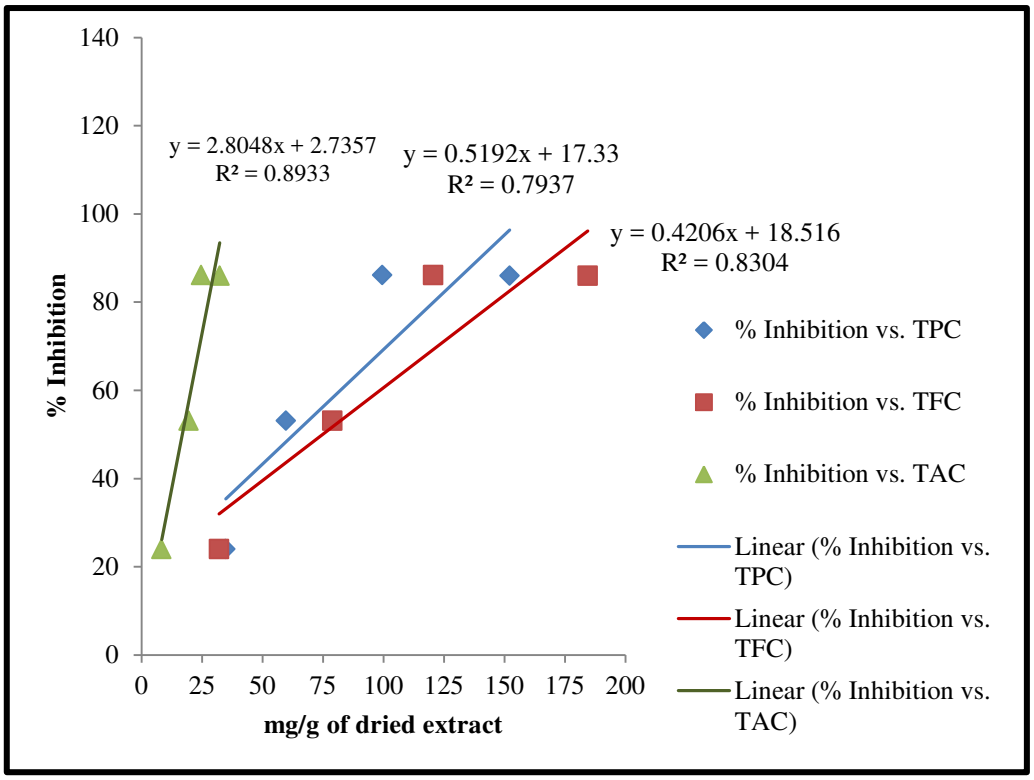


Figure 4.6: Relationship between % Inhibitory activity of DPPH free radical scavengers to that of TPC, TFC and TAC

Interpretation: From the graph, it can be clearly observed that there exists a very strong positive correlation (R^2 = 0.893) between the % inhibitory activity of *C. verrucosa* on DPPH free radical scavengers and total antioxidant capacity (TAC). This indicates that whilst there are greater quantities of flavonoids and polyphenolic compounds in *C. verrucosa*, the ascorbic acid (antioxidant) inhibits the DPPH free radical scavengers more strongly, thereby, reversing the oxidative damage more strongly than the other two pathways. Thus, the total flavonoid content (TFC) of *C. verrucosa* showed a moderate % inhibitory activity (R^2 = 0.830) whilst that of total phenolic content (TPC) showed less than moderate % inhibitory activity (R^2 = 0.793).

4.8. Identification and quantification of Polyphenolic compounds in *C. verrucosa* using HPLC-DAD

4.8.1. HPLC chromatogram of standard mixtures of phenolic antioxidants

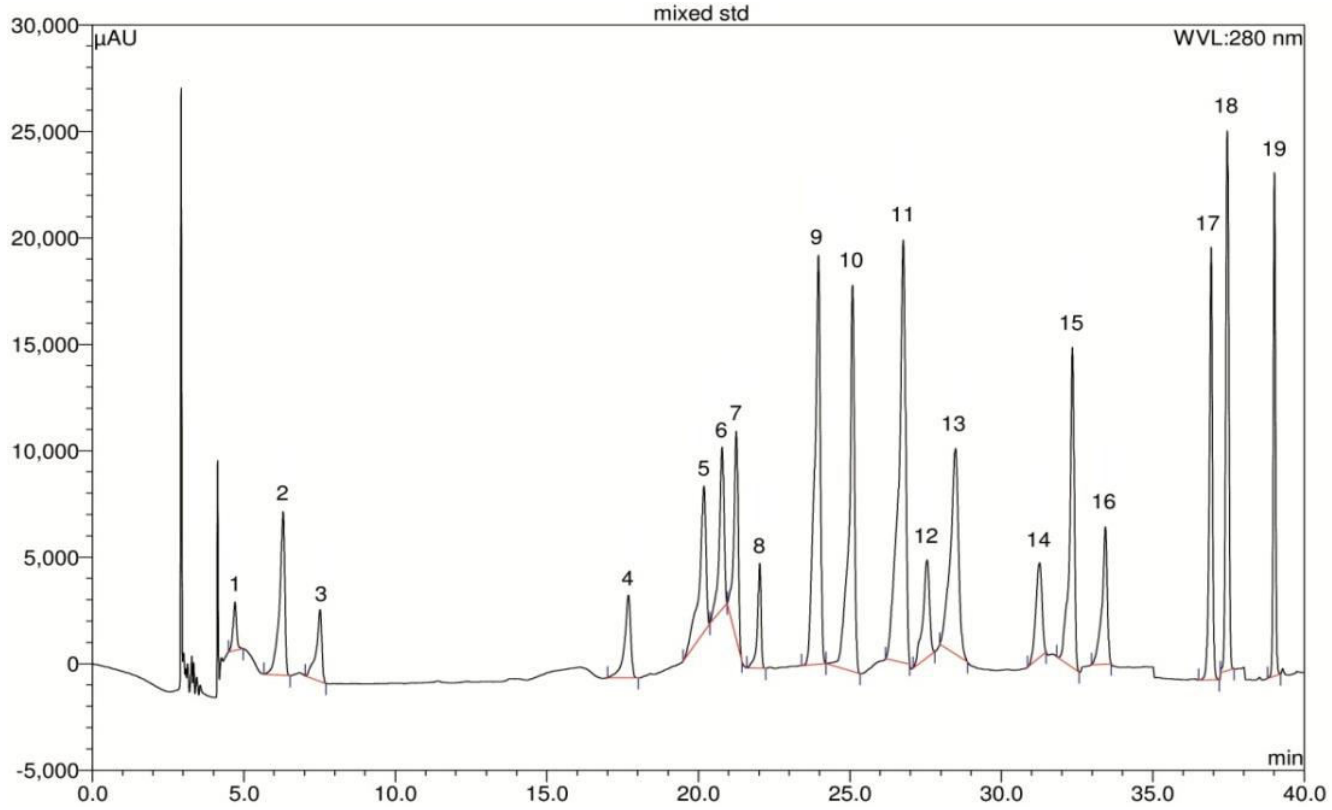


Figure 4.7: HPLC chromatogram of standard mixtures of phenolic antioxidant compounds

Interpretation: The HPLC chromatogram showed the presence of: Peaks: 1, arbutin; 2, gallic acid; 3, hydroquinone; 4, (+)-catechin hydrate; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (–)-epicatechin; 9, vanillin; 10, *p*-coumaric acid; 11, *trans*-ferulic acid; 12, rutin hydrate; 13, ellagic acid; 14, benzoic acid; 15, rosmarinic acid; 16, myricetin; 17, quercetin; 18, *trans*-cinnamic acid; and 19, kaempferol

4.8.2. HPLC chromatogram of methanolic leaf extract of *C. verrucosa*

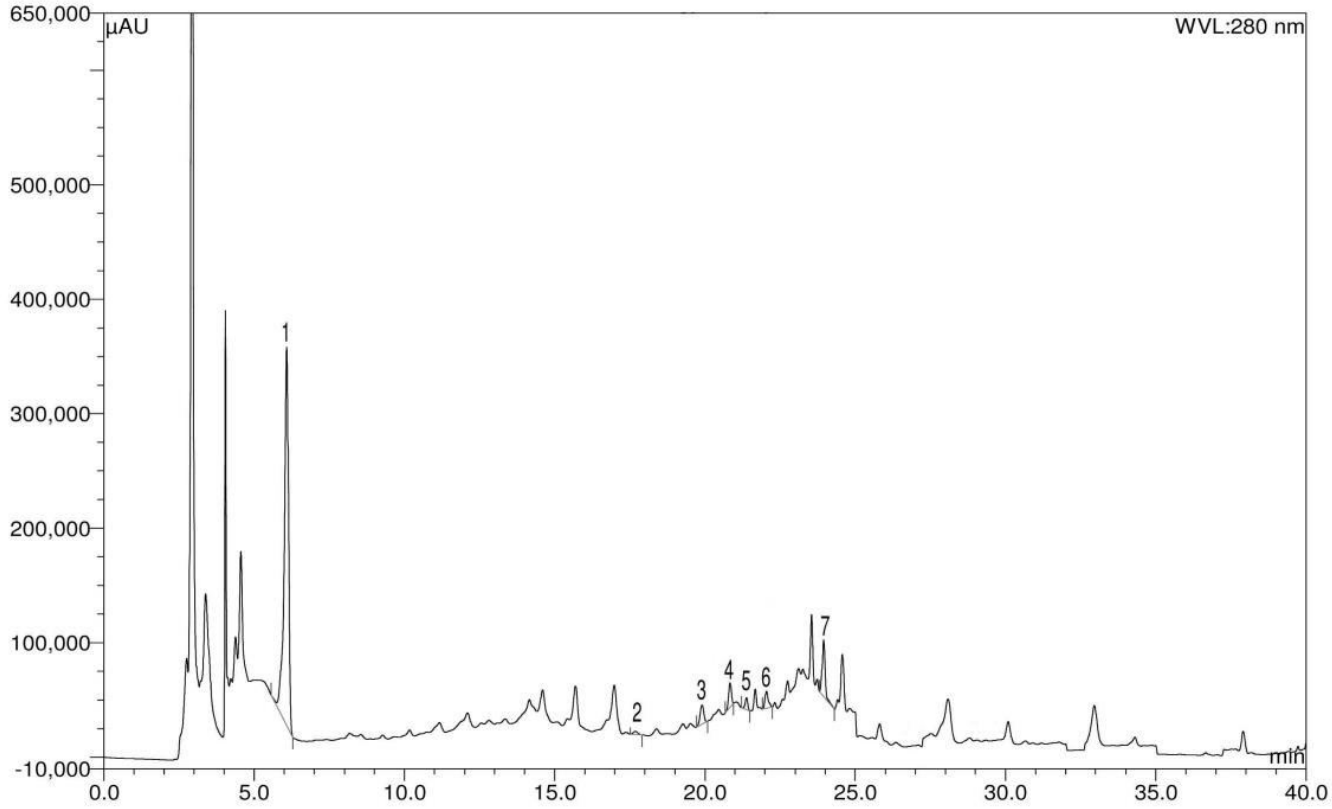


Figure 4.8: HPLC chromatogram of methanolic leaf extract of *C. verrucosa*.

Interpretation: The HPLC chromatogram showed the presence of: Peaks: 1, gallic acid (GA); 2, (+)-catechin hydrate (CH); 3, vanillic acid (VA); 4, caffeic acid (CA); 5, syringic acid (SA); 6, (–)-epicatechin (ECA); and 7, vanillin (VL)

4.8.3. Quantification of polyphenolic antioxidants in *C. verrucosa* via HPLC

Table 4.11: Determining the contents of polyphenolic antioxidant compounds in methanolic extract of *C. verrucosa* using HPLC

Phenolic compound	Methanol extract of *C. verrucosa*	
	Content (mg/100 g of dried extract)	% RSD
GA	19.53	0.57
CH	5.08	0.14
VA	5.97	0.18
CA	6.22	0.22
SA	1.09	0.05
ECA	7.16	0.29
VL	8.05	0.35

Each of the values are the result of mean of five determinations (n=5)

RSD = Relative standard deviation

GA = gallic acid; CH = (+)-catechin hydrate; VA = vanillic acid; CA = caffeic acid; SA = syringic acid; ECA = (–)-epicatechin; VL = vanillin

Interpretation: It can be observed that gallic acid (GA) is most predominantly present in the methanolic leaf extract of *C. verrucosa* than any other polyphenolic compounds as determined by HPLC analysis.

4.8.4. Phytocompounds present in *C. verrucosa* responsible for its antioxidant activity

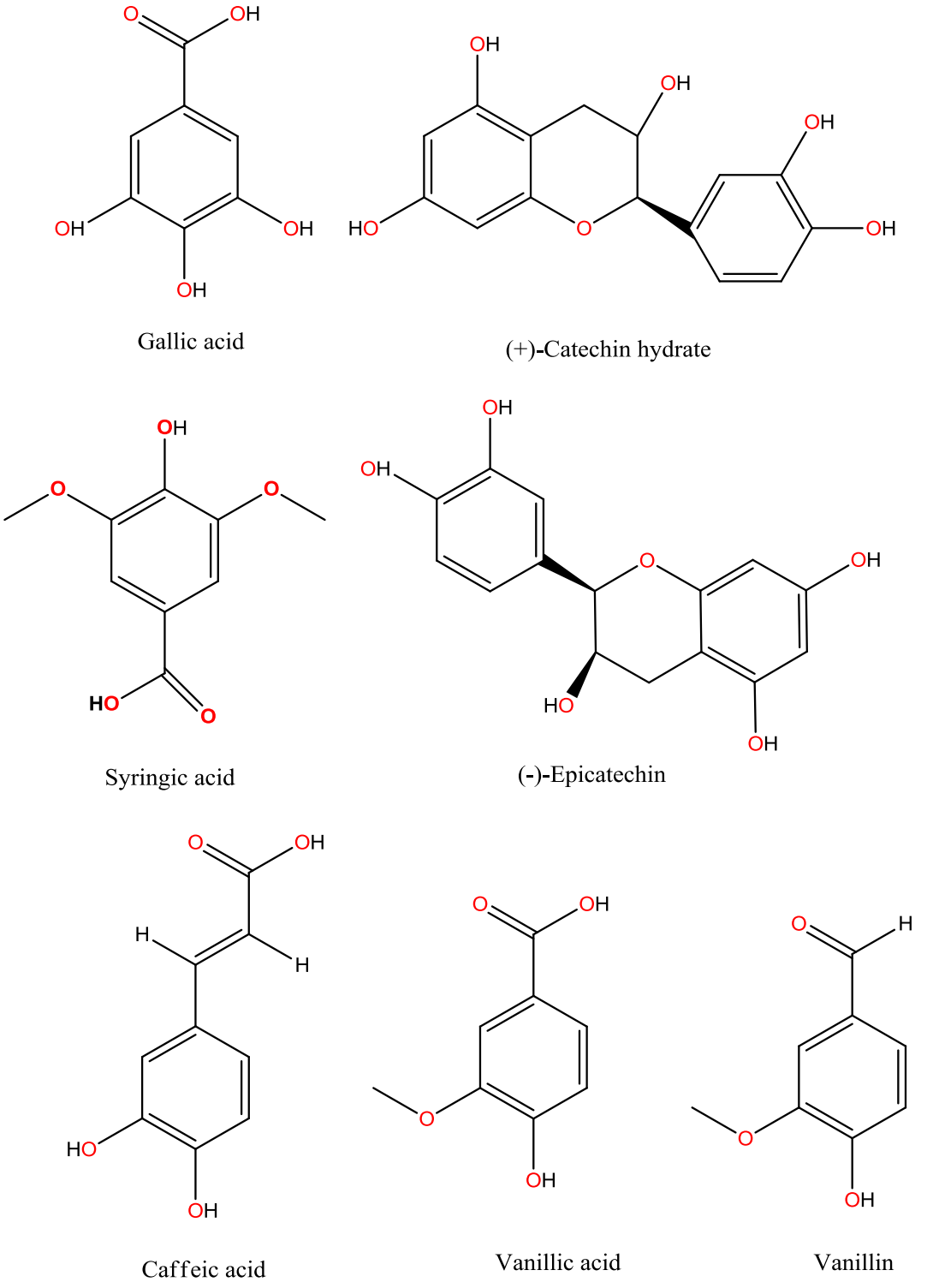


Figure 4.9: Phytocompounds identified in the methanolic leaf extract of *Crotalaria verrucosa* via HPLC analysis

CHAPTER FIVE

DISCUSSION

Chapter 5: Discussion

The project began with a total of 278.30g of powdered plant material, which after maceration in methanol, a percentage yield of 8.084% of dried extract of *C. verrucosa* was obtained. This comprised the 22.50g of methanolic extract of *C. verrucosa* which was subsequently used for investigation into its potential antioxidant properties.

Previous studies on phytochemical screening were performed on the ethanolic extract of *Crotalaria verrucosa* which confirmed the presence of flavonoids and phytosterols in this plant (Nawrin *et al.*, 2015; Riazunnisa, Prasad, Sudha, and Khadri, 2015; Singh *et al.*, 2011; Lekharni *et al.*, 2013). The current study performed a preliminary phytochemical screening of the methanolic leaf extract of *C. verrucosa* which qualitatively confirmed the presence of flavonoids, phenolic compounds and alkaloids amongst many other chemical constituents such as tannins, glycosides, resins, steroids and carbohydrates (Table 4.3). However, according to a study conducted by Kamalakar, Prabhakar and Shailaja (2014) which evaluated the phytochemical constituents present in *C. verrucosa* using various solvent namely, hexane, petroleum ether, ethyl acetate, chloroform, acetone, ethanol, methanol and aqueous, the author reported that the methanol extract of *C. verrucosa* did not possess carbohydrate, steroids, fixed oils and resins. On the contrary, our study on phytochemical screening of this plant revealed the presence of carbohydrate and steroids as indicated in the Table 4.3.

After having qualitatively determined the presence of flavonoids and polyphenolic compounds of dried extract of *C. verrucosa*, the extract was then subjected to various *in-vitro* antioxidant assays.

DPPH free radical scavenging (FRS) assay was performed to determine the total antioxidant potential of *C. verrucosa* as illustrated in Figure 4.2. In DPPH free radical scavenging assay, the IC_{50} values of *C. verrucosa* and standard ascorbic acid were also calculated and was found to be 533.738 ug/mL and 154.916ug/mL, respectively (Table 4.6). IC_{50} value is the concentration at which 50% of total DPPH free radical is scavenged by the antioxidants present in sample. Therefore, it could be surmised that a concentration of 533.738ug/mL of methanolic leaf extract of *C. verrucosa* can inhibit 50% of all the DPPH free radical scavengers (FRS) whereas; a concentration of 154.916ug/mL of ascorbic acid can inhibit 50% of all the DPPH free radical scavenging activity.

Furthermore, the quantitative determination of total phenolic content of this extract was determined by using the Folin-Ciocalteu method which utilizes gallic acid as the standard. The result obtained depicts the amount of gallic acid present in the sample extract, expressed in gallic acid equivalents (GAE) as shown in the Table 4.7. The results showed that at concentrations of 200, 400, 800 and 1200ug/mL of this extract, the total phenolic content obtained were 34.78, 59.72, 99.56, and 152.18mg of gallic acid per gram of dried extract, respectively. It could thus be suggested that *C. verrucosa* possesses sufficient content of gallic acid to exhibit moderate antioxidant properties.

Thereafter, the total flavonoid content (TFC) of *C. verrucosa* extract was investigated to deduce the quantity of flavonoids responsible for antioxidant activity by using quercetin (QU) as the standard. Quercetin equivalents of 32.08, 78.94, 120.60 and 184.51mg/g of dried extract were obtained for concentrations ranging from 200, 400, 800 and 1200ug/mL (Table 4.8).

Afterwards, the total antioxidant capacity (TAC) of dried extract of *C. verrucosa* was evaluated using the phosphomolybdenum method, the results of which were expressed as ascorbic acid equivalent (AAE) as shown in Table 4.9. According to the results obtained, the total antioxidant capacity (TAC) of this extract was observed to be 8.35, 19.58, 24.63 and 32.34 AAE for concentrations of extract 200, 400, 800 and 1200ug/mL, respectively. At a concentration of concentrations of 200, 400ug/mL and 800ug/mL, the TAC of *C. verrucosa* was found to be 8.35, 19.58 and 24.63mg/g of AAE whilst that of *G. jasminoides* was found to be 1.99, 2.80 and 3.41mg/g of AAE, respectively (Uddin *et al*., 2014). Thus proving that the methanolic leaf extract of *C. verrucosa* has a higher total antioxidant capacity than the methanolic leaf extract of *G. jasminoides*,

Using the results of the *in-vitro* antioxidant assays, a relationship was established between the % inhibition of DPPH free radical scavenging activity to that of the three *in-vitro* antioxidant assays i.e. total flavonoid content (TFC), total phenolic content (TPC) and total antioxidant capacity (TAC), in order to determine a correlation (if existed) between them. It was observed that a positive correlation existed between them all, although a very strong positive correlation (R^2 = 0.893) was observed between % inhibition of *C. verrucosa* on DPPH free radical scavengers and TAC. This indicates that whilst the quantity of flavonoids and polyphenolic compounds in *C. verrucosa* are more, the ascorbic acid (antioxidants) inhibit DPPH free radical scavengers more strongly than the other two and thus, reversing the oxidative damage more than the other two. Thus,

the TFC of *C. verrucosa* showed a moderate % inhibitory activity (R^2 = 0.830) whilst that of TPC showed less than moderate % inhibitory activity (R^2 = 0.793) on DPPH free radical scavengers.

Since, the methanolic leaf extract of *Crotalaria verrucosa* showed potential antioxidant activities in all four *in-vitro* antioxidant assays, the next step was to identify and quantitate the amount of individual polyphenolic compounds present in it which was done using HPLC coupled to diode array detector (HPLC-DAD). The chromatographic separations of phenols in standard and methanol extract of *C. verrucosa* are shown in Figure 4.7 & 4.8, respectively. The content of polyphenolic compounds present in the extract was calculated from the corresponding calibration curve and presented as the mean of five determinations (Table 4.11). The HPLC analysis of methanolic leaf extract of *C. verrucosa* identified the presence of gallic acid, (+)-catechin hydrate, vanillic acid, caffeic acid, syringic acid, (–)-epicatechin, and vanillin (Figure 4.9) and also quantified the amount of each of the phenolic compound as 19.53, 5.08, 5.97, 6.22, 1.09, 7.16 & 8.05mg/100g of dried extract, respectively. Amongst the polyphenolic compounds obtained by HPLC analysis, the experimental results indicate that gallic acid (19.53 mg per 100 g of dried extract) was predominantly present in the crude extract of *C. verrucosa.*

On the contrary, HPLC analysis of polyphenols also confirmed the absence of certain flavonoids in this extract such as myricetin, kaempferol and quercetin, and the absence of certain polyphenolic compounds such as ellagic acid, p-coumaric acid, trans-ferulic acid, rutin hydrate, rosmarinic acid and *trans*-cinnamic acid, thus, proving that they are not responsible for any antioxidant activity established in this extract so far.

Therefore, the higher TFC of *C. verrucosa* can be attributed to the fact that flavonoids such as (+)-Catechin hydrate and (–)-epicatechin are present in the crude extract in addition to other unidentified compounds. (+)-Catechin hydrate and (–)-epicatechin are flavonoids and vanillic acid is a catechin-metabolite–all of which have shown high antioxidant activity in tea and green tea drinks (Keen *et al.*, 2005). Nonetheless, flavonoids are one of the largest class of polyphenolic compounds. Gallic acid (pseudotannin), syringic acid, caffeic acid and vanillin are polyphenolic compounds, all of which demonstrate antioxidant activity with syringic acid claiming to possess higher antioxidant potential (Stanikunaite, Khan, Trappe & Ross, 2009; Gulcin, 2006; Rice-Evans, Miller & Paganga, 1997).

On a concluding note, the various *in-vitro* antioxidant assays performed on this plant, established its moderate antioxidant activity comparable to that of ascorbic acid. Our results clearly indicate that amongst the many polyphenolic compounds responsible for the antioxidant activity of *C. verrucosa*, gallic acid, syringic acid, caffeic acid, vanillin, (+)-catechin hydrate, (–)-epicatechin and vanillic acid are few of the polyphenolic compounds which have been identified as being present in it which can be extrapolated to the promising antioxidant activity of the plant. Therefore, the presence of such antioxidant substances in methanolic leaf extract of *C. verrucosa* may justify its use in folkloric remedies in ailing scabies and heart complaints including the treatment of throat and oral diseases.

CHAPTER SIX

CONCLUSION

Chapter 6: Conclusion

This is the first study devised to investigate the antioxidant potential of methanolic extract of *Crotalaria verrucosa* preliminary by screening for desired phytocompounds followed by assessment of its antioxidant potential using previously established *in-vitro* antioxidant methods and finally aiming at identifying and quantifying the polyphenolic compounds present.

The phytochemical screening of *C. verrucosa* tested positive for the presence of flavonoids and phenolic compounds. The total antioxidant capacity (TAC) of the methanolic extract of *C. verrucosa* was found to be very high compared to that of *Gardenia jasminoides*. At a concentration of concentrations of 400ug/mL, the TAC of *C. verrucosa* was found to be 19.58mg/g of AAE whilst that of *G. jasminoides* was found to be 2.800mg/g of AAE, respectively. The IC_{50} values obtained from DPPH free radical scavenging assay was found to be 533.738 and 154.916ug/mL for *C. verrucosa* and ascorbic acid, respectively; thus indicating that the dried extract of *C. verrucosa* possesses moderate antioxidant potential comparable to standard ascorbic acid. The total flavonoid content (TFC) of the sample extract was found to be higher than its total phenolic content, indicating the presence of certain flavonoids responsible for antioxidant function. Using HPLC-DAD analysis, polyphenolic compounds such as (+)-catechin hydrate, (–)-epicatechin, vanillic acid, gallic acid, syringic acid, caffeic acid and vanillin were indentified and quantified with gallic acid being most predominantly present (19.53mg/100g of dried extract). Also a positive correlation was established between the inhibitory potential of DPPH and the reducing potential of gallic acid.

On a concluding note, the various *in-vitro* antioxidant assays performed on this plant, establishes that the methanolic extract of *C. verrucosa* possesses moderate antioxidant activity comparable to ascorbic acid. Our results clearly indicate that gallic acid, syringic acid, caffeic acid, vanillin, (+)-catechin hydrate, (–)-epicatechin and vanillic acid are few of the polyphenolic compounds present in the leaf extract of *C. verrucosa* which extrapolates to its promising antioxidant potential while justifing its role in folkloric remedies.

CHAPTER SEVEN

FUTURE DIRECTIONS

Chapter 7: Future Directions

The result of the present study established that methanolic extract of *C. verrucosa* possesses moderate antioxidant activity relative to that of ascorbic acid, thus, broadening up a dimension full of different scopes for further studies:

- In order to completely identify and comprehend all the antioxidants responsible for the *in-vitro* antioxidant activities of crude extract of *C. verrucosa* plant, other flavonoids and polyphenolic compounds may be employed as reference standards during HPLC analysis.
- The knowledge of the *in-vitro* antioxidant activity of *C. verrucosa* opens up another posibility to carry out *in-vivo* antioxidant study to demonstrate the pharmacological effect on laboratory animal-models.
- Except for necic lactone, no known phytocompounds have yet been isolated from the leaves of *C. verrucosa* nor published. Thus, appropriate research initiatives may be carried out for the isolation of phytocompounds present in the leaves of *C. verrucosa*.
- Further pharmacological studies on cytotoxic, anti-cancer, antihyperlipidemia, and anti-atherosclerotic activities of this plant are yet to be carried out. Such research initiatives into these activities might lead to the drug discovery, drug isolation and may serve this plant as a natural source for the development of novel drug compounds.

CHAPTER EIGHT

APPENDIX

Chapter 8: Appendix

8.1. Phytochemical screening of *C. verrucosa* (Individual test methods)

Table 8.1: The individual test methods used in phytochemical screening of *C. verrucosa*

S.L.#	*Name of test*	*Observation*	*Result*
1.	**Detection of Alkaloid**		
	a) Hager's Test	Light yellow ppt.	+
	b) Mayer's Test	Cream colored ppt.	+
	c) Wagner's Test	Brownish-black ppt.	+
2.	**Detection of Carbohydrate**		
	a) Molisch's Test	Brick-red ppt.	+
	b) Fehling's Test	Violet ring at the junction	+
3.	**Detection of Glycoside**		
	Borntrager's test	Reddish-pink color	+
4.	**Detection of Flavonoids**		
	a) Lead Acetate Test	Deep yellow ppt.	+
	b) Zinc Ribbon Test	Deep brown ppt.	+
5.	**Detection of Phenol/Phenolic compounds**		
	Ferric Chloride Test	Bluish-black ppt.	+
6.	**Detection of Phytosterols**		
	Libermann-Burchard's Test	Clear yellow solution	-
7.	**Detection of steroids**		
	Salkowski Test	Oily greenish-yellow solution	+
8.	**Detection of tannin**		
	a) Lead acetate Test	Yellow ppt.	+
	b) Potassium dichromate Test	Red colored solution with Yellow ppt.	+
	c) Ferric chloride Test	Greenish black ppt.	+
9.	**Detection of resins**	Purple-brown ppt.	+

8.2. DPPH free radical scavenging activity

Table 8.2: Calculations associated with DPPH scavenging activity of standard ascorbic acid (AA); calculation of the mean and standard deviation (SDV) of the absorbance of standard AA, followed by calculation of % inhibition of scavenging activity by ascorbic acid (% Inhibition1, % inhibition2); calculation of mean and standard deviation of % inhibitory activity of AA

Concentration of standard (A.A.) (ug/mL)	Absorbance of standard (AA)				% Inhibition of standard (AA)			
	A1	A2	Mean	SDV	% inhibition 1	% inhibition 2	Mean % Inhibition (AA)	SDV
12.5	0.380	0.390	0.385	0.007	41.176	39.628	40.402	1.095
25	0.254	0.256	0.255	0.001	60.681	60.372	60.526	0.219
50	0.112	0.114	0.113	0.001	82.663	82.353	82.508	0.219
100	0.027	0.028	0.0275	0.001	95.820	95.666	95.743	0.109
200	0.025	0.026	0.0255	0.001	96.130	95.975	96.053	0.109
400	0.023	0.025	0.024	0.001	96.440	96.130	96.285	0.219
800	0.024	0.023	0.0235	0.001	96.285	96.440	96.362	0.109
1200	0.024	0.021	0.0225	0.002	96.285	96.749	96.517	0.328

Table 8.3: Calculations associated with DPPH scavenging activity of the sample *Crotalaria verrucosa* (CV); calculation of the mean and standard deviation (SDV) of the absorbance of sample (CV), followed by calculation of % inhibition of scavenging activity by ascorbic acid (% Inhibition1, % inhibition2); calculation of mean and standard deviation of % inhibitory activity of sample (CV)

Concentration of CV (ug/mL)	Absorbance (CV)				% inhibition (CV)			
	A1	A2	Mean	SDV	% inhibition1	% inhibition2	Mean % Inhibition (CV)	SDV
12.5	0.630	0.632	0.631	0.001	2.477	2.167	2.322	0.219
25	0.615	0.613	0.614	0.001	4.799	5.108	4.954	0.219
50	0.598	0.596	0.597	0.001	7.430	7.740	7.585	0.219
100	0.586	0.584	0.585	0.001	9.288	9.598	9.443	0.219
200	0.492	0.490	0.491	0.001	23.839	24.149	23.994	0.219
400	0.304	0.302	0.303	0.001	52.941	53.251	53.096	0.219
800	0.091	0.089	0.090	0.001	85.913	86.222	86.068	0.219
1200	0.091	0.089	0.090	0.001	85.913	86.222	86.068	0.219

8.3. Determination of Total Phenolic Content (TPC)

Table 8.4: Records of the mean and standard deviation (SDV) of absorbance of sample *Crotalaria verrucosa* (CV), obtained spectrophotometrically (at 765nm), against its respective concentration; the absorbance value of gallic acid (GA) is also recorded

Concentration (ug/mL)	**Abs (GA)**	**Abs (CV) at 765nm**			**Abs (CV) (Mean ± SDV)**
200	1.634	-0.099	-0.105	-0.101	-0.102 ± 0.003
400	3.226	0.039	0.045	0.043	0.042 ± 0.003
800	3.625	0.500	0.538	0.511	0.516 ± 0.020
1200	3.805	1.410	1.360	1.405	1.392 ± 0.027

Table 8.5: Determining the value of "x" (Concentration of gallic acid in mg/mL) using the equation obtained from the calibration curve, $y = 0.0085x - 0.1607$ ($R^2 = 0.9977$)

Concentration (ug/mL)	**y = Mean Abs (CV) at 765nm**	**x = Concentration of GA in plant extract of CV (mg/mL)**
200	-0.102	6.955×10^{-3}
400	0.042	23.888×10^{-3}
800	0.516	79.651×10^{-3}
1200	1.392	182.610×10^{-3}

Table 8.6: Calculation of total phenolic content (TPC) using the formula, C = (c x V)/m, where, C=Total content of phenolic compounds mg/g of gallic acid equivalent (GAE), c=concentration of gallic acid from the calibration curve, V=volume of sample extract (mL) & m=mass of crude plant extract (g)

Concentration of CV (ug/mL)	**Total phenolic content (GAE) (Mean ± SDV)**
200	34.775 ± 1.815
400	59.721 ± 0.900
800	99.564 ± 2.876
1200	152.180 ± 2.688

8.4. Determination of Total Flavonoid Content (TFC)

Table 8.7: Record of the mean and standard deviation (SDV) of absorbance of sample *Crotalaria verrucosa* (CV) obtained spectrophotometrically (at 415nm) against its respective concentration; the absorbance value of quercetin is also recorded

Concentration (ug/mL)	**Abs (Quercetin)**	**Abs (CV) at 415nm**			**Abs (CV) (Mean ± SDV)**
200	0.219	-0.004	-0.006	-0.005	-0.005 ± 0.001
400	0.422	0.025	0.024	0.027	0.025 ± 0.001
800	0.959	0.105	0.104	0.100	0.103 ± 0.003
1200	1.369	0.249	0.253	0.257	0.253 ± 0.004

Table 8.8: Determining the value of "x" (Concentration of quercetin in mg/mL) using the equation obtained from the calibration curve, $y = 0.0012x - 0.0127$ ($R^2 = 0.9982$)

Concentration of CV (ug/mL)	**y = Mean Abs (415nm)**	**x = Concentration of Quercetin in plant extract of CV (mg/mL)**
200	-0.005	6.417×10^{-3}
400	0.025	31.580×10^{-3}
800	0.103	96.480×10^{-3}
1200	0.253	221.420×10^{-3}

Table 8.9: Calculation of total flavonoid content (TFC) using the formula, C = (c x V)/m, where, C=Total content of flavonoid compounds mg/g of quercetin equivalent (QE), c=concentration of quercetin from the calibration curve, V=volume of sample extract (mL) & m=mass of crude plant extract (g)

Concentration of CV (ug/mL)	**Total flavonoid content (QE) (Mean ± SDV)**
200	32.083 ± 4.167
400	78.938 ± 2.700
800	120.600 ± 2.622
1200	184.510 ± 2.778

8.5. Determination of Total Antioxidant Capacity (TAC)

Table 8.10: Record of the mean and standard deviation (SDV) of absorbance of sample *Crotalaria verrucosa* (CV) obtained spectrophotometrically (695nm) against its respective concentration; the absorbance value of ascorbic acid (AA) is also recorded

Concentration (ug/mL)	**Abs (AA)**	**Abs (CV)** at 695nm			**Abs (CV) Mean ± SDV**
200	1.08	0.133	0.137	0.135	0.135 ± 0.002
400	2.101	0.228	0.231	0.230	0.230 ± 0.002
800	3.741	0.408	0.415	0.412	0.412 ± 0.004
1200	4.122	0.702	0.707	0.705	0.705 ± 0.003

Table 8.11: Determining the value of "x" (Concentration of ascorbic acid in mg/mL) using the equation obtained from the calibration curve, y = 0.0046x + 0.1094 (R^2 = 0.9954)

Concentration (ug/mL)	**y = Absorbance of CV at 695nm**	**x = Concentration of AA in plant extract of CV (mg/mL)**
200	0.135	5.565×10^{-3}
400	0.230	26.109×10^{-3}
800	0.412	65.674×10^{-3}
1200	0.705	129.370×10^{-3}

Table 8.12: Calculation of total antioxidant capacity (TAC) using formula, A = (c x V)/m, where, A=Total antioxidant capacity mg/g in ascorbic acid equivalent (AAE), c=concentration of ascorbic acid from the calibration curve, V=volume of sample extract (mL) & m=mass of crude plant extract (g)

Concentration of CV (ug/mL)	**Total antioxidant capacity (AAE) (Mean ± SDV)**
200	8.348 ± 0.652
400	19.582 ± 0.245
800	24.628 ± 0.285
1200	32.342 ± 0.136

Table 8.13: Total antioxidant capacity of methanolic extract of *Gardenia jasminoides Ells*. (Uddin *et al.,* 2014)

Concentration of CV (ug/mL)	**Total antioxidant capacity (AAE) (Mean ± SDV)**
100	1.00 ± 0.15
200	1.99 ± 0.07
400	2.80 ± 0.15
600	3.41 ± 0.14
800	5.37 ± 0.14

(Source: Uddin et al., 2014)

CHAPTER NINE

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